

ABSTRACT

Title of Dissertation / Thesis: **SWIMMING FOR SULFUR: ANALYSIS OF
THE ROSEOBACTER-
DINOFLAGELLATE INTERACTION**

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Marine algae are some of the most productive organisms on earth, and their survival is dependent upon a diverse community of bacteria that consume algal products. The identity of these bacteria and mechanisms used to interact with their algal partner are not well understood. Recently it has been shown that α -Proteobacteria of the *Roseobacter* clade are the primary consumers of the algal osmolyte, dimethylsulfoniopropionate (DMSP). In addition, their production and activity is highly correlated with DMSP producing algal blooms, especially those containing dinoflagellates. To understand more about this relationship, I have studied *Roseobacter*-dinoflagellate interactions in laboratory cultures of *Pfiesteria* dinoflagellates, a ubiquitous group of estuarine, heterotrophic dinoflagellates. The results show that cultures of *P. piscicida* and a similar dinoflagellate, *Cryptoperidiniopsis* sp., harbor a robust DMSP degrading bacterial community that contains members of the *Roseobacter* clade. One of these bacteria, *Silicibacter* sp. TM1040 degrades DMSP by demethylation producing 3-methylmercaptopropionate (MMPA). Interestingly, this bacterium senses and actively

moves toward *P. piscicida* cells. It is highly chemotactic toward amino acids, especially methionine, and DMSP metabolites, including DMSP and MMPA. Chemotaxis of TM1040 toward *P. piscicida* cells is mediated in part by the presence of these compounds in the dinoflagellates. Using a fluorescent tracer dye, this bacterium was found attached and/or within *P. piscicida* cells. The apparent intracellular occurrence of *Silicibacter* sp. TM1040 requires both flagella and motility since mutants lacking motility and/or flagella are not found within the dinoflagellate, although they can be found attached. The presence of *Silicibacter* sp. TM1040 in axenic dinoflagellate cultures enhances dinoflagellate growth, a process that does not require the bacteria to be intracellular. The genome sequence of *Silicibacter* sp. TM1040 indicates that this bacterium contains a large number (20) of chemoreceptors and a full complement of flagellar and other chemotaxis genes. In addition, this bacterium contains all of the genes necessary to produce a type IV secretion system similar to the *vir* pilus of *Agrobacterium tumefaciens*. Taken together, the data suggest that *Silicibacter* sp. TM1040 is an attached and/or intracellular symbiont of *P. piscicida*. The significance of this study to microbial and algal bloom ecology is discussed.

**SWIMMING FOR SULFUR: ANALYSIS OF THE ROSEOBACTER-
DINOFLAGELLATE INTERACTION**

By

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Dedication

To my loving wife Jenn, the source of all that inspires me.

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Chapter 1: Introduction

1.1 Harmful algal blooms (HABs) and toxic dinoflagellates

1.1.1 Harmful algal blooms (HABs).

Marine algae are a diverse group of microscopic organisms that inhabit nearly every ocean environment from polar ice to coral reefs and open-ocean or coastal waters (61, 216, 240, 296). They are some of the most productive organisms on earth, responsible for the majority of all carbon production in the ocean (35). Many algae contain light harvesting pigments that are used to drive energy production while others are also heterotrophic, consuming compounds produced by other organisms. When conditions are appropriate, these single cell eukaryotes may reproduce to high cell densities causing a discoloration of ocean surface water that can extend for miles. Some algal species may obtain high enough cell densities as to prevent the penetration of sunlight or the dissolution of oxygen to lower ocean depths and/or produce toxins harmful to fish, birds, or mammals including humans. Blooms that occur in open-ocean waters are seldom problematic for humans, but near shore blooms are a common problem leading to costly and sometimes hazardous conditions.

The occurrence of these toxic, or nuisance algal blooms in the United States, otherwise known as Harmful Algal Blooms (HABs), represent a growing problem which impacts on ecological, economic, and human health-related issues. Over the last three decades, the distribution and incidence of HABs in coastal states has increased with devastating consequences (234). Over a five-year period from 1987 – 1992, an estimated \$50 million were lost due to HABs in the United States (18). Among the hardest hit areas were commercial fishing, recreation, tourism and health care industries. Economic loss

was mainly due to the death of commercially important marine organisms, but may also have resulted from consumers' fear of tainted seafood. In 1986, a bloom of the dinoflagellate, *Gymnodinium breve* in Texas devastated local oyster production resulting in 2.22 million dollars of lost profits. Blooms of this organism are a common problem in the Gulf of Mexico and have affected a variety of marine life important to humans since the 1800's. Along with these expected and sometimes predictable outbreaks there are new threats emerging. In 1997, a recently discovered dinoflagellate, *Pfiesteria piscicida*, was implicated in the death of 30 – 50,000 menhaden (*Brevoortia tyrannus*), a commercially unimportant, but ecologically relevant fish species in the Chesapeake Bay (18). In this case, news media coverage of the event caused a considerable drop in the demand for seafood even though no commercial fish kills were reported. This unnecessary loss underscores the need for primary scientific research that provides vital information regarding existing HAB species and novel approaches for the detection of new species.

1.1.2 Factors that contribute to algal bloom formation

The initiation of an algal bloom represents an imbalance in microbial community structure. This is best explained by an analysis of the flow of organic carbon through the microbial food web, better known as the microbial loop (Fig. 1.1). In this theoretical scheme, primary production (PP) by algae or phytoplankton is consumed by heterotrophic flagellates or protists (F). These algal grazers are in turn consumed by larger organisms such as ciliates (C). Ciliates and flagellates both consume bacteria (B). All organisms contribute to the pool of dissolved organic matter (DOM). The large pool of DOM is degraded by bacterial communities who regenerate the essential dissolved

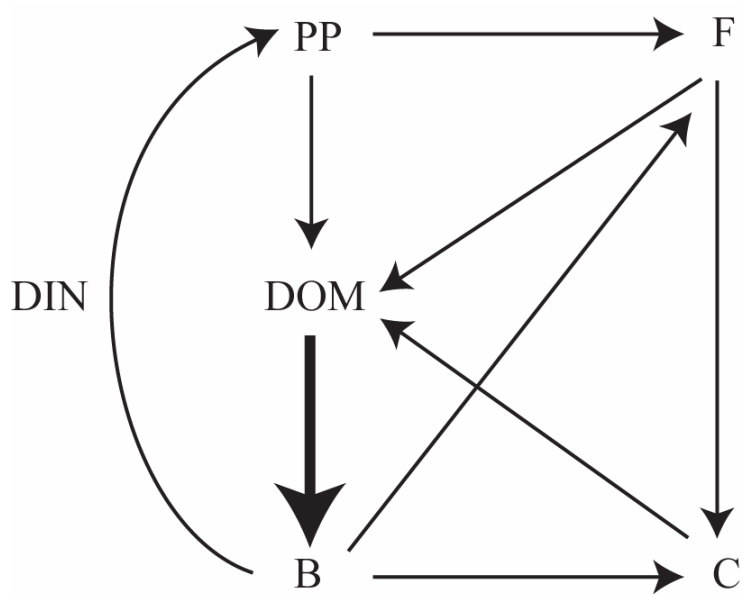


Fig. 1.1. The microbial loop. PP = primary productivity, B = bacteria, DOM = dissolved organic matter, C = ciliates, F = flagellates, DIN = dissolved inorganic nitrogen

inorganic compounds (DIN) to refuel algal primary production. Other factors are also involved including viral or bacterial-induced lysis of other microorganisms. In addition, some algal species bridge multiple trophic levels such as the dinoflagellates, many of which are primary producers as well as heterotrophs, capable of consuming other algal species. Finally, we now know that nearly half of all the carbon fixed by algal cells is consumed by bacteria, surpassing the grazers for their carbon needs (35). Algal - associated bacteria may cause lysis of the algal cells, enhance algal growth through the production of essential nutrients, or provide protection from algal predators by enhancing algal toxin production (see Section 1.3). This is likely to have large impacts upon the distribution and formation of algal blooms.

The physiology of the algal species is also an important aspect of bloom formation. Laboratory studies indicate that the optimal balance of nitrogen and phosphorous for algal growth is 16:1, the Redfield ratio (202, 309). However, this can vary depending upon the algal species, especially its' storage capacity for N and P (202). Iron is also an essential element for algal growth and can be limiting, but this varies considerably depending upon the species in question (97, 98, 202, 206). Salinity, light, and water temperatures are also key variables. Some algae, especially dinoflagellates have a very wide range of salinity tolerances, such as *Pfiesteria* species (2 – 50 psu) (346) and *Oxyrrhis* species (4 – 130 psu), but the optimal salinity for growth of these species is more narrow at 8 – 15 psu and 16 -20 psu, respectively. Generally speaking high light levels are more favorable for algal growth (374). At low levels of light photosynthesis is linear with respect to irradiance, but at higher levels the rate of photosynthesis becomes saturated with respect to light irradiance (45). In addition,

photosynthetic assimilated carbon must be balanced with the availability of other essential nutrients such as N and P. In photosynthetic/heterotrophic (mixotrophic) dinoflagellates, high light intensity induces higher grazing rates, presumably due to the need for N and P to balance photosynthetic carbon assimilation (225). Ultimately, the critical factors that cause bloom formation are determined by the physiology of the algal species, its associated microbial community, nutrient availability and characteristics of the geographic area (17, 356).

1.1.3 Toxic dinoflagellates.

Many harmful algal blooms are caused by one or more species of toxic dinoflagellates, although the majority of dinoflagellates are not toxic (116). The toxins produced by these organisms can be concentrated in filter - feeding shellfish such as clams and oysters or in commercial fish destined for human consumption. These toxins are grouped into five classes based upon both their structural similarities and their pharmacological effects in humans (Table 1.1). Due to an increase in the number of identified toxic algal species, there is also a growing number of currently unclassified toxins.

Amnesic shellfish poisoning is associated with the consumption of mussels contaminated with domoic acid, an amino acid belonging to the kainoid class of compounds (184). This is one of the few algal toxins not produced by a dinoflagellate. Production of this compound is limited to diatoms of the genus *Pseudonitzschia*. Domoic acid binds with high affinity to a subfamily of glutamate receptors in the brain called kainate receptors (159). Binding of domoic acid to these receptors results in over excitation of neurons, which may lead to neuronal cell death, most likely through the

uptake of excess calcium and an imbalance of ion flow (114). This activity can cause a degeneration of brain regions required for memory formation, such as the hippocampus, and over excitation of regions required for visceral function, mainly in the brain stem. As a result, symptoms associated with amnesic shellfish poisoning are gastroenteritis, dizziness, headache, seizures, disorientation, short-term memory loss, respiratory difficulty, and coma (184).

Ciguatera fish poisoning is the most commonly reported marine illness and is caused by one or more types of lipid soluble polyether toxins including ciguatoxin and maitotoxin (100). The dinoflagellate, *Gambierdiscus toxicus*, is most often attributed to the production of ciguatoxins, although other dinoflagellates may be responsible as well including *Amphidinium carterae*, *Coolia monotis*, *Ostreopsis* spp., and *Prorocentrum* spp. (221, 267). The lipophilic toxins produced by these dinoflagellates are transferred throughout the marine food web and bioaccumulate in predatory fishes that are destined for human consumption (221). Ciguatoxin and its derivatives open voltage - gated sodium channels which interfere with the function of neuronal and striated muscle tissue while maitotoxin activates voltage sensitive calcium channels resulting in increased hormonal release from pituitary cells (100, 125). Symptoms of ciguatera fish poisoning include parasthesia, vertigo, and ataxia, in addition to gastrointestinal symptoms such as diarrhea, abdominal pain, nausea, and vomiting (38).

Dinoflagellate toxins within the class of diarrhetic shellfish poisons include okadaic acid and dinophysistoxins both produced by *Dinophysis* sp. (370, 371). okadaic

Table 1.1 Algal toxins and sources

Class	Toxins	Sources
Amnesic shellfish poisoning	Domoic acid	<i>Pseudonitzschia</i> spp.
Ciguatera fish poisoning	Ciguatoxin Maitotoxin	<i>Gambierdiscus toxicus</i> <i>Amphidinium carterae</i> <i>Coolia monoti</i> <i>Ostreopsis</i> spp. <i>Prorocentrum</i> spp. <i>Thecadinium</i> spp.
Diarrhetic shellfish poisoning	Okadaic acid Dinophysistoxins	<i>Dinophysis</i> spp.
Neurotoxic shellfish poisoning	Brevetoxins	<i>Karenia brevis</i>
Paralytic shellfish poisoning	Saxitoxin Tetrodotoxin Gonyautoxin	<i>Alexandrium</i> Bacteria Cyanobacteria Puffer fish Crab Salamanders
Unclassified	Karlotoxin Yessotoxin Azospiracid	<i>Karlodinium micrum</i> <i>Protoceratium reticulatum</i> <i>Proto-peridinium</i> spp.

acid inhibits protein phosphorylase phosphatase 1 and 2A in the cytosol of mammalian cells. This activity is thought to be the cause of diarrhea in humans since phosphatases control sodium pumps, and thus osmotic balance, in cells of the intestinal lining (51). Okadaic acid is also a vasodilator of arteriolar smooth muscle tissue and promotes tumor formation in mouse epidermal tissues. Symptoms of diarrhetic shellfish poisoning include diarrhea without any reported chronic effects.

Neurotoxic shellfish poisoning is caused by brevetoxins most commonly associated with blooms of the dinoflagellate *Karenia brevis* in the Gulf of Mexico. Brevetoxin is structurally similar to ciguatoxin and so also binds to voltage gated sodium channels (37, 100). The symptoms of neurotoxic shellfish poisoning are nearly identical to those of ciguatera fish poisoning, although less severe. In addition, inhalation of aerosols containing brevetoxins results in respiratory illness characterized by conjunctival irritation, copious catarrhal exudates, rhinorrhea, nonproductive cough, and bronchoconstriction (1).

Paralytic shellfish poisoning is a life threatening illness caused by the ingestion of shellfish contaminated with saxitoxin, tetrodotoxin or gonyautoxin including their derivatives (135, 176). These compounds are distributed widely in nature occurring in shellfish, dinoflagellates, dinoflagellate-associated bacteria, cyanobacteria and eubacteria as well as in puffer fish, crab and salamanders among others (7, 20, 102, 134, 230, 326, 364). In marine environments, blooms of the dinoflagellate *Alexandrium* spp. are a significant source of saxitoxin, which is then concentrated in commercially important filter feeding shellfish (369). Paralytic shellfish toxins irreversibly bind to and block sodium channels in a voltage dependent manner such that neurons fail to achieve an

action potential and muscles fail to contract (81). Symptoms of paralytic shellfish poisoning include numbness of the perioral region, nausea and muscular weakness followed by upper extremity weakness, tongue immobilization and in some cases death (89, 137, 303).

There are many other harmful dinoflagellate toxins that have been discovered, but their mode of toxicity is less well known. For example yessotoxin and homoyessotoxin are classified as diarrhetic shellfish toxins because they often occur in tandem with okadaic acid, however, these toxins appear not to cause diarrhea (286). Studies show that yessotoxin causes cardiotoxic as well as other cytotoxic effects in a variety of tissues including neurofilaments, suggesting that yessotoxin may be neurotoxic (123). Another new toxin, azospiracid, is a polyether toxin produced by the dinoflagellate, *Protoperidinium* spp. This toxin has caused severe diarrhea in individuals following consumption of mussels from Ireland (132, 217, 285). Symptoms of azaspiracid poisoning include nausea, vomiting, severe diarrhea, and stomach cramps similar to okadaic acid. However, morphopathological examinations indicate that the mode of toxicity may be different from okadaic acid (178, 179). Finally, *Karlodinium micrum*, a dinoflagellate responsible for fish deaths in the Mid-Atlantic region of the United States produces at least two hemolytic toxins that impair gill function in adult fishes (101). It is not known if these toxins cause human illness. These toxins and others are a growing problem for fisheries management and human health.

1.1.4 Distribution of toxic dinoflagellate blooms in the United States.

The incidence of toxic dinoflagellate blooms and other HAB related events have been on the rise in the United States and in other countries for several decades (157). This

increase in recorded events is undoubtedly the result of an increased number of HAB monitoring programs, but is also due to increased nutrients into our coastal waterways and other human-related activities (reviewed in (157, 166, 295)). There are several areas throughout the coastal United States that experience well-documented, often recurrent, HAB events, mainly due to toxic dinoflagellates. For review purposes, these events can be categorized into four regions within the coastal United States; 1) Gulf of Maine, 2) Mid-Atlantic, 3) Gulf of Mexico, and the 4) West Coast.

Blooms of dinoflagellates in the genus *Alexandrium* spp. frequently occur in the Northeastern United States, especially in the Gulf of Maine. The two species responsible for paralytic shellfish poisoning in this area are *A. tamarense* and *A. fundyense* (19). Field studies indicate that cells of these species are transported in a southwesterly direction along the coast of Maine from the Bay of Fundy within the eastern Maine coastal current to Penobscot Bay (17). Most blooms of *Alexandrium* occur within this current, but do not occur in Penobscot Bay. Here the Eastern Maine Coastal Current deflects eastward due to freshwater flow from numerous inland streams, rivers and tributaries. Cells are carried offshore and may be pushed back into the Eastern Maine Coastal Current feeding further bloom activity. Alternatively cyst bed populations may feed bloom activity in this region (21). During low freshwater flow from the Penobscot Bay and other sources, the Eastern Maine Coastal Current may not deflect eastward in which case cells traveling in the Eastern Maine Coastal Current join the Western Gulf of Maine Coastal Current and are carried into the Massachusetts Bay and on to the Georges Bank to feed the other common site of *Alexandrium* blooms off Georges Bank (17).

The Mid-Atlantic coast of the United States has recently experienced massive fish mortalities in estuarine river systems of the Chesapeake Bay of Maryland and Virginia and the Neuse River and Pamlico Sound Estuary in North Carolina (66, 74). The causative agent is thought to be toxic *Pfiesteria* dinoflagellates, however, it is unclear how this dinoflagellate kills fish since a toxin produced by *Pfiesteria* has not been identified (264). In contrast, *Pfiesteria shumwayae* has been shown to kill fish in the laboratory by consumption of fish epidermal tissue, but death of the fish only occurs when *P. shumwayae* cell density reaches environmentally irrelevant concentrations (>1000 per ml) (382). Other dinoflagellate species indigenous to these areas may be the culprits, including *Karlodinium micrum* which produces at least two different hemolytic toxins (101). In addition, toxic cyanobacteria, including *Microcystis* species, have been problematic in more recent years (Maryland Department of Natural Resources (MDNR) at <http://dnr.maryland.gov/bay/hab/microcystis2.html>). More research is needed to determine the exact cause of fish kills in the Mid-Atlantic region, however, estuarine river systems in this area often experience low levels of dissolved oxygen due to eutrophication and this may be a more common cause of fish deaths in these areas (295, 297).

The Gulf of Mexico near the Florida coast has experienced blooms of the brevetoxin-producing dinoflagellate *Gymnodinium breve* since the 1800's. This dinoflagellate is dependent upon light for photosynthesis and is phototactic remaining at the surface during the day. *G. breve* cells are mainly drifters, transported within three major currents; the Gulf of Mexico Loop current, the Florida Current and the Gulf Stream Current (356). The Gulf of Mexico Loop Current occurs in eastern part of the Gulf of

Mexico circulating water in this region until it exits between the Dry Tortugas and Cuba where it becomes the Florida Current (167). Cells are then transported around Florida and up the Eastern coast of the United States as far North as Cape Hatteras of North Carolina in the Gulf Stream Current (356). The northward extent of the Gulf of Mexico Loop Current and its potential to extend east toward the Florida coast influence the occurrence of *G. breve* blooms along the southwestern coast of Florida (356). The majority of all *G. breve* blooms occur in this area, however, they are also common in the Campeche Bay, and the Texas Coast between Port Arthur and Galveston Bay (288). Blooms of *G. breve* are initiated in these areas due to warmer water temperatures and nutrient upwelling (356).

The West Coast suffers from recurrent outbreaks of paralytic and amnesic shellfish poisoning caused by the dinoflagellate *Alexandrium catenella* and the diatom *Pseudonitzschia australis*, respectively (173). Human deaths related to the consumption of clams and oysters tainted with paralytic shellfish toxins (saxitoxin) and amnesic shellfish toxins (domoic acid) have been documented as early as the 1700's. This large region is characterized by cold, nutrient rich water from Alaska brought to the area in the North Pacific Current which becomes the California Current System as it moves eastward (56). The California Current is a slow moving, cold body of water that flows in a southerly direction along the West Coast of the U.S. toward the Southern California Bight (56, 173). The California Undercurrent transports warmer, less oxygenated water northward from equatorial waters (56). Upwelling of the cold, well-oxygenated nutrient-rich water in the California Current is a major factor that influences blooms in off-shore waters (173, 308, 362). Blooms that occur inland, such as in the Puget Sound, are characterized

by warm water temperatures (14 °C), high concentrations of N and P and stagnant water (172). Although *A. catenella* is generally accepted as the most significant producer of paralytic shellfish toxins in this area, throughout the West Coast region it has been difficult to determine the source of toxins detected in shellfish since in many cases, blooms initiated in one area may be quickly transported several hundred miles northward by fast moving currents (172).

1.2 *Pfiesteria* dinoflagellates

1.2.1 Discovery of *Pfiesteria* dinoflagellates.

The dinoflagellate, *P. piscicida* (Fig. 1.2) was first discovered in an aquarium at North Carolina State University in 1988 where it was blamed for an incident of fish deaths (277). These deaths occurred shortly after the addition of Pamlico River water and were coincident with an increased abundance of the small (ca. 10 µm) dinoflagellates. Interest in *Pfiesteria* heightened in 1991 and 1992 when massive fish mortalities accompanied by open ulcerations on the bodies of the fish were reported in the Pamlico and Neuse estuaries of North Carolina. Water taken from these areas was tested in a fish bioassay in which the abundance of dinoflagellates resembling *P. piscicida* was coincident with fish death (76). From these results and in the absence of alternative explanations, *P. piscicida* was identified as a possible cause for the observed fish mortalities in the Neuse and Pamlico estuaries, as well as in the fish bioassay aquaria (reviewed in (141)). Further research conducted in the laboratory of Dr. JoAnn Burkholder (NCSU) suggested that these dinoflagellates produced a putative water-soluble toxin that causes ulcers, disorientation, and eventually death of fish and other

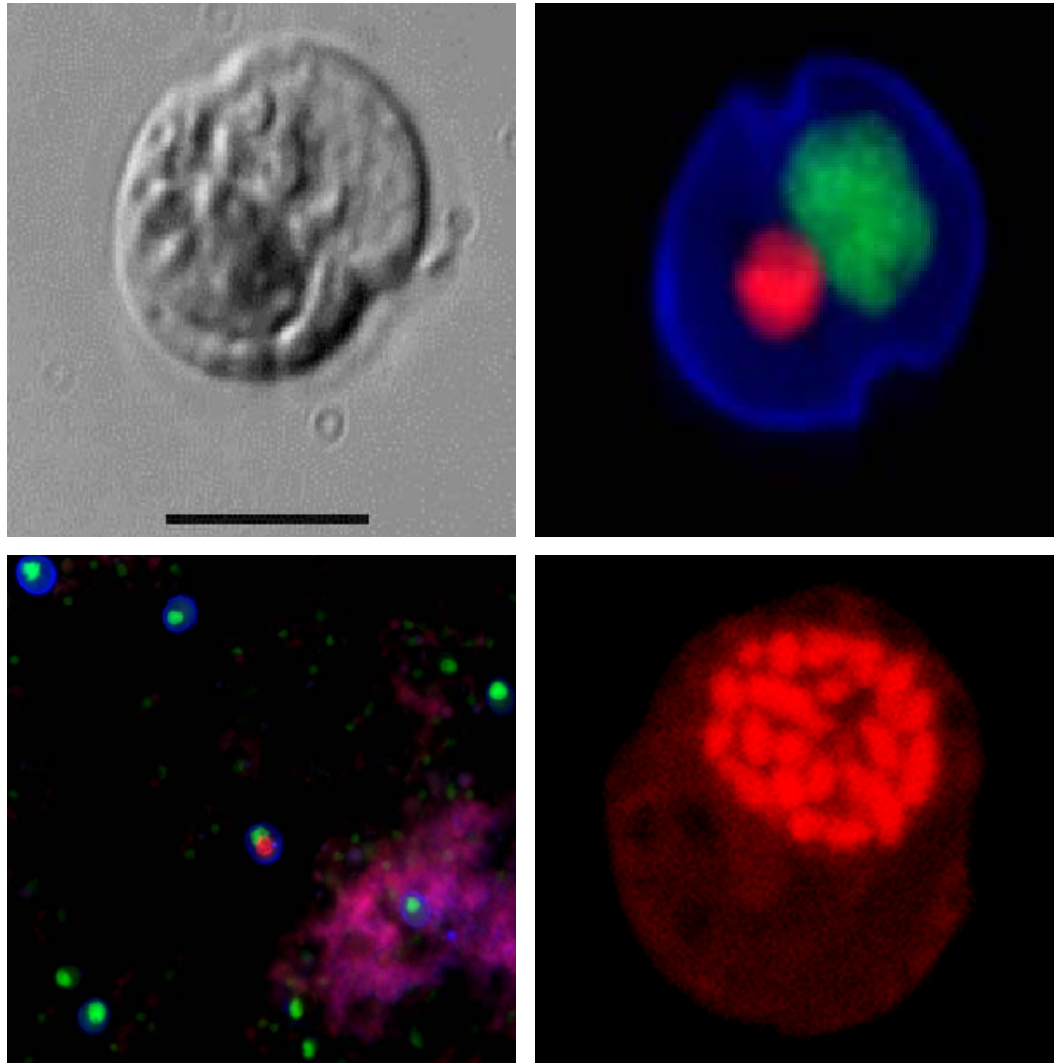


Fig. 1.2 Micrographs of *P. piscicida*. (A) Phase contrast micrograph (bar = 10 μ m). (B) Epifluorescent micrograph in which the dinoflagellate is stained with acridine orange (green) to show the nucleus and calcofluor (blue) to show the thecal plates. Prey chlorophyll is seen in red as autofluorescence. (C) A low magnification view of the field depicted in panel B showing other dinoflagellates without prey chlorophyll. (D) Confocal scanning laser micrograph in which *P. piscicida* is stained with ethidium bromide to show the permanently condensed chromosomes.

marine animals (76, 143). During this period, three scientists reported adverse symptoms attributed to laboratory exposure to *Pfiesteria* (143). These symptoms included extremity paresthesias, circumoral paresthesias, arthralgia, myalgia, asthemia, headache, nausea, abdominal pain, vomiting, perspiration, tearing/eye irritation, dyspnea/respiratory problems, memory problems, emotional changes, and skin lesions (143). Other scientists also working with *Pfiesteria*, as well as watermen working near areas of *Pfiesteria*-related fish deaths, reported a similar suite of symptoms (tentatively named the “possible—estuary associated syndrome” (263, 328). These reports and intense media coverage enhanced concern over *Pfiesteria* and public safety. The “*Pfiesteria* hysteria” reached its apex in 1997 when the MDNR reported *Pfiesteria*-related fish deaths in the Pocomoke and Chicamacomico Rivers. As a result, several waterways were closed for recreational use by MDNR and news media coverage of the event fueled a growing concern among politicians and the general public over the safety of commercial seafood products for human consumption and in general, the health of the Chesapeake Bay. The “*Pfiesteria* hysteria” instigated funding for the development of better monitoring techniques, both for *Pfiesteria* itself and its putative toxin. A direct result of this research has been a greater understanding of mixotrophic dinoflagellates and the complexities of detecting harmful dinoflagellates. Unfortunately, scientists are still in disagreement over issues of toxicity and life stages of *Pfiesteria* species (188).

1.2.2 General characteristics.

Some general characteristics of *Pfiesteria piscicida* are listed in table 1.2. *Pfiesteria* species are phagotrophic dinoflagellates that employ a feeding tube known as a

Table 1.2. General characteristics of *Pfiesteria piscicida*

Characteristic	Result	Reference
Doubling time	2 per day	(117)
Size	5 - 60 μm	(343)
Trophic status	Mixotrophic	(223, 224, 343)
Grazing rate	0.22 prey cells per hour	(112)
Swimming speed	100 - 300 $\mu\text{m s}^{-1}$	(67)
Salinity	2 - 50 psu	(346)
Temperature	20 °C	(325)
Light	150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	(112)
Life stages	3 cyst, 1 zoospore, planozygote, hypnozygote	(228)

peduncle to feed primarily on phytoplankton, such as unicellular Cryptomonads. This type of feeding is known as myzocytosis (228, 382). One *Pfiesteria* species, *P. piscicida*, has also been reported to absorb nitrogen compounds including ammonia (NH₄), nitrate (NO₃), urea, and even glutamate (222). To complicate matters, *P. piscicida* may harbor whole, intact chloroplasts obtained after engulfing prey algae in its food vacuole, a phenomenon called kleptochloroplastidy (223). However, it is unclear how much photosynthesis contributes to the growth of *P. piscicida*, as these dinoflagellates cannot be grown autotrophically (68, 71). Currently, there are two recognized *Pfiesteria* species, *P. piscicida* Steidinger et Burkholder (343) and *P. shumwayae* Glasgow et Burkholder (142). *Pfiesteria*-like organisms include numerous cryptoperidiniopsoid strains, which may superficially look very similar to *Pfiesteria* spp. but are in fact different genetically and ultrastructurally (227, 342). Studies by Marshall et al. (242) suggest that cryptoperidiniopsoid species share some of the same life stages and feeding behaviors as the two-named *Pfiesteria* spp., but are not toxicogenic. Both *Pfiesteria* species were originally placed in the Dinamoebales because it was thought that the dominant stage was amoeboid (343). However, recent data questioning whether *Pfiesteria* has amoeboid life stages (Section 1.2.3 “Life history”) has suggested that a more appropriate taxonomic grouping for these dinoflagellates is in the Peridinales, considering *P. piscicida* readily feeds upon algal prey and is therefore not an obligate parasite of fish, unlike its closest relative of the Blastodinales, *Amyloodinium ocellatum* (88).

1.2.3 Life History

The life cycle originally proposed for *P. piscicida* by Burkholder et al. (67) includes 19 flagellated, encysted, and amoeboid stages. This life cycle has undergone

recent revision to include a palmelloid mass (defined as a group of nonmotile cells in a gelatinous matrix) (72). In contrast, the life cycle described for *P. shumwayae* is somewhat less complex and has fewer stages (72). For both species, transitions between stages, are reported to require the presence of live finfish, which is an integral aspect of toxicity (72, 75). Functional or physiologically distinct strains (i.e., toxic or nontoxic (73)) have been tested using the fish bioassay to determine ichthyotoxicity. Whether these are truly distinct strains, or the result of substrate limitation for toxin synthesis remains uncertain. To date, no toxin has been purified from *Pfiesteria* dinoflagellates making this assessment even more uncertain (264).

The discrepancies and confusion about the number of life stages inherently results in part from the use of cultures of *Pfiesteria* dinoflagellates growing in mixed assemblages with other microorganisms native to its habitat. For example, the fish bioassay used to test for toxicity and identify amoeboid and toxic *Pfiesteria* life stages contains a large consortia of micro- and macro-organisms, including viruses, bacteria, cyanobacteria, coccoid green algae, chrysophytes, protozoan ciliates, amoebae, rotifers, parasitic copepods, nematodes and opportunistic fungi (75). The identification of *Pfiesteria* life stages is made difficult by the presence of these other organisms and because *Pfiesteria* zoospores are remarkably similar to the zoospores of many other nontoxic heterotrophic dinoflagellate species. Indeed, the “*Pfiesteria*” amoebae described are very similar to other known true species of amoebae (70). In addition, newly excysted toxic dinoflagellates that were isolated from *Pfiesteria* fish bioassays have been described as being photosynthetic, a trait not possessed by *Pfiesteria* (76). For these reasons, the 20 or more life stages in the *Pfiesteria* life cycle have remained controversial. Recent efforts

have focused on developing clonal cultures of single cell isolates of *Pfiesteria* isolated by micromanipulators and then confirmed as bone fide *Pfiesteria* sp. by SEM, PCR or Fluorescent In Situ Hybridization (FISH) (68, 70, 71, 287, 313, 314).

Recent research indicates that a much simpler life cycle exists for *Pfiesteria* dinoflagellates, one that is more characteristic of other marine dinoflagellates. In this work, Litaker et al. (228) combined the use of standard light, epifluorescence and video microscopy with fluorescent in situ hybridization using peptide nucleic acid probes (PNA) to describe the life cycle of *P. piscicida*, which is presented in two relatively simple phases: the asexual phase and the sexual phase (Fig. 1.3). Within each phase, the dinoflagellates can be either cysts or zoospores (no amoeboid forms). In the asexual phase, there are three cyst types (division cysts, resting cysts, and temporary cysts) as well as a motile flagellated zoospore. In this phase, mitosis always occurs during encystment. The sexual phase of the life cycle involves the fusion of two daughter cells resulting in the planozygote followed by the hypnozygote and germination. Thus, the entire life cycle consists of six stages.

Are amoeboid stages a part of the *Pfiesteria* life cycle? The existence of amoeboid stages in the *Pfiesteria* life cycle was tested by Litaker et al. (228) using cultures of *P. piscicida* grown in the presence of goldfish (*Carassius auratus*) or tilapia (*Oreochromis niloticus*). Amoebae from these cultures were fixed and probed with different fluorescently labeled small subunit (18S) ribosomal RNA-specific PNA probes. These included a universal eukaryote-specific probe which served as a positive control to ensure the RNA targets were intact and that the probes penetrated the cells, a Eubacteria

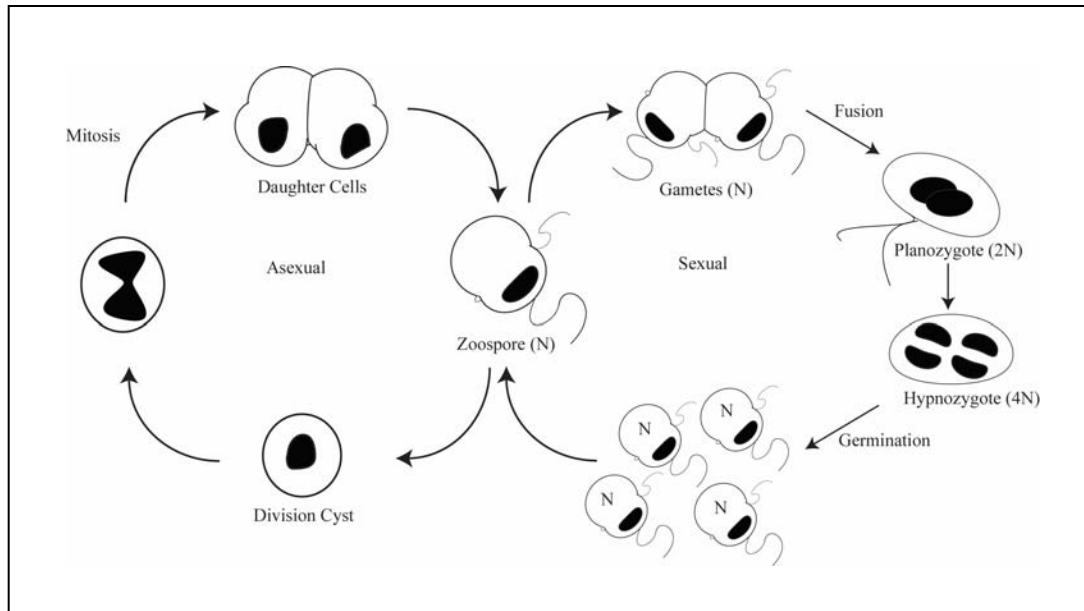


Fig. 1.3. The *Pfiesteria piscicida* life cycle as described by Litaker (2002). The life cycle of *P. piscicida* occurs in either the asexual or sexual phase giving rise to several different cyst stages (division cysts can also become temporary cysts and resting cysts), the biflagellated zoospore, planozygote and hypnozygote for a total of essentially six life

– specific negative probe which served as a nonspecific binding control, and finally amoeba and *P. piscicida* specific probes. The universal eukaryotic probe hybridized to both *P. piscicida* zoospores, as well as to the amoebae isolated from fish cultures, while PNA probes specific to *P. piscicida* did not bind to amoebae, and probes specific for amoebae did not bind to *P. piscicida* zoospores. Using the amoebae specific probe, 98% of the 300 amoebae examined were hybridization positive, while none were positive using the *P. piscicida* probe. Although further research needs to be done, there is, at this time, no conclusive evidence for an amoeboid stage in the life cycle of any *Pfiesteria* dinoflagellate.

1.2.4 Toxicity

Much of the *Pfiesteria* literature has been directed at the production of one or more “toxins” that are induced in the presence of fish (reviewed in (70)). I broadly define *Pfiesteria* “toxin” (or more appropriately, toxicity) as the production of one or more bioactive substances with potency towards fish and other animals that does not involve bioaccumulation in shellfish or finfish. Despite what can best be described as Herculean efforts by several prominent laboratories, to date, no *Pfiesteria* toxin has been isolated, or purified, nor a chemical structure elucidated (264). Methods to detect toxicity have primarily relied upon the standardized fish bioassay, as described by Burkholder et al. (75). In the standardized fish bioassay, a set of guidelines has been developed to help determine if water from an active fish kill event (either natural or laboratory aquaria where fish are dying) contains toxic *Pfiesteria*. The guidelines are:

- (Step 1) Measure field parameters (temperature, salinity, wind/current patterns) and confirm that other factors such as low oxygen or other toxic species are not present.
- (Step 2) Count dinoflagellates that resemble *Pfiesteria* using light microscopy to establish a minimum required density of more than 300 cells per ml.
- (Step 3) Incubate fish with unpreserved water samples with a goal of observing significantly more fish deaths over controls within 21 days.
- (Step 4) Continue the bioassay for an incubation sufficient to produce higher cell densities, permitting identification by a combination of SEM and PCR assays, and isolation followed by growth of the dinoflagellates in axenic culture (free from all other organisms except algal prey and bacterial endosymbionts).
- (Step 5) Expose fish to the axenic culture as in Step 3 to prove a set of standard hypotheses.

These standard hypotheses are tested as one would test Koch–Henle’s Postulates for an infectious organism (75). If all of these criteria are met then the water sample is assumed to contain *Pfiesteria* dinoflagellates that produce a toxic fish-killing substance. This assay has been the basis for all identifications of toxic *Pfiesteria* dinoflagellates (75).

Research by Moeller et al. and others (264) indicates that a toxin may be partially purified from cultures of *Pfiesteria* dinoflagellates growing in the presence of fish where fish are dying. In these experiments, these scientists used a luciferase reporter assay (115) in which rat Neuro2A and GH4C1 cells were transfected with plasmids containing the *c-*

fos-luciferase gene fusion such that light produced by the luciferase enzyme was dependent on the activation of the *c-fos* promoter. The *c-fos* gene is induced by a wide range of extracellular stimuli including convulsant drugs such as picrotoxin and pentylenetetrazole, various growth factors, and other pharmacologically active substances (337, 377). Using water from *Pfiesteria* fish bioassay aquaria, luciferase activity from GH4C1–A1 cells increased 41% over controls. These data suggest that cytotoxic substances (referred to as putative *Pfiesteria* toxin, pPfTx) in the *Pfiesteria* bioassay water sample activate the *c-fos*-luc.

In a second set of experiments, this same group determined that pPfTx acted upon the P2X7 receptor of GH4C1 pituitary cells (199). Several polar, methanol-soluble fractions of water obtained from a *Pfiesteria* fish bioassay aquarium were purified from a C18 column and tested using the *c-fos* luc assay. The lipid soluble *c-fos*-luc-active fractions were found to contain a phthalate ester as their principle active component (264). Such phthalate esters are common contaminants resulting from plastic polymerization processes. In this case, the authors believe that the phthalate ester identified was derived from the manufacturing process to make Instant Ocean artificial sea salt, subsequently used to make up the water in the bioassay aquarium (264). No compounds were identified from the water-soluble *c-fos*-luc-active fractions. Thus, as of this time, no toxin produced by either *P. piscicida* or *P. shumwayae* has been identified.

Efforts to identify toxic materials produced specifically by *Pfiesteria* dinoflagellates grown in fish bioassay aquaria are fraught with problems. As may be expected, there is abundant evidence that the *Pfiesteria* bioassay aquaria are rife with a thriving community of microorganisms, complicating the identification of the agent

responsible for the toxicity (75). For this reason, the toxicity of *Pfiesteria* dinoflagellates has been rigorously examined using experimental procedures designed to separate *Pfiesteria* dinospores from the other microorganisms, and to determine if fish death is the result of a soluble toxin. In an elegant set of experiments, Vogelbein et al. (382) utilizing low protein binding polycarbonate inserts to create two compartments within a culturing vessel, showed that fish mortality occurred only when *P. shumwayae* was in physical contact with the test fish, and then, only at cell densities exceeding 1000 cells per ml (382). In these experiments, *P. shumwayae* zoospores were observed attached to fish epidermal cells, which they ultimately consumed. Interestingly, while *P. shumwayae* quickly attached and consumed fish cells, *P. piscicida* never caused fish death and was not associated with fish epidermal tissue when placed in physical contact with fish. Moreover, an analysis of fish deaths in a 38 l fish bioassay aquarium using Tilapia (*O. niloticus*) as test fish confirmed that only fractions containing whole, intact *P. shumwayae* killed 100% of the larvae within 48 h. In contrast, dinoflagellate cell-free, bacteria enriched, and high ammonia fractions never killed larvae within the 96-h period. Thus, the data indicate that fish deaths require the presence of intact, living *P. shumwayae* cells that then attach to and devour fish epidermal tissues in a process of myzocytosis. Interpretation of these data does not require invoking an extracellular toxic molecule to explain the morbidity and mortality of the test fish.

Most toxic dinoflagellates produce a class of toxins called polyketides, chemically stable molecules synthesized nonribosomally by a polyketide synthase. In a separate study, Berry et al. (50) amplified polyketide synthase (PKS) genes from several known toxin-producing dinoflagellates using PCR and “universal” PKS gene primers, but were

unable to amplify PKS genes from *P. shumwayae* bulk DNA or cDNA. In addition, these researchers conducted an 8-month long fish bioassay using *P. shumwayae* and sheepshead minnows (*Cyprinodon variegatus*) in which fish died on a regular basis. Cell free supernatants, dichloromethane/methanol extracts, raw tank water, cell-free lysates, and a clonal culture of *P. shumwayae* grown on algal prey were tested for their ability to kill *C. variegatus* in six-well plates over a seven-day period. Only the clonal culture and raw water samples from the fish bioassay killed fish within the seven day period. The author's conclusion from these studies is *P. shumwayae* does not kill fish by releasing a toxin into bulk water, but rather consumes fish epidermal tissue by myzocytosis, a phenomenon the authors call micropredation.

In conclusion, the life history and toxicity of *Pfiesteria* species has been called into question. A majority of the 20 or more proposed stages of the *Pfiesteria* life cycle has only been shown in mixed microbial assemblages, a condition prone to the misidentification of life stages. Using advanced molecular tools, researchers have not been able to verify the amoeboid form of *P. piscicida*, even when it is in the presence of fish and other microorganisms. In addition, certain stages of the proposed *Pfiesteria* life cycle are differentiated based on the ability of the dinoflagellate to produce toxins. Thus, it appears that *Pfiesteria* dinoflagellates have six life stages involving three types of cysts, motile zoospores (or dinospores), planozygote, and heterozygote stages. There is little evidence to support amoeboid life stages in this dinoflagellate. Although cytotoxic substances have been partially purified from water containing *Pfiesteria* and other microorganisms, it has not been clearly established that those substances are derived from *Pfiesteria* dinoflagellates, and use of the fish bioassay as a source of toxic material may

prove useless in answering questions of toxin production specifically by *Pfiesteria*. More likely, *Pfiesteria* dinoflagellates, as exemplified by *P. shumwayae*, do not produce toxins, but rather kill fish by micropredation. Indeed, there are no data in the peer-reviewed literature that demonstrate toxin production specifically by *Pfiesteria*.

Do these new findings make *Pfiesteria* dinoflagellates uninteresting? No. Quite the contrary, it appears that, while not the “menace” they originally were imagined to be, *P. piscicida* and *P. shumwayae* are excellent representatives of a very ubiquitous group of small, heterotrophic dinoflagellate species that inhabit estuarine and coastal marine habitats. While this dinoflagellate may still have harmful effects, it is worthy of study because it represents a heretofore ignored, estuarine heterotrophic dinoflagellate found throughout the Atlantic coast of the U.S. and the world over. Table 3 lists all of the *P. piscicida* and *P. shumwayae* strains currently held in the Provasoli-Guillard Center for Cultures of Marine Phytoplankton (CCMP). A large number of strains isolated from the U.S. Atlantic coast region, as well as a new strain from New Zealand, are now available for study and these strains outnumber other species present in culture collections. The availability of these strains and much of the work that has been done up until now provides the basis for use of *Pfiesteria* dinoflagellates as model organisms for basic research in dinoflagellate physiology.

It has been well established that primary production by autotrophic phytoplankton increases due to nutrient loading by non-point source pollution, a process called eutrophication. In comparison, few studies have successfully mapped the response of the small heterotrophic dinoflagellates, such as *Pfiesteria* species, to increased nutrients in estuarine waterways (49, 140). *Pfiesteria* provides an excellent model organism for such

Table 1.3. Strains of *Pfiesteria piscicida* and *Pfiesteria shumwayae* available from the Provasoli-Guillard Center for Cultures of Marine Phytoplankton (CCMP).

Genus / species	Strain	Collection Site	Toxic^a	Axenic^b
<i>Pfiesteria piscicida</i>	1834 ^c	Pokomoke River, MD	Yes	No
	1830 ^c	Chicamacomico River, MD	Yes	No
	1921 ^c	Chicamacomico River, MD	Yes	No
	1831	Chicamacomico River, MD	Yes	No
	1901	Chicamacomico River, MD	Yes	No
	1902	Chicamacomico River, MD	Yes	No
	1928	Wilmington River, GA	Yes	No
	2091	Raleigh, NC	Yes	No
	2354	Marshall Creek, MD	Yes	No
	2361	Newport River, NC	Yes	No
	2362	Neuse River, NC	Yes	No
	2363	Calibogue Sound, SC	Yes	No
	2423	Neuse River, NC	Yes	No
	<i>Pfiesteria shumwayae</i>	2357	Neuse River, NC	Yes
2358		Neuse River, NC	Yes	No
2359		Marshall Creek, MD	Yes	No
2360		Tasman Bay, New Zealand	Yes	No
2089		Pamlico River, NC	Yes	No

^aAs indicated by CCMP

^bNo *Pfiesteria* dinoflagellate has been cultured without associated bacteria (axenic)

^cMixotrophy has been demonstrated in this strain.

studies. In addition, *Pfiesteria* dinoflagellates have a rapid growth rate in culture making them suitable for laboratory studies. They also occupy a unique ecological niche having characteristics of fish parasites, detritivores, and free-living herbivores. What controls the abundance of these dinoflagellates, and how do they affect the overall balance of carbon, nitrogen, and other nutrients in estuarine habitats? How do they sense fish and other types of prey and what molecules are involved? Answers to such questions may provide missing links between fish deaths and the presence of *Pfiesteria*. They may also provide critical information to governmental organizations struggling to manage nutrient overloading in our waterways. Scientists have learned much about the life cycle, physiology, and genetics of *Pfiesteria* dinoflagellates and it is highly likely that many more discoveries will be made. Thus, it is suggested that *P. piscicida* and *P. shumwayae* serve as excellent laboratory model organisms for further and future studies designed to understand more about estuarine dinoflagellates.

1.3 Bacterial interactions with dinoflagellates

1.3.1 Attached and intracellular bacteria.

Understanding how dinoflagellate species interact with other microorganisms is a challenging task. Co-occurring with dinoflagellates is a complex bacterial community that undergoes changes in species composition, abundance and physiology (109, 110). The most detailed studies of dinoflagellate-bacterial interactions have taken place in laboratory grown cultures. Several bacterial species have been shown to physically interact with toxic and non-toxic dinoflagellate species. For example, Silva (1985) detected intranuclear bacteria in *Gymnodinium splendens* and *Glenodinium foliaceum* by

light and electron microscopy (331, 332). These intranuclear bacteria could also be found in the cytoplasm and seemed to increase in number with the age of the culture. At the time of this study, methods were not available for species identification. Three species of intracellular bacteria were also detected in the toxic dinoflagellate *Alexandrium minutum*, and were identified as *Pasteurella haemolytica*, *Pseudomonas vesicularis*, and *Sphingomonas sp.* (231). These bacteria could be removed using antibiotics, however, when added back to the culture, dinoflagellate growth rate was not affected, although maximum cell density obtainable in culture was reduced. Similarly, bacteria-like structures contained inside chloroplasts have been observed in the freshwater dinoflagellate, *Woloszynskia pascheri* (393). These structures were transferred to the offspring through the cytoplasm and had two membranes, a chromosome, and ribosomes. It is not clear what evolutionary advantage these bacteria provided to the host, but other symbiotic interactions in protozoans involve complementing a nutritional deficiency or providing protection (150). Finally, bacteria were recently visualized in the dinoflagellate *Alexandrium catenella* by fluorescent staining and confocal scanning laser microscopy as well as electron microscopy. These bacteria could be cultured on Zobell's nutrient agar and were identified as *Aeromonas salmonicida*, *Flavobacterium breve*, *Pseudomonas diminuta*, *Pasteurella haemolytica*, *Proteus vulgaris*, *Pseudomonas putida*, *Pseudomonas vesicularis*, and a *Moraxella*-like species. The effect of these bacteria on dinoflagellate physiology, if any, is unclear, however, two of the isolates, *Moraxella*-like species and *P. diminuta* were capable of producing small amounts of saxitoxin detectable by HPLC (92).

1.3.2 Physiological interactions.

There are many examples linking the physiology of dinoflagellates to their associated bacteria. Tosteson et al. (1989) found that increases in the number of bacteria associated with dinoflagellate cell surfaces coincided with peak dinoflagellate toxicity in cultures of *Ostreopsis lenticularis* and *Gambierdiscus toxicus* (361). In addition, Nayak et al. (1997) found that the hemolysin titer from cultures of *Amphidinium carterae* increased eight-fold when supplemented with *Aeromonas*, *Vibrio*, *Moraxella*, or *Micrococcus* species (274). In these experiments, hemolysin production from the bacteria alone was ruled out. Nayak et al. (1997) suggests that *A. carterae* may increase hemolysin production in response to non-native microorganisms. According to Plumley (302), this has also been shown for saxitoxin producing species. However, an equally interesting hypothesis is that the bacteria provide necessary carbon or other essential compounds such as acetate or arginine for synthesis of the many saxitoxin derivatives.

Bacteria may also affect the growth of dinoflagellates in culture. As mentioned previously, three intracellular bacteria were shown to reduce the maximum cell density obtained for *Alexandrium* in culture (274). Also, toxicity decreased after stationary phase. It is possible these bacteria were able to utilize saxitoxin as a source of carbon and/or nitrogen as has been shown for bacteria present in bivalve mollusks (336). Interestingly, a *Roseobacter* species, *Jannaschia cystaugens* from Hiroshima bay, Japan has been shown to promote cyst formation of the dinoflagellate, *Alexandrium tamarenses* and 14 *Roseobacter* - like strains isolated during blooms of *A. tamarenses* were shown to possess this ability (2, 4). These bacteria occurred during the peak of bloom activity and during its decay suggesting that the bacteria were partially responsible for bloom senescence (3). A more common theme is that bacteria are necessary for dinoflagellate growth. Sakami et

al. (1999) found that *Gambierdiscus toxicus* would not grow after bacteria were removed by antibiotic treatment (319). Yet, one bacterial isolate was able to significantly improve growth when added back to the culture. High molecular weight fractions from this isolate stimulated growth better than did live intact cells. The authors suggest that the bacterium may provide some substance that is able to chelate zinc, based on evidence that *G. toxicus* growth media contains higher than optimum concentrations of Zn^{2+} . It is thought that, without this bacterium in culture, dinoflagellate growth would be inhibited by excess Zn^{2+} .

In the last few decades several algal lysing bacteria have been described from various genera including *Alteromonas*, *Cytophaga*, *Flavobacterium*, *Pseudomonas*, *Pseudoalteromonas*, *Saprospira* and *Vibrio* (244). A recent review suggests that algal lysing bacteria may be important for the accumulation or decline of dinoflagellate blooms (109). Two bacteria (*Flavobacterium* sp. and *Cytophaga* sp.) were isolated during the decline of a *Gymnodinium mikimotoi* bloom. Each isolate specifically lysed this dinoflagellate. One of these released a high molecular weight killing compound in response to organic matter excreted from *G. mikimotoi* and not from excreta of the other dinoflagellates tested. The identity of this compound is still under investigation. Generalized, or less species-specific algalytic bacteria, could, in some cases, promote the growth of an algal species by inhibiting the growth of competing species. Doucette (1998) cites an example in which a *Cytophaga* species was isolated from a bloom of *Heterosigma carterae* (109). This bacterium lyses all algal species tested, except *H. carterae*. Thus, the unique immunity of *H. carterae* to the *Cytophaga* species may partly explain this particular bloom of *H. carterae*. Addition of algalytic bacteria to affected

waters has been suggested as a possible mitigation strategy, however, algalytic bacteria may be more useful in predicting HAB activity. Doucette et al. (108) isolated two unidentified bacterial species from the Gulf of Mexico that were able to lyse several strains of the toxic dinoflagellate, *Gymnodinium breve*. These bacteria came from waters where the dinoflagellate is rarely detected. Doucette suggests that the natural bacterial assemblage may specifically deter blooms of *Gymnodinium* species in this area and others where the bacterial species are found.

Bacteria that are able to metabolize organic sulfur compounds may influence the physiology of dinoflagellates. In a North Atlantic bloom dominated by *Emiliana huxleyi* the surrounding waters consisted mainly of dinoflagellates (149). Relatives of the *Roseobacter* clade of bacteria were the dominant bacterial species throughout the bloom. These *Roseobacter* species were found to correlate with the algal osmolyte, dimethylsulfoniopropionate (DMSP). This is interesting given that dinoflagellates are known to produce relatively large quantities of DMSP. Keller et al. (1996) measured concentrations of dissolved and particulate DMSP in 26 species of marine phytoplankton representing 7 different classes (191). Concentrations of DMSP were highest in dinoflagellates and prymnesiophytes. Thus bacteria capable of catabolizing DMSP would likely dominate blooms of these phytoplankton (243, 405). Zubkov et al (2001) tested this hypothesis in a 2000 Km² bloom of *Emiliana huxleyi* and *P. minimum* in the North Sea. These researchers found that bacterial production correlated with the degradation of dissolved DMSP. Furthermore, a species related to the genus *Roseobacter* was the most abundant bacterial species present throughout the bloom. The abundance of this

bacterium correlated with both overall bacterial production and DMSP consumption, whereas the abundance of other species did not.

1.4 The *Roseobacter* clade and dimethylsulfoniopropionate

1.4.1 General characteristics of the *Roseobacter* clade

The marine α -Proteobacteria phylogenetically related to *Roseobacter* species, are the second most abundant species of bacteria in the ocean (138). They are found in a diverse range of habitats including coastal (148) and open ocean environments (405), salt marsh ecosystems (65), salt lakes (207), coral reefs (389), within marine sponges (11, 262, 271), and marine sediments (146, 148). They are not indigenous to freshwater (307) and most require sea salts for growth. The majority of strains that have been isolated are planktonic organisms and their abundance has been correlated with blooms of certain planktonic algae, especially algae that produce the algal osmolyte, DMSP (405). This is a tremendously diverse group of taxonomically related marine bacteria.

Based upon substrate utilization and phylogenetic relatedness, approximately 16 different genera have been assigned to the *Roseobacter* clade. These genera are *Antarctobacter* (207), *Sagittula* (147), *Ruegeria* (366), *Silicibacter* (299), *Leisingera* (322), *Sulfitobacter* (306), *Octadecabacter* (60), *Oceanicola* (85), *Jannaschia* (385), *Ketogulonicigenium* (367), *Marinosulfonomonas* (169), *Methylarcula* (106), *Roseobacter* (327), *Roseovarius* (208), *Staleyia* (209) and *Roseovivax* (349). They all possess a large range of metabolic capabilities. Members of the *Roseobacter* clade are capable of carbon monoxide oxidation (265), aerobic anoxygenic photosynthesis (327), oxidation of sulfite and methanesulfonic acid (146, 169), degradation of organic sulfur compounds (146),

Table 1.4. General characteristics of *Roseobacter* species

Species	Motile	Flagella	Length (μm)	Rosettes ^a	Bchl a	Habitat	Salinity (ppt)	Temp. °C	DMSP ^b	Ref.
<i>Antarctobacter heliothermus</i>	Yes	1-3 subpolar	0.8 - 1.2	Yes	No	Hypersaline lake	10-40	16-20	ND	(207)
<i>Sagittula stellata</i>	No	≥ 1 detached	0.9	Yes	No	Coastal sewer	20	27	ND	(147)
<i>Silicibacter lacuscaerulensis</i>	No	ND	9 - 18	ND	No	Silica-rich saline lake	35-40	45	D	(299)
<i>Silicibacter pomeroyi</i>	Yes	1 polar	ND	Yes	No	Coastal seawater	5 - 20	30	L/D	(145)
<i>Silicibacter</i> sp. TM1040	Yes	≥ 3 polar	1.7	Yes	No	Dinoflagellates	5 - 20	30	D	(249)
<i>Sulfitobacter mediterraneus</i>	Yes	1-5 subpolar	1 - 3	Yes	No	Mediterranean Sea	15-20	17-28	ND	(306)
<i>Oceanicola granulosus</i>	No	ND	0.7 - 2.1	ND	No	Sargasso Sea	25-30	28-30	ND	(85)
<i>Jannaschia helgolandensis</i>	No	No	1.9 - 3.2	No	No	North Sea	10-70	25-30	ND	(385)
<i>Jannaschia cystaugens</i>	Yes	ND	1.5 - 2.0	ND	No	Algal bloom	20-70	25-30	ND	(4)
<i>Roseobacter denitrificans</i>	Yes	1 -2 subpolar	1 -2	No	Yes	Seaweed	ND	20-30	ND	(327)
<i>Roseovarius tolerans</i>	Yes	ND	1.1 - 2.2	No	Yes	Hypersaline lake	10-80	8-34	L/D	(145, 208)
<i>Ruegeria algalicola</i>	Yes	1 -2 subpolar	2 - 3	No	No	Dinoflagellates	5 - 60	30	ND	(210)

^aRosettes are groups of cells attached to one another by their cell poles

^b*Roseobacter* species can degrade DMSP by either the lyase pathway (L), the demethylation pathway (D) or both (L/D).

