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Molecular mechanisms underlying roseobacter–phytoplankton symbioses

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Members of the Roseobacter clade of α-proteobacteria are among the most abundant and ecologically relevant marine bacteria. Bacterial isolates and gene sequences derived from this taxonomic lineage have been retrieved from marine environments ranging from sea ice to open ocean mixed layer to tropical coral reefs, and in ecological niches ranging from free-living plankton to sponge symbiont to biofilm pioneer. Although roseobacters are cosmopolitan in the marine environment, their numbers and activity significantly rise with increases in the population density of phytoplankton [1,2], suggesting that these bacteria are highly adapted to engage in these symbioses. This review examines the molecules and phenotypes of roseobacters that are important in establishing and maintaining the symbioses between roseobacters and phytoplankton.

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Introduction

Marine microbes, both prokaryotes and eukaryotes, play important roles in the global biogeochemical cycle owing to their abundance, taxonomic diversity, and high potential metabolic activity [3]. One half of all global primary production occurs in the ocean and is mediated by autotropic drifting organisms referred to as phytoplankton [4]. Half of the ocean's primary productivity is converted into dissolved organic matter (DOM), which is exclusively assimilated by heterotrophic prokaryotes (bacteria and archaea) [3]. Marine bacteria also play a crucial role in the complex global sulfur cycle where they catabolize organic sulfur-containing molecules such as dimethylsulfoniopropionate (DMSP), produced in abundance by phytoplankton species such as prymnesiophytes and dinoflagellates [5]. The breakdown of DMSP by marine bacteria is significant since it is a major source of organic sulfur in the world's oceans and its potential conversion to dimethyl sulfide (DMS) via bacterial metabolism has an impact on the Earth's climate [6]. Many bacterial species, particularly members of the *Roseobacter* taxonomic lineage or clade, inhabit the niche – called the phycosphere – immediately surrounding phytoplankton [3]. Therefore, a more thorough knowledge and appreciation of the molecular mechanisms underlying interactions between roseobacters and their phytoplankton hosts is likely to improve our understanding of many marine biogeochemical processes. In this short review, we will focus on what is known about the molecular mechanisms that roseobacters use to initiate and maintain their symbioses with phytoplankton.

The Roseobacter clade

It is worth stating what should be obvious to all: in nature, marine phytoplankton are not axenic. Instead, these unicellular eukaryotic microbes are surrounded by bacteria that often colonize the outer surface of the larger host cell and may even find their way into interior parts of the alga. Recent studies have revealed that members of the *Roseobacter* clade of α-proteobacteria are a dominate group of bacteria found on surfaces in the marine environment, with the surface of phytoplankton cells being no exception [7,8].

The Gram-negative *Roseobacter* clade contains over 30 genera most of which are marine [9]. The roseobacters are heterotrophic bacteria widely distributed in the marine ecosystem, with their greatest abundance observed in coastal waters [9]. These bacteria possess diverse and flexible metabolic capabilities, including aerobic anoxygenic photosynthesis [10], polyhydroxybutyrate metabolism [11], catabolism of organic sulfur compounds [12], and the synthesis of biologically active secondary metabolites, such as tropodithietic acid (TDA), which has antibacterial properties [13]. Laboratory studies have demonstrated that TDA biosynthesis is most prominent under static nutrient broth cultivation favoring biofilm formation, while bacteria grown in liquid nutrient broths under shaking conditions produce negligible TDA. This and other evidence suggests many species in the *Roseobacter* clade have a biphasic 'swim-or-stick' lifestyle, for example, these bacteria have an apparent life cycle that includes the formation of either motile cells that swim via one or more flagella and respond to attractant and repellent molecules via chemotaxis behavior [14••], or sessile cells that lack flagella and readily attach to surfaces

[15••,16]. It is highly likely that the dual lifestyle of roseobacters greatly influences and may control their symbiosis with phytoplankton.

