To the Graduate Council:

I am submitting herewith a thesis written by Rachael N. Slightom entitled "Characterization of Motility and Surface Attachment in Thirteen Members of the Roseobacter Clade." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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CHARACTERIZATION OF MOTILTY AND SURFACE ATTACHMENT AMONG THIRTEEN MEMBERS OF THE ROSEOBACTER LINEAGE

A Thesis

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Master of Science Degree

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Rachael N. Slightom

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ABSTRACT

The Roseobacter clade is an abundant and biogeochemically relevant group of marine bacteria. Physiological and ecological traits identified in specific representatives of the clade are often universally attributed to all Roseobacter group members, however, culture-dependent studies utilizing phylogenetically distinct members are rare. Other attributes often associated with this clade include motility, biofilm formation and surface attachment, chemotaxis and quorum sensing. This study compared a collection of 13 diverse Roseobacter strains both pheno- and genotypically on the basis of these traits. Motility was determined for seven previously uncharacterized strains, with five of the strains demonstrating motility. Microscopic analysis using both phase contrast and transmission electron microscopy supported this finding. A crystal violet assay was used to assess biofilm formation on plastic and glass surfaces with a range of surface properties and yielded a wide array of phenotypic responses. Taking into account the variety of surface types and media types tested approximately half (54%) of the strains showed pronounced biofilm formation and all motile strains were capable of forming biofilms. Degenerate primer sets were designed to probe strains for which no genome sequence is currently available for genes involved in flagellar synthesis and chemotaxis. Two strains that demonstrated no signs of motility in the laboratory were found to possess a necessary gene for flagellar formation and a flagellar-associated chemotaxis gene. Genome analysis including other sequenced Roseobacter strains revealed that flagellar, chemotaxis and quorum sensing operons are abundant in members of this lineage, with 89% possessing flagellar and chemotaxis operons and 78% possessing genes believed to be involved in quorum sensing. This study underscores the diversity of this clade and emphasizes the difficulty of assigning phenotypic capabilities to all lineage members.

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INTRODUCTION

Roseobacter Clade

Though microbes are recognized to make up the majority of the organisms in the ocean, their diversity and varied roles in this environment are not yet fully appreciated (Fuhrman *et al.*, 1993). Very few marine bacterial groups have been identified and even fewer have been brought into culture (Giovannoni, 2000). The lack of culturability of most marine organisms has made studying marine systems all the more difficult (Ferguson *et al.*, 1984). However, one group of marine bacteria that has recently increased in interest among microbial ecologists is the Roseobacter clade. This clade is one of the most abundant marine prokaryotic lineages known, however, it was not recognized until the early 1990's with the usage of molecular tools to profile marine prokaryotic communities (Shiba, 1991). Since the realization of the abundance of the Roseobacter clade, interest in this group has steadily increased. The members fall within the *Alphaproteobacteria* and members of the Roseobacter clade have 16S rRNA gene similarities of >89% (Buchan *et al.*, 2005). All characterized members of the Roseobacter clade, with the exception of the genus *Ketogulonicigenium*, have been demonstrated to have a salt requirement and Roseobacter 16S rRNA genes have yet to be recovered from non-marine, or at least non-saline, environments. Thus the group is considered to be restricted to marine or saline habitats. As representatives of the clade are relatively easily cultured and demonstrate a variety of interesting pheno- and genotypes, members of this group are considered model organisms for the study of successful marine heterotrophic bacteria (Wagner-Dobler and Biebl, 2006). Due to the increased interest in this clade we now have many sequenced genomes and are beginning to understand and appreciate the metabolic and physiological diversity that could provide insight into their ecological success.

Members of the Roseobacter lineage have been isolated from a wide variety of marine environments. While they are most abundant in coastal areas, composing up to 20% of bacteria, they have also been isolated from a variety of other marine environments including the open ocean, hydrothermal vents, marine snow and arctic ice (Buchan *et al.*, 2005). Members have also been found in various commensal or

symbiotic relationships (Althoff *et al.*, 1998). For example, *Silicibacter* sp. TM1040 was isolated from a *Pfiesteria*-like dinoflagellate and has since been shown to have a mutualistic relationship with that dinoflagellate (Miller and Belas, 2006; Miller and Belas, 2004). Associations with marine phytoplankton appear common as several other Roseobacter strains or 16S rDNA clone sequences have derived from dinoflagellates and marine algae (Rao *et al.*, 2006; Alavi *et al.*, 2001; Gonzalez *et al.*, 2000). Roseobacter members have also been associated with other marine eukaryotes including fish, sponges and marine plants (Lee *et al.*, 2007; Ruiz-Ponte *et al.*, 1998). Finally, though reports are rare, several Roseobacter species have been implicated as disease causing agents in both oysters and corals (Cooney *et al.*, 2002; Boettcher *et al.*, 2000).

Roseobacter clade members possess a wide variety of important metabolic and physiological capabilities. These capabilities are thought to be integral to the particular ecological niches Roseobacters inhabit. Production of secondary metabolites has been demonstrated for several clade members. For example, the antimicrobial compound, tropodithietic acid, is produced by two members of the Roseobacter clade (Bruhn *et al.*, 2005; Brinkhoff *et al.*, 2004). It has been hypothesized that secondary metabolite production by Roseobacters affords these organisms with a selective advantage in certain environments. Experiments have shown Roseobacters are capable of out-competing other strains for nutrients and available space on biotic surfaces (Rao *et al.*, 2005; Brinkhoff *et al.*, 2004). Roseobacter isolate MA03 has been shown to increase the predation of a *Rhodomonas* alga by a *Pfiesteria* dinoflagellate (Alavi, 2004). This further shows the high degree of success of Roseobacters in their ecological niches.

One of the most important aspects of the Roseobacter clade is their contribution to the global biogeochemical cycling of elements, particularly sulfur and carbon. Several strains have been implicated in the global sulfur cycle through their metabolic breakdown of the algal osmolyte dimethylsulfoniopropionate (Miller and Belas, 2004; Gonzalez *et al.*, 2003), as well as the ability to oxidize a variety of reduced sulfur compounds (Moran *et al.*, 2003). Roseobacters contribute to the carbon cycle through their degradation of plant-related aromatic compounds (Buchan *et al.*, 2000; Gonzalez *et al.*, 1997), anaerobic and aerobic anoxygenic phototrophy, as well as carbon monoxide oxidation (Wagner-

Dobler and Biebl, 2006; Allgaier *et al.*, 2003). Photoheterotrophy by Roseobacters is achieved through the production of bacteriochlorophyll-a, whose pink pigment provided the names for the first characterized strains (Wagner-Dobler and Biebl, 2006).

In recent years the genomes of several Roseobacter strains have become available. Currently, completed or draft genomes are available for 23 Roseobacter strains, while the sequencing of approximately another 10 strains is either planned or underway. However, 11 of these strains have not yet been properly characterized. Initial analysis of the available genome sequences suggests the group is metabolically diverse (Moran *et al.*, 2007). Furthermore, the majority of Roseobacter strains that have been sequenced reveal many genes required for flagellar synthesis, chemotaxis proteins, and numerous transcriptional regulators, including quorum sensing systems (Moran *et al.*, 2007). Despite the wealth of information revealed by these genome sequences, there are still many cultured strains for which little to no genetic information is available. However, the availability of sequenced genomes facilitates the generation of tools (e.g. PCR primers, DNA probes) that allow investigators to probe additional strains.

Most of the investigations aimed at gaining an understanding of Roseobacter physiology and ecology have been carried out on a limited number of strains; comparisons of several cultured strains representing the phylogenetic diversity of the lineage are lacking. Culture-dependent and -independent studies suggest that traits such as motility, biofilm formation, attachment to surfaces, chemotaxis and quorum sensing are important aspects of the ecology and success of the members of the Roseobacter clade. A study of these traits among phylogenetically distinct members of the Roseobacter clade is necessary for further understanding of the capabilities of clade members.

Motility

Bacteria explore their world via various forms of motility. Motility is crucial to specific interactions a bacterium forms within an environment in which it resides. For example, motility can contribute to cell attachment to a surface, biofilm formation, chemotaxis and many symbiotic relationships (Harshey, 2003). Without some form of directed movement, most bacteria are unable to participate in these relationships and

interactions (O'Toole and Kolter, 1998). Not only do bacteria use motility to move towards a surface or an organism, motility also plays a role once the surface is encountered. Motility is accomplished through several different modes of action including swimming, swarming, twitching and gliding. Swimming and swarming are flagellar-associated movements, twitching is pili-associated movement, and gliding can occur by one of several different mechanisms (Harshey, 2003). Though swimming and swarming are both flagellar-associated movements, the ability to perform one does not guarantee the ability to perform the other. Swarming is considered to be a concerted effort by an entire population of bacteria and is strictly a surface-oriented ability, while swimming is the action of a single cell and allows a cell to move through a liquid medium (Harshey, 2003). Organisms that possess the ability to move via both swimming and swarming exhibit different cellular morphologies depending upon the movement type. Swarming cells actually differentiate from the swimming cell before beginning to swarm, while swimming requires no cell differentiation (Rather, 2005; Kearns and Losick, 2003). All movement capabilities have important implications for ecological interactions. This type of cell differentiation has been well-characterized in *Pseudomonas aeruginosa*.

Flagellar assembly is a multiple-step process involving many genes. The major components of the flagella include the basal body, flagellar motor, motor switch, hook, flagellar filament, capping proteins, junction proteins and an export apparatus. There are at least 35 proteins designated as flagellar-specific proteins that are required to form a flagellum (Macnab, 2003). The basal body consists of the MS ring, the P ring, the L ring and a rod. The flagellar motor is formed from two proteins, MotA and MotB. Only the flagellar filament and hook are external to the cell. The flagellum is formed in a sequential and orderly manner with construction beginning with components localized to the membrane and extending out of the cell (Macnab, 2003).

Swimming motility has been demonstrated for several members of the Roseobacter clade (Miller and Belas, 2006; Gonzalez *et al.*, 2003; Sorokin, 1995) and occurs via a single, or multiple, polar flagella. Of particular interest, is work done by Robert Belas and colleagues with *Silicibacter* sp. TM1040. In this strain motility has

been shown to facilitate a mutualistic relationship with a *Pfiesteria*-like dinoflagellate by being motile (Miller and Belas, 2006). This is an indication of the importance of motility in this Roseobacter clade member's ecology and raises interest in the role of motility in other strains within the clade.

Chemotaxis

A behavior closely associated to motility is that of chemotaxis. Chemotaxis allows a bacterium to sense its environment utilizing membrane-bound receptors and to alter its movement towards or away from an attractant or repellent, respectively (Budrene and Berg, 1995). There are several types of motility and movement patterns associated with chemotaxis. Change in direction is accomplished using one of several types of flagellar movement. One type of movement is called "run and tumble", whereby a bacterium swims quickly and smoothly in one direction, then "tumbles" by separating its flagellar bundle, allowing the bacterium to quickly assess its environment and move towards a desired location and away from an undesirable one (Berg, 1996). This type of swimming pattern has been best documented in *E. coli*, the first organism for which this behavior was observed (Berg and Brown, 1972). Change in direction can also be characterized by "run and stop" or "run and slow" patterns, as seen in the alphaproteobacterium *Rhizobium leguminosarum* bv. *viciae* (Miller *et al.*, 2007; Johansen *et al.*, 2002).

In most bacteria, chemotaxis is mediated by a specific suite of dedicated proteins; three of which appear to be universally important. CheA, a sensor histidine kinase undergoes autophosphorylation after sensing changes from a chemosensory transmembrane protein, CheY. CheY competes with CheB, a protein that functions as a methylesterase, for the CheA phosphoryl group to control flagellar motor switching, and CheB that controls the adaptation of the chemosensors. (Wadhams and Armitage, 2004). Another imperative set of proteins are the MCPs, or methyl-accepting chemosensory proteins. MCPs are the proteins that receive the chemical sensor and communicate with CheA to induce autophosphorylation while CheB acts to control the adaptation of the MCPs. The CheW protein is also important as it transduces the signal from the MCPs to CheA (Wadhams and Armitage, 2004). While the *che* genes are often found in close

proximity in the genome, MCP genes are often spread throughout the genome.

Bacterial chemotaxis has been documented in several marine species, including *Vibrio fisheri* and *Rhodobacter sphaeroides (*DeLoney-Marino *et al., 2003;* Armitage and Schmitt, 1997*).* Chemotaxis has only been characterized in one roseobacter strain: *Silicibacter* sp. TM1040. This strain has been shown to be chemotactic towards exudate from the *Pfiesteria* dinoflagellate from which it was isolated (Miller *et al.*, 2004).

Biofilm Formation/Surface Attachment

Bacteria are exposed to a variety of surface types in the marine environment. An important aspect of bacterial behavior is the ability to attach to and colonize these surfaces. A biofilm is a collection of adhered cells and their products at a surface (Characklis and Cooksey, 1983). Bacterial biofilms have been compared to multi-cellular organisms due to their high level of organization and ability to divide functions amongst the cells in the community (Stoodley *et al.*, 2002). These structures have a degree of cellular specialization and differentiation that is not observed in planktonic cultures (Stoodley *et al.*, 2002).

Biofilm formation and many of the factors involved in surface attachment have been extensively studied in many diverse microbes, including the non-marine, opportunistic pathogen *Pseudomonas aeruginosa*. It has been shown in *P. aeruginosa* that the genes for flagellar synthesis are essential for surface attachment and biofilm development (O'Toole and Kolter, 1998). In addition, genes coding for proteins involved in the synthesis of type IV pili that are responsible for twitching motility, are also essential for development of a mature, developed biofilm in this organism (Heydorn *et al.*, 2002; O'Toole and Kolter, 1998). Finally, it has been demonstrated that quorum sensing is necessary for cell differentiation to occur during biofilm maturation in *Pseudomonas aeruginosa* (Davies *et al.*, 1998). These findings suggest that biofilm formation is a complex process that invokes a number of cellular processes.

Surface characteristics influence bacterial attachment. Considering the range of substrata with varying surface properties that are present in the marine environment it is interesting, though maybe not surprising, to find that there are differences in preference for adherence by bacteria (Liu *et al.*, 2004; Wiencek and Fletcher, 1995; Fletcher and

Loeb, 1979) . Many experiments performed over a number of years show that surface properties affect the ability of bacteria to attach to and colonize particular surfaces by either delaying the onset of biofilm formation, accelerating colonization or accelerating desorption of cells from the surface (Liu *et al.*, 2004; Dang and Lovell, 2002b; Wiencek and Fletcher, 1995; Fletcher and Loeb, 1979; Dexter *et al.*, 1975). This has many ecological implications for how bacteria interact with both biotic and abiotic surfaces in their environment.

Prior studies suggest surface attachment/biofilm formation may be a common feature of Roseobacter clade members. Dang and Lovell (2002b) used culture-independent approaches to examine bacterial colonization on substrates with various surface properties, including hydrophilicity, hydrophobicity, net surface charge, varying surface free energy and differing surface tension in the coastal salt marshes of the southeastern U.S. (Dang and Lovell, 2002b). Roseobacter species were found to be the dominant primary colonizers (24 -72 hrs) of a variety of these surface types (Dang and Lovell, 2002a; Dang and Lovell, 2000). However, the colonization of these surfaces after 72 hrs has not been evaluated.

Furthermore, studies of several cultured Roseobacter strains have demonstrated attachment and biofilm formation. *Roseobacter gallaeciensis* has been shown to aggressively colonize the surface of the marine alga *Ulva australis*; this strain can disperse established colonization of *Pseudoalteromonas tunicata* (Rao *et al.*, 2006; Rao *et al.*, 2005). Under specific growth conditions, Phaeobacter 27-4 can produce rosette structures and form mature biofilms on a glass surface (Bruhn *et al.*, 2006). Finally, Roseobacter isolate T5 aggressively colonizes marine agar particles by displacing organisms that have already colonized the particles in mesocosm studies (Grossart *et al.*, 2003). Specifically, strain T5 not only colonizes suspended agar particles but is also able to reduce the colonization rate of other strains on the same agar particle. Collectively, these results suggest surface colonization may be a distinguishing feature of the clade.

Another potentially important aspect of biofilm development is the ability of bacteria to communicate with one another. In order to begin forming a structured biofilm, many bacterial species must be able to signal to each other that there is adequate

cell density to initiate colonization (Hammer and Bassler, 2003; Davies *et al.*, 1998). This process is known as quorum sensing and utilizes a chemical signal. There are several different types of chemical signals including acyl homoserine lactones, modified oligopeptides, *Pseudomonas* quinolone signal (PQS) and autoinducer-2 (AI-2) (Camilli and Bassler, 2006). Quorum sensing systems based on N-acyl homoserine lactones (AHLs), found exclusively in gram-negative bacteria, are arguably the best studied density-dependent communication mechanisms found in bacteria. In many well-characterized AHL-producing bacteria, quorum sensing is mediated by the LuxR and LuxI proteins. LuxI proteins, generally referred to as AHL synthetases, are the biosynthetic enzymes responsible for production of the quorum sensing chemicals, often referred to as autoinducers. These autoinducer molecules are signaling molecules and have been found to be necessary for a variety of physiological responses, including biofilm formation, in some bacterial strains (Fuqua *et al.*, 1994). LuxR proteins are transcriptional regulators that mediate luxI expression in a positive feedback manner. Low levels of the AHL molecule bind to LuxR, this stimulates production of LuxI and results in increased production of the autoinducer (Engebrecht and Silverman, 1984). The chemical structure of these AHLs vary among species and can range from having no additional functional groups to many additional side-chains. Subtle chemical variations allow bacteria to distinguish between signals coming from other bacterial species (Camilli and Bassler, 2006). Bacteria may also be able to sense AHL-s given off by other bacterial species which can aid in the development of mixed-species biofilms and allow one bacterial species to sense the density of another species (Keller and Surette, 2006).

Quorum sensing was first genetically described in the marine bacterium *Vibrio fisheri*. In this organism, all *lux* genes, which include the quorum sensing genes luxRI as well as the genes required for light generation, are contained in a single operon (Engebrecht and Silverman, 1984). However, subsequent investigations of quorum sensing in other bacteria suggests it is more common that genes encoding pathways regulated by this cell density-dependent mechanism are located in genetic loci distinct from the luxR/luxI genes, often making identification of regulated genes and pathways using sequence analysis alone difficult. Furthermore, multiple quorum sensing systems

have been identified in many phylogenetically diverse microbes. For example, *Serratia marcescens* MG1 has been observed to invoke two different quorum sensing systems depending on the surface type to which it is attaching (Labbate *et al.*, 2007).

Genomic analysis of representative Roseobacter strains, suggests quorum sensing mechanisms are broadly distributed among clade members. Of the 23 sequenced Roseobacter strains currently available, 15 possess *luxI/luxR* family genes and five appear to have two sets of *luxI/luxR* family genes (Buchan unpub.). The literature contains few reports exploring quorum sensing among clade members. For instance, Gram and colleagues (2002) demonstrated AHL production by 60% of Roseobacter strains isolated from marine snow. Recently it has been found that 55% of isolates from the marine sponges *M. laxissima* and *I. strobilina* were AHL producing Roseobacter strains (Mohamed *et al*., 2007). While Wagner-Dobler et al. (2005) demonstrated that Roseobacter AHLs possess some of the longest acyl side chains characterized to date (i.e. C_8 to C_{18}). Relatively common among a-proteobacteria, long chain AHLs are hydrophobic in nature, often causing them to partition in cell membranes (Schafer et al., 2002), the biological implications of which are not yet fully appreciated (Wagner-Dobler *et al.*, 2005).

Research Objectives

While Roseobacter abundances and diversity in various marine habitats are well appreciated, we are just now beginning to appreciate the underlying basis of why this group is so successful. There are many lines of evidence that suggest motility, chemotaxis, and surface attachment are important features in defining the ecological success of the Roseobacter clade. Much of this evidence is derived from a limited number of studies that have focused on a few representative strains. These activities have yet to be properly characterized for a collection of phylogenetically distinct clade members cultivated from distinct marine habitats. This thesis seeks to fill that knowledge gap by characterizing a group of 13 Roseobacter strains with a variety of degrees of relatedness (as determined by 16S rDNA sequence similarity) ranging from strains which have identical 16S rDNA sequences to far more diverse strains from several genera

(Figure 1) that represent diverse marine environments. The specific objectives of this thesis are to:

- Characterize motility among members of this group
- Characterize surface attachment on a variety of surfaces
- Examine the representative genes encoding for flagellar assembly and chemotaxis

METHODS

Strains

Thirteen different strains belonging to the Roseobacter clade were characterized in this thesis (Table 1, all figures and tables found in Appendix A). These strains were isolated from several distinct marine environments. Eight of the strains were isolated from the Georgia coast; five from coastal seawater, two from decaying salt marsh grass (*Spartina alterniflora*) and one from a marine fungal culture. The remaining strains were isolated from the surface waters of the Caribbean Sea, North Atlantic Ocean, the Black Sea or the Blue Lagoon (geothermal lake) or from the phycosphere of a *Pfiesteria*-like dinoflagellate from the Chesapeake Bay. Strains *Silicibacter pomeroyi* DSS-3 and *Roseovarius nubinhibens* ISM were isolated via enrichment cultures from seawater samples. DSS-3 was isolated from sea-water enriched with dimethylsulfoniopropionate (DMSP) and ISM was isolated using an enriched sea-water medium (peptone, casamino acids and 80% sea water) (Gonzalez *et al.*, 2003). Except where noted, all strains were routinely grown on YTSS [per liter: 2.5g yeast extract, 4g tryptone, 15g sea salts (Sigma-Aldrich, St. Louis, MO)] at 30^{o}C, with agitation.

Motility Assays

All motility assays were performed on semi-solid agar (0.35%) with both complex and minimal media. One-tenth YTSS (complex) media consisted of 0.25 g yeast extract, 0.4 g tryptone, 15 g sea salts (Sigma-Aldrich), and 3.5 g purified agar (Sigma-Aldrich) per liter. Per liter, *Silicibacter* basal media (SBM, minimal) consisted of 50 ml 1M $MgSO_4$, 50 ml 4M NaCl, 50 ml 1M Tris-HCl (pH7.5), 20 ml 0.5M NH_4Cl, 50 ml 1.36mM Fe-EDTA (Sigma-Aldrich), 20 ml 50mM K_2HPO_4, 50 ml 0.2M $CaCl_2$, 50 ml 0.2M KCl (Mallinckrodt, Paris, KY), 1 ml trace metals solution (Henrickson and Whitman, personal communication), 2 ml vitamin mix (Gonzalez *et al.*, 1997), and 3.5 g purified agar. The SBM medium was supplemented with either 10mM sodium acetate or glycerol (for *Sulfitobacter pontiacus* cultures) as a carbon source. Strains were initially grown in liquid medium for at least two transfers prior to inoculation of the semi-solid agar plates with 15 ul of a stationary phase culture at the center of the plate. Plates were

placed in plastic tubs, lined with damp paper towels to retain moisture, and placed at 30^{o}C. Distance migrated from the point of inoculation was measured every 24 hours for three days. Strains were successively transferred onto fresh semi-solid agar plates with 15 ul of cells from the leading edge of growth from the previous plate. This process was repeated three times and in triplicate for each strain.

Growth Curves

Growth rates were determined for all 13 strains in both YTSS and SBM + 10 mM acetate or glycerol (*S. pontiacus* only) media (Table 2). Growth curves were performed in triplicate for each strain. Three single colonies were selected for each strain and grown initially in liquid media until cells reached late logarithmic or stationary phase (~15 hrs). Cells were diluted 100-fold in fresh media and turbidity measured at 540 nm using a Spectronic Genesys20 (Thermo Fisher Scientific, Waltham, MA) throughout the course of the growth curve. In addition, viable cell counts on YTSS were performed at each time point. Culture density and viable plate counts were determined for all strain in both media types every 0.5-6 hrs for at least 30 hrs.

Morphological Characterizations

Transmission electron micrographs (TEMs) were performed using a Hitachi H-800 (Tokyo, Japan) transmission electron microscope at the University of Tennessee's Microscopy Facility. Preparations for motile strains were obtained using cells collected directly from motility plates. Cells (50 ul) embedded in semi-solid agar were diluted in 500 ul of a 1 mM Tris-HCl pH 7.5 and 1% Sea Salts solution. Samples were absorbed onto a 400 mesh copper grids with a collodion and carbon coating and were freshly glow-discharged before use (Electron Microscopy Sciences, West Chester, PA) and stained with 0.75% uranyl formate. Non-motile strains were analyzed directly from liquid cultures grown in YTSS. In brief, cells from 1 ml of late logarithmic phase culture were collected by centrifugation (6,000 for 2 min) and suspended in 500 ul of the Tris-HCl/sea salt solution and subsequent processing was performed in the same manner described for the motile strains.

Unfixed cultures were also visualized using phase contrast microscopy with a Nikon Eclipse TE200-U (Tokyo, Japan). Cultures were grown under each of the following conditions: YTSS liquid, shaking; YTSS liquid, static, SBM liquid, shaking, 1/10 YTSS motility agar and SBM motility agar + 10 mM acetate or glycerol. Strains were analyzed for motility, cellular morphology, and aggregation.

Identification of Genes Involved in Motility and Chemotaxis

Degenerate primer sets were developed for two flagellar genes, *fliF* and *flgH*. In order to identify regions of conservation that would be suitable for degenerate oligonucleotides, putative *fliF* and *flgH* protein sequences were aligned from Roseobacter strains and several closely related strains (Tables 3 & 4). A degenerate primer set (P4P5) targeting the flagellar chemotaxis gene *cheA* was graciously provided by Dr. Gladys Alexandre-Jouline and Burnette Crombie. The expected size of the *fliF*, *flgH* and *cheA* are ca. 756bp, ca. 1032bp and ca. 440bp, respectively. The sequences for all primer sets are shown in Table 5.

Attachment Assays

Strains were analyzed for their ability to attach to surfaces with different properties; namely, polystyrene, polyproylene, polyvinyl chloride, polycarbonate, polyethyene terphthalate, Teflon^{TM} and glass. Polystyrene (Corning, Corning, NY), polypropylene (Abgene, Surrey, UK) and poly-vinyl chloride (Falcon, Franklin Lakes, NJ) plastics were in a 96-well microtiter dish format (370 ul, 370 ul and 250 ul wells, respectively). All assays were performed in triplicate and with cultures grown in both YTSS and SBM + 10 mM acetate or glycerol. Assay procedures for polystyrene, polypropylene and poly-vinyl chloride were slightly modified from those outlined in O'Toole & Kolter (1998). One hundred ul of a 10^6 colony forming units (CFU)/ml culture was added per well to 8 wells and plates were incubated for 13 hours at 30^{o}C. After incubation, 25 ul crystal violet was added to each well, incubated at room temperature for 15 min and was rinsed out with deionized water. Subsequently, 125ul of 95% ethanol was added to each well and allowed to solubilize for 1 hr at room temperature. Optical densities at 600 nm were read with a BioTek Synergy HT-1 plate

reader (BioTek, Winooski, VT). Additional assays with shorter growth periods (5 min, 1 min and 30 sec) were conducted on polystyrene with cellular attachment quantified using the crystal violet assay.

The remaining plastic types (polyethylene terphthalate, polycarbonate, Teflon^{TM}, and glass) were in strip or slide form. All plastic types were ordered from McMaster-Carr Supply Company (Atlanta, GA) and were cut into 2.5 cm x 4 cm strips. Glass microscope slides were obtained from VWR (Bridgeport, NJ). The assay involved aliquoting 15ul of a 10^6 CFU/ml YTSS-grown culture into sterile, 50ml conical tubes (Corning, Corning, NY) adding sterile strips of plastic or glass, and incubating cultures at 30^{o}C for 13 hrs. Following incubation, 3.75 ml of crystal violet was added to each tube and incubated at room temperature for 15 min. Each strip was then removed and gently rinsed with deionized water until all excess crystal violet was removed and only stained attached cellular material remained. Solubilization was perfomed in 13 mm petri dishes (Thermo Fisher Scientific) with 4 ml of 95% ethanol and the optical density at 600nm of 1 ml of solubilized crystal violet was determined using a Spectronic Genesys 20 (Thermo Fisher Scientific).