ND = not determined.

lignin and other aromatic compounds (64), degradation of methylamine (106), methyl bromide, and the production of allelopathic compounds (262).

Table 1.4 lists some of the best studied *Roseobacter* species and their general characteristics. In motile strains, motility is almost always achieved by one to three subpolar to polar flagella. Most are rod shaped although in laboratory cultures strains exhibit a range of morphologies including elongated cells sometimes >33 μm , ovoid shapes or irregular rods (207). Many are lightly colored with either a brownish yellow pigment, a reddish pink pigment or contain bacteriochlorophyll a, giving cells a deep red appearance. As noted above, they can be found in a range of habitats and salinities. Optimal growth for most species is somewhere between 30 °C and 20°C except for the so-called extremophile, *Silicibacter lacuscaerulensis* which can grow at 45 °C. As can be seen, several including *Ruegeria algicola*, *Jannaschia cystaugens* and *Silicibacter* sp. TM1040 (isolated in this study) are associated with dinoflagellates. Several species have also been shown to degrade the algal osmolyte DMSP by one or more pathways.

1.4.2 Pathways involved in the degradation of DMSP

One of the most striking characteristics to be discovered about the *Roseobacter* clade is the ability of these bacteria to consume a number of important sulfur compounds, including DMSP and its catabolites (146). These bacteria and others are known to catabolize DMSP by multiple biochemical pathways and some of the enzymes involved have been studied in detail. The initial biodegradation of DMSP may occur by one of two biochemical pathways called the lyase pathway or the demethylation pathway. To initiate the lyase pathway, bacteria as well as some algae produce an enzyme, dimethylpropiothetin dethiomethylase (DMSP lyase) (EC 4.4.1.3) (Fig. 1.4, reaction 1),

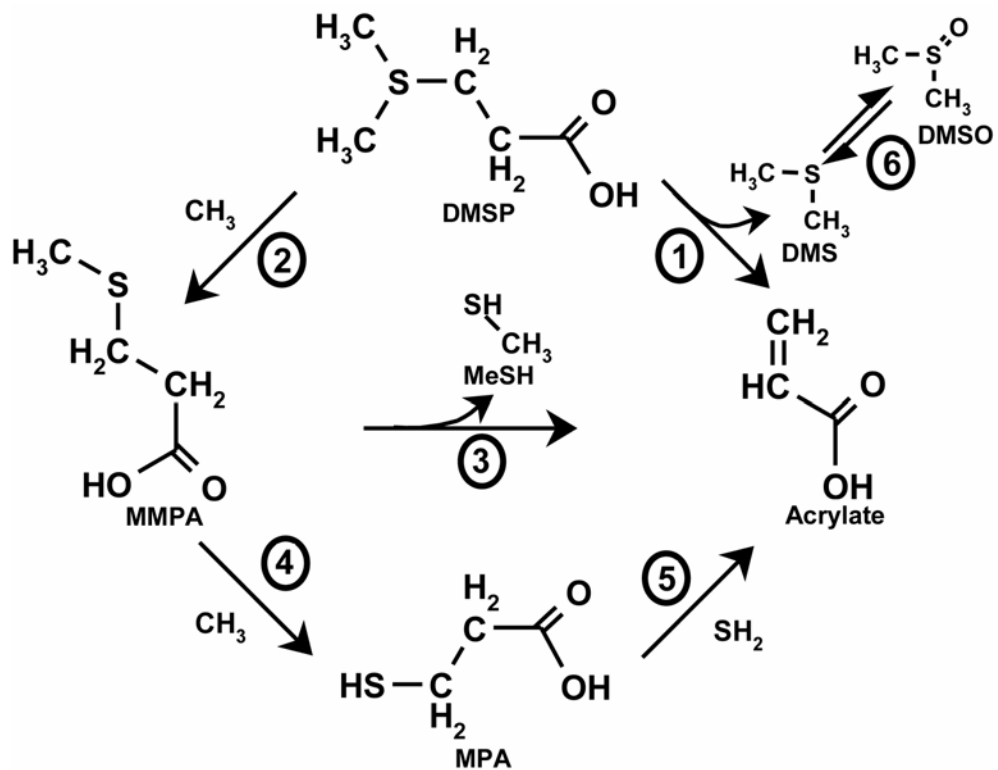


Fig. 1.4 Pathways involved in the catabolism of DMSP. Degradation of DMSP may occur by the lyase pathway, hydrolysis of the C3 carbon producing acrylate and DMS (Rxn 1), or by the demethylase pathway, demethylation of the DMS moiety producing MMPA (Rxn 2). MMPA may be further demethylated to MPA (Rxn 4) followed by elimination of hydrogen sulfide (Rxn 5) or demethylated producing acrylate and MeSH (Rxn 3). In some cases, DMS may be oxidized to DMSO (Rxn 6).

that cleaves DMSP at the C3 carbon producing dimethylsulfide (DMS) and acrylate (399). In the γ -Proteobacteria *Alcaligenes faecalis* M3A and *Pseudomonas duodoroffi* as well as in at least one *Roseobacter* species, strain LFR, the activity of this enzyme is induced when the bacteria are grown in the presence of acrylate or DMSP compared to when the same cells are grown in the presence of glucose (28, 99). Strain LFR accumulates DMSP in the cell followed by the production of DMS and β -hydroxypropionate. Acrylate is also thought to be produced but is immediately converted to β -hydroxypropionate, and is not detected presumably due to its rapid turnover (27). This may be a common trait of other marine bacteria since acrylate is rapidly degraded by marine bacterial communities associated with acrylate-producing algal blooms (278, 279, 290). The β -hydroxypropionate produced from the degradation of acrylate is thought to be decarboxylated producing an unknown compound (27).

The DMS produced by the lyase pathway is an important degradation product since it contains the sulfur atom. Many *Roseobacter* species capable of DMSP lyase activity utilize DMS produced from the lyase reaction. For example, 10 out of 15 *Roseobacter* species isolated from Georgia coastal seawater capable of DMSP lyase activity eventually degraded all of the DMS produced from the reaction (146). It is not clear how DMSP-degrading *Roseobacter* species degrade DMS, but in the taxonomically related anoxygenic phototroph, *Rhodobacter rhodovulum*, DMS is converted to DMSO by a DMS dehydrogenase (248). This reaction donates electrons to support photosynthetic growth in *R. rhodovulum*. In the α -Proteobacterium, *Thiocapsa roseopersicina*, another anoxygenic phototroph, DMS is instead oxidized to sulfate (380). Yet, a more likely route for DMS degradation might be demethylation to produce

methanethiol since this allows the incorporation of sulfur into proteins. For example, in *Hyphomicrobium* and *Thiobacillus* species, DMS is converted to methanethiol and formaldehyde via a DMS monooxygenase (48). This methanethiol is then converted to hydrogen sulfide and formaldehyde by a methanethiol oxidase. The enzyme, cysteine synthetase, incorporates sulfide from this reaction into the amino acid methionine for protein synthesis (48). However, Kiene et al (197) show that a large percentage of marine bacteria directly incorporate methanethiol into methionine through a cystathionine - γ - lyase instead of cysteine synthetase (197). This direct incorporation would not require the conversion of methanethiol to hydrogen sulfide before incorporation into methionine. Assimilation of sulfur through DMSP degradation is a potentially important aspect of *Roseobacter* physiology. While concentrations of sulfur are generally high in marine ecosystems (28 mM as sulfate, SO_4^{2-}), the most oxidized form of sulfur predominates. This makes the assimilation of sulfur from organic sulfur compounds like DMSP energetically more favorable (197).

The second and most predominant pathway for DMSP degradation is demethylation of DMSP at the DMS moiety (Fig. 1.4, reaction 2), producing 3-methylmercaptopropionate (MMPA) (182). This compound may be further demethylated to 3-mercaptopropionate (MPA; Fig. 1.4, reaction 4) (378) or demethylated to produce acrylate (Fig. 1.4, reaction 3) and methanethiol (MeSH) (353). The second demethylation product, 3-mercaptopropionate may be converted to hydrogen sulfide and acrylate. As mentioned previously, methanethiol and hydrogen sulfide can be incorporated into methionine for protein synthesis while acrylate may be converted to β -hydroxypropionate. However, bacteria may instead oxidize hydrogen sulfide to sulfite

and sulfate using these compounds as electron donors to drive energy production (192). In any case, demethylation of thiols, either DMS or DMSP by marine bacteria, especially, *Roseobacter* species provides a direct route for sulfur assimilation.

1.4.3 *Roseobacter* interactions with dinoflagellates

Several studies indicate that *Roseobacter* species are associated with dinoflagellates in the environment and in laboratory cultures. In cultures of the dinoflagellate *Alexandrium tamerense*, a *Roseobacter* species, strain 253-11, attaches to the dinoflagellate cell surface (334). The maximal number of *Roseobacter* attached to *A. tamerense* occurred during stationary phase of the dinoflagellate growth curve (45 cell^{-1}) while the number of other, free living bacteria remained constant through out all dinoflagellate growth phases at ca. 10^3 bacteria per dinoflagellate cell. These experiments took place in cultures lacking other bacteria, so the effect of the *Roseobacter* species on growth of *A. tamerense* could be assessed. Compared to cultures of *A. tamerense* with no bacteria (axenic), the lag phase of growth in cultures with only strain 253-11 was extended by ca. 5 days, but reached stationary phase at the same point as control cultures (334). *A. tamerense* is known to produce the paralytic shellfish toxin, saxitoxin, however, there is variability in toxin production among strains. Interestingly, *Roseobacter* sp. strain 253-11 also produces saxitoxin (134). It is possible that strain 253-11 is responsible for saxitoxin production associated with *A. tamerense* or at least enhances the toxicity of saxitoxin-like molecules produced by *A. tamerense*.

Other studies indicate that *Roseobacter* species are associated with algal blooms in the environment. For example, *Roseobacter* species made up >20% of all bacterial species within a bloom in the North Atlantic consisting of the dinoflagellates

Gymnodinium and *Ceratium* sp., and to a large extent (40 -50%) the coccolithophore, *Emiliania huxleyi* (405). The greatest percentage of 16S rDNA clones related to *Roseobacter* species were found at the sea surface (>50 meters depth). Furthermore, in a separate study, the abundance of *Roseobacter* species within the bloom was correlated with bacterioplankton abundance, the consumption of DMSP, and algal production (406). These results confirm existing reports indicating that *Roseobacter* species are the major consumers of DMSP in the environment and that dinoflagellates are the largest producers of DMSP (146, 398). It is likely that the association of *Roseobacter* with dinoflagellates is mediated by the metabolism of DMSP in marine ecosystems.

1.5 Production of DMSP in the marine ecosystem.

1.5.1 Biochemistry of DMSP production

The biosynthesis of DMSP has been studied in a dinoflagellate, a macroalga and a terrestrial plant. In the dinoflagellate, *Cryptothecodinium cohnii*, the hypothetical pathway proposed for DMSP synthesis begins with decarboxylation of methionine to produce methylthiopropylamine followed by deamination to produce methylmercaptopropionate (MMPA) (Fig. 1.5) (365). The final step is S-methylation of MMPA to form DMSP. Other intermediates cannot be ruled out, such as methylthio-oxobutanoic acid, S-adenosylmethionine and S-methylmethionine. In the photosynthetic dinoflagellates, *Gonyaulax polyedra*, *Amphidinium* sp. and *Pyrocystis lunula*, DMSP is synthesized via a similar pathway as determined by [methyl-¹³C₁]methionine feeding studies (273). However, DMSP is subsequently used as substrate for the synthesis of two other

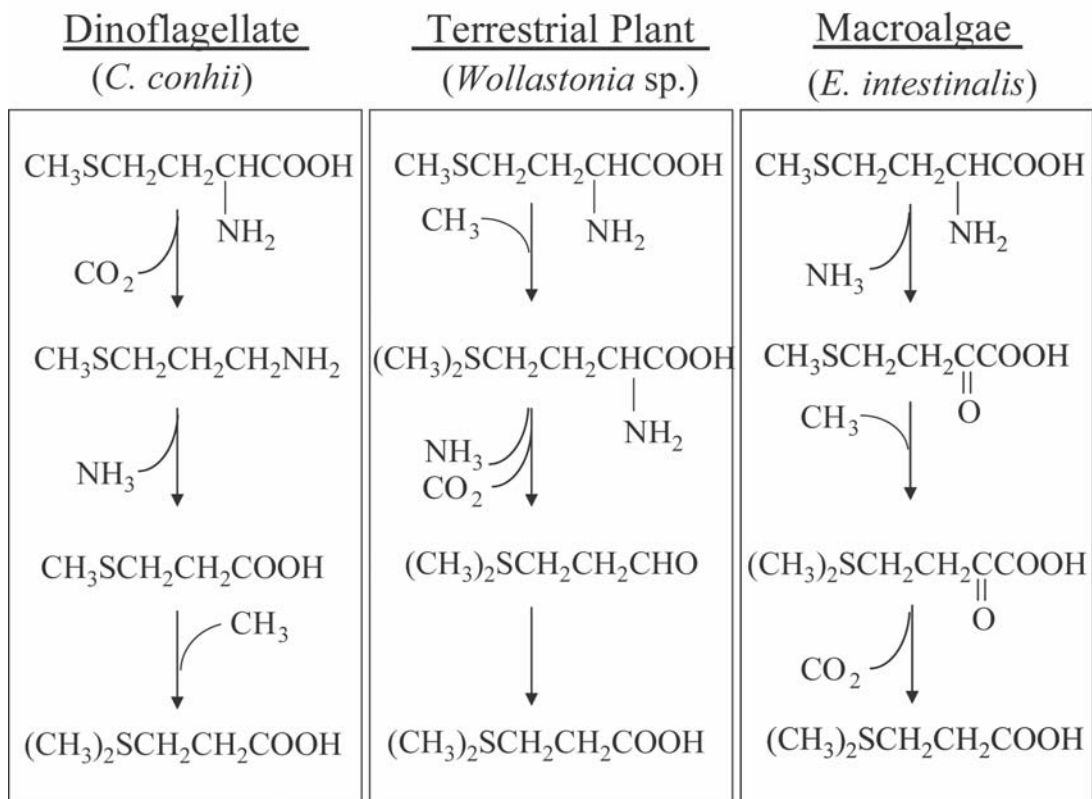


Fig. 1.5. Biosynthesis of DMSP in a dinoflagellate, terrestrial plant and macroalga. In all organisms the synthesis of DMSP begins with methionine and involves the removal of an amine and decarboxylation and/or methylation. The enzymes utilized and the order of reactions varies between organisms (see text for details).

compounds gonyol and gonyauline such that in *P. lunula* gonyol is the major sulfonium compound in this dinoflagellate under normal culture conditions (273). Both gonyol and gonyauline are responsible for maintaining circadian rhythms in *G. polyedra*, suggesting very broad implications for DMSP metabolism in dinoflagellate physiology.

In the macroalga, *Enteromorpha intestinalis* (also called *Ulva lactuca*) the starting substrate for DMSP synthesis is also methionine. However, in this case the first step is to transfer the amino group from methionine to 2-oxo-butyrate forming 4-methylthio-2-oxobutyrate and glutamate (Fig. 1.5)(133). Synthesis continues with reduction of the 2-hydroxyl group of 4-methylthio-2-oxobutyrate to form 4-methylthio-2-hydroxybutyrate. These first two reactions are completely reversible. In the final two steps, 4-methylthio-2-hydroxybutyrate is S-methylated producing 4-dimethylsulfonio-2-hydroxybutyrate which is then oxidatively decarboxylated producing 3-dimethylsulfoniopropionate (133). In this case synthesis follows the same basic steps consisting of deamination, decarboxylation and S-methylation. However, since the pathway begins with deamination instead of decarboxylation, as in the case of the dinoflagellate *C. cohnii*, a different set of chemical intermediates are produced.

High levels of DMSP have been found in angiosperms, or flowering plants, mostly in mesophytic or halophytic herb species (160). In the coastal plant, *Wollastonia biflora* (Asteraceae) synthesis of DMSP begins with S-methylation of methionine to produce S-methylmethionine via a AdoMet:Met S-methyltransferase, EC 2.1.1.12 (Fig. 1.5)(161, 181). S-methylmethionine is then transferred to the chloroplast where it is thought to undergo deamination and decarboxylation to produce DMSP-aldehyde, however the enzyme(s) involved in these reactions is (are) unknown (160, 363). The

Table 1.5. Intracellular DMSP concentrations of plants and algae

Angiosperms^a	DMSP (mM)	Reference
<i>Spartina alterniflora</i>	20 – 70	(91, 94, 292}
<i>Spartina angelica</i>	4 – 48	(105, 212, 268}
<i>Spartina foliosa</i>	7 – 8	(292}
<i>Saccharum sp.</i>	6	(293}
<i>Wollastonia biflora</i>	12 – 30	(162}
Chlorophyceae^b		
<i>Enteromorpha intestinalis</i>	15	(372}
<i>Ulva californica</i>	68	(372}
<i>Acrosiphonia coalita</i>	5	(372}
Prymnesiophytes		
<i>Isochrysis galbana</i>	103	(275}
<i>Gephyrocapsa oceanica</i>	129	(275}
<i>Emiliania huxleyi</i>	157 – 242	(344}
<i>Hymenomonas carterae</i>	120	(368}
Haptophytes		
<i>Phaeocystis sp.</i>	71 – 169	(341}
Bacillariophyceae (Diatoms)		
<i>Thalassiosira pseudonana</i>	5.7	(191}
<i>Skeletonema costatum</i>	16	(191}
<i>Melosira nummuloides</i>	29	(191}
<i>Chaetoceros simplex</i>	1.3	(191}
Dinophyceae^c		
<i>Gymnodinium nelsoni</i>	280	(95}
<i>Heterocapsa triquetra</i>	320	(275}
<i>Scrippsiella trochoidea</i>	573	(275}
<i>Gymnodinium simplex</i>	523	(191}
<i>Prorocentrum micans</i>	172	(191}
<i>Prorocentrum minimum</i>	104	(191}
<i>Cryptocodinium cohnii</i>	13	(191}

^aDMSP concentrations in angiosperms were calculated based upon the assumption that wet wt. is all due to cytoplasmic water.

^bDMSP concentrations in chlorophyceae were calculated based upon the percent of DMSP in fresh algal mass.

^cDMSP concentrations were calculated using either fmoles or pg DMSP per cell from the reference listed and cell diameter measurements from CCMP website (<http://ccmp.bigelow.org/>).

DMSP-aldehyde that is formed is converted to DMSP by oxidation of the aldehyde group.

The intracellular concentration of DMSP in plants, macroalgae and algae vary according to the species or genus (Table 1.5). In *S. alterniflora*, an angiosperm, the average concentration of intracellular DMSP is 20 – 70 mM (292) while in sugarcane (*Saccharum* sp.) it is only 6 mM. Chlorophytes (Macroalgae) also vary with species and in general have intracellular DMSP concentrations similar to those of the angiosperms (4 – 70 mM). Of the microalgae, diatoms produce the least amount of intracellular DMSP (1.3 – 29 mM) whereas some of the prymnesiophytes and dinoflagellates produce the highest concentrations of intracellular DMSP that have been measured (> 0.5M). While these laboratory measurements generally seem to reflect DMSP concentrations in the environment, DMSP production is known to be influenced by a host of variables including salinity, light, dissolved nitrogen and sulfur, temperature and other environmental stressors (269, 347, 368, 397). Changes in intracellular DMSP concentrations may be related to the role of DMSP in algal physiology.

1.5.2 Role of DMSP metabolites in dinoflagellate physiology

There are several possible effects of DMSP production on the physiology of dinoflagellates and other organisms. These include osmoprotection (104, 368) and cryoprotection (189), antiherbivory (395), protection from oxidative stress (347) and cell signaling.

DMSP is structurally related to glycine betaine, a common osmolyte in many different cell types. Glycine betaine, proline and DMSP are all compatible solutes, compounds that can accumulate to high intracellular concentrations without interfering

with normal cellular processes (62). This is due in part to their chemical nature. Glycine betaine, proline and DMSP are zwitterions (“twin ions”), having a positive charge at one end of the molecule and a negative charge at the other making them essentially neutral compounds within the cellular environment. An organism may vary the concentration of these compounds in order to balance osmotic stress or protect themselves from near-freezing or suboptimal temperatures. There is increasing evidence that DMSP is a compatible solute used to regulate osmolarity. In the prymnesiophyte *Hymenomonas carterae*, increasing salinity leads to an increase in DMSP production by this species (368). This effect is not due to a specific ion since both high concentrations of NaCl and sucrose cause an increase in DMSP production by *H. carterae* (368). However, in the diatom *Cylindrotheca closterium*, production of DMSP increases with an increase in salinity up to 44 psu seawater, at which point, no further increases in DMSP production are observed. Instead, at higher salinities, the intracellular concentration of the amino acid, proline, increases as the salinity shifts from 33 – 55 psu. Proline is probably a more important osmoprotectant at higher salinities than is DMSP. Since DMSP concentrations are normally already high within the cell, DMSP probably serves as a buffer for sudden changes in salinity since increases in DMSP production occur too slowly to be of any use in most ‘real-world’ circumstances (200). Some algae do not use DMSP at all to control osmolarity suggesting that DMSP has other roles within the cell (373).

In plants and algae, high light irradiance can increase the production of oxygen radicals due to higher rates of photosynthesis (276). In order to cope with the added stress, the organism may increase the production of enzymes in the antioxidant system and/or the production of antioxidant compounds. Sunda et al (347) report that DMSP and

its breakdown products including DMS and acrylate function as an antioxidant system. The authors report that limitation for CO₂ and iron, two known inducers of oxidative stress, causes an increase in the production of cellular DMSP in the diatom *Thalassiosira pseudonana*. The half-lives of oxygen radicals were shown to be shorter when in the presence of DMSP, acrylate and DMS, suggesting that these compounds are effective antioxidants. Yet, it is not known how this antioxidant system would be affected in environmental situations, especially when in the presence of a robust DMSP degrading bacterial community.

The production of DMSP and its degradation occurs by multiple species in marine environments, such that microbial interactions are likely to be important for DMSP and its effects on algal physiology. A recent report indicates that DMSP lyase activity may protect algae from predators. Wolfe et al. show that strains of *Emiliana huxleyi* possessing high DMSP lyase activity are grazed upon at a much slower rate than strains with low DMSP lyase activity (344, 395). It is not known how high DMSP lyase activity actually deters predation, but acrylate, a principle breakdown product from DMSP lyase activity is toxic to some microorganisms at elevated concentrations (329, 330). In this “poisoned pill” theory, a high DMSP lyase activity would cause high concentrations of acrylate in the microniche surrounding algal cells thus deterring predators. This argument is helped by the fact that DMSP lyase activity in *E. huxleyi* and in the dinoflagellate *A. tamerense* is inducible by agitating the cells or upon the addition of polyamines, known inducers of exocytosis (production of a feeding tube) in some algal predators (396). These studies took place in axenic cultures, so it is not known how DMSP and acrylate

degrading *Roseobacter* species would affect this phenomenon, especially since these bacteria are abundant within blooms of these algal species (77, 405-407).

1.5.3 Distribution of DMSP production in the environment

DMSP can be found in a variety of aquatic environments including coastal and estuarine waters, the open-ocean, coral reefs, microbial mats, salt marsh ecosystems, and marine sediments (61, 317, 381). The occurrence of DMSP in freshwater environments has not been fully investigated (399). The majority of studies that have measured the distribution and concentration of DMSP in the environment have occurred in either open-ocean or coastal environments, and it is thought that these areas are where the bulk of global DMSP is produced. In open-ocean waters, Andrae et al. (24) measured DMSP during a spring bloom in the North Atlantic, near the Western European Basin between the Azores front and the Subarctic front. Blooms of DMSP-producing algae frequently occur in this area during the spring. Before the bloom event occurred small flagellates with low chlorophyll *a* content were responsible for the bulk of DMSP production, producing 20 – 30 nM particulate DMSP and 8 – 10 nM dissolved DMSP (24). In this case there was not a correlation between chlorophyll *a* and DMSP concentrations. During the bloom event, which consisted mainly of diatoms, concentrations of particulate and dissolved DMSP reached highs of 380 and 25 nM, respectively (24). Overall, concentrations of DMSP correlated with chlorophyll *a* and decreased with depth, rapidly declining at 60 m in most places and becoming undetectable after 150 m. In a separate study in the North Sea, a bloom dominated by *E. huxleyi* and dinoflagellates produced an average 1,360 μM per m^2 particulate DMSP and 155 μM per m^2 dissolved DMSP (77). Interestingly, the major DMSP producer in this bloom was not *E. huxleyi* since this

species only accounted for 13% of all particulate DMSP. Therefore, dinoflagellates were likely responsible for the bulk of the DMSP production during this bloom. Again, DMSP concentrations were not correlated with chlorophyll a and decreased with depth.

In coastal waters, Kiene et al measured DMSP concentrations in the northern Gulf of Mexico for a full year (194). Samples collected from Dauphin Island, AL did not show a seasonal correlation or a correlation with temperature or salinity. The yearly average concentration of dissolved DMSP was 3.5 nM while particulate DMSP averaged 49 nM. During a bloom event in July the dissolved and particulate DMSP concentrations spiked to over 10 and 200 nM, respectively. In another coastal study, Ledyard and Dacey (214) measured DMSP concentrations in Monterrey Bay, CA during April and May. These scientists found particulate concentrations of DMSP ranging from 32 – 118 nM and dissolved DMSP ranging from 4.2 – 11.5 nM. The particulate DMSP concentrations were highest near the coast and decreased with depth. A study in the Chesapeake, Delaware and Ochlockonee Bays shows a correlation between salinity and DMSP. Iverson et al (180) measured DMSP concentrations along transects through each bay and found that the dissolved and particulate DMSP concentrations in the Delaware and Ochlockonee Bays increased with increasing salinity while DMSP concentrations decreased with increasing salinity in the Chesapeake Bay. For all bays, maximum particulate DMSP concentrations occurred at the chlorophyll a maximum with values ranging from 40 nM in the Ochlockonee Bay to 70 nM in the Chesapeake Bay and 150 nM in the Delaware Bay. The researchers did not measure DMSP in the rivers, creeks, and backwaters of these bays where many algal blooms occur. Surprisingly little work has been reported concerning DMSP metabolism in these important estuarine ecosystems.

From these studies several conclusions can be made about the distribution of DMSP in the environment. In all cases the particulate concentrations of DMSP are higher than dissolved concentrations suggesting that most DMSP in the environment is intracellular. This could change, for example, during bloom senescence when algal cells lyse and release DMSP. However, dissolved DMSP would most likely be quickly degraded by bacterial and algal enzymes reducing them to background levels, which appear to be in the range of 1 – 10 nM. The majority of DMSP is produced in surface waters and decreases with increasing depth. This may indicate that most DMSP is produced by phototrophs. While some photosynthetic organisms produce high intracellular concentrations of DMSP, there are many, such as diatoms that produce very little intracellular DMSP. In addition, concentrations of DMSP are not always correlated with chlorophyll a. In some coastal regions, DMSP concentrations may be correlated with other variables such as salinity or temperature. However, these variables may be related to conditions that are optimal for growth of a high DMSP-producing algal species. At this time there are no strict set of physical or chemical factors that predict where and when high DMSP concentrations will be found. The occurrence of high DMSP concentrations in any given area most likely involves both physical and chemical factors as well as the composition of the bacterial and/or algal community.

1.5.4 DMSP and the global sulfur cycle

DMSP is the major source of organic sulfur in the world's oceans and plays a significant role in the global sulfur cycle. During blooms of marine algae, cellular DMSP is released due to algal senescence, predation, or stress and is degraded by both algal and bacterial enzymes producing, among other chemicals, DMS. The DMS is readily

volatilized at the air-sea interface adding an incredible amount of gaseous sulfur to the atmosphere. Approximately 39 Tg S are introduced to the atmosphere each year by this reaction, accounting for 50% of all the world's sulfur emissions and 90% of all naturally produced sulfur (23, 25, 87). Within the atmosphere, DMS is readily oxidized forming, among others, methane sulfonic acid and sulfuric acid (83). These compounds act as nuclei for the condensation of water vapor resulting in cloud formation. The sulfur trapped in these clouds eventually falls back to the earth as acid rain (280). More importantly, these atmospheric sulfur molecules cause the backscattering of sunlight, counteracting the effects of global warming (44, 83). It is postulated that cloud formation and the backscattering of sunlight may theoretically influence bloom-formation by reducing mean light intensity and temperature (83). Thus, aspects of bacterial or algal physiology that influence DMSP production and consumption may impact the global sulfur cycle and climate.

1.6 Chemotaxis and motility in non-enteric bacteria

1.6.1 General aspects of motility and chemotaxis in bacteria.

Motile bacteria can change their direction of movement in response to chemical gradients through a process called chemotaxis. In *E. coli* and similar enteric bacteria this is achieved by alternating between smooth swimming and tumbling behavior (5, 6). When the flagella turn in a counter clockwise direction, all flagella turn in a tight helical path consistent with the shape of the flagella forming a bundle (237). This propels the bacterium forward in a smooth swimming motion called a run. When the bacterium senses a change in its' chemical environment the flagella change from a counter

clockwise to a clockwise direction. In this direction the flagella do not form a bundle and the cells tumble, a condition that ultimately causes a reorientation of the cell. After a brief period (1-2 s), smooth swimming resumes and the bacterium moves in a different direction. This process of smooth swimming and tumbling is repeated at a high frequency until the bacterium reaches a stable environment, at which point the frequency of switching returns to a basal level.

The job of sensing the chemical environment is achieved by the chemotaxis proteins (59). This process begins with the methyl - accepting chemotaxis proteins (MCP) that span the inner membrane and bind a compound or suite of compounds (ligands) in the periplasmic space (204). When a ligand is not bound to the MCP receptor, the CheW protein couples CheA to the cytoplasmic protein of the MCP receptor where CheA is stimulated to autophosphorylate at a histidine residue (266). CheA phosphate (CheA-P) then donates this phosphate to both the CheY and CheB proteins at aspartate residues (57, 233, 266). Phosphorylated CheY (CheY-P) binds to a portion of the flagellar motor, FliM, regulating switching from counterclockwise (smooth swimming) to clockwise (tumbling) rotation of the flagella (359, 391). When the MCP receptor is bound with ligand, the autophosphorylation activity of CheA is reduced, which ultimately decreases tumbling frequency (352).

Adaptation to the chemical signal must be achieved in order for the bacterium to sense a gradient of ligand concentrations rather than a single change in its concentration. This is achieved by the methylesterase / deamidase CheB protein that demethylates MCP receptors and the methyltransferase CheR that increases methylation of MCP receptors (220, 357). Receptors not bound with ligand are easily methylated by CheR and

methylated receptors stimulate the autophosphorylation activity of CheA (42, 340). However, phosphorylation of CheB by CheA-P increases demethylation of the receptors by the activity of CheB-P (41, 339). This provides a negative feed back loop for the chemotaxis system. The CheZ protein also provides negative feed back by dephosphorylating CheY such that CheY is less likely to cause switching of flagellar rotation to the clockwise (tumbling) direction (57, 205).

E. coli is chemotactic to carbohydrates using the phosphoenolpyruvate -dependent carbohydrate phosphotransferase system (PTS) (218). There are several of these systems in *E. coli* and they are used to transport various carbohydrates into the cell (304). Transport requires at least three enzymes, Enzyme I and II, and Hpr. Enzyme I is phosphorylated at the expense of phosphoenol pyruvate and donated to Hpr for subsequent phosphorylation of the carbohydrate molecule (235). Studies indicate that when these carbohydrates are transported into the cell the increase in dephosphorylated Enzyme I relays a signal to the MCP-CheW-CheA complex causing a stimulation in autophosphorylation of CheA (185, 235, 236). An important aspect of this signaling mechanism is that chemotaxis through the PTS system requires transport of the attractant molecule while chemotaxis using MCP receptors does not. Yet, while transport is required subsequent degradation of the carbohydrate molecule is not necessary (235, 236). Thus, in *E. coli* and similar bacteria, chemosensory behavior alone is not controlled by the energy status of the cell as it is in non-enteric species (see Section 1.6.2) (355). However, production of a flagellar filament by *E. coli* and most other bacteria is under the control of catabolite repression (333).

1.6.2 Chemotaxis and motile behavior of non-enteric bacteria.

Table 1.6. Motile and chemosensory behaviors of *E. coli* and *R. sphaeroides*

<u>Motility</u>	<u><i>Escherichia coli</i></u>	<u><i>Rhodobacter sphaeroides</i></u>
Swimming motion	run-tumble	dart-stop
Maximum swimming speed	20 μm per s	35 μm per s
Flagellar rotation changes speed	No	Yes
<u>Chemotaxis</u>		
Metabolism dependent	No	Yes
Chemokinesis (flagellar rotation changes speed)	No	Yes
Number of copies of each che gene	One per cell	1 - 4 per cell
Number of mcp genes	5	12
Location of MCP proteins	Membrane	Membrane/cytoplasm

The motility and chemotaxis of *E. coli* and other enteric bacteria are among the best understood systems among bacteria. In contrast, motility and chemotaxis of non-enteric bacteria are not as well understood. These bacteria, such as many marine bacteria, live in chemically diverse habitats that are very different from the intestines of mammals. It is not surprising then that the motile behavior of non-enteric species, especially marine bacteria, does not resemble that of *E. coli* (29, 261). These differences are reflected in the rotation of the flagellar filament and the sensory mechanisms possessed by many of these species (Table 1.6).

Many non-enteric bacteria do not display run and tumble motile behavior. Instead, motility is characterized by a dart-stop motion. *R. sphaeroides* and *Sinorhizobium meliloti*, both α -Proteobacteria are two of the best examples (29). In both species, the flagella, turns in only the clockwise direction and never reverses, however the flagellar motor has the ability to change speeds, something not observed in any enteric bacterial species (164, 402). In *S. meliloti*, changes in swimming direction occur when individual filaments rotate at slow, but asynchronous speeds (301). At high speeds all of the flagella turn at the same rate and the flagella form a tight bundle that propels the cells forward. In *R. sphaeroides*, a single lateral flagellar filament is used for propulsion (30). Rotation of the filament can change speeds and at certain times stop rotating completely (30, 294). Stopped cells tumble due to Brownian motion which causes a reorientation of the cell and thus a change in swimming direction. It is important to note that the relaxed or “stopped” form of the *R. sphaeroides* filament forms a circle wound up close to the cell (30). This is thought to facilitate reorientation of the cell through Brownian motion since a long

extended filament may reduce Brownian movement. The speed of non-enteric bacteria, especially marine bacteria, is often much faster than that of enteric bacteria with some exceptions (260, 261)(see below). The average speed of *E. coli* is $20 \mu\text{m s}^{-1}$ while *R. sphaeroides* has a mean speed of $35 \mu\text{m s}^{-1}$. Amazingly, some marine bacteria exhibit speeds of over $200 \mu\text{m s}^{-1}$ (261). This difference in speed between *E. coli* and marine bacteria is most likely due to differences in the forces used to turn the flagellar motor.

There are two types of forces currently known that bacteria use to turn the flagellar filament, they are the proton motive force and sodium motive force. Both the proton motor and sodium motor associate with the typical flagellar basal body structure in the inner membrane, although the exact mechanism of how they translate energy to turn the filament is not well understood.

The proton motor consists of two proton conducting proteins, MotA and MotB, that interact with each other via their transmembrane regions and are located on either side of the MS and C rings of the basal body structure (55, 58). The passage of protons through these proteins drives the rotation of the flagellar filament by a still unexplained mechanism (54). Both enteric bacteria such as *E. coli* and *Salmonella* as well as non-enteric bacteria such as *S. meliloti* and *R. sphaeroides* have proton motors (29, 239). However, the ability to change the speed of the motor is limited to non-enteric species.

Sodium motors have been identified in several bacterial species including *Aeromonas*, *Azospirillum*, *Rhodospirillum*, and *Vibrio* species (247), and many of these are marine bacteria. Several *Vibrio* and *Halomonas* species have both proton and sodium motors. In the case of *Vibrio*, proton motors generate torque for lateral flagella and are important at high viscosity, while sodium motors generate a much faster motor, but only

function well at low viscosity (247). The sodium pump requires four proteins, PomA, PomB, MotX, and MotY (31, 246). The PomA and PomB proteins share homology with MotA and MotB of the proton motor, however they require the unique proteins MotX and MotY to function (31). It has been suggested that these proteins change the ion specificity of PomA/B, however MotX is known to form a functional sodium channel alone (246). Further research is needed to elucidate the exact role of these proteins in generating sodium motive force. However, since the sodium motor can generate much higher torque than the proton motor it is thought that the sodium motor is responsible for the high swimming speeds observed in many marine bacteria (201).

Differences in the motile behavior of enteric and non-enteric species may also be explained by differences in their chemosensory mechanisms. In *R. sphaeroides* and *S. meliloti* chemotaxis relies on MCP receptors, CheA, CheY, CheB, and CheR proteins as it does in *E. coli*. However, there are several major differences (Table 1.6). Some MCP receptors appear to be cytoplasmic in *R. sphaeroides*, a condition not observed in *E. coli* or *Salmonella* (165). These cytoplasmic receptors are thought to sense the metabolic state of the cell by an unknown mechanism (387). In addition, attraction to a substrate in *R. sphaeroides* requires metabolism of the substrate. For example, *R. sphaeroides* mutants in glucose-6-phosphate dehydrogenase are not chemotactic toward sugars metabolized through the Entner-Doudoroff pathway, but are still chemotactic toward sugars metabolized through the Embden - Meyerhoff pathway, such as fructose (158). Somehow metabolism of the substrate is linked to the chemosensory and/or flagellar switch proteins. *R. sphaeroides* has significantly more chemoreceptors compared to *E. coli* and contains multiple CheA and CheY proteins. These multiple chemosensory proteins are

specific for different subsets of MCP receptors. Having multiple sets of chemosensory proteins may enhance chemotaxis through cooperativity since oligomerization or clustering of receptors and the signal transducing proteins can increase signal sensitivity and gain (229). The complex chemotactic systems of these bacteria, offers increasing evidence that the sensory systems seen in *R. sphaeroides* and *S. meliloti* provide these bacteria with sensory plasticity which matches their diverse metabolic capabilities (29). This may be a common theme for other metabolically diverse, non-enteric bacteria living in chemically complex environments such as soil, lakes, and ocean waters.

1.6.3 Chemotaxis, motility and bacterial interactions with host cells.

Bacteria from a variety of taxa have developed sophisticated mechanisms to interact with eukaryotes, most often through mutualistic or pathogenic relationships. Among these mechanisms are flagella-mediated motility and chemotaxis. Virulence in pathogenic bacteria such as *E. coli*, *S. enterica*, *Proteus mirabilis*, *Burkholderia* species, *Yersinia enterocolitica*, and *Vibrio cholerae* requires, or is enhanced by, one or more components of the flagellar apparatus and/or chemotaxis machinery. In *E. coli*, *Y. enterocolitica*, *B. pseudomallei*, and *B. cepacia*, flagella are required for attachment or invasion of host cells (86, 139, 360, 400). In *P. mirabilis*, a urinary tract pathogen, flagella are important for virulence and are expressed in a coordinated fashion during the differentiation into a swarmer cell along with other virulence factors such as the metalloprotease ZapA. In *Vibrio cholerae*, chemotaxis toward mucosal fluids enhances infection by providing the bacteria with a mechanism to obtain nutrients that support faster growth (126-129). In other pathogenic bacteria, such as *S. enterica*, flagella-mediated motility

and chemotaxis may be important for initial phases of infection, after which time expression of flagellar and chemotaxis proteins decreases (113).

Symbiotic or mutualistic interactions with eukaryotes also require flagellar-mediated motility and/or chemotaxis. The marine bacterium *Vibrio fischeri* colonizes and initiates light organ development in the Hawaiian squid *Euprymna scolopes* (316). The squid light organ is located in the squid mantle cavity and contains two lobes, each with three crypts that contain the symbiotic bacteria (316). The *V. fischeri* cells are not passed to squid offspring through successive generations, rather newly hatched squid are colonized by free-living *V. fischeri* cells in the ambient water (215). This occurs even though *V. fischeri* typically make up < 1% of the bacterial community in surrounding waters (284). Colonization is enhanced by bacterial attachment to mucous secreted by the squid which is then pulled into the light organ (281-284). Interestingly, *V. fischeri* has been shown to be chemotactic toward N-acetylneuraminic acid, a major component of squid mucous, so it is likely that chemotaxis toward squid mucous also enhances colonization (103). Once near the light organ, the bacteria enter the light organ through a pore in the lateral surface of the two lobes and migrate to the crypts (315).

Bacteria in the crypts derive host amino acids, grow and divide and produce light via the luciferase enzyme when at a sufficiently high density (151). Non-motile mutants with and without flagella are not able to colonize the light organ indicating that motility is required for light organ development (152). There are six flagellin subunits in the flagellum of *V. fischeri* and at least one, flagellin A, is required for normal motility and symbiotic development (255). In addition, motile behavior must be tightly regulated since mutants defective in the alternative σ^{54} RNA polymerase and spontaneous hyperswimmer

variants initiate light organ development at a reduced rate or not at all (253, 254). Although it has not been proven, light organ development most likely also requires chemotaxis since the bacteria must have directed movement toward the crypts. As evidence of this, *V. fischeri* is chemotactic toward amino acids, one of the major products secreted by the light organ (103).

Perhaps one of the best studied symbiotic interactions is that of rhizobia and legumes. Bacteria belonging to the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Azorhizobium* grow in the soil as free-living organisms but can also live as symbionts inside root nodules of legume plants (321). The bacterium, *S. meliloti*, elicits the formation of a nodule in the plant root apical meristem where it fixes atmospheric nitrogen (226). The bacterium provides the plant with a source of reduced nitrogen in the form of ammonia. In return, the plant feeds the bacterium organic acids such as malate. The net result is a plant-microbe interaction that replaces nitrogen in the soil from the atmosphere.

There is a complex set of events leading up to the rhizobium-legume symbiosis that involves bacterial motility, chemotaxis and cell-to-host-cell communication. The plant releases signaling molecules called flavonoids, which the bacterium recognizes and moves toward by chemotaxis (156). It is not clear how the bacterium is able to sense and move toward flavonoids since there appears to be two chemotaxis signaling pathways in *S. meliloti*, one for sensing nutrients and one for sensing plant products. A clue might be in the regulation of nod gene transcription.

The *nodABC* gene products produce the major nodulation factor, lipochitooligosaccharide. This compound causes changes in plant root hair morphology

such that the bacteria are able to gain access to the root meristem. Other nod gene products modify the lipochitooligosaccharide molecules to make them plant-specific (111, 338). The nodulation genes *nodD1*, *nodD2* and *nodD3* all activate transcription of the *nodABC* genes and others by binding to the ATCN₉GAT motif, called a nod box (93, 171, 375). These genes are highly conserved and are homologous to the LysR family of regulatory proteins (171). LysR transcriptional activators share common features, including 1) a helix-turn-helix DNA binding motif 2) a domain that binds a co-inducer and 3) a domain that binds co-inducer and DNA. Generally the *nodD* protein binds to the *nodABC* promoter without the co-inducer. Upon binding the co-inducer, a conformational change takes place such that the LysR protein is able to interact with the -35 site to activate transcription (323).

The co-inducer bound in *S. meliloti* is one or more types of plant flavonoids, the major one being luteolin (323, 375). This makes transcription of the nod genes, and thus production of lipochitooligosaccharide plant-dependent and is one of the first sensing mechanisms to take place in the interaction. Interestingly, it appears that the *nodD* genes are also important for chemotaxis toward plant flavonoids since a deletion in the *nodD* genes, causes defects in chemotaxis toward luteolin, while general chemotaxis toward other metabolites such as proline is not affected (78). A mechanism for how loss of these nod genes results in reduced chemotaxis toward plant flavonoids is not known. Not surprisingly, nonmotile mutants of *S. meliloti*, with or without flagella, have a reduced ability to form root nodules (156).

1.7 Bacteria associated with *Pfiesteria* (preliminary data)

1.7.1 Identification of the bacterial community members.

In order to understand how bacteria might affect the physiology of *P. piscicida* dinoflagellates, the bacterial community inhabiting *Pfiesteria* cultures were identified (9). Identifications were made by making 16S rDNA clone libraries using DNA extracted from whole dinoflagellate cultures. Unique clones were identified based upon restriction fragment length polymorphisms (RFLP). The 16S rDNA molecules were then sequenced and analysed through phylogentic comparisons. The results indicate that a diverse community of bacteria is present in *Pfiesteria* cultures with members of the Cytophagales, the α -, β - and γ -Proteobacteria and high GC gram-positive bacteria. The most common bacterial species found were α -Proteobacteria accounting for 50 % of all unique clones. Most sequences were at least 98 % identical to 16S rDNA sequences in GenBank (National Library of Medicine, Bethesda, MD). The most abundant 16S rDNA clones obtained from *P. piscicida* culture were similar to *Flexibacter maritimus* and marine bacterium SRF3 at a frequency of 24 % and 17 %, respectively. In all cultures, the second most abundant clones were phylogenetically related to *Roseobacter* species, an interesting occurrence given that these bacteria are commonly found associated with other dinoflagellates (168, 210). Other clones from *P. piscicida* appeared at a frequency less than 5 %.

The RFLP groups detected in multiple dinoflagellate cultures are shown in figure 1.6. No bacterial species was found to inhabit all four cultures, however, RFLP9, RFLP10 and RFLP26 were found in three of the four cultures. These clones are similar to *Sulfitobacter pontiacus*, *Hyphomonas jannaschianna*, and *Curacaobacter baltica*,

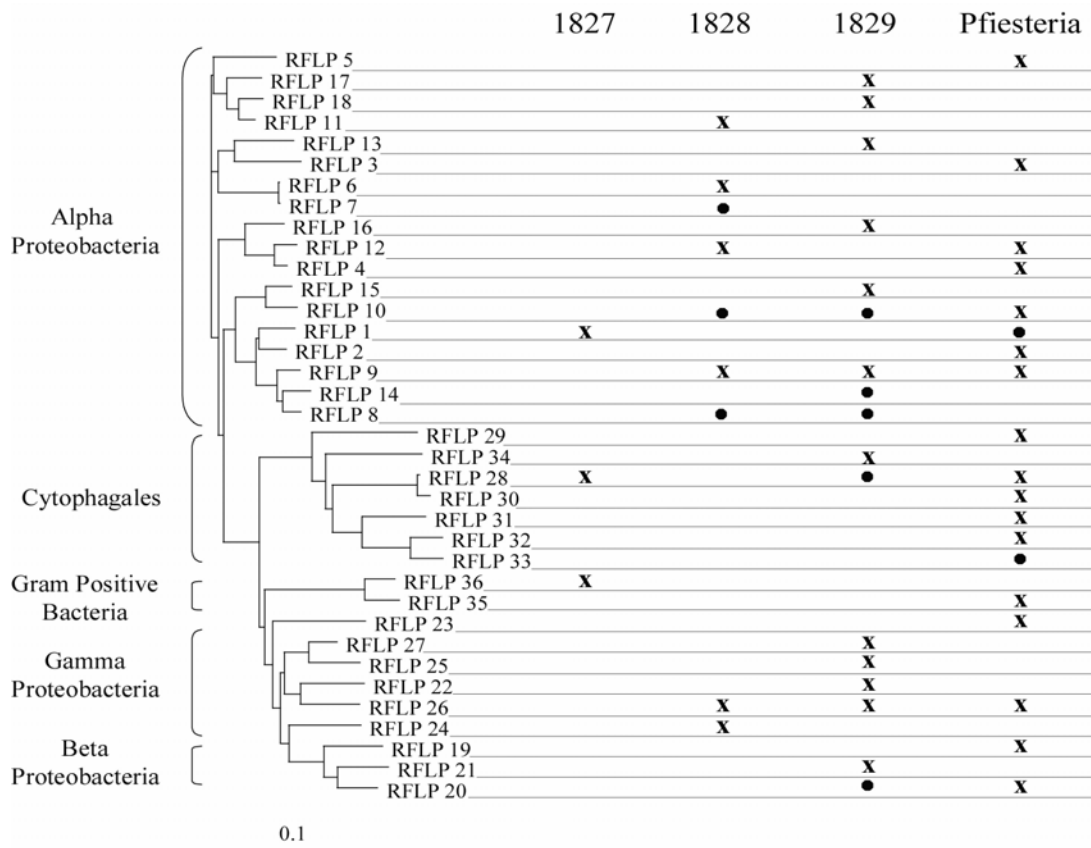


Fig. 1.6. Identification of bacteria in *Pfiesteria* cultures. The 16S rDNA from *P. piscicida* (*Pfiesteria*) and *Pfiesteria*-like (1827, 1828 and 1829) dinoflagellate cultures was cloned and subjected to restriction fragment length polymorphism analysis (RFLP) in order to identify unique clones before DNA sequencing. Clones producing unique RFLP patterns are shown and grouped taxonomically. The “x” indicates a bacterial species occurrence in one or more of the four dinoflagellate cultures and a “•” indicates that this bacterial species occurred at a high frequency within the clone libraries. The bar represents 0.1 units of evolutionary distance.

respectively. The *P. piscicida* culture showed the greatest species diversity with 19 unique 16S rDNA sequences. This descriptive analysis of bacterial communities in *Pfiesteria* cultures is crucial to future experiments that seek to identify key species interacting with the dinoflagellates.

1.7.2 Analysis of Community Structure

The use of 16S rDNA clone libraries to identify bacteria is a valid method for the identification of bacterial communities, however, there are other techniques available that can provide additional information. Denaturing gradient gel electrophoresis following PCR amplification of 16S rDNA (16S rDNA DGGE) was used to compare the bacterial community structure among dinoflagellate cultures. This procedure does not immediately offer a species identification, but rather allows quick comparisons between members of multiple bacterial communities. In this analysis, the PCR amplified 16S rDNA from multiple species within a community is electrophoresed in a polyacrylamide gel containing a gradient of formamide and Urea. The DNA molecules stop moving at different points in the gel, observed as a single band, due to differences in their susceptibility to denaturation from the formamide and urea, a DNA sequence-dependent process. Complete denaturation is prevented with a GC rich fragment of DNA incorporated into each DNA molecule during PCR amplification. Since each band in a denaturing gradient gel can indicate more than one 16S rDNA species, a single band is simply defined as one operational taxonomic unit (OTU).

The 16S rDNA DGGE was performed using cultures of *P. piscicida* and compared to the OTUs to other *Pfiesteria* and non-*Pfiesteria* cultures (Fig. 1.7). A mixture of known sequences was included in the analysis (lanes marked “0”). There are

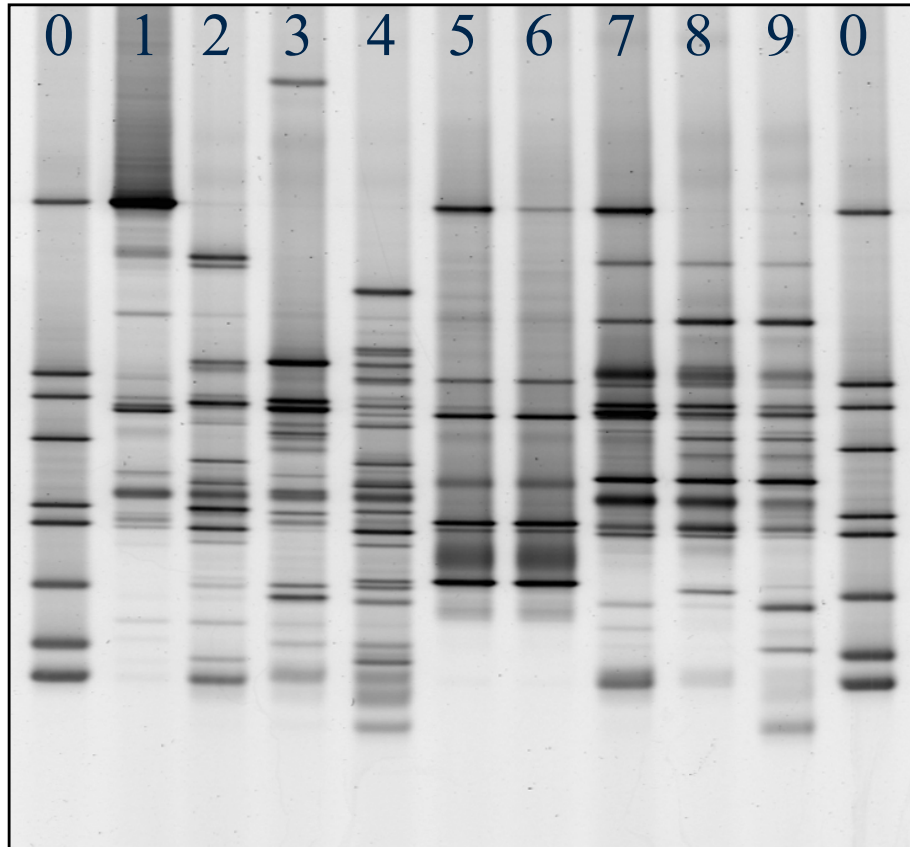


Fig. 1.7. Analysis of bacterial community structure in dinoflagellate cultures using DGGE. To compare bacterial communities among dinoflagellate cultures, the 16S rDNA from each dinoflagellate culture was amplified using PCR and electrophoresed in a 40 – 60% denaturing gel. 0) Known sequences 1) Non-axenic *Rhodomonas* 2) *Prorocentrum minimum* 3) *Oxyrrhis sp.* 4) *Gymnodinium galatheanum* (*Karlodinium micrum*) 5) *Pfiesteria*-like, 1828 6) *P. piscicida*, PPKS 7-9) *P. piscicida*, PPKS, fed non-axenic *Rhodomonas*. Known sequences (Top –bottom): Plastid rDNA, “*Agrobacterium*” *sanquineum*, *Marinobacterium*, *Erythrobacter*, *S. pontiacus*, *R. algalicola*, *P. myrsinacearum* *P. myrsinacearum*, *Cytophaga*.

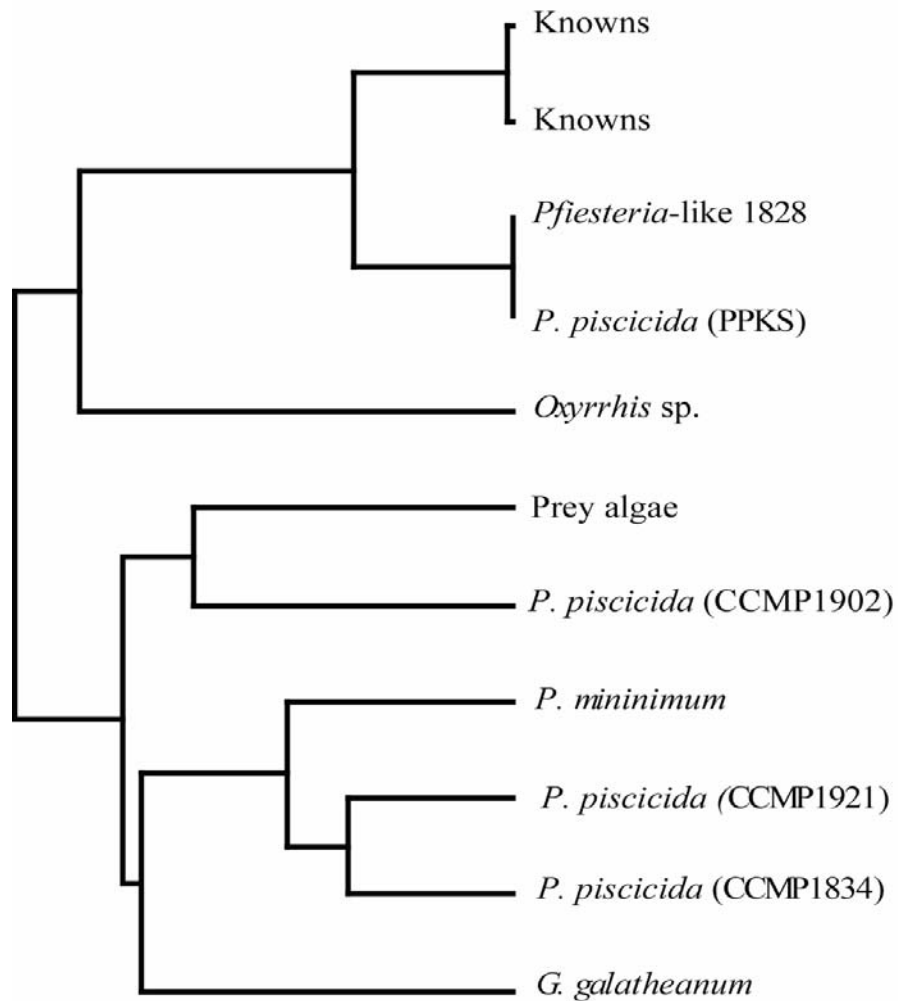


Fig. 1.8. Dendrogram showing 16S rDNA DGGE pattern similarity among dinoflagellate cultures. A quantitative comparison of banding patterns in figure 1.6 was made by measuring the distance bands traveled and using this data to construct a distance matrix. The dendrogram pictured was created from the distance matrix using the UPGMA method. Similarities in banding patterns between cultures of *P. piscicida* (PPKS, CCMP1902, CCMP1921, CCMP1834), *Pfiesteria*-like dinoflagellates (1828), *Oxyrrhis* sp., *Prorocentrum minimum*, *Gymnodinium galatheanum* (*Karlodinium micrum*) and non-axenic *Rhodomonas* sp. (prey algae) and the known sequences are shown. As a control, notice that the two “knowns” lanes group with each other.

at least eight different OTU's in all cultures and most have greater than fifteen, indicating a diverse bacterial community inhabits the dinoflagellate cultures. Interestingly, the least diversity is present in *P. piscicida* cultures that were fed bacteria-free, or axenic, prey algae (*Rhodomonas* sp.).