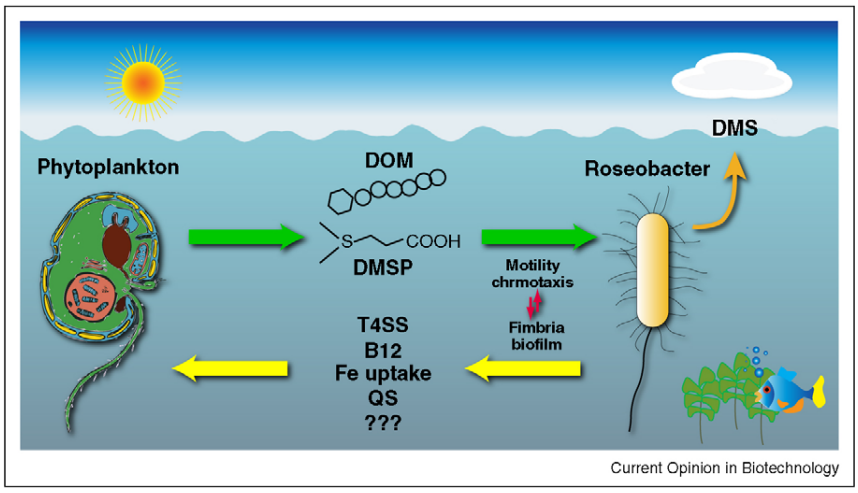
Known and putative bacterial molecular mechanisms crucial to the symbiosis

Our laboratory has analyzed the bacterial community associated with laboratory microcosms of the heterotrophic dinoflagellates, specifically *Pfiesteria piscicida* [17•,18]. The microcosm (CCMP 1830 from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton) contains *P. piscicida*, its prey algae (*Rhodomonas*, CCMP768), and the community of bacteria derived from Chesapeake Bay (Maryland, USA), where the original dinoflagellate sample was obtained in 1998. The bacterial community inhabiting this microcosm is a diverse group of over 30 species numerically dominated by roseobacters. One species, *Silicibacter* sp. TM1040 (referred to as TM1040 for the remainder), is notable as it exists in close physical association with the dinoflagellate forming a biofilm on the surface of the host. Not only does TM1040 develop a biofilm on the surface of *P. piscicida*, but our studies also have shown that axenic dinoflagellate cultures, that is, those lacking bacteria, fail to grow and ultimately die. Surprisingly, adding back TM1040 or mixed bacterial assemblages containing bacteria physiologically like TM1040 restores the growth of *P. piscicida* [17•,19••]. The TM1040:*Pfiesteria* association is the only known 'obligate' association between a dinoflagellate and a culturable bacterium, and fits the definition of a symbiosis as 'the close, permanent relationship between two or more different organisms' [20]. The genome of TM1040 has been annotated [21], and we have developed a large array of genetic tools to probe the molecular mechanisms that aid this roseobacter in its symbiosis with its phytoplanktonic host. Thus, while TM1040 is ecologically extremely relevant, it is also an excellent choice for laboratory studies. Using the TM1040:*P. piscicida* symbiosis as a model for other roseobacter–phytoplankton symbioses, the following mechanisms are probably involved in the processes of initiating and maintaining the symbiosis.

Motility and chemotaxis to phytoplankton

Chemotaxis behavior allows motile bacteria to swim toward an attractant and away from toxic conditions, providing bacteria with a competitive advantage for nutrition in natural environments [22]. More than half (16 out of 28) of the annotated roseobacter genomes harbor homologs to known chemotaxis genes and diverse chemoreceptor proteins that are required for binding different ligands [23••]. The presence of multiple cytoplasmic chemotaxis genes reflects the metabolic versatility of the roseobacters, and is undoubtedly used as part of chemotactic behavior that allows these bacteria to swim toward nutrient sources (Figure 1), such as an algal cell [3].

Figure 1



A model of the molecular mechanisms involved in the symbiosis between roseobacters and phytoplankton. Motile phase roseobacters use chemotaxis behavior to sense and swim toward phytoplankton to form initial attachment to the algal surface. Once close to the surface of the algal cell, a currently unknown molecular 'switch' is turned on resulting in a transition of the bacteria to a sessile life phase whose phenotype includes loss of flagella, formation of fimbrial adhesins, and subsequent biofilm development. Roseobacters attached to a phytoplankton cell have immediate access to dissolved organic matter (DOM) and dimethylsulfoniopropionate (DMSP), which may be used in the synthesis of one or more biologically active molecules, such as tropodithietic acid (TDA), that act to prevent algicidal bacterial species from harming the phytoplankton and enhance to roseobacter symbiosis by limiting competition from other species. In addition to the potential benefit from producing TDA, the roseobacter biofilm may also provide necessary nutrients, for example, Vitamin B12 (cobalamin), iron-binding siderophores, and so on, to the alga. Many of these exchanges and interactions may be mediated through a *vir*-gene-mediated Type 4 Secretion System (T4SS) and/or quorum signaling through known and (currently) unknown chemicals.

