The Role of Antibiotics in Microbial Host Colonization

by

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Abstract

Surface colonization is ubiquitous in marine environments, and bacteria are one of the first, or primary colonizers, to attach to newly formed surfaces. Marine algae are host to a plethora of such bacterial colonizers, one of the most abundant being roseobacters. Most of known pathogenic or mutualistic interactions between algal hosts and roseobacters are mediated by bioactive molecules produced by the organisms. Tropodithietic acid (TDA), an antibiotic made by the roseobacter *Phaeobacter inhibens*, is hypothesized to contribute to the chemical defense for *Emiliania huxleyi*, a globally abundant microalga, against colonization of roseobacter *Ruegeria italica*, a known opportunistic pathogen of *E. huxleyi*. To investigate the role of TDA in the chemical defense, a multi-species culturing system was established with *E. huxleyi*, *R. italica* and *P. inhibens*, as well as a *P. inhibens* transposon mutant lacking TDA production. This system was monitored, with the bacterial-algal surface association visualized via fluorescence microscopy, population densities of the bacteria were measured by colony forming unit and all algal populations monitored using pulse-amplitude modulated fluorometry. Within 72 hours of colonization, algal health was not affected by bacteria inoculated. *R. italica* gained higher density than *P. inhibens* during simultaneous primary colonization of *E. huxleyi* by these two bacteria, though *P. inhibens* can inhibit *R. italica* growth on solid media in a diffusion-dependent manner. If *R. italica* was added as a secondary colonizer after *P. inhibens* primary colonization of *E. huxleyi*, *R. italica* growth was significantly inhibited by 98%. In conclusion, *P. inhibens* was successful at inhibiting the secondary colonization of pathogen *R. italica*, in part due to its production of TDA, which acts as a chemical defense for *E. huxleyi*.

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Sincerely,

Yue Xu

Table of Content

List of Tables

List of Figures

Chapter 1 Introduction

1.1 Microbial colonization in marine systems

Microbial colonization in marine systems is well studied because it results in the accumulation of organisms on surfaces, a phenomenon known as biofouling. This poses huge risks to desalination plants, marine vessels and their water conduits, industrial pipelines, and other underwater constructions (Railkin, Ganf and Manylov 2004). For instance, the accumulation of biofouling organisms on hulls may increase hydrodynamic friction, leading up to 60% increase of dragging force that can severely damage the hulls of ships (Chambers *et al.* 2006). The drag can decrease speeds by 10%, and increase fuel consumption by more than 40% (Chambers *et al.* 2006). Biofouling is a complex multistep process, which needs to be better understood if we are to control it.

A fully developed biofouling system has a sophisticated structure that is also remarkably dynamic, and the system is resistant to disturbance once established (Holmström *et al.* 2002). Besides the visible attachment of barnacles and macroalgae, it was estimated that there can be up to 1700 species contributing to biofouling in marine environments (Almeida, Diamantino and de Sousa 2007). The marine bacterial growth and biofilm formation on natural and artificial surfaces is an essential first step to biofouling (Rao, Webb and Kjelleberg 2006). Once the biofilms are formed, the further attachments of other fouling microorganisms including phytoplankton and invertebrate larvae will be facilitated (Fuhrman, McCallum and Davis 1993). Efficient anti-fouling compounds therefore often target microorganisms. An example is tributyltin, a biocide additive to marine paints targeting the microorganisms responsible for the formation of

an initial bacterial biofilm (Evans, Leksono and McKinnell 1995). However, biocides like tributyltin are highly toxic to larger marine organisms including oysters (defective shell growth) and dog whelk (disorders of sex development), even at low concentrations (< 100 ng/L) (Evans, Leksono and McKinnell 1995). Further understanding the biology of biofilm forming species in marine environments may bring insights into the future development of antifouling agents that are more environmentally friendly.

1.2 Biofilm biology of bacterial colonization

A biofilm consists of cells attached to each other and a surface, held within an extracellular matrix called extracellular polymeric substances (EPS), that are composed of polysaccharides, proteins, and extracellular DNA initially found essential for biofilm formation of *Pseudomonas aeruginosa* (Whitchurch *et al.* 2002; Hall-Stoodley, Costerton and Stoodley 2004). The extracellular DNA compose physical structure of the biofilm while extracellular enzymes serve as an external digestion system and polysaccharides protect the bacteria against desiccation and predation (Jakubovics *et al.* 2013; Ghosh and Maiti 2016).

Such a complex structure provides homeostasis for the bacteria in unstable conditions such as extreme temperature, salinity and pH changes (Hall-Stoodley, Costerton and Stoodley 2004). Compared to single planktonic cells, cells within biofilm has a more suitable environment for cell-to-cell communication signaling pathways and lateral gene transfer which all require close proximity (Stoodley *et al.* 2002). In addition, biofilms also provide protection to the embedded cells from disturbances such as UV exposure, metal toxicity, acid exposure, phagocytosis, eukaryotic grazing and antimicrobial agents (Hall-Stoodley, Costerton and Stoodley 2004).

1.2.1 The formation process of Biofilms

The first step of the formation process of biofilms is the attachment of planktonic bacteria on to a surface (Watnick and Kolter 2000). The bacteria can sense the environmental cues, such as nutrient concentration, temperature, osmolarity, pH, iron and oxygen, and switch from planktonic to sessile lifestyle on a surface (O'Toole, Kaplan and Kolter 2000). It has been suggested that the initial colonizing bacteria attach to the surface through van der Waals forces and hydrophobic effects, which are weak, and the attachment is reversible (Briandet, Herry and Bellon-Fontaine 2001; Takahashi *et al.* 2010).

If the initial colonizers stay attached, the bacteria can form stronger cell adhesion structures, such as pili in *P. aeruginosa*, so the irreversible attachment is established (O'Toole, Kaplan and Kolter 2000). Cells on the attached surface would aggregate and form microcolonies, which are groups of less than 50 cells serving as a core for the growth of biofilm (Zhao *et al.* 2013). The maturation of the biofilm occurs after early attachment. The genes involved in the synthesis of EPS are often upregulated after attachment (O'Toole, Kaplan and Kolter 2000), leading to an increased production of EPS which further facilitates attachment of more cells. Antibiotic resistance genes are also frequently upregulated at this stage (O'Toole, Kaplan and Kolter 2000), protecting the bacteria from competitors in the environment. Other properties, including UV light resistance, lateral gene transfer, and increased secondary metabolite production may also be developed during late maturation of the biofilm (O'Toole, Kaplan and Kolter 2000).