Band positions were measured using Fragment NT Analysis software (Molecular Dynamics, Sunnyvale, CA) and a distance matrix was constructed from the output data. A dendrogram was constructed from this matrix using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) as part of the NTSYSpc software (Exeter Software, NY). The results indicate that culturing methods have a significant impact on bacterial community structure (Fig. 1.8). For example, the banding pattern from *P. piscicida* culture fed axenic *Rhodomonas* prey algae grouped with banding patterns from CCMP1828, also fed axenic *Rhodomonas* prey algae. In fact, the five OTUs from these two cultures align perfectly with each other. This is not surprising since data from our 16S rDNA clone library (discussed above) indicate that these two cultures share at least four species in common, "*Agrobacterium*" *sanguineum*, *Hyphomonas jannaschiana*, *Sulfitobacter pontiacus*, and *Curacaobacter baltica*. Although the clone library analysis did not show that any of these clones were abundant, DGGE has been shown in at least one study to be a less sensitive technique than 16S rDNA clone libraries (388). Banding patterns from other *P. piscicida* cultures fed non-axenic *Rhodomonas* sp. did not group with the *P. piscicida* or *Cryptoperidiniopsis* sp. CCMP1828 cultures. It appears that some of these bands align with OTUs from the non-axenic *Rhodomonas* culture, indicating that bacteria were added exogenously with the prey algae. The banding patterns from three other dinoflagellate cultures representing different genera (*P. minimum*, *G. galatheanum*,

and *Oxyrrhis* sp.) show little similarity to those of the *P. piscicida* or *Cryptoperidiniopsis* sp. CCMP1828 cultures. Since these other dinoflagellates have different modes of nutrition (photosynthetic, and/or mixotrophic) differences in their associated bacterial species composition are expected. Also, in some cases these dinoflagellates were isolated from different bodies of water that likely contain a different bacterial community.

1.7.3 Attached and intracellular bacteria

The general identification of the bacteria in *Pfiesteria* cultures provides useful information, but it does not indicate which species might interact with *Pfiesteria*. To examine bacteria that physically interact with *Pfiesteria*, a washing procedure was used to enrich for attached or intracellular bacteria (9). This procedure takes advantage of the natural tendency of *Pfiesteria* dinoflagellates to actively seek the bottom of the culturing vessel. Therefore, the procedure is called “behavioral washing.” Dinoflagellates are layered over sterile media in a 250 ml tissue culture flask and allowed to migrate to the bottom of the flask for a period of 15 minutes at room temperature. The upper layers of media containing unattached bacteria are removed and the process is repeated in a new flask using the washed dinoflagellates in the bottom layers of media. By repeating the procedure several times, dinoflagellates naturally separate themselves from the unattached bacteria each time they migrate through the sterile media. Aliquots of culture were taken before, during and after washing to enumerate dinoflagellates using a Beckman Coulter Multisizer Particle Counter (Beckman Coulter, Inc. Fullerton CA) and colony forming units (CFU) were determined by spreading dilutions on 1/2X 2216 marine agar.

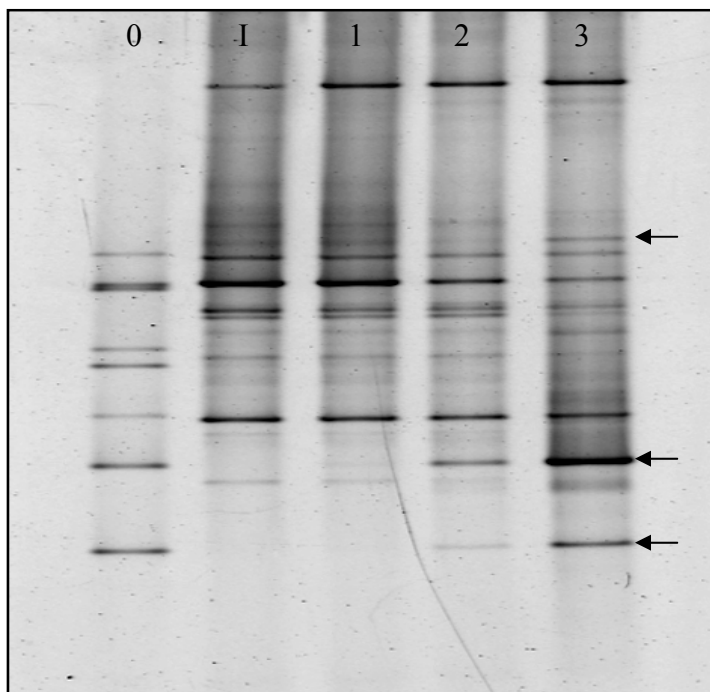


Fig. 1.9. Washing dinoflagellates causes changes in the bacterial community structure as determined by 16S rDNA DGGE. Dinoflagellates were consecutively washed to remove unattached bacteria and samples taken after each wash for 16S rDNA DGGE. New bands are unmasked as the dinoflagellates are washed. 0) knowns, 1) no washes, 1) 1 wash, 2) 2 washes, 3) 3 washes. Three new bands are unmasked in the final wash. Known sequences (Top –bottom): Plastid rDNA, “*Agrobacterium*” *sanguineum*, *Marinebacterium*, *Erythrobacter*, *S. pontiacus*, *R. algalicola*, *Phyllobacterium* sp., *Phyllobacterium* sp., *Cytophaga*.

During the washing procedure, CFUs per ml were reduced from 10^7 per ml to 10^4 per ml while the concentration of dinoflagellates remained constant at 10^4 per ml. Aliquots were also taken for DNA extraction and 16S rDNA DGGE before, during and after washing. Three new OTUs were unmasked as dinoflagellates were consecutively washed, indicating a shift in species composition (Fig. 1.9). This wash procedure followed by 16S rDNA DGGE has been repeated several times giving consistent results. The three unmasked bands were excised, re-amplified and sequenced. The 300 base pair sequences obtained are identical to three of the 16S rDNA clones obtained from our clone libraries using the *Cryptoperidiniopsis* sp. CCMP1828 culture. These clones were identified as *Phyllobacterium myrsinacearum*, *Cytophaga* sp., and *Caulobacter* sp. Thus, these species are candidates for attached and/or intracellular bacteria associated with *Cryptoperidiniopsis* sp. CCMP1828.

There is evidence that bacteria are physically associated with *Pfiesteria* dinoflagellates. Attached and intracellular bacteria associated with *P. piscicida* have been visualized using fluorescent in situ hybridization. A universal oligonucleotide probe (EUB338) specific to most bacteria (13-16) was labeled with Texas Red fluorophore (Molecular Probes, Eugene, OR) and hybridized to paraformaldehyde-fixed, washed and unwashed aliquots of *P. piscicida* culture (Fig. 1.10). The results indicate that about 50 % of the dinoflagellates show attached bacteria after washing with about 5 –10 per cell. This offers direct evidence that bacteria are intimately associated with *P. piscicida*.

1.7.4 Physiological effects of bacteria

Many bacteria have been shown to affect the physiology of dinoflagellates. There is evidence that one or more bacteria are required for the growth of *P. piscicida* in culture

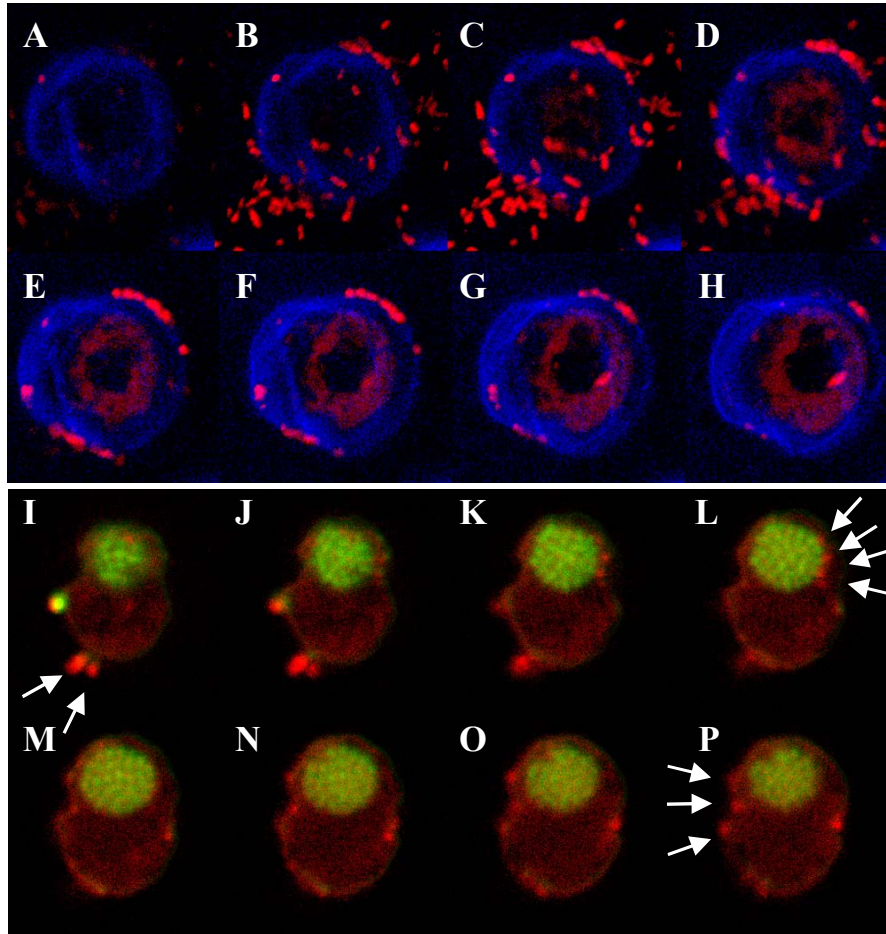


Fig. 1.10. Localization of attached and intracellular bacteria in and on unwashed / washed dinoflagellates. Bacteria probed with EUB338 labelled with Texas Red fluorophore were false colored in red. A-H) Unwashed dinoflagellates were background stained with calcafluor to highlight thecal plates I-F) Washed dinoflagellates were background stained with SYBR Green to show the nucleus. Arrows indicate attached and intracellular bacteria.

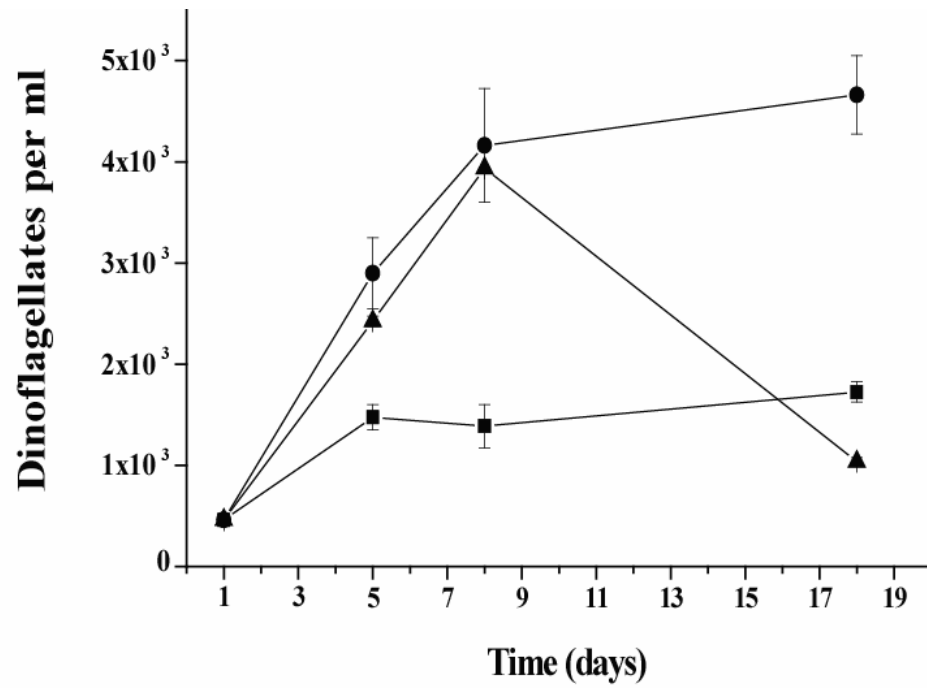


Fig. 1.11 Effect of bacteria on dinoflagellate growth. ●) *P. piscicida* with bacteria and prey algae, ▲) *P. piscicida* with bacteria only, ■) *P. piscicida* with axenic prey algae.

(9). A process has been developed to make axenic dinoflagellate cultures to show bacterial-dependent dinoflagellate growth. In this experiment, dinoflagellates that had been previously encysted are treated with a dilute bleach solution and then washed with sterile media to induce excystment (9). *Pfiesteria* dinoflagellates processed in this way will not grow unless both prey algae and bacteria are added back to the culture (Fig.1.11). One bacterium related to *Roseobacter* species was able to complement growth without other bacteria present (9).

1.8 Specific aims of this study

1.8.1 Metabolism of DMSP in *Pfiesteria* cultures

In chapter two the metabolism of DMSP by *Pfiesteria* dinoflagellates and its subsequent degradation by bacterial species within the dinoflagellate cultures is quantified. It is hypothesized that the dinoflagellates produce DMSP and harbor a robust DMSP-degrading bacterial community that includes one or more *Roseobacter* species.

1.8.2 Chemotaxis of *Silicibacter* sp. TM1040 toward dinoflagellates

In chapter three the chemotaxis of a DMSP-degrading *Roseobacter* species isolated from *P. piscicida* culture in chapter one is studied in detail. This bacterium, *Silicibacter* sp. TM1040, is hypothesized to be chemotactic toward *Pfiesteria* cells and certain dinoflagellate products such as DMSP.

1.8.3 Motility and interactions with *P. piscicida*

In chapter four physical and physiological interactions of *Silicibacter* sp. TM1040 with *P. piscicida* are studied. The bacterium is hypothesized to interact physically with *P. piscicida* through attachment or invasion of the dinoflagellate cell surface and to interact

physiologically by enhancing dinoflagellate growth. Furthermore, it is hypothesized that motility and/or flagella will be important for these interactions. To that end, genes involved in motility and/or flagellar biosynthesis are indentified using transposon mutagenesis and rescue cloning of the affected genomic DNA.

1.8.4 Annotate flagellar and chemotaxis genes

The recent completion of the *Silicibacter* sp. TM1040 genome provides a rich source of data that may guide future experiments and may also validate existing conclusions about the physiology of this bacterium. Since motility and chemotaxis are hypothesized to be important for establishing interactions with *P. piscicida*, the flagellar and chemotaxis genes from *Silicibacter* sp. TM1040 are annotated using the genome sequence of this bacterium. It is hypothesized that the flagellar and, especially the chemotaxis genes of TM1040 will be highly developed relative to other bacteria, including those of other *Roseobacter* species.

Chapter 2: Dimethylsulfoniopropionate (DMSP) metabolism

by *Pfiesteria*-associated *Roseobacter*

2.1 Summary

The *Roseobacter* clade of marine bacteria is often found associated with dinoflagellates, one of the major producers of dimethylsulfoniopropionate (DMSP). In this study, I tested the hypothesis that *Roseobacter* species have developed a physiological relationship with DMSP-producing dinoflagellates mediated by the metabolism of DMSP. DMSP was measured in *Pfiesteria* and *Pfiesteria*-like (*Cryptoperidiniopsis*) dinoflagellates, and the identities and metabolic potentials of the associated *Roseobacter* species to degrade DMSP were determined. Both *Pfiesteria piscicida* and *Pfiesteria shumwayae* produce DMSP with an average intracellular concentration of 3.8 μM . Cultures of *P. piscicida* or *Cryptoperidiniopsis* sp. that included both the dinoflagellates and their associated bacteria rapidly catabolized 200 μM DMSP (within 30 h), and the rate of catabolism was much higher for *P. piscicida* cultures than for *P. shumwayae* cultures. The community of bacteria from *P. piscicida* and *Cryptoperidiniopsis* cultures degraded DMSP with the production of dimethylsulfide (DMS) and acrylate, followed by 3-methylmercaptopropionate (MMPA) and methanethiol (MeSH). Four DMSP-degrading bacteria were isolated from the *P. piscicida* cultures and found to be taxonomically related to *Roseobacter* species. All four isolates produced MMPA from DMSP. Two of the strains also produced MeSH and DMS, indicating that they are capable of utilizing both the lyase and demethylation pathways. The diverse metabolism of DMSP by the dinoflagellate-associated *Roseobacter* spp. offers evidence consistent with a hypothesis that these bacteria benefit

from association with DMSP-producing dinoflagellates.

2.2 Introduction

Pfiesteria piscicida, *Pfiesteria shumwayae*, and *Pfiesteria*-like organisms, such as *Cryptoperidiniopsis* sp., are estuarine, heterotrophic dinoflagellates with a global distribution (reviewed in reference (251)). Reports have implicated *Pfiesteria* sp. as the causative agent of massive fish deaths along the Atlantic Coast of the United States, especially in the estuaries of Pamlico Sound, N.C., and the Chesapeake Bay of Maryland and Virginia (76). Certain *Pfiesteria* species are thought to kill fish by excreting potent ichthyotoxins, similar to those of other well characterized dinoflagellate species (143). However, no toxins from this organism have been identified (264), and recent reports show that *P. shumwayae* can kill fish by consuming epithelial cells, a process that does not require toxin production (50, 382). While the toxicity of *Pfiesteria* species is an important question, other physiological aspects of these dinoflagellates deserve further attention.

Dimethylsulfoniopropionate (DMSP) is the major source of organic sulfur in the world's oceans and plays a significant role in the global sulfur cycle (reviewed in reference (398)). During blooms of marine unicellular algae, cellular DMSP is released due to algal senescence, predation, or stress and is degraded by both algal and bacterial enzymes. In marine environments, dinoflagellates and prymnesiophytes are the major producers of DMSP, with intracellular concentrations as high as 0.5 M (398). Although the exact function of DMSP is unclear, it has possible roles in osmoprotection (104, 368) and cryoprotection (189), antiherbivory (395), and protection from oxidative stress (347).

Bacteria and certain species of phytoplankton produce an enzyme, dimethylpropiothetin dethiomethylase (DMSP lyase) (EC 4.4.1.3) (Fig. 2.1, reaction 1), which degrades DMSP to produce dimethylsulfide (DMS) and acrylate (399). As shown in Fig. 2.1, DMS produced from this reaction is oxidized by some bacterial species to form dimethyl sulfoxide (Fig. 2.1, reaction 6) (354). Acrylate is readily consumed by bacteria and is converted to β -hydroxypropionate by α - and γ -Proteobacterial species (26, 27). Bacteria may also demethylate DMSP at the DMS moiety (Fig. 2.1, reaction 2), producing 3-methylmercaptopropionate (MMPA) (182), which may be further demethylated to 3-mercaptopropionate (MPA; Fig. 2.1, reaction 4) (378) or demethylated to produce acrylate (Fig. 2.1, reaction 3) and methanethiol (MeSH); (353). The products of these reactions provide a rich source of carbon and sulfur for bacterial production (195, 379). It is estimated that ~15% of the DMSP produced by marine phytoplankton is degraded by the lyase pathway and 85% or more is degraded by bacterial demethylation of DMSP, suggesting an essential bacterial role in controlling DMS emissions from the world's oceans (275, 405).

In marine surface waters, α -Proteobacteria phylogenetically related to *Roseobacter* spp. are predominantly responsible for the degradation of DMSP, its catabolites, and other sulfonium compounds (146). Although *Roseobacter* spp. are cosmopolitan in nature, their production and activity are significantly correlated with DMSP-producing algae, including dinoflagellates and prymnesiophytes (148, 405). Furthermore, some *Roseobacter* spp. exhibit close physical or physiological relationships with toxic, DMSP-producing dinoflagellates, including *Prorocentrum* spp. (210), *Alexandrium* spp. (96, 134), and *Pfiesteria* spp. (9).

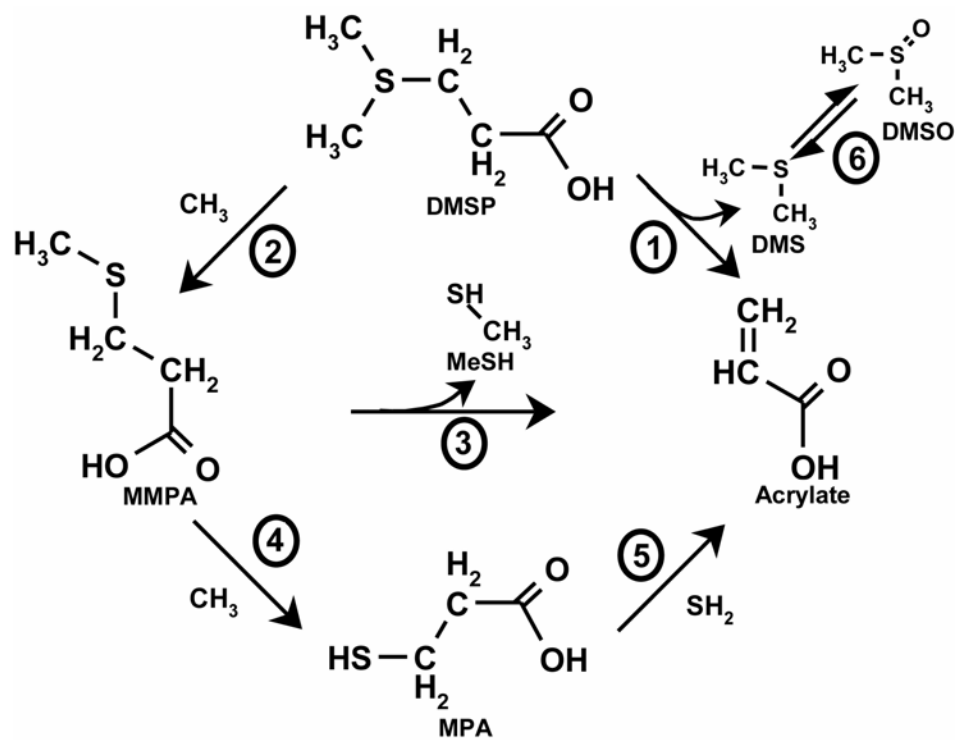


Fig. 2.1 Pathways involved in the catabolism of DMSP. Degradation of DMSP may occur by the lyase pathway, hydrolysis of the C3 carbon producing acrylate and DMS (Rxn 1), or by the demethylase pathway, demethylation of the DMS moiety producing MMPA (Rxn 2). MMPA may be further demethylated to MPA (Rxn 4) followed by elimination of hydrogen sulfide (Rxn 5) or demethylated producing acrylate and MeSH (Rxn 3). In some cases, DMS may be oxidized to DMSO (Rxn 6).

In the environment, dinoflagellates coexist and interact with a diverse community of bacteria and other microorganisms. These interactions can be studied in monocultures of dinoflagellates obtained from environmental samples. Within these cultures, bacteria native to the algal niche assimilate dinoflagellate-derived nutrients and are intrinsically propagated with the dinoflagellates in continuous subcultures. In an earlier study, we characterized the bacterial community inhabiting several *Pfiesteria* dinoflagellate cultures isolated from the Chesapeake Bay, MD. (9). All of the dinoflagellate cultures examined contained one or more *Roseobacter* spp. representing the second most abundant clone obtained from 16S ribosomal DNA (rDNA) clone libraries. In addition, several bacteria were found attached to these dinoflagellates by using fluorescent in situ hybridization and confocal scanning laser microscopy. After a stringent washing procedure to remove unattached bacteria, the predominant bacterial species present was a bacterium closely related to *Sulfitobacter pontiacus*, a *Roseobacter* clade organism. Also, a *Roseobacter* sp. was found to be necessary for the growth of *Pfiesteria* in culture (9).

In this study, we measured the production of DMSP by *P. piscicida* and *P. shumwayae* and then assessed DMSP catabolism by the *Pfiesteria* cultures and the bacterial communities associated with them. New dinoflagellate-associated roseobacters capable of DMSP degradation by both the lyase and demethylation pathways were isolated and identified.

2.3 Materials and Methods

2.3.1 Dinoflagellate strains and culturing.

Dinoflagellate cultures of *P. piscicida* CCMP1830, CCMP1921, and CCMP1834;

Cryptoperidiniopsis sp. strain CCMP1829; and *P. shumwayae* CCMP2089 (Provasoli-Guillard National Center for Culture of Marine Phytoplankton) were grown as previously described (See Appendix B.2) (9). The dinoflagellates were fed the prey alga *Rhodomonas* sp. strain CCMP768 continuously as needed. *Rhodomonas* sp. was grown in 35 practical salinity units (psu) F/2 medium lacking silica at 20°C under a 14-h light, 10-h dark cycle (155). For all assays, the dinoflagellates were grown to a maximum cell density of $\sim 10^5$ per ml, whereupon feeding was stopped for 36 h, which allowed complete removal of the *Rhodomonas* algae (monitored by inverted microscopy).

2.3.2 Bacterial strains and media.

Bacteria were isolated from *P. piscicida* CCMP1830 culture by first spreading a 10-fold dilution series of the dinoflagellate culture on 0.5x Zobell marine agar 2216 (18.7 g of Difco marine broth 2216, 15 g of Difco Bacto Agar, and 1,000 ml of distilled H₂O), hereafter referred to as marine agar. After 5 to 7 days of incubation at 30°C, colonies with unique morphologies were picked at random and streaked to purity on marine agar, resulting in the strains TM1034 to TM1042. The bacterial cultures were used after incubation in marine broth (same as marine agar but lacking the agar) at 30°C in a shaking water bath for 1 to 3 days. *Escherichia coli* INV α F' was grown in Luria-Bertani (LB) broth (32) or on LB agar containing 1.5% Bacto Agar (Becton Dickinson, Franklin Lakes, N.J.).

2.3.3 Chemicals.

DMSP was synthesized from acrylate and DMS according to the method of Chambers et al. (82). The purity of the resulting DMSP was confirmed by chemical analyses, including flash point, melting point, and total C, H, O, N, S, and Cl (Galbraith

Laboratories, Inc., Knoxville, Tenn.). MMPA was synthesized by alkaline hydrolysis of its methyl ester, methyl-3-(methylthio)propionate (Aldrich, Milwaukee, Wis.) (183). Other organic sulfur compounds were purchased from Aldrich. All chemicals used were of the highest purity commercially available.

2.3.4 DMSP content and metabolism.

To measure the DMSP contents of the dinoflagellates *P. piscicida* CCMP1830 and *P. shumwayae* CCMP2089 and the prey alga *Rhodomonas* sp. strain CCMP768, cells were grown to a maximum density of $\sim 10^5$ per ml in 500-ml batch cultures, except for *Rhodomonas* sp., which was grown in 1-liter batch cultures to a maximum density of 10^6 cells per ml. The abundance and cellular volume of the cells were measured in the 7- to 20- μ m-diameter particle range from three or more 1-ml samples of culture using a Coulter Multisizer II particle counter (Becton-Dickinson). Because the particle counter does not distinguish between *Pfiesteria* dinoflagellates and *Rhodomonas* prey algae, all dinoflagellate cultures were starved to reduce the *Rhodomonas* population to below detectable limits (as described above).

DMSP was measured in 2-ml whole-culture aliquots and in concentrated cell lysates. To obtain concentrated cell lysates, 200-ml culture aliquots were centrifuged at 4,000 x *g* for 15 min and the cell pellets were resuspended in 2 ml of sterile distilled water on ice. For *Rhodomonas* sp. strain CCMP768, multiple cell pellets were combined and resuspended in a final 2-ml aliquot of sterile distilled water. A cell homogenate of each sample was then obtained using a Sonic Dismembrator sonicator (Fisher, Hampton, N.H.). DMSP was measured in 2-ml samples as described in “Analytical techniques” below.

To measure the degradation of DMSP by dinoflagellate and prey algal cultures, cells were grown as described above and the cell density was normalized across all cultures to 10^4 per ml by diluting the cultures with sterile medium. DMSP was added to the culture from a sterile neutralized stock at a final concentration of 200 μ M, and its degradation was measured at intervals throughout the duration of the experiment, as described in “Analytical techniques” below.

The catabolism of DMSP by the bacterial component of each culture was measured in suspensions containing a mixture of dinoflagellate-associated bacteria that were isolated as follows. A 10-fold dilution series of each dinoflagellate culture at peak dinoflagellate density ($\sim 10^5$ cells per ml) was spread on marine agar and incubated at 30°C for 5 days. The resulting colonies from plates containing 50 to 200 colonies were resuspended from the agar surface using sterile 10-psu artificial seawater (Instant Ocean, Mentor, Ohio) and washed twice by centrifugation at 14,000 $\times g$, whereupon the optical density was normalized to 0.6 at 600 nm for each suspension. An aliquot of DMSP was then added from a sterile neutralized stock to a final concentration of 1 mM, and DMS, MeSH, MMPA, and acrylate were measured as described in “Analytical techniques” below.

DMSP catabolism was also measured in four bacterial strains (TM1035, TM1038, TM1040, and TM1042) isolated from the dinoflagellate culture. Each strain was grown in a 50-ml marine broth culture amended with 1 mM DMSP to induce the production of enzymes necessary for DMSP catabolism. The cultures were grown to an optical density at 600 nm of 0.6 and washed twice with sterile 10-psu artificial seawater. DMSP was added to a final concentration of either 0.1 or 1 mM, and 1-ml aliquots of the bacterial

cultures were dispersed into 26-ml serum bottles. The bottles were immediately capped with a butyl rubber septum and incubated at 30°C with shaking. At intervals throughout the experiment, samples were sacrificed for measurement of DMSP, DMS, MeSH, acrylate, and MMPA as described below.

2.3.5 Analytical techniques.

DMSP was measured as DMS following alkaline hydrolysis. An aliquot of the sample was added to a 26-ml serum bottle with the addition of an equal volume of either 5 M NaOH or distilled water, and the bottle was capped with a butyl rubber septum. Solutions of pure DMSP at 1 to 500 μM dissolved in distilled water were prepared in exactly the same manner in parallel with each experiment. After overnight incubation, DMS resulting from alkaline hydrolysis of DMSP was measured in 500 μl of headspace gas using a Hewlett-Packard 5890 gas chromatograph equipped with flame ionization detection (GC-FID) and a Poropak R 80/100 column (Agilent, Palo Alto, CA) maintained at 160 °C. The carrier gas was helium delivered at a flow rate of 30 ml per min. DMS produced without alkaline hydrolysis was subtracted from the total, and the result was compared to DMS produced from the hydrolysis of pure DMSP standards to obtain the final molar concentration of DMSP in the unknown sample. The retention time of DMS was determined by injecting 50 μl of headspace gas from a capped serum bottle containing 5 μl of pure DMS that had completely volatilized.

DMS and MeSH production in the cultures was measured by direct sampling of 500 μl of headspace gas without prior alkaline hydrolysis of the sample. The concentration of DMS was determined using standard curves generated from known concentrations of DMS produced by complete alkaline hydrolysis of known amounts of

DMSP. The concentrations of gaseous MeSH in the cultures were determined from standard curves using a dilution series of pure MeSH gas. The presence and concentrations of acrylate and MMPA in the cultures were measured by high-performance liquid chromatography (Agilent [Palo Alto, Calif.] 1100 equipped with diode array detection) and a Zorbax XDB C18 column (2.1 by 150 mm; 5- μ m pore size) (Agilent) according to the method of Ansedè et al. (26).

2.3.6 Statistics.

The Mann-Whitney test for two independent samples was used to compare the DMSP contents of *P. piscicida* CCMP1830 and *P. shumwayae* CCMP2089. The apparent first-order rate constants for DMSP degradation and catabolite production by bacterial strains TM1035, TM1038, TM1040, and TM1042 was calculated using a linear least-squares regression analysis of the data, where the slope of the line equals the first-order rate constant ($r > 0.90$).

2.3.7 DNA methods.

Chromosomal DNA was extracted from bacterial cells by routine methods (36) and used as a template in a PCR to amplify the near-full-length (~1,300-bp) 16S rDNA gene. The PCR conditions were as previously described (9). The resulting PCR products were analyzed by electrophoresis using a 1.0% agarose gel in 1x TAE (32) to confirm the presence of a single 1,300-bp product, which was then excised from the gel using a sterile razor blade, purified using the QIAGEN gel extraction kit, and cloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif.) under the ligation conditions recommended by the manufacturer. Plasmid DNA was transformed into *E. coli* INV α F' competent cells (Invitrogen). Transformants were selected and screened for DNA

insertion using LB agar containing kanamycin (80 µg per ml) plus X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 µg per ml). White colonies, i.e., those harboring recombinant plasmids, were picked at random and grown overnight with antibiotic selection. Plasmid DNA was extracted by alkaline lysis and purified by standard methods using a cesium chloride gradient (32). The presence of a near-full-length 16S rDNA insert was confirmed by agarose gel electrophoresis analysis of EcoRI-digested plasmid DNA. The nucleotide sequence of each 16S rDNA was determined as previously described (1).

2.3.8 Nucleotide sequence analysis and phylogenetic-tree construction.

The construction of phylogenetic trees was done as described by Alavi et al. (9). Briefly, evolutionary trees were generated using the neighbor-joining (318), Fitch-Margoliash (121), and maximum-parsimony (203) algorithms in the PHYLIP package (118). Evolutionary-distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated as described by Jukes and Cantor (187). The confidence in tree topology was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data, and only values of ≥ 500 were shown on the tree.

2.3.9 Nucleotide sequence accession numbers.

The GenBank accession numbers for the 16S rDNA sequences used to generate phylogenetic trees are as follows: *Roseovarius tolerans*, Y11551; *Roseovarius* sp. strain DFL-24, AJ534215; marine bacterium ATAM407-61, AF359525; *Sagittula stellata*, U58356; α-proteobacterium GMD29C12, AY162070; *Roseovarius nubinhibens*, AF098495; uncultured *Rhodobacter* LA1-B32N, AF513928; *Roseobacter* sp. strain LA7, AF513438; marine bacterium HP29w, AY239008; α-proteobacterium MBIC1887,

AB026492; *Silicibacter lacuscaerulensis*, U77644; *Silicibacter pomeroyi*, AF098491; marine bacterium P20, AY082668; *Reuveria atlantica*, AF124521; *Reuveria algocolus*, X78313; *Roseobacter gallaciensis*, Y13244; *Sulfitobacter mediterraneus*, Y17387; *Roseobacter denitrificans*, X69159; *Roseobacter litoralis*, X78312; *Sulfitobacter* sp. strain GAI-37, AF007260; *Sulfitobacter* sp. strain GAI-21, AF007257; *Sulfitobacter pontiacus*, Y13155; and *Sulfitobacter* sp. strain EE36, AF007254. The nucleotide sequences incorporating 1,300 bp of the 16S rDNA gene from strains TM1035, TM1038, TM1040, and TM1042 have been deposited in the GenBank database under accession numbers AY332660, AY332661, AY332662, and AY332663, respectively.

2.3 Results

2.3.1 DMSP content of *P. piscicida* and *P. shumwayae*.

Figure 2.2 shows representative chromatograms of DMSP in cell lysates from each *Pfiesteria* species. The internal cell volumes for *P. piscicida* and *P. shumwayae* were determined to be 0.69 and 0.55 nl, respectively; thus, the average intracellular DMSP concentration of *P. piscicida* is ~3.44 μM , while that of *P. shumwayae* is estimated to be 4.25 μM (Table 2.1). Statistical analyses show that the mean DMSP concentrations and the mean intracellular volumes for the species are not significantly different ($P > 0.05$).

Intracellular DMSP was not detected in the *Rhodomonas* prey algal cultures, even when 1,000-fold more cells were used for the analysis. Thus, *Rhodomonas* sp. strain CCMP768 is not a significant source of DMSP in the *Pfiesteria* culture. While DMSP was readily detectable in *Pfiesteria* cell lysates, it was not found in either supernatants or

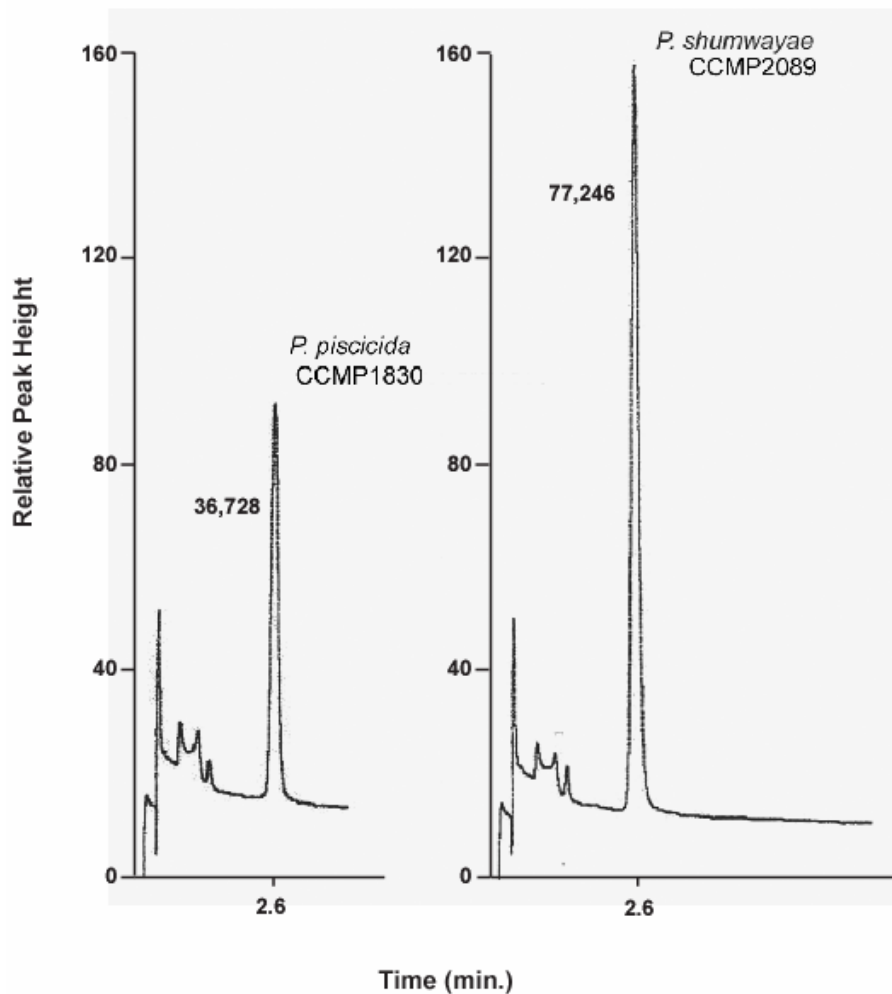


Fig. 2.2. Representative GC-FID chromatograms. DMSP in *Pfiesteria* dinoflagellates is detected as DMS, the major peak in each chromatogram, after alkaline hydrolysis. Numbers indicate the relative peak area. The minor peaks ahead of the DMS peak are not associated with DMSP, DMS, or other DMSP catabolites.

Table 2.1. DMSP content of *P. piscicida*, and *P. shumwayae*

Intracellular DMSP						
Strain	Species	pg / cell	Cell Volume (nL)	S.E.^a	DMSP (mM)	S.E.
1830	<i>P. piscicida</i>	0.41	0.69	0.12	3.44	1.00
2089	<i>P. shumwayae</i>	0.4	0.55	0.02	4.25	1.47

^a S.E. = Standard error of the mean.

whole-cell samples.

2.3.2 Degradation of DMSP by dinoflagellate cultures. Cultures of *P. piscicida*, *P. shumwayae*, and a taxonomically similar dinoflagellate, *Cryptoperidiniopsis* sp., lacking *Rhodomonas*, as well as a culture of the prey algae, were analyzed for the ability to degrade DMSP. While *Rhodomonas* cultures failed to degrade DMSP (data not shown), *P. piscicida*, and *Cryptoperidiniopsis* cultures degraded exogenously added DMSP within 20 to 30 h of incubation (Fig. 2.3). In contrast, the *P. shumwayae* culture was much slower to degrade DMSP, requiring >72 h to achieve complete degradation of the DMSP (data not shown). *P. piscicida* CCMP1830, *Cryptoperidiniopsis* sp. strain CCMP1829, and *P. shumwayae* CCMP2089 were chosen for further analysis.

2.3.2 DMSP catabolism by the dinoflagellate-associated bacterial consortium.

The contribution of the bacterial community in the dinoflagellate cultures to degrading DMSP was assessed. Three separate mixed communities of culturable heterotrophic bacteria from cultures of *P. piscicida* CCMP1830, *Cryptoperidiniopsis* sp. strain CCMP1829, and *P. shumwayae* CCMP2089 were isolated, and their abilities to degrade DMSP were measured. The bacterial suspensions from the *P. piscicida* and *Cryptoperidiniopsis* cultures catabolized DMSP, initially producing DMS and acrylate, followed by the production of MMPA and MeSH (Fig. 2.4A and B). The concentrations of MeSH and DMS were consistently much higher (~100-fold) than the concentration of either MMPA or acrylate. In general, the concentrations of the DMSP catabolites eventually decreased over 20 h, except for MeSH gas, which continued to increase throughout the period. In contrast to these results, the bacterial suspension obtained from the *P. shumwayae* culture produced only DMS and acrylate and failed to produce either

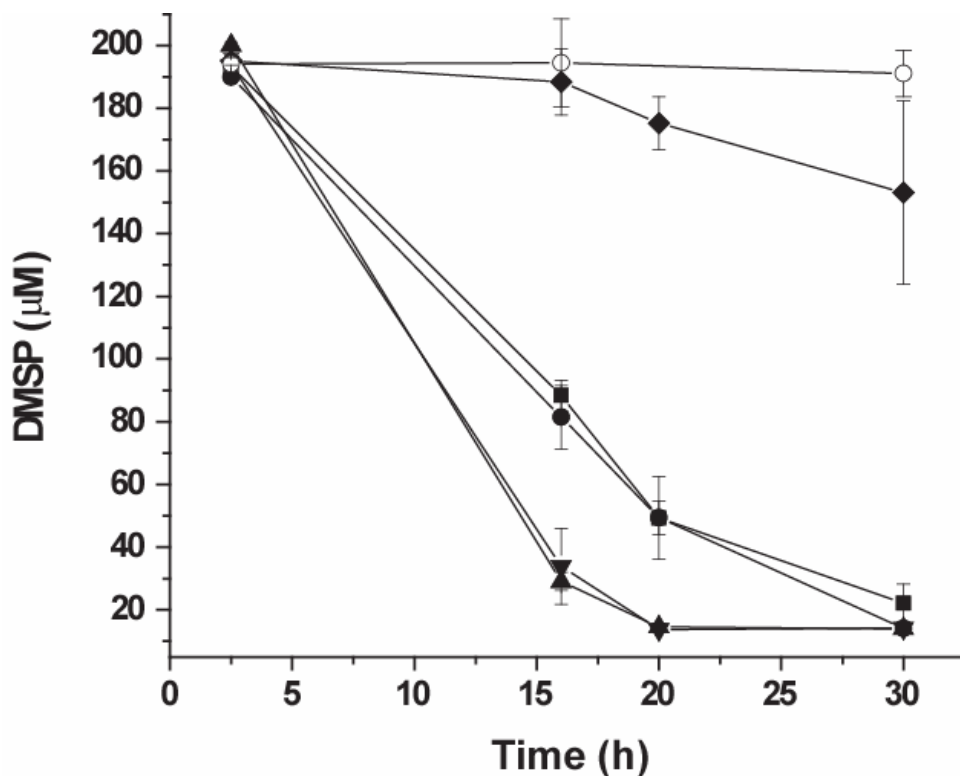


Fig. 2.3. Degradation of DMSP by *Pfiesteria* dinoflagellate cultures. DMSP was added to dinoflagellate cultures (containing both the dinoflagellates and their associated bacteria) and its degradation measured over time using GC-FID. Results are presented for *Cryptoperidiniopsis* sp. CCMP1829 (●), *P. piscicida* CCMP1830 (■), CCMP1921 (▲), CCMP1834 (▼) and *P. shumwayae* CCMP2089 (◆) and the negative control is medium alone (◄). Dinoflagellate cell density is 10^4 cells/ml for all cultures. The error bars represent the standard error in three separate experiments with each culture.

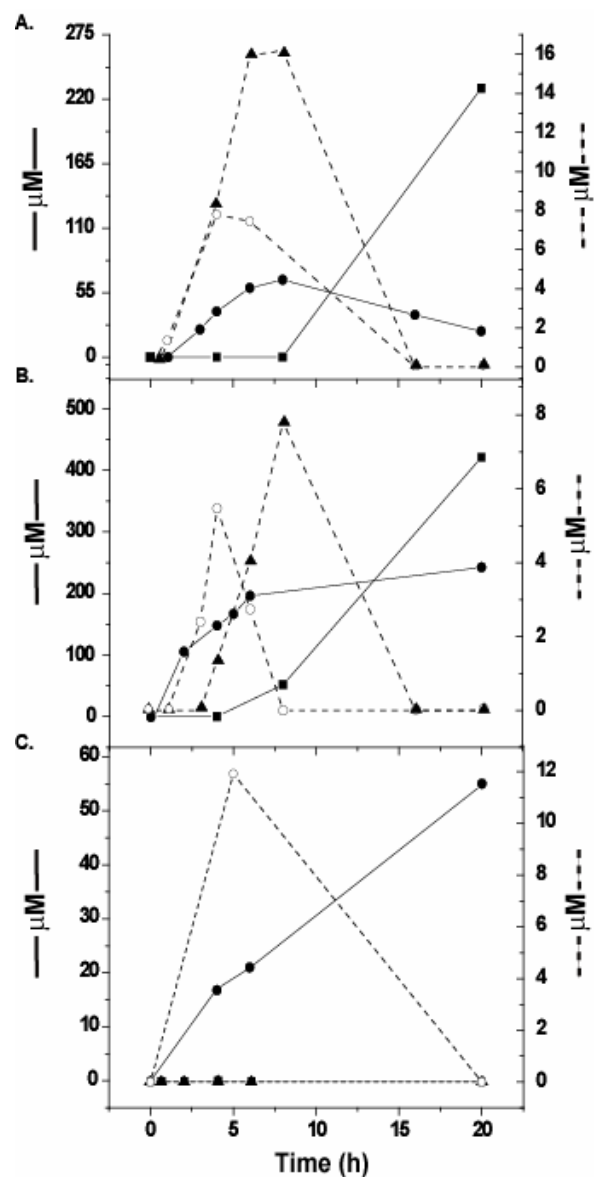


Fig.. 2.4. Production of DMSP catabolites from 1 mM DMSP by the mixed community of heterotrophic bacteria associated with dinoflagellate cultures. (A) *P. piscicida* CCMP1830, (B) *Cryptoperidiniopsis* sp. CCMP1829, and (C) *P. shumwayae* CCMP2089. The production of DMSP catabolites, MeSH (■) and DMS (●) were measured using GC-FID (left axis), while the production of MMPA (□) and acrylate (○) were measured using HPLC (right axis).

MMPA or MeSH. These data demonstrate that one or more species of DMSP-degrading bacteria are associated with the *P. piscicida* and *Cryptoperidiniopsis* dinoflagellates and that both the demethylase and lyase pathways are active. Since the bacterial communities from both the *P. piscicida* CCMP1830 and *Cryptoperidiniopsis* sp. strain CCMP1829 cultures showed similar profiles of DMSP catabolism, only bacteria from the *P. piscicida* CCMP1830 culture were selected for further analysis.

2.3.3 DMSP catabolism by isolated dinoflagellate-associated bacteria.

Using inocula from the *P. piscicida* CCMP1830 culture, nine bacterial strains were isolated, representing a range of colony phenotypes. An analysis of the rate of DMSP degradation showed that four of the nine strains degraded DMSP within a 4-h period (Fig. 2.5A) while the other five showed little or no ability to degrade DMSP (data not shown). Strain TM1040 appeared to degrade DMSP fastest, followed by strains TM1035 and TM1042, with strain TM1038 being the slowest DMSP degrader. Three distinct colony morphologies were apparent in the four DMSP-degrading strains. Strains TM1035 and TM1042 were similar and produced small (1- to 1.5-mm-diameter), translucent, smooth colonies with a light-pink pigment. Strain TM1038 also produced light-pink colonies with a translucent appearance, but they were much smaller (0.2- to 0.7-mm diameter). TM1040, on the other hand, gave rise to colonies that were larger (4- to 6-mm diameter), translucent, and smooth with a brownish-yellow pigment that diffused throughout the agar medium.

The production of DMSP catabolites by each of the four bacterial isolates was assessed 3 h after addition of exogenous DMSP (Fig. 2.5B). All four strains produced the primary demethylation product of DMSP, MMPA, while three strains (TM1035,

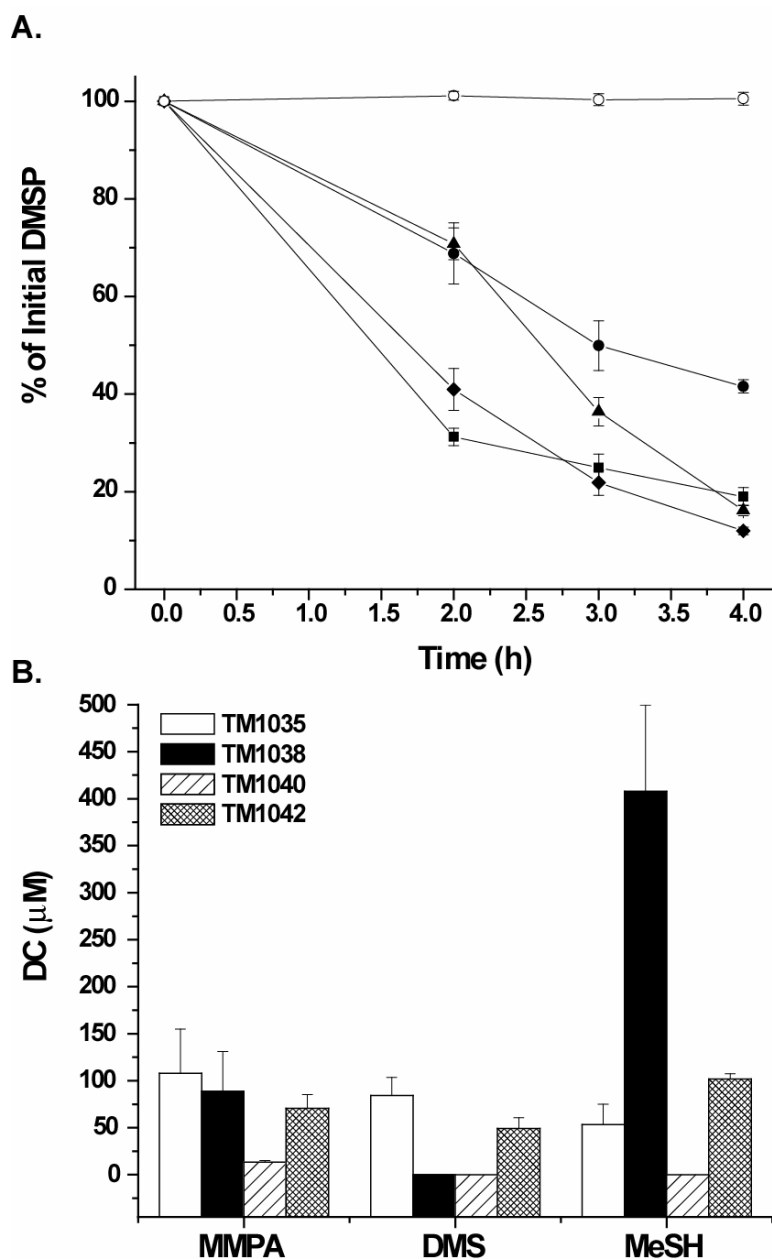


Fig. 2.5. (A) Degradation of DMSP by bacteria isolated from cultures of *P. piscicida* CCMP1830. Bacteria isolated on marine agar from *P. piscicida* CCMP1830 dinoflagellate culture were grown in marine broth supplemented with 1 mM DMSP and washed 2X with seawater. DMSP was then added to a final concentration of 100 μ M and its degradation measured over time using GC-FID. The data are presented as the percent of DMSP remaining in cultures of bacterial strains TM1035 (♦), TM1038 (▲), TM1040 (■) and TM1042 (●). (B) The bar graph compares the production of DMSP catabolites across all isolates at a single time-point, three hours, after the addition of 1 mM DMSP. Error bars represent the standard error in the production of DMSP catabolites during three separate experiments.

TM1038, and TM1042) also produced the secondary demethiolation product, MeSH. Among the three MeSH-producing strains, TM1038 produced significantly more MeSH (Fig. 2.5B), while TM1035 and TM1042, but not TM1038, produced DMS from DMSP in addition to MMPA. These data indicate that TM1035 and TM1042 possess both the DMSP lyase and demethylation pathways.

The kinetics of DMSP degradation and catabolite production were examined in greater detail (Fig. 2.6). Strains TM1035 and TM1042 have similar colony morphologies (medium size; pink), suggesting that they may be taxonomically closely related. The two strains also produce some of the same DMSP catabolites; however, they differ in both the rate of DMSP degradation and the rate of production of DMS (Fig. 2.6A and B). Strain TM1035 removed ~33% (330 μM) of the added DMSP during a 3-h period and produced ~65 μM DMS, 60 μM MMPA, and 16 μM MeSH as a result (Fig. 2.6A). Production of DMS and MMPA occurred within 30 min, with MeSH production following 1 h later. On the other hand, strain TM1042 removed 19% (190 μM) of the added DMSP during the same 3-h period, producing 236 μM MeSH, 38 μM DMS, and 73 μM MMPA (Fig. 2.6B). Production of these compounds occurred simultaneously and within 30 min. The first-order rate constants for DMSP degradation and catabolite production were calculated from linear regions of Fig. 2.6 and are presented in Table 2.2. TM1035 and TM1042 produced MMPA at similar rates (19.5 and 17.5 μM per h, respectively). MeSH production by strain TM1035 was highly variable across replicate experiments. Therefore, it was not possible to compare the rates of MeSH production in these two strains, although TM1042 always produced higher levels of MeSH (Fig. 2.5B). TM1042 also had a lower rate of DMSP degradation (32.6 μM per h) than TM1035 (95 μM per h)

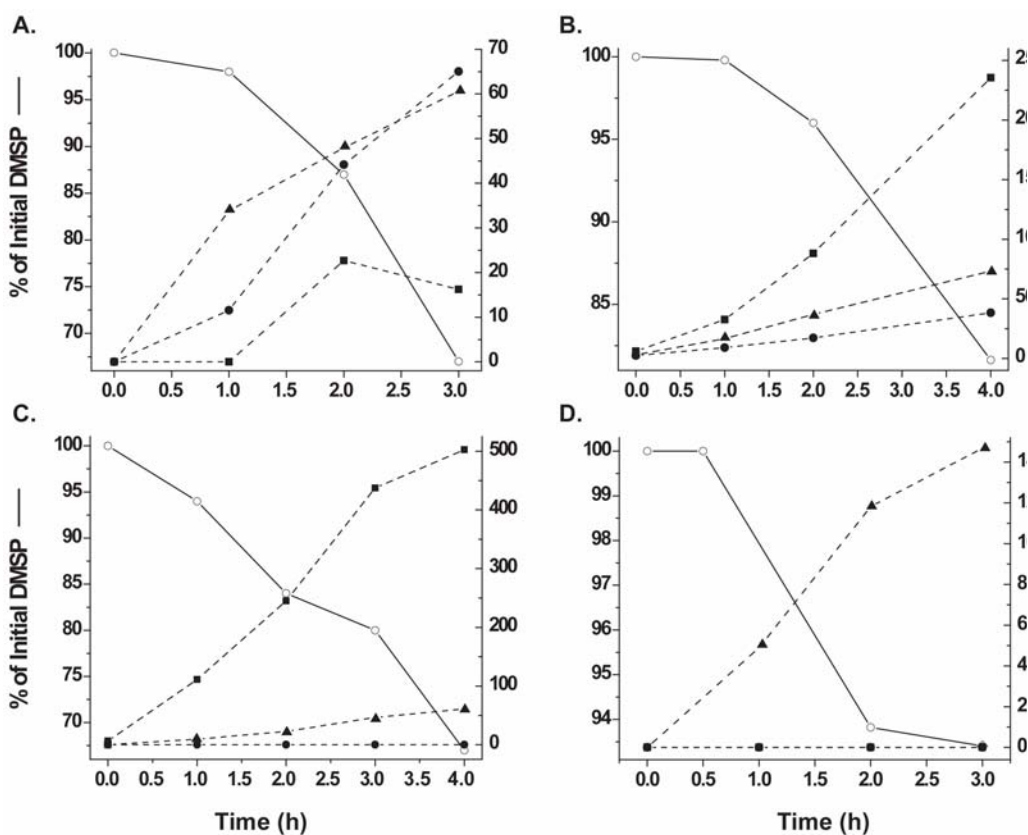


Fig. 2.6. DMSP metabolism and the production of DMSP catabolites over time by four bacterial isolates from cultures of *P. piscicida* 1830. Results are presented for strains (A) TM1035, (B) TM1042, (C) TM1038, and (D) TM1040. Each isolate was grown to stationary phase in marine broth supplemented with DMSP and washed 2X in seawater containing 1 mM DMSP. The washed cultures were then incubated at 30°C and the degradation of DMSP (◇) and production of DMSP catabolites including MMPA (□), MeSH (■), and DMS (●) measured over time. Graphs represent a single experiment and each assay was repeated three or more times with similar results.

and a higher rate of DMS production (59.0 μM per h) than TM1035 (22.7 μM per h). These data suggest that, while they produce similar colony phenotypes, TM1035 and TM1042 are physiologically unique, at least in their metabolism of DMSP.