Our results demonstrate that bacterial motility is an important factor in the symbiosis [19••]. Three transposon-insertion mutants with motility defects were analyzed for their ability to form biofilms on and support the growth of *P. piscicida*. Mutation of *flaA* (a novel flagellar gene whose function is unknown) or *cckA* results in the loss of flagella (Fla^-) and non-motile cells (Mot^-), while $CtrA^-$ cells possess flagella, but are Mot^-. All three flagellar mutants are defective in attaching to and forming biofilms on the dinoflagellate, and the growth of the dinoflagellates is reduced compared to symbiosis with wild-type cells [19••]. Thus, motility and subsequent biofilm development are linked to the obligate physiological requirement of the dinoflagellate for the bacteria.

The TM1040 genome is replete with genes with the potential to encode over 20 methyl-accepting chemotaxis membrane transducers proteins (MCPs; also known as chemoreceptors) and multiple copies of cytoplasmic chemotaxis (Che) proteins [21], suggesting that TM1040 is proficient in sensing and responding to chemical attractants. TM1040 responds specifically to heat-labile compounds found in dinoflagellate homogenates, while homogenates from *Rhodomonas* or culturable heterotrophic bacteria isolated from *P. piscicida* do not elicit a similar response [14••]. TM1040 also responds strongly to DMSP and metabolic byproducts of DMSP metabolism, and is attracted to all amino acids, with the greatest response observed with methionine. TM1040 responds positively to sugars, with sucrose, *N*-acetylglucosamine (NAG), galactose, glucose, and maltose, and to tricarboxylic acid cycle (TCA or Krebs cycle) intermediate compounds, for example, citrate and fumarate [14••]. These results suggest that TM1040's physiology, motility, and chemotaxis machinery is adapted to ensure that the bacteria are able to find the dinoflagellate in oligotrophic seawater and initiate the symbiosis through sensing, response, and swimming to the dinoflagellate surface.

Biofilm formation on phytoplankton

As described earlier, marine bacteria often cluster around phytoplankton for metabolic requirements, thereby increasing the density of the bacterial population beyond what is typical for open ocean waters [24]. As they come closer to the surface of the phytoplankton, various molecular mechanisms that promote bacterial attachment are activated thereby allowing the bacteria to attach to the surface of their host.

Bacterial attachment to surfaces is a complex phenomenon involving numerous molecular mechanisms that are often species-dependent; however, there is strong evidence that the presence of extracellular appendages, such as fimbriae or pili, are frequently used by bacteria to stick to surfaces (Figure 1) [25]. The genome of many species of roseobacters harbors key homologs to the *Caulobacter crescentus cpaBCEF* genes, which play important roles in the biofilm formation in this α-proteobacterium [26]. Additionally, ORFs with homology to *Actinobacillus actinomycetemcomitans* TadB and TadC and *flp* fimbrial proteins are also present [21]. Tad proteins form fimbriae that cause the cells to autoaggregate, while *flp* fimbriae are often involved in tight adherence of bacteria to eukaryotic cells [27]. It is possible that the homologs in TM1040 function similarly in its colonization of surfaces on phytoplankton, although experimental evidence is lacking to support this hypothesis.

It should be noted that bacteria-like particles or structures have been observed within phytoplankton, suggesting that some bacteria may exist as intracellular symbionts in these unicellular eukaryotes. Endocytoplasmic and endonuclear bacteria have been observed by electron microscopy in cells of the phytoplankton *Gymnodinium splendens*, *Glenodinium foliaceum*, and *Gyrodinium instriathum* [28,29]. Rigorous tests to confirm the presence of viable and physiologically relevant intracellular bacteria have not been reported, and a degree of caution should prevail when interpreting these findings.

Acquisition of nutrients derived from phytoplankton

There is an obvious benefit for a bacterium to attach to an algal cell: it obtains a readily available source of organic matter from the eukaryote, the bacterium is in close proximity to other bacteria, which permits more effective transfer of genes and response to chemical inducers, and forming a biofilm on the alga makes it more difficult for bacteriovores to prey on the bacterium (Figure 1). Of these benefits, the first – utilization of phytoplankton-derived nutrients – has been extensively studied.

One of the most conspicuous molecules produced by phytoplankton, such as *Emiliania huxleyi* (a Coccolithophore) and many dinoflagellate species, is DMSP [30]. DMSP is produced from methionine and functions as an osmolyte [31] and antioxidant [32], and is a major source of organic sulfur in the world's oceans [33]. The principal organisms responsible for conversion of DMSP are bacteria, including roseobacters in the coastal waters and *Pelagibacter ubique* SAR11 in the open ocean [6]. This is significant, since DMSP is a preferred source of reduced sulfur for roseobacters, despite it being nearly 10^7-fold less abundant than sulfate in seawater [34,35•].