Hydrophobicity and hydrophilicity of the surfaces were determined by contact angle measurement of each substrate with a Rani-Hart model 100-00 goniometer (Rani-Hart, Inc., Mountain Lakes, NJ). Purified water was used to measure a sessile drop contact angle with each substrate.

All statistical analyses on surface attachment data were performed using the SPSS 15.0 statistical package (SPSS Inc, Chicago, IL). All data sets were initially tested for equal variance using a homogeneity test and Welch's test which also determines the equality of means. If the data was not found to possess equal variances, Dunnett's T3 test was then used to analyze the data set. This test does not assume data with equal variances and is a pairwise comparisons test based on the Studentized maximum modulus. For data sets with equal variances one-way ANOVA analyses were performed. This analysis is robust to departures from normality, but data must be symmetric and it assumes equal variances of data. Once differences were found to exist between the means, Tukey's honestly significant difference (HSD) test was performed to determine

which means differ from one another. Tukey's HSD test is a multiple comparison test which assumes equal variances of the data.

Glass Attachment

Attachment to glass was observed at the cellular level by filling 50ml conical centrifuge tubes (Corning) with 25 ml of 10^6 CFU/ml (shaking) culture, adding a sterile glass slide to the tube, and incubating the cultures on the benchtop (22^oC) for 4, 8, and 24 hr. At each time point the glass slide was removed and observed using phase contrast microscopy with a Nikon Eclipse TE200-U.

Genome Analysis

Representative Roseobacter genome sequences were analyzed using the Integrated Microbial genomes (IMG) system (http://img.jgi.doe.gov). Gene diagrams were constructed using gene designations provided. Nucleic acid and protein sequences were aligned using ClustalW software and edited using the SeaView program. All phylogenetic trees were constructed using Mega4 software with the neighbor joining method including 1000 bootstrap iterations. All tree distances represent probable evolutionary distance (*p*-distance).

RESULTS

The thirteen Roseobacter strains examined in this study were chosen to represent the diversity of the various subgroups within the Roseobacter clade, as defined in Buchan *et al*. (2005). These strains represent a variety of the metabolic capabilities and phenotypic characteristics that are commonly ascribed to members of this lineage (Table 1). This group represents a variety of relatedness levels within this group of organisms (Figure 1).

Motility

The thirteen Roseobacter strains (Table 1) were tested on both complex and minimal media motility plates in order to determine the ability of each strain to swim through semi-solid agar. Under tested conditions, strains ISM, DSS-3, E-37, SE62, PSPC2 and S. lac were non-motile (data not shown). Strains TM1040, Y3F, Y4I, EE-36, NAS-14-1, S. pont and SE45 demonstrated various levels of proficiency at moving through the motility plates (Figure 2 a & b). Growth on complex medium (1/10 YTSS) showed strains TM1040, Y3F and Y4I increased in movement from the site of inoculation upon subsequent transfer. This is common when cells have been removed from the motile front and used to inoculate a new plate. This is indicative of the ability of these strains to adapt to their environment and has been documented in other bacteria (Kearns and Losick, 2003). In contrast, strains SE45, EE-36 and NAS-14-1 remained consistent in their movement from the site of inoculation (Figure 2a). Growth on minimal (SBM) medium showed increased movement from the inoculation site upon transfer for strains TM1040, Y3F, Y4I and SE45 (Figure 2b). As with the complex media, strains EE-36, NAS-14-1, and S. pont did not increase in movement from the site of inoculation upon subsequent transfer.

Microscopic Characterization (Phase Contrast & TEM)

Microscopic analysis was performed using phase contrast microscopy on all strains and using transmission electron microscopy (TEM) for strains that have not been previously characterized in published reports (Table 1) to determine features commonly

associated with cell-directed movement. Several growth conditions were used to identify potential morphological differences that may come to light as a consequence of substrate type and level of agitation. Each strain was grown under static and shaking conditions in YTSS broth and under shaking conditions in SBM broth and then viewed using phase contrast microscopy (Table 6, Figures 3-4). Both Y4I and Y3F were found to be motile in both shaking and statically grown cultures. Motility was evident in SBM shaking broth cultures of strains SE45, EE-36 and NAS-14-1. Strain TM1040 was only motile in shaken YTSS broth cultures. Strains Y4I, Y3F, SE45, EE-36, NAS-14-1, S. pont and TM1040 were found to have motile cells on motility plates of both media types. TEMs were performed on all motile strains not previously characterized and all were found to have one flagellum or multiple flagella when isolated from motility agar, except SE45 for which flagella were not readily apparent (Figure 5a, b, e, g, h, j). TEMs were also performed on non-motile representatives of this collection (Figure 5c, d, f, i).

The 13 Roseobacter strains analyzed demonstrated a variety of cellular morphologies when viewed by phase contrast and TEM (Figures 3-5). Long, rod-shaped cells were visualized for strains ISM, SE62 and S. lac. Strains PSPC2, S. pont, Y4I, SE45, NAS-14-1 and EE-36 were small, ovoid cells, while strain Y3F has a slightly more elongated shape. Strains Y4I, Y3F, S. pont and SE45 were also commonly found in a doublet or dumbbell formation. It is not clear whether this is a true morphological type or is simply an indication of dividing cells. However, previous studies involving Roseobacter species S. pont have observed the dumbbell-shaped bacteria, also termed “matreshkas” due to the polarity typically evident in these structures (Sorokin, 1995).

Rosette formation has been reported for a number of Roseobacter strains and is often more prevalent in cultures grown under static conditions (Bruhn *et al.*, 2005; Ruger and Hofle, 1992). Six of the 13 strains formed rosettes under the tested conditions. Strains Y4I, TM1040, ISM, E-37 and S. pont were all found to form rosettes under shaking SBM culture conditions (Figure 4). Y4I, Y3F, TM1040, DSS-3, NAS-14-1, S. pont and E-37 were also found to form rosettes under shaking YTSS liquid culture conditions (Figure 3).

Surface Attachment Assays

As mentioned earlier, many bacteria are capable of attaching to and forming biofilms on a wide variety of biotic and abiotic surfaces. As some Roseobacter species have been demonstrated to adhere to several types of biotic surfaces (Rao *et al.*, 2006; Grossart *et al.*, 2003), we sought to determine the ability of this set of Roseobacter strains to attach to surfaces as this may be an indication that these strains can establish productive biofilms. Seven substrates with varying surface properties were selected for analysis: Teflon^{TM}, polycarbonate, polyethylene terephthalate (PET), glass, polyvinyl chloride, polystyrene and polypropylene. These substrates have varying degrees of hydrophobicity as measured by their sessile drop contact angles with purified water (Table 7). Glass is the most hydrophilic and Teflon^{TM} the most hydrophobic; the remaining surfaces fall within a small range between these two. Due to an uneven surface, we were unable to make a measurement of polystyrene and obtained contact angle measurements of hydrophobicity from the literature. None of the substrates are known to have a net surface charge. Relative adherence of cellular material was measured using a crystal violet assay. Due to variation in growth rates among the strains, comparisons were drawn between strains with similar growth rates and those with a high degree of relatedness, as determined by 16S rDNA sequence analysis.

The collection of strains demonstrated various degrees of colonization on all surface types (Figures 6 a-c, 7 a-d, 8a-c). As commonly reported for bacteria in general (Fletcher and Loeb, 1979), colonization by the Roseobacter strains was greatest on the most hydrophobic surfaces. A statistical analysis of all substrates, using strain type, media type and whether the substrate format was a strip or a plate as covariates, shows statistical significance of all factors in this experimental design, including substrate type ($p = 0.001$, *D*= Dunnett's T3 test). Not surprisingly, the strain type, media type and format of the substrate results in significant differences in the degree of surface attachment.

Surface attachment in microtiter dishes was performed with both complex and minimal media to draw comparisons between relatively nutrient-rich and nutrient-limited environments. For most strains, surface attachment in minimal medium was reduced

compared to the complex medium (Figure 8a-c). This is not surprising given the decreased growth rates, and thus lower amount of biomass, in minimal compared to complex media that was found for most strains (Table 2). However, in comparison to the other strains, SE45 demonstrated the opposite response in the two media tested. This strain did not strongly attach to surfaces in complex medium but in minimal medium formed the strongest attachment in comparison to the rest of the strain collection grown on similar media ($p = <0.042$, *D*). Interestingly, SE45 is one of the few strains that had comparable growth rates in complex and minimal media (Table 2). An analysis of the remaining 12 strains on all substrate types in minimal media, revealed that Y4I and Y3F were all statistically similar in their surface attachment as determined by the crystal violet assay, while being significantly different from the remaining strains ($p = <0.030$, *D*).

In all cases where the two media types were tested (i.e. microtiter dishes and strips), all strains formed more extensive surface attachments in complex rather than in minimal medium (Figure 6a-c). However, both PSPC2 and ISM developed relatively poor surface attachments in complex medium in comparison to the rest of the strain collection and showed significantly less surface attachment than 9 of the 12 remaining strains ($p = <0.023$, *D*). For PSPC2, this is not surprising given the relatively slow growth rate of this strain (Table 2). However, as ISM has a growth rate comparable to many of the other strains this difference may hold more significance. Strains Y4I, TM1040 and EE-36 formed substantial surface attachments and were significantly greater in attachment from the seven of the remaining Roseobacter strains ($p = <0.040$, *D*). These four strains represent a fairly broad range in terms of their doubling times (i.e. 79 to 95 min).

Statistical analysis of all three plastic microtiter dish types shows there is a significant difference between the ability of strains attachment to polystyrene and polyvinyl chloride substrates ($p = 0.049$, *D*), with greater surface attachment observed on polystyrene. These plastic surfaces both have no net surface charge and polystyrene is slightly more hydrophobic than polyvinyl chloride.

Surface attachment to plastic and glass strips was tested with cells grown in complex medium (Figure 7a-d). Among the strip types there was a significant difference

between Teflon™ and glass (p = 0.05, *D*) and between PET and glass (p = 0.024, *D*). This is not surprising given the differences between these two surfaces. Glass is very hydrophilic with a contact angle measurement of $<10^{o}$ and Teflon™ is very hydrophobic with a contact angle measurement of 94.5^{o}. For most strains, no significant differences were found for a given isolate on the suite of substrates tested. The exceptions were PSPC2 and ISM. For strain PSPC2 there is a difference between glass and polystyrene (p = 0.018, *D*) polypropylene and glass (p = 0.003, *D*) and polycarbonate and glass (p = 0.016, *D*). Strain ISM demonstrated a difference between polypropylene and Teflon™ (p = 0.043, *D*), polycarbonate and PET (p = 0.038, *D*), Teflon™ and glass (p = 0.021, *D*) and Teflon™ and PET (p = 0.012, *D*).

In addition to the collection of 13 strains, three additional strains, transposon mutants of either TM1040 or Y4I, were assayed to explore the question of whether motility and/or quorum sensing is fundamental to surface attachment. TM2014 is a non-motile mutant of TM1040 with a Tn5 transposon insertion in the *flaA* gene (Miller and Belas, 2006). Y403BE8 is a motility-deficient mutant of Y4I with a Tn5 insertion in the phosphoadenosine phosphosulfate reductase gene and Y402AE5 is a quorum-sensing impaired mutant with a Tn5 transposon insertion in a *luxR* gene (Buchan, unpub.). Y403BE8 and Y402AE5 did not have statistically significant differences in surface attachment compared to wildtype Y4I when grown in complex medium (p = 1.000, *D*) but strain Y402AE5 (*luxR*$^{-}$ mutant) differed significantly from Y4I when grown on minimal medium (p = 0.013, *D*). Additionally, strain TM2014 showed diminished surface attachment to the microtiter dishes when grown in complex medium and an increased surface attachment to glass when compared to TM1040, however this was not found to be significant. Similarly, Y403BE8 demonstrated a diminished attachment to polystyrene relative to the wildtype strain but this was not significant.

Comparison of the motility-impaired mutants revealed significant differences between substrate types within a strain. It is important to note that the wild-type strain of the motility-deficient mutants did not have significant differences on the various substrates for a given media type (complex). With seven substrates, 21 pairwise comparisons can be drawn for a given strain. For TM2014 there was a difference

between polycarbonate and Teflon™ (p = 0.032, *T* = Tukey's HSD test), polycarbonate and glass (p = 0.004, *T*), polystyrene and glass (p = 0.001, *T*) and polypropylene and glass (p = 0.001, *T*). Y403BE8 had substantial significant differences with 16 of the pairwise comparisons, the five exceptions that did not show significant differences were polyvinyl chloride and polystyrene, polystyrene and polypropylene, polycarbonate and Teflon™, polycarbonate and polyethylene terphthalate, and Teflon™ and polyethylene terephthalate.

Initial Attachment Assays

To determine if strains differ in the earliest stages of attachment on polystyrene at 5 min, 1 min, and 30 sec was tested (Figure 9a-c). Interestingly, there were several strains with differences between the time points within the strain. Strains TM2014, S. lac and NAS14-1 had significant differences between at least two of the time points. TM2014 has a difference between the 1 min and 5 min time point (p=0.009, *T*) and the 30 sec and 5 min time point (p=0.010, *T*). A difference was observed for S. lac between the 1 min time point and the 5 min time point (p=0.030, *T*). Strain NAS14-1 demonstrated a difference between the 5 min and 30 sec time point (p=0.033, *T*). There were no differences between the mutant strains and their wild-type counterparts.

Glass Attachment

Surface attachment on glass slides was viewed by phase microscopy in order to characterize cellular arrangements as a result of attachment to, and growth on, a surface. Strains S.lac, SE45, SE62, ISM, DSS3, PSPC2 and E-37 did not visibly attach to the glass slide during the incubation period. The remaining six strains did attach to the glass surface and demonstrated different cellular organizations. Viewing cells at different stages of surface attachment suggests that initial attachment is similar among the strains and occurs via one pole followed by settling of the cells along a longitudinal axis (Figure 10). The greatest density of attached cells occurs at the air-liquid interface. Y4I attached to the glass slide in aggregates or rosettes (Figure 10a). Y3F attached in long chains along the slide surface (Figure 10b). S. pont, NAS14-1 and EE36 all attached as single

cells along the slide surface (Figure 10c, d & e). TM1040 also attached to the slide surface in mostly rosette shapes (Figure 10f).

Gene Analysis

Eighteen publicly available Roseobacter genome sequences were analyzed to identify genes related to motility and chemotaxis. Of these 18 genomes, four have been closed (*Jannaschia* sp. CCS1, *Roseobacter denitrificans* OCh114, *Silicibacter pomeroyi* DSS-3 and *Silicibacter* sp.TM1040) while the remaining genomes are available as draft assemblages. Of the seven strains in our collection with sequenced genomes, two are closed (TM1040 and DSS-3) and five are draft assemblages (Y4I, ISM, E-37, EE-36 and NAS14-1). Efforts were focused on four genes, *flgH*, *fliF*, *cheA* and *luxI* that are important for flagellar assembly, chemotaxis or quorum sensing, respectively. The *flgH* gene encodes for the flagellar p-ring protein, the *fliF* gene encodes for the flagellar m-ring protein, the *cheA* gene encodes for a flagellar chemotaxis histidine kinase protein, and the *luxI* gene encodes a synthetase required for AHL production. The ring proteins encoded by *flgH* and *fliF* are essential in building a functional flagellum. The chemotaxis histidine kinase protein CheA is necessary to establish flagellar chemotaxis movement, while quorum sensing is dependent upon production of AHLs via the *luxI* gene product.

To explore the genetic potential of the six strains for which genome sequences are not currently available (Y3F, S. pont, S. lac, PSPC-2, SE62 and SE45), degenerate primers targeting the *fliF*, *flgH* and *cheA* genes were employed (Table 3, 4 & 5). The primer sets targeting *fliF* and *flgH* appear to be fairly non-specific as many non-target products were routinely amplified (Figure 11). However, products of the expected size were gel excised and directly sequenced. Sequences were analyzed by homology searches (tBLASTx). The FliF primer set amplified the expected product from strains SE45 and S. pont (Table 8). The PCR product derived from SE45 showed greatest sequence similarity to *fliF* from *Roseovarius* sp. HTCC2601 and the *Sulfitobacter pontiacus* sequence with both *Sulfitobacter* sp.EE-36 and *Sulfitobacter* sp.NAS-14-1. The FlgH primer set amplified product from both S. lac and Y3F (Table 8). The *Silicibacter lacuscaerulensis* sequence had greatest homology to *flgH* from *Silicibacter pomeroyi* DSS3 as did the sequence from Y3F.

The P4P5 primer set that targets *cheA* yielded fewer non-specific amplicons and products of the expected size were obtained for five of the six strains tested: Y3F, S. lac, S. pont, SE45 and SE62 (Table 8). Sequence analysis of these products revealed that *cheA* from SE45, S. lac and S. pont show greatest sequence homology to *cheA* from *Roseovarius* sp. HTCC2601, while the genes from Y3F and SE62 have greatest homology to *cheA* from *Silicibacter* sp. TM1040 and *Loktanella vestfoldensis* SKA53, respectively.

To compare the relatedness of the genes, phylogenetic trees were constructed using the protein sequences (Figures 12-15). The phylogeny of sequences obtained using the degenerate primer sets were also examined, however, as these sequence are much shorter the comparisons are not as strong and will not be discussed here (Appendix Figures 1-3). After reviewing the trees comparing the FlgH, FliF and CheA protein sequences, several commonalities become apparent (Figures 12, 13 & 14). *Silicibacter* sp. TM1040 and *Roseobacter* sp. SK209-6-2, *Silicibacter pomeroyi* DSS-3 and *Roseobacter denitrificans* OCh114, *Sagitulla stellata* E-37 and *Roseovarius* sp. HTCC2601, *Oceanicola batensis* HTCC2597 and *Oceanicola granulosus* HTCC2516, and *Sulfitobacter* sp. EE-36 and *Sulfitobacter* sp.NAS-14-1 were all found close together on the three trees. The gene content and orientation surrounding the *flgH* and *fliF* genes were nearly exactly the same for all of the above listed pairings (Figures 16 & 17). Interestingly, strains EE-36 and NAS-14-1 which have identical 16S rRNA sequences, show remarkable variation in the genes flanking both the *flgH* and *fliF* genes, while strains which are more distinct at the 16S rRNA level, such as DSS-3 and *R. denitrificans*, are identical in gene composition and orientation surrounding both genes. The *cheA* gene was not found in strains DSS-3, EE-36, NAS-14-1 and *O. batensis* so comparison for these strains is not possible. However, for TM1040 and SK209-6-2 the *cheA* operon shows more diversity between these two strains suggesting a lack of synteny where the chemotaxis genes are involved (Figure 18). The *cheA* operons of E-37 and HTCC2601 are nearly identical. The LuxI tree also showed interesting groupings among the strains, particularly due to the five strains that possessed two *luxR-luxI* genes. For the strains with two *luxR-luxI* gene sets, the protein sequences are usually distant from one

another in the tree (DSS-3, Y4I, *Dinoroseobacter shibae*, *Roseovarius* sp.217 and *Roseobacter* sp.SK209-2-6) (Figure 15). Analysis of the gene synteny between strains reveals a tremendous amount of variety regarding genes flanking the *luxR-luxI* operon (Figure 19).

DISCUSSION

This thesis sought to characterize the phenotypic and genotypic capabilities of 13 members of the Roseobacter clade with regards to motility, chemotaxis and surface attachment. Prior to this work, little was known of the relationship between motility and surface attachment in members of the Roseobacter clade and studies comparing a range of phylogenetically distinct strains was lacking. Previous studies have characterized motility, chemotaxis and surface attachment/biofilm formation in select members of the clade and made inferences to the broad distribution of these phenotypes among lineage members (Miller and Belas 2004; Miller and Belas 2005, Wagner-Dobler and Biebl, 2006). However, this study reveals a significant amount of variation in these phenotypes among a select group of isolates and emphasizes the difficulty of making generalized conclusions regarding these traits in all lineage members. The heterogeneity revealed in this study mirrors findings from a recent genome analysis of a specific set of functional genes expected to be important in the biogeochemical cycling of carbon and sulfur in a collection of Roseobacters (Moran *et al.*, 2007). Taken together, this work suggests that few metabolisms or physiologies are universally found in all lineage members.

Motility has been demonstrated in Roseobacter clade members previously by both phenotypic characterization and/or the observation of a single or multiple polar flagella (Miller and Belas, 2006; Gonzalez *et al.*, 2003). The composition of Roseobacter flagella is poorly understood, and only a rudimentary analysis has been reported for *Silicibacter pomeroyi* DSS-3. This strain was found to possess a complex flagellum that rotates exclusively in the counter-clockwise direction. Complex flagella are found to be more rigid and have a coarse surface of grooves and ridges that serve to helically propel the bacterium. DSS-3 flagella did not react with protein antibodies from the *Alphaproteobacteria Rhizobium lupini* H13-3 and *Sinorhizobium meliloti* (Gonzalez *et al.*, 2003). Interestingly, and as discussed in more detail below, DSS-3 motility is not readily apparent for this strain under a variety of laboratory conditions. In this project we observed several previously uncharacterized motile strains and corroborated motility in strains where it has been previously observed (Table 6). Motility has been well characterized in *Silicibacter* sp. TM1040 where it has been shown to play a crucial role in

this bacterium's symbiotic relationship with a marine dinoflagellate (Miller and Belas, 2006). However, the remaining six motile strains examined in this study have not previously been characterized and these findings lead to questions as to what importance the ability to be motile has on their respective ecological niches. Differences in motility were observed between strains when grown on different media. Specifically, in minimal nutrient conditions, TM1040 and *Phaeobacter* sp. Y4I showed reduced motility relative to the complex medium, while *Phaeobacter* sp. Y3F and *Citricella* sp. SE45 demonstrated enhanced movement. These findings may suggest that under relatively nutrient replete conditions at least some Roseobacter strains may invoke motility as a mechanism to search for nutrients outside of their immediate area. This type of behavior has been described for other bacterial species, including species several marine isolates (Miller *et al.*, 2004; Mueller, 1996).

Six strains demonstrated no motility under the various conditions tested (Table 6). However, these results must be interpreted with some caution given published reports that indicate motility may be feasible by at least two of these strains. An attached flagellum has been observed in DSS-3, (Gonzalez *et al.*, 2003) and in the report characterizing *Sagitulla stellata* E-37 loose flagella were evident by transmission electron microscopy (Gonzalez *et al.*, 1997). These reports reveal that motility is rarely (DSS-3) or yet to be (E-37) observed under laboratory conditions. This may indicate that motility in these two strains, and possibly the remaining four "non-motile" strains, require special growth conditions not tested in this study. This has been seen in *E.coli,* which has been found to not produce flagella when grown under certain circumstances such as high temperature, high levels of various nutrients including carbohydrates and high salt content (Li *et al.*, 1993).

Many of the Roseobacter strains with sequenced genomes have been found to possess both motility and chemotaxis operons. Flagellar operons were found in 81% (13 of 16) of the sequenced strains and chemotaxis operons were found in 50% (8 of 16) of the sequenced strains. Interestingly, DSS-3 does not have the chemotaxis suite of genes but does possess all of the necessary flagellar genes. This leads to the question of what might entice DSS-3 to be motile? Since this strain does not appear to be performing

chemotaxis towards molecules, something else might cause this strain to be motile. One possible suggestion is that DSS-3 may be capable of performing a component of energy taxis. Energy taxis is the movement of an organism towards areas where cellular energy generation is most favorable and encompasses aerotaxis, phototaxis, chemotaxis to oxidizable substrates, redox taxis and taxis to alternative electron acceptors (Alexandre *et al.*, 2004; Alexandre *et al.*, 2000). This behavior has been observed as the dominant form of taxis in several bacteria, including *Azospirillum brasilense* (Alexandre *et al.*, 2000). No form of taxis has yet been demonstrated in DSS-3.

As mentioned earlier, Roseobacter strains can be involved in many interactions with other organisms and surfaces in their environment. The importance of motility and chemotaxis in order to establish a symbiotic relationship with a dinoflagellate has been demonstrated for TM1040 (Miller and Belas, 2006; Miller *et al.*, 2004). This correlation allows for speculation that other Roseobacter strains may be using motility and chemotaxis to interact with specific organisms or surfaces. The seven strains that demonstrated motility in this set of experiments likely utilize motile behavior to establish themselves in nutrient rich niches in the environment. This may be through interaction with another organism (such as a dinoflagellate), attachment to a surface, or searching out a transient deposit of nutrients (such as marine snow).

The formation of a flagellum and the ability to be motile has been implicated in surface attachment and biofilm development (O'Toole *et al.*, 2000; Pratt and Kolter, 1998). *Pseudomonas aeruginosa*, which forms a developed biofilm, has been found to be unable to form a biofilm when it is not able to produce a flagellum (O'Toole and Kolter, 1998). This same result has been found for *Vibrio cholerae* and *E. coli* (O'Toole *et al.*, 2000). In *E. coli*, it has been found that genes associated with flagellar and Type I pili formation are necessary for surface attachment and biofilm development (Pratt and Kolter, 1998). Importantly, no correlation between motility and surface attachment/biofilm formation in any member of the Roseobacter lineage has been established to date. Though we did not specifically address this question here, we found that all seven motile strains formed strong surface attachments. Alternatively, the six remaining non-motile strains did not form substantial surface attachments under the

conditions tested. As with the motility assays, it is feasible that surface attachment by these non-motile strains may require specific growth conditions not tested in this study.

In some bacterial species, attachment to a surface initiates production of secondary metabolites with antimicrobial capabilities that are not produced when the organism grows as planktonic cells (Liu *et al.*, 2004). The production of antimicrobial compounds upon attachment can allow strains to preferentially colonize a biotic surface or to be economically useful by protecting organisms from potentially harmful bacteria (Liu *et al.*, 2004; Westerdahl *et al.*, 1991). Previous studies have demonstrated that Roseobacter clade members can be proficient at attaching to and colonizing marine algae and marine snow particles (Grossart, Kiorboe et al. 2003; Rao, Webb et al. 2006). Many of these strains are also able to displace other colonized bacterial strains which may indicate the production of antimicrobial compounds (Rao, Webb et al. 2006). Roseobacters live in coastal ocean areas where there are many surfaces present as well as transient nutrient deposits such as marine snow. The ability of a strain to be motile and attach to a surface enables it to exploit many sources of nutrients.

Many surface types are present in marine environments. This observation paired with the information that certain bacteria will only attach to certain surface types indicates that these interactions are important for elucidating environmental niches for bacterial strains. Previous studies have shown intriguing relationships between bacteria and certain surface types. In one culture-independent study, bacteria were found to delay succession on chemically treated hydrophobic surfaces while accelerating succession on a chemically treated moderately hydrophilic surface (Dang and Lovell 2000). This study supports the idea that hydrophobicity and hydrophilicity can influence the colonization of bacterial strains. Additional tests have emphasized the importance of uniformity and creation of roughness when manufacturing or chemically treating surfaces for experimental purposes. These have been found to alter substrate wettability, causing some unexplained differences in surface attachment and biofilm assays (Wiencek and Fletcher 1995). In our surface attachment assays we chose not to chemically treat the surfaces so as to avoid the chance of impurities or non-uniformity in the surface types. Our experiments supported previous evidence that moderately hydrophobic or

hydrophilic surfaces seem to allow for the best attachment of Roseobacter cells. Polystyrene had the greatest level of surface attachment among the 96-well plates and Teflon^{TM} the highest among the strip assays. Interestingly, surface attachment was greatest for some strains on Teflon^{TM}, however, this substrate also showed the most variable response among the strains, with five strains not colonizing this surface type to any measurable degree within the incubation period (Fig. 7c). Polyethylene terephthalate had surface attachment levels reaching nearly as high as Teflon^{TM} but maintained more consistent results among all the strains; evidence of colonization was evident for nearly all strains (Fig. 7b). These results demonstrate the variability in attachment to certain surface types evident among this strain collection.