During later biofilm maturation, the surface colonizing bacteria can communicate via quorum sensing (QS) signaling molecules such as acylhomoserine lactones (acyl-HSLs) (Davies *et al.* 1998). It has been shown that mutations in gene *lasI* (acyl-HSLs synthase) does not affect either the surface attachment or early cell growth, but disrupt later biofilm maturation in *P. aeruginosa*, because QS is not involved in regulation of gene expression when a population has low cell density in the early stages of biofilm formation (Davies *et al.* 1998). The population density has to be high enough for sufficient amount of signaling molecules to activate QS to regulate genes involved in later stages of biofilm formation (Davies *et al.* 1998). For example, *pel* operon, essential for the production of a glucose-rich matrix exopolysaccharide, has been shown to be highly regulated by QS signaling in *P. aeruginosa* (Sakuragi and Kolter 2007).

Introduction of secondary colonizers and dispersal of cells from biofilm can be observed after the biofilm has fully matured. Dispersal allows the bacteria composing the biofilms to spread and colonize new habitats. The enzymes dispersin B (disrupts polysaccharide) and deoxyribonucleases (degrades DNA) can be secreted by bacteria to degrade the biofilm EPS matrix (Izano *et al.* 2008). Interestingly, the cells dispersed from a biofilm, tend to have a different physiology than planktonic cells. For example, cells shed from a by *P. aeruginosa* biofilm have stronger virulence against macrophages and they are highly responsive to iron starvation, compared to cells residing biofilm or those in a planktonic lifestyle (Chua *et al.* 2014).

1.3 Relationships between marine bacteria and their eukaryotic hosts

Biofilms can form on non-living surfaces, such as the hulls of marine vessels, as well as on the surfaces of living organisms, forming sophisticated interactions with the host, and such host-symbiont interactions are commonly found between marine algae and bacteria. The significance of the role bacteria play in altering algal hosts physiology, such as growth dynamics, has been recognized over the last decade, and the ecological interactions among the algal and bacterial partners are mostly mediated by different novel chemicals (Grossart *et al.* 2005; Labeeuw, Bramucci and Case 2017). Interactions between organisms are typically classified as competition, amensalism, commensalism, mutualism, parasitism, and pathogenesis (Martin and Schwab 2012). The definition of these interactions is based on whether the organisms are harmed, benefited, or unaffected, as summarized in Table 1-1.

Table 1-1: Interspecific biological interactions between two organisms X and Y.

Type	Effect on X	Effect on Y
Competition	harm	harm
Amensalism	harm	no effect
Commensalism	no effect	benefit
Mutualism	benefit	benefit
Parasitism	harm	benefit
Pathogenesis	harm	benefit

Competition occurs between organisms when both organisms are harmed due to limited supply of resources (Sahney, Benton and Ferry 2010). In microbial competition, the populations of microorganisms may excrete chemicals that are inhibitory to its competitors in the environments (Gram, Melchiorsen and Bruhn 2010). In amensalism, only one population is negatively affected (Fredrickson and Stephanopoulos 1981). Commensalism describe an interaction where one organism gain benefits while the other remains unaffected. For example, *Staphylococcus epidermidis* is a commensal human skin resident (Chiller, Selkin and Murakawa 2001). In mutualism, two organisms both gain benefits from the interaction (Boucher, James and Keeler 1982). Pathogenic interactions are often confused with parasitism (Seyedsayamdost *et al.* 2011a; Gram *et al.* 2015). Parasites like helminths and nematodes colonize hosts, causing reduced host fecundity and limited growth by physical invasion (Frank 1996; Hurd 2001; Chylinski *et al.* 2009; Newton *et al.* 2010). When parasites scavenge nutrients from the host, parasites tend to prolong infection and avoid killing the host (Frank 1996). In contrast, pathogens like bacteria and viruses usually cause disease and severe damage to the host for their own benefits without facing the trade-off between host exploitation and rapid reproduction like parasites (Frank 1996; Newton *et al.* 2010).

Regarding pathogenesis in the marine environment, researchers used to be mostly focusing on pathogens of human and aquaculture (Blake 1994; Liu and Lee 1999; Karlsen *et al.* 2014). The importance of the roles bacteria play in inducing marine pathogenesis were emphasized in other natural systems including coral and macroalgae (Rosenberg *et al.* 2007; Knowlton *et al.* 2010; Case *et al.* 2011). The system of microalgae haptophytes and bacteria roseobacters is also worth intensive

investigation because of the high abundance of haptophytes in the environment, and roseobacters are the most common bacteria found in association with microalgae (Gonzalez *et al.* 2000; Goecke *et al.* 2013).

1.3.1 Description of haptophytes and *Emiliania huxleyi*

Haptophyta is a class of microalgae well known for its bloom forming ability (Alvain *et al.* 2008), for which the placement in the tree of life remains unsolved. One hypothesis is that the class Haptophyta belongs to supergroup Chromalveolates, where the members (heterokonts, stramenopiles, alveolates, cryptophytes, and haptophytes) of the group evolved through endosymbiosis events of a red algae ancestry lineage (Andersen 2004; Janouskovec *et al.* 2010), and the other hypothesis is that the haptophytes belong to Hacrobians, a unique group distinct from the SAR supergroup (stramenopiles, alveolates, and Rhizaria) (Keeling 2013). One of the most widespread types of haptophytes is called coccolithophores, which are unicellular algae capable of fixing a huge amount of carbonate via calcification to produce calcium carbonate ($CaCO_3$) discs surrounding the cells called coccolith (Satoh *et al.* 2009). Not only are coccolithophores important in global climate via fixing carbon dioxide, but they are also important for carbon transportation from surface water to the deep ocean (Rost and Riebesell 2004). As the organisms become naturally senescent or die of infection by pathogens, the coccoliths are shed and sink down to the ocean floor where the carbon is essentially removed from the carbon cycle (Wilson *et al.* 2002; Schmidt *et al.* 2013; Chow, Lee and Engel 2015).

Emiliania huxleyi is one of the most abundant coccolithophore species, commonly found in various locations across the North Atlantic and Pacific oceans, and in coastal areas like the Black and Tasman seas, which are low in phosphate and high irradiance environments that are not tolerable for most other phytoplankton (Paasche 2001). The ability *E. huxleyi* to grow in various environmental conditions makes it a good model organism for many physiological studies conducted in laboratory environments (Satoh *et al.* 2009). This unicellular microalga has a typical haptophyte life cycle where it alternates between diploid coccolith bearing non-motile cells (C), and haploid motile non-calcified cells (S) which have organic scales, and each cell type is capable of reproducing through mitosis (Paasche 2001). It has also been observed that C cells could lose their ability to form coccolith in laboratory conditions and became bald cells, which are diploid non-motile N cells (Frada *et al.* 2012). The mechanism mediating the switch between cell types during the life cycle remains unsolved (Frada *et al.* 2012). However, recent studies have provided possible explanations for why the switch of the cell type could be a benefit to the algae. It has been shown that different cell types have different gene transcription profiles, as well as different response and susceptibility to viral and bacterial pathogens (Frada *et al.* 2008; Von Dassow *et al.* 2009; Mayers *et al.* 2016).

