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.

Alison Buchan, Major Professor

We have read this thesis and recommend its acceptance:

Gladys Alexandre-Jouline

Steven Wilhelm

Accepted for the Council:

Carolyn R. Hodges, Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
CHARACTERIZATION OF MOTILITY AND SURFACE ATTACHMENT AMONG
THIRTEEN MEMBERS OF THE ROSEOBACTER LINEAGE

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee

Rachael N. Slightom
May 2008
ACKNOWLEDGEMENTS

First I would like to acknowledge Alison Buchan for serving as my advisor and mentor over the past two years. Her guidance, insights and patience have been invaluable. She has showed me, through example, what it means to be a scientist. I would also like to acknowledge Gladys Alexandre-Jouline and Steve Wilhelm for serving on my committee and providing me with great ideas and new avenues of research to pursue. My fellow lab members (Mary, Burnette, Jason and Charles) have helped me in ways I cannot begin to count. I will never forget their encouragement and support. I would also like to thank John Dunlap from the University of Tennessee's Microscopy Facility for being so knowledgeable and helpful with all of my TEM data. Finally I absolutely could not have completed this without the never-ending love and support of my parents, Richard and Phyllis Slightom, and my wonderful family and friends.
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.
# TABLE OF CONTENTS

INTRODUCTION .............................................................................................................. 1  
Roseobacter Clade .......................................................................................................... 1  
Motility ........................................................................................................................... 3  
Chemotaxis ..................................................................................................................... 5  
Biofilm Formation/Surface Attachment ......................................................................... 6  
Research Objectives ........................................................................................................ 9  

METHODS ....................................................................................................................... 11  
Strains ........................................................................................................................... 11  
Motility Assays ............................................................................................................. 11  
Growth Curves .............................................................................................................. 12  
Morphological Characterizations .................................................................................. 12  
Identification of Genes Involved in Motility and Chemotaxis ..................................... 13  
Attachment Assays........................................................................................................ 13  
Glass Attachment .......................................................................................................... 15  
Genome Analysis .......................................................................................................... 15  

RESULTS ......................................................................................................................... 16  
Motility ......................................................................................................................... 16  
Microscopic Characterization (Phase Contrast & TEM) .............................................. 16  
Surface Attachment Assays ........................................................................................ 18  
Initial Attachment Assays ............................................................................................ 21  
Glass Attachment .......................................................................................................... 21  
Gene Analysis ............................................................................................................... 22  

DISCUSSION ................................................................................................................... 25  
REFERENCES ................................................................................................................. 37  

APPENDICES .................................................................................................................. 45  
APPENDIX A: Tables and Figures .............................................................................. 46  
APPENDIX B: Additional Figures ................................................................................. 107  
VITA ............................................................................................................................... 116
LIST OF TABLES

Table 1. List of thirteen Roseobacter strains included in this study .........................47
Table 2. Doubling times for all strains grown in YTSS nutrient-rich medium
and in SBM minimal medium .............................................................................48
Table 3. Strains used in alignment for FliF degenerate primer set .............................49
Table 4. Strains used in alignment for FlgH degenerate primer set .............................50
Table 5. Nucleotide sequences for the degenerate primer sets ..................................51
Table 6. Thirteen Roseobacter strains analyzed with TEM and phase contrast
microscopy in five different growth conditions ..................................................52
Table 7. Contact angle measurements for all surface types used in attachment
assays ..................................................................................................................55
Table 8. Products amplified using the three degenerate primer sets .............................56
LIST OF FIGURES