Surface attachment assays were also performed in minimal medium to provide further insight into the influence of growth conditions on certain phenotypic traits. In most cases, growth rates were slower in minimal relative to complex media (Table 2) making comparisons between the two media types difficult. However, one strain that stood out in the minimal medium attachment assay was SE45. This strain formed the most significant surface attachment in minimal medium and was statistically significant from all other strains ($p=<0.042$, *D*). SE45 also was most motile when grown on minimal medium motility plates. The growth rates of this strain on minimal and complex media (87 and 80 min, respectively) are comparable and suggests this may be one of many variables that contributes to colonization success.

The three mutants included in these assays, TM2014 (*flaA*$^{-}$, non-motile mutant of TM1040), Y402AE5 (*luxR*- mutant of Y4I) and Y403BE8 (PAP reductase, non-motile mutant of Y4I) formed surface attachments indistinguishable from their wildtype counterparts in complex medium. The FlaA mutation in TM2014 is known to inhibit motility (Miller and Belas, 2006), but it was not known whether it hindered flagellar production. In a TEM analysis performed during this study, TM2014 was found to possess flagella in number and orientation similar to wildtype (Appendix Figure 4). Flagella are thought to aid in surface attachment and surface attachment, thus, it is feasible that as TM2014 is still capable of forming a flagellum, attachment may not be impeded. Y403BE8, a motility-impaired mutant of Y4I, is expected to possess wildtype

flagellar machinery as it is motile, it simply seems unable to utilize it in wildtype manner. This may explain why this non-motile strain attaches to a surface similar to wildtype. Interestingly, Y402AE5, the *luxR*$^{-}$ mutant, behaves similarly to wildtype in regards to surface attachment in complex medium. However, the *luxR*$^{-}$ mutant attached significantly greater than wildtype in minimal medium ($p = 0.013$, *D*). Since the product of *luxR* genes has been implicated in surface attachment and/or biofilm formation (Davies et al., 1998) in other bacteria, one would suspect that this strain should be impaired. However, after the attachment assays were performed the Y4I genome sequence became available and homology searches indicate that Y4I contains two quorum sensing systems. This may explain why a mutation in one *luxR* gene did not affect surface attachment. Interestingly, the attachment assay on polystyrene was conducted on yet another Y4I transposon mutant, Y412AH12, a two-component response regulator mutant (Mooney and Buchan, unpub.) which does exhibit impairment in colonization (Figure 5a).

Initial attachment assays were conducted to complement the attachment studies. This allowed comparison between the ability to form attach to surfaces and the ability to quickly attach to a surface. Roseobacter strains have been previously found to attach to different particles, for example E-37 possesses a holdfast structure that appears to be involved in the strain's selective attachment to cellulose and lignocellulose particles (Gonzalez *et al.*, 1997). Interestingly, this strain did not form a strong surface attachment on any of the substrates tested. This suggests the possibility that E-37 is better adapted to attach to surface types that fall outside of the range of surfaces tested here. It is of interest that strains TM1040, Y4I, and Y3F which formed the most developed surface attachments on all surfaces tested, did not attach quickly in the initial attachment assay. In contrast, SE62 and PSPC2, which did not form a substantial attachment, had the most cells attaching in the first 5 minutes. NAS14-1 is of particular interest due to its ability to not only attach to surfaces but also significantly attach within the first 5 minutes of exposure to a surface. This suggests an ecological role for this organism where it not only colonizes sessile surfaces but may also be a strong competitor for transient nutrient particles.

Another interesting aspect of biofilm formation and surface attachment involves studies in which exopolysaccharide (EPS) has been found to influence a cell's ability to attach to certain surfaces. Three Roseobacter strains, SE45, S. lac and Y4I produce visible amounts of EPS in broth cultures and strains SE45 and Y4I floc when grown in nutrient rich liquid medium. It has been found that the marine biofouling bacterium, *Deleya marina*, utilizes EPS to preferentially attach to hydrophilic surfaces (Shea *et al.*, 1991). The EPS-deficient mutant of *D. marina* was significantly reduced in its ability to attach to the tested surfaces compared to wildtype. This suggests there may be a role for the production of EPS in attachment for some members of the Roseobacter clade. It is important to note that in the crystal violet attachment assay, EPS material would be stained as readily as the cells themselves and would contribute to the biomass measured. However, in this study, the EPS-producing strain S. lac was not proficient in surface attachment while strains SE45 and Y4I were rather competent at strongly attaching to most surfaces.

Quorum sensing has been found to be important in the ability of many medically relevant bacteria to attach to surfaces and form biofilms (Labbate *et al.*, 2007; Hammer and Bassler, 2003; Davies *et al.*, 1998). Evidence for quorum sensing systems has been found in some members of the Roseobacter lineage (Wagner-Dobler *et al.*, 2005; Gram *et al.*, 2002). Representative Roseobacters have been found to produce acyl-homoserine lactones (AHLs) similar to those produced by other well-characterized *Proteobacteria* and investigators have suggested that these signaling molecules may be important in interspecies communication and in the formation of mixed-species biofilms by Roseobacters (Wagner-Dobler *et al.*, 2005). Homology searches of completed genome sequences suggests that quorum sensing in this lineage appears to be regulated by a LuxR-type transcriptional regulator and AHLs produced by a LuxI-like AHL-synthetase. Thirteen of 18 (72%) sequenced Roseobacter strains possess these genes and three strains (17%), DSS-3, *Dinoroseobacter shibae* DFL12 and Y4I harbor two sets of *luxI/luxR* genes. These findings may suggest that quorum sensing and the activities most often associated with quorum sensing may play important roles in defining population dynamics of members of this lineage. Conversely, surface colonization by Roseobacters

may not be dependent upon possession of a classic quorum sensing system, but later stages of biofilm development might utilize this system. For example, one of the most prolific surface colonizers in this study, TM1040, does not appear to harbor a quorum sensing system.

Morphological differences may be important in motility and the colonization of surfaces. Analyses of Roseobacters grown under various conditions revealed that cellular morphology is highly plastic in many strains. Isolates grown under static conditions formed more aggregates and rosettes than those grown with agitation (Table 6). This behavior has been demonstrated in Roseobacter strains previously. For example, *Phaeobacter* 27-4 was found to produce rosettes as well as a pigmented antimicrobial compound when grown under static conditions (Bruhn *et al.*, 2005). These phenotypes were not observed when cultures were grown under shaking conditions. Rosette formation is common among lineage members and has been shown to vary with growth conditions (Petursdottir and Kristjansson, 1997; Sorokin, 1995; Ruger and Hofle, 1992). Observations made during this thesis are consistent with earlier findings; for a given strain, certain phenotypes are only seen under certain growth conditions. For example, TM1040, Y4I and Y3F were found to be motile not only on motility plates but also in both minimal and complex media broth cultures (in the absence of "conditioning" on motility plates). This suggests that motility plays a large role in the lifestyle of these strains regardless of the nutrient content of their environment. Other strains including NAS14-1, EE-36, SE45 and S.pont were motile on semi-solid agar plates but not in minimal medium broth. This leads to the possibility that motility may be important to these strains when they encounter a relatively nutrient-limited environment and in nutrient-rich environments they may not utilize their ability to be motile.

Strain differences were also apparent in rosette formation. Strains SE45 and S. lac were never found to form rosettes under any growth condition. Due to this finding, rosette formation is not a trait that can be applied to the entire lineage. Strain NAS14-1 was found to produce an increased number of rosettes in minimal medium compared to complex medium. S. pont formed relatively few rosettes in all media types with the exception of complex medium motility plates. In these plates, S. pont showed enhanced

rosette production, a marked difference from its growth on other motility plates and in liquid culture. In contrast, strain *Roseovarius nubinhibens* ISM showed rosette production under all growth conditions. Also, strain PSPC2 showed increased rosette production on motility agar compared to liquid media. Variation in rosette production by lineage members further highlights the influence of environment on the ability of these strains to demonstrate certain phenotypes. Rosette formation is not limited to Roseobacter lineage members and has been found in a number of aquatic bacteria, including several marine *Agrobacterium* species found to form "star-shaped" aggregates (Ruger and Hofle, 1992). Environmental conditions have also been demonstrated to dictate rosette formation in other bacteria, for example, in the freshwater bacterium *Nevskia ramose*, rosette formation is influenced by nitrogen concentration (Pladdies et al., 2004).

An analysis of the genes that encode for proteins essential to motility, chemotaxis and quorum sensing further supports the concept that the lineage represents significant heterogeneity at both phenotypic and genotypic levels. Of the six strains for which genome sequences are not yet available, partial *cheA* genes were recovered from five (Table 8). PSPC2 is the only strain for which an amplicon of the expected size was not recovered. This is not necessarily surprising as this strain never demonstrated motility and no flagella were ever seen via microscopy. However, given the degenerate nature of the primer sets used, the inability to amplify one or more of the flagellar or chemotaxis genes from a given strain may not necessarily indicate the absence of a homolog in that strain. The recovery of *cheA* homologs from S. lac and SE62 are intriguing as neither strain demonstrated motility nor were flagella ever evident under conditions tested in this study. Similarily intriguing was the finding of a *flgH* homolog in S. lac. These findings lend further support to the idea that some strains may require unique growth conditions in order to be motile.

Although no degenerate primers were constructed to probe for *luxI* genes, comparison of known *luxI* genes and the genetic content surrounding these genes from fully sequenced genomes was performed (Figure 15, Figure 17). As mentioned above and of particular interest, strain TM1040 has no known *luxI* genes, which is somewhat

surprising considering the wide-range of phenotypes it possesses that are often associated with quorum sensing, including surface colonization and production of antimicrobial compounds. Genes that fall within the *luxI* family and have sequence homology with *luxI* genes are often classified with different nomenclature including *rhlL* and *traI* genes. Though these genes are named differently they closely identify with *luxI* sequences and seem to be responsible for producing an autoinducer as well. The alignment of the *luxI* and surrounding genes from representative Roseobacter strains shows a variety of gene content and organizations, however, a few trends are apparent. In most cases, the autoinducer gene and the transcriptional regulator are positioned close to one another with only a few other genes surrounding in close proximity. In almost all cases, the genetic context in which *luxR-luxI* are found does not provide clues as to the pathways that may be regulated by quorum sensing. One possible exception is the non-motile, non-attaching strain ISM, which has two distinct two-component diguanylate cyclase genes flanking its *luxI-luxR* operon (Figure 17). These genes have been found to catalyze the formation of a secondary messenger compound believed to control multicellular behavior (Chan *et al.*, 2004). This has obvious implications for quorum sensing in strain ISM.

The observation that strains with more divergent 16S rRNA sequences have identical flagellar gene operons raises the question of horizontal gene transfer (HGT). HGT has been shown experimentally to occur in nutrient-limited, artificial seawater conditions, as well as in natural marine bacterial populations, using green fluorescent protein as a marker (Dahlberg *et al.*, 1998a). Plasmid transfer has also been implicated in HGT in natural marine populations, lending support to the theory that HGT by these plasmids is responsible for similar genetic sequences in distantly related strains (Dahlberg *et al.*, 1998b). It seems possible HGT is actively occurring in Roseobacter clade members leading to phylogenetically distinct strains possessing identical flagellar operons. The exact protein sequence may have changed over time, but the gene content and orientation have remained the same, such as for the *fliF* gene set in strains DSS-3 and *R. denitrificans* (Figure 17).

The gene analysis performed here highlights the large amount of diversity which can be found in flagellar and chemotaxis operons. One of the few commonalities is the

co-localization of the *fliL* and *fliF* genes, they are typically located adjacent to, but divergently transcribed from, one another. Also, flagellar hook construction genes were always found in conjunction with the flagellar p-ring construction protein. In all but one strain, the *fliF* gene was found in conjunction with flagellar motor genes. The *cheA* gene set was more variable in its consistency. Often the genes *cheB* and *cheD* were found near *cheA,* but this was not always the case. A transcriptional regulator was also often found near the *cheA* gene, but again this was not always the case. Strains that did possess a chemotaxis operon also possess methyl-accepting chemotaxis proteins (MCPs) that were spread throughout the genome. The number of MCPs in Roseobacter strains range in number from 1-10 with an average of 5 MCPs per genome. In comparison to the flagellar and chemotaxis operons, organization of *lux* genes demonstrated more variability between genomes (Figures 19)

The characterization provided by this thesis lays the necessary foundation for future work in the areas of motility, chemotaxis and cell-to-cell signaling in representative members of the Roseobacter clade. Interesting future work on this project includes further investigation of flagellar genes in strains that do not have sequenced genomes. For example, here it was demonstrated that *Silicibacter lacuscaerulensis* possesses both *flgH* and *cheA* homologs. This is of particular interest due to the fact that this strain has never demonstrated motility or flagellar production in this set of laboratory experiments. The genome of S. lac is currently being sequenced, this will undoubtedly provide more insight into the genetic capabilities of this strain. Additionally, the genome of *Citricella* sp. SE45 is also being sequenced which will only further elucidate the presence of flagellar and chemotaxis operons within these members of the Roseobacter clade.

It would also be interesting to further investigate the role of the quorum sensing in surface colonization and biofilm development for this set of Roseobacter clade members. TM1040 does not appear to possess a quorum sensing system yet was a prolific surface colonizer. Additionally, other strains such as ISM and E-37 were not significant surface colonizers and do possess canonical quorum sensing genes. It would be interesting to investigate whether quorum sensing molecules are commonly used in this lineage in

biofilm development or if biofilm development occurs via another form of signaling behavior or no signaling behavior at all.

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APPENDICES

APPENDIX A: Tables and Figures

Table 1**.** List of thirteen Roseobacter strains included in this study.

STRAINS	ISOLATION SITE	CHARACTERIZATION	SEQUENCED GENOME	GENOME ACCESSION NO.
Citricella sp. SE45	Georgia Coast - decaying salt marsh grass	No	In Progress	
Phaeobacter sp. Y3F	Georgia Coast	In progress	No	
Phaeobacter sp. Y4I	Georgia Coast	In progress	Yes*	
Rhodobacteraceae sp. PSPC-2	Georgia Coast – fungi	No	No	
Rhodobacteraceae sp. SE62	Georgia Coast - decaying salt marsh grass	No	No	
Roseovarius nubinhibens, ISM	Caribbean Sea	Yes	Yes	NZ_AALY00000000
Sagitulla stellata, E-37	Georgia Coast	Yes	Yes	NZ_AAYA00000000
Silicibacter lacuscaerulensis (S. lac)	Geothermal Lake	Yes	In Progress	
Silicibacter pomeroyi DSS-3	Georgia Coast	Yes	Yes	NC_003911
Silicibacter sp. TM1040	dinoflagellate	Yes	Yes	NC_008044
Sulfitobacter sp. EE-36	Georgia Coast	No	Yes	NZ_AALV00000000
Sulfitobacter sp. NAS-14-1	North Atlantic Ocean	No	Yes	NZ_AALZ00000000
Sulfitobacter pontiacus (S. pont)	Black Sea	Yes	No	

*Genome scaffolds are available but have not yet been submitted to the NCBI database.

Table 2**.** Doubling times for all strains grown in YTSS nutrient-rich medium and in SBM minimal medium (NA = not available).

DOUBLING TIME	**YTSS**	**SBM**
STRAIN	**TIME (MIN)**	**TIME (MIN)**
Citricella sp.SE45	90	87
Phaeobacter sp. Y3F	62	80
Phaeobacter sp.Y4I	79	94
Roseobacteraceae sp.PSPC2	104	NA
Roseobacteraceae sp.SE62	96	NA
Roseovarius nubinhibens ISM	81	188
Sagittula stellata E-37	84	94
Silicibacter lacuscaerulensis	95	95
Silicibacter pomeroyi DSS3	100	162
Silicibacter sp.TM1040	80	NA
Sulfitobacter sp. EE-36	95	108
Sulfitobacter sp.NAS14-1	79	100
Sulfitobacter pontiacus	95	246

Table 3. Strains used in alignment for FliF degenerate primer set.

FliF ALIGNMENT	
STRAIN	ACCESSION NO.
Sulfitobacter sp. NAS14-1	ZP_00963769
Sulfitobacter sp. EE-36	ZP_00956642
Roseovarius sp. HTCC2601	ZP_01444395
Oceanicola granulosus HTCC2516	ZP_01156099
Silicibacter sp. TM1040	YP_614941
Silicibacter pomeroyi DSS-3	YP_165469
Roseobacter denitrificans Och 114	YP_680680
Roseovarius sp. HTCC2601	ZP_01443283
Oceanicola granulosus HTCC2516	ZP_01157139
Paracoccus denitrificans PD 1222	ZP_00630361
Dinoroseobacter shibae DFL 12	ZP_01583705
Rhodobacter sphaeroides ATCC17029	ZP_00917031
Roseovarius sp. 217	ZP_01037249
Jannaschia sp. CCS1	YP_512122
Agrobacterium tumefaciens str. C58	NP_353552

Table 4. Strains used in alignment for FlgH degenerate primer set.

FlgH ALIGNMENT	
STRAIN	ACCESSION NO.
Silicibacter sp. TM1040	YP_614936
Silicibacter pomeroyi DSS-3	YP_165465
Roseobacter denitrificans Och 114	YP_680967
Uncultured marine bacterium Ant24C4	ABC25339
Roseovarius sp. 217	ZP_01037240
Oceanicola granulosus HTCC2516	ZP_01155033
Roseovarius sp. HTCC2601	ZP_01441642
Dinoroseobacter shibae DFL 12	ZP_01583811
Rhodobacter sphaeroides ATCC17029	ZP_00914854
Jannaschia sp. CCS1	YP_512118
Sulfitobacter sp. EE-36	ZP_00956625
Sulfitobacter sp. NAS-14-1	ZP_00963787
Roseovarius sp. HTCC2601	ZP_01444383
Oceanicola batensis HTCC2597	ZP_01000508
Oceanicola granulosus HTCC2516	ZP_01156110
Sagittula stellata E-37	NZ_AAYA00000000
Jannaschia sp. CCS2	NZ_AAYB01000001

Table 5. Nucleotide sequences for the degenerate primer sets.

DEGENERATE PRIMERS	
FliF for	5' GCI AWR GAR GGI GAR YTI GCI MG 3'
FliF rev	5' YC RTT IAC IAR IAC IGC IAC 3'
FlgH for	5' GGI TAY GGI YTI RTI GTI GG 3'
FlgH rev	5' AR ITC RGC RTG IAR IGC ICC 3'
CheA P4P5 for	5' CAY YTI ITI MGI AAY ISI GAY CAY GG 3'
CheA P4P5 rev	5' CCR TCI CCI ARI ATI GTI GC 3'

Table 6. Thirteen Roseobacter strains analyzed with TEM and phase contrast microscopy in five different growth conditions.

			GROWTH CONDITIONS		
STRAINS	**Liquid YTSS, shaking growth**	**Liquid YTSS, static growth**	**Liquid SBM, shaking growth**	**1/10 YTSS motility plates growth**	**SBM motility plate growth**
Citricella sp. SE45	PC – no motility, ovoid cells, no rosettes, some doublets, many contain black dots TEM – doublet cells, no flagella present	PC – no motility, ovoid cells, no rosettes, increased doublet cells	PC – few motile cells, ovoid cells, no rosettes, fewer doublets than in YTSS	PC – VERY motile cells, no aggregates, lots of doublets TEM – Doublet and ovoid cells, flagella present though not attached	PC – very motile cells, ovoid cells, no rosettes, no doublets
Phaeobacter sp. Y3F	PC – motile cells, ovoid cells and a few long chains, some rosettes, few doublets TEM – mostly doublets, chains, rosettes, flagella present	PC – motile cells, long cells, some small rosettes, few doublets	PC – increase motility from YTSS shaking, ovoid cells, no rosettes, few doublets	PC – motile cells, ovoid cells, no rosettes, many doublets TEM - Doublet and ovoid cells, multiple, polar flagella	PC – increased motility from 1/10 YTSS, ovoid cells, some black dots in cells, some rosettes, some doublets
Phaeobacter sp. Y4I	PC - motile cells, short and plump cells, chains present, some rosettes, many doublets TEM – doublet and ovoid cells, flagella present	PC – motile cells, oval cells & long, thin cells, some rosettes, many doublets	PC – increased motility from YTSS, short and plump cells with fat and short chains, some rosettes, no doublets	PC – very motile cells, mostly doublets, some ovoid and rod cells, some rosettes TEM – ovoid and doublet cells, multiple polar flagella	PC – motile cells, very large ovoid cells, some rosettes, some doublets present
Roseobacteraceae sp. PSPC-2	PC – no motility, small ovoid cells, no rosettes, bat shaped cells also present TEM – short, ovoid shaped cells, no flagellum	PC – no motility, mostly bat shaped cells, few rosettes	PC – no motility, very small ovoid cells, various other shapes as well, no rosettes	PC – no motility, large variety of cell shapes, ovals & long cells, huge clusters, some doublets	PC – no motility, cells smaller than seen before, ovoid shaped cells, small rosettes

Table 6, continued.

			GROWTH CONDITIONS		
STRAINS	**Liquid YTSS, shaking growth**	**Liquid YTSS, static growth**	**Liquid SBM, shaking growth**	**1/10 YTSS motility plates growth**	**SBM motility plate growth**
Sagitulla stellata, E-37[f]	PC – no motility, ovoid cells, several rosettes, lots of doublets	PC – no motility, ovoid cells, some rosettes, some doublets	PC – no motility, larger ovoid cells, some clusters, some doublets, some cells with black dots inside	PC – no motility, ovoid cells, some rosettes, some doublets	PC – no motility, ovoid cells, increased clumping some ublets, black dots inside cells
Silicibacter lacuscaerulensis[c]	PC – no motility, long, thin rod cells, no rosettes, some doublets TEM – long, thin rod shaped cells, no flagella	PC – no motility, large variety of cell shapes and sizes, no rosettes	PC – no motility, long thin cells, no rosettes	PC – no motility, variation in cell shape and size, no rosettes, doublets and ovoid cells present	PC – no motility, long, thin cells, no rosettes
Silicibacter pomeroyi DSS3[b]	PC – no motility, mostly doublet shaped cells, some single ovoid cells, rosettes	PC – no motility, ovoid cells, rosettes, doublets	PC – no motility, smaller ovoid cells, no rosettes, some doublets	PC – no motility, some ovoid cells, rosettes, longer doublet cells	PC – no motility, many ovoid cells with some small chains, clusters, no rosettes, doublets
Silicibacter sp. TM1040[a]	PC – motile cells, small, ovoid cells, rosettes, doublets	PC – no motility, ovoid cells, many rosettes, doublets	PC – no motility, most cells are doublet shaped, many rosettes	PC – very motile cells, cells are larger than YTSS shaking growth, few rosettes, many doublets	PC – motile cells, small, ovoid cells, few rosettes
Sulfitobacter sp. EE-36	PC – no motility, mostly doublet shaped cells, no rosettes	PC – no motility, ovoid as well as longer cells, many chains, rosettes, doublets	PC – motile cells, small, ovoid cells, large, rosette-shaped cluster, some doublets	PC – motile cells, mostly doublet cells, some ovoid cells, small rosettes TEM – Small, ovoid cells, at least one polar flagellum	PC – no motility, small, ovoid cells, rosettes, doublets

Table 6, continued.

			GROWTH CONDITIONS		
STRAINS	**Liquid YTSS, shaking growth**	**Liquid YTSS, static growth**	**Liquid SBM, shaking growth**	**1/10 YTSS motility plates growth**	**SBM motility plate growth**
Sulfitobacter sp. NAS-14-1	PC – no motility, several small ovoid cells, few rosettes, mostly doublet shaped cells	PC – no motility, mixture of large and small ovoid cells, few rosettes, few doublets	PC – motile cells, ovoid cells, large, rosette-shaped clusters, many doublets	PC – motile cells, ovoid cells, very few rosettes, many doublets TEM – Ovoid and doublet cells, two polar flagella	PC – very motile cells, ovoid cells with some chains, no rosettes, many doublets
Sulfitobacter pontiacus[d]	PC – no motility, ovoid shaped cells, few rosettes, few doublets TEM – larger ovoid and doublet shaped cells, many flagella present, difficult to see attachment point	PC – no motility, slightly rounder than shaking culture, some rosettes	PC – no motility, ovoid cells, few dumbbells, few rosettes	PC – few motile cells, no rosettes and aggregates, many long cells, some doublets TEM –small, round cells with at least one polar flagellum	PC –some motile cells, some doublets mostly elongated ovoid cells

* Several strains had been previously characterized using TEM. [a]*Silicibacter* sp. TM1040 was grown in ½ strength 2216 marine broth for 20hrs and visualized using TEM. It possessed three polarly attached flagella and was an oval shaped cell (Miller, 2004). [b]*Silicibacter pomeroyi* DSS3 was grown in ½ strength YTSS broth overnight and visualized to be rod cells with surface "blebs". A flagellum was visualized using SEM (Gonzalez, 2003). [c]Silicibacter lacuscaerulensis was grown on agar overnight embedded in Spurr resin, sectioned, and was viewed with TEM to be long, rod-shaped and contain white dots speculated to be vacuoles. No flagellum was present (Kristjansson, 1994 ;Petursdottir, 1997). [d]Sulfitobacter pontiacus cells were grown on acetate agar , acetate-limited culture and culture with acetate + sulfite then visualized using TEM. Cells were dumbell shaped and contained PHB-like inclusions (Sorokin, 1995). [e]Roseovarious nubinhibens ISM was grown in ½ strength YTSS broth overnight and visualized to be rod-shaped with a suspected separation of the cytoplasm at the poles. No flagellum was present (Gonzalez, 2003). [f]Sagittula stellata E-37 was grown in BM containing 0.2% glucose and 0.001% yeast extract and when visualized using TEM was shown to have polarity in shape, one end is larger than the other. The cells were found to have a holdfast structure and no attached flagellum but a detached flagellum was observed in the sample (Gonzalez, 1997).

Table 7**.** Contact angle measurements for all surface types used in attachment assays.

SUBSTRATE	CONTACT ANGLE MEASUREMENT	SURFACE PROPERTY
Polypropylene	87.3^{o}	Slightly hydrophobic
Polyvinyl chloride	76.5^{o}	Slightly hydrophilic
Polyethylene terephthalate (PET)	68.2^{o}	Slightly hydrophilic
Polycarbonate	67.4^{o}	Slightly hydrophilic
Polystyrene*	86^{o}	Slightly hydrophobic
Teflon^{TM}	93.8^{o}	Hydrophobic
Glass	<10^{o}	Hydrophilic

*Fletcher * Loeb, 1979

Table 8. Products amplified using the three degenerate primer sets.

ORGANISM	GENE DESIGNATION	NUMBER OF NUCLEOTIDES SEQUENCED	CLOSEST BLAST HIT	E-SCORE
SE45	CheA P4P5	859	*Roseovarious* sp. HTCC2601	5E-52
	FliF	645	*Roseovarious* sp. HTCC2601	1E-74
SE62	CheA P4P5	458	*Loktanella vestfoldensis* SKA53	8E-47
S. lac	CheA P4P5	844	Roseovarious sp. HTCC2601	2E-67
	FlgH	946	*Silicibacter pomeroyi* DSS-3	4E-130
S.pont	CheA P4P5	705	*Roseovarious* sp. HTCC2601	1E-57
	FliF	667	*Sulfitobacter* NAS-14-1 & EE-36	2E-106
Y3F	CheA P4P5	449	*Silicibacter* sp.TM1040	3E-68
	FlgH	989	*Silicibacter pomeroyi* DSS-3	1E-126

Figure 1. Phylogenetic tree of Roseobacter members based on 16S rDNA sequences. The tree was constructed using Mega 4 and the neighbor-joining method. The tree is based on positions 80 to 1365 of the 16S rRNA gene (*E. coli* numbering system). GenBank accession numbers are provided in parentheses, with the exception of *Roseovarious* sp.HTCC2601 for which the sequence was obtained from https://research.venterinstitute.org/moore/. Asterisks show strains that were examined in this study. The bar represents *p*-distance (evolutionary). Bootstrap values > 50% are shown at branch nodes (1000 iterations).