Strain TM1038 produced both the demethylation and demethiolation products MMPA and MeSH, respectively, but no products of the lyase pathway. The strain removed $\sim 33\%$ (330 μM) DMSP during a 4-h period, producing 502 μM MeSH and 61 μM MMPA (Fig. 2.6C). Of the four strains, TM1038 was slowest in degrading DMSP (22.3 μM per h) but had the highest rate of MeSH production (132 μM per h), which was at least an order of magnitude above that of TM1042 (Table 2.2). The rate of production of MMPA by TM1038 (15.9 μM per h) was quite similar to those of TM1035 (19.5 μM per h) and TM1042 (17.5 μM per h). The fourth strain, TM1040, catabolized DMSP to produce only MMPA. The cells removed $\sim 7\%$ (70 μM) DMSP in a 3-h period while producing only 15 μM MMPA (Fig. 2.6D). Thus, strain TM1040 has a high rate of DMSP degradation (91 μM per h), but a very low rate of MMPA production (5.1 $\mu\text{M}/\text{h}$), compared to the other three DMSP-degrading strains (Table 2.2).

2.3.4 Taxonomic identification of DMSP-degrading bacteria.

A taxonomic analysis of the DMSP-metabolizing strains placed the four isolates in the α -*Proteobacteria*, closely related to the *Roseobacter* clade (Fig. 2.7). Strains TM1042 and TM1035 are closely related to each other (99% identity; 1,306 of 1,310 bp) and cluster with another dinoflagellate-associated bacterium, strain ATAM407_61, isolated from the dinoflagellate *Alexandrium lustanicum* (168). Both TM1035 and TM1042 are also more distantly related to *R. tolerans* from Ekho Lake (96% [1,246 of 1,294 bp] and 96% [1,248 of 1,294 bp] identity, respectively), which, like strains

Table 2.2. Rate of DMSP metabolism of four bacterial strains isolated from *P. piscicida* CCMP1830 culture.

Strain	MMPA	MeSH	DMS	DMSP
TM1035	19.5	NA ^b	22.7	-95
TM1038	15.9	132	NP ^c	-22.3
TM1040	5.1	NP	NP	-91
TM1042	17.9	9.1	59	-32.6

^aThe rate of DMSP degradation and production of DMSP catabolites calculated based upon the slope of the linear portion of each curve from Fig. 6.

^bNA: Correlation coefficient < 0.90

^cNP: Catabolite not detected or produced by this strain.

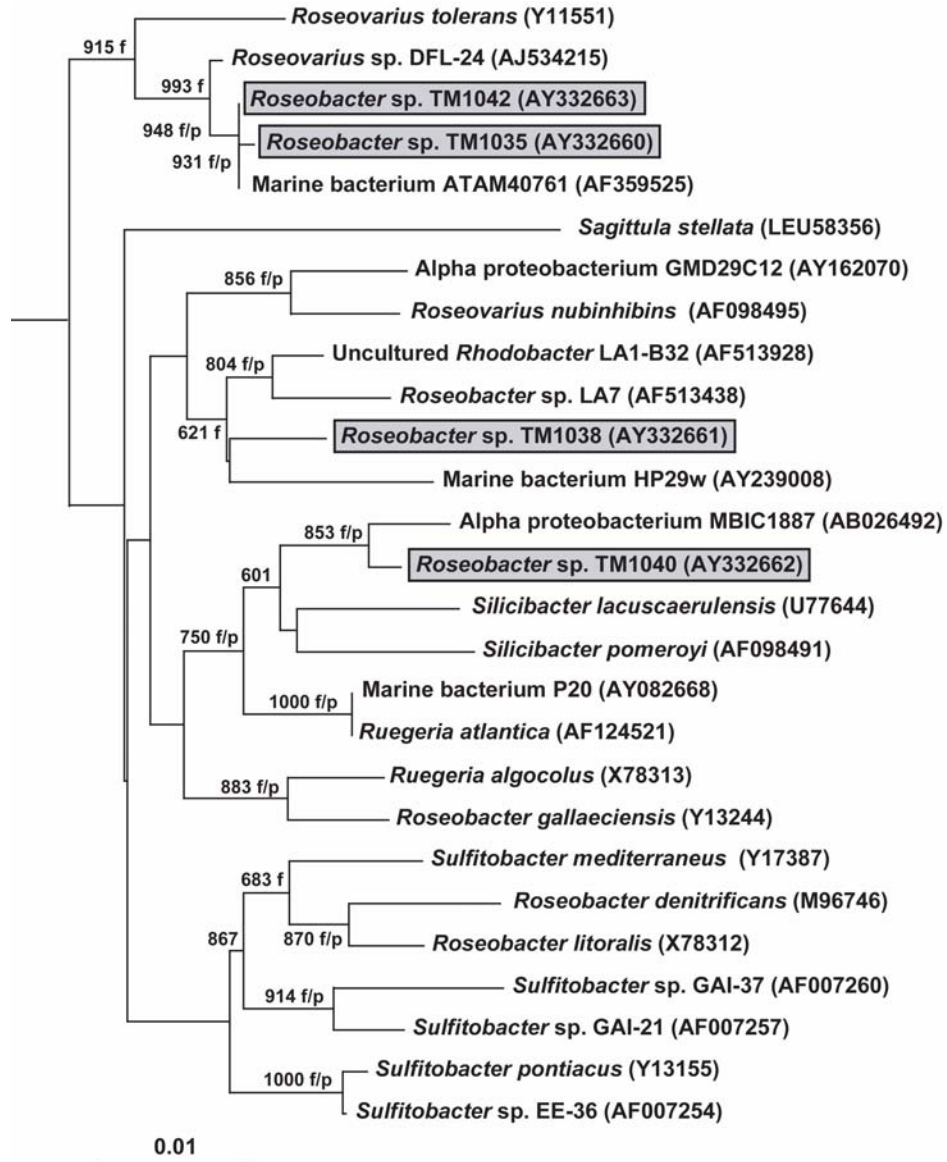


Fig. 2.7. Taxonomic analysis of the four DMSP-degrading bacteria isolated from *P. piscicida*. Shown is the phylogenetic tree inferred from comparative sequence analysis using 1,047 bp of 16S rDNA generated by the neighbor-joining method and Jukes Cantor distance algorithm. The resulting tree shows the relationships between the *Roseobacter* species associated with *P. piscicida* CCMP1830 and nucleotide sequences from other *Roseobacter* clade bacteria (obtained from the GenBank database). Bootstrap values (n = 1000 replicate resamplings) are indicated for the neighbor-joining method where values are greater than 500. An 'f' or 'p' indicates where the Fitch and parsimony methods are in agreement with the neighbor-joining tree. The bar represents 0.01 Units of evolutionary distance.

TM1035 and TM1042, is capable of producing both DMS and MeSH from DMSP (145). The sequences of TM1038 and TM1040 16S rDNAs show the greatest similarity to 16S rDNA sequences obtained from bacteria within the *Roseobacter* clade, yet they did not cluster well with any cultured organisms within this clade and are unrelated to any of the well-characterized *Roseobacter* species listed in GenBank. As shown in Fig. 2.7, the 16S rDNA from TM1038 was 95% (1,230 of 1,282 bp) identical to rDNA obtained from an uncharacterized bacterium isolated from marine snow, HP29w (153). Similarly, strain TM1040 grouped with an uncharacterized α -proteobacterium, MBIC1887 (99% sequence identity; 1,275 of 1,284 bp), and to a lesser extent showed some relatedness to two well-characterized *Silicibacter* species, *S. pomeroyi* and *S. lacuscaerulensis* (Fig. 2.7). Both of these *Silicibacter* species are capable of DMSP degradation (153).

2.4 Conclusions

In this study, *Pfiesteria* dinoflagellates and members of the bacterial community cooccurring with them were used as a model system to study DMSP production and degradation as it may influence prokaryote-eukaryote interactions. *Pfiesteria* dinoflagellates are mainly heterotrophic organisms that acquire nutrients through the consumption of algal prey and at certain times may utilize prey chloroplasts for energy. Not only have *Pfiesteria* species been implicated in causing human illness and fish mortality, these dinoflagellates are dominant species in nutrient-rich estuaries and are thus important to the greater understanding of nutrient cycling and microbial interactions in coastal marine habitats (251).

The results show that both *P. piscicida* and *P. shumwayae* contain significant

levels of DMSP, which to our knowledge is the first report of DMSP in *Pfiesteria* and only the second observation of DMSP in a heterotrophic dinoflagellate (201). The DMSP contents of the two *Pfiesteria* species (3.44 to 4.25 μM) (Table 2.1) are similar to those measured in other dinoflagellates. For example, the intracellular DMSP concentrations in photosynthetic species, such as *Prorocentrum*, *Gymnodinium*, and *Amphidinium* species, are reported to be 1 to 10 μM (191). Interestingly, the concentration of DMSP in another heterotrophic dinoflagellate, *Cryptocodinium cohnii*, has been reported to be 10 pg per cell (200), a value that is much higher than those observed in either *Pfiesteria* species (~ 0.4 pg per cell). This difference undoubtedly reflects physiological and taxonomic differences between *Pfiesteria* and *Cryptocodinium* and underscores the difficulty in making general statements about DMSP physiology among taxonomically diverse dinoflagellate species.

Both *P. piscicida* and *P. shumwayae* contain significant levels of DMSP, yet the *P. piscicida* cultures that include dinoflagellates plus associated bacteria degrade DMSP at significantly higher rates (Fig. 2.3). Previous data have demonstrated differences in the bacterial flora associated with *Pfiesteria* cultures (9). Thus, it is conceivable that differences in the compositions of the bacterial communities may affect the rates of DMSP decomposition in *P. piscicida* and *P. shumwayae* cultures. The data in Fig. 2.4 showing the difference between DMSP catabolite kinetics in the mixed culturable heterotrophic bacteria support this idea. Other factors may also be important, including the possibility that slow-growing bacteria in the *P. shumwayae* culture may have a low rate of DMSP degradation or that the concentration of DMSP used may have a deleterious effect specific to the *P. shumwayae* bacterial community.

Four DMSP-degrading bacterial isolates were obtained from *P. piscicida* cultures and were found to be phylogenetically related to members of the *Roseobacter* clade (Fig. 2.7). As shown in the taxonomic tree, two of the isolates (TM1035 and TM1042) are most closely related to *Roseovarius* species, while TM1038 and TM1040 are unique *Roseobacter* species not related to known roseobacters. Despite their taxonomic differences, all four bacteria shared the common trait of demethylating DMSP to MMPA, while strains TM1035 and TM1042 further metabolize DMSP to produce DMS, indicating that demethylation is a major pathway by which *Pfiesteria*-associated roseobacters degrade DMSP. Equally interesting are the DMSP demethylation pathways used by these strains. As an example, TM1038 demethylates DMSP to produce MMPA and MeSH, a pathway that appears to be commonly used by marine bacteria (193). In contrast, TM1040 strictly demethylates DMSP to produce MMPA without MeSH, a pathway reported to be used by one other aerobic marine bacterium, strain BIS-6, isolated from Biscayne Bay, Fla. (378). This bacterium demethylates DMSP to MMPA (Fig. 2.1, reaction 2) followed by a further demethylation to MPA (Fig. 2.1, reaction 4). Although not measured in this study, strain TM1040 is likely also to produce MPA instead of MeSH, as has been observed for strain BIS-6.

The two *Roseovarius*-related strains, TM1035 and TM1042, are capable of both demethylation and lyase cleavage of DMSP. The presence of dual demethylation-lyase pathways in the same organism is a recently discovered phenomenon. Gonzalez et al. (146) reported that 5 out of 15 DMSP-catabolizing bacteria isolated from Georgia coastal seawater and the Caribbean Sea catabolized DMSP to produce both DMS and MeSH, as well as converted MMPA to MeSH. One of these five isolates was taxonomically

identified as *R. nubinhibens* ISM (146). The capacity to use both DMSP pathways may provide these bacteria with a survival advantage, especially in environments where DMSP concentrations are high, such as the phycosphere surrounding DMSP-producing dinoflagellates. Bacteria that utilize the lyase cleavage pathway are capable of growing on DMSP as a sole carbon source (398). In contrast, while the demethylation pathway does not always lead to increased growth (182), much of the sulfur obtained from this pathway is utilized for protein synthesis and seems to be preferred over other sources of sulfur abundant in seawater (197). Thus, coupling of both DMSP-degradative pathways in the same organism may satisfy both the carbon and sulfur requirements of these dinoflagellate-associated marine bacteria.

In analyses of DMSP catabolism, it was occasionally observed that the sum of the DMSP catabolites produced did not always equal the amount of DMSP lost from the culture. There are several possible explanations for this. First, bacterial enzymes may have degraded the DMSP catabolites shortly after they were produced. This is a strong possibility in light of the high reaction rates observed. A good example supporting this is the failure to detect acrylate production in either strains TM1035 or TM1042, despite the presence of detectable levels of DMS, which constitutes the other half of lyase cleavage of DMSP. The absence of acrylate is most likely due to rapid conversion, a finding that was also noted by Ansedé et al. (26, 27), who, using nuclear magnetic resonance analysis, were unable to detect acrylate production by a *Roseobacter* species even though DMS was produced. The present results also agree with environmental studies that show rapid degradation of acrylate by bacterial communities associated with algal cells or debris (289). A similar case could also be made for the high rate of DMSP degradation by strain

TM1040 which produces only low levels of MMPA. In at least one other marine bacterium MMPA was shown to be converted to MPA

Another possibility to explain the imbalance in DMSP catabolites is that these chemicals may have been degraded or lost due to abiotic factors, such as oxidation of a compound or the adherence of a catabolite to inanimate surfaces. For example, MeSH is readily oxidized to form dimethyl disulfide and may be lost from water samples due to binding with humic acids (193). In our experiments, a minor loss of MeSH due to sticking to inanimate surfaces was observed (data not shown) and may have resulted in a slight overestimation of total MeSH gas production, which in turn may have contributed to the imbalance between MeSH produced and DMSP degraded.

Pfiesteria and other heterotrophic dinoflagellates are in intimate association with a community of bacteria, many of which are members of the *Roseobacter* clade, which interact in a myriad of ways with their eukaryotic partner. *Roseobacter* clade bacteria have been observed attached to or physically associated with dinoflagellate cells, while other *Roseobacter* species are required for dinoflagellate growth (9). It is tantalizing to think that DMSP is involved in these associations, particularly in view of the diversity of DMSP pathways and the high rates of reactions seen in the *Pfiesteria* cultures and the *Roseobacter* isolates obtained from them. These results also bring up new questions about roseobacters, DMSP, and dinoflagellate interactions. Are *Roseobacter* clade bacteria attracted to DMSP or one of its catabolites, which may bring them into close proximity to dinoflagellate cells? Do the catabolites of DMSP have physiological functions in dinoflagellate metabolism, behavior, or growth? In the long term, answers to

these questions will provide significant clues about the molecular and cellular natures of the interactions between bacteria, dinoflagellates, and other single-cell eukaryotes.

Chapter 3: Chemotaxis of *Silicibacter* sp. TM1040 toward dinoflagellate products

3.1 Summary

The α -Proteobacteria phylogenetically related to the *Roseobacter* clade are predominantly responsible for the degradation of organosulfur compounds, including the algal osmolyte dimethylsulfoniopropionate (DMSP). *Silicibacter* sp. strain TM1040, isolated from a DMSP-producing *Pfiesteria piscicida* dinoflagellate culture, degrades DMSP, producing 3-methylmercaptopropionate. Strain TM1040 possesses three lophotrichous flagella and is highly motile, leading to a hypothesis that TM1040 interacts with *P. piscicida* through a chemotactic response to compounds produced by its dinoflagellate host. A combination of a rapid chemotaxis screening assay and a quantitative capillary assay were used to measure chemotaxis of TM1040. These bacteria are highly attracted to dinoflagellate homogenates; however, the response decreases when homogenates are preheated to 80°C. To help identify the essential attractant molecules within the homogenates, a series of pure compounds were tested for their ability to serve as attractants. The results show that TM1040 is strongly attracted to amino acids and DMSP metabolites, while being only mildly responsive to sugars and the tricarboxylic acid cycle intermediates. Adding pure DMSP, methionine, or valine to the chemotaxis buffer resulted in a decreased response to the homogenates, indicating that exogenous addition of these chemicals blocks chemotaxis and suggesting that DMSP and amino acids are essential attractant molecules in the dinoflagellate homogenates. The implication of *Silicibacter* sp. strain TM1040 chemotaxis in establishing and maintaining its interaction with *P. piscicida* is discussed.

3.2 Introduction

In the marine environment, unicellular algae and other eukaryotic microorganisms, such as dinoflagellates, coexist with a diverse community of heterotrophic bacteria. Much of the carbon and nitrogen required for the growth of these bacteria is supplied by the unicellular bloom-forming eukaryotes (90). During feeding, stress, and lysis, nutrients are released from these microbes and made available to the bacteria. The nutrient plumes emitted occur on a microscale and are quickly dispersed and diluted by diffusion and turbulence (10). Over space and time not all species of bacteria are equally likely to obtain these nutrients (53). While some bacterial species rely upon high-affinity uptake mechanisms, the ability to sense and move towards increasing chemical gradients, known as chemotaxis, provides motile bacteria with a distinct advantage over their nonmotile counterparts (53). Knowledge of how marine bacteria sense and respond to algal cells is therefore important to our understanding of bacterial physiology and the interactions between these prokaryotes and their eukaryotic hosts and ultimately impacts our greater understanding of nutrient cycling in marine ecosystems.

Algae-derived compounds that are likely to serve as chemoattractants for marine heterotrophic bacteria include sugars, amino acids, nucleosides, and other small diffusible organic compounds. Many of these molecules are common to all living cells; however, some are algae specific. For example, most marine unicellular algae, especially dinoflagellates and prymnesiophytes, produce large quantities of the organosulfur compound dimethylsulfoniopropionate (DMSP) (191). This compound is degraded by

both algae and bacteria, resulting in the production of dimethylsulfide and acrylate, or demethylated by bacteria only, producing demethylation products including 3-methylmercaptopropionate (MMPA) and 3-mercaptopropionate (MPA) (30). All of these compounds can be utilized as a source of bacterial carbon and sulfur and are candidates for algae-specific chemoattractants (146, 196, 335, 403).

The α -, β -, and γ -Proteobacteria, as well as the Cytophaga-Flexibacter-Bacteroides spp., are important members of the marine picoplankton and are often found within algal communities (138). Of these groups, the α -Proteobacteria genera phylogenetically related to the *Roseobacter* clade are of particular interest. These bacteria are abundant in algal cultures as well as within blooms of algal species (9, 168, 405). They are also predominantly responsible for the degradation of DMSP (146), and their activity has been correlated with blooms of high-DMSP-producing phytoplankton, including dinoflagellates and prymnesiophytes (405). Interestingly, *Roseobacter* clade species are involved in both physical (attached or intracellular) and physiological (toxin production and growth-enhancing) interactions with dinoflagellates (9, 52, 134). Mechanisms for these interactions may rely upon bacterial motility and chemotaxis behavior that allow these bacteria to sense and move towards their eukaryotic host, where they derive beneficial nutrients, once in close physical proximity to the dinoflagellate. While much is known about the cooccurrence of motile *Roseobacter* species and bloom-forming dinoflagellates, little or no data describing chemotactic behavior in any of the *Roseobacter* clade bacteria have been reported.

We have used monocultures of the DMSP-producing dinoflagellates *Pfiesteria piscicida*, *Pfiesteria shumwayae*, and *Pfiesteria*-like (*Cryptoperidiniopsis*) dinoflagellates

as our model system for studying *Roseobacter*-dinoflagellate interactions (9). Within these cultures, bacteria native to the algal niche assimilate dinoflagellate-derived nutrients and are intrinsically propagated with the dinoflagellates in continuous subcultures. The bacterial community inhabiting *Pfiesteria* cultures is diverse, containing at least 19 different species depending upon the strain examined (9). Some of these bacteria can be found physically attached to and aiding in the growth of the dinoflagellates, and a major component of this bacterial community consists of bacteria related to *Roseobacter* species (9).

In the previous chapter (250), I isolated and identified four DMSP-degrading *Roseobacter* species from a *P. piscicida* culture. All of the *Roseobacter* species isolated from these dinoflagellates degrade DMSP (250), and one, strain *Silicibacter* sp. strain TM1040, is highly motile in semisolid agar and in liquid media. In this chapter, I tested the hypothesis that strain TM1040 is chemotactic towards specific molecules produced by the dinoflagellate.

3.3 Materials and Methods

3.3.1 Bacteria and media.

Silicibacter sp. strain TM1040 was isolated from a culture of the dinoflagellate *P. piscicida* CCMP1830 (Provasoli-Guillard National Center for Culture of Marine Phytoplankton) and was maintained on either HIASW agar (25 g of heart infusion broth [Difco], 15 g of artificial seawater [ASW; Instant Ocean], 16 g of Bacto Agar per liter) or half-strength 2216 marine agar (9). Liquid broth cultures were made with half-strength 2216 marine broth. Marine motility agar was prepared by supplementing half-strength

2216 marine broth with 3.0 g of Bacto Agar per liter. For the chemotaxis plate assay, a basal minimal (BM) medium (12.1 g of Tris HCl, 1.0 g of NH₄Cl, 0.0075 g of K₂HPO₄, 15 g of ASW, 3.0 g of Bacto Agar per liter; pH 7.6) was used. After autoclaving, the BM medium was cooled to 50°C and supplemented with 0.2 g of FeSO₃ and 1 ml of Balch's vitamins (39), and a single carbon source (glycerol, glucose, succinate, or alanine) was added to a final concentration of 10 mM.

3.3.2 Dinoflagellates and cultivation.

P. piscicida CCMP1830 was grown as previously described (9). Dinoflagellates were fed a diet of the cryptomonad prey alga *Rhodomonas* sp. CCMP768, supplied as described by Alavi et al. (9).

3.3.3 Chemotaxis plate screening assay.

A qualitative chemotaxis screening method, described by DeLoney-Marino et al. (103), was used with only slight modification. An isolated colony of strain TM1040 was inoculated in the center of a BM-glycerol motility agar plate. After 3 days, a point where the bacteria had swam outwards 5 cm from the site of inoculation, an inoculum taken from the outer ring of the motile colony was used to inoculate the center of a second fresh BM-glycerol motility agar plate. Following 36 h of incubation, a putative attractant was placed approximately 5 cm from the site of inoculation (or 2.5 cm in front of the periphery of motile cells). Possible chemoattractant compounds were administered as either a sterile solid or concentrated stock solutions. The cells were further incubated for 16 h to allow for additional outward movement, at which time measurement of diameter, shape, and chemotaxis rings internal to the motile colony were made. The resulting data were scored on a plus-minus scale, where minus indicates no change compared to

distilled water or no attractant controls and either one or two pluses indicates moderate to strong alteration in motile colony phenotype (respectively). All chemotaxis assays were performed in a 30°C walk-in incubator at 65% relative humidity.

3.3.4 Quantitative capillary chemotaxis assay.

The capillary method of Adler (5), as modified by Palleroni (298), was used to quantitatively measure the chemotactic response of TM1040 toward a subset of compounds screened by the plate method. A broth culture of TM1040 was grown overnight in half-strength 2216 marine broth at 30°C to an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.4, which corresponds to the mid-exponential phase of the *Silicibacter* sp. strain TM1040 growth cycle. The cells were pelleted by centrifugation at 4,000 x g in a tabletop centrifuge (Centra-CL2; International Equipment Company), the supernatant was discarded, and the pellet was resuspended in chemotaxis buffer (CB) (15 g of ASW, 6 g of Tris-HCl per liter [pH 7.6]) to the same OD. Five hundred microliters of washed cells was placed in one of the wells of the Palleroni chamber, followed by a 1- μ l capillary (Microcaps; Drummond) filled with a putative attractant diluted in CB. This process was repeated for each of the four wells in the chamber. A capillary filled with CB only was included in each experiment as a negative control. Depending on the experiment, capillaries remained in the chambers for 0.5 to 2 h, after which time they were removed, and the contents of each capillary were serially diluted in CB. Final dilutions were then spread on HIASW agar plates and incubated for 16 h at 30°C. The concentration of TM1040 in each capillary was derived by counting the CFU on each plate and multiplying by the appropriate dilution factor. To normalize all data, a “response factor” as described by Wei and Bauer (390) was calculated by dividing the mean number of

bacteria in the attractant-filled capillaries by the mean number of bacteria in control capillaries.

The capillary assay was also used to measure the reduction in chemotaxis through competition when a known attractant was supplied exogenously (i.e., outside the capillary). In this variation, the quantitative capillary method was used, but a known attractant was included in the CB used to suspend the washed cells. A capillary containing a mixture of potential chemoattractant molecules was then placed in the chamber and incubated as described earlier. At the completion of the assay, the percent reduction in chemotaxis was calculated using the following formula: % reduction = $[1 - (\text{CFU ml}^{-1}_{\text{exogenous}} / \text{CFU ml}^{-1}_{\text{control}})] \times (100)$.

3.3.5 Preparation of dinoflagellate homogenates.

Dinoflagellates and *Rhodomonas* sp. were grown to late exponential phase (ca. 10^5 to 10^6 cells per ml), and the cell density was measured using a Coulter Counter (model M/SZR II; Beckman Coulter). Dinoflagellates were harvested after starvation of the culture to remove *Rhodomonas* prey algae, which was confirmed by microscopy. The cell densities in *P. piscicida* and *Rhodomonas* cultures were normalized to 10^5 cells per ml, and each culture was then pelleted by centrifugation at $4,000 \times g$ and 4°C for 10 min. The supernatant was removed, and the pellets were separately resuspended with either 1 ml of ice-cold distilled water or CB. The cells were homogenized by sonication (Sonic Dismembrator; Fisher), and cell disruption was assessed by microscopy. To examine the heat sensitivity of chemotaxis elicitors, an aliquot of each homogenate was heated to 80°C for 15 min prior to the assay. All homogenates were then stored at -20°C . When

required, the homogenates were thawed, held on ice, and used in the capillary assay as described.

Bacterial homogenates were prepared in a similar manner. Briefly, an aliquot from several 10-fold dilutions of the *P. piscicida* culture was inoculated onto half-strength 2216 marine agar and incubated until bacterial colonies were evident. Approximately 1,000 mixed colonies were then resuspended from the agar surface using 10 ml of CB, and the cells were collected by centrifugation at 4,000 x g for 10 min. The bacterial pellet was resuspended to an OD₆₀₀ of 0.3 in CB. Aliquots of the bacterial suspension were sonicated, and a portion was heated, as described above.

3.3.6 Video microscopy.

To compare the motility of strain TM1040 with and without an attractant, cells were grown and prepared as described for in the capillary assay. A sample of the washed cells was incubated at 30°C, with and without the addition of 10 mM succinate (in CB, pH 7.0). Cell swimming and behavior over a 5-h period were measured using phase-contrast microscopy (Nikon Optiphot BX60) equipped with a digital video camera (Canon Elura, Lake Success, N.Y.). After examination of the entire video, three separate fields were chosen, and 1-min intervals of each were transferred to a computer for further analysis, using Adobe (San Jose, Calif.) Premier version 6.0 software. Individual 0.33-s frames were exported as a tagged image file format sequence and reassembled with IPLab version 3.55 (Scanalytics, Fairfax, Va.) as a sequence of ordered frames (t-series). The numbers of motile and nonmotile cells per field were determined to determine the average percentage of motile cells per field.

3.3.7 Transmission electron microscopy (TEM).

Silicibacter sp. strain TM1040, taken from the periphery of a motile colony growing in semisolid BM glycerol motility agar, was inoculated in half-strength 2216 marine broth and incubated at 30°C to an OD₆₀₀ of 0.3. A 400- μ m-mesh carbon-coated parlodion copper grid was floated over a 30- μ l aliquot of this culture for 1 to 2 min and blotted dry. Bacteria adhering to the grid were stained two times for 30 s with 1% uranyl acetate and 0.04% tylose in distilled water. Negatively stained cells were viewed using a Philips BioTwin CM120 transmission electron microscope at an operating voltage of 20 kV. The resulting images were recorded on film and scanned into a computer, and the brightness and contrast were changed for optimum viewing using Adobe Photoshop 7.

3.3.8 Analytical techniques.

The concentration of DMSP in 1 ml-samples of heated or boiled 200 μ M DMSP and in heated and untreated *P. piscicida* homogenates was measured using gas chromatography with flame ionization detection, as previously described (250).

3.3.9 Chemicals.

DMSP was synthesized from acrylate and dimethylsulfide as previously described (82). MMPA was synthesized by alkaline hydrolysis of its methyl ester, methyl-3-(methylthio)propionate (Aldrich, Milwaukee, Wis.) (183). All other compounds were of at least reagent grade quality and were purchased from Sigma-Aldrich (Milwaukee, Wis.).

3.4 Results

3.4.1 TM1040 possesses three lophotrichous flagella.

Prior to beginning studies of chemotaxis, motile cells of TM1040 were visualized by TEM to confirm the presence, number, and location of flagella. As shown in Fig. 3.1,

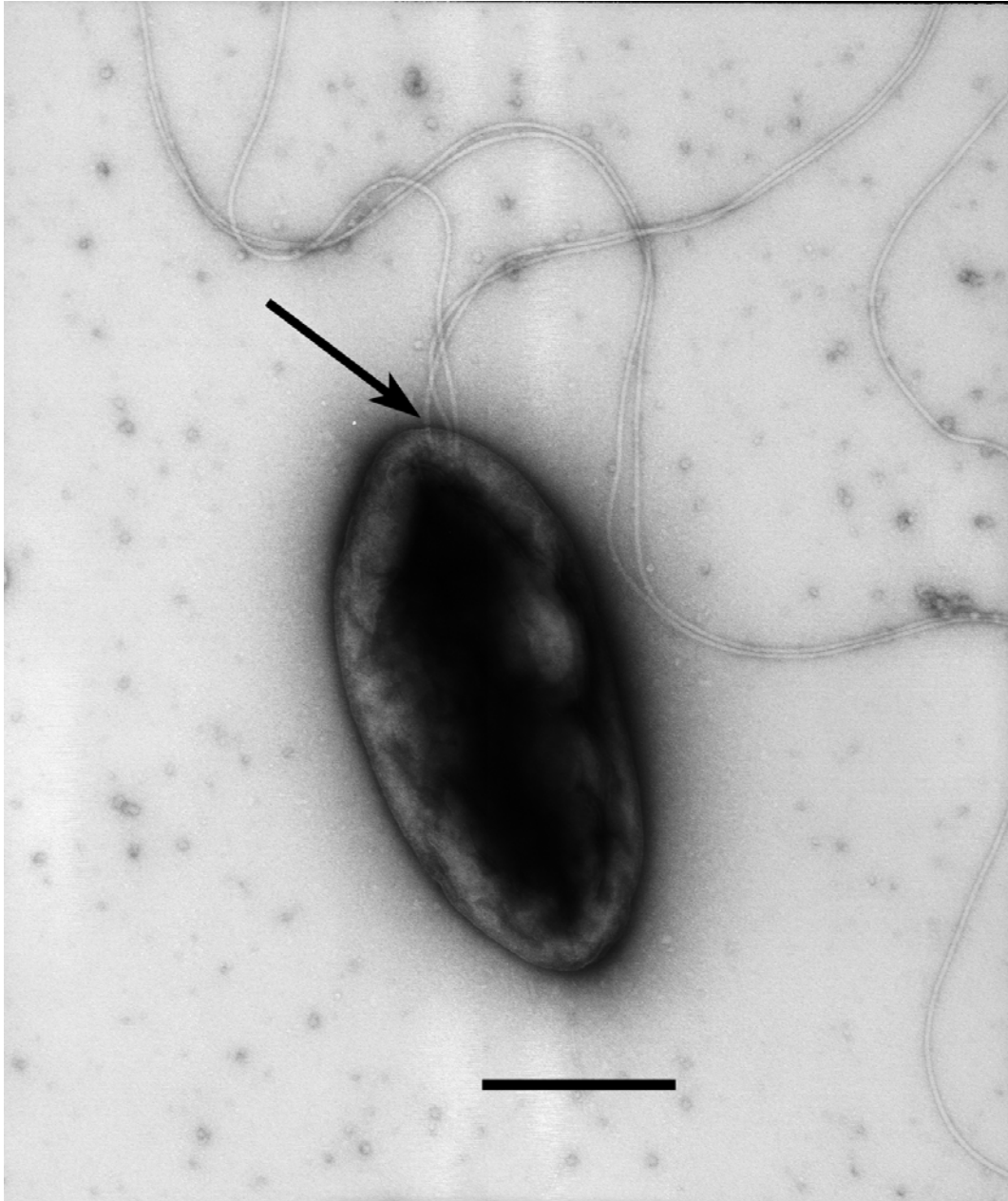


Fig. 3.1 Transmission electron micrograph of *Silicibacter* sp. TM1040. A culture of TM1040 was prepared in ½ strength 2216 marine broth and grown for 20 h at 30 °C without agitation. Motile cells were blotted onto copper discs, stained with 1% uranyl acetate and visualized by TEM. Three simple lophotrichous polar flagella are apparent (arrow). (Bar = 0.5 µm)

strain TM1040 is a small (ca. 1.0-to 1.5- μm) rod-or oval-shaped bacterium with at least three lophotrichous flagella. These flagella are located at one end of the cell, slightly off-center from the cell pole. An analysis of the structure of filaments suggests that they are simple filaments, rather than the complex forms found in *Silicibacter pomeroyi* DSS-3 (145) or other α -Proteobacteria (324).

3.4.2 Chemotaxis of TM1040 is enhanced by prior starvation.

Initial observations suggested that TM1040 cells held for 1 to 2 h in CB (a starvation condition) were more responsive to methionine than cells that had not been starved (data not shown). To examine this more closely, chemotaxis of starved and unstarved TM1040 toward methionine was measured by using a quantitative capillary assay. As shown in Fig. 3.2, starving the bacteria of nutrients prior to the assay resulted in a greater maximal response to the attractant, which was maintained for at least 2 h. This can be seen by comparing the starved-cell response to methionine to the unstarved response to methionine in Fig. 3.2. After 0.5 h, the mean concentration of bacteria in methionine-filled capillaries was 5.3×10^7 CFU per ml and increased thereafter, with increasing exposure time rising from 1.8×10^8 CFU per ml at 1h to a maximum of 2.4×10^8 CFU per ml at 1.5 and 2 h. By contrast, without starvation the maximum mean concentration of bacteria in methionine-filled capillaries reached a peak of 2.1×10^8 CFU per ml at 1.5 h and decreased thereafter. The bacterial concentration in control capillaries for either starved or unstarved cells reached an equilibrium of 2×10^7 CFU per ml after 0.5 h.

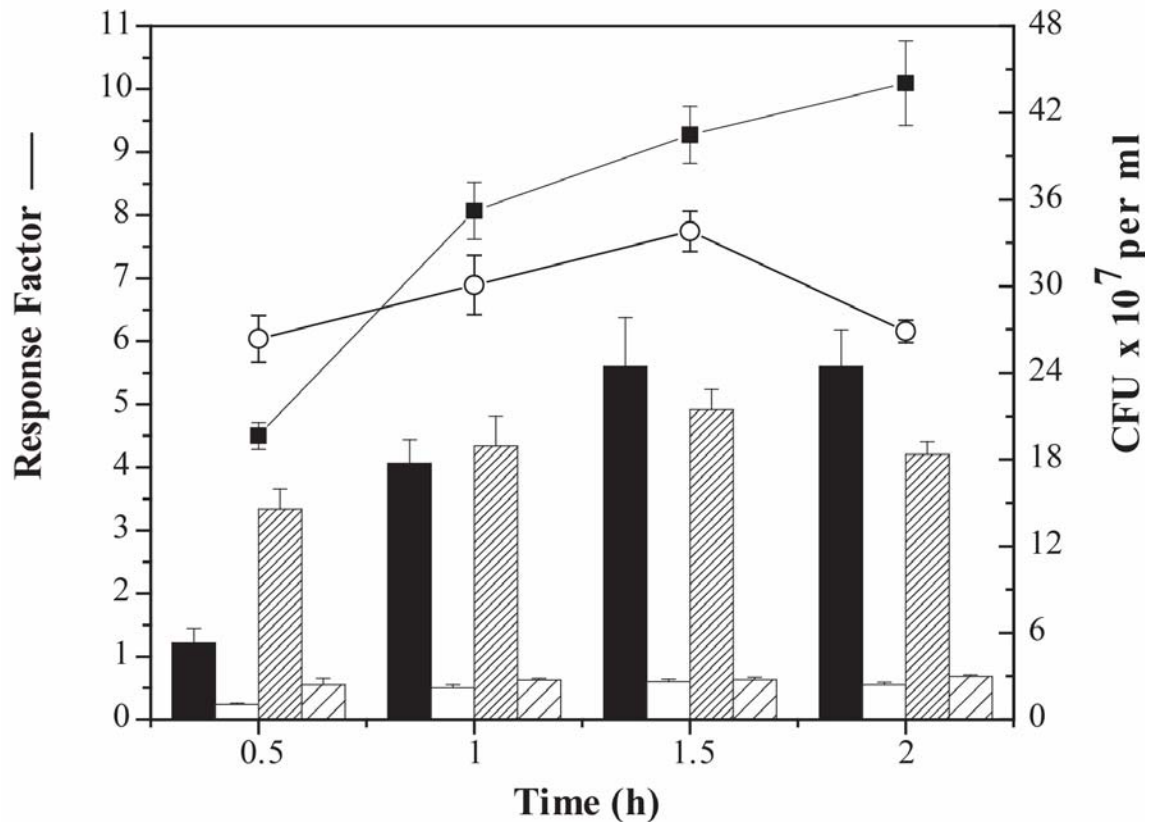


Fig.3.2. The effects of starvation on the chemotactic response of *Silicibacter* sp. TM1040. The chemotaxis of unstarved vs. starved cells to 200 μ M methionine was measured using the capillary assay (Materials and Methods). The chemotaxis of starved cells to either methionine (black bars) or buffer lacking nutrient (white bars) is compared to the response of unstarved cells to either methionine (dense cross-hatched bars) or to the buffer lacking nutrient (sparse cross-hatched bars). The response factor, calculated by dividing the number of bacteria in methionine-filled capillaries by the number of bacteria in buffer-filled capillaries, is significantly increased in the starved cells (■) compared to the unstarved cells (○). Values shown are the mean and S.D. (n=12).

The difference between the chemotactic responses of starved and unstarved cells is also shown in Fig. 3.2. The response factor is calculated by dividing the concentration of bacteria in attractant-filled capillaries by the concentration of bacteria in control capillaries (390). The response factor of starved cells to methionine is higher than the response of unstarved cells at all times except 0.5 h (where equilibrium has yet to be reached). Significantly, a comparison of the response factors of starved cells and unstarved cell confirms that starvation improves the chemotactic response of strain TM1040 and maintains it longer.

3.4.3 Motility is affected by starvation.

The motility of the starved TM1040 cells was measured by light microscopy. While starvation enhanced the chemotactic response, it also reduced swimming motility, and prolonged starvation resulted in a majority of the cells becoming nonmotile. After 2 h, the percentage of motile cells under starvation conditions decreased drastically to a mean of 5.93% (n = 69), and by 4 h all of the cells were nonmotile (n = 66). In comparison, the percentage of motile cells under nonstarvation conditions did not change after 5 h and was similar to the percentage of motile cells after 1 h (mean, 26.64%; n = 69). Thus, while starvation stimulates chemotaxis, it also depletes the energy supplies required to rotate the flagella. Based on these results, a starvation period of 1.5 h was considered optimal and was used in all further capillary assays.

3.4.4 TM1040 is attracted to dinoflagellate homogenates.

Does *Silicibacter* sp. strain TM1040 sense and respond to dinoflagellates? To determine this, chemotaxis of strain TM1040 toward cell homogenates of *P. piscicida*

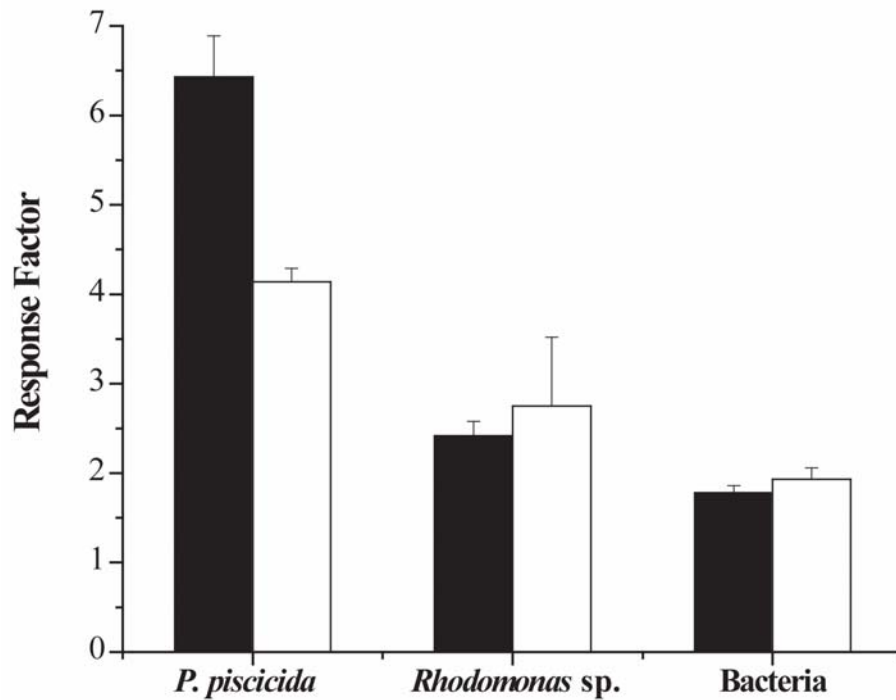


Fig. 3.3. Chemotaxis of *Silicibacter sp.* TM1040 toward cell homogenates. Chemotaxis of TM1040 toward heated (80 °C), indicated by the white bars, and untreated (black bars) homogenates was measured using the capillary chemotaxis assay (Materials and Methods). The response factor was calculated by dividing the number of bacteria in homogenate-filled capillaries by the number of bacteria in buffer-filled capillaries. The chemotactic response of TM1040 to the untreated dinoflagellate homogenate is > 2.5x greater than the response to either algal or bacterial homogenates, and heating the dinoflagellate homogenate reduces the response factor by ca. 40%. Values shown are the mean and S.D. (n=8).

and other constituents of the dinoflagellate culture was measured using the capillary assay. It is important to emphasize that the *P. piscicida* culture normally contains three different types of organisms: the dinoflagellates, the prey algae (*Rhodomonas* sp.), and a diverse bacterial population that includes TM1040 (9). *Rhodomonas* can be virtually eliminated from the cultures through attrition from dinoflagellate feeding, but the bacterial community cannot be removed without adversely affecting the dinoflagellates themselves (9). So, a strategy was developed to measure the chemotaxis of TM1040 towards three cell homogenates: dinoflagellates plus associated bacteria, *Rhodomonas*, and a mixture of heterotrophic bacteria obtained from the same dinoflagellate culture (see Materials and Methods). In this manner, the relative contribution of each population towards eliciting a chemotactic response from TM1040 could be assessed.

As is shown in Fig. 3.3, *Silicibacter* sp. strain TM1040 responded strongly to *P. piscicida* homogenates, producing a response factor of 6.4. This response is mediated in part by heat-labile compounds because heating of the same homogenates prior to the assay reduced the response by 36% (response factor = 4.1) (Fig. 3.3). TM1040 cells are substantially less chemotactic to *Rhodomonas* homogenates, and this response did not change upon heating of the algal homogenate, remaining at a mean of ca. 2.5 (Fig. 3.3). Equally, bacterial homogenates were also poor elicitors of TM1040 chemotaxis, giving a mean response factor of only 1.9 and 1.7, with or without heating, respectively. These results indicate that TM1040 is more strongly attracted to components present in *P. piscicida* cell homogenates, some of which are heat labile.

3.4.5 DMSP compounds and amino acids are strong chemoattractants of TM1040.

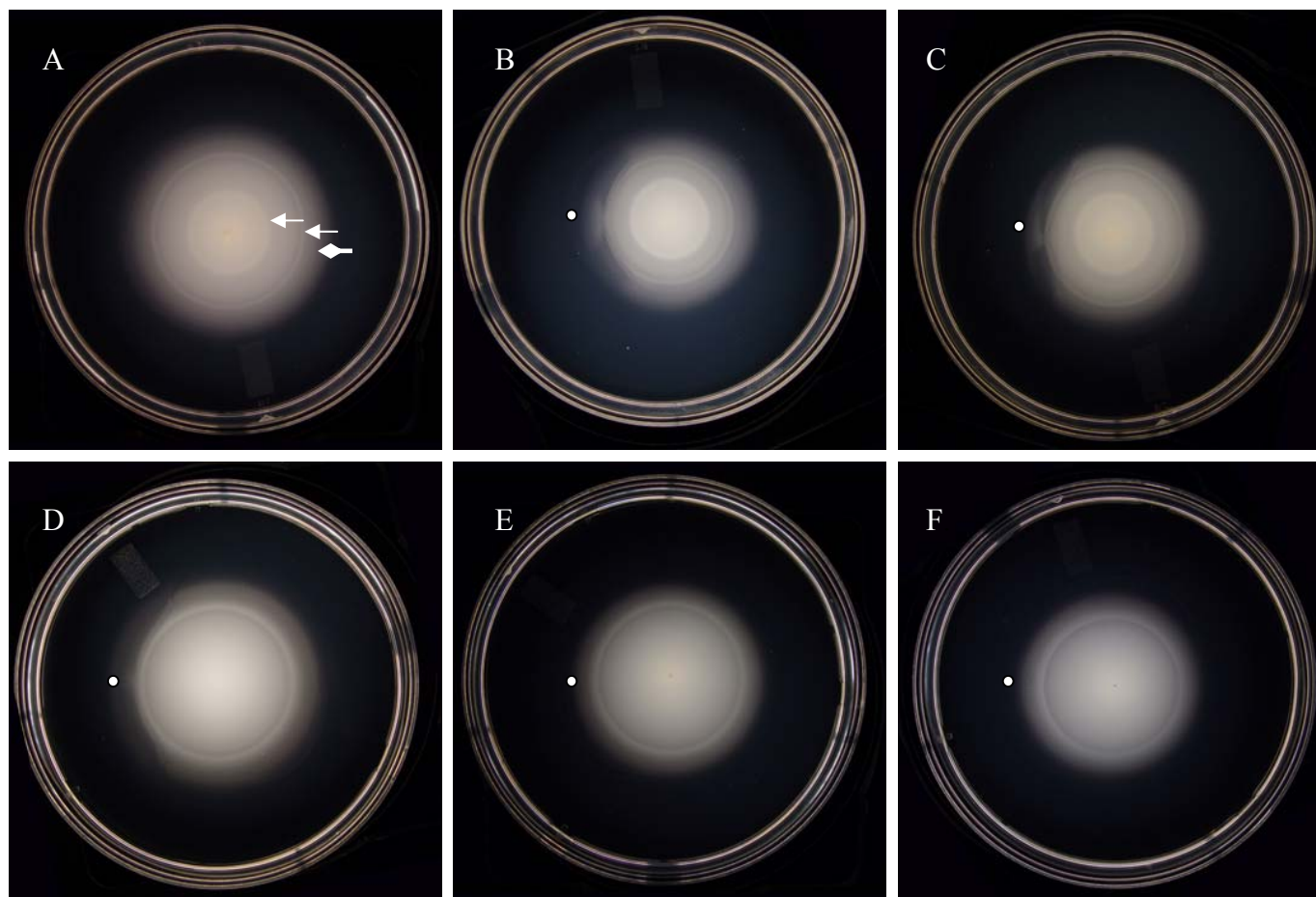


Fig. 3.4. Screening of putative attractant compounds using the qualitative chemotaxis assay. Chemotaxis behavior of TM1040 was assessed using BM glycerol motility agar on which a compound to be tested is placed (indicated by the dot to the left of the swimming colony). (A) Chemotaxis of *Silicibacter* TM1040 through BM glycerol motility agar results in an ever-increasing colony of motile bacteria that is punctuated by one or more internal bands of bacteria (indicated by the arrows) and the periphery (diamond head arrow). Shown is the response of TM1040 to (B) DMSP, (C) methionine, (D) valine, (E) arabinose, and (F) fructose. In the presence of either DMSP, methionine, or valine the outer ring is disturbed and becomes asymmetrical, indicating a chemotactic response from the cells. In contrast, neither arabinose or fructose cause a change in the symmetry of the colony, suggesting that TM1040 does not respond to these compounds.

Table 3.1. The change in motile colony morphology of TM1040 in response to attractants, compared to the control.

<u>Amino Acids</u>	<u>Response^a</u>
Alanine, Arginine, Asparagine, Aspartic acid, Glutamic acid, Methionine, Phenylalanine, Proline, Threonine, Valine	++
Glycine, Histidine, Cysteine, Isoleucine, Leucine, Lysine, Serine, Tryptophan, Tyrosine	+
<u>DMSP Metabolites</u>	
DMSP, Acrylate, MMPA	++
MPA	+
DMSO	-
<u>Sugars</u>	
Galactose, N-acetylglucosamine, Sucrose	++
Glucose, Maltose, Glycerol	+
Lactose, Arabinose, Fructose, Fucose, Mannose, Ribose, Xylose	-
<u>TCA Intermediates</u>	
Citrate, Fumurate	++
Succinate	+
Alpha - ketoglutarate	-

^aThe strength of the response indicates the change in motile colony morphology when a chemical is spotted near the colony periphery compared to the control, no spotted chemical.

Table 3.2. The change in motile colony morphology of TM1040 in response to putative attractants when grown in BM motility agar

Chemical	Carbon Source ^a			
	Glycerol	Glucose	Succinate	Alanine
Acrylate	++	+	++	+
Alanine	++	-	+	-
Alpha - ketoglutarate	-	-	+	-
DMSO	-	-	-	-
DMSP	++	-	+	+
Galactose	++	-	+	-
Glucose	+	-	+	+
Glutamic Acid	++	-	+	-
Lactose	-	-	-	-
Lysine	+	-	-	-
Maltose	+	-	++	++
N - acetylglucosamine	++	-	+	+
Succinate	+	+	-	++
Valine	++	-	+	+

^aTM1040 was Inoculated into the center of a BM motility agar plate containing a single carbon source (glycerol, glucose, succinate or alanine) and allowed to grow and move outwards. Chemicals were then spotted near the periphery of the motile colony and the change in colony appearance compared to the control (no spotted chemical) recorded on a plus/minus scale.

A chemotaxis plate assay (103) was used to screen a large number of pure compounds thought to be in dinoflagellate cell homogenates for their ability to affect the chemotactic behavior of TM1040. This assay utilizes a minimal medium with 0.3% agar (BM glycerol motility agar) that allows the bacteria to swim through the agar matrix. Bacteria inoculated into the center of the agar consume nutrients and create a concentration gradient that increases outward from the point of inoculation. The bacteria sense the increasing gradient and swim outwards, seeking higher concentrations of nutrients, which is manifested as a symmetrical “motile” colony. If an attractant is placed at a short distance in front of the advancing motile colony, it forms a second gradient moving towards the oncoming cells that will affect the symmetry of the colony by reducing the net outward swimming of the bacteria. This is shown in Fig. 3.4. When inoculated in BM glycerol motility agar (Fig. 3.4A), TM1040 cells swim outward from the point of inoculation to form a colony that often contains one or two internal bands of cells (Fig. 3.4A). These bands have been associated with subpopulations of bacteria that are responding to different attractants (394).

As measured by this method, TM1040 is chemotactically responsive to a number of different chemicals, most notably DMSP and its catabolites, as well as amino acids (Fig. 3.4). For example, addition of DMSP (Fig. 3.4B) caused a marked deformation in the periphery of the motile colony, highlighted by flare of bacteria and disruption of the outer ring. Responses to methionine (Fig. 3.4C) and valine (Fig. 3.4D) also had significant effects on the periphery of the motile colony. In comparison, there was no detectable change in the appearance of the motile colony when either arabinose (Fig.

3.4E) or fructose (Fig. 3.4F) was tested, suggesting that these two sugars do not affect TM1040 chemotaxis. The response to other chemicals was scored on a plus-minus scale (Table 3.1). TM1040 is attracted to all amino acids, similar to the response observed with methionine or valine. The bacterium responded strongly to the DMSP metabolites, acrylate and MMPA, while only weakly to MPA. These responses were similar to that of methionine and valine. Of the 12 sugars that were tested, strain TM1040 responded positively to (in order of response) sucrose, N-acetylglucosamine (NAG), galactose, glucose, and maltose. The tricarboxylic acid cycle (TCA) intermediates, citrate and fumarate, also elicited a strong positive response from TM1040, while only a mild response to succinate and no response to α -ketoglutarate was seen.

As described, BM glycerol medium was used because TM1040 can utilize glycerol as a sole carbon source, as has been observed for other roseobacters (327). However, it is known that chemotaxis behavior in other bacterial species can be affected by the availability of background nutrients (103). Indeed, when glucose was used in place of glycerol as the sole background carbon source, TM1040 chemotaxis was severely affected, and many of the chemicals that were attractants using BM glycerol failed to produce an effect in BM glucose (Table 3.2). The two exceptions were acrylate and succinate, which both produced a moderate response in the glucose background (Table 3.2). In contrast to glucose, when either succinate or alanine was used as the sole carbon source, TM1040 chemotactic behavior was similar to what had been observed in BM glycerol medium, albeit the response was often less intense. Not surprisingly, since chemotaxis relies on the establishment of chemical gradients, TM1040 did not respond to

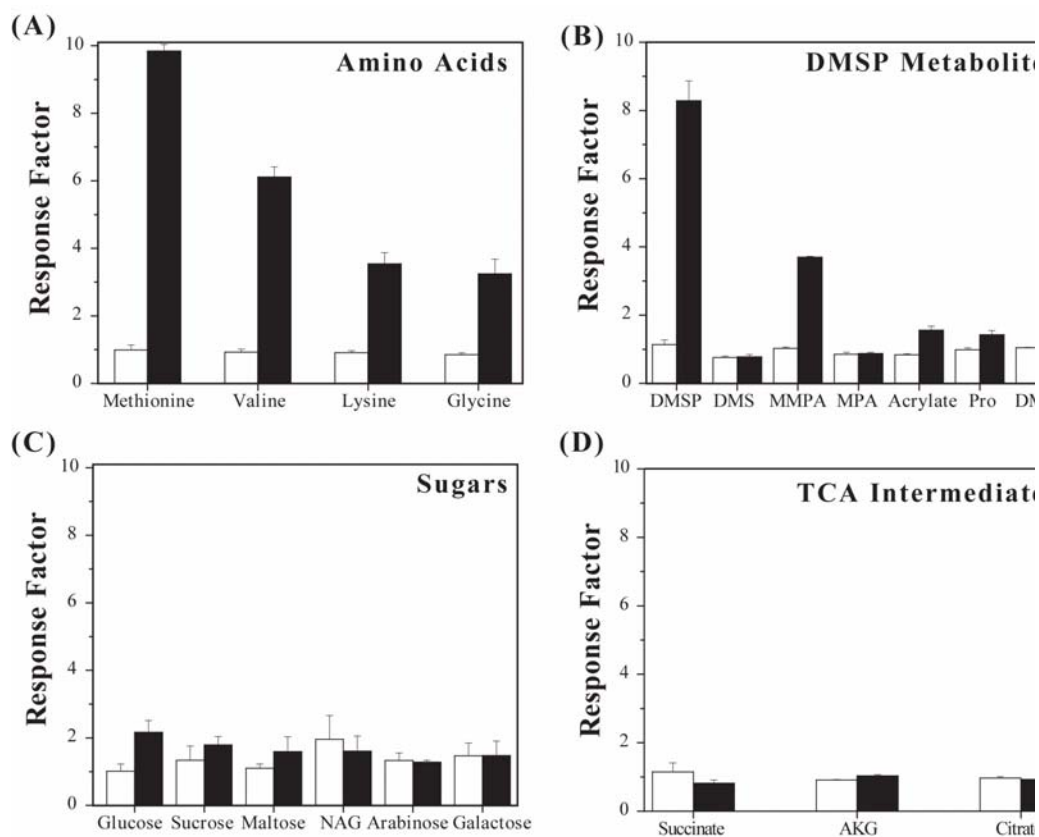


Fig. 3.5. Quantitative measurement of the chemotactic response of *Silicibacter sp. TM1040* to pure compounds. Chemotaxis of TM1040 was assessed using the capillary assay with a subset of potential attractants discovered using the qualitative assay. Capillaries were filled with either buffer (as a control), 2 μM (white bars), or 200 μM (black bars) of an attractant. The response factor was calculated by dividing the number of bacteria in attractant-filled capillaries by the number of bacteria in buffered-filled capillaries. (A) amino acids, (B) DMSP metabolites, (C) sugars, and (D) TCA intermediates. DMSP and amino acids produced the strongest chemotactic response from TM1040. NAG = N - acetylglucosamine, Pro = Propionate, AKG = α -ketoglutarate. Values shown are the mean and S.D. (n=12).

any chemical when the same chemical was incorporated into the BM motility medium (Table 3.2).