Figure 1.  Phylogenetic tree of Roseobacter members based on 16S rDNA sequences ..................57
Figure 2.  Motility graphs ..............................................................................................................59
Figure 3.  Phase contrast microscope images of Roseobacter strains grown in YTSS (complex) broth .............................................................62
Figure 4.  Phase contrast microscope images of five Roseobacter strains grown in SBM (minimal medium) broth ........................................................................66
Figure 5.  Transmission electron micrographs of ten flagellated and non-flagellated Roseobacter strains ..................................................................................69
Figure 6.  Microtiter dish surface attachment assays of thirteen Roseobacter strains and three mutant strains grown on YTSS (complex) medium ....................72
Figure 7.  Strip surface attachment assay graphs of thirteen Roseobacter strains and three mutant strains grown in YTSS (complex) medium ........................................................................75
Figure 8.  Microtiter dish surface attachment assays of twelve Roseobacter strains and one mutant strain grown in SBM (minimal) medium .....................................................78
Figure 9.  Initial attachment assays for thirteen Roseobacter strains and three mutant strains .............................................................................................................82
Figure 10. Phase contrast microscope images of strains grown in YTSS (complex) broth attached to a glass slide ..................................................................................85
Figure 11.  Gel showing non-specific amplification of degenerate primer set FlgH .................87
Figure 12.  Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on FlgH protein sequences ...........................................................................89
Figure 13.  Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on FliF protein sequences .............................................................................91
Figure 14.  Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on CheA protein sequences ..............................................................................93
Figure 15.  Phylogenetic tree of Roseobacter members and select non-Roseobacter strains based on LuxI protein sequences ..............................................................................95
Figure 16.  Color key for all gene diagrams ..................................................................................97
Figure 17.  Gene diagrams for flgH and flanking genes of interest ............................................99
Figure 18.  Gene diagram for fliF and flanking genes of interest .............................................101
Figure 19.  Gene diagram for cheA and flanking genes of interest ...........................................103
Figure 20.  Gene diagram for luxI and flanking genes of interest ............................................105
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 dimethylsuloniopropionate (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 multicellular 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 (Engebrecth 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 (Engebrecth 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. C₈ to C₁₈). 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 30ºC, 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$_4$Cl, 50 ml 1.36mM Fe-EDTA (Sigma-Aldrich), 20 ml 50mM K$_2$HPO$_4$, 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°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, \textit{fliF} and \textit{flgH}. In order to identify regions of conservation that would be suitable for degenerate oligonucleotides, putative \textit{fliF} and \textit{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 \textit{cheA} was graciously provided by Dr. Gladys Alexandre-Jouline and Burnette Crombie. The expected size of the \textit{fliF}, \textit{flgH} and \textit{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, polypropylene, polyvinyl chloride, polycarbonate, polyethylene terephthalate, Teflon™ 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°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, TeflonTM, 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°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 performed 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 ($22^\circ$C) 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™, 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™ 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° and Teflon™ is very hydrophobic with a contact angle measurement of 94.5°. 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 terephthalate, 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
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 attachment 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. Similarly 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 rhlI and tral 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.
REFERENCES
REFERENCES


APPENDICES
APPENDIX A: Tables and Figures
### Table 1. List of thirteen Roseobacter strains included in this study.

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

*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).