In addition to its important role in global carbon cycling, *E. huxleyi* is also known for its great impact on the atmosphere-ocean sulfur cycle. *E. huxleyi* is a major producer of dimethylsulfoniopropionate (DMSP), which is a very abundant and accessible organic sulfur source in marine ecosystems (Seymour *et al.* 2010; Alcolombri *et al.* 2015). DMSP can also be degraded to dimethylsulfide (DMS) in *E. huxleyi* cells via DMSP

lyase Alma1, and Alma1-like proteins are also found in roseobacter (Alcolombri *et al.* 2015). DMS is a major contributor of cloud condensation nuclei (CCN) which regulates the climate (Charlson *et al.* 1987; Ayers and Cainey 2007). DMSP can also serve as a potent foraging cue for zooplankton and chemotactic signal for bacteria (Seymour *et al.* 2010; Garren *et al.* 2014). For example, roseobacter possess DmdA, a DMSP demethylase, that initiates the first step of DMSP degradation to methanethiol and thereby direct substantial amount of the primary production into marine food web (C Howard et al. 2006).

1.3.2 Description of the *Roseobacter* clade

Roseobacters have been known to function as probiotics and pathogens in various systems of aquaculture (D'Alvise et al. 2012) and coral (Luo and Moran 2014), and they are also the most abundant α-proteobacteria associated with *E. huxleyi* blooms, which potentially produce coccolith as solid surfaces for the bacteria to colonize (Gonzalez et al. 2000). In addition, roseobacters can also be highly motile, because of their biphasic lifecycle in which the can either be sessile and form biofilms on solid surfaces or swim in response to signaling molecules (Miller et al. 2004; Wadhams and Armitage 2004). The *Roseobacter* clade accounts for 15-20% of the marine bacterial communities in various marine habitats, and they represent the majority of the heterotrophic bacteria in the upper photic layer of the oceanic water column (Rappé, Vergin and Giovannoni 2000). The first two roseobacters identified, *Roseobacter litoralis* and *Roseobacter dentrificans*, were named for their distinctive pink pigment and bacteriochlorophyll (Bchl) a (Shiba 1991). Roseobacters are common primary colonizers of surfaces in the marine

environment (Dang and Lovell 2000). Their ability to colonize surfaces in early succession provides them with advantages such as increased access to nutrients, protection against antimicrobial agents and potential competition and invasion of secondary colonizers (Rao, Webb and Kjelleberg 2006).

Phaeobacter inhibens DSM 17395 is the first bacteria described as a pathogen of *E. huxleyi* (Seyedsayamdost *et al.* 2011b; Bramucci *et al.* 2018). It has been implicated that *P. inhibens* produces algaecidal molecule roseobacticide in response to the production of the senescence signal *p*-coumaric acid, a breakdown product of the aging *E. huxleyi* cell wall (Seyedsayamdost *et al.* 2011b). In a co-culture of *E. huxleyi* and *P. inhibens* under laboratory conditions, the algal health of *E. huxleyi* starts to decline at day 6, and *E. huxleyi* is fully killed by *P. inhibens* around day 12 (Bramucci *et al.* 2018). In addition to the pathogenicity of *P. inhibens*, it has been suggested that there is also a symbiotic phase of the interactions between *E. huxleyi* and *P. inhibens* (Seyedsayamdost *et al.* 2011b). Indeed, *P. inhibens,* like other roseobacters, is known to produce a sulfur-containing antimicrobial compound, tropodithietic acid (TDA), during its sessile phase, and the sulfur source is likely from DMSP (Brinkhoff *et al.* 2004; Bruhn *et al.* 2006; Wang, Gallant and Seyedsayamdost 2016). It is possible that *P. inhibens* protects the algal host against other potential pathogens via the production of TDA. The two novel bioactive molecules, roseobacticide and TDA, can mediate a dual phase lifestyle of *P. inhibens* as both pathogen and symbiont to *E. huxleyi*. The hypothetical ecological interactions among the organism are illustrated in Figure 1-1.

The only other bacteria demonstrated as a pathogen of *E. huxleyi* is another roseobacter, *R. italica* (Mayers *et al.* 2016). It was known to cause bleaching disease in

Delisea pulchra, an important habitat-forming red macroalga (Case *et al.* 2011). The pathogenesis of *R. italica* to *D. pulchra* is only present at elevated temperature either in the laboratory or natural environment (Case *et al.* 2011). *R. italica* was originally isolated from the Tasman Sea off the coast of Australia, where *E.huxleyi* can form massive blooms. Since *R. italica* is closely related to the known pathogen of *E. huxleyi*, *P. inhibens*, the pathogenesis of *R. italica* towards *E. huxleyi* was investigated, and it was found that there was a temperature-enhanced virulence causing bleaching in *E. huxleyi* (Mayers *et al.* 2016). At 18 °C, *R. italica* starts to cause a decline in algal health at day 10 in the co-culture experiment of *R. italica* and *E. huxleyi* (Mayers *et al.* 2016). In comparison, the decline happens much earlier by day 2 at 25 °C (Mayers *et al.* 2016). The specific mechanism of the pathogenesis remains unsolved, but *R. italica* is known to produce ammonia and cytolytic toxins, which inhibits photosynthesis and lyses cells, respectively (Fernandes *et al.* 2011). It was also recently found that *R. italica* is able to produce repeats-in-toxin (RTX) like proteins that mediate colonization and host damage (Gardiner *et al.* 2017). *R. italica* also produces other secondary metabolites, such as glutathione peroxidase, which enables it to resists oxidative bursts from the host (Gardiner *et al.* 2015).