3.4.6 Quantitative chemotaxis toward pure compounds.

The results of the chemotaxis screening assay were used to select a subset of chemicals for further testing, using a quantitative capillary assay. As the data in Fig. 3.5 show, the response of strain TM1040 to a given attractant is concentration dependent, e.g., concentrations below 2 μM failed to elicit a significant response, while a 200 μM concentration of an attractant produce strong chemotaxis (Fig. 3.5A). Among the amino acids tested, methionine and valine gave the strongest response factors (means of 9.8 and 6.1, respectively), while lysine and glycine produced a similar response (mean, ca. 3.5) from TM1040. TM1040 also showed strong chemotaxis toward DMSP, which produced a response factor of 8.2 (Fig. 3.5B). The only breakdown product of DMSP that is a significant chemoattractant for TM1040 is MMPA (mean response factor of 3.8). In comparison, all the sugars tested produced only a weak to mild chemotactic response from TM1040 (means of < 3 [Fig. 3.5C]). Of these sugars, TM1040 cells were most attracted to glucose, producing a mean response factor of 2.1. Figure 3.5 also shows the response of strain TM1040 to TCA intermediates. Of the three TCA intermediates that were tested (succinate, α -ketoglutarate, and citrate), none were found to be significant chemoattractants for TM1040, suggesting that these chemicals play a minor role in the overall response of TM1040 toward dinoflagellates. Overall, the results from the capillary assay agree with the data obtained from the qualitative assay using BM motility agar.

Table 3.3. Reduction in chemotaxis of TM1040 toward untreated and heated *P. piscicida* homogenates by external addition of known attractants.

Treatments ^a	Bacteria per capillary (CFU x 10 ⁴)						
	Control ^c	Attractants Added to External Buffer ^b					
		DMSP	% ^d	Methionine	%	Valine	%
Untreated	7.24 (+/- 1.39)	3.33 (+/- 0.15)	54.01	3.03 (+/- 0.32)	58.15	1.53 (+/- 0.15)	78.87
Heated	3.51 (+/- 0.35)	3.10 (+/- 0.16)	11.68	1.80 (+/- 0.11)	48.72	0.91 (+/- 0.24)	74.07

^a Chemotaxis of TM1040 toward heated (80°C for 15 min.) or untreated *P. piscicida* homogenates was measured using the capillary assay (see Materials and Methods).

^b Prior to the assay DMSP, methionine and valine were added to external buffer containing motile cells of TM1040 at a final concentration of 200 μM and mixed to homogeneity.

^c The control was buffer only.

^d The percent reduction in CFU per capillary compared to the control.

3.4.7 Chemotaxis toward *P. piscicida* homogenates is inhibited by externally supplied attractants.

As was noted in the data presented in Table 3.2, in the presence of an externally supplied attractant distributed homogeneously throughout the medium, chemotaxis toward a point source of the same attractant is inhibited because the gradient is deflated. Using this knowledge, experiments were designed to identify the heat-labile attractants found in the dinoflagellate homogenates. As shown in Table 3.3 and in accord with the data in Fig. 3.3, the response of TM1040 to untreated dinoflagellate homogenate in the absence of any externally supplied attractant was greater than its response when the homogenate was preheated to 80°C. This relationship was altered, however, when DMSP, methionine, or valine was supplied externally in the buffer (Table 3.3). In these cases, the mean number of bacteria in the untreated capillaries was 3.33×10^4 , 3.03×10^4 , or 1.53×10^4 CFU per ml, giving a reduction in chemotaxis of 54, 58, or 78%, respectively (Table 3.3). An externally added attractant also reduced TM1040 chemotaxis to heated homogenates, with external valine producing the greatest reduction (74%), followed by methionine (48%) and DMSP (11%).

3.4.8 DMSP in *P. piscicida* homogenates is destroyed by heating.

These results suggest that while DMSP is an attractant in untreated homogenates, its attractant quality is reduced upon heating, possibly due to degradation of the molecule. Destruction of 200 µM DMSP in CB was assessed after heating the samples to either 80 or 100°C for 15 min. At both temperatures, negligible (<2%) loss of DMSP was observed. These results indicate that pure DMSP is not a heat-labile molecule.

An alternative explanation for why heat reduced the chemoattractant quality of the homogenates is that heat acts indirectly through an intermediate to inactivate DMSP. This hypothesis predicts that the concentration of DMSP decreases when a homogenate is heated. The DMSP concentration in untreated and heated homogenates was measured. In untreated *P. piscicida* homogenates the mean concentration of DMSP was $8.21 \pm 1.2 \mu\text{M}$. After the homogenate was heated, the concentration of DMSP fell below the level of detectability ($<1 \mu\text{M}$). These data indicate that heat-activated components of *P. piscicida* homogenates enhance the degradation of DMSP and may explain the reduction in chemotaxis of TM1040 toward heated homogenates.

3.5 Conclusions

The results of the present study show that *Silicibacter* sp. strain TM1040, originally isolated from *P. piscicida* dinoflagellate cultures, senses and responds chemotactically to compounds produced by the dinoflagellate cells. Thus, this represents the first report of chemotaxis behavior of a *Roseobacter* clade bacterial species. Since *Roseobacter* species are prevalent in marine environments and abundant within blooms of DMSP-producing phytoplankton, chemotaxis to DMSP by these bacteria is likely to be an important mechanism in establishing close interactions with the dinoflagellate at both the physical and physiological levels.

Several conclusions may be drawn from these data. First, *Silicibacter* sp. strain TM1040 senses and responds chemotactically to dinoflagellate cell homogenates, and the response is reduced when the homogenate undergoes heat treatment prior to testing. Second, DMSP, MMPA, and amino acids elicit a strong, positive chemotactic response

from *Silicibacter* sp. strain TM1040. Third, the concentration of DMSP in dinoflagellate cell homogenates is significantly reduced upon heating. Fourth, addition of DMSP (or amino acids) into the buffer suspending the bacteria inhibits chemotaxis to unheated dinoflagellate cell homogenates but not heat-treated homogenates. Fifth, prior starvation of the bacteria enhances their chemotactic response but also results in an ultimate decrease in swimming motility.

Cell homogenates of *P. piscicida* produce a strong chemotactic response from *Silicibacter* sp. strain TM1040 (Fig. 3.3). This response is specific to the dinoflagellate homogenate, which elicits a response factor that is >2.5 times greater than the response factor from either *Rhodomonas* algal cell homogenates or homogenates of a mixture of bacteria obtained from the dinoflagellate culture. The simplest interpretation of these results is that the dinoflagellate cell homogenates contain chemoattractant molecules that are specific to the dinoflagellate, such as DMSP. This suggestion is reinforced by the results from the chemotaxis assays, showing a strong response from TM1040 when pure DMSP is used (Fig. 3.4 and 3.5). Moreover, when DMSP is homogeneously applied in the chemotaxis assays, thus eliminating the concentration gradient of this attractant required for chemotactic sensing, its presence results in a significant reduction in the chemotaxis of strain TM1040 to a DMSP point source (in the capillary or a spot on BM motility agar). The ability to reduce the response of TM1040 cells to DMSP by deflating the DMSP gradient is strong evidence for chemotaxis of this roseobacter to the dinoflagellate product.

DMSP is not the only compound that elicits a response from *Silicibacter* sp. strain TM1040. MMPA and amino acids such as methionine and valine also produce a strong

response, which is also reduced by exogenous application of the respective attractant molecule, suggesting that TM1040 is capable of sensing more than just DMSP. This is reasonable, since most motile bacteria can sense and chemotactically respond to multiple elicitors (29) and having this capacity increases the survival of the bacteria by providing the cell with multiple inputs to control chemotaxis towards complex nutrient sources.

The response of TM1040 to heat-treated dinoflagellate cell homogenates was significantly reduced compared to the response to untreated homogenates (Fig. 3.3 and Table 3.3). One explanation for the reduced response is that heat treatment causes the loss of an important attractant molecule, such as DMSP. This explanation, however, is complicated by the knowledge that *in vitro* pure DMSP is not degraded by elevated temperatures (>80°C), yet heating a dinoflagellate cell homogenate reduces the concentration of DMSP in the homogenate to below detectable levels. Thus, some other component in the homogenates must, when heat treated, act to destroy DMSP. Since most enzymes are sensitive to heating, it is not likely that the loss of DMSP at 80°C is due to enzymatic cleavage, but at elevated temperatures DMSP does react with halides to produce volatile methyl halides (174). This process occurs naturally in algal cultures and is significantly enhanced by heating (174). Such a mechanism would have the overall effect of lowering the concentration of DMSP in heated homogenates and could help explain the reduced chemotaxis of TM1040 toward heated homogenates.

The loss of DMSP in the dinoflagellate cell homogenates upon heating also provides clues as to why exogenously added DMSP reduces TM1040 chemotaxis to untreated homogenates but not to heat-treated ones. In considering this, it is important to keep in mind that TM1040 responds to several attractants, not just DMSP, and bacteria

respond to a gradient of the respective attractant. Anything that reduces that attractant gradient also reduces chemotaxis. Therefore, the application of exogenous DMSP adversely affects TM1040 chemotaxis to the untreated cell homogenates (Table 3.3), which contain DMSP (250), because the DMSP gradient is disrupted. In contrast, the heat-treated dinoflagellate cell homogenates lack DMSP, so the exogenous application of DMSP has no effect because the cells are not responding to DMSP attraction. We speculate that the bacteria are instead responding to other attractants that are still present and active in the heat-treated samples. This is borne out by the data in Table 3.3 showing the effect of methionine and valine addition on chemotaxis of TM1040 to the heat-treated homogenate. In both cases, addition of the exogenous amino acid results in a significant reduction in chemotaxis, suggesting that the bacteria are sensing methionine and/or valine gradients, and not DMSP gradients, when they swim towards heat-treated homogenates.

The chemotactic response of *Silicibacter* sp. strain TM1040 is increased when the cells are starved prior to measuring chemotaxis (Fig. 3.2). These results are interesting and potentially significant to the survival of TM1040 cells, since they suggest that chemotactic behavior of this roseobacter in the oligotrophic marine environment may be modulated by the concentration of nutrients surrounding the cells. This is not unusual, and similar results have been reported with other bacteria. As an example, Wei and Bauer (390) observed a sixfold increase in the chemotactic response of *Rhizobium meliloti* L5-30 to 10 mM glutamine when the cells were starved for 3 h. Similarly, chemotaxis of *Pseudomonas aeruginosa* to inorganic phosphate is dependent upon starvation of the cells for phosphate (190).

The enhancement of chemotaxis when TM1040 is starved is a two-edged sword, however, because prolonged starvation ultimately results in the loss of swimming motility. While the mechanism responsible for the loss of motility is not known, starvation almost certainly causes the energy reserves in the cell to be depleted, and this is likely to adversely affect the rotation of the flagella on TM1040. This phenomenon, i.e., loss of motility under prolonged starvation, is frequently observed with motile marine bacteria. While most marine bacteria isolates are motile in culture (404), only a small fraction (<10%) of bacteria in natural assemblages are motile at any given time (260). This motile fraction increases to ca. 80% after 15 to 30 h of enrichment with suitable nutrients (259), presumably through prolonging the percentage of time the bacteria are motile (154). This phenomenon may be an adaptation to save energy when marine bacteria experience nutrient-depleted conditions, while also enhancing chemotactic sensitivity to nutrients if and when they become available.

Many physiological characteristics of the *Roseobacter* clade of bacteria, such as *Silicibacter* sp. strain TM1040, make them well suited for life in close proximity to dinoflagellates and algal cells. Since the area immediately surrounding a dinoflagellate is hypothesized to be a habitable niche for some marine bacteria (47), it is not surprising to find that a dinoflagellate-associated bacterium like strain TM1040 has mechanisms to exploit this niche. At present, these mechanisms include swimming motility and the chemotactic response of the cells to DMSP and other dinoflagellate molecules, as well as the enzymatic mechanisms to degrade and utilize DMSP (250). These are but two obvious physiological functions that enhance the survival of these bacteria when associated with a dinoflagellate cell. It comes as no surprise, therefore, that a preliminary

analysis of the genome of *Silicibacter* sp. strain TM1040 reveals several genetic loci that may serve to enhance the interaction of this bacterium with its dinoflagellate host (Chapter 5). These data suggest that TM1040 is a very good model for studying bacterium-dinoflagellate interactions.

Chapter 4: Motility and Invasion of Dinoflagellates by

Silicibacter sp. TM1040

4.1 Summary

Marine algae, especially dinoflagellates, co-occur with a diverse community of bacteria that may affect algal physiology and take on attached or intracellular lifestyles. *Pfiesteria piscicida* is an estuarine dinoflagellate that produces the organosulfur compound, dimethylsulfoniopropionate (DMSP). *Silicibacter* sp. TM1040 was isolated from *P. piscicida* cultures and catabolizes DMSP by demethylation producing 3-methylmercaptopropionate (MMPA). Interestingly, this bacterium is also chemotactic toward DMSP, MMPA, and amino acids produced by *P. piscicida*. We tested the hypothesis that *Silicibacter* sp. TM1040 also physically interacts with *P. piscicida* via attachment and/ or intracellular behavior, and that the bacterium influences growth of the dinoflagellates in laboratory cultures. Furthermore, this interaction was hypothesized to require motility and/or flagella. Transposon mutants defective in motility (Mot⁻) were constructed and their phenotypes characterized. Two of the Mot⁻ mutants do not produce flagella, while one mutant produces flagella, but is poorly motile in broth and semi-solid agar media. All mutants show reduced ability to attach to abiotic surfaces, and are wild-type for growth rate and rosette formation. When fluorescently-labelled wild-type *Silicibacter* sp. TM1040 were added to washed *P. piscicida* dinoflagellates, bacterial cells were observed to be attached and (apparently) within *P. piscicida*. In contrast, the Mot⁻ mutants less frequently co-localized with the dinoflagellate cytoplasm, and did not attach to the dinoflagellate cells. In the presence of wild-type or Mot⁻ mutants, growth of *P.*

piscicida was enhanced compared to growth in bacteria-free cultures. Overall, the results indicate that flagellar-mediated motility is important for the physical interaction between *Silicibacter* sp. TM1040 and *P. piscicida*.

4.2 Introduction

Interactions between marine algae, especially dinoflagellates and their associated bacteria are important factors controlling carbon and nutrient cycling in marine ecosystems. Nearly half of all carbon fixed by photosynthetic algae is consumed by bacteria and transferred back into the microbial food web (35). Essential nutrients for algal growth, such as nitrogen and phosphorous, are regenerated by associated bacterial communities (34). In other situations, bacteria may compete for nutrients, such as iron, or cause algal cell death (244, 245). These interactions significantly influence the growth of algal populations in the environment and may modulate harmful (i.e. toxic) effects of some algal species (107, 134, 144). The species of bacteria involved in these interactions and mechanisms they use to establish intimate relationships with their algal partner are not well studied (110).

Bacteria phylogenetically related to *Roseobacter* species are an abundant group of marine bacteria that are associated with algae in both laboratory and field studies (168, 210, 305, 405). These bacteria are the major consumers of the algal osmolyte, dimethylsulfoniopropionate (DMSP), which may serve as both a sole carbon and sulfur source for these bacteria (146). In addition, the production and activity of *Roseobacter* species are significantly correlated with DMSP-producing algal blooms, especially those of dinoflagellates and prymnesiophytes (148, 405). Furthermore, some *Roseobacter*

species exhibit close physical (attached and intracellular) or physiological (growth enhancing) relationships with toxic, DMSP-producing dinoflagellates, including *Prorocentrum* (210), *Alexandrium* (96, 134), and *Pfiesteria* species (9, 250, 252).

We have used monocultures of the DMSP-producing dinoflagellates *Pfiesteria piscicida*, *Pfiesteria shumwayae*, and *Pfiesteria*-like (*Cryptoperidiniopsis*) dinoflagellates as our model system for studying *Roseobacter*-dinoflagellate interactions (9). Within these cultures, bacteria native to the algal niche assimilate dinoflagellate-derived nutrients and are intrinsically propagated with the dinoflagellates in continuous subcultures. The bacterial community inhabiting *Pfiesteria* cultures is diverse, containing at least 19 different species depending upon the strain examined (9). A major component of this bacterial community consists of bacteria related to *Roseobacter* species and some of these bacteria can be found physically attached to and aiding in the growth of the dinoflagellates (9).

The metabolism of DMSP has been examined in *Pfiesteria* cultures. Both the *Pfiesteria* type species, *Pfiesteria piscicida* Steidinger et Burkholder and *Pfiesteria shumwayae* Steidinger et Burkholder produce DMSP (250). In addition, bacterial communities associated with *P. piscicida* degrade DMSP producing the majority of all known DMSP catabolites (250). Four *Roseobacter* species isolated from *P. piscicida* culture are capable of degrading DMSP by demethylation of the DMS moiety while two are also capable of dual DMSP lyase and demethylation pathways (250). One of these bacteria, *Silicibacter* sp. TM1040 is chemotactic toward dinoflagellate homogenates, DMSP, the DMSP demethylation product MMPA, and nearly all amino acids (249). Furthermore, this bacterium responds chemotactically to these molecules within

dinoflagellate homogenates indicating that these compounds are important chemoattractants produced by *P. piscicida*.

It is likely that motility and chemotaxis are important for *Silicibacter* interactions with *P. piscicida*. In this study, I tested the hypothesis that TM1040 interacts with *P. piscicida* through close physical associations (attached and/or intracellular) as well as by aiding in the growth of the dinoflagellates. In addition, transposon mutants defective in motility (Mot⁻) were constructed in order to determine if motility and/or flagella are important for *Silicibacter* sp. TM1040 interactions with *P. piscicida*.

4.3 Materials and Methods

4.3.1 Bacteria and media.

Silicibacter sp. TM1040 was routinely grown in HIASW broth or agar (25 g Heart Infusion broth or agar (1.5% Bacto-agar) (Becton-Dickinson, Franklin Lakes, NJ) supplemented with 10 g of artificial seasalts per liter (Instant Ocean, Mentor, Ohio)) (249) or in half-strength 2216 marine broth (Becton-Dickinson) (9). Marine motility agar was made by supplementing half-strength 2216 marine broth with 3.0 g of Bacto-agar per liter. A basal minimal (BM) broth or agar (1.5% Bacto-agar) was also used containing glycerol as the sole carbon source (249). *Escherichia coli* DH5 α λ *pir* was grown in Luria-Bertani (LB) broth or agar (1.5% Bacto-agar)(320). Where indicated, kanamycin was used at 120 μ g per ml for *Silicibacter* strains and 50 μ g per ml for *E. coli* DH5 α λ *pir*.

4.3.2 Dinoflagellates and cultivation.

P. piscicida CCMP1830 was grown as previously described (9). Dinoflagellates were fed a diet of the axenic (bacteria-free) cryptomonad prey alga *Rhodomonas* sp.

CCMP768, supplied as described by Alavi et al. (9). All culturing was accomplished in a Laminar flow hood to prevent unintentional bacterial contamination.

4.3.3 Transposon mutagenesis and screening assays

Electrocompetent *Silicibacter* sp. TM1040 was prepared following the procedures of Garg et al. with some changes (136). A 2 ml overnight culture of TM1040 in HIASW broth was transferred to 250 ml of the same medium and grown to an O.D.₆₀₀ of 0.5 at 30°C with shaking. Cells were placed on ice for 30 min. and then centrifuged at 8000 x g for 10 min. at 4°C. Cells were then washed four times in 200 ml of ice-cold distilled water using the same centrifugation conditions. The cells were finally resuspended in 0.1 ml of 10% ice-cold glycerol and used immediately for electroporation or flash frozen in a dry-ice ethanol bath and stored at -80 °C.

For transposon mutagenesis, a 1 µl (25 ng) aliquot of the EZ::TN <R6K γ ori/KAN-2> Transposome (Epicentre, Madison, WI) was added to 50 µl of electrocompetent cells in an ice-cold 0.1 cm cuvette and held on ice for 30 min. The transposon was then electroporated into TM1040 cells at 2.4 kV, 400 Ohms and 25 µF which resulted in a final 16 kV per cm and a time constant of ~ 9.2 ms. Electroporation conditions other than these produced negligible results (data not shown). Electroporated cells were held on ice for 10 min. then resuspended in 2 ml of HIASW broth and incubated overnight at 30 °C with shaking. A 1.5 ml aliquot of the overnight culture was then centrifuged at 12,000 x g. for 15 s, resuspended in 100 µl of the supernatant and spread onto HIASW agar containing kanamycin. Following 1 – 2 days of incubation at 30°C, individual colonies were transferred to 7 - by - 7 arrays using sterile toothpicks. The arrays were then replica plated onto fresh HIASW agar and marine motility agar to

screen for motility defects as well as BM plus glycerol to screen for auxotrophs. Motility mutants and auxotrophs were culled from the initial bank and re-tested two more times in 3 - by - 3 arrays to confirm the selected phenotypes.

4.3.4 Cloning and sequencing of EZ:TN flanking DNA

The EZ:TN transposon and flanking DNA were rescue cloned through the use of the *oriR6K* origin of replication on the EZ:TN transposon. A 1 ml overnight culture of the transposon mutants in HIASW broth with kanamycin was transferred to 50 ml of the same medium and grown to an O.D.₆₀₀ of 1.0 at 30 °C with shaking. Genomic DNA was extracted by phenol - chloroform extraction using CTAB following standard methods (32). Extracted DNA was then digested with *NcoI* restriction endonuclease for 1.5 h at 37 °C followed by 20 min. at 80 °C to kill the enzyme. The digested DNA was then treated with T4 DNA ligase for 4 h at 16 °C to circularize the molecules. A 20 µl aliquot of the ligated DNA was desalted with the addition of tRNA and 7.5 M ammonium acetate and washed with 100% ethanol followed by 70% ethanol. The desalted DNA was then transformed into *E. coli* DH5α λ *pir* by electroporation using standard methods (32). The transformed cells were spread onto LB containing kanamycin and incubated at 37 °C overnight. Resulting colonies were streaked onto fresh medium and grown in LB broth. These cells were used to extract plasmid DNA using a Qiagen Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Plasmid DNA was sequenced using the KAN-2 FP-1 and R6KAN-2 RP-1 outward facing primers provided with the EZ::TN <R6Kγori/KAN-2> Transposome kit. These primers anneal to either end of the EZ:TN transposon allowing for sequencing across the transposon insertion sites.

4.3.5 Analysis of transposon insertion site

The chromosomal location of the transposon insertions and identification of mutated genes was aided by the nearly complete genome sequence of *Silicibacter* sp. TM1040 provided by the Joint Genome Institute (JGI), U.S. Department of Energy (Walnut Creek, CA; available at the following URL: http://genome.ornl.gov/microbial/rose_tm1040/). The genome sequence was deposited in GenBank (Bethesda, MD) under accession number, NZ_AAFG00000000. Nucleotide sequences flanking the transposon were aligned with the TM1040 genome using BLAST to identify the chromosomal region containing the transposon (12). Open reading frames (ORF) were identified using the GeneMark program (232) and a BLAST alignment of the translated ORF was used to identify potential gene products. Further characterization of the putative gene products was made through the identification of protein family domains using the Simple Modular Architecture Research Tool (SMART 4.0) (219).

4.3.5 Phenotypic analysis of mutants

Transposon mutants defective in motility in marine motility agar were tested for additional phenotypes. Motility in broth cultures using HIASW, BM plus glycerol and half-strength marine broth was determined microscopically. Mutant and wild-type TM1040 were grown at 30 °C with shaking to the stationary phase of growth (1 -2 days) and a 25 µl aliquot was used to inoculate 10 ml of the same medium in a 125 ml flask. Each culture was shaken at 30 °C for up to 8 h and motility was examined microscopically in 10 µl wet mounts at 200 x magnification using a Nikon Optiphot BX60 upright microscope. Motility of the mutants was also examined in cultures grown statically at 30 °C using identical procedures. Motility was scored on a plus/ minus scale

to indicate the percentage of cells motile, e.g. no motile cells = -, at least one motile cell = + and several motile cells = ++.

The growth rate of mutants and wild-type TM1040 growing in HIASW broth or BM plus glycerol broth was determined. A 25 μ l aliquot from cultures in HIASW or BM plus glycerol grown for 1 or 2 days, respectively, was used to inoculate 10 ml of the same medium in a 125 ml flask. These cultures were then incubated in a 30°C shaking water bath and the O.D.₆₀₀ of each culture measured at regular intervals for 1 – 2 days. The increase in O.D.₆₀₀ of mutants over time was compared to that of the wild-type.

The ability of the mutants to form rosettes (groups of cells bound to each other at their cell poles) was determined by light microscopic examination of 10 μ l wet mounts, using cells grown in BM plus glycerol broth with shaking at 30 °C for 2 days.

To determine cell length, wild-type and mutant cells were grown overnight in 2 ml of half-strength marine broth and a 25 μ l aliquot used to inoculate 10 ml of half-strength marine broth in a 125 ml flask. After four hours of growth at 30°C with shaking, a 10 μ l aliquot of cells was viewed in a wet mount at 1000 x magnification using an Olympus BX60 upright microscope. Digital photos of random microscopic fields were captured using a Quantix CCD camera (Photometrics, Tucson, AZ) and cell lengths measured using IPLab computer software (Scanalytics, Fairfax, VA). Measurements were converted from pixels to micrometers using digital images of 5 μ m beads (Ted Pella, Redding, CA) as standards.

The presence or absence of flagella was determined using silver staining according to the protocol of West et al. (392).

The ability of mutant and wild-type cells to attach to glass was determined by first growing cells in half-strength marine broth overnight at 30°C with shaking. Glass coverslips were then placed into the wells of a 6-well plate (BD, Franklin Lakes, NJ) and covered with 3 ml of culture. After 15 min. the coverslips were removed and washed with copious amounts of distilled water. The cover slips were allowed to dry and mounted to glass slides with 10 µl of 10 ppt ASW. Cells remaining attached to the glass cover slips were visualized at 1000 x magnification and the average number of cells attached per field in 20 fields was determined.

The ability of mutant and wild-type TM1040 to form a biofilm on glass, polypropylene and polystyrene tubes was determined using the crystal violet staining method of Fletcher (122), as modified by O'Toole and Kolter (291). Individual colonies of mutant and wild-type cells were inoculated into 10 ml of half-strength marine broth and grown at 30°C with shaking to the stationary phase of growth (1 -2 days). Cells were pelleted by centrifugation at 4000 x g at room temperature and resuspended in the same medium to an O.D.₆₀₀ of 0.6. Several 0.3 ml aliquots of the culture were added to borosilicate glass (12 x 75 mm, Fisher), polypropylene (Falcon #352063), or polystyrene (Falcon #352058) test tubes and incubated at 30°C over night. Tubes were sacrificed in triplicate and washed with copious amounts of distilled water. A 0.5 ml aliquot of crystal violet solution (0.4% crystal violet, 12 % ethanol) (Fisher) was then added to the washed tubes. After 30 min. excess crystal violet was washed away with distilled water and 1 ml of a 50:50 mixture of dimethylsulfoxide: reagent ethanol was added to solubilize stained cells. After 30 min. the absorbance of the solution at 570 nm was measured using a Beckman DU460 spectrophotometer (Beckman-Coulter, Fullerton, CA).

4.3.6 Assay for attached/intracellular *Silicibacter* sp. TM1040

Attachment and invasion of mutant and wild-type *Silicibacter* sp. TM1040 to *P. piscicida* was determined after staining the bacteria with 5-(and 6-)-carboxyfluorescein diacetate, succinimidyl ester (CFDA/SE, Molecular Probes, Eugene, OR) fluorescent tracer dye according to the method of Fuller et al (131). A 5 ml aliquot of half-strength marine broth was inoculated with mutant and wild-type cells using an inoculum from the edge of colonies that had grown in marine motility agar for 2 days. After overnight incubation at 30 °C, cells were pelleted by centrifugation at 4,000 x g for 10 min. at room temperature and resuspended in 10 ppt ASW containing 1 mM succinate to an O.D.₆₀₀ of 0.6. A 10 µl aliquot of 4 mM CFDA/SE tracer dye dissolved in DMSO was added to 1 ml of cells and the tubes inverted several times to mix. After 4 hrs of staining in the dark, cells were washed 3 x with 10 ppt ASW by gentle centrifugation at 8000 x g for 10 min. After staining and prior to the final washing step an aliquot of stained wild-type cells was killed by heating at 65°C for 10 min.

Dinoflagellates were prepared by behavioral washing as previously described with minor changes (9). A 10 ml aliquot of *P. piscicida* culture at maximal density (~10⁵ cells per ml) was added to a 14 ml polypropylene test tube (Falcon #352059) and allowed to remain undisturbed for 30 min. During this time dinoflagellates actively swim to the bottom of the container. A 1 ml aliquot of dense cloudy material containing dinoflagellates was removed from the bottom of the tube and added to 10 ml of sterile 10 ppt ASW in a separate tube. This process was repeated three more times to remove the majority of the unattached or free-living bacteria from the dinoflagellates. A 1ml aliquot

of culture was taken before and after washing to measure dinoflagellate and bacterial density.

The washed dinoflagellates were added to the wells of a 6-well plate containing 1 ml of 10 ppt ASW. Aliquots of mutant, wild-type and heat-killed TM1040 cells stained with the CFDA/SE tracer dye were diluted in 10 ppt ASW to ca. 10^5 cells per ml and a 20 μ l aliquot of each dilution was added to 2 ml of the washed dinoflagellates for a final 10^4 bacteria per ml. After 2 h to allow for attachment/invasion of bacteria to the dinoflagellates, a 1 ml aliquot of each suspension was fixed with 2.5% paraformaldehyde (w/v) on ice for 15 min. The fixed cells were pelleted by centrifugation at 8,000 x g for 10 min. at 4°C and resuspended in 100 μ l of 10 ppt ASW. A 50 μ l aliquot of the cells was added to a clean glass slide and the cells allowed to adhere naturally to the glass. After 10 min, excess fluid was removed and the cells covered with 10 μ l of Prolong anti-fade solution (Molecular Probes, Eugene, OR) and a glass cover slip. The number of CFDA/SE - stained cells attached to or inside *P. piscicida* cells was determine using epifluorescence and confocal scanning laser microscopy (CSLM).

4.3.7 Epifluorescence microscopy

Dinoflagellates and attached or intracellular CFDA/SE - stained *Silicibacter* sp. were visualized at 1000 x magnification using an Olympus BX60 upright microscope equipped with filter cubes giving excitation and emission maximums of 480 and 525 nm, respectively (Chroma, Rockingham, VA). Phase contrast and fluorescent images were captured using a Quantix CCD camera and IPLab computer software. Phase contrast and epifluorescent micrographs from individual dinoflagellates were merged using ImageJ software (Rasbad, NIH, MD) and the co-localization plugin (Bourdoncle, Institut Jacques

Monod, Paris). A preliminary assessment of intracellular bacteria could be made by focusing through the z-axis of the dinoflagellate to observe bacteria within the cell.

4.3.8 Confocal scanning laser microscopy

To confirm the existence of intracellular bacteria, dinoflagellates were visualized using a confocal scanning laser microscope and the accompanying software (Biorad, Hercules, CA). The CFDA/SE stain was excited using the 488 nm line of the Argon laser and detected using the 500 - 535 nm band pass filter. Individual dinoflagellates with attached and intracellular bacteria were optically sectioned in 0.5 μm sections across the Z-axis.

4.3.9 Add-back experiments

Wild-type and mutant strains were added back to cultures of *P. piscicida* that had been made bacteria-free, or axenic, in order to determine the effect of each strain upon dinoflagellate growth. Dinoflagellates were made axenic using bleach - resistant cysts as previously described with some changes (9). *P. piscicida* dinoflagellates were grown to a cell density of 10^4 - 10^5 cells per ml whereupon several 1 ml aliquots were removed for determination of cell density. A 60 μl aliquot of 0.5 M 5-methoxytryptamine (5-MOT) diluted in DMSO was added to 30 ml of the culture with gentle mixing. The culture was then incubated at room temperature for 4 h to allow encystment of the dinoflagellates, which was monitored periodically by inverted microscopy, then a 1.2 ml aliquot of 5.25% sodium hypochlorite (Chlorox bleach) was added to a final concentration of 0.1%, to kill vegetative bacteria. After 20 min. at room temperature, the bacteria-free cysts were centrifuged at 800 x g for 5 min. and the pellet resuspended in 30 ml of 10 ppt ASW. A 1-ml aliquot of the bleached cysts was then added to 20 ml of 10 ppt ASW in several 70

ml tissue culture flasks (Corning, Corning, NY) in a laminar flow hood. After overnight incubation, a 10 ml sample was removed and the number of excysted zoospores determined by counting chemically fixed bilobed cells (i.e. zoospores) in a hemacytometer. The percentage of dinoflagellates surviving was calculated by dividing the number of cells present before bleach treatment by the number of cells present after bleach treatment multiplied by 100. The dinoflagellates were then fed with a 100 μ l aliquot of axenic *Rhodomonas* sp. prey algae (final density of $\sim 10^5$ prey algae per ml).

Bacterial were prepared by growing each strain in HIASW broth overnight at 30°C with shaking and washing the cells twice in 10 ppt ASW. Each culture was then normalized to O.D.₆₀₀ 0.2 and placed on ice. A portion of the wild-type TM1040 culture was also heat - killed at 65°C for 10 min. and placed on ice. A 50 μ l aliquot of each bacterial culture or 10 ppt ASW (negative control) was then added to the axenic dinoflagellate cultures with gentle mixing to give ca. 10^4 bacteria per ml. Cultures were incubated at 20°C on a 14:10 light:dark cycle for 9 days. A sample of each culture was taken on days 1, 3, 5, 7 and 9 for determination of dinoflagellate and prey algal cell densities and samples taken on days 1 and 9 for the analysis of bacterial species using denaturing gradient gel electrophoresis (DGGE).

4.3.10 Cell counts

Dinoflagellates and *Rhodomonas* sp. were fixed in 10% (v/v) Bouin's fixative (Sigma, St. Louis, MO) and held on ice pending determination of cell density. A 1 – 10 ml aliquot of fixed cells was then pelleted at 10,000 x g for 1 min. and resuspended in 10 - 100 μ l of 10 ppt ASW depending upon the cell density. A 10 μ l aliquot was then deposited onto a hemacytometer (Corning) and cells counted in areas large enough to

give between 30 – 300 cells. In some cases this was not possible since cell densities were extremely low, in which case the cell density was assumed to be the starting concentration, which was determined before dilution into the treatment cultures.

4.3.11 Denaturing gradient gel electrophoresis

During the add-back experiments, dinoflagellate cultures were analyzed using DGGE in order to determine the bacterial species composition of each culture and to ensure that all cultures either contained no bacteria (control) or only *Silicibacter* strains (TM1040, TM2014, TM2017, TM2038 and heat killed cells). The amplification of 16S rDNA was accomplished according to the method of Ferris et al, with some changes (120) and DGGE was accomplished according to Wang et al (386). A 1 ml sample of each culture was pelleted at 14,000 x g at 4°C for 10 min. and resuspended in 100 µl of sterile distilled water. The suspensions were then boiled for 15 min. and placed on ice. A portion of the 16S rDNA gene from most bacteria was amplified using primers 1055f (ATGGCTGTCGTCAGCT) and 1392rGC (CGCCCGCCGCGCCCCGCGCCCCGGCCC-GCCGCCCCCGCCCCACGGGCGGTGTGTAC) containing the GC-clamp (underlined portion) (120). Each reaction contained 3 µl of boiled template, 10 pmoles of each primer, 2.5 mM dNTP's and 1 unit of Taq DNA polymerase (New England Biolabs, Beverly, MA). After an initial denaturation step at 95 °C for 5 min. amplification continued for 30 cycles with denaturation at 95 °C for 5 min., annealing starting at 65°C for 30s, and extension at 72 °C for 1 min. Every other cycle the annealing temperature was reduced by 1 °C until 55°C at which point the cycle was repeated 10 more times followed by a final extension step at 72°C for 10 min. The PCR products were electrophoresed in a 1.0% agarose gel to ensure a single 323 bp band.

For DGGE, DNA concentrations of PCR products were normalized according to band intensities in the agarose gel. The diluted PCR products were then mixed with 4 μ l of loading dye (40% sucrose, 0.25% Bromophenol blue, and 0.25% Xylene cyanol) and electrophoresed at 100 v for 16 h at 65°C in an 8% polyacrylamide gel (acrylamide:bisacrylamide 37.5:1) containing a 30 -70% linear vertical denaturing gradient formed with urea and formamide. A 100% denaturing gradient is defined as 40% (v/v) formamide plus 7 M urea. Bands were visualized in the gel after staining with ethidium bromide.

4.4 Results

4.4.1 Random mutagenesis of *Silicibacter* sp. TM1040

Transposon mutagenesis of *Silicibacter* sp. TM1040 was performed using the EZ::TN <R6K γ ori/KAN-2> transposome with a transformation efficiency of ca. 1.5×10^{-4} total Kan resistant cells obtained/ total cells electroporated. A bank of 3,724 mutants was screened for the loss of motility in marine motility agar and auxotrophy on BM plus glycerol. Non-motile (Mot⁻) mutants occurred at a frequency of 0.75% while auxotrophs occurred at a frequency of 0.16%.

4.4.2 General characteristics of *Silicibacter* sp. TM1040 mutants

Three Mot⁻ mutants, designated TM2014, TM2017 and TM2038, were selected for further characterization. Strain TM2014 is completely non-motile in marine motility agar (Fig. 4.1) and in all liquid media tested, and does not produce visible flagella upon silver staining (Fig. 4.2). However, it is capable of forming rosettes similar to wild-type (an

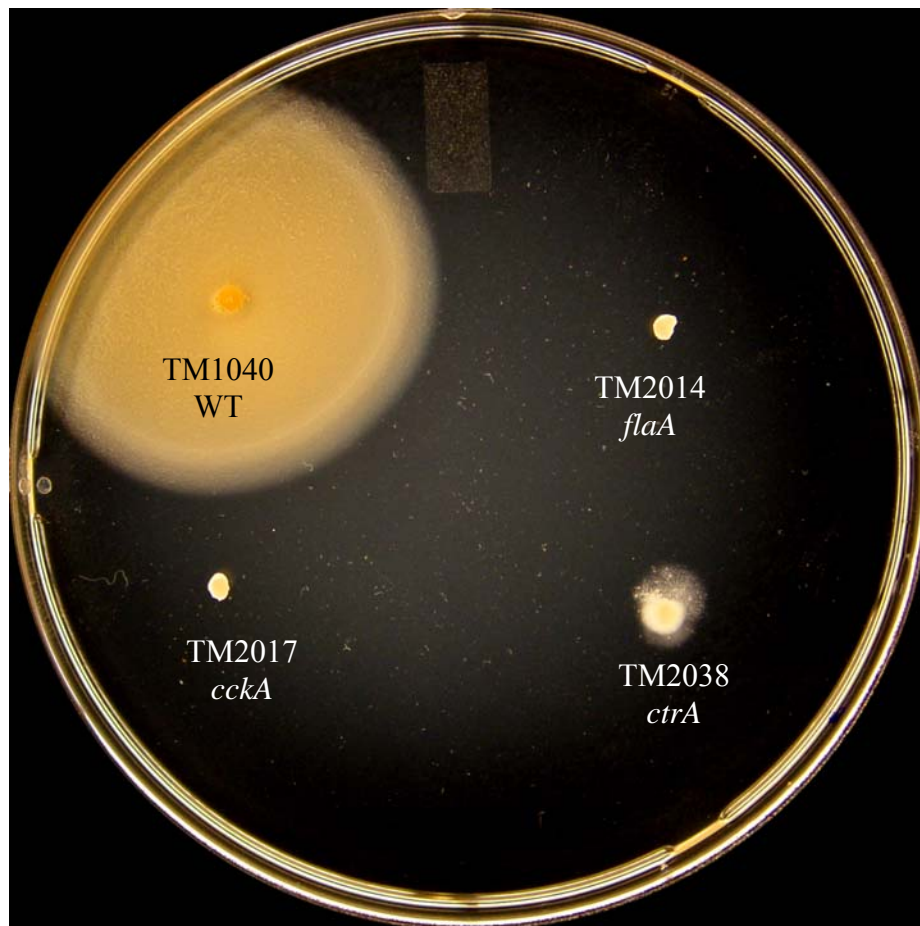


Fig. 4.1. Screening for motility mutants on marine motility agar. Following transposon mutagenesis, kanamycin resistant transformants of *Silicibacter* sp. TM1040 were inoculated onto $\frac{1}{2}$ x 2216 marine motility agar and allowed to grow at 30 °C for 2 days. Shown are wild type TM1040 plus three motility mutants TM2014, TM2017, and TM2038 with transposon insertions in a novel *fla* gene, the *cckA* and *ctrA* genes, respectively. Wild-type TM1040 moves outward from the site of inoculation about 5 cm within 2 days while mutant strains TM2014 and TM2017 do not move at all and TM2038 moves out only slightly.

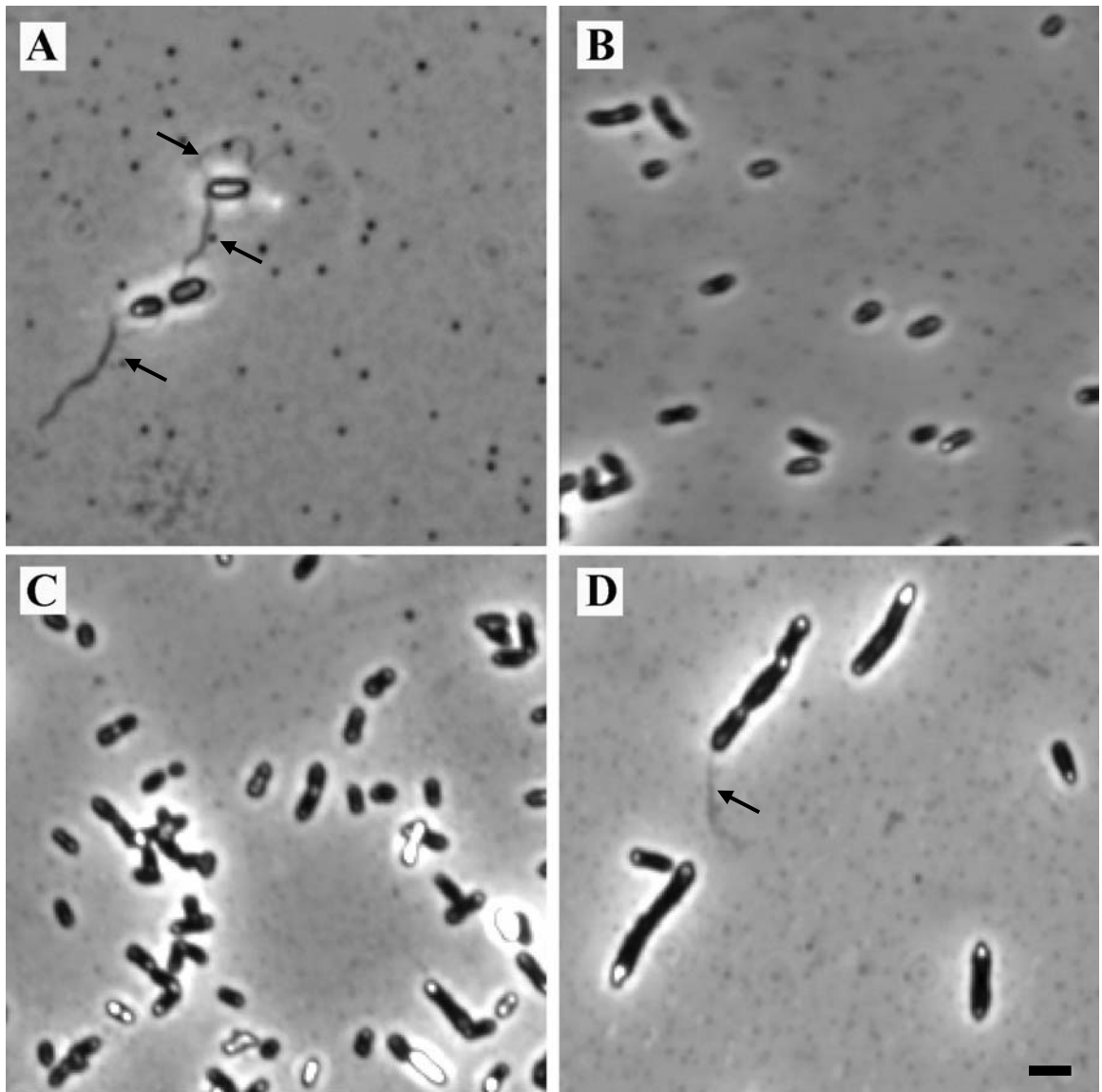


Fig. 4.2 Detection of flagella using a silver stain. Motile bacteria were stained with silver nitrate according to the method of West et al. (392) in order to visualize flagella on wild type and mutant cells. A) TM1040 (wild type) B) TM2014 (*flaA*) C) TM2017 (*cckA*) and D) TM2038 (*ctrA*). Both wild type TM1040 and TM2038 produce flagella, although much fewer cells of TM2038 produce flagella while TM2014 and TM2017 do not produce any visible flagella. (Bar = 1 μ m)

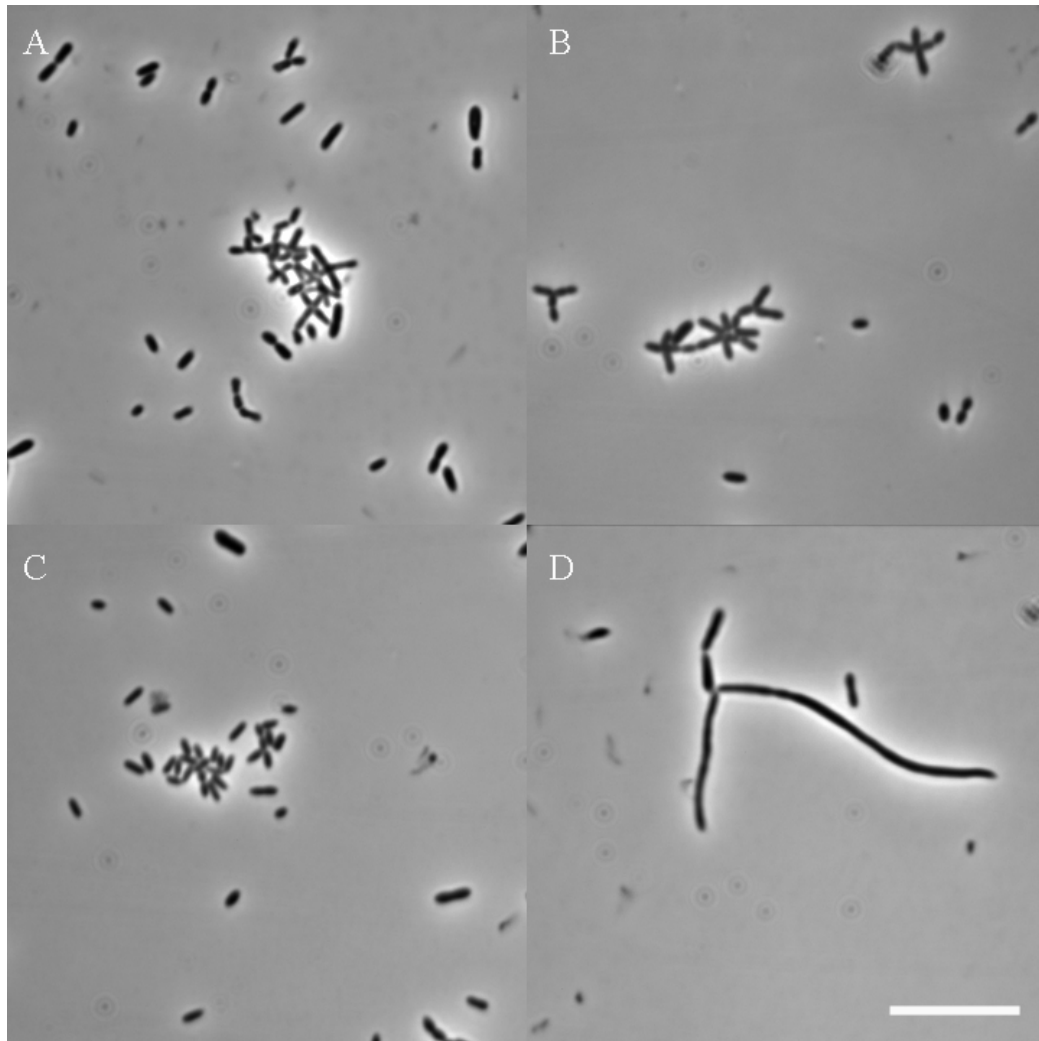


Fig. 4.3. Phase contrast micrographs of wild type *Silicibacter* sp. TM1040 and motility mutants. Bacteria were grown in marine broth and 5 μ l aliquots used to make wet mounts. Shown are A) Wild-type TM1040, B) TM2014, C) TM2017, D) TM2038. TM2038 has an elongated phenotype while TM2014 and TM2017 are similar in length to wild type. Magnification = 1000 x, bar = 5 μ m.

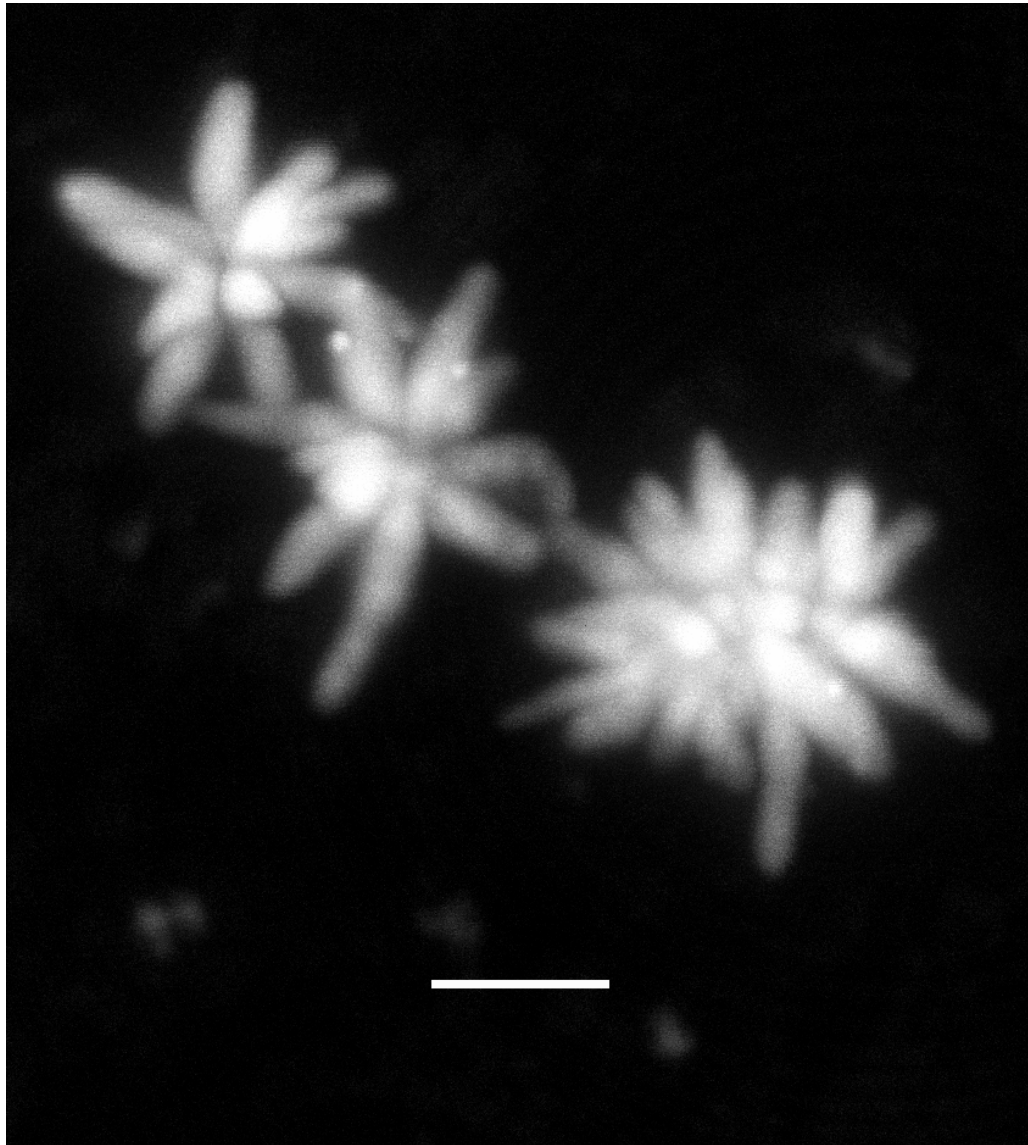


Fig. 4.4. Wild type *Silicibacter* sp. TM1040 rosette formation. Wild type *Silicibacter* sp. TM1040 was grown in BM plus glycerol broth and stained with SYBR Green nuclear stain (Molecular Probes, Eugene, OR). Cells were visualized using confocal scanning laser microscopy. All motility mutants formed rosettes similar to wild type. (Bar = 1 μ m)

Table 4.1 General phenotypes of wild-type *Silicibacter* sp. TM1040 and motility mutants

Phenotypic Test	TM1040	TM2014	TM2017	TM2038
Motile in broth ^a	++	-	-	+
Motile in agar ^b	++	-	-	+
Flagella ^c	Yes	No	No	Yes
Growth rate ^d	Wild type	Wild type	Wild type	Wild type
Cell length ^e	1.79 ± 0.01 µm	1.57 ± 0.01 µm	1.50 ± 0.01µm	6.40 ± 0.03µm
Rosette formation	Yes	Yes	Yes	Yes

^aThe percentage of motile cells per miscorscopic field was scored on a plus/minus scale where no motile cells = “-,” at least one motile cell = “+” and several motile cells = “++.”

^bMotility in agar was assessed using marine motility agar. The distance cells traveled outwards in motility agar was scored on a plus/minus scale where a “-“ indicates no outward movement, “+” indicates a small amount of movement and “++” means the cells traveled outwards similar to wild type.

^cFlagella were stained with a silver stain according to West et al (392). A strain was considered to be capable of flagellar biosynthesis if at least one cell was observed to have a flagellum.

^dThe growth rate of motility mutants was determined by comparing changes in the O.D.₆₀₀ of each strain grown in HIASW or BM plus glycerol to that of wild type TM1040 grown in the same media. Results were scored as “wild type” or “non-wild type”.

^eThe cell length of wild type and motility mutants was determined using cells grown to exponential phase in marine broth.

example of wild-type cells is shown in Fig. 4.4) and its growth rate and cell size are indistinguishable from wild-type (Table 4.1). Strain TM2017 is also completely non-motile in marine motility agar (Fig. 4.1) and in all liquid media tested and does not produce visible flagella upon silver staining (Fig. 4.2). In addition it is capable of forming rosettes and its growth rate and cell size are similar to wild-type (Table 4.1). Conversely, strain TM2038 is slightly motile in marine motility agar (Fig. 4.1), with the majority of cells appearing non-motile in all broths tested. Upon silver staining a few cells produced visible flagella (Fig. 4.2). The majority of cells of this mutant are elongated (Fig. 4.2, and Table 4.1) having an average cell length of 6.91 μm , nearly four times the size of wild-type (1.74 μm) (Table 4.1).

4.4.3 Transposon insertion location

Transposon insertion sites were determined by rescue cloning the EZ:TN transposon and flanking DNA followed by DNA sequencing with outward facing primers specific to either end of the transposon. The identification of the genes disrupted by insertion of the transposon was aided by the recent completion of the *Silicibacter* sp. TM1040 genome sequence including an automated annotation of predicted open reading frames (ORFs) (personal observation). Since the preliminary ORFs have been assigned, all alignments with GenBank were accomplished with the translated protein sequence.

Strain TM2014 harbors the transposon in a gene with little to no homology to other genes in GenBank (Table 4.2). However, this novel gene, *flaA*, is downstream of the predicted stop codon for a gene that is homologous to *Caulobacter crescentus motA* ($E = 2.0 \times 10^{-87}$), which overlaps *flaA* by three base pairs (Fig. 4.5). In addition, there is a 335 bp gap between the *flaA* stop codon and the start site for a

Table 4.2. Annotation of genes harboring the transposon

Strain	Gene ^a	ORF ^b	BLAST	Probability ^c	Identity (%) ^d	Conserved Protein Family Domains (C' - N' terminus)
TM2014	<i>flaA</i>	1857	No significant matches	>10	NA	Signal peptide
TM2017	<i>cckA</i>	3662	Signal transduction histidine kinase [Rhodobacter sphaeroides]	0.00E+00	71	Signal peptide, Transmembrane, PAS, PAS, Histidine kinase, Histidine kinase, CheY-like receiver domain
TM2038	<i>ctrA</i>	3516	Response regulator homolog [Rhodobacter capsulatus]	1.00E-162	89	CheY-like receiver domain, DNA binding/transcriptional regulatory domain

^aDNA flanking the transposon insert was aligned with the TM1040 genome using nucleotide BLAST (12) to identify mutated chromosomal regions. The putated ORF within this region was then aligned with GenBank using BLAST to identify the gene products. The gene name and putative function was finally assigned after annotation of conserved protein family domains using SMART 4.0 (219) and comparison with similar proteins from other organisms.