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>YTSS TIME (MIN)</th>
<th>SBM TIME (MIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citricella sp.SE45</td>
<td>90</td>
<td>87</td>
</tr>
<tr>
<td>Phaeobacter sp. Y3F</td>
<td>62</td>
<td>80</td>
</tr>
<tr>
<td>Phaeobacter sp.Y4I</td>
<td>79</td>
<td>94</td>
</tr>
<tr>
<td>Roseobacteraceae sp.PSPC2</td>
<td>104</td>
<td>NA</td>
</tr>
<tr>
<td>Roseobacteraceae sp.SE62</td>
<td>96</td>
<td>NA</td>
</tr>
<tr>
<td>Roseovarius nubinhibens ISM</td>
<td>81</td>
<td>188</td>
</tr>
<tr>
<td>Sagittula stellata E-37</td>
<td>84</td>
<td>94</td>
</tr>
<tr>
<td>Silicibacter lacuscaerulensis</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>Silicibacter pomeroyi DSS3</td>
<td>100</td>
<td>162</td>
</tr>
<tr>
<td>Silicibacter sp.TM1040</td>
<td>80</td>
<td>NA</td>
</tr>
<tr>
<td>Sulfitobacter sp. EE-36</td>
<td>95</td>
<td>108</td>
</tr>
<tr>
<td>Sulfitobacter sp.NAS14-1</td>
<td>79</td>
<td>100</td>
</tr>
<tr>
<td>Sulfitobacter pontiacus</td>
<td>95</td>
<td>246</td>
</tr>
</tbody>
</table>
Table 3. Strains used in alignment for FliF degenerate primer set.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ACCESSION NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sulfitobacter</em> sp. NAS14-1</td>
<td>ZP_00963769</td>
</tr>
<tr>
<td><em>Sulfitobacter</em> sp. EE-36</td>
<td>ZP_00956642</td>
</tr>
<tr>
<td><em>Roseovarius</em> sp. HTCC2601</td>
<td>ZP_01444395</td>
</tr>
<tr>
<td><em>Oceanicola granulosus HTCC2516</em></td>
<td>ZP_01156099</td>
</tr>
<tr>
<td><em>Silicibacter</em> sp. TM1040</td>
<td>YP_614941</td>
</tr>
<tr>
<td><em>Silicibacter pomeroyi</em> DSS-3</td>
<td>YP_165469</td>
</tr>
<tr>
<td><em>Roseobacter</em> denitrificans Och 114</td>
<td>YP_680680</td>
</tr>
<tr>
<td><em>Roseovarius</em> sp. HTCC2601</td>
<td>ZP_01443283</td>
</tr>
<tr>
<td><em>Oceanicola granulosus HTCC2516</em></td>
<td>ZP_01157139</td>
</tr>
<tr>
<td><em>Paracoccus</em> denitrificans PD 1222</td>
<td>ZP_00630361</td>
</tr>
<tr>
<td><em>Dinoroseobacter shibae</em> DFL 12</td>
<td>ZP_01583705</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em> ATCC17029</td>
<td>ZP_00917031</td>
</tr>
<tr>
<td><em>Roseovarius</em> sp. 217</td>
<td>ZP_01037249</td>
</tr>
<tr>
<td><em>Jannaschia</em> sp. CCS1</td>
<td>YP_512122</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em> str. C58</td>
<td>NP_353552</td>
</tr>
</tbody>
</table>
Table 4. Strains used in alignment for FlgH degenerate primer set.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ACCESSION NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Silicibacter</em> sp. TM1040</td>
<td>YP 614936</td>
</tr>
<tr>
<td><em>Silicibacter pomeroyi</em> DSS-3</td>
<td>YP 165465</td>
</tr>
<tr>
<td><em>Roseobacter denitrificans</em> Och 114</td>
<td>YP 680967</td>
</tr>
<tr>
<td>Uncultured marine bacterium Ant24C4</td>
<td>ABC25339</td>
</tr>
<tr>
<td><em>Roseovarius</em> sp. 217</td>
<td>ZP 01037240</td>
</tr>
<tr>
<td><em>Oceanicola granulosus</em> HTCC2516</td>
<td>ZP 01155033</td>
</tr>
<tr>
<td><em>Roseovarius</em> sp. HTCC2601</td>
<td>ZP 01441642</td>
</tr>
<tr>
<td><em>Dinoroseobacter shibae</em> DFL 12</td>
<td>ZP 01583811</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em> ATCC17029</td>
<td>ZP 00914854</td>
</tr>
<tr>
<td><em>Jannaschia</em> sp. CCS1</td>
<td>YP 512118</td>
</tr>
<tr>
<td><em>Sulfitobacter</em> sp. EE-36</td>
<td>ZP 00956625</td>
</tr>
<tr>
<td><em>Sulfitobacter</em> sp. NAS-14-1</td>
<td>ZP 00963787</td>
</tr>
<tr>
<td><em>Roseovarius</em> sp. HTCC2601</td>
<td>ZP 01444383</td>
</tr>
<tr>
<td><em>Oceanicola batensis</em> HTCC2597</td>
<td>ZP 01000508</td>
</tr>
<tr>
<td><em>Oceanicola granulosus</em> HTCC2516</td>
<td>ZP 01156110</td>
</tr>
<tr>
<td><em>Sagittula stellata</em> E-37</td>
<td>NZ_AAYA00000000</td>
</tr>
<tr>
<td><em>Jannaschia</em> sp. CCS2</td>
<td>NZ_AAYB01000001</td>
</tr>
</tbody>
</table>
Table 5. Nucleotide sequences for the degenerate primer sets.