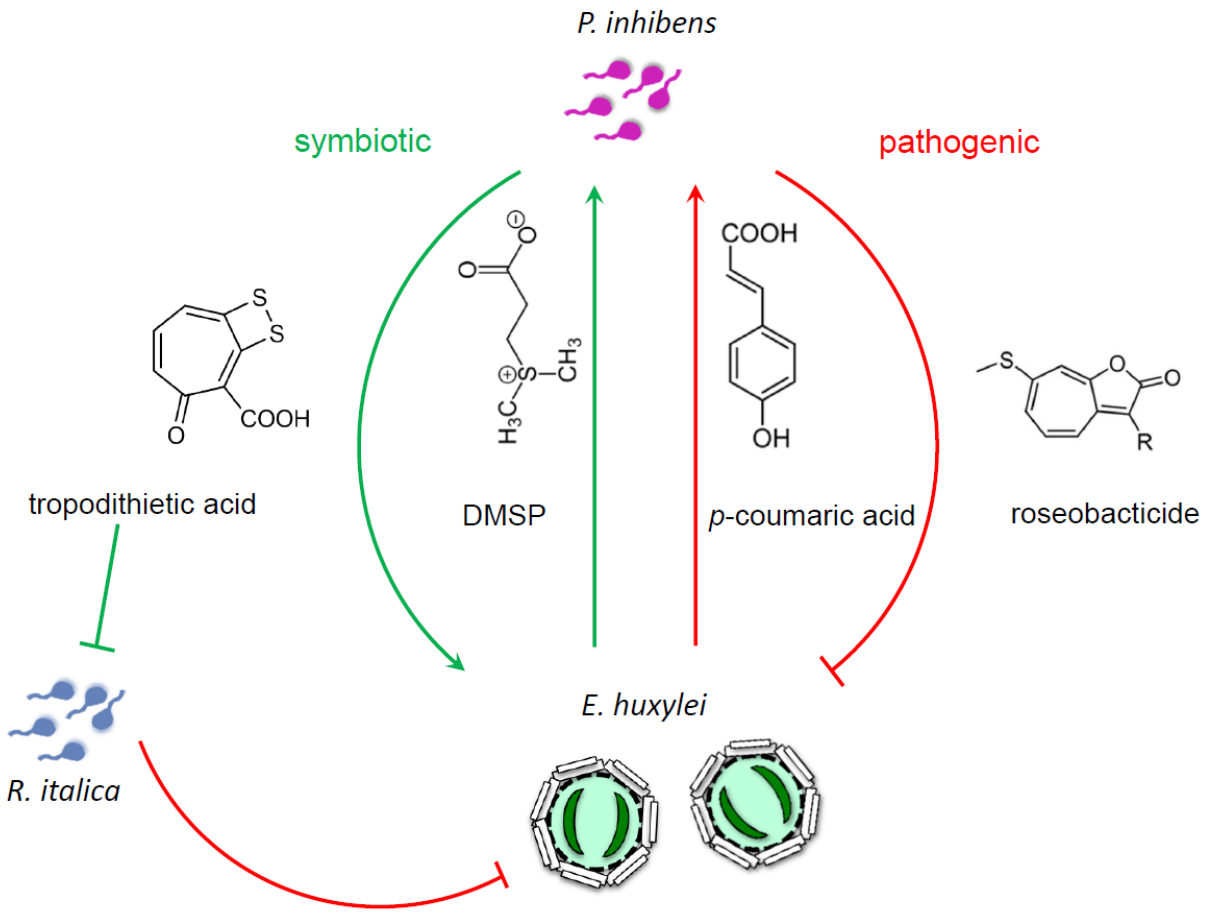

Figure 1-1: Proposed model for algal-bacterial interactions among *P. inhibens*, *R. italica* and *E. huxleyi*. The interactions are composed of two phases mediated by novel molecules, a symbiotic phase (green) and a pathogenic phase (red). During the symbiotic phase, *P. inhibens* utilize DMSP produced by *E. huxleyi* to synthesize tropodithietic acid (TDA) which is an antimicrobial against pathogen *R. italica*; In the pathogenic phase, the senesced algal host releases p-coumaric acid from broken cell walls. In response, *P. inhibens* produce algaecide roseobacticides to kill *E. huxleyi* (Wang, Gallant and Seyedsayamdost 2016). Adapted from "Investigation of the genetics and biochemistry of roseobacticide production in the *Roseobacter* clade bacterium *Phaeobacter inhibens*," by Wang R, Gallant É, Seyedsayamdost MR., 2016, MBio;7:1–10.

1.4 The biochemistry of antimicrobial compound tropodithietic acid

1.4.1 Chemical structures and functions of TDA

Penicillin, the first antibiotic to be discovered, was found by Alexander Fleming in 1928, and its clinical use began in the early 1940s due to the demand from wounded soldiers during World War II (Tan and Tatsumura 2015). Antibiotics saved countless lives since then, and it was widely propagandized that antibiotics were “miracle drugs”, even for viral infections (Zarb and Goossens 2012). However, bacteria have a high capacity to mutate and acquire resistance genes, which are commonly carried by plasmids and transposons, through horizontal transfer (Kasanah and Hamann 2004). Due to the heavy use of antibiotic treatments in the last century, certain bacterial pathogens have been shown to have broad resistance to many antibiotics currently available. There have been significant resources devoted to the discovery of new antimicrobial natural compounds or the development of novel antibiotic drugs. The marine environment has been considered a rich source of such compounds, because of novel antimicrobials have been found in marine microorganisms associated with sponge, coral, and algae (Kasanah and Hamann 2004).

As mentioned in the previous Section 1.3.2, TDA is an antimicrobial compound produced by roseobacter *P. inhibens* and its close relatives (Brinkhoff *et al.* 2004; Geng *et al.* 2008). It has has a unique 7-membered-ring structure of tropone-2-carboxylic acid with a dithiet moiety fused to it (Figure 1-2 a) (Geng *et al.* 2008; Rabe *et al.* 2014). In *Phaeobacter*, TDA can be found with a hydroxyl radical in the form of hydroxy-TDA (Figure 1-2 b) (Rabe *et al.* 2014). The tautomer of TDA, thiotropocin, was found in *Pseudomonas* (Figure 1-2 c) (Greer *et al.* 2008; Rabe *et al.* 2014).