There are two possible pathways for the breakdown of DMSP: (1) the lyase or cleavage pathway leads to the production of dimethyl sulfide (DMS), a volatile gas [36–38] and (2) the demethylation/demethiolation pathway through which sulfur contained in DMSP is retained by the bacteria and not released as DMS [6]. DMS generated from the lyase pathway further reacts within the atmosphere and is readily oxidized to sulfate aerosols that

increase the abundance of cloud condensation nuclei leading to greater backscattering and cloudiness [39], which counteracts the effects of global warming [39,40]. It has also been postulated that cloud formation and the backscattering of sunlight may theoretically affect primary productivity in the ocean by reducing mean light intensity and temperature [39]. So, the symbiosis between roseobacters and phytoplankton may play a pivotal role in regulating global climate on Earth.

In addition to the sulfur cycle, roseobacters also may contribute a significant source of carbon to coastal waters owing to their ability to degrade complex organic compounds, such as cellulose and lignin [41,42]. This is ecologically significant since coastal runoff distributes much plant-derived cellulose and lignin from coastal marshes into the nearby ocean. Furthermore, genomic analyses have shown that the roseobacters contain numerous pathways for degradation of aromatic hydrocarbons, which may include phytoplankton-derived molecules that function as antioxidants, toxins, and predator deterrents [21].

The role of bioactive compounds produced by roseobacters

Several reports have highlighted that members of the *Roseobacter* clade produce numerous molecules whose biological activity is of potential importance to marine phytoplankton [16,43•]. Specifically, the production of TDA is a common feature of certain roseobacters [16]. TDA is a novel dual-sulfur tropolone compound, which is synthesized by an oxidative ring expansion of phenylacetic acid, from the shikimate–chorismate pathway [44]. Biosynthesis of TDA by roseobacters is sessile-phase dependent: high TDA activity is correlated with an overall change in the physiology of roseobacters that includes the production of a yellow-brown pigment, in addition to formation of rosettes and biofilms, and the loss of flagella and motility [45]. TDA inhibits a broad range of marine pathogens [16]; perhaps indicating that TDA may help to prevent harmful bacteria from colonizing the alga when it is in symbiosis with TM1040 or other physiologically similar roseobacters [15••].

We have reported on a random mutagenesis used to construct loss-of-function mutants defective in TDA activity (Tda^-) [15••]. Twelve genes were identified that are required for Tda activity including genes encoding phenylacetate degradation proteins, supporting earlier work that determined that TDA is derived from the phenylacetate degradation pathway [44], which is one of five aromatic compound degradation pathways in roseobacters [21]. This is relevant, since, as mentioned previously, roseobacters are able to use complex hydrocarbon compounds including those derived from terrestrial sources, for example, lignin derivatives. Some of these compounds are degraded via the phenylacetate pathway, which may be one source of precursors for TDA synthesis.

The effect of bacteria on the physiology of phytoplankton

Within the phycosphere, multiple bacterial mechanisms may come into play that benefit the host cell. For example, symbiotic bacteria may help phytoplankton regenerate inorganic nutrients [46] and may provide essential compounds that the microalgae themselves cannot synthesize. A powerful example of this is Vitamin B12, cobalamin, which is required but not synthesized by many phytoplankton, and is instead provided by the bacterial community associated with the unicellular alga [47]. Bacteria associated with phytoplankton also help the microalgae acquire iron (Figure 1) through the synthesis of bacterial siderophores that increase iron solubility [48], thus conferring to the algae an advantage under iron-limiting conditions such as are found in the open ocean. These studies show that micronutrients acquired by phytoplankton through bacterial metabolism benefit the alga and are an important facet in the symbiosis between phytoplankton and bacteria.

While the previous example provides substantial empirical evidence of the benefits to the phytoplankton, there are many other examples of how bacteria positively affect the physiology of marine unicellular algae. For example, bacteria taxonomically related to the *Roseobacter* lineage have been isolated from a culture of the dinoflagellate *Alexandrium*, and were shown to have beneficial effects on the growth of this dinoflagellate [49,50]. This beneficial effect is supported by laboratory experiments with axenic cultures of *P. piscicida*, whose zoospores grow poorly (and ultimately die), compared to cultures with their associated bacterial assemblages [17•]. Adding back TM1040 or a mixture of bacteria obtained from the original *P. piscicida* microcosm restores normal growth of the dinoflagellate [17•]. It would not be surprising to find that many of the interactions between roseobacters and phytoplankton are beneficial to both partners.