<table>
<thead>
<tr>
<th>DEGENERATE PRIMERS</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FliF for</td>
<td>5’ GCI AWR GAR GGI GAR YTI GCI MG 3’</td>
</tr>
<tr>
<td>FliF rev</td>
<td>5’ YC RTT IAC IAR IAC IGC IAC 3’</td>
</tr>
<tr>
<td>FlgH for</td>
<td>5’ GGI TAY GGI YTI RTI GTI GG 3’</td>
</tr>
<tr>
<td>FlgH rev</td>
<td>5’ AR ITC RGC RTG IAR IGC ICC 3’</td>
</tr>
<tr>
<td>CheA P4P5 for</td>
<td>5’ CAY YTI ITI MGI AAY ISI GAY CAY GG 3’</td>
</tr>
<tr>
<td>CheA P4P5 rev</td>
<td>5’ CCR TCI CCI ARI ATI GTI GC 3’</td>
</tr>
</tbody>
</table>
Table 6. Thirteen Roseobacter strains analyzed with TEM and phase contrast microscopy in five different growth conditions.

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

52
<table>
<thead>
<tr>
<th>STRAINS</th>
<th>GROWTH CONDITIONS</th>
<th>GROWTH CONDITIONS</th>
<th>GROWTH CONDITIONS</th>
<th>GROWTH CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sagitulla stellata, E-37f</em></td>
<td>PC – no motility, ovoid cells, several rosettes, lots of doublets</td>
<td>PC – no motility, ovoid cells, some rosettes, some doublets</td>
<td>PC – no motility, larger ovoid cells, some clusters, some doublets, some cells with black dots inside</td>
<td>PC – no motility, ovoid cells, some rosettes, some doublets</td>
</tr>
<tr>
<td><em>Silicibacter lacuscaerulensis</em></td>
<td>PC – no motility, long, thin rod cells, no rosettes, some doublets TEM – long, thin rod shaped cells, no flagella</td>
<td>PC – no motility, large variety of cell shapes and sizes, no rosettes</td>
<td>PC – no motility, long thin cells, no rosettes</td>
<td>PC – no motility, variation in cell shape and size, no rosettes, doublets and ovoid cells present</td>
</tr>
<tr>
<td><em>Silicibacter pomeroyi DSS3</em></td>
<td>PC – no motility, mostly doublet shaped cells, some single ovoid cells, rosettes</td>
<td>PC – no motility, ovoid cells, rosettes, doublets</td>
<td>PC – no motility, smaller ovoid cells, no rosettes, some doublets</td>
<td>PC – no motility, some ovoid cells, rosettes, longer doublet cells</td>
</tr>
<tr>
<td><em>Silicibacter sp. TM1040a</em></td>
<td>PC – motile cells, small, ovoid cells, rosettes, doublets</td>
<td>PC – no motility, ovoid cells, many rosettes, doublets</td>
<td>PC – no motility, most cells are doublet shaped, many rosettes</td>
<td>PC – very motile cells, cells are larger than YTSS shaking growth, few rosettes, many doublets</td>
</tr>
<tr>
<td><em>Sulfitobacter sp. EE-36</em></td>
<td>PC – no motility, mostly doublet shaped cells, no rosettes</td>
<td>PC – no motility, ovoid as well as longer cells, many chains, rosettes, doublets</td>
<td>PC – motile cells, small, ovoid cells, large, rosette-shaped cluster, some doublets</td>
<td>PC – motile cells, mostly doublet cells, some ovoid cells, small rosettes TEM – Small, ovoid cells, at least one polar flagellum</td>
</tr>
</tbody>
</table>
## Table 6, continued.

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

* Several strains had been previously characterized using TEM.  

1. *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).  

2. *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).  

3. *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).  

4. *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).  

5. *Roseovarious nubinhens* 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).  

6. *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.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>CONTACT ANGLE MEASUREMENT</th>
<th>SURFACE PROPERTY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypropylene</td>
<td>87.3°</td>
<td>Slightly hydrophobic</td>
</tr>
<tr>
<td>Polyvinyl chloride</td>
<td>76.5°</td>
<td>Slightly hydrophilic</td>
</tr>
<tr>
<td>Polyethylene terephthalate (PET)</td>
<td>68.2°</td>
<td>Slightly hydrophilic</td>
</tr>
<tr>
<td>Polycarbonate</td>
<td>67.4°</td>
<td>Slightly hydrophilic</td>
</tr>
<tr>
<td>Polystyrene*</td>
<td>86°</td>
<td>Slightly hydrophobic</td>
</tr>
<tr>
<td>Teflon™</td>
<td>93.8°</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Glass</td>
<td>&lt;10°</td>
<td>Hydrophilic</td>
</tr>
</tbody>
</table>