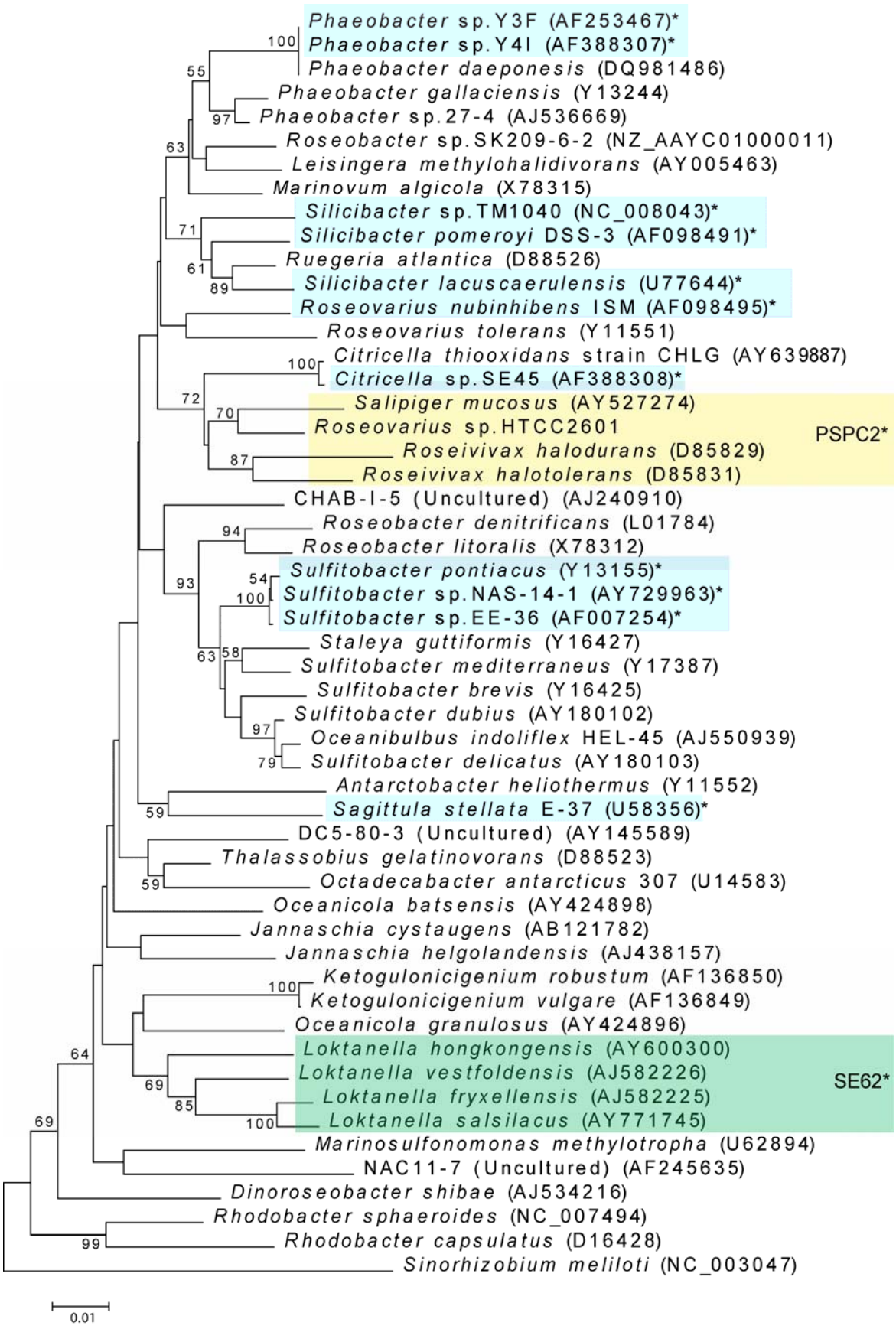
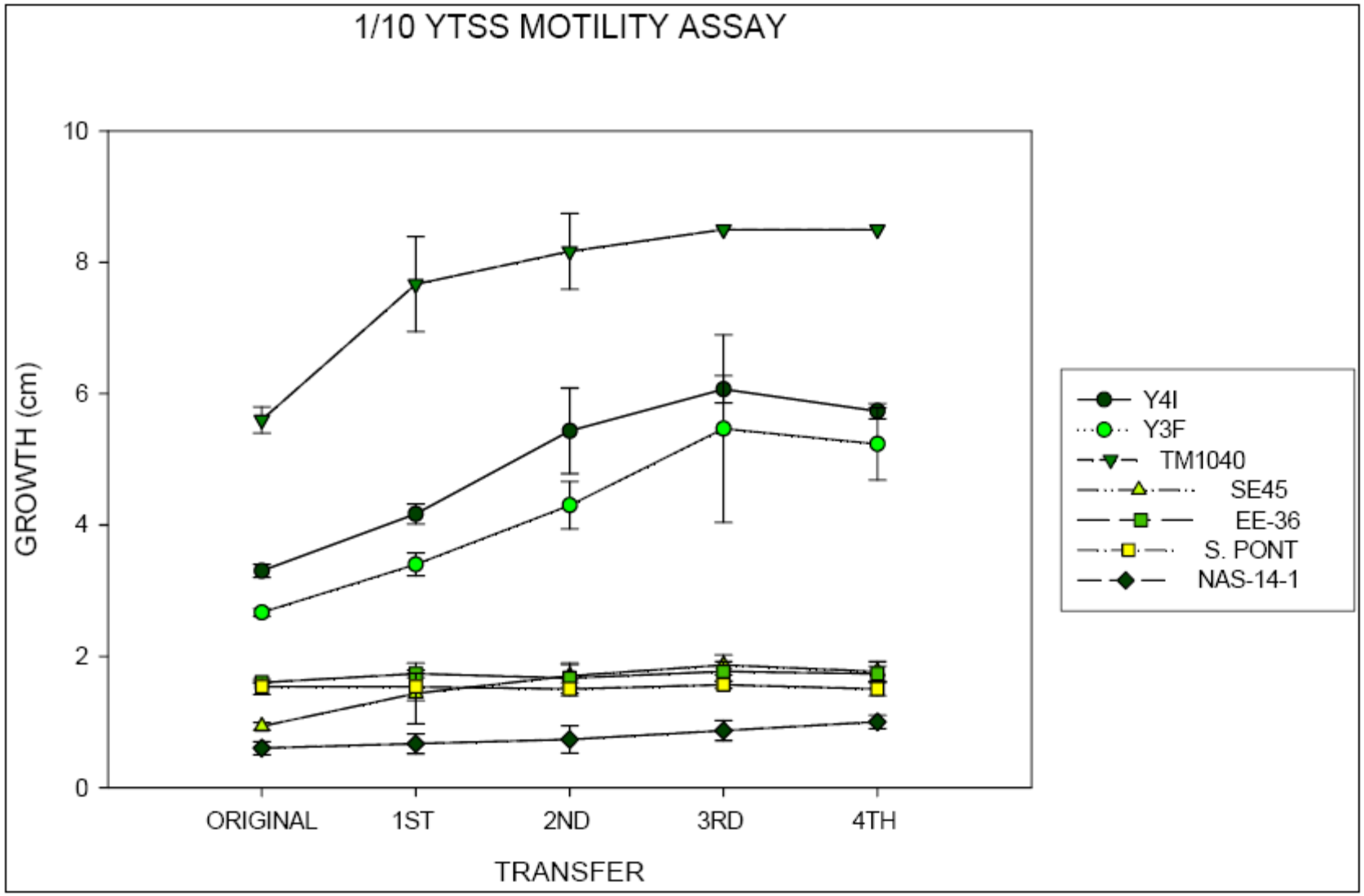

Phaeobacter sp.Y3F (AF253467)*
Phaeobacter sp.Y4I (AF388307)*
Phaeobacter daeponesis (DQ981486)
Phaeobacter gallaciensis (Y13244)
Phaeobacter sp.27-4 (AJ536669)
Roseobacter sp.SK209-6-2 (NZ_AAYC01000011)
Leisingera methylohalidivorans (AY005463)
Marinovum algicola (X78315)
Silicibacter sp.TM1040 (NC_008043)*
Silicibacter pomeroyi DSS-3 (AF098491)*
Ruegeria atlantica (D88526)
Silicibacter lacuscaerulensis (U77644)*
Roseovarius nubinhibens ISM (AF098495)*
Roseovarius tolerans (Y11551)
Citricella thiooxidans strain CHLG (AY639887)
Citricella sp.SE45 (AF388308)*
Salipiger mucosus (AY527274)
Roseovarius sp.HTCC2601
Roseivivax halodurans (D85829)
Roseivivax halotolerans (D85831)
PSPC2*
CHAB-I-5 (Uncultured) (AJ240910)
Roseobacter denitrificans (L01784)
Roseobacter litoralis (X78312)
Sulfitobacter pontiacus (Y13155)*
Sulfitobacter sp.NAS-14-1 (AY729963)*
Sulfitobacter sp.EE-36 (AF007254)*
Staleya guttiformis (Y16427)
Sulfitobacter mediterraneus (Y17387)
Sulfitobacter brevis (Y16425)
Sulfitobacter dubius (AY180102)
Oceanibulbus indoliflex HEL-45 (AJ550939)
Sulfitobacter delicatus (AY180103)
Antarctobacter heliothermus (Y11552)
Sagittula stellata E-37 (U58356)*
DC5-80-3 (Uncultured) (AY145589)
Thalassobius gelatinovorans (D88523)
Octadecabacter antarcticus 307 (U14583)
Oceanicola batsensis (AY424898)
Jannaschia cystaugens (AB121782)
Jannaschia helgolandensis (AJ438157)
Ketogulonicigenium robustum (AF136850)
Ketogulonicigenium vulgare (AF136849)
Oceanicola granulosus (AY424896)
Loktanella hongkongensis (AY600300)
Loktanella vestfoldensis (AJ582226)
Loktanella fryxellensis (AJ582225)
Loktanella salsilacus (AY771745)
SE62*
Marinosulfonomonas methylotropha (U62894)
NAC11-7 (Uncultured) (AF245635)
Dinoroseobacter shibae (AJ534216)
Rhodobacter sphaeroides (NC_007494)
Rhodobacter capsulatus (D16428)
Sinorhizobium meliloti (NC_003047)
100
55
97
63
71
61
89
100
72
70
87
94
93
54
100
63
58
97
79
59
59
100
64
69
85
100
69
99
0.01

Figure 2. Distances migrated from the site of inoculation after four transfers on 1/10 YTSS motility agar plates (A) and SBM + 10mM acetate motility agar plates (B).

A


1/10 YTSS MOTILITY ASSAY
GROWTH (cm)
0
2
4
6
8
10
ORIGINAL
1ST
2ND
3RD
4TH
TRANSFER
Y4I
Y3F
TM1040
SE45
EE-36
S. PONT
NAS-14-1

B

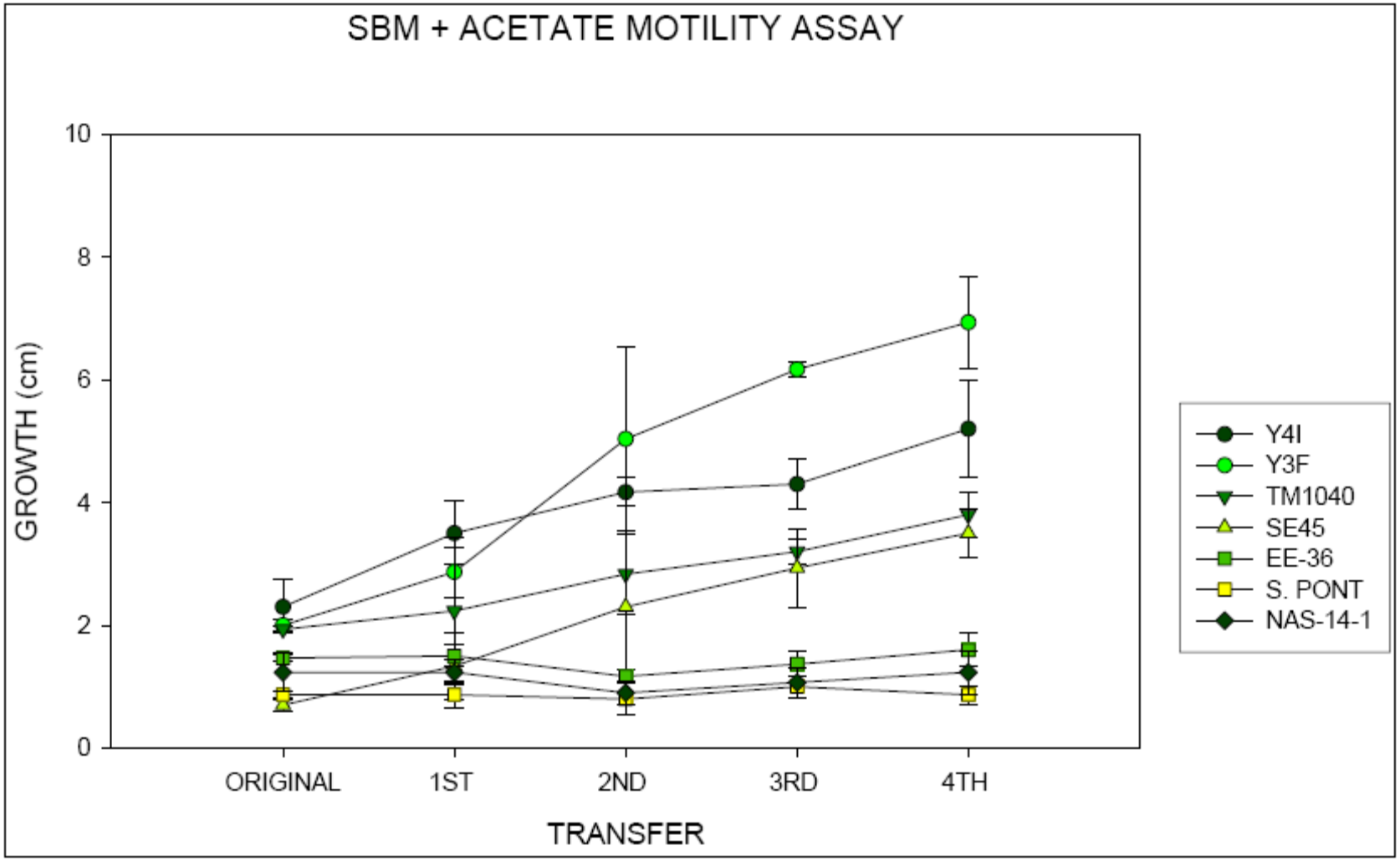
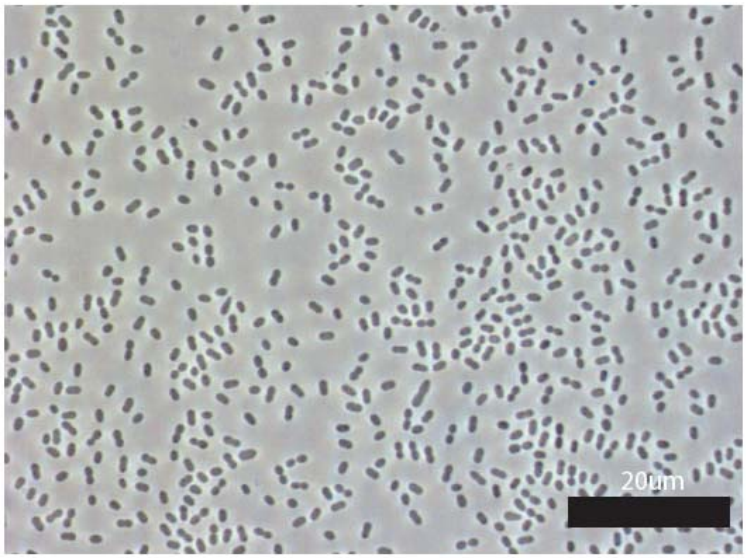
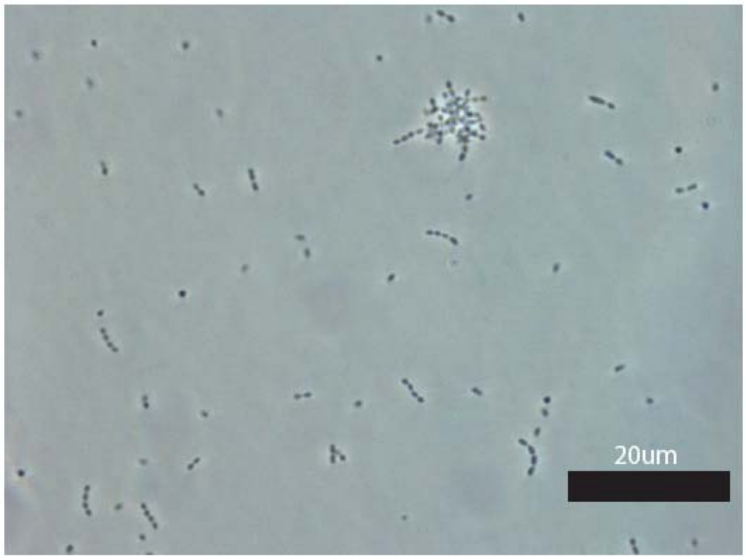

SBM + ACETATE MOTILITY ASSAY
GROWTH (cm)
10
8
6
4
2
0
ORIGINAL
1ST
2ND
3RD
4TH
TRANSFER
Y4I
Y3F
TM1040
SE45
EE-36
S. PONT
NAS-14-1

Figure 3. Phase contrast microscope images of Roseobacter strains grown in YTSS (complex) broth. Strains demonstrate a variety of phenotypes including, strain SE45 doublet cellular morphology (A), Y3F chain formation (B), Y4I rosette formation (C), PSPC2 asymmetrical cellular morphology(D), SE62 slender, rod cellular morphology(E), E-37 small rosette formation(F), DSS-3 elongated doublet cellular morphology (G) and EE-36 doublet and ovoid cellular morphology (H).

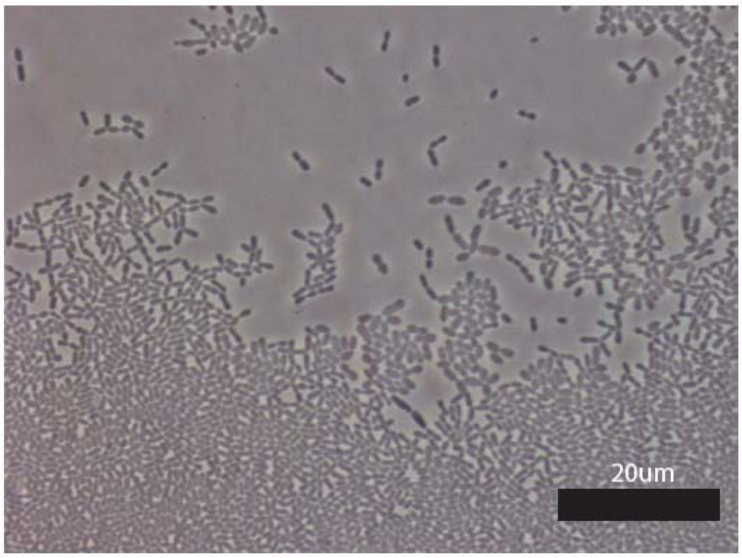
A.



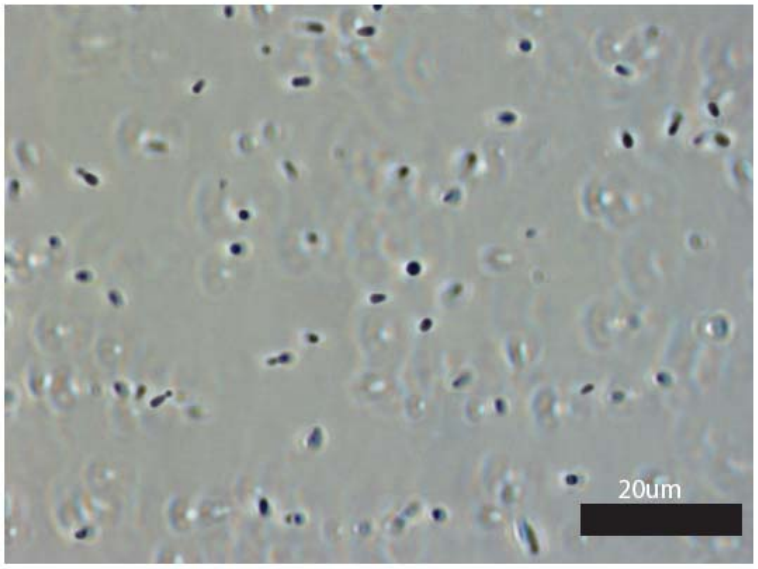
B.



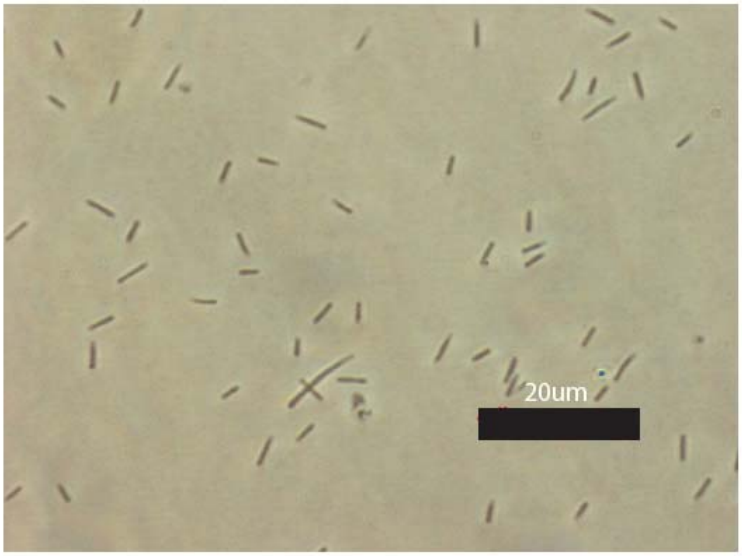
C.



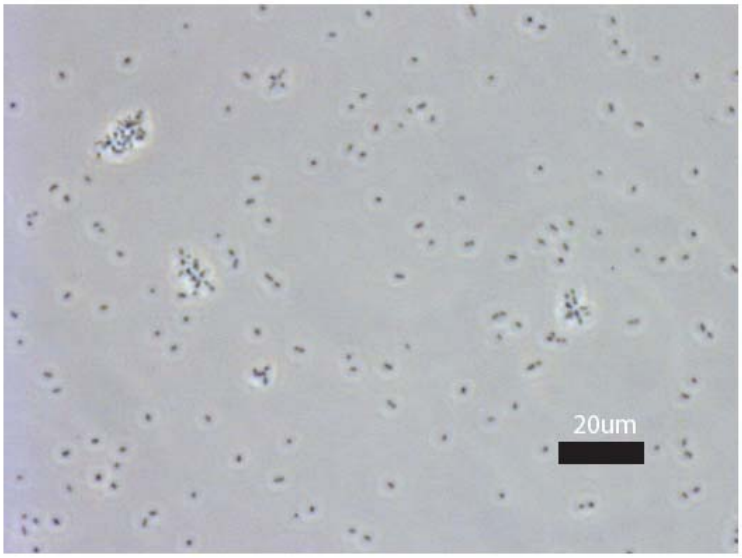
D.



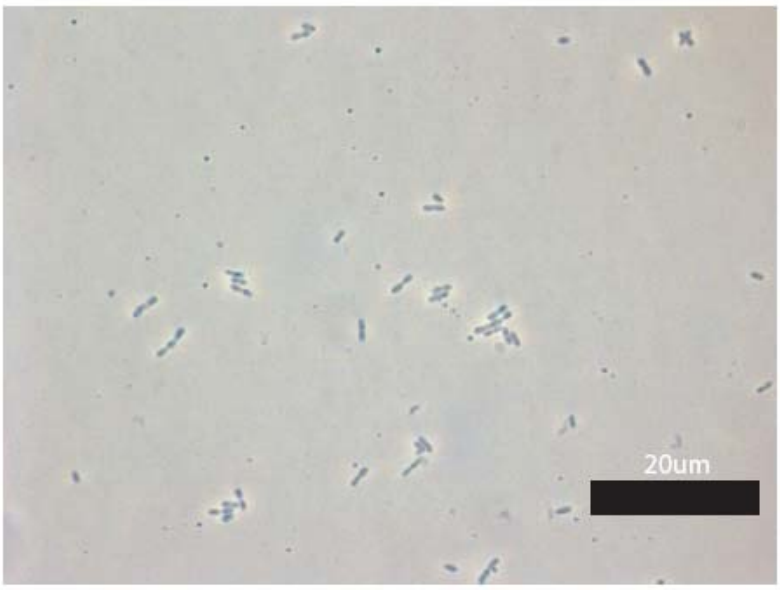
E.



F.



G.



H.

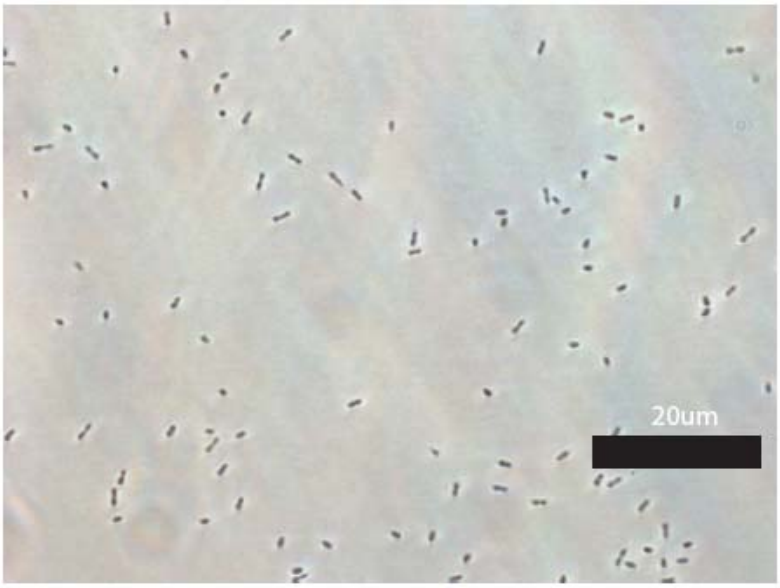
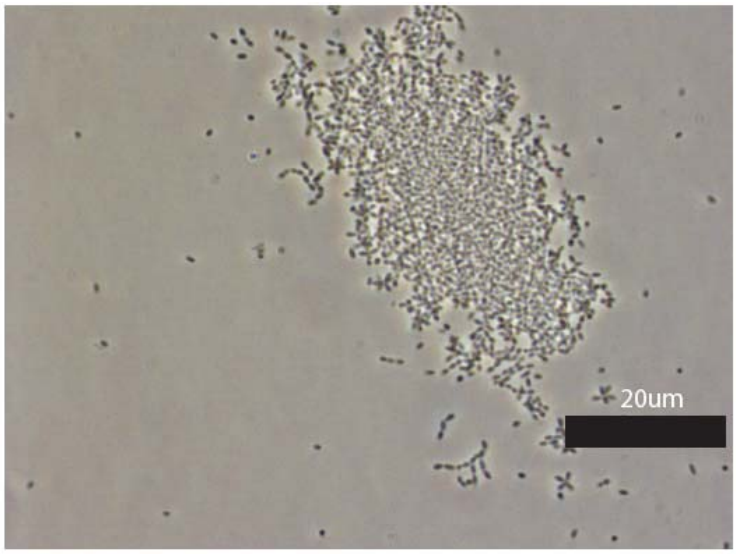
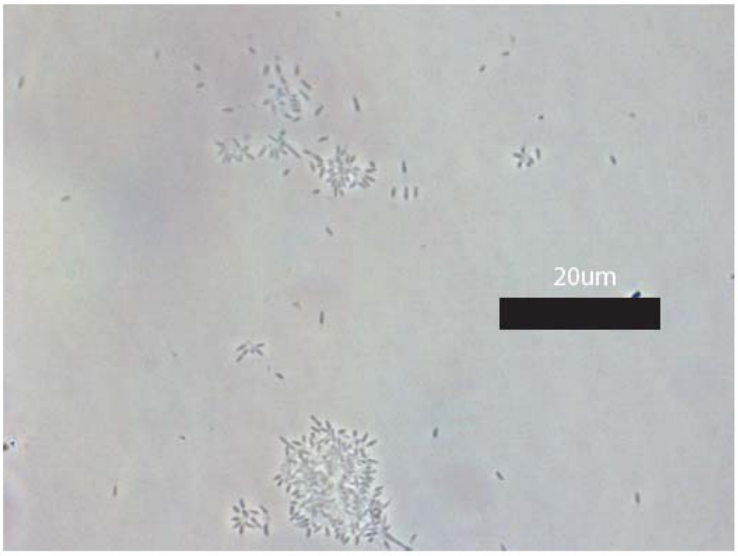


Figure 4. Phase contrast microscope images of five Roseobacter strains grown in SBM (minimal medium) broth. Strains Y4I (A), ISM (B), E-37 (C), TM1040 (D), EE-36 (E) and NAS-14-1 (F) demonstrate a wide range of aggregate formation.