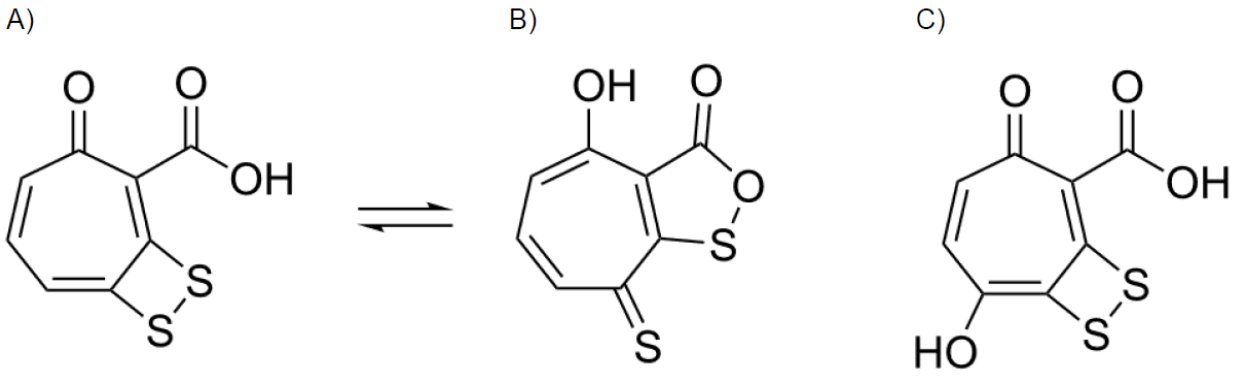

Figure 1-2: Tropodithietic acid and closely related compounds produced by bacteria. A) tropodithietic acid (TDA) with a dithiet moiety fused to tropone-2-carboxylic acid in *Phaeobacter inhibens* (Liang 2003); **B)** TDA with a hydroxyl radical, hydroxy-TDA in other *Phaeobacter* species (Rabe *et al.* 2014); **C)** the tautomer of TDA, thiotropocin, reported in *Pseudomonas* (Cane, Zhen and Vanepp 1992). Adapted from "Synthesis and bioactivity of analogues of the marine antibiotic tropodithietic acid," by Rabe et al., 2014, *Beilstein J Org Chem*,10:1796–801.

TDA is a broad spectrum antibiotic with the mode of action similar to polyether antibiotics that disrupt the permeability of cell membranes to cations such as K^+, Na^+, and Ca^{2+} (Kevin II, Meujo and Hamann 2009; Wilson *et al.* 2016). It has been implicated that TDA is an effective antibiotic on a wide range of human pathogens, including Gram-positive and Gram-negative bacteria (Porsby *et al.* 2011; Wilson *et al.* 2016). As there was also anticancer cell toxicity previously reported in polyether drugs, the anticancer activity of TDA was investigated (Huczyński 2012). It was shown that TDA is a potent anticancer agent with high lethality or a growth inhibitory effect towards a number of cancer cell lines, such as renal cancer cell, lung cancer cells and melanomas (Wilson *et al.* 2016).

In light of this ability to produce TDA, several roseobacters have been used as probiotics in aquaculture (Porsby *et al.* 2011). It was shown that TDA producing bacteria *Phaeobacter* sp. 27-4 (formally *Roseobacter* sp. strain 27-4) could kill fish pathogenic bacteria like *Vibrio anguillarum*, but a mutant of the same strain, which was deficient in TDA production, could not kill *V. anguillarum* (D'Alvise *et al.* 2010). The same TDA producing roseobacter strain was also shown to have an inhibitory effect on another common fish pathogen, *Vibrio splendidus* (Bruhn *et al.* 2005). It has been proposed that TDA is a potential fish larval probiotic, because it not only can decrease the mortality in the larvae of commercial fish (including turbot and cod) but also has no toxic effects on *C. elegans* and *Artemia* sp., which are commonly used as models for toxicity towards eukaryotes (Harrington *et al.* 2014).

In addition to its antibiotic activities, TDA can also function as an autoinducer controlling its own production in the roseobacter *Silicibacter* sp. TM1040 (Geng and

Belas 2010). It was also shown TDA production in *P. inhibens* is controlled by N-acylhomoserine lactones (AHLs), that are intercellular signaling molecules responsible for QS, a population-density-dependent chemical communication (Berger *et al.* 2011). Further molecular studies confirmed the role of TDA as an autoinducer in *P. inhibens*, and it was demonstrated that TDA regulates not only its own production, but also that of 297 other genes involved in motility, chemotaxis, and biofilm formation, which are down-regulated by the TDA-mediated QS (Beyersmann *et al.* 2017).

1.4.2 TDA biosynthesis in *P. inhibens*

A ^{13}C isotope feeding experiment has previously shown that the carbon source of the unique carbon backbone of TDA was the amino acid phenylalanine (Phe) and its catabolite phenylacetic acid (PAA) from PAA catabolon, which is a functional unit of different pathways that catalyzes the transformation of Phe, PAA and other structurally related compounds into phenylacetyl-CoA (Luengo, García and Olivera 2001; Berger *et al.* 2012). The involvement of the PAA catabolon, primary sulfur metabolism, and primary TDA biosynthetic gene cluster (*tdaA-F*) in TDA biosynthesis was further confirmed in a functional genomics study based on transposon mutagenesis (Thole *et al.* 2012). First, Phe is converted to phenylpyruvate (PP) via TyrB, which shows similarity to aminotransferase of archea. In the next step, oxidoreductase Ior1 converts PP to phenylacetyl-CoA (compound 1), and then multicomponent oxygenase PaaABCDE converts compound 1 to ring-1,2-epoxyphenylacetyl-CoA (compound 2) (Thole *et al.* 2012). In the next steps, PaaG and PaaZ convert compound 2 to 2,5-cycloheptadiene-1-one-2-formyl-CoA (compound 3) (Berger *et al.* 2011; Thole *et al.* 2012). Compound 3

would serve as the substrate for enzymes TdaA-F for TDA formation, while putative cystathionine-β-lyase (PatB) insert sulfur from sulfur-containing amino acid cysteine, which is converted from algal-produced DMSP into TDA (Wang, Gallant and Seyedsayamdost 2016). This proposed biosynthetic pathway is illustrated in Figure 1-3.