^bPutative coding regions were determined using GeneMark (232) and are given ORF numbers which serve as identifiers during the on-going annotation process.

^cThe probability value describes the number of times the amino acid sequence will produce significant alignments to any protein in the GenBank database just by chance. Thus a lower value indicates a better match.

^dThe percent identity indicates the number of amino acids in the correct position shared by the TM1040 amino acid sequence and the protein from GenBank. NA = not applicable because the amino acid sequence did not produce a significant match with any protein in GenBank.

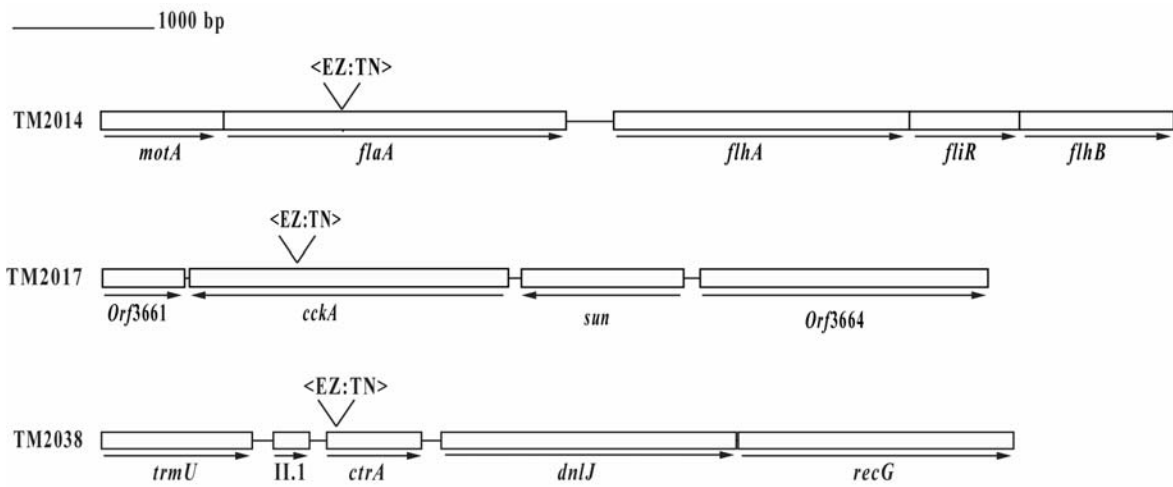


Fig. 4.5. Genomic regions containing transposon insertions. The region of the chromosome containing transposon insertions was annotated after the identification of ORFs using GeneMark (232), alignment of the putative ORF with GenBank using BLAST (12) and annotation of conserved protein family domains using SMART 4.0 (219). Shown are the transposon insertion sites in motility mutants TM2014, TM2017 and TM2038. Notice that insertions occur in genes separated from downstream genes by several hundred base pairs or in genes where the next downstream gene is transcribed in an opposite direction.

gene with perfect homology to *flhA* from *Rhodobacter sphaeroides* ($E = 0.0$). Both *flaA* and *motA* are transcribed in the same direction, toward *flhA*, which suggests that *flaA* is co-transcribed with *motA*, and that *flaA* is involved in flagellar biosynthesis or motor function.

Strain TM2017 harbors the transposon in a gene with significant homology to *R. capsulatus cckA* ($E = 1 \text{ e-}162$) (Table 4.2). Approximately, 30 bp upstream of *cckA* is a gene with similarity to an unknown gene from *R. sphaeroides* ($E = 6.0 \text{ e-}32$) and 97 bp downstream of *cckA* is a gene with similarity to a cytosine methylase or SUN protein from *Bradyrhizobium japonicum* ($E = 6.0 \text{ e-}57$) (Fig. 4.5).

In strain TM2038, the transposon is in a gene with a high degree of similarity to *ctrA* of *R. capsulatus* ($E = 1.0 \text{ e-}116$) (Table 4.2). Approximately 400 bp upstream is a gene with similarity to the II.I protein of *R. capsulatus* ($E = 2.0 \text{ e-}28$) and 154 bp downstream is a gene with homology to an *R. sphaeroides* NAD-dependent DNA ligase ($E = 0.0$) (Fig. 4.5). The *ctrA* and *cckA* genes regulate motility and genetic exchange in *R. capsulatus* (211) perhaps offering clues as to the homologous gene functions in *Silicibacter* sp. TM1040.

4.4.4 Attachment and biofilm development of mutants

Since flagella are important for attachment to surfaces in other bacteria, I sought to determine if the TM1040 Mot⁻ strains were defective in general attachment to abiotic surfaces. All mutants examined had a reduced attachment to glass cover slips compared to the wild type. Attachment to glass by the TM2017 (*cckA*) and TM2038 (*ctrA*) mutants

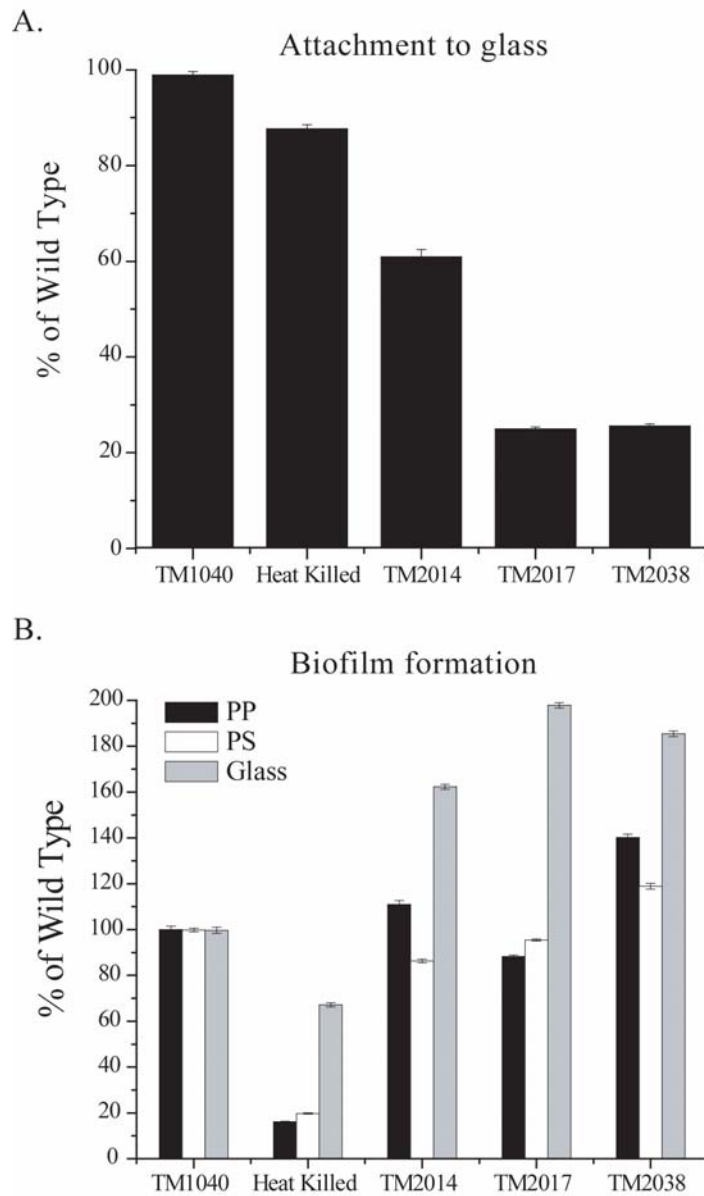


Fig. 4.6. Attachment and biofilm formation on abiotic surfaces. A) The average number of bacteria attaching to glass coverslips compared to wild type was measured. B) The ability of mutants and wild type bacteria to form biofilms on polypropylene (PP), polystyrene (PS) and glass was measured.

was reduced by 75% and 74%, respectively (Fig. 4.6A), while attachment of TM2014 (*flaA*) mutant was reduced by >50%. In contrast, heat killed cells were reduced in attachment to glass by only 11% suggesting that attachment to glass does not require a metabolically active cell.

Biofilm formation by the Mot⁻ mutants was measured on borosilicate glass, and polypropylene (PP) and polystyrene (PS) plastic surfaces. All mutants formed biofilms on glass surfaces equal to or better than wild-type TM1040, while biofilm formation on PS and PP was influenced by the nature of the mutation (Fig. 4.6B). On PP, the FlaA⁻ and CtrA⁻ mutants formed slightly more extensive biofilms than did the wild type (110 and 135% of wild-type biofilm formation, respectively), whereas the CckA⁻ mutant formed biofilms just as well as the wild type. On PS, biofilm formation by the FlaA⁻ and CckA⁻ mutants was approximately equal to the wild type (87 and 95% of wild-type, respectively) while biofilm formation by the CtrA⁻ mutant was slightly better than the wild type (118%). Biofilm formation by heat killed, wild-type cells on PP and PS surfaces was reduced compared to the wild-type (16 and 20% of wild-type biofilm formation, respectively) while biofilm formation on glass was 67% of wild-type levels. It is clear from these data that the mutations affect biofilm formation and was dependent upon the substratum.

4.4.5 Attached and intracellular *Silicibacter* sp. TM1040

My previous results suggest that bacteria may be found attached or within *P. piscicida* (9), which prompted an investigation to determine if *Silicibacter* sp. TM1040 physically interacts with *P. piscicida* through attachment to or invasion of the dinoflagellate cell surface. Since flagella and motility are required by many bacteria for

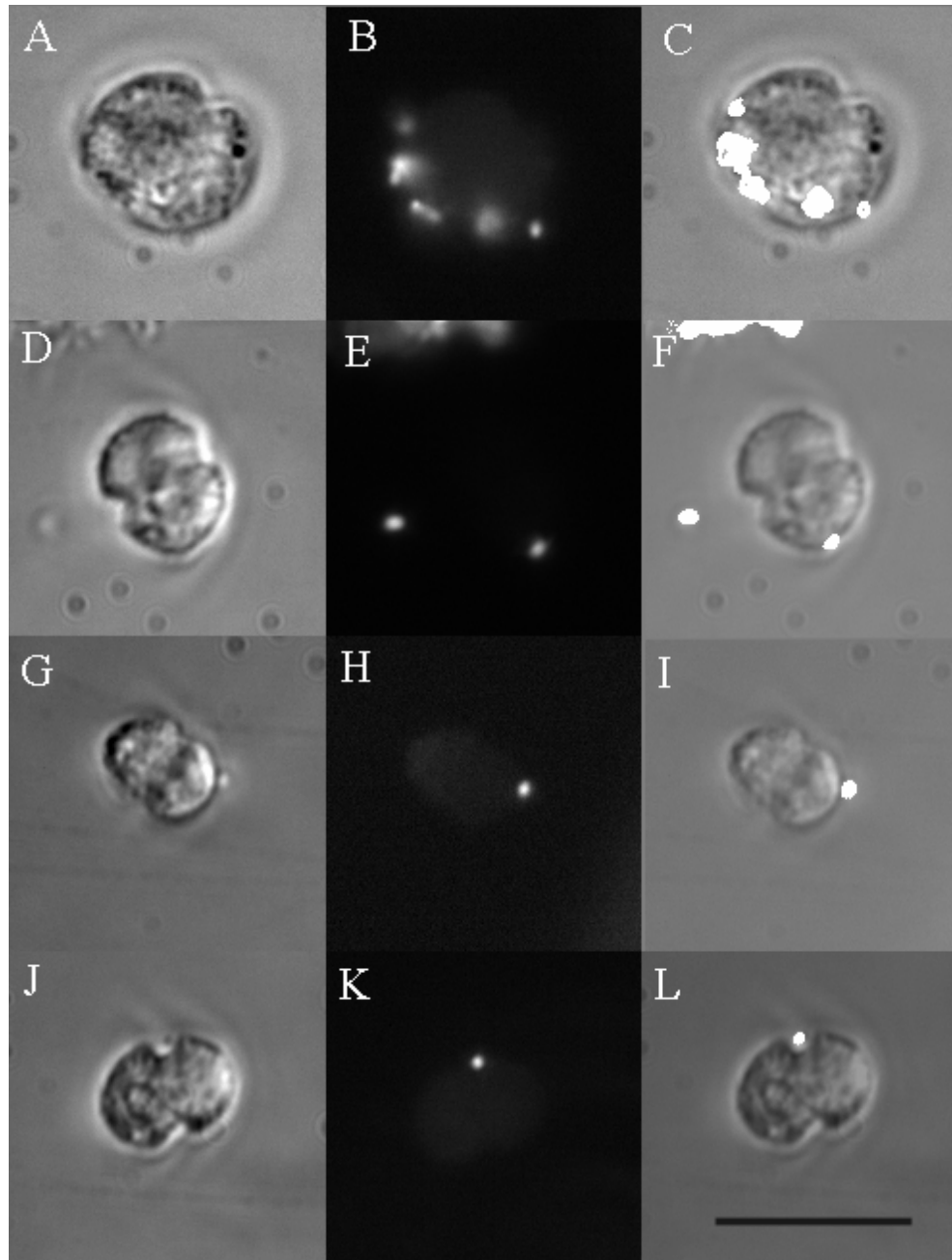


Fig. 4.7. Colocalization of Attached and/or intracellular *Silicibacter* sp. TM1040 (wild type) and motility mutants to *P. piscicida*. Bacteria were pre-stain with a fluorescent tracer dye and added to washed *P. piscicida* dinoflagellates. After two hours samples were removed, chemically fixed and viewed at 1000 x magnification using phase contrast (First column) and epifluorescence microscopy (second column). Bacteria observed under epifluorescence were co-localized with the dinoflagellates on phase contrast images using ImageJ software (Rasbad, NIH, MD) and the Co-localization plugin (Bourdoncle, Institut Jacques Monod, Paris)(third column). (A-C) TM1040, (D-F) TM2014, (G-I) TM2017, (J-L) TM2038. Scale bar = 10 μ m.

attachment and invasion of cell surfaces, the Mot⁻ mutants were also tested for their ability to physically interact with the dinoflagellates. Mutant and wild-type TM1040 were pre-labelled with the CFDA/SE tracer dye (a cell-permeant non-fluorescent dye that freely enters living cells where it is cleaved by non-specific esterases becoming a cell-impermeant fluorescent molecule). After incubation with the dinoflagellates, samples were visualized using epifluorescence and phase contrast microscopy. The extent to which bacteria physically interact with the dinoflagellate was determined by superimposing the fluorescent images with the phase contrast image. As shown in Fig. 4.7A-C, several wild-type, fluorescently - labeled bacteria are visible (panel B) and colocalize with the dinoflagellate (panel C). Analysis of these images revealed that the bacteria were in close proximity to the sides, as well as within or on top of the dinoflagellates, perhaps indicative of being intracellular within the dinoflagellate cytoplasm or in deep grooves within the outer surface of the dinoflagellate cell. Mot⁻ mutants were never observed to colocalize within or on top of the dinoflagellates (Fig. 4.7 panels D-L), and only appeared to be attached to its side.

To provide further evidence to support an intimate contact between TM1040 cells and *Pfiesteria*, dinoflagellates were optically sectioned using confocal scanning laser microscopy (CSLM). CSLM imagery further confirmed that wild-type TM1040 could be found to co-localize with cytoplasmic elements within the dinoflagellates. These bacteria were also observed to co-localize with the surface of the dinoflagellate. For example, in Fig. 4.8A, at least five wild-type bacteria are visible in the the center and near the edges of the dinoflagellate within optical sections scanned >2 μm below the dinoflagellate surface, suggesting that the bacteria are intracellular. These bacteria are not in the food

vacuole, which is evident by an absence of dinoflagellate autofluorescence in the area marked by a “v” in Fig. 4.8A. Conversely, the FlaA⁻ (TM2014) and CtrA⁻ (TM2038) cells were never found to co-localize to positions designated as intracellular to the dinoflagellates, however these bacteria could be seen near the edges of the dinoflagellate (like TM1040), as well as in optical sections < 2 μm below the surface of the dinoflagellate (Fig. 4.8B and D). The CckA⁻ (TM2017) mutant was also predominantly found at the edges of the dinoflagellate and in optical sections less than 2 μm below the dinoflagellate cell surface, however, one bacterium was observed in the center of the dinoflagellate at a depth >2 μm below the dinoflagellate cell surface (Fig. 4.8C), suggesting that the CckA⁻ defect may not result in a complete loss of the ability of TM1040 to co-localize to positions apparently within the cytoplasm of the dinoflagellate.

In order to view the series of Z-sections through the dinoflagellates in one image, the optical sections were color coded according to depth and the series of images merged into one. Green pixels were used to indicate objects that are close to the proximal side of the dinoflagellate cell surface, blue denotes objects on the distal side of the dinoflagellate, and red indicates objects in the center of the eukaryote. Optical sections processed in this way are shown in Fig. 4.9. Autofluorescence from the dinoflagellate is visible as a mixture of all three colors and serves to counterstain the dinoflagellate. Wild-type *Silicibacter* sp. TM1040 can be seen attached to the dinoflagellate cell surface (green) and also co-localized with the cytoplasm of the dinoflagellate (red) (Fig. 4.9A). An amorphous red or pink area in this dinoflagellate is also visible and is most likely due to autofluorescence from prey algal chlorophyll within the food vacuole. In contrast to the wild type, the Mot⁻ mutants were only co-localized with the dinoflagellate

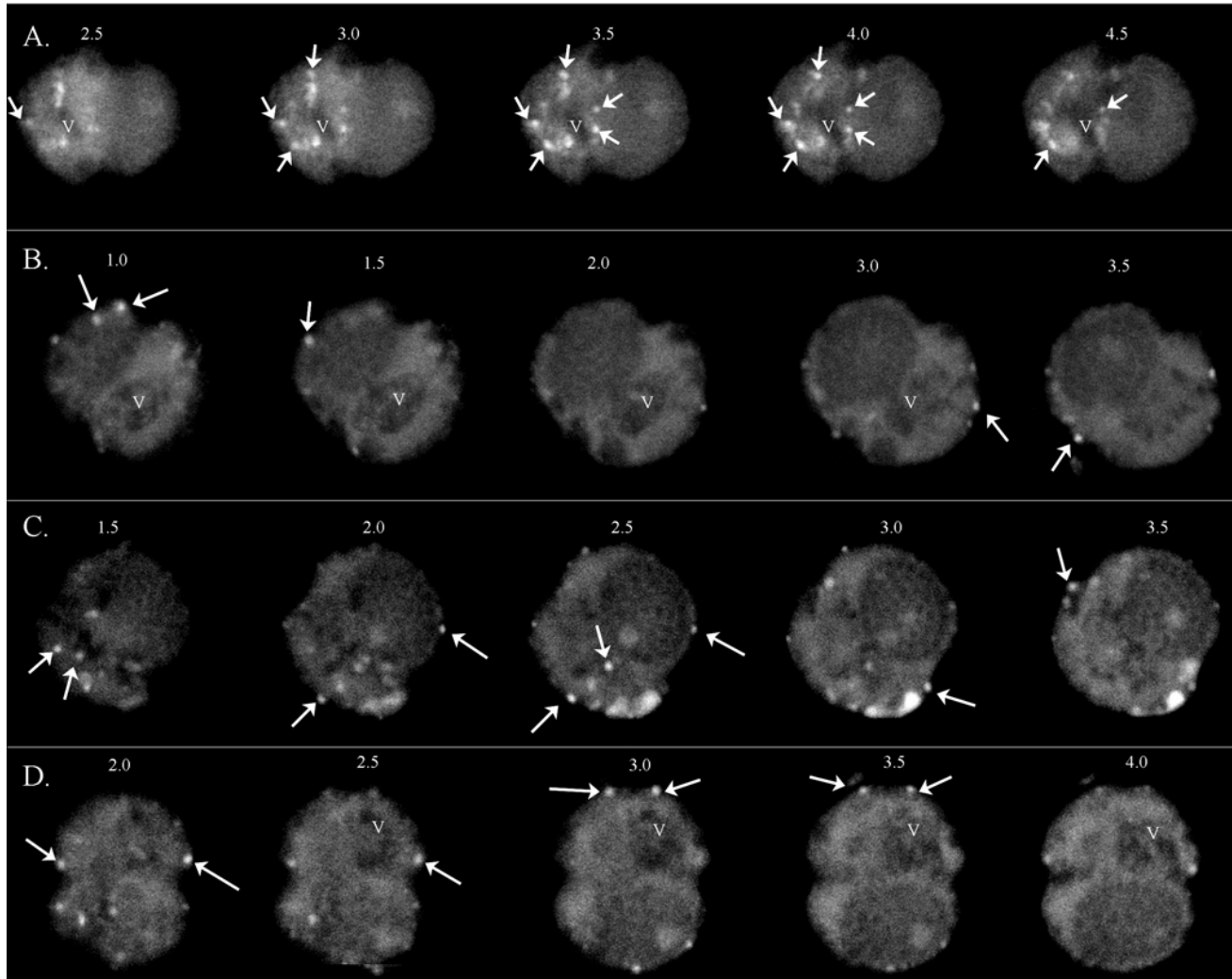


Fig. 4.8. Confocal scanning laser microscopy showing intracellular bacteria. *Silicibacter* sp. TM1040 and motility mutants were prelabelled with a fluorescent tracer dye, washed 3 x in 10 ppt seawater and incubated with washed *P. piscicida* dinoflagellates. Samples were chemically fixed after two hours and optical sections of individual dinoflagellates captured using confocal scanning laser microscopy. Numbers indicate the depth from the surface of the dinoflagellate towards the cytoplasm. Arrows show the presence of bacteria attached and inside the dinoflagellate cell, and around the vacuole (V). (A) TM1040, (B) TM2014, (C) TM2017, and (D) TM2038.

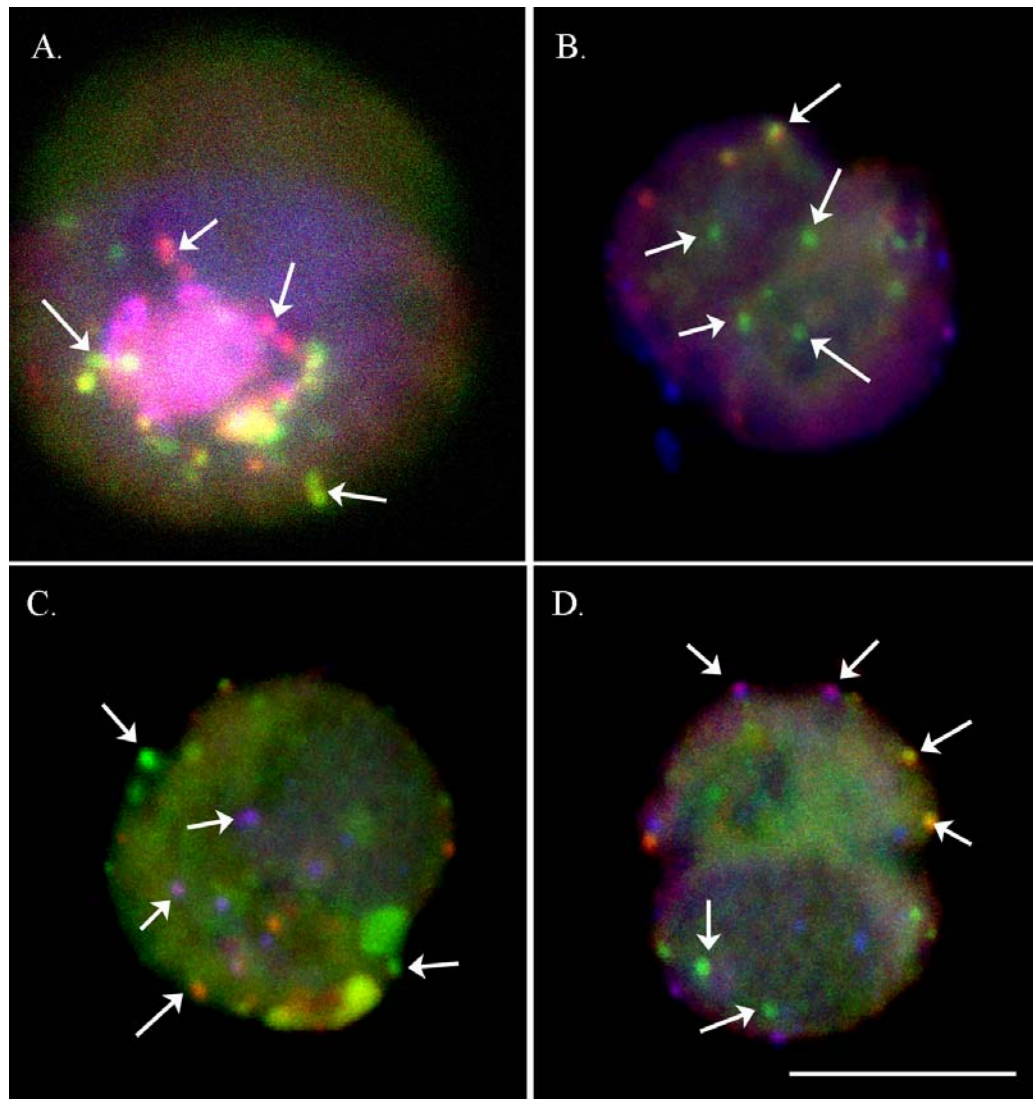


Fig. 4.9. Depth color coded images showing intracellular *Silicibacter* sp. TM1040. *Silicibacter* sp. TM1040 and Mot⁻ mutants were pre-labeled with a tracer dye and incubated with *P. piscicida* dinoflagellates. Samples were chemically fixed and tracer dye fluorescence visualized using CSLM. Optical slices through individual dinoflagellates were captured in 0.5 μ M increments creating a series of images through the z-axis of the cell. The resulting stack of images was then color coded according to the distance from the top to the bottom of the dinoflagellate (front to back) and merged into a single projection image using ImageJ software. Depth is color coded such that green = top, red = middle and blue = bottom of the dinoflagellate. Arrows show intracellular and attached *Silicibacter* sp. TM1040 pre-labeled exhibiting tracer dye fluorescence. Line = 10 μ m. A) wild-type TM1040, B) *FlaA*⁻ TM2014, C) *CckA*⁻ TM2017, and D) *CtrA*⁻ TM2038.

cell surface (Fig. 4.9B – C; green and blue pixels). None of the three Mot⁻ mutants co-localized within *P. piscicida* (indicated by red pixels), although a few bacteria could be observed at the surface of the dinoflagellate.

In order to determine quantitative differences in attached or intracellular wild-type and mutant strains, as well as heat - killed wild-type cells, a total of 52 out of ~10⁴ dinoflagellates (0.5 % of the population) were observed from each treatment using epifluorescence microscopy. By focusing up and down through the specimen to observe colocalization, it was possible to determine if the bacteria were present within the dinoflagellates or attached to the cell surface. Approximately 55 and 21% of the dinoflagellates incubated with wild-type bacteria displayed attached or intracellular bacteria, (Fig. 4.10B) and, on average, there were 1.5 and 0.5 wild-type bacteria attached or inside *P. piscicida* cells, respectively (Fig. 4.10A). The percentage of dinoflagellates showing attached TM2014, TM2017 and heat killed cells was similar to that of dinoflagellates incubated with the wild type (Fig. 4.10B) and the number of attached TM2014, TM2017 and heat killed cells per dinoflagellate cell was not significantly different from the wild type (Fig. 4.10A). In contrast, while the percentage of dinoflagellates showing attached TM2038 bacteria was not different from wild-type (Fig. 4.10A), there was a significant reduction in the number of TM2038 cells attached per dinoflagellate cell (Fig. 4.10B). For all mutants and heat - killed cells, the number of bacteria found inside the dinoflagellates and the percentage of dinoflagellates with intracellular bacteria was reduced compared to wild-type. For example, the mean number of intracellular TM2014 (*flaA*), TM2017 (*cckA*) and TM2038 (*ctrA*) mutant cells per dinoflagellate was reduced by 82, 59, and 59%, respectively (Fig. 4.10A) and the

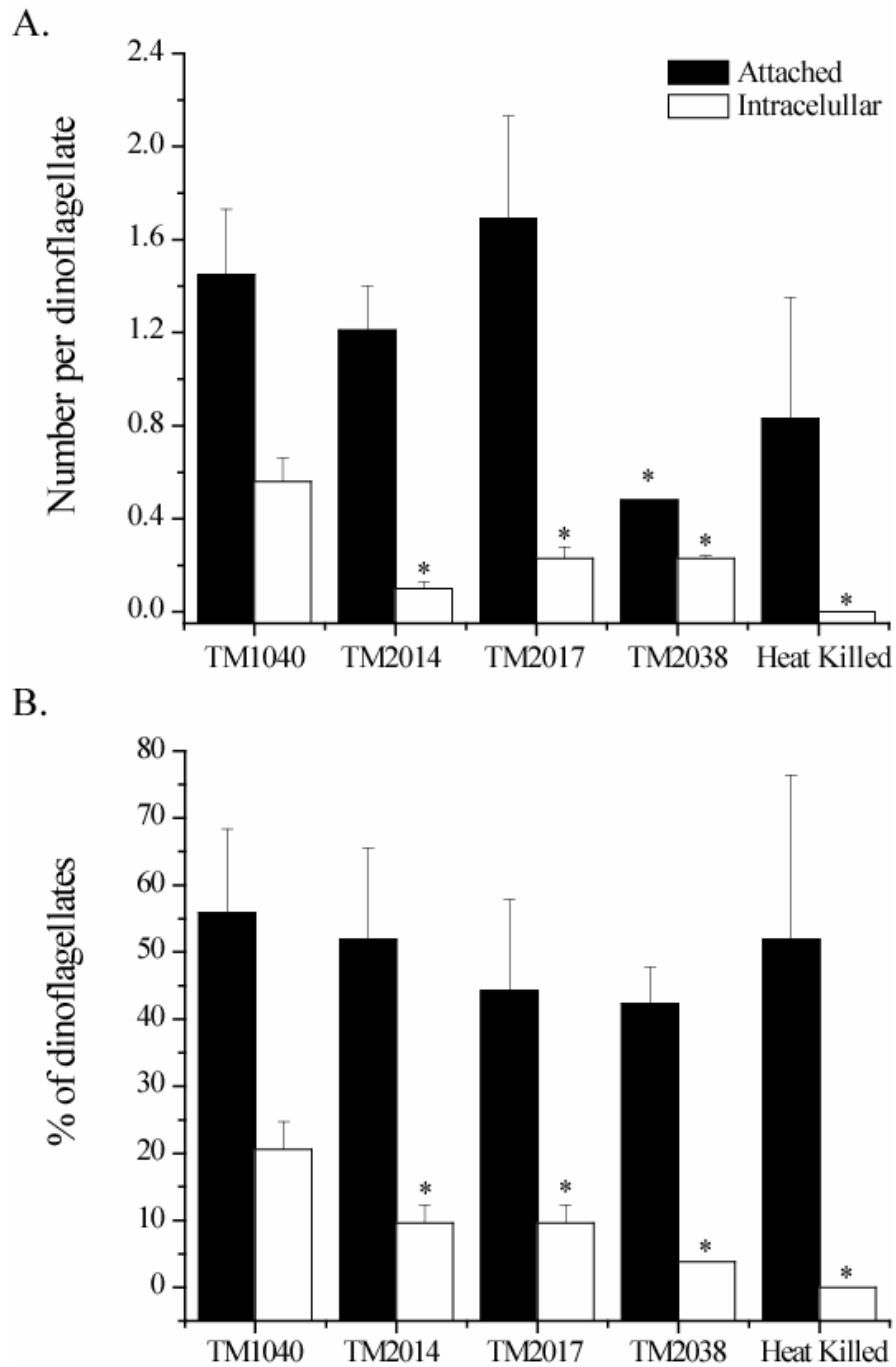


Fig. 4.10. Quantitation of attached and intracellular bacteria in *P. piscicida*. A) The average number of wild type and motility mutants attached (black bars) or inside *P. piscicida* (white bars). B) The percent of dinoflagellates observed showing attached (black bars) or intracellular (white bars) bacteria. Asterisks indicate that the mean data point is significantly different from the wild type ($P < 0.05$).

percentage of dinoflagellates with intracellular bacteria was reduced by 8, 7 and 15%, respectively (Fig. 4.10B). No heat killed cells were found in any of the dinoflagellates.

4.4.6 Complementation of *P. piscicida* growth in axenic culture

Previous results show that *P. piscicida* does not grow well in cultures lacking bacteria (i.e. axenic), and this defect in growth can be overcome by the addition of one or more *Roseobacter* species (9). I sought to determine if *Silicibacter* sp. TM1040 can aid or complement growth of *P. piscicida* when added to axenic cultures of this dinoflagellate. Dinoflagellates were made axenic by treating cysts with bleach, washing and allowing the dinoflagellates to excyst. After 24 h, 0.1 ± 0.04 % of the total encysted and bleached dinoflagellates excysted forming the motile, bi-flagellated zoospores. These cells were then cultured in either the absence of bacteria, in the presence of wild-type TM1040 bacteria or in the presence of each of the Mot⁻ mutants. Bacteria were added at a final concentration of between 10 – 100 cells per ml. As shown in Fig. 4.11A, in the absence of bacteria (control, open circles), dinoflagellates began to divide on day five of culturing reaching densities of only 1.2×10^3 cells per ml on day seven and 2.9×10^3 cells per ml on day nine. Conversely, in the presence of wild-type TM1040 (closed circles), dinoflagellate density reached 3.4×10^3 cells per ml on day seven and 9.5×10^3 cells per ml on day nine, a 65 – 69% improvement in growth (Fig. 4.11A). In the presence of the TM2014 (*flaA*), TM2017 (*cckA*) and TM2038 (*ctrA*) mutants dinoflagellate growth was reduced only slightly or not at all compared to the wild-type giving 7×10^3 , 2×10^3 , 7×10^3 and 9.4×10^3 cells per ml on day nine, respectively. In the presence of heat - killed cells dinoflagellate growth was poor, similar to growth in the absence of bacteria (control).

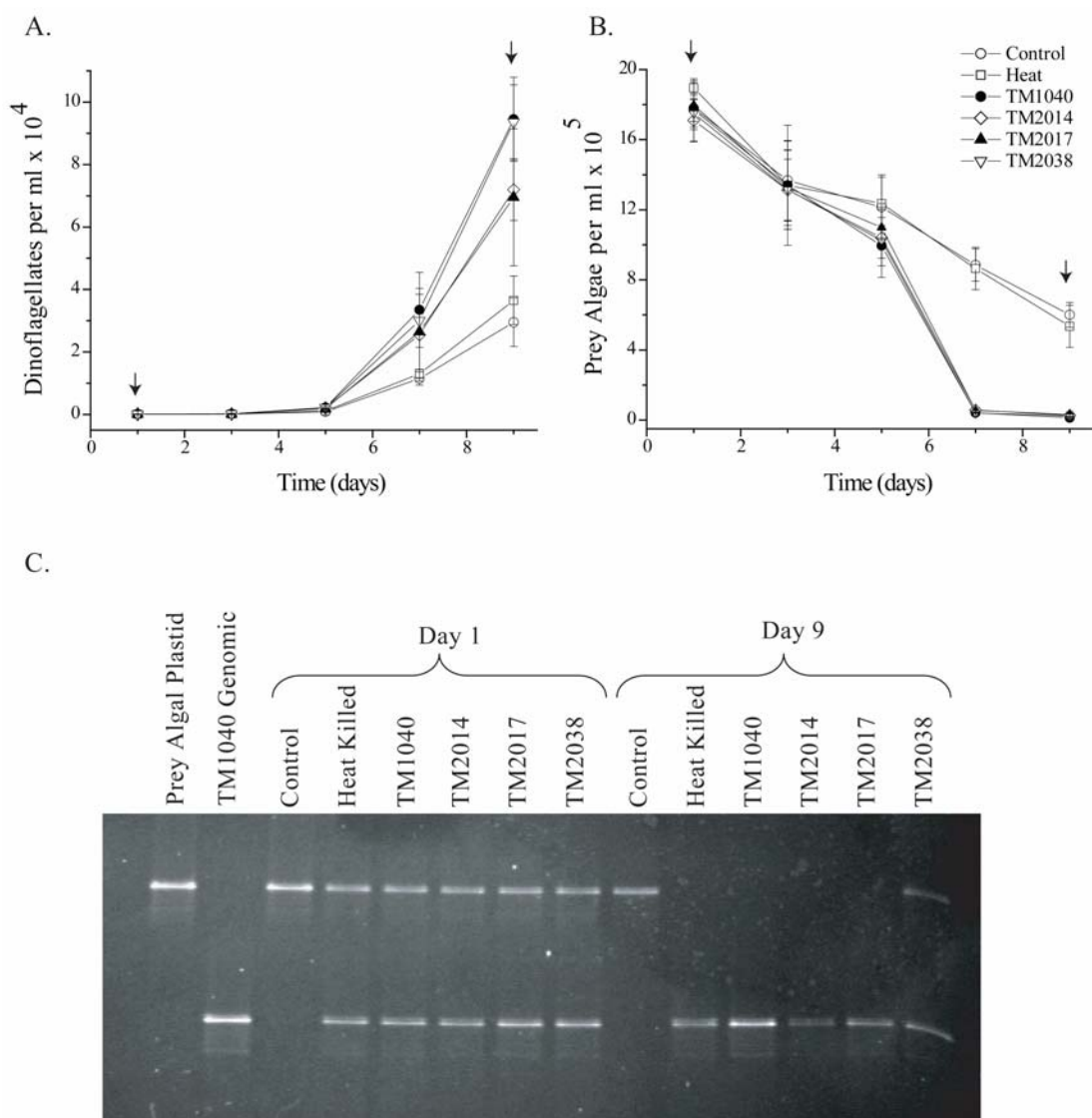


Fig. 4.11. Growth of *P. piscicida* with and without *Silicibacter* sp. TM1040 or motility mutants in axenic cultures. Dinoflagellates were made axenic by a previously described procedure involving bleach treatment of cysts (see Materials and Methods). Wild type *Silicibacter* sp. TM1040 and motility mutants were added to the axenic culture and dinoflagellate growth and grazing upon prey algae (*Rhodomonas* sp.) measured. A) Dinoflagellate cell density over time and B) *Rhodomonas* sp. prey algal density over time. Arrows indicate days of sampling for DGGE. C) 16S rDNA DGGE (30-70% denaturing gel) was performed in order to ensure that the cultures did not contain other bacteria besides *Silicibacter* sp. TM1040 and motility mutants. The primers for amplification of 16S rDNA are able to amplify products from the prey algal plastid DNA, which serves as an internal positive control. A maximum of two bands are present in all cultures corresponding to TM1040 genomic DNA and the axenic *Rhodomonas* sp. prey algae.

With the increase in dinoflagellate cell density there was a concomitant reduction in prey algal cell densities. In the presence of wild-type and mutant bacteria, prey algal densities decreased at a similar rate from ca. 1.7×10^5 per ml on day one to ca. 9×10^4 cells per ml on day five followed by a dramatic decrease resulting in ca. 1.5×10^3 cells per ml on day seven (Fig. 4.11B). Without the addition of any bacteria or in the presence of heat - killed cells, prey algal densities decreased slowly from 1.7×10^5 per ml on day one to 1.2×10^5 cells per ml on day five and finally to 6×10^4 per ml at the completion of the experiment.

The bacterial species composition of the cultures was determined using molecular. From previous reports, it is known that the primers used to amplify 16S rDNA in the PCR for DGGE, also amplify prey algal plastid DNA, which serves as an internal positive control (9). On day one of culturing, in the control culture, only one band aligning with prey algal plastid 16S rDNA was present indicating that the culture was truly axenic following bleach treatment of dinoflagellate cysts (Fig. 4.11C). In all other cultures on day one, including cultures containing (from left to right in Fig. 4.11C) the heat killed cells, wild-type and the three mutant TM1040 strains, two bands are present, one aligning with prey algal plastid 16S rDNA and another aligning with products amplified from TM1040 genomic DNA. On day nine, 16S rDNA from plastids was detected in control cultures and a light band in cultures containing the TM2038 mutant. Cultures containing wild-type TM1040, TM2014, TM2017 and TM2038 as well as heat - killed cells contained only one band aligning with TM1040 16S rDNA. Other bands, besides those aligning with TM1040 and plastid 16S rDNA are not visible in the gel on any day of sampling.

4.5 Conclusions

Marine bacteria, especially *Roseobacter* species, have been shown to interact with dinoflagellates both in the laboratory and in the environment. However, little is known about the genetic and phenotypic determinants of these interactions. Previous studies show that *Silicibacter* sp. TM1040 is chemotactic toward *P. piscicida* homogenates, as well as toward DMSP and amino acids produced by *P. piscicida*. I hypothesized that TM1040 might also interact physically (attached or intracellular) and/or physiologically (growth enhancing) with the dinoflagellate. In addition, motility and/or flagella were hypothesized to be necessary for these interactions. To test this hypothesis, I constructed transposon mutants of *Silicibacter* sp. TM1040 defective in motility and/or flagellar biosynthesis. The data presented here indicate three major conclusions concerning the hypothesis. First, *Silicibacter* sp. TM1040 attaches to and may be found internal to the dinoflagellate, *P. piscicida*. Second, the “intracellular” co-localization of *Silicibacter* sp. TM1040 with the dinoflagellate requires flagellar-mediated motility and a metabolically active cell. Third, *Silicibacter* sp. TM1040 aids the growth of *P. piscicida* in axenic cultures, but this does not require intimate contact between the bacteria and the dinoflagellate.

In the analysis of transposon insertion sites, it appears that downstream or polar effects of transposon insertions are not likely since each mutated gene is at the end of a putative operon (Fig. 4.5), or transcribed as a single mRNA separated from the downstream gene by >300 bp. However, polar effects cannot be ruled out and should be reconciled by constructing nonpolar mutations in these genes. Yet, current methods for introducing mutations in *Silicibacter* species are limited since I and others have had little

or no success introducing autonomously replicating elements or any closed circular molecules into *Roseobacter* species (data not shown, (63)). Future work will include development of molecular methods for use with TM1040 and other roseobacters.

Cloning and annotation of genes harboring transposon insertions in *Silicibacter* sp. TM1040 identified a sensor kinase and response regulator pair involved in motility (Fig. 4.1, Table 4.1 and 4.2). The *cckA* and *ctrA* genes of TM1040 are nearly identical to the same genes in other α -Proteobacteria with respect to sequence homology and conserved protein domains. In *R. capsulatus* and *C. crescentus* these genes encode a sensor kinase and response regulator two component system controlling cell differentiation, motility and genetic exchange. In *R. capsulatus*, CckA functions as a class I flagellar regulator such that *cckA* mutations result in a lack of transcripts from class II, class III or class IV genes (211). The *cckA/ctrA* genes seem to regulate flagellar biosynthesis according to the growth stages of the culture, becoming most active in the stationary growth phase (211). In *C. crescentus* and *Rhizobium meliloti* *ctrA* is essential for viability and controls the differentiated cell cycle of *C. crescentus*, including flagellar biosynthesis (43, 310). The CtrA protein is functionally analogous to the FlhDC master regulator for flagellar synthesis in *E. coli*. In *Silicibacter* sp. TM1040, disruption of *ctrA* resulted in mutants that produced elongated cells (Fig. 4.3, Table 4.1) and were poorly motile (Fig. 4.1) due to impairment of flagellar synthesis (Fig. 4.2). On the other hand, disruption of *cckA* resulted in mutants that were completely non-motile (Fig. 4.1, Table 4.1) and lacked flagella (Fig. 4.2). Neither of these mutants showed differences in growth rate or other major defects (Table 4.1), but given the role these genes play in other bacteria it is possible that other cryptic phenotypes are also regulated by these proteins. It

will be interesting to determine if these proteins regulate other functions required for interactions with the dinoflagellate. One possibility is that this two component system also regulates DMSP degradation by sensing the presence of DMSP or DMSP breakdown products. This might also serve as a signal to induce the expression of other genes necessary for interactions with the dinoflagellate, including genes for motility and chemotaxis.

A novel flagellar gene, *flaA* was identified in this study. Disruption of this gene caused a complete loss of motility (Fig. 4.1) and flagellar biosynthesis (Fig. 4.2). In addition, this gene is located within a ~24 Kb segment of the chromosome containing the majority of all flagellar biosynthetic genes (Fig. 4.5). This novel *flaA* gene is conserved in at least two other *Roseobacter* species. The discovery of this gene emphasizes the power of a genetic approach and the novelty of this gene hints that motility and/or flagellar biosynthesis in *Roseobacter* species may be different from that of many other bacteria.

The ability of these motility mutants to interact with *P. piscicida* on a physical basis compared to wild-type cells was tested. The data indicate that the co-localization of *Silicibacter* sp. TM1040 with the dinoflagellate cytoplasm is enhanced by flagellar-mediated motility, and requires a metabolically active bacterial cell. Given fluctuations in the shape of the dinoflagellate cell surface, the identification of an intracellular location of *Silicibacter* sp. TM1040 is limited. However, in all the results presented in this Chapter, the depth of wild-type cells relative to the dinoflagellate cell surface was consistently lower than the depth of Mot⁻ mutants or the heat-killed cells, suggesting that the depth of *Silicibacter* sp. TM1040 relative to the dinoflagellate's surface requires both

bacterial motility and a metabolically active cell (Fig. 4.8, 4.9 and 4.10). It is unlikely that the “intracellular” occurrence of *Silicibacter* sp. TM1040 is simply due to dinoflagellate feeding upon the bacteria since many dinoflagellates have been shown to feed upon heat-killed cells (79, 119), and heat-killed wild-type *Silicibacter* sp. TM1040 was never found within *P. piscicida* dinoflagellates. In addition, *Silicibacter* sp. TM1040 appeared to present both in areas containing food vacuoles and in areas devoid of food vacuoles. A more likely hypothesis is that the bacteria gain access to intracellular spaces of the dinoflagellate through the dinoflagellate feeding apparatus or peduncle.

There are several possible scenarios that could explain why motility enhances the apparent intracellular occurrence, but not attachment, of *Silicibacter* sp. TM1040 to *P. piscicida*. It is possible that *Silicibacter* sp. TM1040 may attach through simple random contact with the highly motile dinoflagellate zoospores. The intracellular life style may then require the attached bacteria to move over the dinoflagellate cell surface to specific areas suitable for entrance into the dinoflagellate cell. However, this would require a swarming behavior and *Silicibacter* sp. TM1040 does not appear to swarm on agar surfaces. Alternatively, while attachment may occur through random collisions, invasion may only occur at specific times during the dinoflagellate life cycle when the dinoflagellate is most vulnerable to bacterial invasion. For example, the dinoflagellate life cycle involves both sexual and asexual division and both of these involve stationary life stages and morphological changes including changes in the dinoflagellate cell membrane (228). *Silicibacter* sp. TM1040 may use directed movement, or chemotaxis, toward these stationary dinoflagellate cells to invade the dinoflagellates at specific times during dinoflagellate cell division. Another possibility is that the bacteria invade the

dinoflagellate through the peduncle as it is extended outwards, but before it is attached to the prey algal cell. Motility may be required for the bacteria to move up the feeding tube and into the dinoflagellate cell.

The apparent intracellular occurrence of *Silicibacter* sp. TM1040 could suggest either parasitism or a symbiotic, possibly mutualistic relationship with the dinoflagellate. As evidence for a symbiotic type of relationship, the growth of *P. piscicida* in axenic cultures is aided by the presence of *Silicibacter* sp. TM1040 added back to the culture. This effect was reduced only slightly in the presence of the motility mutants. It is not entirely clear what effect *Silicibacter* sp. TM1040 imparts upon *P. piscicida* in order to alleviate growth defects of the dinoflagellates in axenic cultures, but it can either occur without a physical interaction, or simply through attachment and not invasion. This is because the motility mutants, *flaA*, *cckA*, and *ctrA* that are not found within the dinoflagellate were still able to complement growth of the dinoflagellate, though to a somewhat lesser extent than wild-type TM1040. As with invasion, since heat-killed cells did not complement growth of the dinoflagellates, the increased growth in the presence of *Silicibacter* sp. TM1040 is not simply due to the bacteria serving as a food source.

In other dinoflagellate species, bacteria are thought to remove the build up of toxic waste products, regenerate essential nutrients, or produce other compounds necessary for dinoflagellate growth (110). In the case of *P. piscicida*, whole algal cells are supplied as the food source. The consumption and digestion of these algal cells could result in the build up of excess carbon or other compounds in the culture that limit dinoflagellate growth. The bacteria may function to degrade those compounds as they accumulate, acting as a living garbage disposal. Alternatively, bacteria may stun, or

partially digest the algal prey making capture and thus feeding less energetically expensive. This does not seem to be the case however since in axenic cultures, without the addition of any bacteria, feeding by *P. piscicida* on prey algae occurred concomitantly with small increases in dinoflagellate density. The rate of feeding was reduced compared to cultures containing wild-type TM1040, but this reduced rate of consumption can simply be explained by a decreased rate of dinoflagellate growth.

Alternatively, in any cell, balanced growth is important and achieving balanced growth may require supplemental nutrients from the bacterial community. This is the basis for a number of symbiotic and/or syntrophic relationships among organisms in nature. In the phylogenetically related *Rhizobium* species, nitrogen is supplied to the plant host in return for carbon (33). While any such relationship in the *Silicibacter* sp. TM1040 and *P. piscicida* interaction is currently unknown, *Silicibacter* sp. TM1040 appears to be an attached and/or intracellular bacterium whose presence enhances the growth of *P. piscicida* dinoflagellates in culture. This phenomenon is likely to be important for dinoflagellate physiology in nature.

Chapter 5: Genomic analysis of genes important for *Silicibacter* sp. TM1040 interactions with *P. piscicida*

5.1 Summary

The genome of *Silicibacter* sp. TM1040 was sequenced by the Joint Genome Institute (Department of Energy) and an automated annotation of all open reading frames (ORFs) completed. Chemotaxis and flagellar-mediated motility are important characteristics for physical interactions with *P. piscicida* and an analysis of the *Silicibacter* sp. TM1040 genome suggests that other characteristics of this bacterium may also play a key role. To better understand these characteristics, open reading frames (ORFs) with homology to proteins involved in chemotaxis and flagellar biosynthesis or energetics were manually annotated and compared to similar proteins in other α -Proteobacteria. In addition, ORFs with homology to proteins required for producing a pilus structure, nodulation factors, and a type IV secretion system (or *vir* pilus) similar to that of *Agrobacterium tumefaciens* were manually annotated. The analysis indicates that *Silicibacter* sp. TM1040 is equipped with a large number of chemotaxis proteins including 20 different chemoreceptors, as well as proteins for adapting to varying concentrations of chemoattractant compounds. In addition, the flagellar apparatus of *Silicibacter* sp. TM1040 appears to be complex, with at least five different genes encoding flagellin proteins, and two genes encoding flagellar motor proteins. The existence of ORFs with homology to a *vir* pilus and nodulation factors suggest a hypothesis in which this bacterium communicates with its dinoflagellate host through chemical signaling and DNA transfer. Overall, the genomic data agree with previous

observations showing that this is a highly motile bacterium that is chemotactic toward dinoflagellate products and displays symbiotic-like interactions with the dinoflagellate, *P. piscicida*.

5.2 Flagellar proteins

Previous work shows that *Silicibacter* sp. TM1040 is highly motile and chemotactic toward dinoflagellate products (Chapter 3). In addition, flagellar-mediated active motility is important for *Silicibacter*-dinoflagellate interactions, including a life style that intimately associates the bacteria with the dinoflagellate (Chapter 4). Thus, an analysis of *Silicibacter* sp. TM1040 flagellar and chemotaxis proteins is warranted.

The genome of *Silicibacter* sp. TM1040 contains all of the genes necessary to produce a functional flagellum, including those required for synthesis of the hook and basal body, flagellar filament, cap protein, and motor/switch proteins (reviewed in (84)). The hook basal body (HBB) consists of the export pathway, C - ring, MS- ring, proximal rod, P-ring, L-ring, distal rod and hook. Genes in the TM1040 genome that code for proteins forming these structures were annotated to understand more about the functional flagellum of this bacterium.

As can be seen in Table 5.1, the proteins composing the structural components of the flagellum of *Silicibacter* sp. TM1040 are homologous to their equivalents from other α -Proteobacteria, particularly *R. sphaeroides*. The motility of *R. sphaeroides* is unusual when compared to *E. coli* and other enteric bacteria, and probably more representative of non-enteric bacteria such as strain TM1040. *R. sphaeroides* moves in a dart and stop motion instead of a run and tumble motion (30). In addition, it changes the speed

Table 5.1. Flagellar and chemotaxis proteins from *Silicibacter* sp. TM1040

Export Pathway				
Gene #	Protein	Size (da)	Blast Result	Probability
1858	FliA	76086	COG1298: Flagellar biosynthesis pathway, component FliA [Rhodobacter sphaeroides]	1e-152
1860	FliB	40,080	COG1377: Flagellar biosynthesis pathway, component FliB [Rhodobacter sphaeroides]	8e-60
1849	FliP	29869	COG1338: Flagellar biosynthesis pathway, component FliP [Rhodobacter sphaeroides]	2e-67
1867	FliQ	9825	FliQ [Treponema denticola]	1e-10
1859	FliR	34726	COG1684: Flagellar biosynthesis pathway, component FliR [Rhodobacter sphaeroides]	1e-34
1871	FliI	51791	COG1766: Flagellar biosynthesis/type III secretory pathway lipoprotein [Rhodobacter sphaeroides]	2e-73
1851	FliO	21729	COG0318: Acyl-CoA synthetases forming/AMP-acid ligases II [Rhodobacter sphaeroides]	5e-20
3414	FliM	43921	COG1868: Flagellar motor switch protein [Rhodobacter sphaeroides 2.4.1].	5e-23
1850	FliN		flagellar motor switch protein fliN [Brucella melitensis]	1e-9
3100	FliG	41210	COG1536: Flagellar motor switch protein [Rhodobacter sphaeroides]	
MS ring				
Gene #	Protein	Size (da)	Blast Result	Score
1852	FliF	60779	COG1766: Flagellar biosynthesis/type III secretory pathway lipoprotein [Rhodobacter sphaeroides]	2e-73
Rod				
1870	FlgB	14065	flagellar basal-body rod protein FlgB, putative [Caulobacter crescentus]	1e-11
1869	FlgC	14655	flagellar basal-body rod protein flgC [Brucella melitensis]	
1866	FlgF	25455	flagellar basal-body rod protein FlgF [Caulobacter crescentus]	7e-34
1865	FlgG	27910	flagellar basal-body rod protein FlgG [Caulobacter crescentus]	1e-70
1868	FliE	10620	flagellar hook-basal body protein; FleE [Mesorhizobium loti]	2e-9

L-, P- ring				
Gene #	Protein	Size (da)	Blast Result	Probability
1863	FlgH	53030	COG2063: Flagellar basal body L-ring protein,Rhodobacter sphaeroides	2e-60
1848	FlgI	42166	COG1706: Flagellar basal-body P-ring protein [Rhodobacter sphaeroides]	1e-117
Hook				
Gene #	Protein	Size (da)	Blast Result	Score
1009	FlgD	24279	Probable basal-body rod modification protein flgD [Ralstonia solanacearum]	5e-20
1845	FlgE	46524	COG1749: Flagellar hook protein FlgE [Rhodobacter sphaeroides]	9e-86
1008	FliK			
1846	FlgK	51750	COG1256: Flagellar hook-associated protein [Rhodobacter sphaeroides]	7e-54
1847	FlgL	35,294	COG1344: Flagellin and related hook-associated proteins [Rhodobacter sphaeroides]	3e-23
Filament				
Gene #	Protein	Size (da)	Blast Result	Score
1005	FliC	34700	COG1344: Flagellin and related hook-associated proteins [Rhodobacter sphaeroides]	7e-47
1352	FliC	30834	COG1344: Flagellin and related hook-associated proteins [Rhodobacter sphaeroides]	3e-39
3126	FliC	29361	COG1344: Flagellin and related hook-associated proteins [Rhodobacter sphaeroides]	1e-44
3127	FliC	29961	COG1344: Flagellin and related hook-associated proteins [Rhodobacter sphaeroides]	8e-42
3128	FliC	34467	COG1344: Flagellin and related hook-associated proteins [Rhodobacter sphaeroides]	2e-46
Unknown				
1857	FlaA	87577	Required for flagellar synthesis	NA
Motor				
Gene #	Protein	Size (da)	Blast Result	Score
470	MotA1	42756	MotA/TolQ/ExbB proton channel family [Brucella melitensis]	4e-50
1856	MotA2	32101	COG1291: Flagellar motor component [Rhodobacter sphaeroides]	4e-76
469	MotB1	86112	similar to flagellar motor protein; MotB [Mesorhizobium loti]	9e-61
1844	MotB2	32178	COG1360: Flagellar motor protein [Rhodobacter sphaeroides]	4e-55

Chemotaxis				
Gene #	Protein	Size (da)	Blast Result	Probability
2231	CheA	77894	cheA homolog [Agrobacterium tumefaciens]	1e-160
948	CheY1	91450	COG0784: FOG: CheY-like receiver [Rhodobacter sphaeroides]	2e-75
2230	CheY2	125636	sensory box sensor histidine kinase/response regulator [Vibrio parahaemolyticus]	1e-131
2194	CheD1	19172	Chemotaxis protein CheD[Clostridium acetobutylicum]	6e-11
2227	CheD2	29779	Chemotaxis protein CheD, Caulobacter crescentus CB15, Score = 169 bits (428), Expect = 7e-41	7e-41
2228	CheB	38307	protein-glutamate methyltransferase (EC 3.1.1.61) cheB [Rhizobium meliloti]	6e-43
598	CheR1	34326	CheR1 protein [Rhodobacter sphaeroides]	2e-67
2195	CheR2	31932	Methylase of chemotaxis methyl-accepting proteins [Leptospira interrogans]	6e-35
2233	CheR3	32052	CheR1 protein [Rhodobacter sphaeroides]	2e-66
2382	cheR4	92792	conserved hypothetical chemotaxis protein [Methanosarcina acetivorans]	1e-120
2193	cheW1	58776	CheW2 [Rhodospirillum centenum]	3e-34
2232	cheW2	17964	Chemotaxis protein chew2 [Agrobacterium tumefaciens]	4e-29

of its flagellar motor in response to chemical attractants (164). A similar form of motility has been observed in TM1040 cells and it is likely that this is due, in part, to the similarity in flagellar genes between these two organisms.