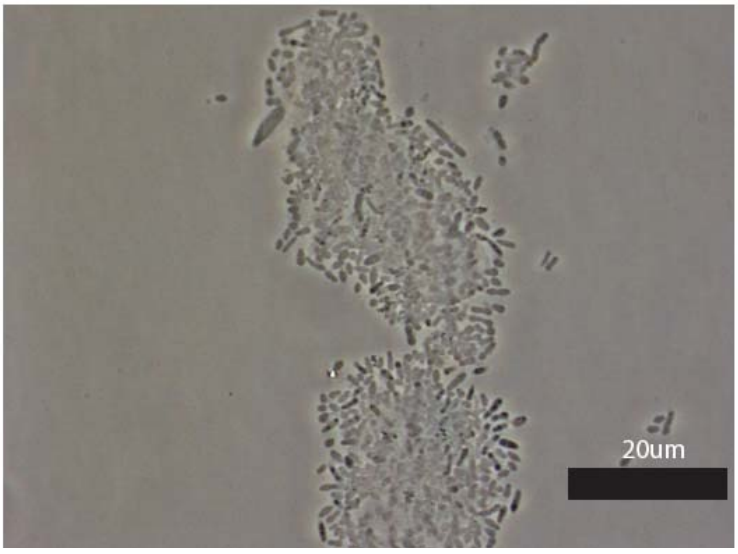
A.



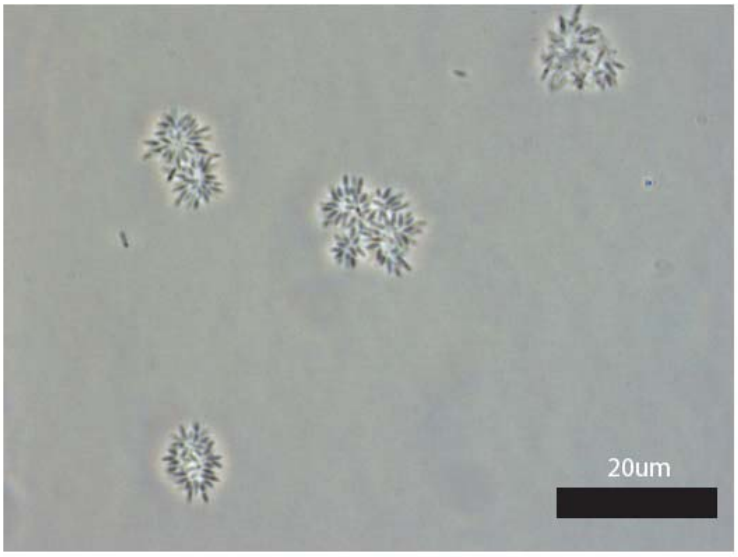
B.



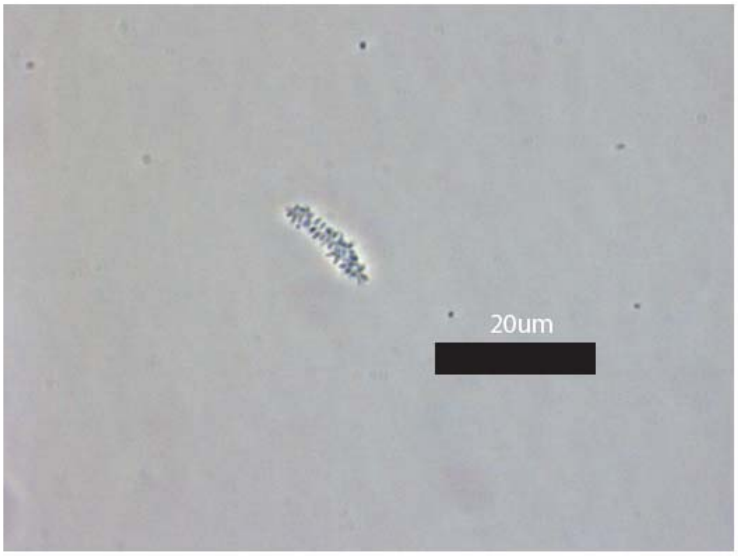
C.



D.



E.



F.

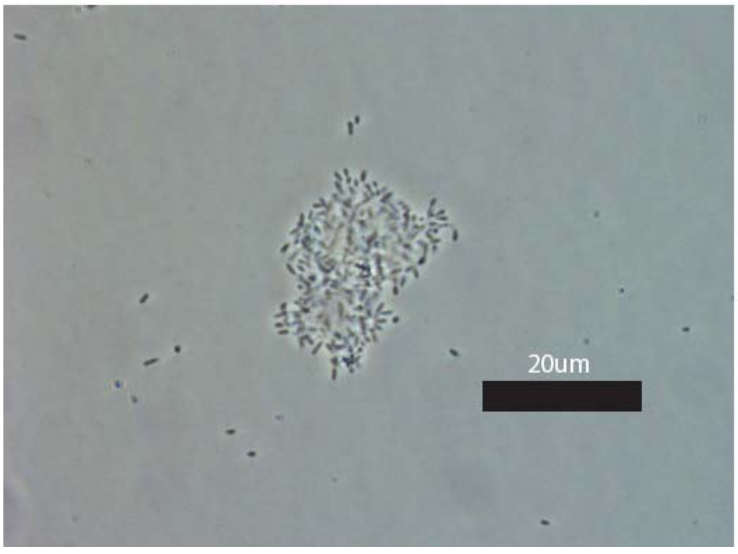
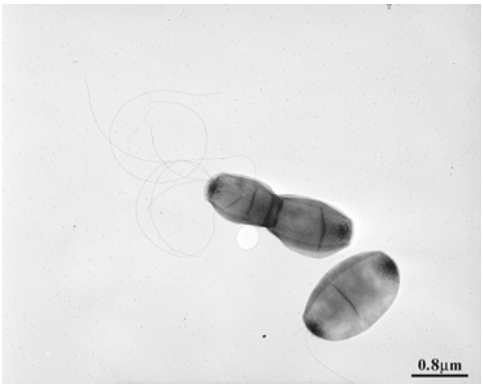
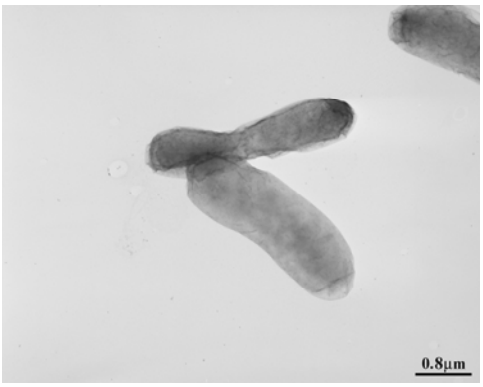


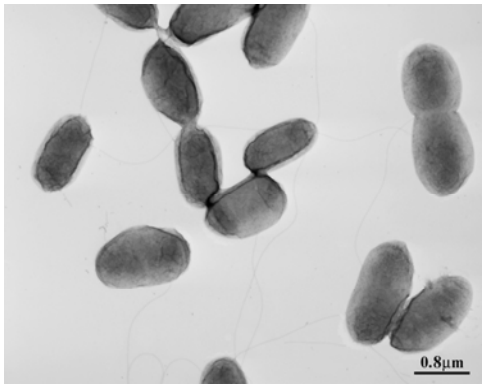
Figure 5. Transmission electron micrographs of ten flagellated and non-flagellated Roseobacter strains. Strains shown here Y3F (A), Y4I (B), ISM (C), S. lac (D), S. pont (E), SE62 (F), SE45 (G), NAS14-1 (H), PSPC2 (I) and EE-36 (J).

A. 

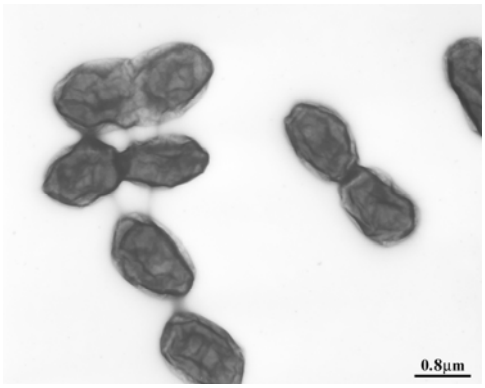
B. 

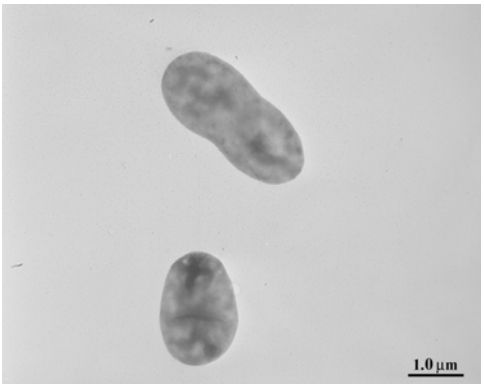
C. 

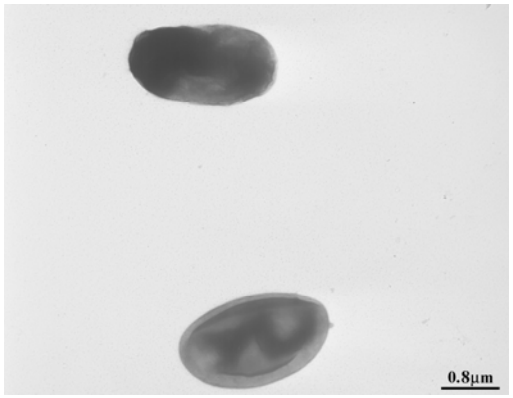
D. 

E. 

F. 

G. 

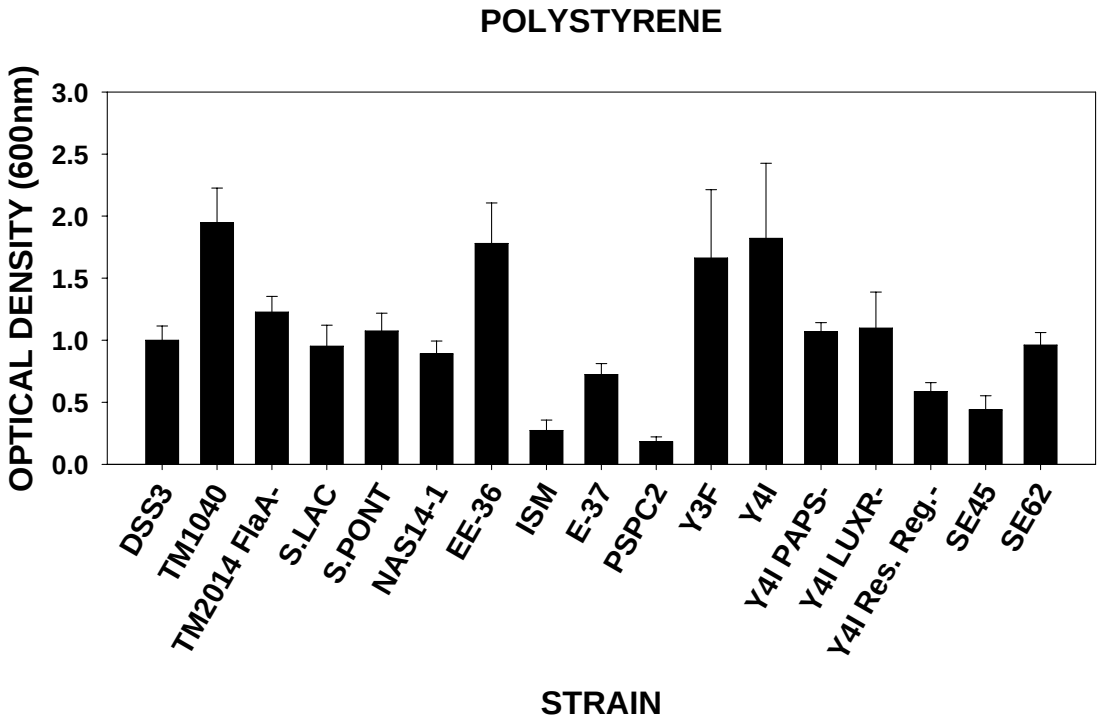
H. 

I 

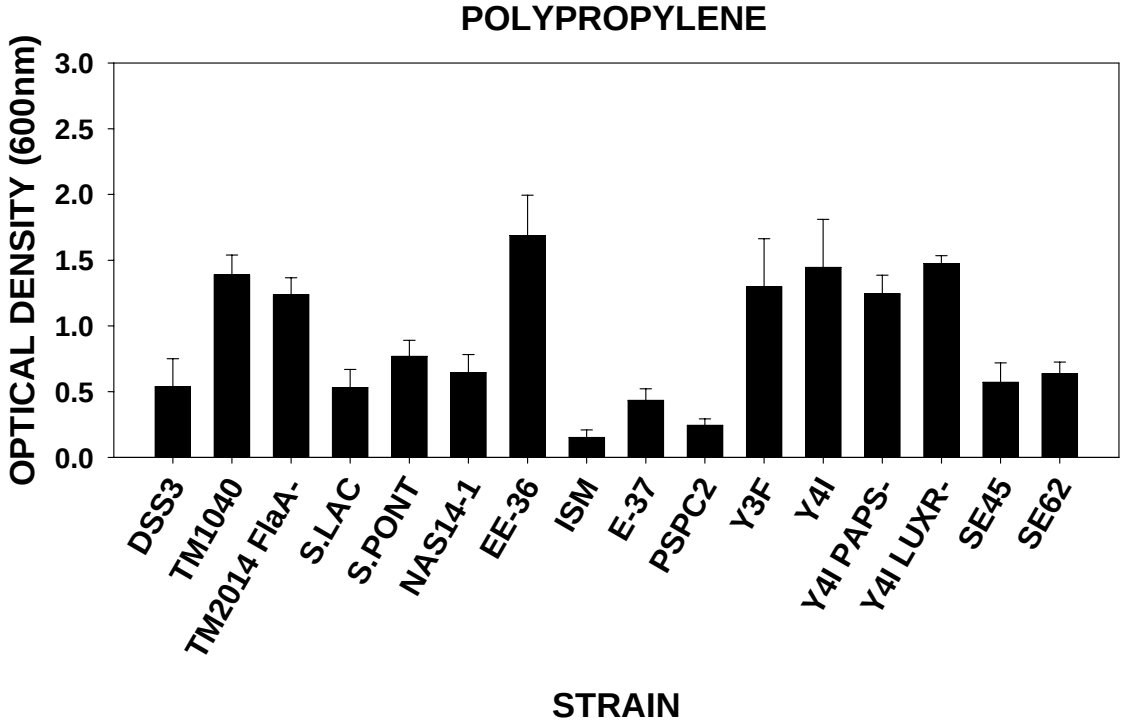
J 

Figure 6. Microtiter dish surface attachment assays of thirteen Roseobacter strains and three mutant strains grown on YTSS (complex) medium in polystyrene (A), polypropylene (B) and polyvinyl chloride (C) dishes.

A.


POLYSTYRENE
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DSS3
TM1040
TM2014 FlaA-
S.LAC
S.PONT
NAS14-1
EE-36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
Y4I Res. Reg.-
SE45
SE62
STRAIN

B.


POLYPROPYLENE
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DSS3
TM1040
TM2014 FlaA-
S.LAC
S.PONT
NAS14-1
EE-36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

C.

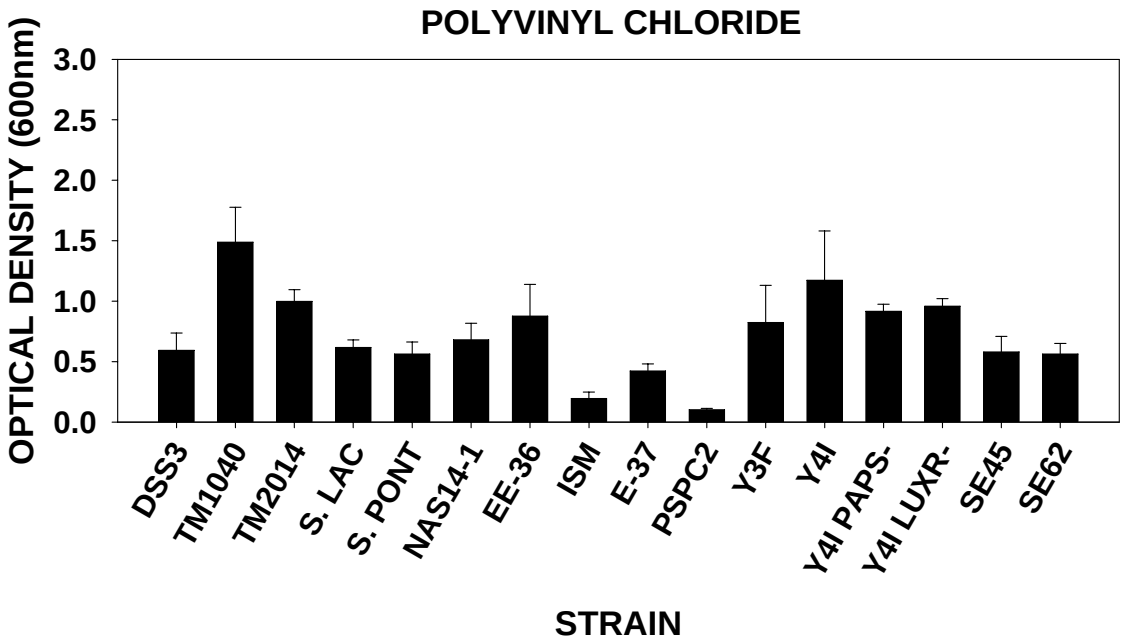
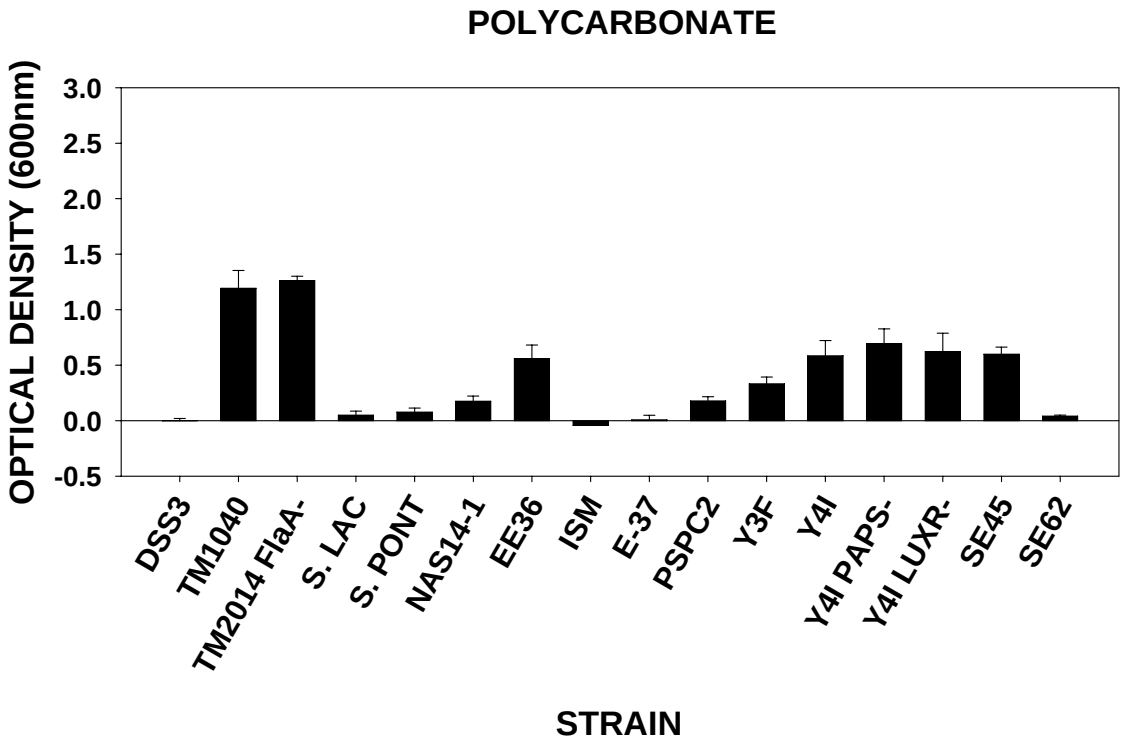
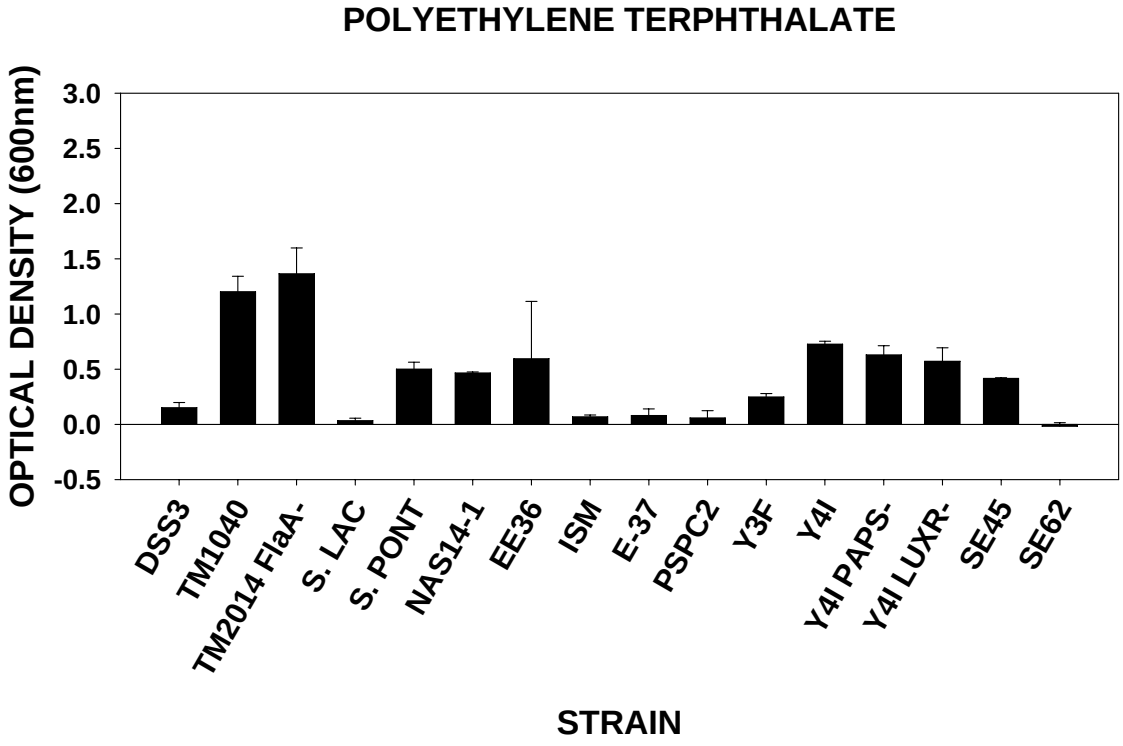

POLYVINYL CHLORIDE
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DSS3
TM1040
TM2014
S. LAC
S. PONT
NAS14-1
EE-36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

Figure 7. Strip surface attachment assay graphs of thirteen Roseobacter strains and three mutant strains grown in YTSS (complex) medium on polycarbonate strips (A), polyethylene terphthalate strips (PET) (B), Teflon™ strips (C) and glass slides (D).

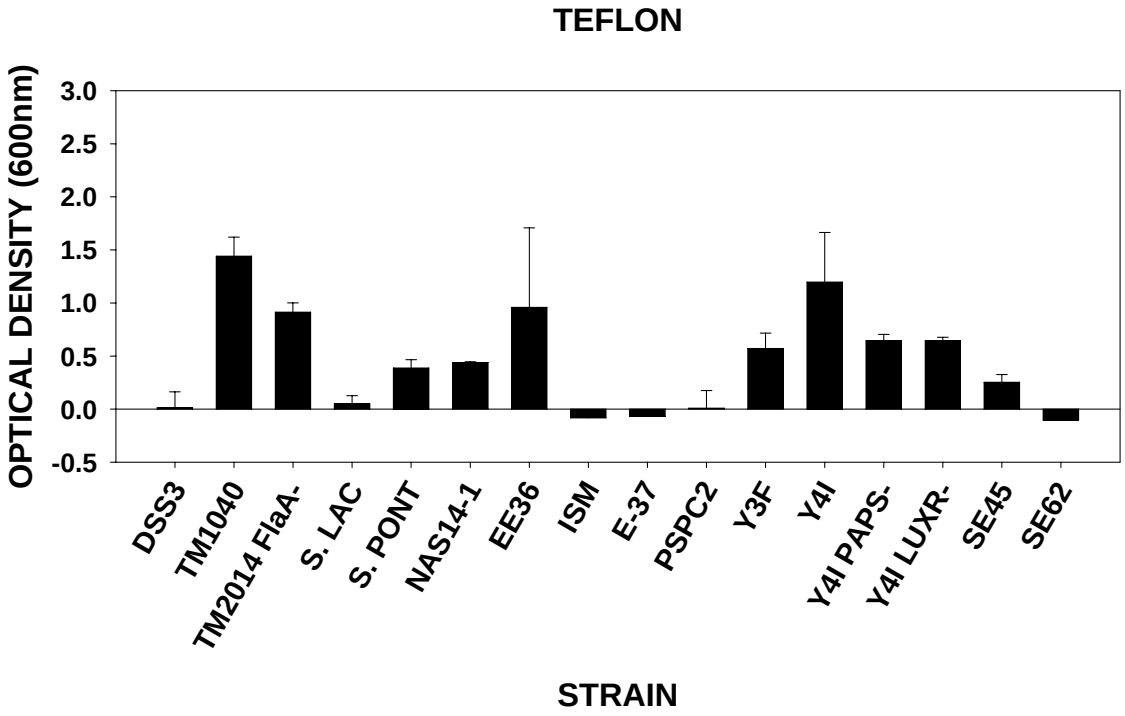
A.


POLYCARBONATE
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
DSS3
TM1040
TM2014 FlaA-
S. LAC
S. PONT
NAS14-1
EE36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

B.


POLYETHYLENE TERPHTHALATE
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
DSS3
TM1040
TM2014 FlaA-
S. LAC
S. PONT
NAS14-1
EE36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

C.