Vir (Type IV secretion) system

Genomic analyses have also hinted that roseobacters may be able to interact with their eukaryotic microalgal hosts through cell-to-cell direct transfer of molecules. One of the many surprises that has come from analyses of roseobacter genomes is that half of the roseobacter species with annotated genomes harbor a putative Type IV secretion system (T4SS) [21,23••]. T4SS is an important secretion mechanism in many Gram-negative bacteria [51]. For example, *Agrobacterium tumefaciens*, another α-proteobacterium, uses a set of *vir* genes products to transfer DNA (referred to as T-DNA) from the bacteria into plant cells, thereby initiating the formation of crown galls in terrestrial dicot plants. A similar set of *vir* genes is found in TM1040 (and other roseobacters) [21]. The TM1040 '*vir*'

genes have homology to *virD2* and *virD4*, which encode for the relaxase and coupling proteins (providing the energetics for export of DNA), and *virB1-11* (excluding *virB7*), which encode the inner membrane channel and pilus structure of the transfer machinery [21,52]. The symbiosis between TM1040 and dinoflagellate *P. piscicida* tempts speculation that Vir proteins enable roseobacters to transfer DNA or proteins directly to phytoplankton cells. While this has yet to be experimentally proven, roseobacters have been found inside of galls formed on the red macroalgal genus *Prionitis* [53,54]. These galls are morphologically similar to crown galls formed by *A. tumefaciens*, hinting that the roseobacter T4SS may have a similar function to the *vir* system of *Agrobacterium*.

Quorum signaling

Quorum signaling (QS) is a form of population-density-dependent chemical communication used by bacteria to control cellular functions through excreted small molecules that interact directly to regulate the expression of sets of genes within certain bacterial species [55]. QS is widespread among bacteria and has been shown to be involved in pathogenesis, for example, in the virulence of *Pseudomonas aeruginosa* in the lung's of cystic fibrosis patients [56], and symbioses, such as the symbiosis between the bioluminescent marine bacterium *Vibrio fischeri* and *Euprymna scolopes*, a squid [57]. Many other species of marine bacteria produce QS molecules, particularly acylated homoserine lactones (AHLs), including many roseobacters [16,58•]. AHLs have been detected in roseobacters obtained from samples of marine snow [59] and elsewhere, emphasizing the importance of AHL QS as one of the most common chemical communication mechanisms in roseobacters [57].

However, not all members of the *Roseobacter* lineage possess the capacity to synthesize AHLs. Our work has shown that TM1040 lacks genes encoding known QS systems and does not produce the most common AHL molecules [16,21], yet some of the phenotypes of this species, such as biofilm development, rosette formation, and TDA biosynthesis, appear to be population-density-dependent, suggesting that molecules other than canonical AHLs may be used by TM1040.

Although QS has been studied primarily as a prokaryote-to-prokaryote signal system, eukaryote hosts have also developed strategies that modulate QS. For example, research has shown that higher plants produce furanone and AHL-like chemicals that mimic AHL compounds and affect specific bacteria [60]. Conversely, the production of bacterial QS chemicals can also affect the behavior of higher plants, such that bacterial biofilms producing AHLs cause planktonic zoospores of *Enteromorpha* to settle down on or near those same bacteria [61]. Although yet to be experimentally proven, it is likely that QS controls many bacterial behaviors that are essential for roseobacter–phytoplankton symbioses, including formation of biofilms and production of biologically active compounds, that ultimately influence the initiation and establishment of the symbiosis.

Conclusions

Members of the *Roseobacter* clade of α-proteobacteria are among the most abundant and ecologically relevant marine bacteria. One of the most salient features of these bacteria from aspects of marine ecology, carbon and sulfur cycling, and global climate is their ability to metabolize DMSP, a major source of organic sulfur produced by phytoplankton, specifically dinoflagellates. Bacterial reactions that degrade DMSP influence the fate of sulfur, and determine whether it is volatilized to the atmosphere or retained in the ocean in organic form. Roseobacters such as *Silicibacter* sp. TM1040 exhibit close physical and physiological relationships with DMSP-producing dinoflagellates suggesting that these bacteria are highly adapted to engage in this symbiosis. The molecular mechanisms used by these bacteria to initiate and maintain the symbiosis include flagellum-dependent swimming and chemotaxis behavior, biofilm formation, degradation of phytoplankton-derived compounds, and subsequent synthesis of biologically active chemicals, including those that have antibacterial and potential cell–cell communication functions (respectively). Modulating many of these bacterial processes is a currently unknown molecular mechanism that determines if these biphasic bacteria are in their motile or sessile phase. Knowledge of the molecular mechanisms underlying the symbioses between roseobacters and their phytoplankton hosts is important, as these symbioses influence the health of the oceans and ultimately affects on global climate.

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