The genomes of two other *Roseobacter* species have recently become available (265). *Silicibacter pomeroyi* DSS-3 and *Jannaschia* sp. CCS1 are both *Roseobacter* clade species isolated from coastal seawater (146). *S. pomeroyi* DSS-3 also has an unusual form of motility in that it is only motile under a strict set of culturing conditions and is not motile at all times of its growth curve (145). The early automated annotation of the TM1040 genome did not make use of these sequences for similarity searches because they were not yet available. During the manual annotation presented here, the flagellar gene system of TM1040 has been compared to these other *Roseobacter* species.

The *Silicibacter* sp. TM1040 genome contains all of the structural proteins that make up the export pathway including the six integral membrane proteins FlhA, FlhB, FliO, FliP, FliQ, and FliR, as well as the cytoplasmic ATPase protein FliI (Table 5.1). The genome of strain TM1040 does not contain the FliH or FliJ proteins involved in flagellar export (257). *E. coli* FliH negatively regulates FliI ATPase activity, while FliJ is a chaperone that prevents flagellar proteins from folding prematurely in the cytoplasm before they are exported (258). *Silicibacter* sp. TM1040 might have other, as of yet, unknown proteins that provide these functions for export. Interestingly, the *R. sphaeroides* genome contains homologs of both of these proteins (40). A homology search of the TM1040, *S. pomeroyi* and *Jannaschia* sp. genomes using the FliH protein from *R. sphaeroides* did not find a significant match ($E < 0.1$) to this protein. In addition, other α - Proteobacteria such as *R. meliloti*, *A. tumefaciens*, and *C. crescentus* also lack

this protein. Thus, it may be that the absence of FliH and FliJ homologs is a common theme among many α -Proteobacteria, including *Roseobacter* species.

Genes for the export pathway in *Silicibacter* sp. TM1040 are arranged within a large ~ 28 kB flagellar gene locus (Fig. 5.2). Currently, this locus resides on contig 52 starting with *motB2* and ending with *fliI*. Based upon the direction of transcription and overlapping coding regions, the flagellar export genes are contained in three gene clusters, the *flhA* cluster (*flhA*, *fliR*, *flhB*), the *fliF* cluster (*fliF*, *fliO*, *fliN*, *fliP*) and the *fliQ* gene is in the *flgA* cluster (*flgA*, *flgG*, *flgF*, *fliQ*, *fliE*, *flgC*, *flgB*) (Fig. 5.2). This arrangement is consistent with expression of class II flagellar genes.

The C-ring is made up of the FliG, FliN and FliM proteins, which comprise the switch complex that is responsible for changes in flagellar rotation (124, 351, 358). All three of these genes are present in the genome of strain TM1040, but are spread out among different regions of the chromosome. Both the *fliM* and *fliG* genes occur by themselves and are not near any other flagellar or chemotaxis genes. The *fliM* gene homolog is flanked by 77 bp at its 5' end by an ORF that is similar to a two-component sensor protein of *R. sphaeroides* ($E = 0.0$) and located 157 bp on its 3' end by a homolog of a conserved protein from *R. capsulatus* with unknown function ($E = 4.0e-24$). The latter, 3' end protein has some homology to a sulfide dehydrogenase from *Brucella melitensis* ($E = 3.0e-15$) and other predicted, conserved NAD-dependent dehydrogenases from a large number of recently sequenced organisms. FliM is especially important for motility and chemotaxis because it is thought that the CheY-P protein interacts with FliM to cause changes in the rotation of the flagellum (359). Deletions in the N-terminus of the FliM

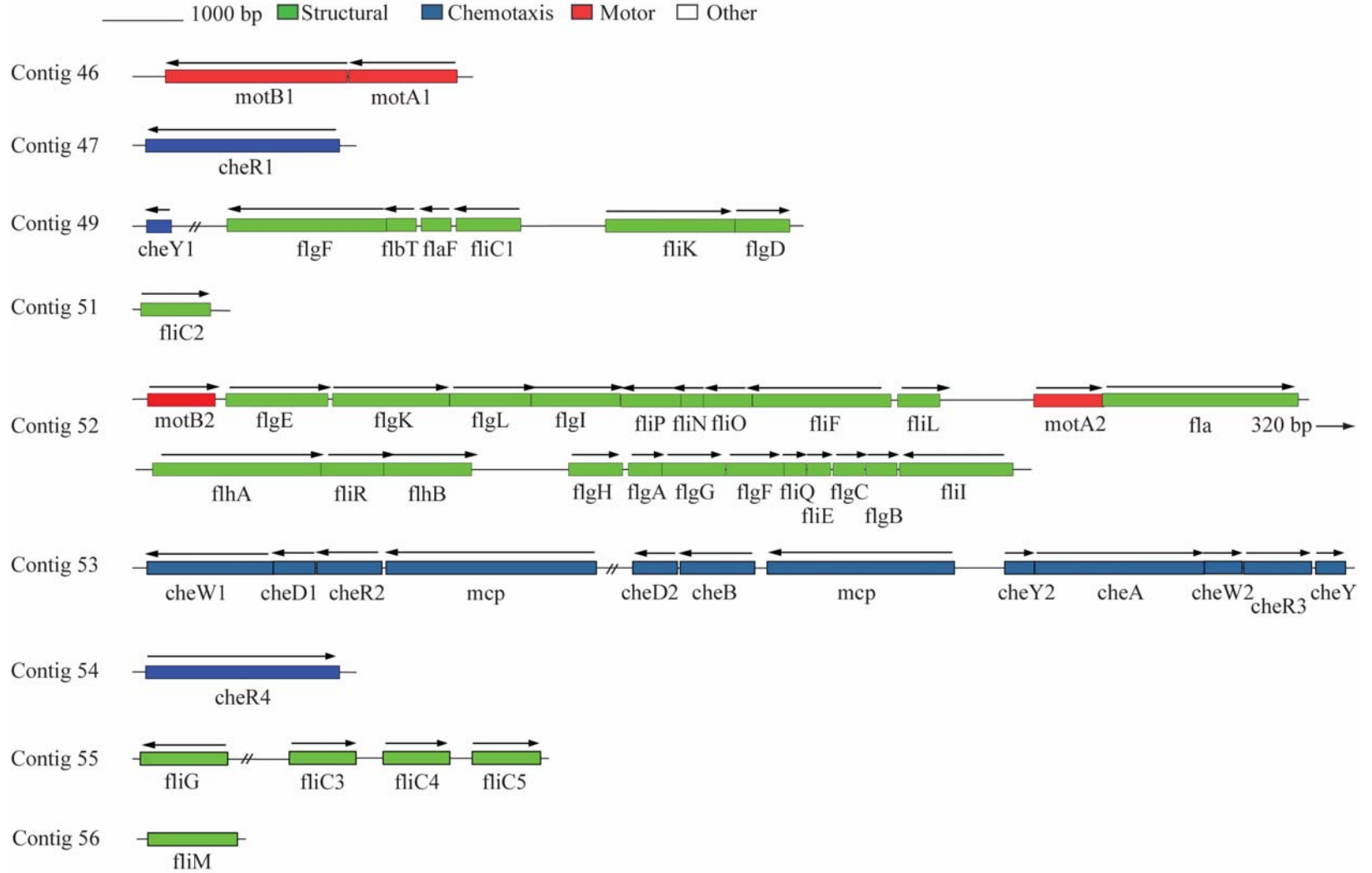


Fig 5.1. Genomic organization of the flagellar and chemotaxis genes in *Silicibacter* sp. TM1040.

protein of *Salmonella typhimurium* cause a counterclockwise switch bias, while in *R. sphaeroides* they completely abolish motility (358). The difference in mutant phenotypes from these two species may be related to the fact that *R. sphaeroides* flagellum turns in only one direction, with complete stopping of rotation causing changes in swimming direction, unlike that of *S. typhimurium* which is constantly moving, and reverses direction to cause changes in swimming direction (238).

The *fliG* gene is flanked 154 bp at its 5' end by an ORF with homology to LipA from *Brucella suis* for lipid biosynthesis ($E = 3.0e-23$), and 79 bp at its 3' end by an ORF with homology to PilF, a pilus assembly protein from *Mesorhizobium loti* ($E = 2.0e-18$). FliG is part of the rotor and is stabilized by FliF, the MS-ring component (348). FliG is an important component of the switch mechanism since without this protein switching does not occur or only rarely occurs such that cells are stuck in a run condition (177). The *fliN* gene is part of the *fliF* gene cluster occurring between *fliP* and *fliO*. FliN is involved in torque generation or export, although its role is less well known (351, 376).

The MS-ring is made by the FliF protein and is probably a structural element that binds FliG allowing for a gating mechanism of rotation (348, 376). This protein is also found in the *Silicibacter* sp. TM1040 genome (Table 5.1) and is at the start of the *fliF* gene cluster that contains *fliF*, *fliO*, *fliN*, and *fliP* of the export pathway. The MS-ring is necessary for translocation of proteins across the outer membrane (198).

The proximal rod and distal rods are made by proteins FlgB/FlgC and FlgF/ FlgG, respectively, forming a flexible rod that passes through the periplasmic space and is connected at one end to the motor and at the other end to the hook proteins (8, 170). Homologs to all four of these proteins are found in strain TM1040 in addition to the FliE

protein (Table 5.2, Fig. 5.1). In *E. coli* and others, the FliE protein is required for assembly of the rod, although its exact function is still not known (8, 270). In *Silicibacter* sp. TM1040, the genes for the rod are organized in the *flgA* gene cluster which includes *flgA*, *flgG*, *flgF*, *fliQ*, *fliE*, *flgC*, and *flgB*. However, there is a second *flgF* gene, presently on contig 49 that occurs with *flbT*, a post-transcriptional regulator of flagellin synthesis in *C. crescentus* (22)(Fig 5.1). Both the contig 52 *flgF* and contig 49 *flgF* gene products have about equal homology to known FlgF proteins (*C. crescentus* FlgF, E = 7.0 e-34 and *Brucella melitensis* FlgF, E = 2 e-18). The *fliQ* gene, occurring between *flgF/G* (distal rod) and *flgB/C* (proximal rod) on the major flagellar biosynthetic gene cluster (contig 52) makes a protein that has homology to proteins involved in the export of virulence factors, suggesting that it is involved in the export of flagellar proteins, similar to FliR (401).

The L- and P-rings are made by the FlgH and FlgI proteins, respectively. These proteins form a pore in the outer membrane anchoring the complex in place (186, 257). These proteins are present in the genome of strain TM1040 and occur separately. The *flgI* gene is contained within the *flgK* gene cluster (*flgK*, *flgL*, *flgI*) which makes the *flgK* and *flgL* proteins for the hook, while the *flgH* gene occurs either by itself, or as part of the *flgA* gene cluster.

The hook is made up of the FlgD, FlgE, FliK, FlgK, FlgL and FliD proteins. All of these except for the FliD protein are present in strain TM1040's genome. Normally the first hook proteins to be assembled are the FlgD and FlgE proteins and genes for these proteins are found in separate locations. The *flgE* gene occurs by itself or as part of the *flgK* gene cluster while the *flgD* gene occurs with one other gene, *fliK*, which is the next

protein to be assembled. With the addition of FliK, the full length hook is essentially finished. The FlgK and FlgL proteins form the first and second hook-to-filament junction zones, respectively, and are located next to each other within the *flgK* gene cluster. The *fliD* gene is not present, suggesting that there is no hook capping protein or that the hook cap protein is not similar to that of other bacteria.

The flagellar filament is composed of proteins (flagellin) encoded by *fliC*. In *Silicibacter* sp. TM1040, there are five proteins with homology to flagellin from *R. sphaeroides* (Table 5.1). Three of these genes are located together on contig 55 and all appear to be transcribed in the same direction, however there are short 100 – 200 bp gaps between them. Another flagellin gene is found on contig 51 by itself and is flanked by 658 bp on its 5' end by *mauG*, encoding a methylamine utilizing protein ($E = 1.0 \times 10^{-72}$) and 143 bp on its 3' end by a homolog of choline sulfatase from *Mesorhizobium loti* ($E = 0.0$). In other bacteria with multiple flagellin genes, such as *Vibrio fischeri* and *Proteus mirabilis*, a subset of the flagellin genes are always expressed and additional flagellin genes are expressed at either a low frequency or only at specified times in the bacterium's life cycle (46, 241, 256, 272). In these bacteria, multiple flagellin genes are important for symbiotic or pathogenic interactions. Interestingly, both *S. pomeroyi* and *Jannaschia* sp. appear to have only one flagellin gene since a BLAST search of their genomes using flagellin genes from *C. crescentus*, *R. loti*, *A. brasilenses* and *B. suis* returns only one flagellin homolog with significant similarity ($E < 0.1$).

The genes that code for proteins of the flagellar motor in the genome of strain TM1040 consist of two *motA* genes and two *motB* genes. The *motA1* and *motB1* genes occur next to each other on contig 46. At the 5' end of *motA1*, separated by ca. 80 bp is a

gene whose ORF is homologous to a cation transport channel, ChaC from *A. tumefaciens* ($E = 8.0 \times 10^{-36}$). The *motA2/B2* genes are located within the major flagellar gene locus of contig 52, but are separated from one another by ca. 12 kb. The *motB2* gene occurs 433 bp from the 3' end of a methyl-accepting chemotaxis protein (MCP) and is separated from the downstream *flgE* gene by 131 bp. The *motA2* gene overlaps the novel *flaA* gene (see Chapter 4) on its 3' end and has an unknown gene at its 5' end, separated by 75 bp. Using the *motA1/B1* and *motB1/B2* proteins from *Silicibacter* sp. TM1040 in a BLAST search of the *S. pomeroyi* and *Jannaschia* sp. genomes indicates that these species also contain these two sets of motor proteins.

The motor proteins form channels that conduct either protons or sodium ions and these channels can be inhibited specifically by FCCP or phenamil, respectively. These chemical inhibitors have been used to determine the type of motor possessed by certain bacterial species. When added to separate cultures of *Silicibacter* sp. TM1040, both FCCP and phenamil caused TM1040 to stop moving in a dose-dependent manner. A complete loss of motility was observed within 10 min in the presence of 500 μM FCCP or 250 μM phenamil. These data suggests that the flagellar motor of *Silicibacter* sp. TM1040 is sensitive to both compounds or that both ions are used to generate force needed to rotate the flagellum.

5.3 Chemotaxis proteins

Cytoplasmic chemotaxis proteins receive signals from the methyl-accepting chemoreceptors and pass those signals to the flagellar motor causing changes in swimming direction. In TM1040 there are multiple copies of genes that code for these

proteins, except for one copy of *cheA*, which codes for the response regulator, and one copy of *cheB*, encoding an enzyme that demethylates receptors to cause sensory adaptation and no copy of the *cheZ* gene (Table. 5.1). Most notably there are four *cheR* genes in the TM1040 genome. The CheR protein works to oppose the activity of CheB as it methylates receptors not bound with ligand. This causes an increase in the autophosphorylation activity of CheA and thus promotes tumbling or stopping in *E. coli* and *R. sphaeroides*, respectively. There are also two each of the *cheD*, *cheW* and *cheY* genes. In *Bacillus subtilis*, CheD increases the methylation activity of CheR. In *E. coli* and other bacteria, the CheW protein couples CheA to the chemoreceptor where it autophosphorylates. CheY accepts a phosphate from CheA such that it is active and can bind to FliM of the flagellar motor to inhibit rotation or cause a change in the direction of rotation. Multiple cytoplasmic chemotaxis proteins are not present in the genomes of the two other *Roseobacter* species, *S. pomeroyi* and *Jannaschia* sp. Others have suggested that multiple chemotaxis proteins increase the potential for signal sensitivity since cooperativity among multiple groups of chemosensory protein complexes is thought to enhance chemotaxis. Another possibility is that metabolic diversity, a characteristic of many α -Proteobacteria demands a complex chemosensory system (29).

The methyl-accepting chemotaxis proteins or MCPs are the chemoreceptors that bind chemical attractants or repellants and initiate the signal transduction pathway. Besides homology to known proteins, the MCPs can be identified based upon conserved protein domains including one or more transmembrane regions, a HAMP domain, possibly a PAS domain, and a methyl-accepting domain. Most MCP proteins are located

Table 5.2. Methyl-accepting chemotaxis proteins from *Silicibacter* sp. TM1040

#	Gene#	Size (da)	Location ^a	Blast	Probability
1	120	53,760	C	Methyl-accepting chemotaxis protein [<i>Agrobacterium tumefaciens</i>]	5.00E-71
2	366	52,453	C	Methyl-accepting chemotaxis protein[<i>Novosphingobium aromaticivorans</i>]	4.00E-58
3	385	56,243	M	Methyl-accepting chemotaxis protein McpA [<i>Caulobacter crescentus</i>]	1.00E-97
4	834	83,579	M	Methyl-accepting chemotaxis protein [<i>Agrobacterium tumefaciens</i>]	1.00E-93
5	1308	93,049	M	Methyl-accepting chemoreceptor mcpA [<i>Rhodobacter capsulatus</i>]	1.00E-111
6	1310	79,024	M	Methyl-accepting chemotaxis protein McpX [<i>Agrobacterium tumefaciens</i>]	1.00E-143
7	1842	78,572	M	Methyl-accepting chemotaxis protein B [<i>Rhodobacter capsulatus</i>]	1.00E-107
8	2196	97,112	C	Methyl-accepting chemotaxis protein [<i>Pseudomonas syringae</i>]	1.00E-126
9	2229	84,440	M	Methyl-accepting chemotaxis protein McpC [<i>Caulobacter crescentus</i>]	1.00E-95
10	2241	64,902	M	Methyl-accepting chemotaxis protein McpC [<i>Caulobacter crescentus</i>]	2.00E-89
11	2367	63,638	M	Methyl-accepting chemotaxis protein [<i>Rhodobacter capsulatus</i>]	1.00E-121
12	2408	82,944	M	Chemoreceptor gene similar to orf2 gene in pTi15955 [<i>Rhizobium rhizogenes</i>]	2.00E-83
13	2474	98,963	M	Methyl-accepting chemotaxis protein McpA [<i>Rhodobacter capsulatus</i>]	1.00E-103
14	2712	46,110	C	Aerotaxis probable chemoreceptore [<i>Rhodobacter capsulatus</i>]	2.00E-74
15	2943	98,846	M	Methyl-accepting chemoreceptor mcpA [<i>Rhodobacter capsulatus</i>]	1.00E-102
16	3079	64,575	M	Methyl-accepting chemotaxis protein McpA [<i>Caulobacter crescentus</i>]	1.00E-100
17	3116	86,215	M	Methyl-accepting chemotaxis protein [<i>Sinorhizobium meliloti</i>]	1.00E-87
18	3166	62,832	M	Methyl-accepting chemotaxis protein [<i>Sinorhizobium meliloti</i>]	8.00E-72
19	3432	49,672	C	Methyl-accepting chemotaxis protein McpH [<i>Caulobacter crescentus</i>]	1.00E-103
20	3530	76,917	M	Methyl-accepting chemotaxis protein McpA [<i>Caulobacter crescentus</i>]	5.00E-92

^aLocation, C = cytoplasm, M = inner membrane

within the inner membrane, however some, such as *tlpC* in *R. sphaeroides* are cytoplasmic (384).

The *Silicibacter* sp. TM1040 genome encodes at least 20 identifiable MCP proteins based upon their conserved protein domains and homology to known MCP proteins (Table 5.2). Each MCP protein follows the general arrangement of having one or more of the following: 1) a signal peptide and transmembrane region (unless cytoplasmic), 2) a PAS or PAC domain, 3) a HAMP domain, and 4) a methyl-accepting domain. Five of the 20 MCPs of TM1040 may be cytoplasmic, since they do not contain the transmembrane region and signal peptide. One of these putative cytoplasmic proteins is similar to the Aer receptor for aerotaxis, suggesting that strain TM1040 may be chemotactic to oxygen and does so by sensing oxygen within the cytoplasm. As evidence of this, I have observed accumulation of TM1040 cells around air bubbles by microscopy. Another cytoplasmic MCP with homology to an MCP from *Pseudomonas syringae* is also quite interesting because of its complexity. This MCP contains five PAS domains, four PAC domains, no HAMP domain and a methyl-accepting domain. The PAS domains are conserved protein domains found in signaling proteins and may be responsible for sensing a signal from the environment, usually involving a co-factor. The PAC domain is sometimes found associated with PAS domains as they contribute to correct folding of the PAS protein region. Given the large number of PAS domains in this MCP, it suggests that this protein can sense more than one type of signal.

The other MCP proteins are probably membrane associated since they contain either a signal peptide sequence and/or a transmembrane (hydrophilic) domain, usually overlapping one another. Most are closely related to those of other α -Proteobacteria such

as MCP's from *C. crescentus*, *S. meliloti*, *R. sphaeroides*, *R. capsulatus*, and *A. tumefaciens*. Most have only one or two identifiable transmembrane regions, although one (*mcp 5*) has five.

Silicibacter sp. TM1040 contains a relatively large number of MCP proteins compared to other bacteria. For example, *E. coli* has only five MCPs (Tar, Tap, Tsr, Trg and Aer) (345) while *R. sphaeroides* has 12 (383). In the closely related *Jannaschia* sp. there are only 7 while in *S. pomeroyi* there are none that can be readily identified. This suggests that strain TM1040 has adapted strategies for sensing a variety of chemical characteristics in its surroundings. The bacterium is chemotactic to a wide range of substrates, but of those tested only amino acids and DMSP metabolites produced a significant response. Considering the large number of *mcp* genes, it is probable that there are, as of yet unknown attractants for this bacterium.

Some of these attractants might be algal specific. One obvious example is DMSP, a compound that is produced by no other marine organism except algae and terrestrial plants. Another example is dinosterol, a compound specific to dinoflagellates, including *P. piscicida* (213). The ability to sense these compounds may then require unique chemoreceptors or other chemotaxis proteins. This concept is analogous to the ability of rhizobial species to sense and move towards plant flavonoids. Flavonoids are recognized by the NodD1 protein, which bind the flavonoids as co-inducers during transcriptional activation of genes containing the conserved *nod* box, including genes to produce nodulation factors. In *Silicibacter* sp. TM1040, there is a *nodD* homolog (gene # 2180, E = 3e-26) suggesting the possibility that TM1040 recognizes plant or algal - specific

Table 5.3. Proteins for a type IV secretion system from *Silicibacter* sp. TM1040

Gene	T ^a	Protein	Function	Top Blast			<i>A. tumefaciens</i> ^d	
				Species	Score ^b	Identity ^c	Score	Identity
1220	F	VirD2	Relaxase	<i>Reugeria</i> sp.	0.00E+00	79	5.00E-21	52
1221	F	VirD4	Coupling protein	<i>Reugeria</i> sp.	0.00E+00	91	6.00E-76	54
1243	R	VirB2	T-pilus subunit	<i>A. tumefaciens</i>	1.00E-09	36	1.00E-09	36
1242	R	VirB4	ATPase	<i>Reugeria</i> sp.	0.00E+00	93	2.00E-69	29
1241	R	VirB1	Transglycosylase	<i>Reugeria</i> sp.	0.00E+00	94	1.00E-12	37
1240	R	VirB5	T-pilus lipoprotein	<i>Reugeria</i> sp.	1.00E-140	97	1.00E-08	21
1239	R	VirB8	Channel component	<i>Reugeria</i> sp.	1.00E-117	99	8.10E-12	26
1238	R	VirB9	Outer membrane protein	<i>Reugeria</i> sp.	5.00E-95	93	3.00E-19	28
1237	R	VirB10	Channel component	<i>Reugeria</i> sp.	0.00E+00	94	2.90E-20	31
1236	R	VirB11	ATPase	<i>Reugeria</i> sp.	1.00E-175	96	1.50E-33	29
1234	R	VirB6	Channel component	<i>Reugeria</i> sp.	1.00E-173	88	0.004	27

^a The relative direction of transcription, F = forward, R= reverse

^b The Expect value from a BLAST search using the translated nucleotide sequence against Genbank

^c The percent identity in amino acid sequence

^d Results from a BLAST search specifically against the *A. tumefaciens* C58 genome (<http://depts.washington.edu/agro/genomes/c58/c58homeF.htm>)

products. In addition, there are several other genes with similarity to nodulation genes, including *nodT* for nod factor export (gene#378, E = 3.0e-61), as well as *nodB* (gene#2502, E = 3.0e-40), and *nodE* (gene#3669, E = 1.0e-106) for nod factor synthesis.

5.4 The *vir* pilus

All of the genes necessary for the production of a type IV secretion system with homology to the *Agrobacterium tumefaciens* *vir* pilus are present in the *Silicibacter* sp. TM1040 genome (Table 5.3). While the majority of these genes are most closely related to genes from an uncharacterized, marine *Reuveria* sp., the second most similar genes are from bacteria known to form symbiotic or pathogenic interactions with plants and animals, including *A. tumefaciens* and *R. meliloti*. These genes are located within two groups that are separated by ca. 16 kb. The first group consists of genes with homology to *virD2* and *virD4* (Table 5.3, gene#1220 and 1221, respectively) which encode for the relaxase and coupling proteins providing the energetics for export of transfer DNA (T-DNA). The second grouping consists of genes with homology to *virB1-11* excluding *virB3* and *virB7*, which are minor components of the transfer machinery (163). The VirB proteins are responsible for producing the inner membrane channel and pilus structure (80). In sum, all genes required to produce a type IV secretion system are present in the TM1040 genome. A BLAST search of the *S. pomeroyii* or *Janasschia* sp. CCS1 genomes using the VirD4 protein sequence from *Silicibacter* sp. TM1040, the most evolutionarily conserved component among type IV secretion systems (80) suggests that a type IV secretion system is not present in these other *Roseobacter* species. This may be a unique

characteristic of *Silicibacter* sp. TM1040 that is important for interactions with its dinoflagellate host.

5.5 Conclusion

The genome sequence of *Silicibacter* sp. TM1040 should provide a wealth of information to guide future experiments, especially those experiments that seek to understand more about the *Silicibacter*-dinoflagellate interaction. Motility and, most likely, chemotaxis are required for developing these relationships with the dinoflagellate. The genomic data indicates that there is a full and complete set of flagellar and chemotaxis genes present in the TM1040 genome. While many of the flagellar structural genes are similar to those of other *Roseobacter* species, the chemotaxis system is very different. Both *Jannaschia* sp. and *S. pomeroyi* do not contain multiple motor genes, flagellin genes, or chemotaxis genes, including a large number of MCP receptors, nor the genes for a type IV secretion system. Neither of these organisms known to be chemotactic toward algal products nor are they thought to maintain physical interactions with an algal host. Some of these sensory proteins may be especially important for establishing an intracellular lifestyle. It is likely that further analysis of the genome sequence and comparison with other *Roseobacter* species, will indicate further attributes unique to this dinoflagellate associated bacterium.

Chapter 6: Discussion

6.1 A model of the *Silicibacter* sp. TM1040 and *P. piscicida* interaction

It is a long standing hypothesis that marine algae harbor attached and/or intracellular bacteria that affect algal physiology. However, rarely have these bacteria been identified, nor is it known how they establish interactions with their algal host. While some bacteria associated with marine algae have been described, the *Silicibacter* sp. TM1040 and *P. piscicida* interaction provides one example of an algal-bacterial relationship that can be described at the genetic and molecular levels. Much of this puzzle is still missing, however, the pieces are coming together and can provide the framework for a model that can be dissected in future research (Fig. 6.1). Currently, this model involves aspects of DMSP metabolism, flagellar-mediated motility, chemotaxis, signal transduction and other cell-to-cell interactions inferred from genomic data. The following discussion will focus on this model.

Most marine bacteria phylogenetically related to genera of the *Roseobacter* clade catabolize DMSP and *Silicibacter* sp. TM1040 is one of several DMSP-degrading roseobacters associated with *P. piscicida*. However, not all of these roseobacters degrade DMSP by the same mechanism. While there are two major pathways known for the initial degradation of DMSP there are multiple pathways for the degradation of DMSP break down products. *Silicibacter* sp. TM1040 produces MMPA from DMSP without the production of methanethiol, acrylate or DMS, making it unique among the roseobacters identified in *P. piscicida* cultures. Thus, most, if not all of the DMSP consumed by *Silicibacter* sp. TM1040 is converted to MMPA. At least some of this MMPA is extracellular since it is present within supernatants of *Silicibacter* sp. TM1040 cultures.

Production of MMPA without the production of other DMSP breakdown products could be an important aspect of the *Silicibacter* sp. TM1040 and *P. piscicida* interaction. This compound, MMPA, is involved in several different eukaryotic biochemical pathways including an intermediate in DMSP synthesis and in the methionine salvage pathway (311, 365). In addition, the lyase pathway of DMSP degradation, which is not exhibited by *Silicibacter* sp. TM1040, leads to the production of acrylate, an inhibitor of bacterial and algal growth (350). One possibility is that *Silicibacter* sp. TM1040 consumes DMSP such that acrylate cannot be produced by other bacterial or algal lyases. This would benefit both the bacterium and the dinoflagellate by preventing elevated concentrations of acrylate. Future research should consider the possibility that DMSP demethylation by *Silicibacter* sp. TM1040 is involved in the bacterial - dinoflagellate interaction.

The bacterium, *Silicibacter* sp. TM1040, must be able to find its dinoflagellate host amongst a crowded population of microbial cells. This may be achieved through chemotaxis toward DMSP metabolites and amino acids. Both classes of compounds are produced by the dinoflagellate and *Silicibacter* sp. TM1040 is highly attracted to these compounds. The question arises whether or not the dinoflagellate excretes amino acids and/or DMSP and if the bacterium is able to sense low concentrations ($< 3 \mu\text{M}$) of these compounds as they diffuse away from the dinoflagellate cell. Studies measuring extracellular DMSP in other dinoflagellate cultures indicate that DMSP is in fact present, albeit at low concentrations in the extracellular milieu ($< 1\%$ of total DMSP) (191). Additionally, cytoplasmic contents, including amino acids and DMSP, could be excreted during dinoflagellate feeding, cellular division or from environmental stress.

As these compounds diffuse away from the dinoflagellate cell, the chemical gradient produced quickly becomes smaller making it necessary for the bacterium to sense very low concentrations of these attractants. Studies in a similar α -Proteobacterium, *Rhodobacter sphaeroides*, show that when single cells are observed by microscopy attractants delivered to the cells at < 1 nM concentration produce a chemotactic response. Furthermore, *Silicibacter* sp. TM1040 contains multiple homologs of chemotaxis proteins, including those involved in adaptation to the chemical signal, which would have the effect of increasing the range of concentrations that the bacterium is able to sense. Also, rather than sensing just one compound produced by the dinoflagellate, the bacterium actually senses multiple compounds including DMSP, methionine and valine which ultimately enhances chemotaxis toward the dinoflagellate cell. Therefore, even though concentrations of intracellular DMSP are relatively small (< 4 μ M), *Silicibacter* sp. TM1040 would still be attracted to the dinoflagellate cell surface through chemotaxis to the sum total concentration of all attractants produced by the dinoflagellate.

These attractant compounds or others may be sensed by other signal transduction systems as well, upregulating the expression of genes required for the interaction. For example, the occurrence of apparently intracellular *Silicibacter* sp. TM1040 requires several genes involved in flagellar-mediated motility, some of which are also involved in many other cell functions. The *flaA* gene product is required for the apparent intracellular occurrence of *Silicibacter* sp. TM1040 and is most likely involved in flagellar biogenesis or energetics which may allow the bacterium to access intracellular spaces of the dinoflagellate (i.e. swimming up the feeding tube or through openings in the

dinoflagellate cell wall). The *ctrA/cckA* gene products are also required for this phenotype, but may actually be required much sooner in the interaction since they are involved in sensing environmental signals which ultimately results in the expression of several different genes, including those for flagellar biogenesis or motility, chemotaxis, pili, and other cell cycle dependent processes. The signal(s) sensed by this sensor kinase/response regulator pair are unknown. One hypothesis might be that these proteins sense some products of dinoflagellate metabolism such as DMSP or amino acids which ultimately upregulate the transcription/translation of genes required for the physical interaction, such as those genes involved in chemotaxis, motility or other cell-to-host-cell communication proteins.

The *Silicibacter* sp. TM1040 genome contains several groups of genes that may allow the bacterium to secure a foothold on the dinoflagellate cell surface and/or to communicate with its dinoflagellate host. For example, most of the genes required to make a pilus can be found in the *Silicibacter* sp. TM1040 genome. A bacterial pilus would allow for a more permanent and secure attachment to the dinoflagellate cell surface beyond any initial connections. In addition, a type IV secretion system, or *vir* pilus appears to be encoded by the *Silicibacter* sp. TM1040 genome. One hypothesis may be that this *vir* pilus allows the bacterium to communicate with the dinoflagellate host by injecting bacterial DNA into the dinoflagellate cell encoding for host modifying proteins. These proteins might then alter dinoflagellate physiology to the benefit of the bacterium (i.e. increased production of DMSP) or both the bacterium and the dinoflagellate. In *A. tumefaciens* the *vir* pilus induces abnormal plant cell growth and the synthesis of opines by plant cells, which the bacterium utilizes as a carbon source. Such an interaction

between *Silicibacter* sp. TM1040 and *P. piscicida* is unknown, but provides a reasonable model that can be tested.

Finally, there are some proteins encoded by the *Silicibacter* sp. TM1040 genome similar to nodulation genes present in *Rhizobium* species. These include genes for the synthesis and export of nod factors. One hypothesis might be that the dinoflagellate responds to these nod factors as they diffuse away from the bacterium. In plants these Nod factors cause changes in root hair morphology which allow the bacteria access to an infection thread. In the bacterial-dinoflagellate interaction an analogous situation may occur in which Nod-like factors cause changes in the dinoflagellate cell wall structure, or induce formation of the feeding tube (i.e. peduncle) allowing *Silicibacter* sp. TM1040 access to intracellular spaces. To carry this analogy even further, *Rhizobia* produce Nod factors in response to plant flavanoids. *Silicibacter* sp. TM1040 may produce Nod-like factors in response to dinoflagellate products, including DMSP, MMPA and one or more amino acids. Such a sensing mechanism would most likely rely upon a two-component signal transduction system, a good candidate being the CckA/CtrA proteins.

6.2 Significance to microbial ecology

In this study, the microbial community associated with *P. piscicida* and *Pfiesteria*-like dinoflagellates was shown to be representative of marine bacterial communities in the environment. This offers good evidence that data obtained using laboratory dinoflagellate cultures can approximate what occurs in the environment. Bacteria foreign to the marine habitat, or bacteria that could have come from anthropogenic sources such as enteric bacteria were not detected and many of the bacteria

that were isolated and characterized, namely *Roseobacter* species, displayed characteristics similar to those of other *Roseobacter* species observed in ocean water samples (i.e. DMSP degradation).

While the bacterial community is representative of what is generally found in the marine environment there is a strong relationship in community structure between *Pfiesteria*-associated bacterial communities and those associated with other dinoflagellates. For example, α -Proteobacteria are the most abundant bacterial species associated with both *Alexandrium* and *Pfiesteria* dinoflagellate cultures, with *Roseobacter* species being the most prevalent. In the environment, *Roseobacter* species are also prevalent but normally other α -Proteobacterial species dominate, such as the SAR86, SAR83, SAR11 and SAR116 clusters of marine bacterioplankton (138). The reason for a high abundance of *Roseobacter* species in these dinoflagellate cultures is interesting and suggests that they possess unique abilities that help to promote their survival within the dinoflagellate cultures. Indeed, *Roseobacter* species abundance does increase during some dinoflagellate blooms (405). The studies of DMSP metabolism and mechanisms for *Roseobacter*-dinoflagellate interactions presented here may offer an example of how these bacteria have come to dominate environments surrounding dinoflagellates and other algal cells.

Several bacteria isolated from *P. piscicida* culture were shown to degrade DMSP and these bacteria were identified as *Roseobacter* species. A total of nine different bacteria were isolated and tested for DMSP degradation, but only the *Roseobacter* species degraded DMSP. This extends an earlier hypothesis by Gonzalez et al. that *Roseobacter* are the primary consumers of DMSP in marine environments (146).

However, unlike the study by Gonzalez et al. the bacteria described here were isolated from a dinoflagellate culture originally obtained from the estuarine waters at less than full strength seawater. These bacteria grow best at salinities less than full strength seawater (10 - 15 psu). Therefore, these unique estuarine DMSP-degrading *Roseobacter* species will be useful in comparative studies of DMSP catabolism.

6.3 Significance of this work to algal bloom ecology

The development of an algal bloom is a complicated process that is regulated by a variety of factors, not the least of which is the physiology of the algal species and its associated bacteria. While physical factors such as wind, light, and currents influence bloom production, nutrients are a major contributor to algal growth. A diverse community of heterotrophic bacteria exists in marine surface waters and these bacteria are able to rapidly influence nutrient concentrations. Heterotrophic bacteria consume dissolved and particulate organic matter and regenerate essential nutrients for algal growth including ammonia, nitrate and phosphorous (36). In some circumstances bacteria may compete for available resources such as for iron (175). While these processes are generally well-studied at the population level, there are significant impacts upon algal growth that take place at the species level. For example, it has become increasingly clear that the production and activity of DMSP-degrading *Roseobacter* species is correlated with blooms of DMSP-producing algae, especially dinoflagellates.

The *Silicibacter* sp. TM1040 and *P. piscicida* relationship provides one example to explain how *Roseobacter* species may be involved in bloom formation. In the laboratory, the presence of *Silicibacter* sp. TM1040 in *P. piscicida* culture aids in

dinoflagellate growth. This may also be important for the initiation of a *P. piscicida* bloom event. Blooms of *P. piscicida* generally occur on a small scale having low cell densities ($< 10^4$ per ml) and occur in years of low rainfall during July and August (75, 143). Assuming that salinity, temperature and other physical factors are appropriate then *Silicibacter* sp. TM1040 is most likely to influence other factors including prey capture, nitrogen availability and the action of viruses or other bacteria. During prey capture, if the prey source produces DMSP and contains a DMSP lyase then the conversion of DMSP to acrylate and DMS may lead to prey avoidance by *P. piscicida* as it does for some other heterotrophic dinoflagellates (395). *Silicibacter* sp. TM1040 could prevent this by consuming DMSP before it is converted to acrylate and DMS.

Nitrogen is often a limiting factor for algal bloom formation (300). *P. piscicida* has been shown to absorb nitrogen in the form of ammonia from its surroundings in order to balance carbon assimilation from prey algae (224). *Silicibacter* sp. TM1040 does not fix atmospheric nitrogen, but could provide ammonia as a waste product from the degradation of nitrogen containing compounds including amino acids. While many other bacteria may also produce nitrogenous compounds, *Silicibacter* sp. TM1040 physically associates with the dinoflagellate and is thus in closer proximity to provide such compounds.

Viruses may also affect bloom formation. While no viruses of *P. piscicida* have been identified there are viruses that infect some *Roseobacter* species (312). In this case, if the abundance of *P. piscicida* is dependent upon the presence of *Silicibacter* sp. TM1040 for the reasons stated above or others, then the presence of a bacteriolytic virus (i.e. *Roseophage*) may cause a drop in the abundance of *Silicibacter* sp. TM1040 such

that the bacterium is not present to aid in dinoflagellate growth. Viruses could be a significant regulator of *Roseobacter* species abundance given that up to 50% of bacterial production in marine surface waters is converted to dissolved organic matter by lytic bacteriophages (130).

An intimate relationship, perhaps a symbiotic relationship, exists between *Silicibacter* sp. TM1040 and *P. piscicida*. In laboratory cultures, *P. piscicida* does not grow without this bacterium or other as yet unidentified *Roseobacter* species. There is no reason to assume that such a relationship could not exist between other *Roseobacter* species and algal cells, nor is it assumed that *Silicibacter* sp. TM1040 is specific for only *P. piscicida* dinoflagellates. A similar relationship might exist for other, toxic dinoflagellate species, such as saxitoxin-producing *Alexandrium* species. Indeed, a *Roseobacter* species has been shown to attach to *A. tamerenses* and aid in dinoflagellate growth (52, 96, 134, 168). This illustrates what might be a more global picture of bacterial-dinoflagellate interactions, reminiscent of bacterial - plant pathogens interactions where individual strains specifically infect only a given range of plant species, a process governed by chemical signaling. This should prove to be a useful ecological model for future studies of the *Silicibacter* sp. TM1040 and *P. piscicida* interaction. The involvement of *Roseobacter* upon algal bloom formation holds major consequences for how researchers view dinoflagellate-associated bacteria. It might be predicted that without *Silicibacter* sp. TM1040, *P. piscicida* does not reach bloom densities, perhaps explaining some fluctuations in bloom activity. Confirmation of such a prediction can only be made through further experimentation both in laboratory and field settings.

Appendix A: Media and Buffers

A.1. Media

A.1.1. Luria Bertani (LB) Broth

1. 10 g Tryptone
2. 5 g NaCl
3. 5 g Yeast Extract
4. 1 Liter RODI water

Autoclave sterilize

A.1.2. Luria Bertani (LB) Agar

1. 10 g Tryptone
2. 5 g NaCl
3. 5 g Yeast Extract
4. 15 g Bacto-agar
5. 1 Liter RODI water

Autoclave sterilize

A.1.3. Marine Broth (½ x 2216)

1. 17.5 g 2216 marine broth powder
2. 1 Liter RODI water

Autoclave sterilize

A.1.4. Marine Agar (½ x 2216)

1. 17.5 g 2216 marine broth powder
2. 15 g Bacto-agar
3. 1 Liter RODI water

Autoclave sterilize

A.1.5. Marine Motility Agar (½ x 2216)

1. 17.5 g 2216 marine broth powder
2. 3 g Bacto-agar
3. 1 Liter RODI water

Autoclave sterilize

A.1.6. HIASW Broth

1. 25 g Heart infusion powder
2. 15 g Instant ocean sea salts
3. 1 Liter RODI water

Autoclave sterilize

A.1.7. HIASW Agar

1. 25 g Heart infusion powder
2. 15 g Instant ocean sea salts
3. 15 g Bacto-agar
4. 1 Liter RODI water

Autoclave sterilize

A.1.8. Basal Minimal Medium

1. Prepare the following solutions separately.

Base Medium:

Chemical	Amount
Tris-HCl	12.1 g
NH ₄ Cl	1.0 g
K ₂ HPO ₄ • 7H ₂ O	0.0075 g
Instant Ocean (ASW)	15 g
Distilled H ₂ O	1 L

pH ~ 7.5 using HCl and autoclave in a glass bottle. Cool to 50°C.

FeSO₄ Solution:

Chemical	Amount
FeSO ₄	20 g
Distilled H ₂ O	100 ml

Filter sterilize into a 125 ml bottle and store at room temp.

Balch's Vitamins:

Chemical	Amount (mg)
D-Calcium Pantothenate	5
Vitamin B12	0.1
p-Aminobenzoic Acid	5
Biotin	2
Folic Acid	2
Pyridoxine HCl	10
Thiamine HCl	5
Riboflavin	5
Nicotinic Acid	5

Filter sterilize into 1 ml aliquots and store at -20°C or -80°C.

A carbon source: sterile 100% glycerol:

Pour ~100 ml of glycerol from the stock bottle into a glass beaker and microwave for 45s, then pour into a 125 ml glass bottle. Autoclave for 20 minutes.

2. When the base medium has cooled to 50 °C then add 1 ml Balch's vitamins, 1 ml of FeSO₄ solution and 20 ml 100 % sterile glycerol. Cap and gently swirl to mix.
3. For BM agar or BM motility agar, add 20 g or 3.0 g of bacto-agar to the base medium before autoclaving, respectively. Autoclave in an Erlenmeyer flask and leave a stir bar in the flask during sterilization. Complete all procedures as for the broth except pour medium into petri dishes after all additions (vitamins, glycerol and FeSO₄) have been made.

A.1.9. F/2 Metals

To 950 ml ddH₂O add:

1. 3.15 g FeCl₃ • 6H₂O
2. 4.36 g Na₂EDTA • 5H₂O
3. 1.0 ml CuSO₄ • 5H₂O (Stock: 9.8 g/L dH₂O)
4. 1.0 ml Na₂MoO₄ • 2H₂O (Stock: 6.3 g/L dH₂O)
5. 1.0 ml ZnSO₄ • 7H₂O (Stock: 22.0 g/L dH₂O)
6. 1.0 ml CoCl₂ • 6H₂O (Stock: 10.0 g/L dH₂O)
7. 1.0 ml MnCl₂ • 4H₂O (Stock: 180.0 g/L dH₂O)
8. Make the final volume 1.0L in dH₂O and filter sterilize

A.1.10. F/2 Vitamins

To 950 ml dH₂O add:

1. ml Vitamin B12, Cyanocobalamin (Stock: 1.0 g/L dH₂O)

2. 10.0 ml Biotin (Stock: 0.1 g/L dH₂O)
3. 200.0 mg Thiamine HCL
4. Make the final volume 1.0L in dH₂O and filter sterilize

A.1.11. 35 ppt F/2 Medium

1. Add 900 ml of de-ionized water to a 2 L Erlenmeyer flask.
2. Weigh out 40 grams of Instant Ocean Sea-salt. Add this to the water.
3. Stir with a stir bar for at least 5 minutes.
4. Add 1 ml of stock trace metals (see formula), 500 µl of stock vitamins (see formula), 1 ml of 75 g/L NaNO₃ and 1 ml of 5g/L NaH₂PO₄. Stir with the stir bar and bring the volume up to 1000 ml.
5. Bring the media and all other materials to the Laminar Flow Hood. Follow all necessary procedures for working in the Laminar Flow Hood.
6. Using two 0.22 µm Nalgene Bottle Top Filters, filter sterilize the media into two 500 ml bottles. Flame the lip of the bottle before opening and hold the cap in your fingers to prevent contamination
7. Cap and wrap foil around the bottle as the metals and vitamins are sensitive to light. Label the bottle with your name, date and “Sterile 35 ppt F2.”
8. Store the media at 4°C.

A.2. Solutions

A.2.1. 10 ppt Artificial Seawater (ASW)

1. Add 900 ml of distilled water to a 2 L Erlenmeyer flask.

2. Weight out 11 grams of Instant Ocean Sea-salts.
3. Add the Sea-salts to the water and stir with a stir bar for at least 5 minutes. Bring the volume up to 1 L with de-ionized water.
4. Dispense the 10 ppt ASW into two 500 ml bottles and autoclave.

A.2.2. 50X TAE

Reagent	Amount	Final Concentration
Tris Base (Boehringer Mannheim cat.# 604205)	242 g	2M
Glacial Acetic Acid (Fisher cat.# A38-212)	57.1 ml	1M
Na ₂ EDTA (Fisher cat.# BP120-1)	37.2 g	50 mM
ddH ₂ O	Bring to volume	1 Liter

pH the solution to 8.0 with concentrated HCl

A.2.3. Tris/EDTA (TE) (0.5 M)

Reagent	Amount (ml)	Final Concentration (mM)
1 M Tris, pH 7.6	10	10
0.5 M EDTA	2	1
ddH ₂ O	Bring to volume	1 Liter

A.2.4. Potassium acetate

1. 20 ml ddH₂O
2. 29.5 ml of Glacial acetic acid
3. Potassium hydroxide pellets until pH = 4.8
4. Bring to 100 ml with ddH₂O

A.2.5. Deionized Formamide

Add 20 g of bead resin (AGX501-XB, Bio-Rad 143-6424) to 500ml of molecular grade formamide and stir overnight at 4°C. Remove resin by pouring through filter paper (Whatman #1 or similar). Discard in the trash and store deionized formamide at 4°C in a glass bottle wrapped in aluminum foil.

A.2.6. 40% Acrylamide:bisacrylamide (37.5:1)

(Pure acrylamide, Bio-Rad, 161-0101)

Warning: Acrylamide is a neurotoxin, where gloves, coat, goggles and a respirator.

Reagent	Amount	Final Concentration
Acrylamide	194.8 g	38.96 %
Bisacrylamide	5.19 g	1.04 %
ddH ₂ O	Bring to volume	
Final Volume	500 ml	

Filter through Whatman #1 analytical grade paper. Store in glass bottle at 4°C, wrapped in foil.

A.2.7. DGGE Denaturant Stock Solution A (DSSA) (0% Denaturant)

Reagent	Amount	Final Concentration
40% Acrylamide	20 ml	8%
50X TAE	2 ml	1X
ddH ₂ O	Bring to volume	
Final Volume	100 ml	

De-gas for at least 30 minutes and store at 4° C wrapped in foil. Use fresh.

A.2.8. DGGE Denaturant Stock Solution B (DSSB) (100 % Denaturant)

Reagent	Amount	Final Concentration
40% Acrylamide	20 ml	8%
50X TAE	2 ml	1X
Urea	42 g	42%
Formamide	40 ml	40%
DdH ₂ O	Bring to volume	

Final Volume	100 ml	
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Heat slowly at low temperature and stir to dissolve urea. Filter through Whatman

#1 analytical grade paper. De-gas for at least 30 minutes and store at 4° C

wrapped in foil. Use fresh.

A.2.9. Ammonium Persulfate (APS)

Reagent	Amount	Final Concentration
Ammonium Persulfate	0.1 g	10%
ddH ₂ O	1ml	

Make fresh when needed and store at 4°C.

A.2.10. DGGE Running Dye

Reagent	Amount	Final Concentration
Sucrose (Fisher cat. #S5-3)	4 g	40%
Bromophenol blue (Sigma cat.# B-7021)	0.025 g	.25%
Xylene cyanol (Sigma cat.# X-0377)	0.025 g	.25%
ddH ₂ O	10 ml	

Filter sterile w/syringe and disc, aliquot and store at 4°C.

A.2.11. Chemotaxis Buffer

1. 10 g instant ocean sea salts
2. 50 ml 1 M Tris HCL, pH 7.7
3. Bring to 1 L ddH₂O
4. Autoclave sterilize

A.2.12. HPLC Running Buffer (buffer A)

1. 25 ml acetonitrile (HPLC grade)

2. 1.8 ml 58% Phosphoric acid
3. Bring to 1 L with ddH₂O

A.2.13.HPLC Storage Buffer (buffer B)

1. 800 ml acetonitrile (HPLC grade)
2. Bring to 1 L with ddH₂O

A.2.14. FISH Hybridization Buffer

<u>Chemical</u>	<u>Amount</u>	<u>Final</u>
Formamide	20 ml	20%
NaCl	5.3 g	.9M
SDS	.1 g	.1%
1M Tris-HCl	2 ml	20 mM
Water		100 ml

Filter sterilize

A.2.15. FISH Wash Buffer

<u>Chemical</u>	<u>Amount</u>	<u>Final</u>
NaCl	.4 g	70 mM
SDS	.1 g	.1%
.5 M EDTA	1 ml	5 mM
1M Tris-HCl	2 ml	20 mM
Water		100 ml

Filter sterilize

Appendix B: Detailed Protocols

B.1. Culturing CCMP 768 (*Rhodomonas* sp.)

Protocol for culturing new samples received from CCMP:

1. Remove sterile 35 ppt F2 from the refrigerator.
2. Bring all necessary materials to the Laminar Flow Hood and perform all necessary procedures to begin working there.
3. Add 35 ml of sterile 35 ppt F2 to a 75 ml vented cap tissue culture flask using a 25ml plastic pipette. Use sterile technique.
4. Transfer 5 ml of a two-week-old culture of *Rhodomonas* sp. (CCMP 768). Using a 5 ml glass or plastic pipette. Keep the flask tilted.
5. Immediately cap the flask and label it with your name, the date and “*Rhodomonas* (CCMP 768), AXENIC.”
6. Using sterile technique spread a 100 μ l aliquot of the original culture of 768 on $\frac{1}{2}$ x 2216 marine agar to make sure the culture we have received is axenic. Keep the flask tilted. Incubate the agar plate at 20 °C for 3 days, examine, and if there are no bacterial colonies discard the plate. If there are colonies, the *Rhodomonas* was not axenic and the cultures are contaminated. Discard them immediately.
7. Incubate the culture at 20 °C, 80% humidity in the 14-hour light, 10-hour dark, environmental room. Leave for 2 weeks, occasionally checking for growth under the inverted microscope.

Preparation of Stock culture:

1. After two weeks, prepare the Laminar Flow Hood and bring the culture and all other necessary materials to it.

2. Add 35 ml of sterile 35 ppt F2 to a 75 ml vented cap tissue culture flask using a 25 ml plastic pipette. Keep the flask tilted.
3. Transfer 5 ml of the two-week-old culture to the new flask using a 5 ml glass or plastic pipette. Keep the flask tilted.
4. Cap each flask immediately and label the new culture with your name, the date and “Rhodomonas (CCMP 768) AXENIC, Stock.” Label the old culture as working or experimental.
5. Using sterile technique spread a 100 μ l aliquot of the two-week-old culture of 768 on $\frac{1}{2}$ x 2216 marine agar to make sure the culture is axenic. Keep the flask tilted. Incubate the agar plate at 20C for 3 days, examine, and if there are no bacterial colonies discard the plate. If there are colonies, the Rhodomonas was not axenic and the cultures are contaminated. Discard them immediately.
6. Dip a glass spreader into 100% ethanol and flame it to remove the ethanol. Allow the spreader to cool in the hood and use it to spread the sample on the agar plate.
7. Incubate the plate at 20 °C for 3 days.
8. If after three days no growth has occurred, then use the old culture for feeding and/or experiments. Repeat steps 1 through 4 for sub-culturing the stock culture.
9. If after three days growth has occurred, then immediately dispose of all cultures with bleach and obtain new axenic cultures.

B.2. Culturing CCMP 1828

Protocol for culturing new samples received from CCMP:

1. Prepare the Laminar Flow Hood and bring all necessary materials to it. Observe BSL-3 procedures.
2. Transfer 20 ml of sterile 10 ppt ASW to a 75 ml vented cap tissue culture flask using a 25 ml plastic pipette. Keep the flask tilted and recap immediately.
3. Transfer 15ml of CCMP 1828 to the flask using a 25 ml plastic pipette. Keep the flask tilted.
4. Obtain an axenic Rhodomonas culture marked “FEEDING” and transfer 20 ml to a 50 ml sterile conical tube using a sterile pipette. Keep the flask tilted.
5. Centrifuge the conical tube at 2500 r.p.m. (970 x g) for 2 minutes, 30 seconds, on the I.E.C. centrifuge.
6. Using sterile technique, remove the supernatant and add 5 ml of sterile 10 ppt ASW using a glass or plastic pipette.
7. Vortex the tube to resuspend the algal pellet.
8. Add all 5 ml to the flask containing CCMP 1828 (“Pfiesteria-like) using a 5 ml glass or plastic pipette. Keep the flask tilted and observe BSL-3 precautions.
9. Immediately cap the flask and label it with your name, the date and “CCMP 1828, Stock.”
10. Dispose of the remaining Rhodomonas culture or mark it as EXPERIMENTAL.
11. Incubate CCMP 1828 at 20 °C, 80% humidity in the 14-hour light, 10-hour dark, environmental room (5003). Incubate for 2 weeks, viewing periodically for growth using the inverted microscope. A healthy culture should have actively motile dinoflagellates.
12. Preparation of Stock culture:

13. After two weeks prepare the Laminar Flow Hood and bring all necessary materials to it.
14. Using sterile technique, transfer 15 ml of sterile 10 ppt ASW to a 75 ml vented cap tissue culture flask using a 25 ml plastic pipette. Keep the flask tilted.
15. Add 20 ml of stock CCMP 1828 to the flask using a 25 ml plastic pipette.
16. Carefully cap and label the flask with your name, the date and “CCMP 1828, Stock.” Label the old stock culture as experimental, or dispose of with bleach. Observe BSL-3 safety precautions.
17. Feed with axenic Rhodomonas following the protocol listed above in steps D through I.
18. Repeat sub-culturing the stock culture every two weeks.

Notes:

Always use the Laminar flow hood for working with stock cultures.

Always clean the working area before and after use with disinfectant.

Always keep flasks angled when transferring liquids and try to recap as soon as possible.

Be careful not to touch the inside of the cap to any surface including your hands and try to minimize its exposure to open air.

Take precautions when working with a fish-killing organism. Follow BSL-3 safety precautions.

Check the axenic Rhodomonas and culture media for contamination every week by plating on 2216 ½ marine agar.

The final volume of all stock cultures is 40 ml.

B.3. Chemotaxis assay for *Silicibacter* sp. TM1040 using Palleroni chambers

References:

1. Adler, J. 1966. Chemotaxis in bacteria. *Science* 153:708-16.
2. Palleroni, N. J. 1976. Chamber for bacterial chemotaxis experiments. *Appl. Environ. Microbiol.* **32**:729-730.