*Fletcher * Loeb, 1979
Table 8. Products amplified using the three degenerate primer sets.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>GENE DESIGNATION</th>
<th>NUMBER OF NUCLEOTIDES SEQUENCED</th>
<th>CLOSEST BLAST HIT</th>
<th>E-SCORE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE45</td>
<td>CheA P4P5</td>
<td>859</td>
<td><em>Roseovarious</em> sp. HTCC2601</td>
<td>5E-52</td>
</tr>
<tr>
<td></td>
<td>FliF</td>
<td>645</td>
<td><em>Roseovarious</em> sp. HTCC2601</td>
<td>1E-74</td>
</tr>
<tr>
<td>SE62</td>
<td>CheA P4P5</td>
<td>458</td>
<td><em>Loktanella vestfoldensis</em> SKA53</td>
<td>8E-47</td>
</tr>
<tr>
<td>S. lac</td>
<td>CheA P4P5</td>
<td>844</td>
<td><em>Roseovarious</em> sp. HTCC2601</td>
<td>2E-67</td>
</tr>
<tr>
<td></td>
<td>FlgH</td>
<td>946</td>
<td><em>Silicibacter pomeroyi</em> DSS-3</td>
<td>4E-130</td>
</tr>
<tr>
<td>S.pont</td>
<td>CheA P4P5</td>
<td>705</td>
<td><em>Roseovarious</em> sp. HTCC2601</td>
<td>1E-57</td>
</tr>
<tr>
<td></td>
<td>FliF</td>
<td>667</td>
<td>*Sulfitobacter NAS-14-1 &amp; EE-36</td>
<td>2E-106</td>
</tr>
<tr>
<td>Y3F</td>
<td>CheA P4P5</td>
<td>449</td>
<td><em>Silicibacter</em> sp.TM1040</td>
<td>3E-68</td>
</tr>
<tr>
<td></td>
<td>FlgH</td>
<td>989</td>
<td><em>Silicibacter pomeroyi</em> DSS-3</td>
<td>1E-126</td>
</tr>
</tbody>
</table>
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).
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).
SBM + ACETATE MOTILITY ASSAY

GROWTH (cm)

TRANSFER

ORIGINAL  1ST  2ND  3RD  4TH

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).
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.
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).
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

B. POLYPROPYLENE
C.

POLYVINYL CHLORIDE

OPTICAL DENSITY (600nm)


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 terephthalate strips (PET) (B), Teflon™ strips (C) and glass slides (D).
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.
C. POLYVINYL CHLORIDE (SBM)

![Graph showing optical density (600nm) for various strains of Polyvinyl Chloride (SBM)]
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).
C.

THIRTY-SECOND INITIAL ATTACHMENT

OPTICAL DENSITY (600nm)

STRAIN

DSS3
TM1040
TM2014 FlaA.
S. LAC
S. PONT
NAS14-1
EE36
ISM
E-37
PSPC2
Y3F
Y4I
Y4I/PAP5
Y4I/LUXR
SE45
SE62
Figure 10. Phase contrast microscope images of strains grown in YTSS (complex) broth attached to a glass slide. Strains Y41 (A), Y3F (B), S. pont (C), NAS14-1 (D), EE-36 (E) and TM1040 (F) all demonstrated attachment to glass during the incubation period.
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.
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).
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).
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).
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).
Figure 16. Color key for all gene diagrams (Figures 17-20).
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)
Sulfotobacter sp. EE-36
Sulfotobacter sp. NAS14-1
Loktanella vestfoldensis
Figure 18. Gene diagram for *fliF* and flanking genes of interest.
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.
Figure 20. Gene diagram for luxI and flanking genes of interest.
Dinoroseobacter shibae
Jannaschia sp. CCS1
Roseovarius nubinhibens ISM
Sulfitobacter sp. NAS14-1
Sulfitobacter sp. EE-36
Roseobacter sp. MED193
Roseovarius sp. 217
Roseobacter sp. SK209-2-6
Oceanicola granulosus
Loktanella vestfoldensis
Roseobacter sp. CCS2
Roseobacter sp. SK209-2-6 (2)
Roseovarius sp. 217 (2)
Silicibacter pomeroyi DSS-3
Dinoroseobacter shibae (2)
Roseobacter sp. MED193 (2)
Sagittula stellata E-37
Silicibacter pomeroyi DSS-3 (2)
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.
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).
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).
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.