TEFLON
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
DSS3
TM1040
TM2014 FlaA-
S. LAC
S. PONT
NAS14-1
EE36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

D.

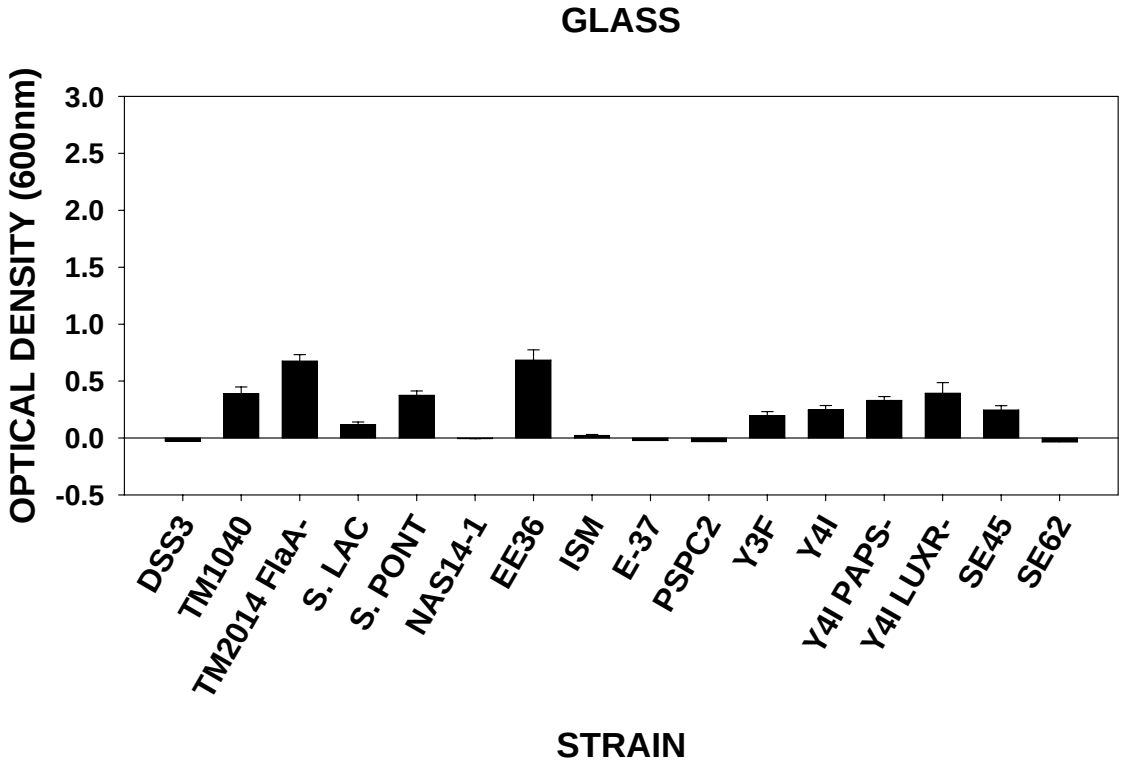
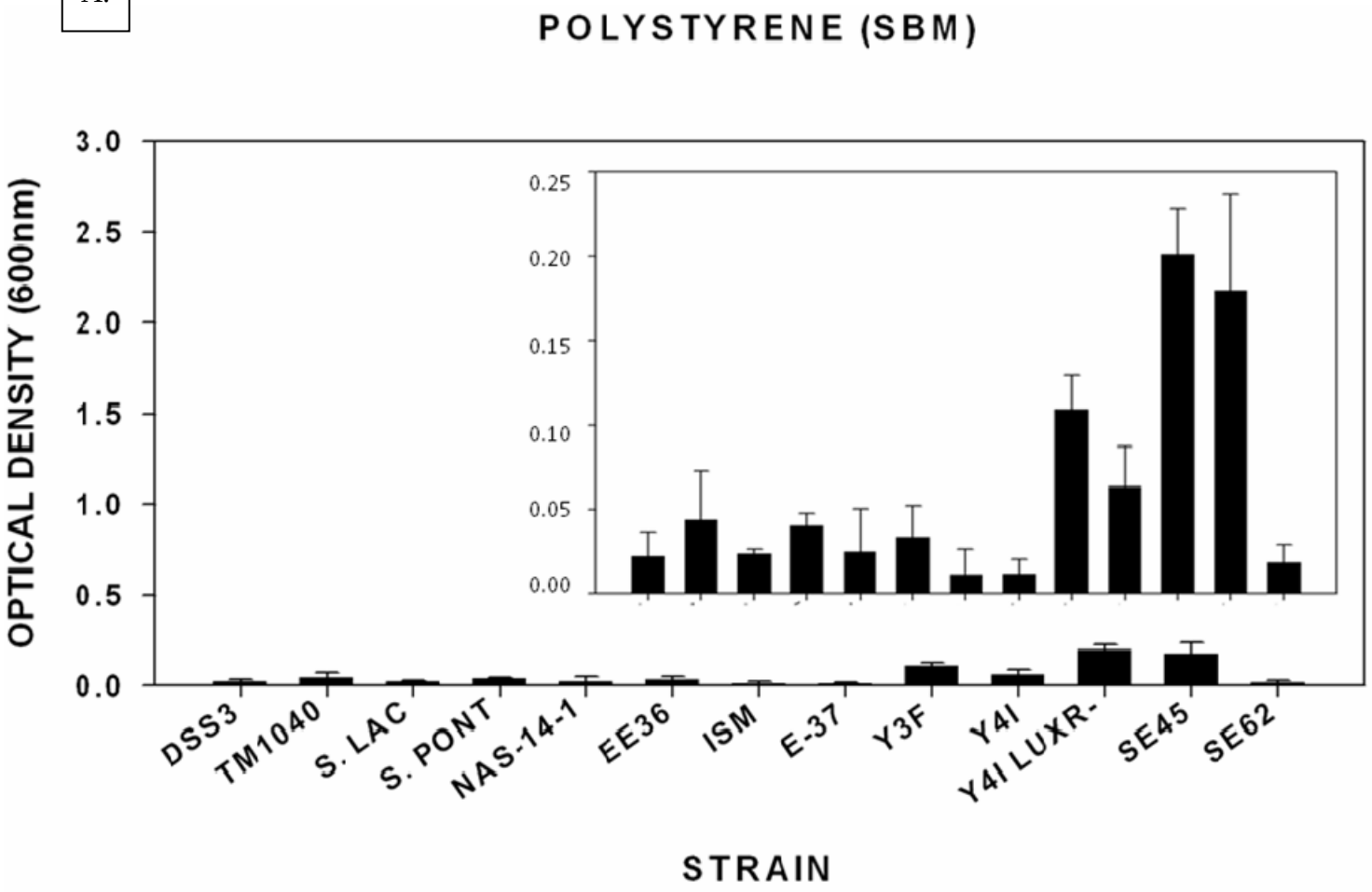
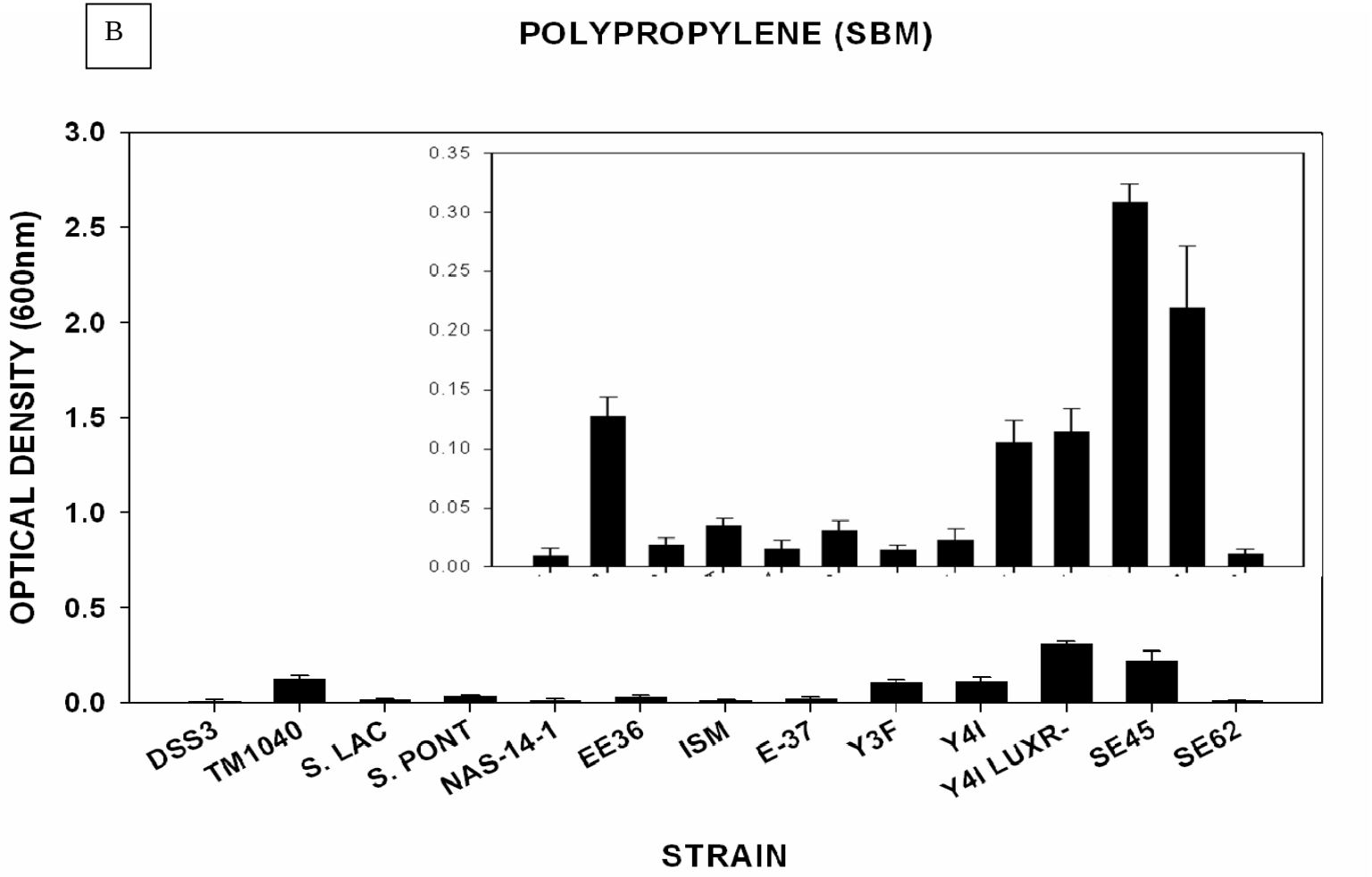

GLASS
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
DSS3
TM1040
TM2014 FlaA-
S. LAC
S. PONT
NAS14-1
EE36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

Figure 8. Microtiter dish surface attachment assays of twelve Roseobacter strains and one mutant strain grown in SBM (minimal) medium + 10mM acetate (10mM glycerol for S. pont) on polystyrene (A), polypropylene (B) and polyvinyl chloride (C). Graphs scaled to show differences between the strains are incorporated in the upper right corner of each graph.

A.


POLYSTYRENE (SBM)
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.25
0.20
0.15
0.10
0.05
0.00
DSS3
TM1040
S. LAC
S. PONT
NAS-14-1
EE36
ISM
E-37
Y3F
Y4I
Y4I LUXR-
SE45
SE62
STRAIN

B


POLYPROPYLENE (SBM)
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
DSS3
TM1040
S. LAC
S. PONT
NAS-14-1
EE36
ISM
E-37
Y3F
Y4I
Y4I LUXR-
SE45
SE62
STRAIN

C.

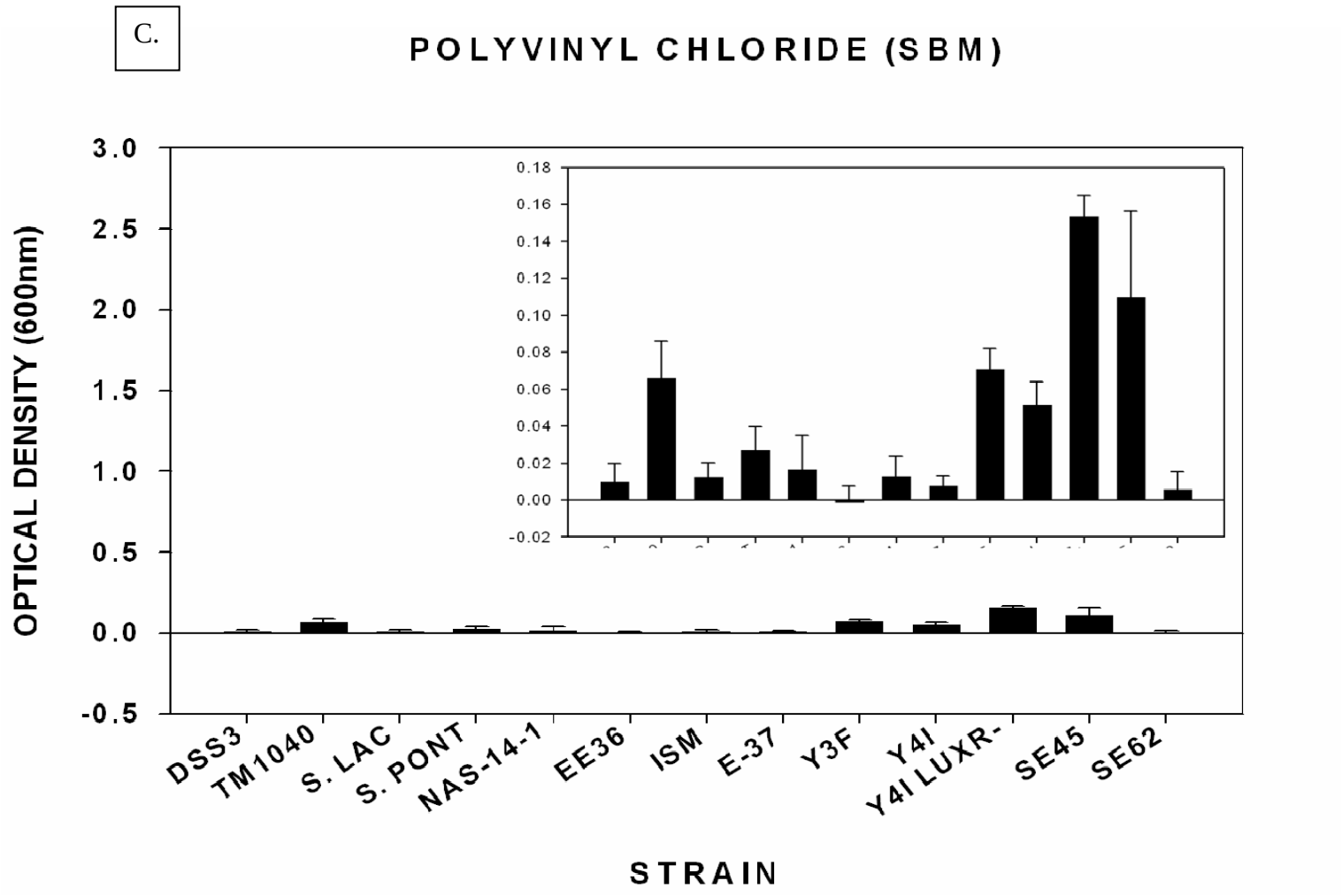
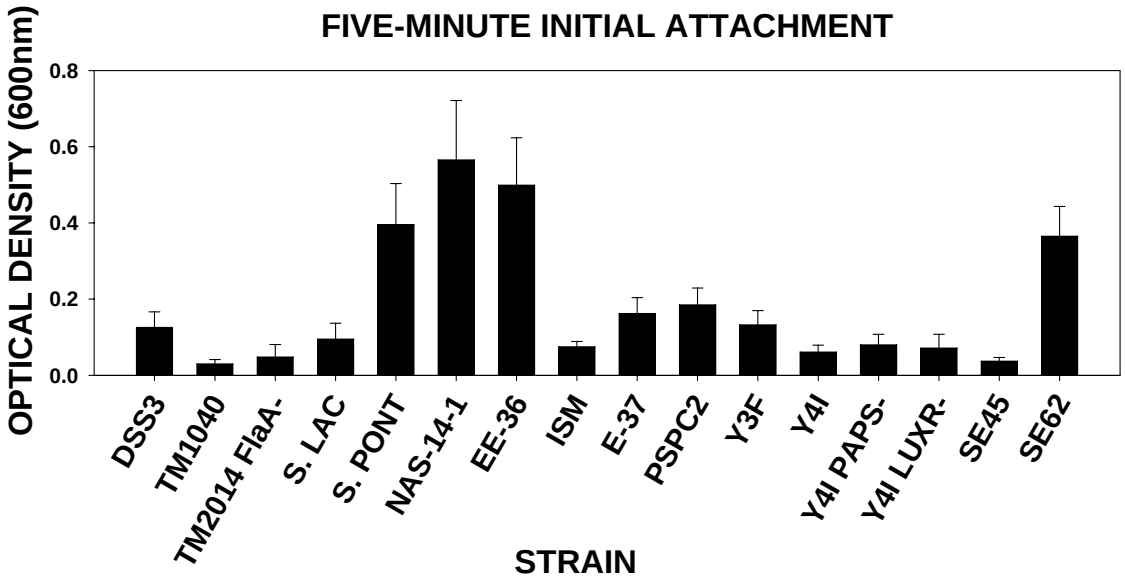
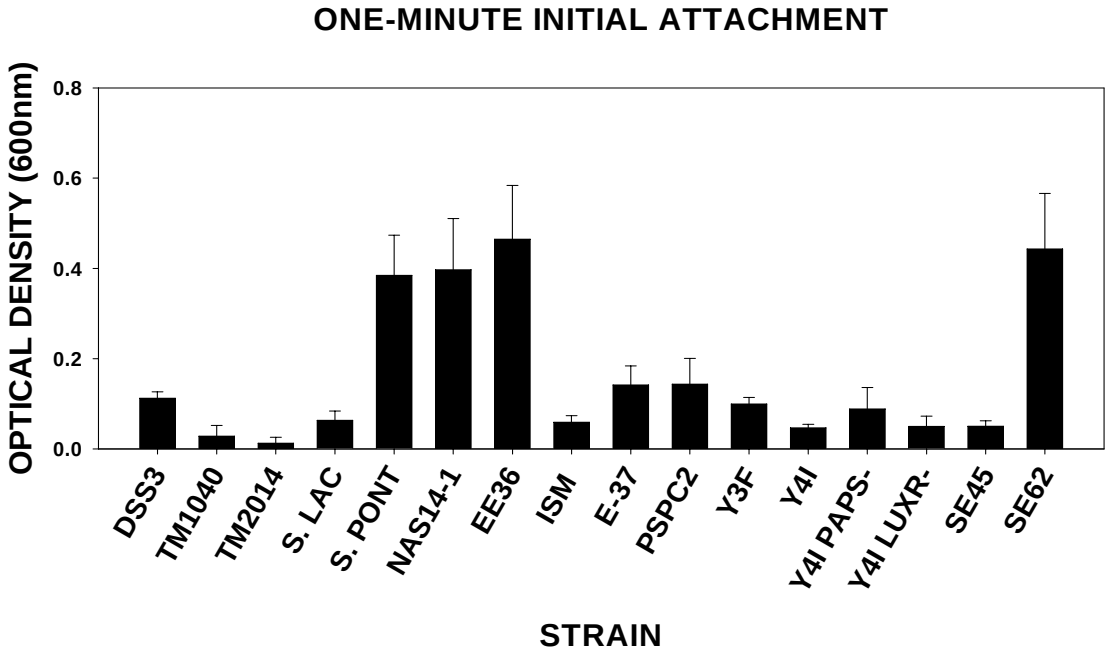

POLYVINYL CHLORIDE (SBM)
OPTICAL DENSITY (600nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
DSS3
TM1040
S. LAC
S. PONT
NAS-14-1
EE36
ISM
E-37
Y3F
Y4I
Y4I LUXR-
SE45
SE62
STRAIN

Figure 9. Initial attachment assays for thirteen Roseobacter strains and three mutant strains grown in YTSS (complex) medium in polystyrene microtiter dishes for 5 min (A), 1 min (B) and 30 sec (C).

A.


FIVE-MINUTE INITIAL ATTACHMENT
OPTICAL DENSITY (600nm)
0.0
0.2
0.4
0.6
0.8
DSS3
TM1040
TM2014 FlaA-
S. LAC
S. PONT
NAS-14-1
EE-36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

B.


ONE-MINUTE INITIAL ATTACHMENT
OPTICAL DENSITY (600nm)
0.0
0.2
0.4
0.6
0.8
DSS3
TM1040
TM2014
S. LAC
S. PONT
NAS14-1
EE36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

C.

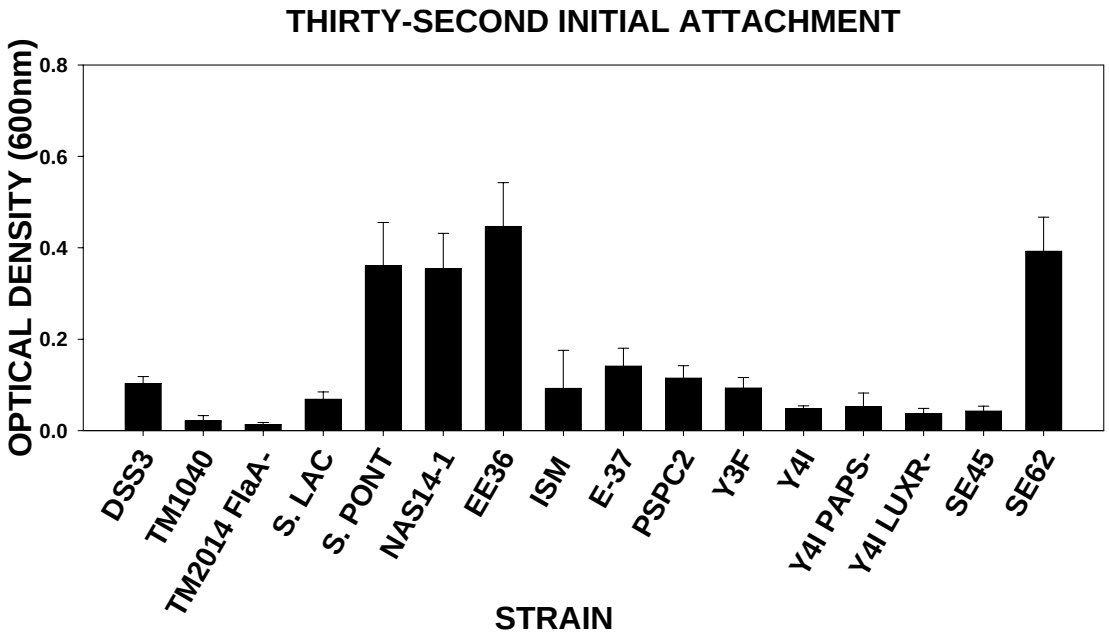

THIRTY-SECOND INITIAL ATTACHMENT
OPTICAL DENSITY (600nm)
0.0
0.2
0.4
0.6
0.8
DSS3
TM1040
TM2014 FlaA-
S. LAC
S. PONT
NAS14-1
EE36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I PAPS-
Y4I LUXR-
SE45
SE62
STRAIN

Figure 10. Phase contrast microscope images of strains grown in YTSS (complex) broth attached to a glass slide. Strains Y4I (A), Y3F (B), S. pont (C), NAS14-1 (D), EE-36 (E) and TM1040 (F) all demonstrated attachment to glass during the incubation period.

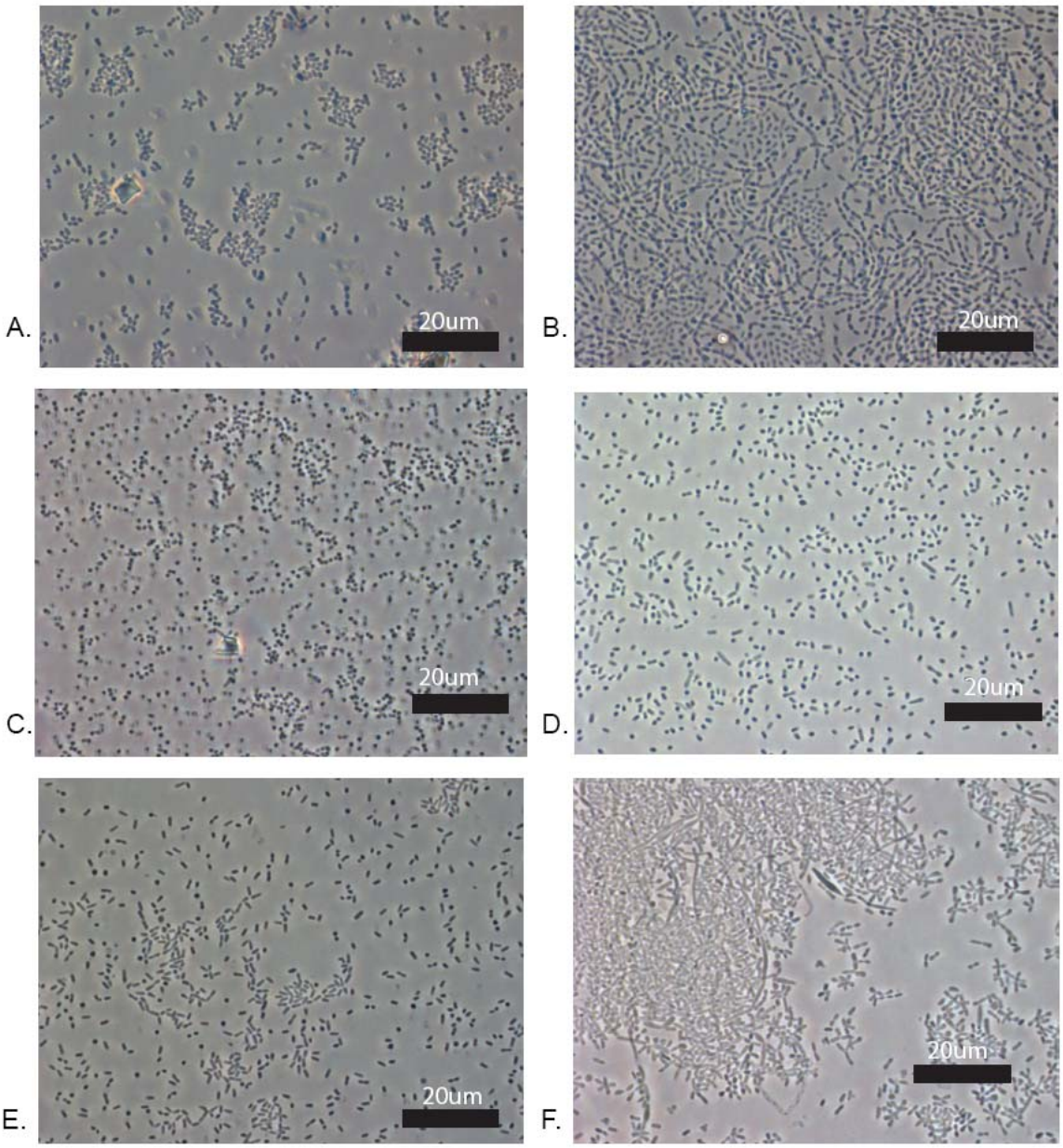

A.
20um
B.
20um
C.
20um
D.
20um
E.
20um
F.
20um

Figure 11. Gel showing non-specific amplification of degenerate primer set FlgH. Lanes 1-12 show Failsafe buffers A-L using PSPC2 as template. Lanes 13-24 show Failsafe buffers A-L using SE45 as template. Marker lanes (M) contain a 1kb ladder. Faint product of expected size of 1000bp.