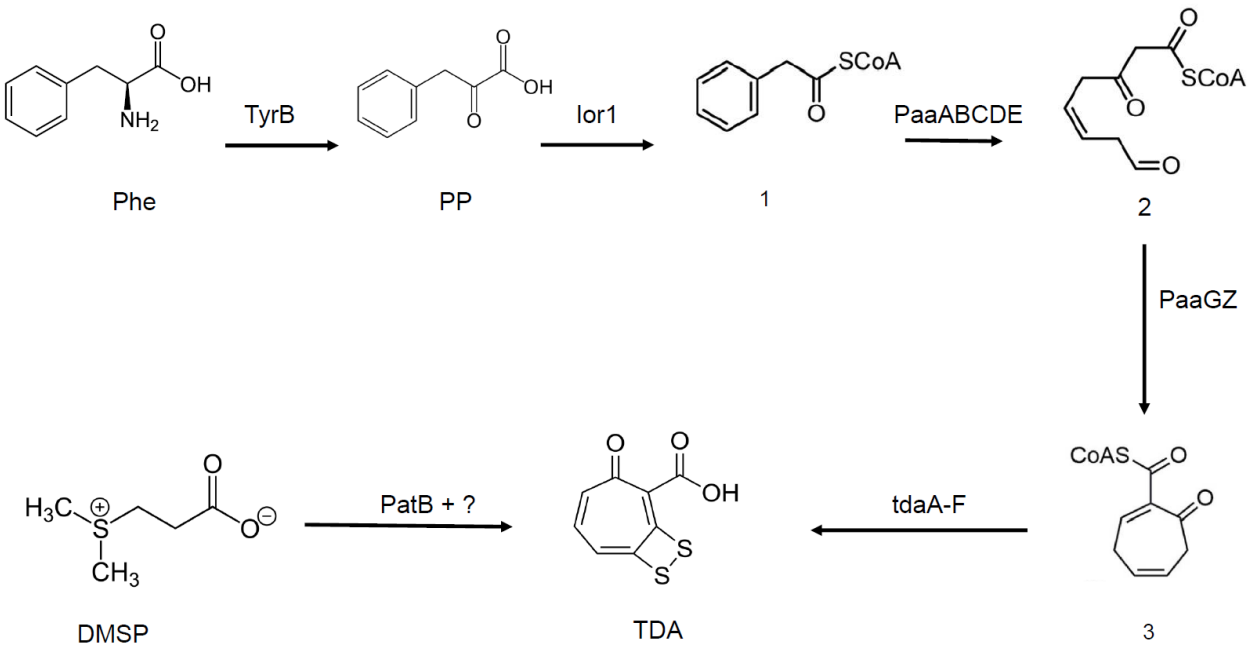

Figure 1-3: Biosynthesis of tropodithietic acid in *Phaeobacter inhibens*. Phe, phenylalanine; PP, phenylpyruvate; compound 1, phenylacetyl-CoA; compound 2, ring-1,2-epoxyphenylacetyl-CoA; compound 3, 2,5-cycloheptadiene-1-one-2-formyl-CoA; TDA, tropodithietic acid; DMSP, dimethylsulfoniopropionate. The enzymes involved in the process of converting sulfur from DMSP to TDA remain unknown, except for the PatB, a cystathionine-β-lyase insert sulfur from sulfur-containing amino acid cystine potentially converted from algae-produced DMSP (Wang, Gallant and Seyedsayamdost 2016). Adapted from "Genetic analysis of the upper phenylacetate catabolic pathway in the production of tropodithietic acid by *Phaeobacter gallaeciensis*," by Berger et al., 2012, *Appl Environ Microbiol*; 78:3539–51.

1.5 Thesis objectives

There have been several studies focusing on direct interactions between one bacterial symbiont and one algal host (Rao, Webb and Kjelleberg 2006; Case *et al.* 2011; Seyedsayamdost *et al.* 2011b; Labeeuw *et al.* 2016; Mayers *et al.* 2016; Bramucci *et al.* 2018). However, a single algal host is typically colonized by multiple bacteria species in natural marine system (Burmølle *et al.* 2006), and interactions among the bacterial symbionts have received little attention. This topic can be difficult to investigate, due to the complexity of co-culturing multiple species in laboratory settings. Based on *R. italica* pathogenicity against microalga *E. huxleyi* and the probiotic activity of *P. inhibens* linked to its production of TDA (Section 1.2.3), I hypothesized that *P. inhibens* provides chemical defense against pathogen *R. italica* via TDA production during early bacterial colonization of *E. huxleyi*. A tri-culture system of roseobacters *P. inhibens*, *R. italica*, and the alga *E. huxleyi* was established to test this hypothesis. It is important to understand how novel bioactive molecules mediate biological interactions, not only because of the ecological impacts of bacteria and algae on global cycle of essential chemical elements like carbon and sulfur, but also because of industrial and pharmaceutical values of novel natural products produced by these microorganisms.

To answer this hypothesis, this thesis had two main objectives:

1. To test if *P. inhibens* is a better primary colonizer than *R. italica* of the algal host *E. huxleyi* by forming a higher population density.
2. To assess the role that tropodithietic acid production by *P. inhibens* plays in deterring colonization by the pathogen *R. italica* on their host, *E. huxleyi*.

Chapter 2 Bacterial Comepetition over Host Microalga *Emiliania huxleyi*

2.1 Introduction

Surfaces are prone to colonization by microorganisms in marine environments. For example, typical bacterial population densities on surfaces of a microalga can reach up to about 10^7 cells per cm^2 (Armstrong *et al.* 2000). Many previous studies have been focused on the interactions between marine algae and bacteria and the beneficial effects provided by algal cells to associated bacteria (Lange 1967; Jones and Cannon 1986), or the impacts of bacterial bioactive molecules on the behavior and survival of the host (Seyedsayamdost *et al.* 2011b; Labeeuw *et al.* 2016; Bramucci *et al.* 2018). In some cases, bacteria are essential for maintaining the health of algae, providing crucial vitamins and growth hormones (Bolch, Subramanian and Green 2011), while there can be also bacterial pathogens releasing virulence factors that cause disease or kill their algal host (Case *et al.* 2011; Seyedsayamdost *et al.* 2011b).

Emiliania huxleyi, a ubiquitous marine microalga, belongs to phylum haptophyta and forms massive coccolithophore blooms that influence the global carbon and phosphate cycles (Paasche 2001). *E. huxleyi* lives in close association with diverse groups of microorganisms (Green *et al.* 2015). The *Roseobacter* clade is one the most abundant bacterial groups commonly isolated from *E. huxleyi* blooms (Gonzalez et al. 2000; Green et al. 2015). Roseobacters makes up to 30% of marine bacterial communities during algal blooms (Bentzon-Tilia and Gram 2017), and many of the members of the clade are known as probiotics and pathogens in different aquaculture systems (D'Alvise *et al.* 2012). Interactions among members of the microbial

communities are usually directed by chemical exchange and communication (Sapp *et al.* 2007).

One example of a roseobacter forming a symbiotic interaction with *E. huxleyi* is *Phaeobacter inhibens* (Seyedsayamdost *et al.* 2011b; Segev *et al.* 2016). The colonization strategy *P. inhibens* 2.10 (formerly known as *Roseobacter gallaeciensis and Phaeobacter gallaeciensis*) was previously described in association with the green algae *Ulva australis* (Rao, Webb and Kjelleberg 2006). It was demonstrated that *P. inhibens* is an aggressive colonizer, as its colonization process is not negatively affected by either low inoculum cell density, light, or carbon source limitation (Rao, Webb and Kjelleberg 2006). *P. inhibens* was even able to invade and out-compete a pre-established biofilm of *Pseudoalteromonas* tunicate, a known symbiont of *U. australis* (Rao, Webb and Kjelleberg 2006). Normally, once the biofilms of one bacterial population are established, the protective structures would hold against other competing bacteria (Rao, Webb and Kjelleberg 2006). *P. inhibens is the* superior competitor during multi-species colonization, which is attributed to its ability to produce an extracellular antibacterial compound, tropodithietic acid (TDA) (Rao, Webb and Kjelleberg 2005).