Procedure:

1. Using a flamed loop, insert it into the edge of a motile colony of TM1040 growing on ½ X2216 motility agar and transfer to 5 ml of ½ X2216 in a large test tube.
2. Incubate at 30 °C for 20 h or until the O.D._{.600} 0.3 - 0.4.
3. Transfer the culture to a 15 ml Falcon tube and centrifuge the culture for 10 min. at 3400 r.p.m. using the I.E.C. Centra CL2 tabletop centrifuge and the 215 rotor (4000 x g).
4. Meanwhile, dry agar plates at 42 °C for 30 min. and label them. Add 1 ml of sterile chemotaxis buffer to several centrifuge tubes (one for each capillary) and 0.9 ml to two more centrifuge tubes per capillary for dilutions.
5. When centrifugation is complete, aspirate the supernatant with a 20 gauge needle carefully removing all of spent medium without removing the pellet.
6. Resuspend the pellet in 3 ml sterile chemotaxis buffer in a small test tube and vortex vigorously for 2 minutes.
7. Add additional chemotaxis buffer until the O.D._{.600} is exactly 0.3.
8. Place the culture at 30°C for 1.5 hours of starvation.

9. Meanwhile, make dilutions of attractants in chemotaxis buffer and label plates.
10. Make a 1:10 dilution of the starved culture in chemotaxis buffer. Immediately place 500 μl of washed culture into Palleroni chambers, filling the canals first followed by the wells. There are four chambers per plate so a total of 2 ml washed culture will be needed per plate.
11. Fill clean 1 μl capillaries with sterile attractants and wipe the capillary off with a Kimwipe. Place filled capillaries into the canal of each chamber, be sure to include a control, chemotaxis buffer only.
12. Immediately start a timer and incubate the chambers with Petri dish covered for 2 hours.
13. Remove the capillaries one at a time using clean forceps and wipe the capillary clean using a Kimwipe. Insert one end into the capillary holder and dispense the contents of the capillary into 1 ml of chemotaxis buffer in a labeled centrifuge tube for an initial 10^{-3} dilution. Repeat for each capillary.
14. Make two ten-fold dilutions on the initial 10^{-3} dilution of capillary contents by placing 100 μl into 900 μl of chemotaxis buffer. Be sure to vortex the initial capillary contents and in between dilutions thoroughly.
15. Spread 100 μl of the ten-fold dilutions onto HIASW agar plates for a final 10^{-4} and 10^{-5} plate dilutions. Spread 2 plates per dilution = 4 plates needed per capillary.
16. Incubate the plates at 30°C for 20 hours.

17. Count the number of colonies on each plate and calculate the concentration of bacteria in the capillary as $\text{CFU / ml} = \text{CFU/plate} \times \text{dilution factor}$ (either 10^4 or 10^5).

B.4. Test for DMSP Catabolism by Bacterial Isolates

Procedure:

1. Inoculate 35 ml of 1/2X 2216 broth containing 40 μl of 0.5 M DMSP in a 250 ml Erlenmeyer flask with a colony of bacteria using a sterile toothpick. Shake overnight or until cells achieve stationary phase at an appropriate temperature. A maximum of five isolates plus one control can be tested in this experiment.
2. Harvest cells by centrifugation and wash 2X with 30 ml of 10 ppt ASW. Resuspend in 30 ml of 10 ppt ASW.
3. Label two sets of 15 ml amber bottles, 15 per set. Label one set “DMS/DMSP” and the other “MMPA/MPA/Ac.” Label all of the bottles with the strain number and time points I – 13.
4. Add 60 μl of DMSP to the first culture and mix well by inverting several times. Aliquot 2 ml into each of the amber vials using a 35 ml pipette. Immediately cap all bottles, place them in the 30 °C shaker and start two timers, one counting up and the other counting down from 15 minutes.
5. Measure DMS in 500 μl of headspace gas in an amber bottle from the “DMSP/DMS” set. Immediately add 200 μl of 5M NaOH to the bottle through the septum using the liquid Hamilton syringe. Briefly shake to mix and place the bottle at 30 °C for incubation overnight.

6. Remove an amber bottle from the “MMPA/MPA/Ac” set and uncap. Remove 1 ml and place into a microcentrifuge tube, centrifuge on max speed (10,000 x g) for 20 seconds, place supernatant into a GC vial and freeze both the pellet and supernatant at –20 °C. Be sure to label them with the strain and time the sample was taken according to the timer counting up.
7. After 15 minutes stop the timer, reset it to 15 minutes and repeat step 4 – 6 for the next isolate to be tested. Every 15 minutes then, two new sets of samples will be added to the incubator until all amber bottles are in the incubator.
8. Every 30 minutes after the start of the experiment (according to the timer counting up), repeat steps 5 and 6 for the next set of amber bottles from each isolate in the incubator until all bottles have been sampled. Just as each set of bottles from each isolate was started sequentially, so will they end sequentially.
9. Thaw the supernatants and measure MMPA, MPA and acrylate in 50 µl injections by HPLC using the Chemstation method file Miller1.m. In addition, measure MMPA, MPA and acrylate in dilutions of standards of these chemicals from 1 – 1000 µM.

B.5. Cloning of EZ:TN <KAN R6K γ ori> and Flanking Genomic DNA

1. Extract DNA by the CTAB method.
2. Digest the extracted DNA with Nco I:

Reagent	Amount	Final
Genomic DNA	1 - 4 µl	2 µg
Nco I (10 U/ µl)	1 µl	10 U
NEBuffer 1,2,3 or 4	2 µl	1 X
ddH ₂ O	13 - 16 µl	20 µl

Incubate at 37 °C for 90 min., then heat inactivate for 20 min. at 80 °C.

3. Ligate the digested DNA back on itself by adding the following to the digestion:

Reagent	Amount	Final
T4 DNA Ligase	1 μ l	
10 X ligase buffer	4 μ l	1 X
ddH ₂ O	15 μ l	40 μ l

Incubate the ligation at 16 °C for 4 hours.

4. Desalt the ligated DNA:
- Add 20 μ l of the ligation to a microcentrifuge tube
 - Add 10 μ l of tRNA (10 μ g per μ l in dH₂O) followed by 20 μ l of 7.5 M Ammonium acetate.
 - Add 100 μ l of absolute ethanol and incubate on ice for 15 min.
 - Centrifuge at 14,000 x g for 15 min. at 4 °C. Decant the supernatant.
 - Wash the pellet with 1 ml 70 % ethanol. Centrifuge again, decant the supernatant.
 - Dry the pellet in speed vac for 15 min. and resuspend the pellet in 20 μ l sterile RODI water.
5. Remove electrocompetent DH5 α λ pir *E. coli* from the -80 °C freezer and transfer 50 μ l to pre-chilled microcentrifuge tubes on ice containing 1 μ l of desalted DNA and vortex.
6. Add cell/DNA mixture to chilled cuvettes (0.2 cm gap) and electroporate at 25 μ F and 200 Ohms for ~ 4.2 – 4.8 ms.
7. Immediately add 1 ml of pre-warmed LB to the cuvette and transfer to a small test tube.
8. Incubate at 37 °C for at least 4 h hours (no more than 20 h) with shaking.

9. Centrifuge 1 ml of culture and resuspend in ~100 μ l of the supernatant. Spread onto LB+Kan50 agar plates. Incubate at 37 °C overnight.
10. Pick and streak several individual colonies onto fresh LB + Kan50 and extract plasmid DNA using Qiagen.
11. Sequence genomic DNA flanking the transposon using EZ:TN primers supplied with the transposome kit (Epicentre catalog# TSM08KR).

B.6. Complete Digital Video Analysis of Cell Motility

Export a Tiff Sequence

1. Open the video clip in Adobe Premiere.
2. Set the in and out points in the video approximately 1 second apart.
3. File > Export Clip > Movie.
4. Choose a destination folder and a name. The name will be appended with a two-digit serial number for each frame.
5. Click on settings. Under general, the file type is .tiff sequence and the range is in/out points. Under video, choose the 4:3 aspect ratio and set the image size to 754 x 510. Choose a frame rate of 15 fps (this means that every other frame from the original clip will be exported. Thus, the time between each frame is not 1/30th of a second (0.03 s), but 1/15th of a second (0.067 s). Press Okay.
6. Press Save and the Tiff sequence will be exported to the destination folder you chose with your file name and a two-digit serial number.
7. Repeat for each section of video to be analyzed.

“Save without Alpha” Batch Action Command

1. Open Adobe Photoshop, open any image and choose Window > Actions.
2. At the bottom of the window choose the folder icon to create a new action. Name the new action , Save without Alpha. Press record.
3. Choose File > Save as. Choose .tiff for the file format and click the box , Save as copy. Press save.
4. Choose File > close. Click the Stop button on the bottom of the actions window.

Remove Adobe Alpha Channel From Tiff Sequence

1. Choose File > Automate > Batch. In default actions choose the Save without Alpha action. The source should be folder. Press choose and go to the folder with the Tiff sequence exported from Premiere.
2. Click the box ‘suppress color profile warnings.’
3. The destination should be folder. Press choose and go to the folder with the Tiff sequence exported from Premiere. Make this also the destination folder, the files will be overwritten.
4. Click the box ‘Override action “save as” commands.’ The file name should be document name and extension. Click Okay.
5. The tiff sequence will be opened, saved and closed one frame at a time automatically. When it is finished, repeat for each tiff sequence.

Determine Percent Population Motile

1. Open IPLab v 3.5.5, be sure the dongle is in place if you have it.

2. Choose Window > Frame to sequence > Browse. Find the first file of the tiff sequence saved without the α channel in Adobe Photoshop. Click on it and press open. Press OK.
3. The tiff sequence will be reassembled into a movie that you can click through one frame at a time using the time buttons on the time of the movie window.
4. Choose Enhance > Normalization. Increase/decrease brightness and contrast to achieve the best image of the cells such that they can easily be distinguished from the background.
5. Choose Edit > Define ROI. Make the ROI the same size for every video you analyze and make it about $\frac{1}{4}$ the size of the full video size or small enough to only include 20 – 50 cells. Try 15 (Left) x 350 (Right) x 5 (Top) x 250 (bottom) in pixels.
6. Zoom in one click by pressing the magnifying glass one time.
7. Choose the paint brush tool and a color. Focus on one cell and click through the video to determine if the cell is moving. It should be pre-determined how far a motile cell moves in one frame to distinguish floating cells from motile cells. Determine if spinning cells are to be considered motile.
8. Draw a circle around motile cells and a line through stationary cells.
9. Input the data (Total number of cells and motile cells) into the Excel spreadsheet “Template for motility analysis.” Calculations are made automatically.
10. Choose Overlay > Segments > Delete, to remove all markings.

Determine Cell Velocity and Distance Traveled

1. Focus on one cell and determine if it is motile (see above).
2. Place a dot on the cell using the paint brush tool and a chosen color (ex. Red).
3. Press the time advance button one time to go the next frame (0.067 s in real time).
4. Place a dot on the cell in its new position.
5. Repeat this procedure following the cell for 6 – 10 frames to create a series of cell tracks.
6. When finished, draw an ROI around the dot in the first position of the cell tracks.
7. Choose Analyze > Set Measurements, and click on the Position tab. Only choose the X- and Y- centroid measurements. All other measurements on all other tabs are not needed. Press OK.
8. Choose Analyze > Measure Seg/ROI. Choose the color of the dot (ex. Red) segments in ROI. Click OK.
9. Draw an ROI around the next dot in the series of cell tracks and simply press Ctrl R to repeat the last command (i.e. the Measure Seg/ROI command). Repeat for each dot in the cell track series. The measurements results window will record the X/Y position (X – Y- centroid) of each dot in the cell track series as you proceed.
10. Repeat these steps for each cell to be analyzed.
11. Click on the Measurement Results window. Choose File > Save, make the file type text and choose an appropriate destination. Click Save. (Note this requires the dongle key for the full version of IPLab 3.5.5)
12. Open the text document in Excel and transfer the X/Y coordinates from the X- Y- centroid results to the excel document “Template for motility analysis.”

Calculations of velocity and distance traveled will be done automatically. Save the excel document under a different name.

B.1. Denaturing Gradient Gel Electrophoresis (DGGE)

Completion Time: 2 Days

Equipment:

1. Dcode Universal Mutation Detection System (Biorad 16CM 120V cat.# 1709080)
2. Power Supply (Biorad 3000xi)
3. Thermocycler (MJ Research PTC-200)
4. Peristaltic pump and tubing (Millipore cat.# XX80 ELO 85)
5. 22 gauge filling needle 5 “ long (Becton Dickinson)
6. Hamilton Syringe, 50 μ l (No. 705)

Materials:

1. 10X PCR buffer (Provided with Taq)
2. Primers, working stock at 2.5 picomoles / μ l (one must have GC clamp)
3. dNTP's at 100mM each diluted to 2.5 mM in sterile dH₂O each nucleotide for the working stocks. (Promega, cat.# U1202,U1212,U1222 and U1232)
4. HotStartTaq DNA Polymerase, 5 Units / μ l (Qiagen, cat.# 202203)
5. 50X TAE
6. Deionized formamide (Fisher cat.# BP227-500)
7. Urea (Gibco cat.# 15505050)
8. 40% Acrylamide:bisacrylamide (37.5:1) (Biorad cat.# 161-0101 and 161-0201)

9. Denaturant Stock Solution A (DSSA) (see below)
10. Denaturant Stock Solution B (DSSB) (see below)
11. 10% Ammonium Persulfate (APS) (Biorad cat.# 161-0700)
12. TEMED (Biorad cat.#161-0801) Store at 4°C
13. Loading Dye (see below)

Amplification of DNA via PCR

1. Prepare template DNA as described in the DNA extraction protocol.
Concentration of DNA should be 20-50 ng/μl.
2. Set up general 50 μl PCR reactions according to the following table:

Note: If PCR product will be used for several analyses, then double all the amounts for a 100 μl RX

Reagent	A	B	C	Etc.	Final
10X Buffer	5	5	5	5	1X
dNTP	4	4	4	4	.2 mM, or 50uM each
Primer 1	1	1	1	1	2.5 pmoles
Primer 2	1	1	1	1	2.5 pmoles
Taq	.2	.2	.2	.2	1 unit
DNA	0	1	1	1	1 – 50 ng
DdH ₂ O	39	38	38	38	

A=negative control (sterile water)

3. Make a master mix and use 11.5 μl/reaction.
4. Start the thermocycler protocol, which should include heating to 95 °C for 15 minutes at the start to initiate Hotstart Taq Polymerase.

Running the agarose gel

1. Make a 1.5% agarose gel (Gibco cat.# 15510-027). Prepare the following samples from the PCR Reactions.

Reagent	100bp ladder	A	B	C	D
TE Buffer	9	0	0	0	0
DNA	1	10	10	10	10
Loading Dye	3	3	3	3	3

A=negative control

Note: Determine concentration of PCR products with the spectrophotometer.

2. Briefly spin samples on the mini centrifuge, vortex and spin again.
3. Pour 1X TAE into the agarose bed, clean the wells and load the samples
4. Run the gel at constant voltage at the gel box's recommended time and constant voltage.
5. Stain the gel with SYBR gold in 1X TAE (1 in 10000; 10ul concentrated to 100ml 1X TAE).
6. View the gel with the fluorimager and confirm that the product is of the correct size and that there is no product for the negative control.

Dcode Universal Mutation Detection System: Casting and electrophoresis

1. Clean glass plates with soap and water, rinse and dry them well. Place on paper towel.
2. Assemble glass plates according to Dcode manual.
3. Make 12ml of both the low and high denaturant solutions according to the following chart.

% Denaturant	DSSA	DSSB
0	12	0
5	11.4	.6
10	10.8	1.2
15	10.2	1.8
20	9.6	2.4
25	9	3
30	8.4	3.6
35	7.8	4.2
40	7.2	4.8
45	6.6	5.4
50	6	6
55	5.4	6.6
60	4.8	7.2
65	4.2	7.8
70	3.6	8.4
75	3	9
80	2.4	9.6
85	1.8	10.2
90	1.2	10.8
95	.6	11.4
100	0	12

Add 120 μ l of APS and 6 μ l of TEMED to each denaturant solution just prior to pouring the gel.

Note: Temed should be stored at 4°C.

4. Make sure all valves are closed on the gradient maker and pour the low denaturant solution into the entrance chamber of the gradient maker. Slowly open the entrance chamber valve and then quickly close it before fluid begins to fill the exit chamber, but after it fills the space between chambers. This will eliminate air bubbles between the two chambers. With a glass pasteur pipette and bulb, pull out any fluid in the exit chamber and place it back into the entrance chamber. Pour the high denaturant solution into the exit chamber.

5. Turn on the peristaltic pump with vacuum pressure set to its lowest setting and open the exit valve. Turn the vacuum pressure up to the setting of 50. It will take about one or two minutes for fluid to start coming out and about 5 minutes to finish.
6. Use the 22 gauge filling needle to pour the gel, keeping it close to the side of the gel plates and about an inch above the surface of the solution as it fills.

Alternatively, a 22 gauge syringe needle may be used, but you will not be able to keep the needle at the surface of the solution as it fills.
7. When all the fluid has been poured into the sandwich, remove the needle from the gel. Rinse the tubing with distilled water. Place the comb in the sandwich and add the stacking gel with a syringe, or pour the stacking gel into the exit chamber with the exit valve closed and allow the pump to pour the stacking gel (set pump to 30). **POUR THE STACKING GEL SLOWLY** (4 ml stacking gel, 40 μ l APS and 2 μ l TEMED). Allow the gel to solidify for at least two hours.
8. Remove the comb by gently pulling it straight up. Rinse the wells with 1X TAE .

Use 1X TAE Buffer for running gels.
9. Set up the Dcode apparatus as instructed in the manual. Fill the electrophoresis tank with 6.5 L of 1X TAE running buffer.
10. Place the temperature control module on top of the electrophoresis tank. Attach the power cord to the temperature control module and turn the power, pump and heater on. The clear loading lid should be on the temperature control module during pre-heating.

11. Set the temperature controller to 60°C. Set the temperature ramp rate to 2000 C/hr to allow the buffer to reach the desired temperature the quickest.
12. Place the gel in the tank and add 350ml of preheated buffer in the upper chamber
13. Prepare samples for loading, use 1 µl of loading dye per 6 µl of solution. An equal mass of DNA should be loaded into each well.

As an example:

Reagent	Blank	A	B	C	D
TE Buffer	17	Varies	Varies	Varies	Varies
DNA	0	100ng	100ng	100ng	100ng
Running Dye	3	3	3	3	3

Note: It may be useful to include a standard containing known sequences that are expected in the mixed PCR products.

14. Turn off the Dcode system and remove the top. Add samples to the wells using a micro pipetter or Hamilton syringe. Add a sample or blank to every well.
15. Replace top and turn the power, pump and heater back on. Attach the electrical leads from the power supply and set the constant voltage to 70 volts. Press start.
16. Run the gel overnight or for at least 16 hours.
17. After the run, turn off the power, pump and heater and remove the top.
18. Remove the gels and disassemble according to the Dcode manual. Use the plate separator to pull apart the plates. Be very careful at this stage not to rip the gels, separate slowly. Leave the gel on top of the larger plate and place this plate into staining solution.
19. Stain gels in 1:10,000 solution of SYBR gold, slowly rocking for 20 minutes.
20. Visualize the stained gel on a fluorimager or transilluminator. Convert the gel to a digital image.tiff file or save as the original .gel file from Image Quant. Analyze

the gel by measuring band positions and band densities. See the DGGE Band Pattern Analysis Protocol using NTSYSpc software and UPGMA.

21. If bands are to be excised, immediately place plastic wrap over the gel and place at 4°C. See protocol for excising bands from denaturing gels.

B.2. Fluorescent In situ Hybridization

Materials:

1. Hybridization solution
2. Wash solution
3. Formaldehyde (16% made fresh)
4. Coverslips
5. Glass slides
6. Aspirator
7. DAPI, SYBR Green or equivalent nucleic acid stain
8. Fluorescently-labeled Oligonucleotide Probe, 100 - 1000 ng/ml (For example EUB338 – FITC labeled at the 5' end)

Fixation

1. Obtain a culture of *Pfiesteria*-like dinoflagellates in late log phase of growth such that the population is at a peak density of at least 10,000 cells /ml.
2. Remove 1 ml of cells and add to an Eppendorf tube on ice.
3. Make 16% paraformaldehyde and adjust the pH to 7.5 – 8.0. Filter the solution through a syringe filter and place the solution on ice for at least 15 minutes.

4. Add 200 μ l of 16% paraformaldehyde to the 1 ml of dinoflagellate culture, dropwise, for a final concentration of 2.7% paraformaldehyde (This step assumes that the dinoflagellates cells are in artificial seawater which has some buffering capacity).
5. Allow the dinoflagellates to fix overnight at 4°C.

Note: It is a good idea at this stage to include several replicates for the experimental sample, positive control (universal probe), and negative control (probe complimentary to the test probe).

Mounting

1. Lay several coverslips (as many as you will need) on a flat paper towel and add 100 μ l of 1% Poly-l-lysine. Allow the lysine to set for 1 hour.
2. Dip the coverslips into distilled water briefly using forceps and allow them to dry on another flat paper towel. These coverslips can now be kept for several days before use.
3. Obtain the fixed dinoflagellate cells and centrifuge for 20 seconds on high (14,000 rpm on the Eppendorf centrifuge).
4. Aspirate off the fluid using a 22 gauge syringe needle at the tip of the aspirator. Be very careful not to suck up the pellet. This is best avoided by tilting the tube almost vertical while sucking out the fluid.
5. Resuspend the pellet in 100 μ l of 10 ppt artificial seawater (ASW) and vortex briefly. Centrifuge as previously and repeat step 4.

6. Resuspend the washed pellet in 50 μ l of 10 ppt ASW and vortex briefly.
7. Add 20 – 50 μ l of the washed cells to a coverslip and place them in a covered container (such as a Petri dish) for no more than 1 hour.
8. Briefly dip the coverslip into distilled water using forceps and allow it to dry. Immediately place into the covered container.

Hybridization and Wash

1. Obtain a six well plate and add all coverslips to be treated equally in the same plate (i.e. all experimental coverslips in one plate and all controls in another.)
2. Label Eppendorf tubes and add 1 ml of hybridization solution to each.
3. Add the probe (fluorescently labeled oligonucleotide) to appropriate tubes at a final concentration of 10 ng / μ l.
4. Add 200 μ l of solution to each coverslip in the wells and cover the dish.
5. Incubate the samples at 46°C (for universal bacterial probe EUB338) for at least 2 hrs.
6. Remove the samples and dip the coverslips into pre-heated wash solution (48°C). Place the coverslip back into the well and add 3 ml of pre-heated wash solution.
7. Cover the dish and incubate at 48°C for at least 20 minutes.
8. Repeat steps 6 and 7 two more times.
9. Add 1 ml of a background staining solution such as DAPI or SYBR Green at 0.1 – 10 μ M and incubate for the appropriate time period.
10. Aspirate off the staining solution and rinse with several 1 ml aliquots of 10 ppt ASW.

11. Remove the coverslip and dip it into distilled water briefly. Allow the coverslip to dry on a paper towel, cover it with a small box to keep out light.
12. Make 1 ml of Prolong Antifade Mounting solution and add 10 μ l to a clean glass coverslip.
13. Apply a coverslip containing the fixed and stained cells face down on top of the Prolong. Wait 15 minutes before viewing.

B.7. *Silicibacter sp.* TM1040 Electroporation Method

Materials:

1. Pre-chilled, sterile, 0.15 cm cuvettes
2. Pre-chilled 250 ml centrifuge bottles
3. HIASW broth
4. HIASW agar plates
5. Sterile ice-cold ddH₂O
6. Ice-cold 10% glycerol
7. Purified, desalted, plasmid or other DNA diluted in sterile ddH₂O
8. Pre-chilled and labeled microcentrifuge tubes

Procedure:

1. Grow 2 ml culture of TM1040 in HIASW o/n at 30°C with shaking.
2. Transfer to 250 ml HIASW in a 1 L flask incubate at 30°C with shaking to an O.D.₆₀₀ of 0.5 (~3.5 h).

3. Place cells on ice for 30 min., cool down centrifuge.
4. Spin down cells in a 250 ml pre-chilled centrifuge bottle at 9000 r.p.m. for 10 min. at 4 °C.
5. Wash cells four times in ~200 ml sterile, ice-cold, distilled water, using centrifugation conditions as previous.
6. Resuspend cells in 0.1 ml of 10% glycerol.
7. Add 1 – 3 µl of DNA to 100 µl of cells in a pre-chilled microcentrifuge tube on ice and vortex for 10s.
8. Hold on ice for 30 min.
9. Electroporate in chilled 0.15 cm cuvettes at 2.4 Kv, 400 Ohms and 25 µF (16 Kv/cm, 9.2 ms).
10. Put cuvettes on ice for 10 min. immediately following electroporation.
11. Add ~2 ml of room temperature HIASW broth to cuvette and incubate at 30°C o/n in a shaking water bath.
12. Spread 100 µl onto HIASW + antibiotic (for example Kanamycin 120 µg/ml) and incubate for ~ 2 days at 30°C.

B.8. Genomic Extraction of *Roseobacter* sp. TM1040 Using CTAB

Materials:

1. HIASW, 250 ml sterilized in a 1L Erlenmeyer flask
2. Lysozyme (100 mg/ml , dH₂O)
3. Proteinase K (10 mg/ml, dH₂O)
4. 5M NaCl
5. 10% SDS

6. CTAB/NaCl (Dissolve 4.1 g of NaCl in water and slowly add 10 g CTAB while heating to 65°C. Adjust the final volume to 100 ml and autoclave sterilize.)
7. Ribonuclease A/TE (Dissolve 100 mg of ribonuclease A in 10 ml of TE. Dispense in 1 ml aliquots into microcentrifuge tubes and boil for 10 minutes. Allow to cool at room temperature, store at -20 °C.)
8. 70% Ethanol (room temperature)
9. 100% Isopropanol (-20 °C)

Procedure:

1. Inoculate 2 ml of HIASW in a large test tube using a freshly streaked, well - isolated colony.
2. Incubate at 30°C for 18 hours in a shaking water bath.
3. Remove 0.5 ml from the overnight culture and use it to inoculate 25 ml of HIASW in a 250 ml Erlenmeyer flask. Incubate at 30°C in a shaking water bath for 8 hours.
4. Add 10 ml of culture to 250 ml of HIASW in a 1 L flask and incubate for 14 hours at 30°C in a dry shaker.
5. Before starting the next steps get a bucket of ice and place fresh stocks of lysozyme, proteinase K and ribonuclease A on ice. Also get the necessary centrifuge tubes and bottles ready so they are available when needed.
6. Pour the culture into a 250 ml centrifuge bottle leaving some space at the top and pellet cells at 10,000 r.p.m for 5 min at 4°C.

7. Remove the supernatant and resuspend the pellet in ~10 ml of TE. Transfer to a 300 ml beaker.
8. Add additional TE to the beaker until the O.D.₆₀₀ is 1.0. Pour ~ 5 ml into a large test tube after adding TE to check the O.D.₆₀₀.
9. Transfer 30 ml to each of two separate centrifuge bottles.
10. Centrifuge the bottles for 5 min. at 10,000 r.p.m. at 4°C.
11. Remove supernatants and resuspend the pellets in 15 ml of TE.
12. Transfer the suspensions to two Oakridge tubes.
13. Add 400 µl of lysozyme and incubate at room temperature for 5 minutes.
14. Add 800 µl of 10% SDS and mix well.
15. Add 160 µl of proteinase K and mix well.
16. Incubate for 1 hour at 37°C.
17. Add 2 ml of 5 M NaCl and mix well.
18. Add 2 ml of CTAB/NaCl (heated to 65 °C) and mix well.
19. Incubate at 65°C for 10 min.
20. Add 10 ml of chloroform/isoamylalcohol (24:1) and mix well.
21. Centrifuge at 10,000 r.p.m. for 10 min. at room temperature.
22. Transfer the upper layer to a clean tube.
23. Add 10 ml of phenol/chloroform/isoamyl alcohol (25:24:1) and mix well.
24. Centrifuge at 10,000 r.p.m. for 10 min. at room temperature.
25. Transfer the upper layer to a clean tube and add 0.6 volumes of ice cold isopropanol.
26. Incubate at room temperature for 30 min.

27. Centrifuge at 10,000 r.p.m. for 30 min.
28. Wash the pellets with 5 ml of 70% ethanol, centrifuge at 10,000 r.p.m. for 5 min.
29. Carefully pour off the supernatant and let the pellets dry for 5 – 10 min upright on a paper towel.
30. Resuspend one of the DNA pellets in 0.1 – 1.0 ml of ribonuclease A/TE followed by the next pellet, combining the pellets into one aliquot.
31. Incubate at 37°C for 20 min.
32. Run a 0.8% agarose gel of the DNA undigested and digested with *EcoRI*.

B.9. Synthesis of Dimethylsulfoniopropionate (DMSP)

Reference: Chambers et al, 1987. J. Bacteriol. 169:4845

Note: Chambers et al. calls for “methyl disulfide”, this is incorrect. It should be dimethylsulfide.

Materials:

1. Dimethyl Sulfide (Methyl sulfide, Aldrich)
2. Acrylic Acid (Aldrich)
3. HCl gas, anhydrous (lecture bottle, Aldrich)
4. Methylene chloride, reagent grade (Dichloromethane, Aldrich)
5. Methanol, reagent grade
6. Diethyl ether, reagent grade
7. 250 ml round-bottom flask and holder
8. Ring stand and clamps to secure flask
9. Stainless steel valve (for ¼” inner diameter tubing on lecture bottle)
10. Stir plate

11. Egg shaped stir bar
12. Teflon lined tubing (1/4" inner diameter, 5/16" thickness)
13. Pasteur pipette
14. Scintered glass funnel, 80 mm diameter (medium grade 20 – 40 μm) and stopper
15. Sideneck flask and adapter to fit scintered glass funnel (2 clean)

Procedure:

1. Clear out a space in a fume hood sufficient to accommodate the HCl tank, round-bottom flask and other reagents. Secure the lecture bottle to the ring stand with clamps.
2. Wear safety glasses, lab coat, and gloves
3. Set up the round-bottom flask on the stir plate, holding it in place with the clamp. Add stir bar.
4. Fit the HCl tank with the Teflon tubing and connect tubing to Pasteur pipette. This will be used to bubble HCl gas in the flask.
5. Place 30 ml methylene chloride in the flask and add 5 ml Dimethyl sulfide and 2 ml acrylic acid. Turn the stir plate on to a gentle, low stir.
6. Place the Pasteur pipette tip under the surface of the methylene chloride. Secure its position with a second clamp or by other means.
7. Close the sash of the fume hood, but allow sufficient room for your hands.
8. Turn on the main valve of the HCl tank slowly and then open the stainless steel valve to allow dispersed bubbles into the methylene chloride solution.

9. Allow the HCl to bubble through the mixture for 20 min. You should see a white crystalline solid form in the flask: this is DMSP.
10. After 20 min, turn off the HCl gas and carefully remove the Pasteur pipette tip from the flask. Allow 5 min for the HCl gas to disperse and be carried off by the fume hood flow.
11. Pour the contents of the flask into the scintered glass funnel, apply a weak vacuum and wash the crystals with additional methylene chloride (~300 ml) to remove residual traces of the reagents. Allow the contents to dry in an open dish in the fume hood. This is now crude DMSP.
12. At this point the crystals should be weighed on an analytical balance and recoveries calculated.
13. To recrystallize the DMSP dissolve it in methanol with gentle heating and stirring. Use just enough methanol to fully dissolve the DMSP, more methanol will be required for larger amounts of DMSP.
14. When the DMSP is fully dissolved, remove the stir bar and add diethyl ether drop-wise until crystals begin to form. Place the solution at 4°C to aid this process.
15. When all the DMSP is recrystallized, pour the solution into a clean scintered glass funnel attached to a clean side neck flask.
16. Apply a vacuum and wash the crystals with copious amounts of methylene chloride. Allow the crystals to dry in an open container in the fume hood.

17. Collect the dry DMSP, weigh, and calculate recoveries. Store in a brown bottle in the freezer. Label with the date, your initials and lot number.
18. Make a 1 mM stock in water and analyze by HPLC to confirm the absence of reactants or other impurities. Send crystals out for elemental analysis of H, C, O, Cl and S (Galbraith Laboratories, Inc. Knoxville, TN).

B.3. Test for Chemotaxis of Roseobacter on Motility Agar

1. Make motility agar (0.3% agar) using an appropriate medium such as BM with glycerol or glucose as the carbon source. Before pouring the medium into Petri plates, place a dot directly in the center of the bottom of each plate (4.5 cm radius) using a marker and another dot 2 cm from the center dot.
2. Allow plates to cool and use them within 72 hours.
3. Inoculate the center of the plates (using center dot) with the edge of a motile colony using a sterile toothpick.
4. Incubate the plates until a motile colony appears to have spread approximately 1 cm, or half the distance to the second dot.
5. Inject 10 μ l of putative chemoattractant (pH \sim 7.5, 0.3 – 1M) into the motility agar at the location of the second dot, 2 cm from the site of Inoculum. Include a positive control attractant, for example, the carbon source present in the motility agar (i.e. glucose or glycerol).
6. Incubate until obvious deformations are seen in the ring structure of the motile colony compared to the negative control (sterile water or solvent used to dissolve attractants).

7. Photograph the plates on a colony counter. The current setup is a macro lense attached to the analog video camera. A digital video camera is used to record images of the plates from the analog video camera to tape. The video is uploaded to a PC from the digital video camera and still photos in .tiff format are extracted from the uploaded video using Adobe Premiere.
8. Analyze photos in Adobe Photoshop. The image should be set to gray scale and then posterized into 12 shades of grey or more. Decide which shade of grey will consitute the outer edge of the colony. Measure the distance between the two dots in pixels (use the “info” dialog box to determine the location of the pointer in pixels). Multiply this number by 0.5 to determine the number of pixels per cm.
9. Measure the distance from the site of Inoculum towards the site of attractant (towards) and measure the distance from the site of Inoculum directly away from the site of attractant (away) in pixels and convert to cm. The ratio of the distance towards : distance away will determine the “power” of putative chemoattraction for each compound.

Potential Chemoattractants:

1. Amino acids (all 20)
2. Sugars
3. TCA cycle intermediates
4. DMSO
5. DMSP and its catabolites including propionate
6. Other special substrates metabolized by the species

B.10. Add Back Experiment with TM1040 and Mutants

Make *Pfiesteria axenic*:

1. Grow *Pfiesteria* dinoflagellates to a cell density of 10^4 - 10^5 cells per ml as assessed by Coulter Counts.
2. Remove a 30 ml aliquot of the culture and add 60 μ l of 0.5M 5-methoxytryptamine (5-MOT) diluted in DMSO. Invert the culture several times to mix.
3. Incubate the culture in the dark at room temperature for 4 h to allow for encystment.
4. Agitate the culture gently while adding 1.2 ml of 5.25% hypochlorite (Chlorox) for a final concentration of 0.1%, to kill bacteria. Incubate at room temperature for 20 min.
5. Carefully pipet the bleached culture into sterile Oakridge tubes and centrifuge at 2600 r.p.m. for 5 min. at room temperature using the JA-20 rotor in the Beckman floor centrifuge (= 800 x g).
6. Remove the supernatant with a sterile pipet and add 30 ml of sterile 10 p.p.t. ASW, resuspend with gentle tituration. Centrifuge again as described previously.
7. Remove the supernatant with a sterile pipet and add 10 ml of sterile 10 p.p.t. ASW, resuspend with gentle tituration.
8. Add 1 ml of the washed cells to 20 ml of 10 p.p.t ASW in six separate sterile tissue culture flasks. Use immediately for the add back experiment.

Preparation of Axenic *Rhodomonas*

1. Obtain an axenic culture of *Rhodomonas* CCMP768 that is at peak cell density.
2. Measure the concentration of axenic *Rhodomonas* by Coulter Counts. Dilute the culture in sterile 35 p.p.t F/2 medium to obtain a concentration of 10^5 cells per ml.
3. Centrifuge 30 ml of the *Rhodomonas* culture in the table top I.E.C centrifuge and resuspend the pellet in 3 ml of 10 p.p.t ASW.

Add bacteria to axenic culture

1. Grow *Silicibacter* sp. TM1040 and transposon mutants in 5 ml of $\frac{1}{2}$ x 2216 marine broth in large test tubes overnight, statically at 30°C for optimum motility.
2. Centrifuge cultures at 3400 r.p.m. in the I.E.C. table top centrifuge for 10 minutes at room temperature.
3. Remove the supernatant and resuspend cultures to an O.D.₆₀₀ of 0.3 which is equivalent to ca. 1.5×10^8 cells per ml.
4. Perform several ten-fold dilutions of the bacterial cultures in 1 ml of sterile 10 p.p.t. ASW to obtain 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} dilutions.
5. Add 200 μ l of the diluted bacterial cultures to the axenic dinoflagellates in tissue culture flasks (prepared above) followed by 1 ml of concentrated axenic *Rhodomonas* (prepared above). In addition, add 200 μ l of the 10^{-4} bacterial dilution to a fifth flask followed by 1 ml of concentrated axenic *Rhodomonas*. Finally, add 1 ml of concentrated axenic *Rhodomonas* to a sixth flask, but do not add bacteria.
6. Incubate cultures at 20°C in the light room for 1 week.

Algal Cell Counts

1. At the start of the experiment and everyday at approximately the same time remove three, 1000 μ l aliquots of culture and place directly into 100 μ l of Bouin's fixative (Sigma # HT101128) while on ice. Fix the cells for 15 minutes on ice.
2. Concentrate using centrifugation as necessary. Count dinoflagellates (zoospores only) and *Rhodomonas* in 10 μ l of fixed cells using a hemacytometer and the Olympus BX60 upright microscope at 400x magnification. Determine the number of dinoflagellates and *Rhodomonas* per ml in each treatment according to hemacytometer instructions.

PCR and DGGE

1. For the analysis of SSU ribosomal DNA, remove 1 ml of sample immediately after making dinoflagellates axenic, after adding bacteria to the cultures and at the completion of the experiment.
2. Pellet cells at max speed in the microcentrifuge for 5 min.
3. Resuspend in 100 μ l of ddH₂O, vortex and boil for 15 min. Place on ice.
4. Use 2 – 5 μ l of the extracted DNA to perform PCR for DGGE according to the standard procedure. Include TM1040 and axenic *Rhodomonas* genomic DNA in the analysis as well as bulk DNA extracted from the original, bacterized *Pfiesteria* culture.

B.11. Staining *Silicibacter* sp. TM1040 with CFDA/SE Tracer Dye

Reference: Fuller, M. E. et al. (2000). 66: 4486-4496.

Materials:

1. $\frac{1}{2}$ x 2216 broth
2. 5-(and 6-)-carboxyfluorescein diacetate, succinimidyl ester crystal (CFDA/SE; molecular probes, #V12883)
3. DMSO (anhydrous, reagent grade)
4. 10 ppt Artificial Seawater (ASW)

Prepare 4 mM CFDA/SE/DMSO stock solution:

1. The CFDA/SE molecule is an uncharged and non-fluorescent compound that enters cells and is cleaved by non-specific esterases to become a charged, cell impermeant fluorescent compound. When in water the compound will undergo some spontaneous cleavage, thus, the CFDA/SE crystal should be stored in a desiccant jar at -20 °C.
2. Dissolve 2.4 mg of CFDA/SE crystal in 1 ml of DMSO in a microcentrifuge tube to make 4 mM CFDA/SE/DMSO. Vortex until dissolved (may take some time to do). Dispense into 50 μ l aliquots and store at -20 °C. Good for several months.

Staining procedure:

1. Grow a 5 ml culture of TM1040 in $\frac{1}{2}$ x 2216 marine broth to an O.D.₆₀₀ of 0.2 – 0.3. Normalize all cultures to 0.2

2. Remove 1 ml from the culture at ca. 5 cm under the surface of the medium and place into a 1.5 ml microcentrifuge tube. Centrifuge at 8,000 x g for 5 min. and resuspend pellet in 1 ml of 10 ppt ASW.
3. Add 12.5 μ l of 4 mM CFDA/SE/DMSO and vortex briefly. Cover with tinfoil and stain at room temperature for at least 1 h.
4. Wash cells at least 3 x with 10 ppt ASW by centrifugation at 8,000 x g for 5 min.
5. Resuspend in 100 μ l of 10 ppt ASW and use cells as desired.

B.12. Flagella Silver Stain Method for *Silicibacter* sp. TM1040

Materials:

Solution 1:

1. Add 3.5 g of aluminum potassium sulfate to 25 ml of dH₂O and stir for 30 min.
2. In a separate beaker add 5 g of tannic acid to 50 ml of dH₂O and stir until dissolved.
3. Filter the aluminum potassium sulfate solution through whatman filter paper into the tannic acid solution with stirring.
4. Dissolve 0.25 g of ferric chloride in 5 ml of dH₂O and add to the aluminum-tannic acid solution.
5. Solution is stable when stored in the dark at 4°C for several months.

Solution 2:

1. In the hood, add 2 g of silver nitrate to 40 ml of dH₂O in a 100 ml beaker and stir until dissolved. Remove 5 ml and set aside.

2. Continue to stir and add 1 – 3 ml of concentrated ammonium hydroxide (25%) to the silver solution. A brown precipitate will form. Continue to add ammonium hydroxide drop-wise until the solution becomes clear.
3. Add remaining silver nitrate drop-wise just until solution becomes faintly cloudy.
4. Solution is stable when stored in the dark at 4°C for several months.

Procedure:

1. Grow TM1040 in 5 ml of ½ x 2216 marine broth in a large test tube overnight at 30 °C, statically.
2. Remove 1 ml of culture and centrifuge at 3400 r.p.m. for 10 minutes at room temperature in the table top I.E.C centrifuge. Remove all of the supernatant by aspiration and resuspend the pellet in 100 µl of 15 ppt ASW by gentle titration.
3. Transfer 30 µl of the washed cells to a coverslip and wait 15 min.
4. Tilt the cover slip and allow excess fluid to run off onto a paper towel. Allow the slide to dry, do not heat fix.
5. Cover the cover slip with solution 1 for 4 min.
6. Wash off solution 1 with copious amounts of dH₂O.
7. Cover the cover slip with solution 2. Pass over a flame quickly, but smoothly three times, waiting 2 seconds between each pass. Stain for 4 min.
8. Wash off the cover slip with copious amounts of dH₂O.
9. Allow the cover slip to dry and invert onto a drop of 15 ppt ASW on a clean microscope slide.

10. View cells at 1000x magnification. Bacterial cells will appear black and may refract light to a large extent. Flagella will appear as grey, thread-like structures either emanating from the cells or may be detached and lying on the cover slip.

B.4. High Pressure Liquid Chromatography (HPLC)

Sample Preparation:

1. Obtain at least 500 μl of each sample for the analysis and filter through a .22 μm syringe filter into 1 ml GC vials and cap. Can also centrifuge at max speed in microcentrifuge for at least 15 minutes, but filtration is preferable.
2. Make standards of acrylic acid and 3-mercaptopropionic acid from 1 μM to 1 mM in ddH₂O and filter them into GC vials, cap the vials.
3. Place the GC vials into the autosampler, place the standards in line before the unknown samples and start with position 2. Add 1 ml of the running buffer to a GC vial, cap, and place it into position 1.

Running Parameters:

1. Turn on the HPLC, press all of the power buttons (Degasser, Quaternary pump, Autosampler, Column thermostat, and the Diode Array Detector). Be sure that the computer is on and logged into Windows.
2. Make fresh running buffer (2.5% acetonitrile/ 0.2% phosphoric acid) and storage buffer (80% acetonitrile). Place running buffer into the "A" line and storage buffer into the "B" line.

3. Pull 60 ml of each buffer through the quaternary pump using the syringe provided with the instrument.
4. The run parameters and method are saved in the file MILLER1.m and run with the HP Chemstation software v A.0.8.03 or later. If this file is available then simply load the file from within Chemstation while in the Methods view and go to step 5.
5. All of the run parameters are listed in appendix A. Launch the chemstation software to setup the method.
6. Go to the help menu and follow the tutorials for setting up the method and data analysis. Enter all parameters as they are listed in the appendix and save the method with any file name you wish.
7. Within the methods and run parameters view, be sure you are looking at the system diagram and that the method is loaded. If not, go to method and load method. Go to view and click system diagram.
8. Click the symbol for running a sequence (Top left) and then click sequence and NEW.
9. Click sequence and samples, the samples dialog box will appear. Click insert vial range and enter the required information (method to be used, the samples to be injected, amount to be injected etc.). This information will supersede anything listed in the loaded method. You should end the sequence with one BLANK run (no injection) with the method, SHUTDOWN.m. This is a gradient method that brings up the ACN concentration to 100% of the "B" buffer (80% ACN) over 10 min. and flushes with 100% "B" buffer for 10 min.

10. Click sequence and sequence parameters. Name the folder where files will be stored. A general path will already be setup, you only need to indicate the folder name, which need not be a folder that you have already actually created. The program will ask if it should create the folder, click yes. Since there are character limits for this folder name, a good folder name would be your initials, the month and a letter indicating the run for that month (ex. Todd R Miller's third run in May, TRM05C). If the run will go overnight then click in the drop down box under shutdown and select MACROSHUTDOWN, to have the machine power down on its own when the run is completed. Click OK.
11. Click sequence and save sequence as. Save the sequence in the folder where run files will be saved.
12. The pump, column compartment, and diode array detector should all be on and highlighted in green on the system diagram. If not, click on each one and turn them on. Allow the pump to run buffer through the system for at least 15 minutes before starting a run.
13. Watch the first few samples to be sure the machine does not have problems.
14. When the run is finished, view the chromatograms in the Data Analysis view.

Record peak areas at approximately 1.2 minutes, but corroborate this information with the acrylate and MMPA standards. In order to call an acrylate or MMPA peak correctly, the retention time in the unknown sample should be within the range of retention times for the acrylate standards. If there is any doubt, then spike unknown samples with a small amount of acrylate or MMPA and re-run the samples along with the standards. Ensure that the peak areas of the standards vs.

molarity form a straight line (analyze using Origin graphing software). Determine the equation for this straight line and use it to calculate the concentration of acrylate in unknown samples.

Miller1.m HPLC Method Parameters

Quaternary Pump

1. Column Flow = 0.750 ml/min
2. Stoptime = 25.00 min
3. Posttime = Off

Solvents

1. Solvent A = 100.0 % (0.1% H₃PO₄, 2.5% ACN)
2. Solvent B = 0.00% (80% ACN)
3. Solvent C = Off
4. Solvent D = Off

Pressure Limits

1. Minimum Pressure = 0 bar
2. Maximum Pressure = 365 bar

Time Table:

Zime (min.)	Buffer A (%)	Buffer B (%)	Flow (ml/min.)
0	100	0	0.750
10	100	0	0.750
15	0	100	0.750
20	100	0	0.750

Diode Array Detector

1. Signals and band width = 210 nm, 8 nm
2. Reference and band width = 360 nm, 100 nm
3. Spectrum = store 190 nm to 400 nm
4. Range step = 2.00 nm
5. Threshold = 1.00 mAmp Units
6. Prerun balancing = Yes
7. Postrun balancing = Yes
8. Margin for negative Absorbance = 100 mAmp Units
9. Peakwidth > 0.1 min
10. Slit = 8 nm

Wellplate Autosampler

1. Injection Volume = 50.0 μ l
2. Injection Mode = Needle with wash
3. Wash Mode = wash in flushport
4. Wash Time = 15.0 s

Column compartment temperature = 30.0°C

B.13. Cell Measurements Using the Coulter Counter

1. Before starting, fill the reservoir marked “Fill Solution” with Isoton II and empty the waste reservoir, marked “Waste.”
2. Turn on the Coulter sampling stand and electronics box. Be sure the computer is on and logged into Windows.
3. Check the setup screen on the electronics box and be sure all parameters are set correctly. The following is a list of those parameters:
 - a. Orifice = 100 μm , length = 75 – 00, setup = manual, analysis = sample, calibration = recall, Kd = 925-18, size = 10-05E, size units = μm
 - b. Current/gain = auto, aperture current = 1600 μA , gain = 2, polarity = +, instrument control = siphon, time = 30, channel count = 500, total = 640E².
 - c. Channels = 256, auto scale = on, coincidence correction = on, analytical volume = 2000, partial relative density = 200, differential values = %
4. Fill the glass beaker with Isoton II and place the glass sampling rod into the beaker. Turn the fill dial to “fill.” Wait for 30s and turn it off.
5. When the light turns on in the sampling stand, press “full” on the electronics box. Allow current to be set and then remove the glass beaker.
6. Add 1 ml of algal culture to plastic coulter sampling cups followed by 19 ml of Isoton solution. Cap and invert to mix.

7. Remove the cap and place the glass rod into the sampling cup. Look in the viewing window on the top of the sampling stand and be sure that the sampling cup is placed such that the orifice is visible.
8. Shut the door and turn the dial on the sampling stand to count.
9. Wait for the machine to finish counting. Look in the viewing window to be sure no particles clog the orifice. If this happens, press stop on the electronics box and turn the dial from count to the neutral, or off, position. Press clear on the electronics box and clear the orifice with a brush or Kimwipe.
10. When counting is completed, on the computer with MultiComp computer program started go to acquire/sample info and enter the name of the sample, sample volume (1 ml), analytical volume (2 ml) and total volume (20 ml).
11. Click on acquire/acquire data. Press print on the electronics box and wait for the data to appear on the computer (takes < 5 s).
12. Save the file in a suitable location.
13. To determine the cell density, click on graph/number per ml and then click on either end of the cell peak and drag to the opposite end. The total cells per ml under the cell peak is shown at the bottom of the window. Cell volume can be determined by leaving the cell peak delimited and clicking on graph/volume per ml. The total cell volume per ml is displayed at the bottom of the window. Divide the total cells per ml by the total volume to get volume per cell.

B.14. Instructions for Making 16% Paraformaldehyde (Formaldehyde)

Materials:

1. Paraformaldehyde (Sigma, P-6148) (stored at 4°C)
2. 1M NaOH
3. Distilled water
4. Glass pipette
5. Glass Beaker
6. Hotplate in fume hood
7. pH meter

Procedure:

1. Add 50 ml of distilled water to a 100 ml glass beaker and heat on the hotplate to 60°C (hint: turn the heat dial to just above the first notch and this will slowly heat the water to 60°C.).
2. In the mean time, add 1.6 g of paraformaldehyde to a 50 ml glass beaker on the analytical balance. Tear the beaker, then carefully add the paraformaldehyde to the beaker from a small container such as a 50 ml conical tube. Do not attempt to add the paraformaldehyde from the large glass container that it is supplied in.
3. Add a very small stir bar (flea) to the beaker and cover with tin foil and carry it to the heated water in the fume hood.
4. Add 10 ml of the 60°C water to the paraformaldehyde, cover and set the beaker on the hotplate. Begin stirring.
5. After 5 – 10 minutes, add 1-2 drops of 1M NaOH using the glass pastuer pipette and continue stirring. The solution should gradually become clear. The beaker

should be moved around on the hotplate, allowing the flea to bump the sides of the beaker. This will dislodge any paraformaldehyde stuck to the sides of the container.

6. After the formaldehyde has become clear, turn off the heat and allow the solution to cool while stirring in the fume hood.
7. When the formaldehyde has reached room temperature, bring it to the pH meter and adjust the pH to 7.5 – 8.0.
8. Filter-sterilize the formaldehyde and place on ice until ready to use; it should be used within one day.

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- PhD. Marine, Estuarine, and Environmental Sciences Program, specialization: Marine Molecular Biology and Biotechnology: University of Maryland, College Park, MD. Advisor: Robert Belas.
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Employment History:

- Graduate Research Assistant, Laboratory of Bob Belas, University of Maryland Biotechnology Institute, Center of Marine Biotechnology, Baltimore, MD. January 1999 – Present.
 - Responsibilities: Conduct scientific research in the areas of microbial genetics, microbial ecology, and algal and bacterial physiology as it applies to studies of the bacterial community associated with marine dinoflagellates. Mentor high school and undergraduate students, write manuscripts for publication in peer-reviewed journals and present data at scientific conferences.
- Chemist, Envirodyne, Inc., Boca Raton, FL. October 1998 – December 1998.
 - Responsibilities: Extraction of organic pollutants from water and soil for environmental testing services.
- Laboratory and Teaching Assistant, St. Norbert College, Department of Biological Sciences, DePere, WI. August 1997 – August 1998.
 - Responsibilities: Care and maintenance of freshwater and saltwater aquaria. Establish and run tutoring sessions for students in general biology courses.
- Counselor and Tutor, Upward Bound Program, St. Norbert College, DePere, WI May 1996 – August 1996.
 - Responsibilities: Advise and tutor “at risk” high school students, teach mammalian anatomy section for general biology course, chaperone students during educational field trips.

Awards:

- International Conference on Harmful Algal Blooms, 2002 Student Travel Award
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- Dean’s Honors List, St. Norbert College, DePere, WI, Spring 1998

Mentoring Experience:

2002 – 2003 Kristin Hnilicka: Howard High School, Gifted & Talented Program.

“Sensing of DMSP by *Roseobacter* species,” Second Place, 48th Annual Baltimore Science Fair, Sponsored by The American Society for Microbiology, March 15 – 16, 2003, Towson University.

2001 – 2002

Hayley Nelson: Riverhill High School, Howard County Schools Technology Magnet Program. “Identification of *Roseobacter* Species in the Chesapeake Bay.”

Computer Literacy:

MS Office Suite, Adobe Photoshop, Adobe Illustrator, Adobe Premiere, IPLab Image Analysis, NTSYSpc Numerical Taxonomy System, Agilent Chemstation, Origin, Coulter AccuComp., VectorNTI, Artemis for Genome Annotation.

Activities:

The American Society for Microbiology 1999 - Present, President COMB Fitness Association 2003 - Present, Advanced NAUI Scuba Diver 1999, Montgomery County Parks Marathon 2003, Underwater research at Shoals Marine Laboratory, Appledore Island, ME, 1996.

Publications:

1. **Moran, M. A., A. Buchan, J. M. González, J. F. Heidelberg, W. B. Whitman, R. P. Kiene, J. R. Henriksen, G. M. King, R. Belas, C. Fuqua, L. Brinkac, M. Lewis, S. Johri, B. Weaver, G. Pai, J. A. Eisen, E. Rahe, W. M. Sheldon, W. Ye, T. R. Miller, J. Carlton, D. A. Rasko, I. T. Paulsen, Q. Ren, S. C. Daugherty, R. T. Deboy, R. J. Dodson, A. S. Durkin, R. Madupu, W. C. Nelson, S. A. Sullivan, M. J. Rosovitz, D. H. Haft, J. Selengut, and N. Ward.** In Press. Genome sequence from an ecologically significant marine bacterial taxon. *Nature*.
2. **Miller, T. R.,** and R. Belas. 2004. Chemotaxis of *Silicibacter* sp. TM1040 toward dinoflagellate products. *Applied and Environmental Microbiology*. 70:4692-4701.
3. **Miller, T. R.,** and R. Belas. 2004. Dimethylsulfoniopropionate (DMSP) metabolism by *Pfiesteria*-associated *Roseobacter*. *Applied and Environmental Microbiology*. 70:3383-3391
4. **Miller, T. R.,** and R. Belas. 2003. *Pfiesteria piscicida*, *P. shumwayae*, and other *Pfiesteria*-like dinoflagellates. *Research in Microbiology* 154:85-90.

5. Quesenberry, M. S., K. Saito, D. Krupatkina, J. Robledo, T. Drgon, W. Pecher, N. O'Leary, M. Alavi, **T. Miller**, R. Schneider, R. Belas, J. Deeds, A. Place, Y. Zohar, and G. Vasta. 2003. Bioassay for ichthyocidal activity of *Pfiesteria piscicida*: characterization of a culture flask assay format. *Journal of Applied Phycology* 14:241-254.
6. Alavi, M., **T. Miller**, K. Erlandson, R. Schneider, and R. Belas. 2001. Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures. *Environmental Microbiology* 3:380-396.

Poster Presentations:

1. **Miller, T. R., and R. Belas.** 2004. Chemotaxis of a *Roseobacter* species to dinoflagellate-derived compounds, 104th Annual Meeting of the American Society for Microbiology, Abstract N-219. ASM Press, Washington, D.C.
2. **Miller, T. R., and R. Belas.** 2003. Characterization of dimethylsulfoniopropionate (DMSP) metabolism by dinoflagellate-associated bacteria. 103rd Annual Meeting of the American Society for Microbiology, Abstract N-39. ASM Press, Washington, D.C.
3. **Miller, T. R., and R. Belas.** 2002. Dimethylsulfoniopropionate metabolism in cultures of *Pfiesteria* dinoflagellates. Xth International Conference On Harmful Algae, St. Pete Beach, FL.
4. **Miller, T. R., and R. Belas.** 2001. Bacterial population complexity in cultures of *Pfiesteria* dinoflagellates, 101st Annual Meeting of the American Society for Microbiology, Abstract N-39. ASM Press, Orlando, FL.
5. **Miller, T. R., and R. Belas.** 2000. Visualization and identification of attached and intracellular bacteria within several strains of *Pfiesteria piscicida*. Symposium on Harmful Marine Algae in the U.S., Woods Hole, MA.

Invited Presentations:

1. **Miller, T. R., R. Belas, and Q. Wang.** 2002. Dinoflagellate associated bacteria and the importance of an algal osmolyte, Horn Point Seminar Series, Horn Point Biological Laboratory, Cambridge, MD.

2. **Miller, T. R.,** R. Belas, and Q. Wang. 2002. The microbiology of *Pfiesteria*. Harmful Algal Technical Advisory Committee Meeting, World Trade Center, Baltimore, MD.
3. **Miller, T. R.,** R. Belas, and Q. Wang. 2001. Bacterial interactions with *Pfiesteria*. Harmful Algal Technical Advisory Committee Meeting, Annapolis, MD.