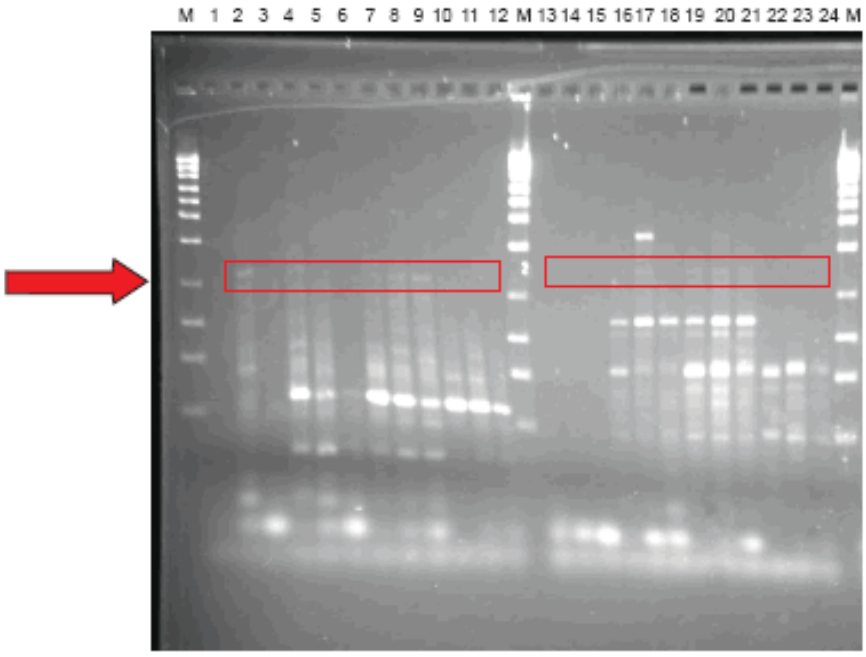

M 1 2 3 4 5 6 7 8 9 10 11 12 M 13 14 15 16 17 18 19 20 21 22 23 24 M

Figure 12. Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on FlgH protein sequences. The tree is based on positions 17 to 364 of the flagellar p-ring protein (*E. coli* numbering system). The tree was constructed using Mega 4 and the neighbor-joining method. GenBank accession numbers are provided in parentheses. Asterisks show strains that were examined in this study. The bar represents p-distance (evolutionary). Bootstrap values > 50% are shown at branch nodes (1000 iterations).

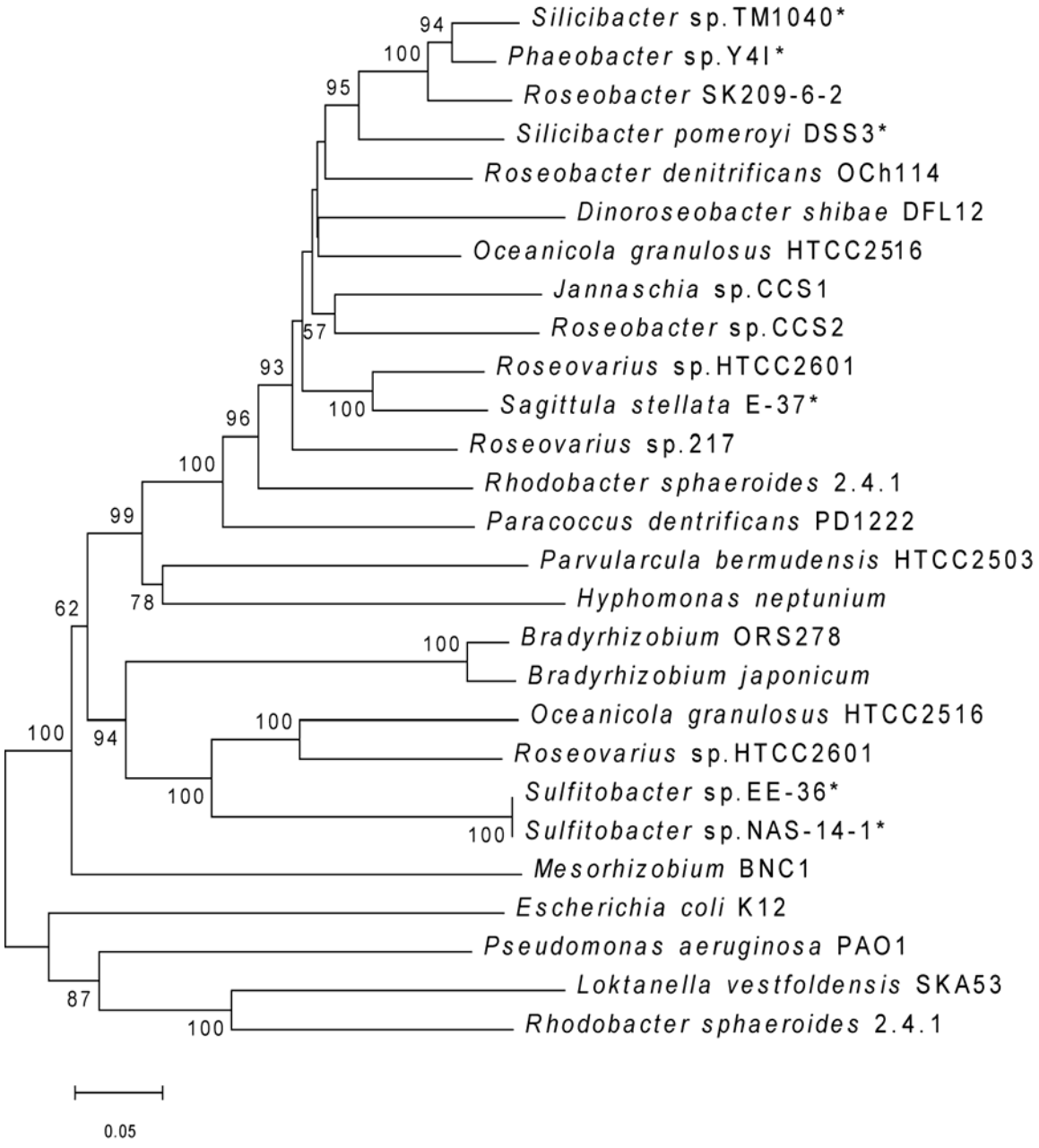

94
100
95
57
93
100
96
100
99
78
62
100
94
100
100
100
100
87
100
Silicibacter sp.TM1040*
Phaeobacter sp.Y4I*
Roseobacter SK209-6-2
Silicibacter pomeroyi DSS3*
Roseobacter denitrificans OCh114
Dinoroseobacter shibae DFL12
Oceanicola granulosus HTCC2516
Jannaschia sp.CCS1
Roseobacter sp.CCS2
Roseovarius sp.HTCC2601
Sagittula stellata E-37*
Roseovarius sp.217
Rhodobacter sphaeroides 2.4.1
Paracoccus dentrificans PD1222
Parvularcula bermudensis HTCC2503
Hyphomonas neptunium
Bradyrhizobium ORS278
Bradyrhizobium japonicum
Oceanicola granulosus HTCC2516
Roseovarius sp.HTCC2601
Sulfitobacter sp.EE-36*
Sulfitobacter sp.NAS-14-1*
Mesorhizobium BNC1
Escherichia coli K12
Pseudomonas aeruginosa PAO1
Loktanella vestfoldensis SKA53
Rhodobacter sphaeroides 2.4.1
0.05

Figure 13. Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on FliF protein sequences. The tree is based on positions 51 to 384 of the flagellar m-ring protein (*E. coli* numbering system). The tree was constructed using Mega 4 and the neighbor-joining method. GenBank accession numbers are provided in parentheses. Asterisks show strains that were examined in this study. The bar represents p-distance (evolutionary). Bootstrap values > 50% are shown at branch nodes (1000 iterations).

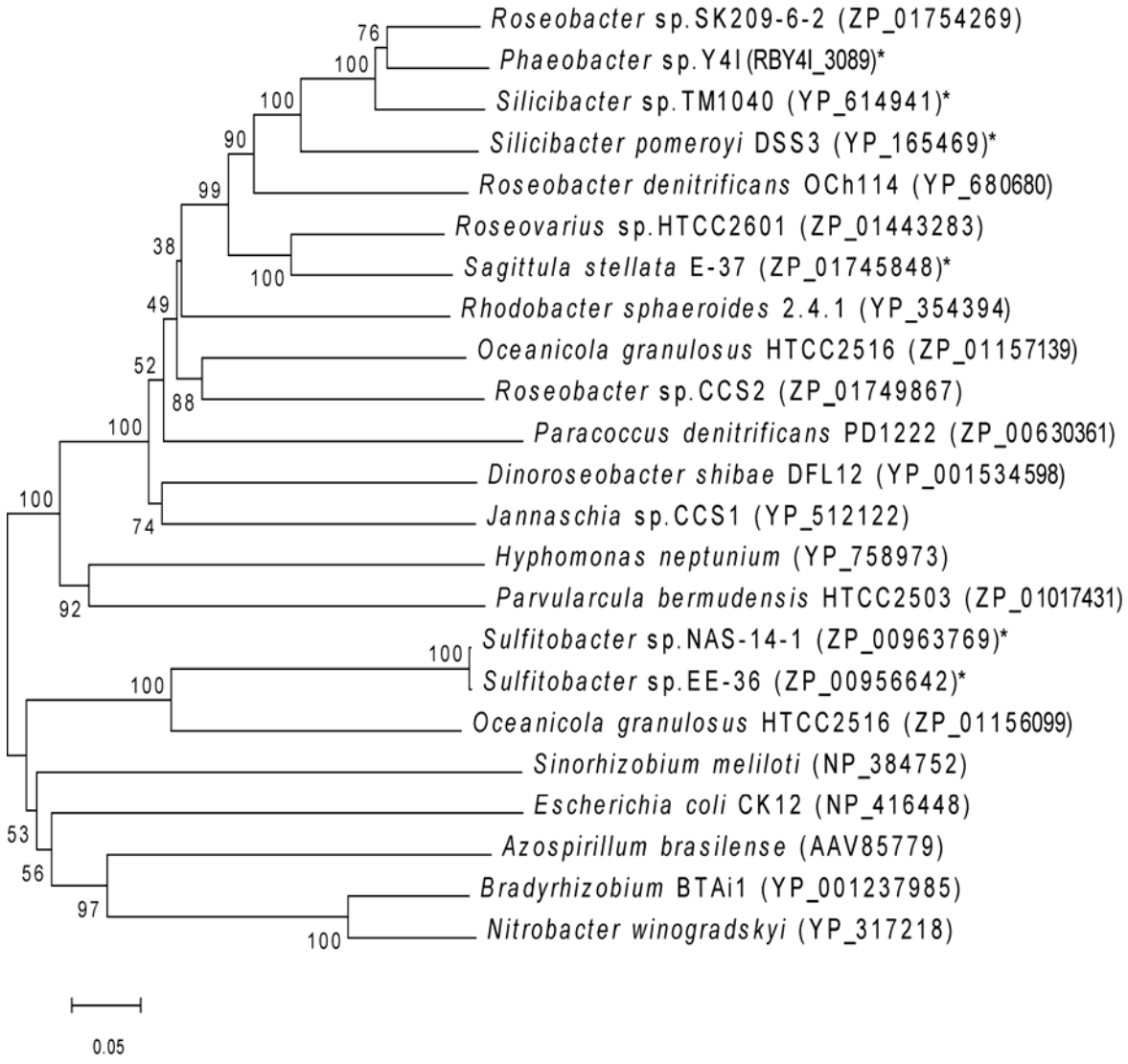

Roseobacter sp.SK209-6-2 (ZP_01754269)
Phaeobacter sp.Y4I (RBY4I_3089)*
Silicibacter sp.TM1040 (YP_614941)*
Silicibacter pomeroyi DSS3 (YP_165469)*
Roseobacter denitrificans OCh114 (YP_680680)
Roseovarius sp.HTCC2601 (ZP_01443283)
Sagittula stellata E-37 (ZP_01745848)*
Rhodobacter sphaeroides 2.4.1 (YP_354394)
Oceanicola granulosus HTCC2516 (ZP_01157139)
Roseobacter sp.CCS2 (ZP_01749867)
Paracoccus denitrificans PD1222 (ZP_00630361)
Dinoroseobacter shibae DFL12 (YP_001534598)
Jannaschia sp.CCS1 (YP_512122)
Hyphomonas neptunium (YP_758973)
Parvularcula bermudensis HTCC2503 (ZP_01017431)
Sulfitobacter sp.NAS-14-1 (ZP_00963769)*
Sulfitobacter sp.EE-36 (ZP_00956642)*
Oceanicola granulosus HTCC2516 (ZP_01156099)
Sinorhizobium meliloti (NP_384752)
Escherichia coli CK12 (NP_416448)
Azospirillum brasilense (AAV85779)
Bradyrhizobium BTAi1 (YP_001237985)
Nitrobacter winogradskyi (YP_317218)
76
100
100
90
99
38
100
49
52
88
100
100
74
92
100
100
53
56
97
100
0.05

Figure 14. Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on CheA protein sequences. The tree is based on positions 263 to 640 of the flagellar chemotaxis CheA protein (*E. coli* numbering system). The tree was constructed using Mega 4 and the neighbor-joining method. GenBank accession numbers are provided in parentheses. Asterisks show strains that were examined in this study. The bar represents p-distance (evolutionary). Bootstrap values > 50% are shown at branch nodes (1000 iterations).

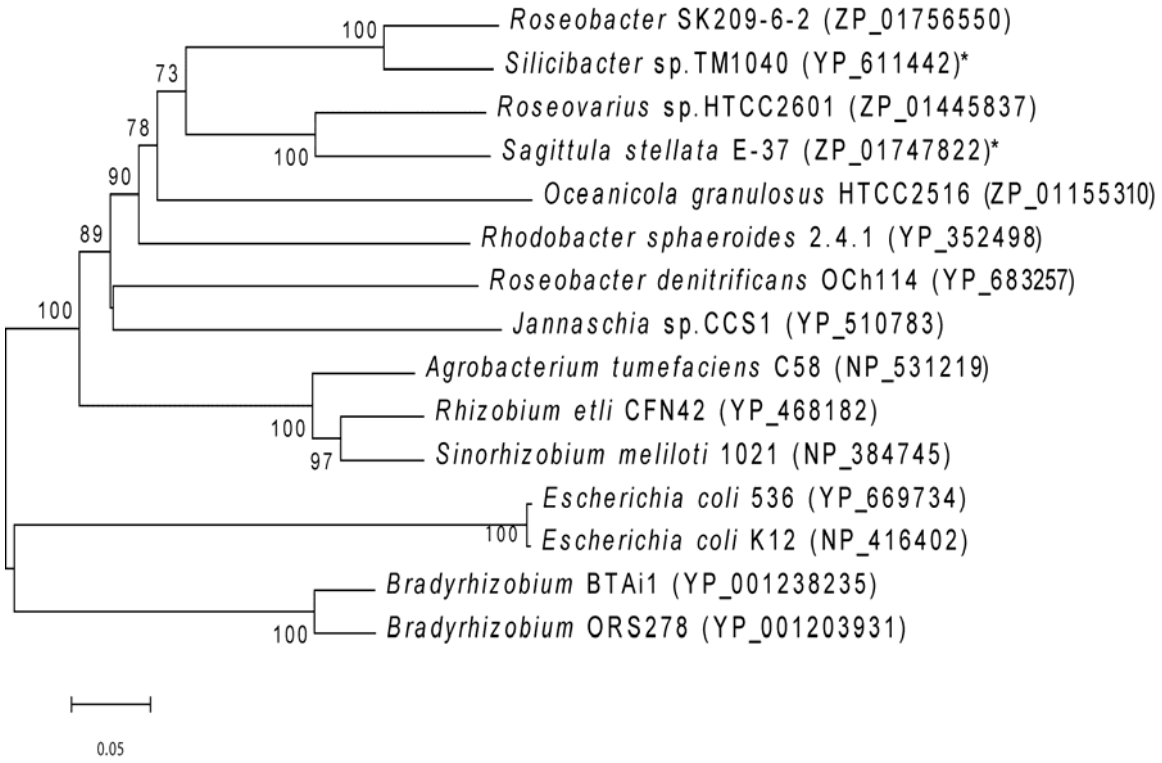

Roseobacter SK209-6-2 (ZP_01756550)
Silicibacter sp.TM1040 (YP_611442)*
Roseovarius sp.HTCC2601 (ZP_01445837)
Sagittula stellata E-37 (ZP_01747822)*
Oceanicola granulosus HTCC2516 (ZP_01155310)
Rhodobacter sphaeroides 2.4.1 (YP_352498)
Roseobacter denitrificans OCh114 (YP_683257)
Jannaschia sp.CCS1 (YP_510783)
Agrobacterium tumefaciens C58 (NP_531219)
Rhizobium etli CFN42 (YP_468182)
Sinorhizobium meliloti 1021 (NP_384745)
Escherichia coli 536 (YP_669734)
Escherichia coli K12 (NP_416402)
Bradyrhizobium BTAi1 (YP_001238235)
Bradyrhizobium ORS278 (YP_001203931)
100
73
78
100
90
89
100
100
97
100
100
0.05

Figure 15. Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on LuxI protein sequences. The tree is based on positions 22 to 109 of the LuxI protein (*Vibrio fisheri* numbering system). The tree was constructed using Mega 4 and the neighbor-joining method. GenBank accession numbers are provided in parentheses. Asterisks show strains that were examined in this study. The bar represents p-distance (evolutionary). Bootstrap values > 50% are shown at branch nodes (1000 iterations).

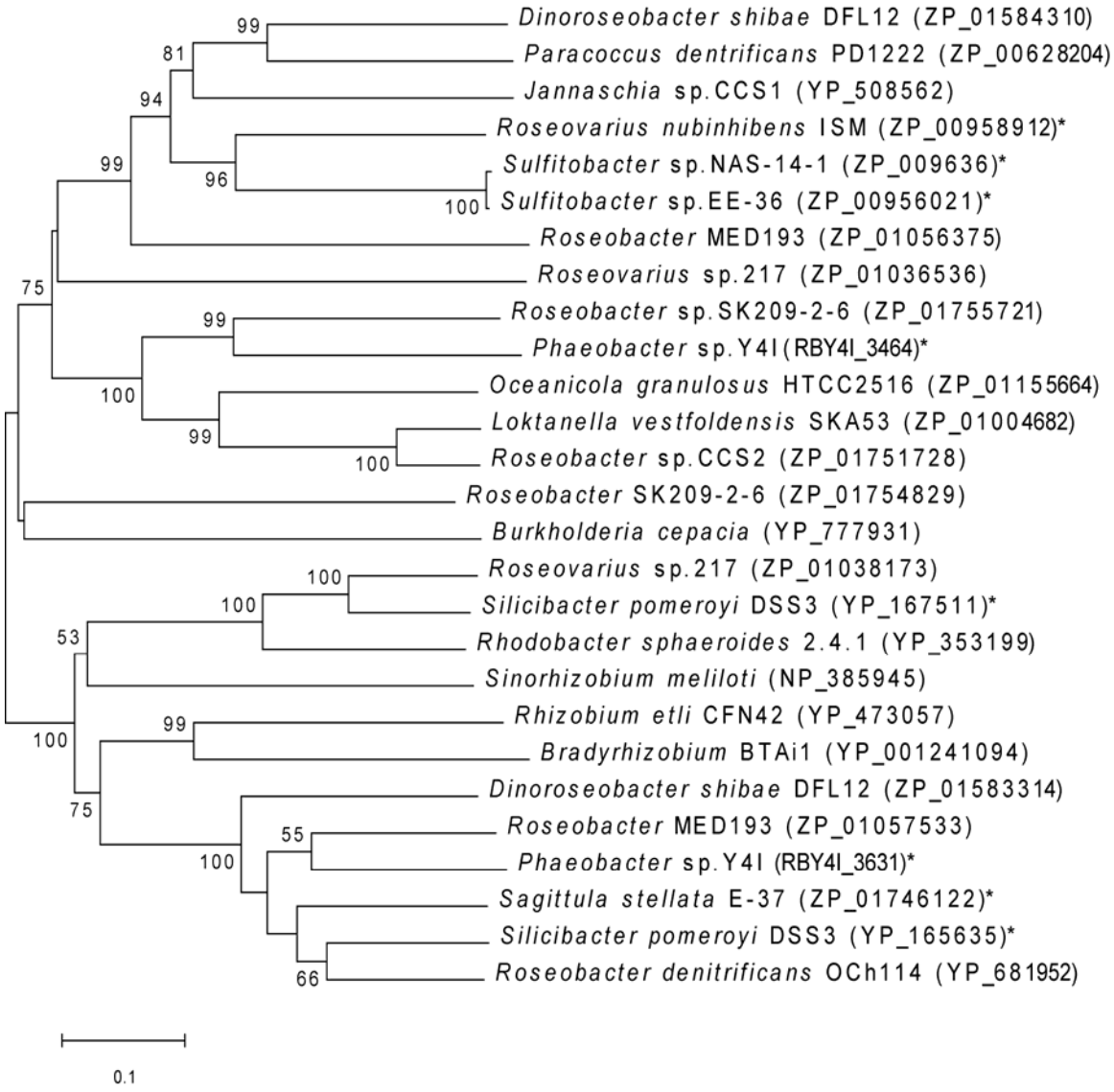

Dinoroseobacter shibae DFL12 (ZP_01584310)
Paracoccus dentrificans PD1222 (ZP_00628204)
Jannaschia sp.CCS1 (YP_508562)
Roseovarius nubinhibens ISM (ZP_00958912)*
Sulfitobacter sp.NAS-14-1 (ZP_009636)*
Sulfitobacter sp.EE-36 (ZP_00956021)*
Roseobacter MED193 (ZP_01056375)
Roseovarius sp.217 (ZP_01036536)
Roseobacter sp.SK209-2-6 (ZP_01755721)
Phaeobacter sp.Y4I(RBY4I_3464)*
Oceanicola granulosus HTCC2516 (ZP_01155664)
Loktanella vestfoldensis SKA53 (ZP_01004682)
Roseobacter sp.CCS2 (ZP_01751728)
Roseobacter SK209-2-6 (ZP_01754829)
Burkholderia cepacia (YP_777931)
Roseovarius sp.217 (ZP_01038173)
Silicibacter pomeroyi DSS3 (YP_167511)*
Rhodobacter sphaeroides 2.4.1 (YP_353199)
Sinorhizobium meliloti (NP_385945)
Rhizobium etli CFN42 (YP_473057)
Bradyrhizobium BTAi1 (YP_001241094)
Dinoroseobacter shibae DFL12 (ZP_01583314)
Roseobacter MED193 (ZP_01057533)
Phaeobacter sp.Y4I (RBY4I_3631)*
Sagittula stellata E-37 (ZP_01746122)*
Silicibacter pomeroyi DSS3 (YP_165635)*
Roseobacter denitrificans OCh114 (YP_681952)
99
81
94
99
96
100
75
99
100
99
100
100
100
53
100
99
75
55
100
66
0.1

Figure 16. Color key for all gene diagrams (Figures 17-20).

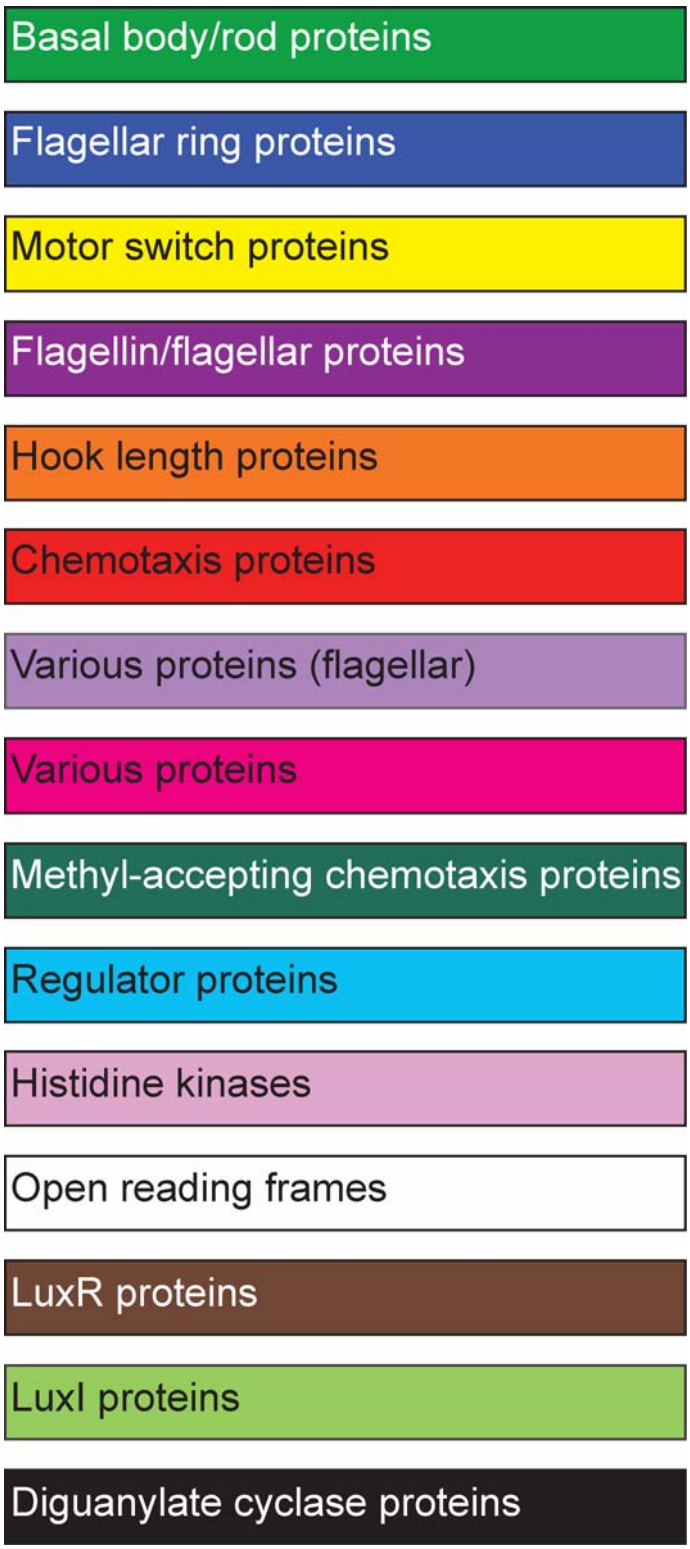
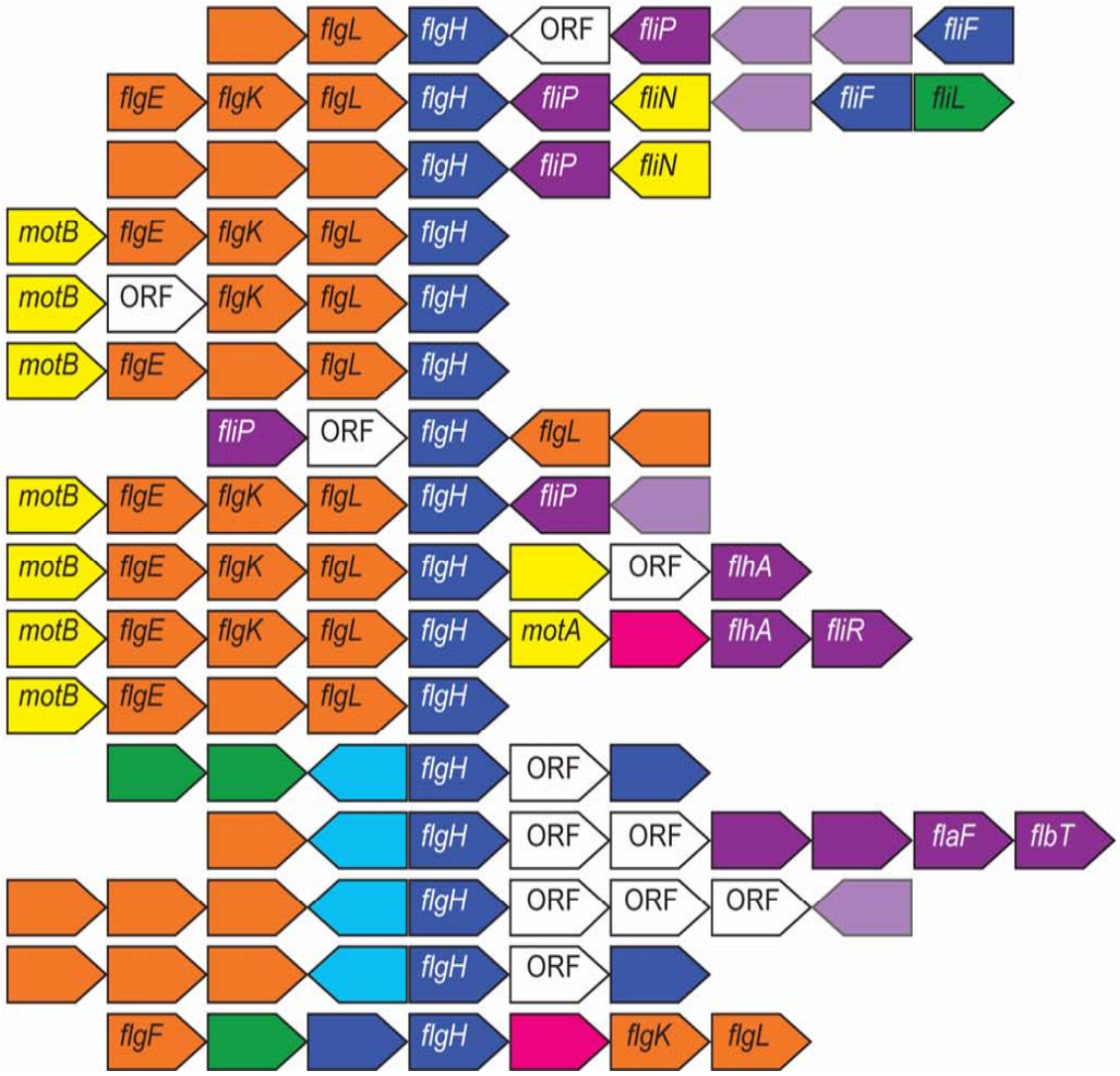

Basal body/rod proteins
Flagellar ring proteins
Motor switch proteins
Flagellin/flagellar proteins
Hook length proteins
Chemotaxis proteins
Various proteins (flagellar)
Various proteins
Methyl-accepting chemotaxis proteins
Regulator proteins
Histidine kinases
Open reading frames
LuxR proteins
LuxI proteins
Diguanylate cyclase proteins

Figure 17. Gene diagrams for *flgH* and flanking genes of interest.