TDA is a sulfur-containing compound with a unique 7-membered-ring structure which co-exists with its tautomer, thiotropocin, in *Pseudomonas* (Greer *et al.* 2008). It has been shown that TDA inhibits a wide range of marine bacteria, including fish-pathogenic bacteria *Vibrio anguillarum* and *Vibrio splendidus* (Porsby *et al.* 2011). Although TDA production is a distinguishable feature of several *Roseobacter* species, this compound was first isolated from a soil bacterium, *Pseudomonas* sp. (Kintaka *et al.* 1984; Cane, Zhen and Vanepp 1992). Therefore, it is not surprising that the

antibacterial effects of TDA can act outside the marine environment, including common Gram-negative and Gram-positive human pathogens (Porsby *et al.* 2011).

Due to its broad antimicrobial spectrum and its novel chemical structure, the biosynthetic pathway of TDA was intensively investigated in *P. inhibens* DSM 17395, which is a close relative to *P. inhibens* 2.10 with 97% average nucleotide identity across their shared genes (Thole *et al.* 2012). 26 genes were identified via screening for transposon mutants with reduced brown pigmentation, which correlates with TDA production. (Thole *et al.* 2012). The identified key TDA production genes *tdaABCEF, which* were also previously described in another roseobacter, *Silicibacter* sp. TM1040, which has the same set of *tda* genes as *P. inhibens*, and defects in any of the key genes would cause the loss of TDA production (Geng *et al.* 2008).

In a later study about TDA production in TM1040 (Geng and Belas 2010), a cross-feeding experiment between TDA^- mutants and exogenous TDA demonstrated this compound is a density-dependent autoinducer that regulates expression of its own biosynthetic genes. In *P. inhibens*, TDA production is not only controlled by itself, but also by *N*-acyl homoserine lactones (AHL), which is family of intercellular signal molecules for quorum sensing (QS) (Rao, Webb and Kjelleberg 2006; Berger *et al.* 2011). Based on these previous studies, the role of TDA as a signaling molecule and its correlation to QS has been further investigated. It was indicated that TDA in sub-inhibitory concentration (1.5 μM) can interact with AHL regulators, thus controlling the expressions of genes involved in TDA synthesis, motility, surface attachment, and AHL synthesis which are regulated by typical AHL-based QS. (Beyersmann *et al.* 2017).

In addition to *P. inhibens*, a recent study demonstrated that another member of the roseobacter clade, *Ruegeria italica* R11, is a pathogen of *E. huxleyi* C (diploid coccolith bearing) and S (haploid swarming cells) (Mayers *et al.* 2016). Although the specific mechanisms of the pathogenesis have not been revealed, there are several possible virulence factors that could play a role. *R. italica* has a novel gene, *varR,* a homolog to quorum sensing regulators, that was not found in closely related non-pathogenic strains (Fernandes *et al.* 2011). It has been shown that mutation in *varR* leads to a deficiency in surface attachment, biofilm formation, and virulence to algal host (Gardiner *et al.* 2015). Proteomics data also revealed the role of VarR protein in LuxR-type quorum sensing regulation on the expression of biofilm-associated proteins involved in nutrient scavenging, chemotaxis, and energy reserves that provide advantages to *R. italica* for growth and colonization (Gardiner *et al.* 2015). In their later study the extracellular proteome (exoproteome) of this bacterium, eight proteins were found to have high identity to repeats-in-toxin (RTX) proteins that mediate colonization and host damage in other pathogens (Fernandes *et al.* 2011; Vigil *et al.* 2012; Gardiner *et al.* 2017). Little is known about the ability of *R. italica* to compete with other bacteria during colonization of algal hosts.

Based on previous studies (Seyedsayamdost *et al.* 2011b; Wang, Gallant and Seyedsayamdost 2016), a model of ecological interactions among the three organisms is proposed in this study: *E. huxleyi* is the algal host, and *P. inhibens* and *R. italica* are bacterial symbionts. *E. huxleyi* produces dimethylsulfoniopropionate (DMSP), a sulfur-containing molecule that either serves as a source of nutrients or one of the substrates required for TDA production in *P. inhibens*, providing antimicrobial defense against

pathogens such as *R. italica*. In this study, I aimed to investigate the colonization dynamics of marine bacteria *R. italica* and *P. inhibens*, which both colonize the surface of the marine algae *E. huxleyi*. I hypothesized that *P. inhibens* provides chemical defense against pathogen *R. italica* via TDA production during early bacterial colonization of *E. huxleyi*. Despite its production of the antimicrobial compound TDA, we found that *P. inhibens* is only more abundant than *R. italica* when the latter is inoculated as a secondary colonizer of *E. huxleyi*. TDA does significantly reduce *R. italica* population density, demonstrating that it plays a role in the chemical defense of its host, but other ecological factors, such as fitness traits involved in colonization, can also play a role in this inhibitory effect.

2.2 Materials and Methods

2.2.1 Bacterial and algal culturing

The bacteria *Phaeobacter inhibens* DSM 17395 and *Ruegeria italica* R11 were maintained on half marine broth agar medium (hMB, 18.7 g Difco Marine Broth 2216n, supplemented 9 g NaCl, and 15 g Difco agar in 1 L). Δ*tdaB P. inhibens* mutant, received from the Join Genome Institute (USA), is defective in a single gene *tdaB* required for the production of TDA, and it was maintained on hMB agar medium supplemented with 100µg/mL kanamycin (Geng *et al.* 2008; Thole *et al.* 2012; Wetmore *et al.* 2015). Prior to experiments, the bacterial cultures were grown in 12.5 mL half yeast extract, tryptone and sea salt liquid medium (hYTSS, 2 g yeast extract, 1.25 g tryptone, and 20 g sea salt in 1L) in 125 mL Erlenmeyer flasks in a shaking incubator (160 rpm) at 25 °C for 24 h to reach stationary phase.