Silicibacter sp. TM1040

Roseobacter sp. SK209-2-6

Silicibacter pomeroyi DSS-3

Roseobacter denitrificans

Dinoroseobacter shibae

Oceanicola granulosus

Jannaschia sp. CCS1

Roseobacter sp. CCS2

Roseovarius sp. HTCC2601

Sagittula stellata E-37

Roseovarius sp. 217

Oceanicola granulosus (2)

Roseovarius sp. HTCC2601 (2)

Sulfitobacter sp. EE-36

Sulfitobacter sp.NAS14-1

Loktanella vestfoldensis

Figure 18. Gene diagram for *fliF* and flanking genes of interest.

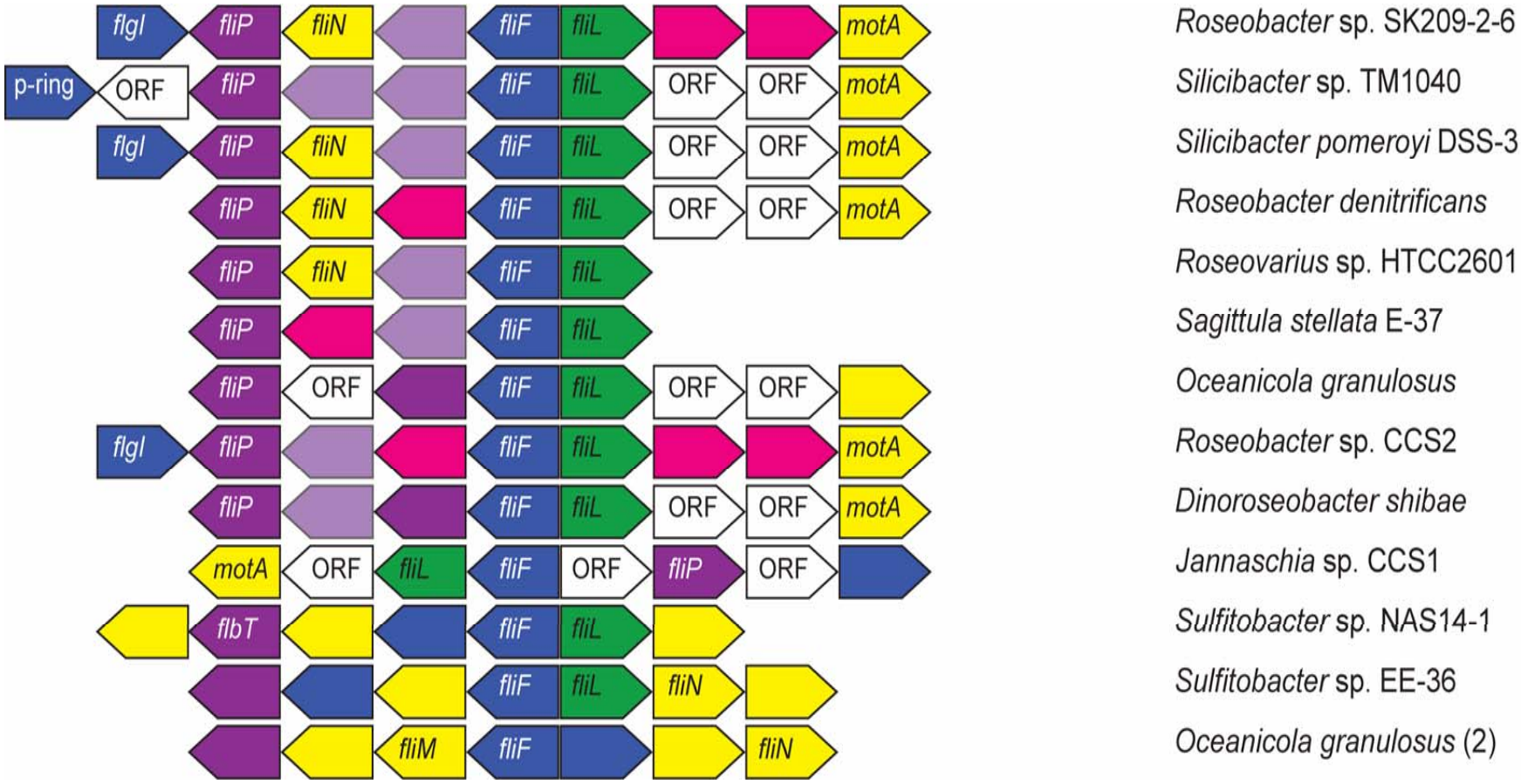

flgI fliP fliN fliF fliL motA
p-ring ORF fliP fliF fliL ORF ORF motA
flgI fliP fliN fliF fliL ORF ORF motA
fliP fliN fliF fliL ORF ORF motA
fliP fliN fliF fliL
fliP fliF fliL
fliP ORF fliF fliL ORF ORF
flgI fliP fliF fliL motA
fliP fliF fliL ORF ORF motA
motA ORF fliL fliF ORF fliP ORF
flbT fliF fliL
fliF fliL fliN
fliM fliF fliN
Roseobacter sp. SK209-2-6
Silicibacter sp. TM1040
Silicibacter pomeroyi DSS-3
Roseobacter denitrificans
Roseovarius sp. HTCC2601
Sagittula stellata E-37
Oceanicola granulosus
Roseobacter sp. CCS2
Dinoroseobacter shibae
Jannaschia sp. CCS1
Sulfitobacter sp. NAS14-1
Sulfitobacter sp. EE-36
Oceanicola granulosus (2)

Figure 19. Gene diagram for *cheA* and flanking genes of interest.

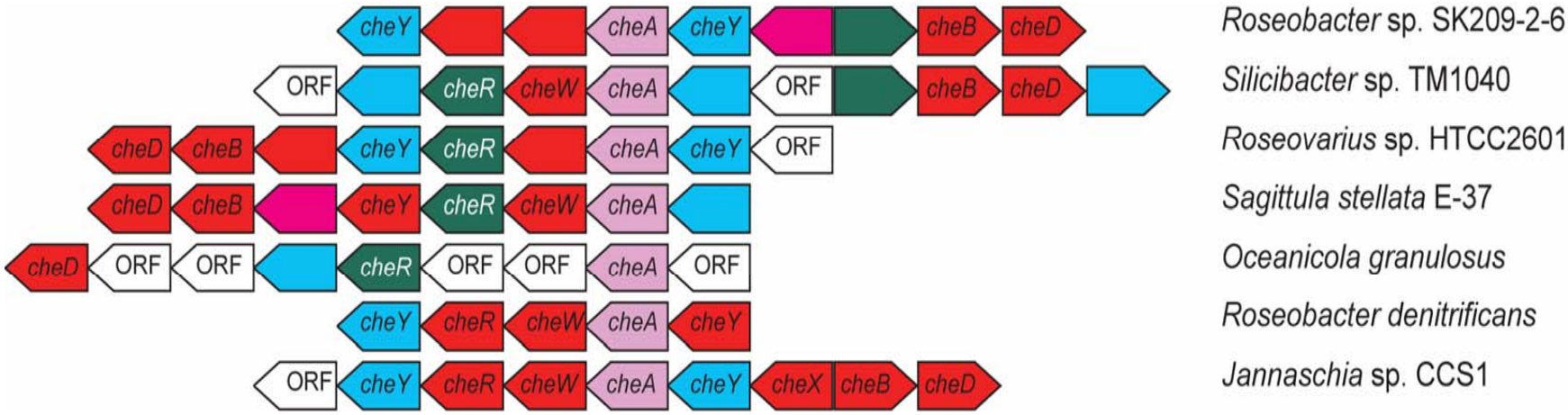
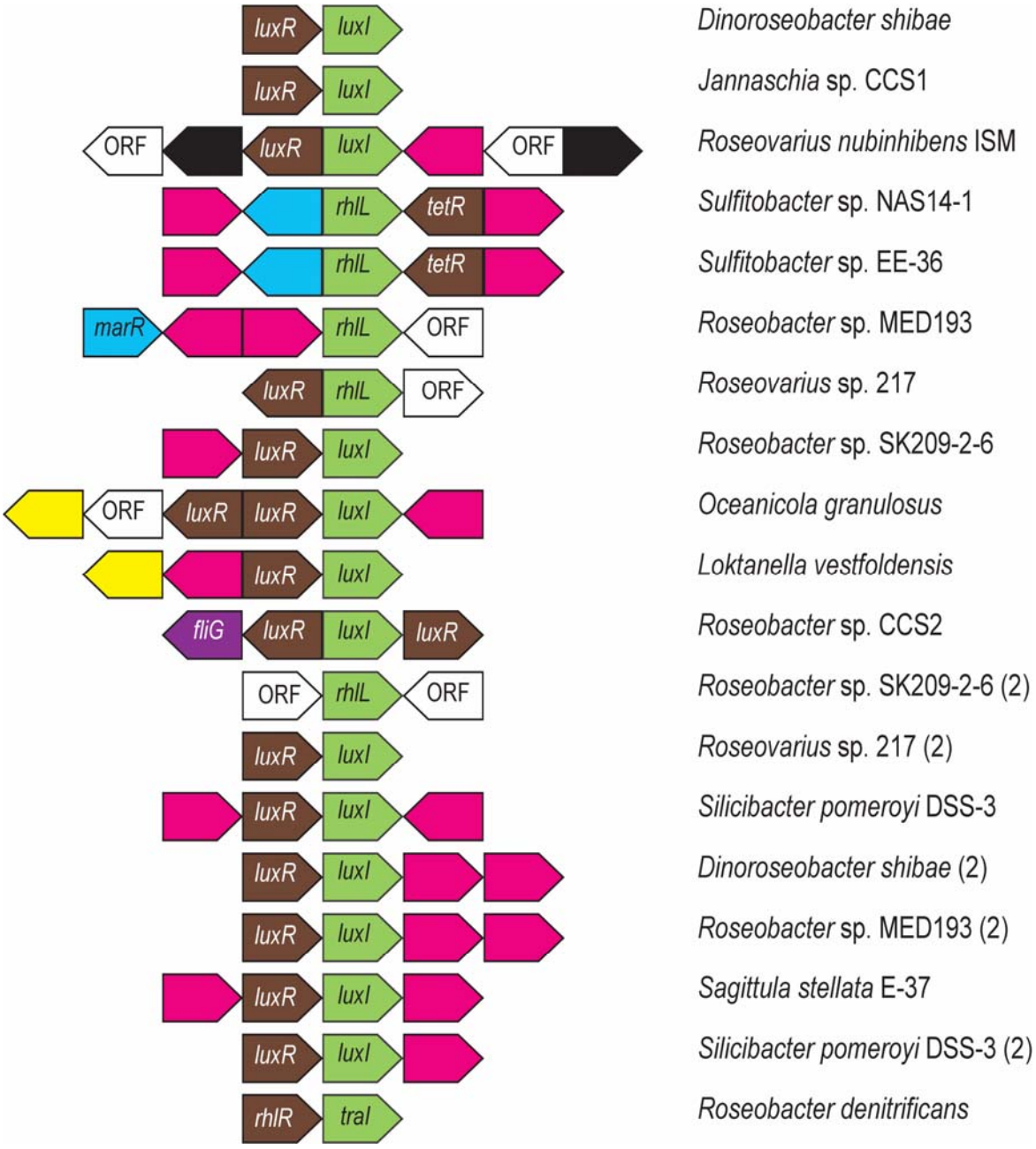
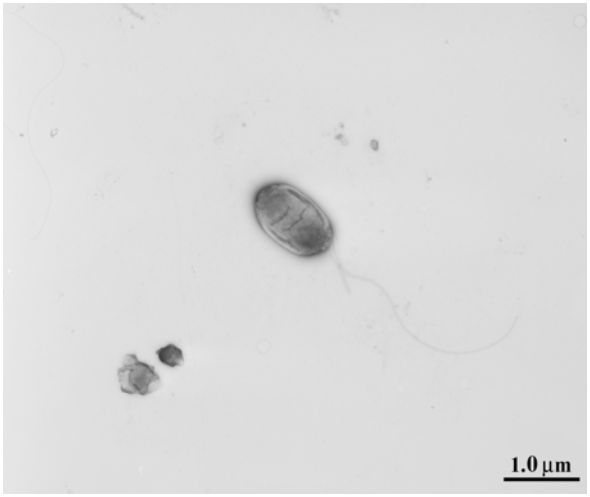

cheY cheA cheY cheB cheD
ORF cheR cheW cheA ORF cheB cheD
cheD cheB cheY cheR cheA cheY ORF
cheD cheB cheY cheR cheW cheA
cheD ORF ORF cheR ORF ORF cheA ORF
cheY cheR cheW cheA cheY
ORF cheY cheR cheW cheA cheY cheX cheB cheD
Roseobacter sp. SK209-2-6
Silicibacter sp. TM1040
Roseovarius sp. HTCC2601
Sagittula stellata E-37
Oceanicola granulosus
Roseobacter denitrificans
Jannaschia sp. CCS1

Figure 20. Gene diagram for *luxI* and flanking genes of interest.


luxR luxI
Dinoroseobacter shibae
luxR luxI
Jannaschia sp. CCS1
ORF luxR luxI ORF
Roseovarius nubinhibens ISM
rhlL tetR
Sulfitobacter sp. NAS14-1
rhlL tetR
Sulfitobacter sp. EE-36
marR rhlL ORF
Roseobacter sp. MED193
luxR rhlL ORF
Roseovarius sp. 217
luxR luxI
Roseobacter sp. SK209-2-6
ORF luxR luxR luxI
Oceanicola granulosus
luxR luxI
Loktanella vestfoldensis
fliG luxR luxI luxR
Roseobacter sp. CCS2
ORF rhlL ORF
Roseobacter sp. SK209-2-6 (2)
luxR luxI
Roseovarius sp. 217 (2)
luxR luxI
Silicibacter pomeroyi DSS-3
luxR luxI
Dinoroseobacter shibae (2)
luxR luxI
Roseobacter sp. MED193 (2)
luxR luxI
Sagittula stellata E-37
luxR luxI
Silicibacter pomeroyi DSS-3 (2)
rhlR traI
Roseobacter denitrificans

APPENDIX B: Additional Figures.

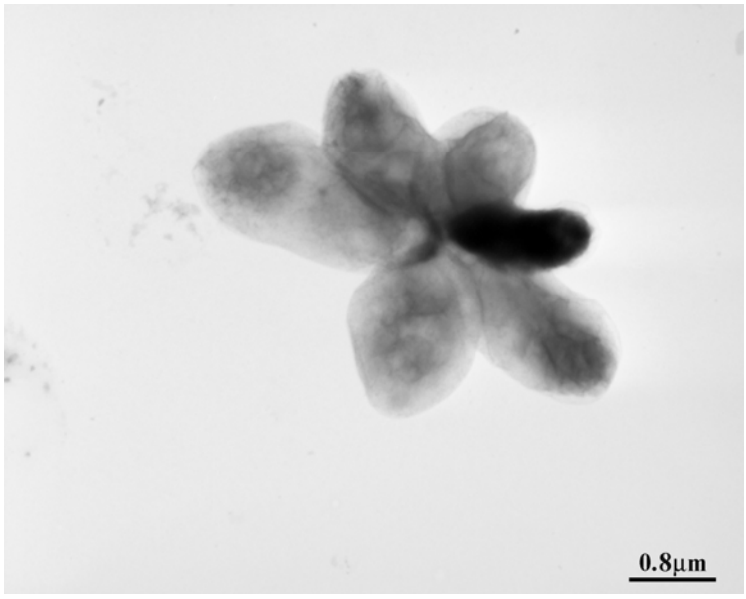
Figure 1a. Transmission electron micrographs of *Silicibacter* sp.TM1040 *flaA*- mutant strain, TM2014. Though this strain is non-motile, flagella are present.


1.0 µm

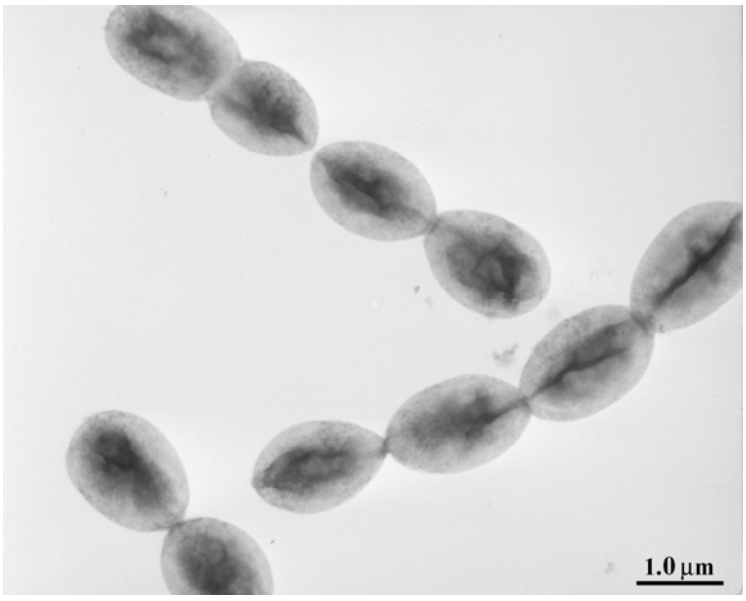

0.8µm

Figure 2a. Additional transmission electron micrographs of Roseobacter strains with varying morphologies and isolated from differing growth conditions. Pictures of strains isolated from complex broth medium Y3F rosette (A), Y3F chain and doublet (B), Y3F multiple flagella (C) and Y4I multiple flagella (D).

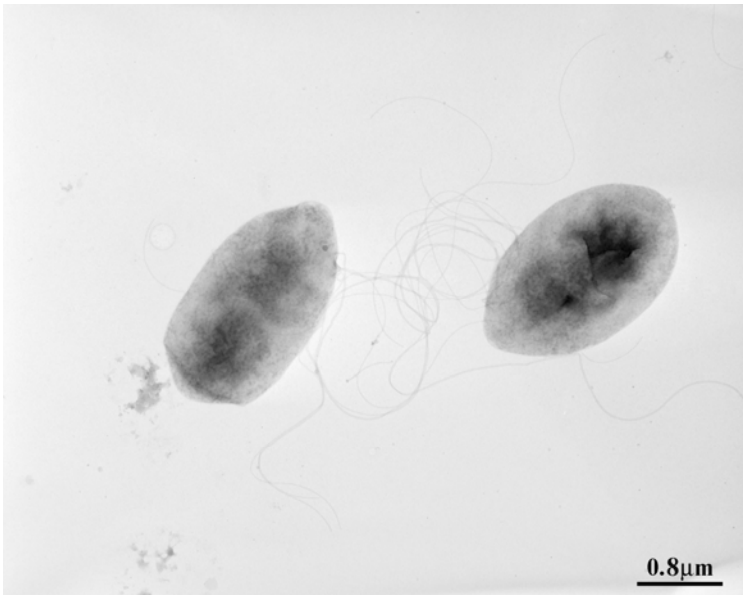
A.



B.



C.

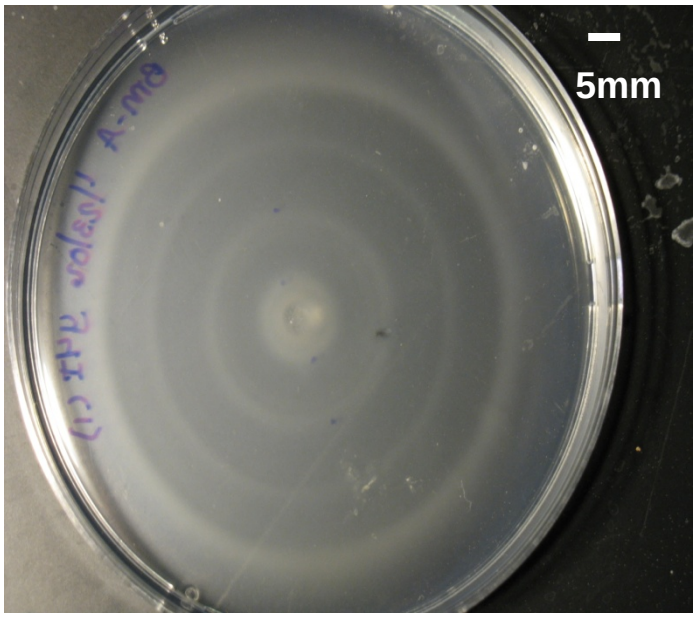


D.

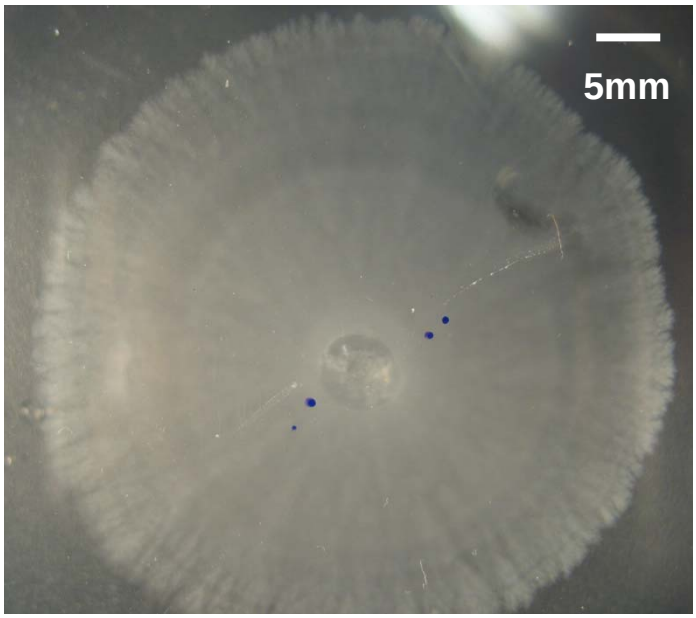


Figure 3a. Photographs of motile strains on motility agar. Y4I on SBM+10mMacetate (minimal medium) motility plate (A), EE-36 on 1/10 YTSS (complex medium) motility plate (B), NAS14-1 on 1/10 YTSS motility plate (C), ISM (non-motile strain) on 1/10 YTSS motility plate (D).

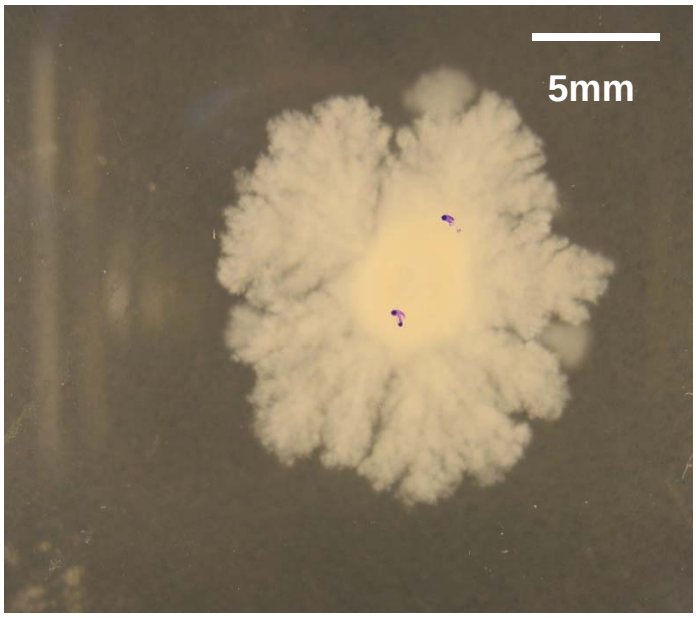
A.



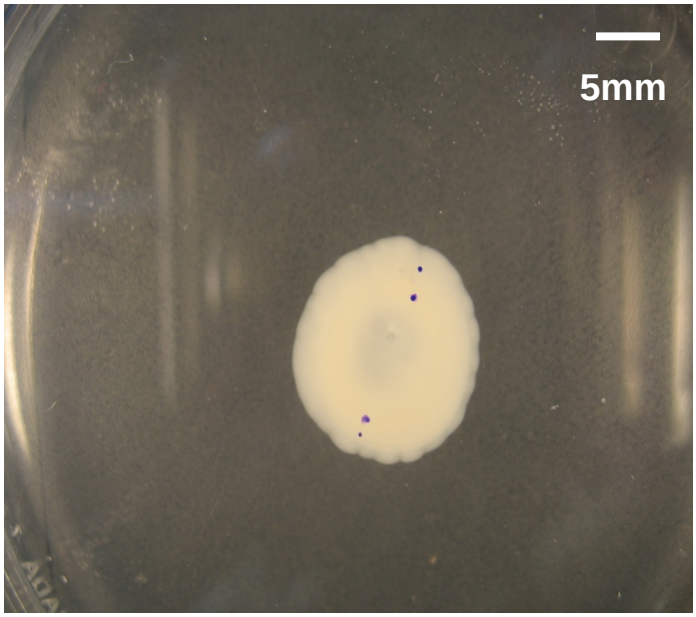
B.



C.



D.



VITA

Rachael Niccole Slightom was born in Harrisburg, Ill., on April 6, 1982. She was also raised Harrisburg and graduated from Harrisburg High School in 2000. She then went on to earn her B.S. degree in biology with a minor in chemistry in 2004 from the University of Evansville in Evansville, Ind., and her M.S. degree in Microbiology from the University of Tennessee in 2008.

Rachael is currently deciding on what career path she would like to pursue and is most interested in science opportunities involving environmental and ecological work.