Emiliania huxleyi CCMP3266, a coccolith bearing strain (C cell type), was obtained from the Provasoli-Guillard National Centre for Marine Algae and Microbiota (NCMA). *E. huxleyi* was maintained in L1-Si media ($NaNO_3$ 8.82 x 10^{-4} M, NaH_2PO_4 3.62 x 10^{-5} M, trace element solution, and vitamin solution) with filtered seawater that obtained from Bamfield Marine Science Center (R. R. L. Guillard and P. E. Hargraves 1993) at 18 °C in a diurnal incubator (8:16 h dark–light cycle). *E. huxleyi* cultures and L1-Si media were checked for bacterial contamination before experiments. Microscopic confirmation of the absence of bacterial cells was required. Also, samples of pure cultures and media were inoculated onto hMB agar followed by incubation at 30 °C for 2 d. In order to obtain early-log phase cultures for experimental use, *E. huxleyi* cultures

were grown statically for 5 d in the diurnal incubator under the same conditions as for maintenance.

2.2.2 Soft agar antibiotic assay

hMB agar was inoculated with an *R. italica* culture, which was grown for 24 h at 25 ◦C, 160 rpm, before solidifying. 20 µL of *P. inhibens* wild type and Δ*tdaB* mutant liquid cultures, which was grown under the same condition as *R. italica*, were plated onto the *R. italica*-inoculated hMB agar. The plates were incubated at 30 ◦C and checked for zones of inhibition after 24 h (Brown and Kothari 1975).

2.2.3 Control cultures, co-cultures, and tri-cultures

Control cultures of axenic *E. huxleyi*, co-cultures of *R. italica* and *E. huxleyi*, co-cultures of *P. inhibens* wild type (or Δ*tdaB* mutant) and *E. huxleyi*, were prepared as described in a previous study with the exception of having the initial bacterial concentration one magnitude higher in order to shorten the exponential phase of growth and having a more stable culture for the studying the early bacterial colonization (Bramucci *et al.* 2015). For *E. huxleyi* in all experiments, a starter culture was grown in L1-Si medium for 5 days to early-exponential phase (about 10^4 cells/mL) at 18 ◦C in a diurnal incubator (8:16 h dark-light cycle). Bacterial cultures were washed twice by centrifugation and re-suspended in L1-Si media prior to serial dilutions in L1-Si to the correct order of magnitude and the exact initial cell concentrations were calculated using colony forming units (cfu) counts.

For co-culture experiments, two starter cultures of *P. inhibens* and *R. italica* were grown for 24 h at 25 °C, 160 rpm in a shaking incubator to 10^9 cfu/mL, and then were diluted down to 10^3 cfu/mL for a final bacterial concentration in bacteria-algae co-cultures of either *P. inhibens–E. huxleyi* or *R. italica -E. huxleyi*. For tri-culture experiments of *R. italica*, *P. inhibens* wild type (or Δ*tdaB* mutant) and *E. huxleyi*, bacterial starter cultures were prepared in the same manner. The first tri-culture experiment had the same initial concentration for both bacteria at 10^3 cfu/mL, and the second tri-culture experiment had the initial concentration of *P. inhibens* at 10^4 cfu/mL while that of *R. italica* was 10^3 cfu/mL. In the last triculture experiment, *R. italica* (10^3 cfu/mL) was added 24 h post *P. inhibens* (initially at 10^3 cfu/mL) inoculation to *E. huxleyi*.

Each culture sample was aliquoted into 48-well microtiter plates, 1 mL per well, with triplicate wells for each sample (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). All microtiter plates were sealed with double parafilm and incubated for 72 h at 18 °C in a diurnal incubator (8:16 h dark-light cycle).

2.2.4 Microscopy

Algal control cultures, co-cultures, and tri-cultures were visualized at 24 h post inoculation using an Axio Imager M2 microscope (Zeiss, Oberkochen, Germany). Differential interference contrast (DIC) images were obtained in the bright field with optics enhancing the contrast of the transparent cell structures in grayscale (Contrast 2003). Both algal and bacterial DNA were stained with SYBR Green I (SG). SG binds to DNA to form a DNA-dye-complex, and the complex absorbs blue light (λ_{max} = 497 nm) results in emission of green light (λ_{max} = 520 nm) (Zipper *et al.* 2004). Live samples of

cultures were stained in dark for 20 min and observed immediately on the microscope. Images were obtained simultaneously in both the DIC and SG channels, and the images were overlaid in the software Zen 2012 (Zeiss).

2.2.5 Enumerating bacterial population density

Bacterial population density was measured by cfu, which is also used for calculation of growth rates between time points (Table S-5 to S-8). For each culture sample in triplicate, a serial dilution was performed in sterile phosphate-buffered saline (PBS) allowing for cfu enumeration on agar plates. Samples were vigorously vortexed for at least 2 min before dilution to break cell aggregates and remove attached bacterial cells from *E. huxleyi*. For each replicate, 100 µL diluted culture sample was spread onto a hMB agar plate. As *R. italica* colonies are white, colonies of *P. inhibens* wild type on hMB agar are dark brown which is a pigmentation phenotype strictly correlating to TDA production in roseobacters (Geng and Belas 2010; Berger *et al.* 2011). This difference in color between the colonies allows them to enumerate the cfu of *R. italica* and *P. inhibens* separately on the same agar plate for tri-cultures. However, the Δ*tdaB* mutants do not produce TDA, thus do not show the indicative brown color and it would be hard to differentiate them from *R. italica* based on colony morphology. Therefore, for tri-tricultures with *R. italica* and Δ*tdaB* mutants, the cfu of the two bacteria together was enumerated on hMB agar for total counts, while the cfu of Δ*tdaB* mutants was enumerated on hMB agar supplemented with 100 µg/mL kanamycin (Wetmore *et al.* 2015), and the cfu of *R. italica* could be calculated by subtracting the cfu of Δ*tdaB* mutants from the total counts. The plates were incubated for 48 h at 30 °C.

2.2.6 Fluorescence measurements

To measure the Photosystem II (PSII) health, photosynthetic yield was measured using a pulse-amplitude-modulation (PAM) fluorometer (WATER-PAM, Waltz, Effeltrich, Germany). Samples were measured at the mid-point of their dark cycle. A saturating pulse was applied to each sample after dark adaptation for 3 min as previously determined (Bramucci *et al.* 2015; Mayers *et al.* 2016). The fluorescence intensities were then taken to calculate the PSII potential quantum yield as a measurement of the efficiency of PSII (van Kooten and Snel 1990). Each culture sample in triplicate would be measured and then discarded at each time point so that all measurements are independent. This sampling procedure allowed a time course experiment to be performed without re-sampling and reduced culture volume sampling (Bramucci et al., 2015).

2.2.7 Data processing and statistical analysis

All quantitative data were processed using SigmaPlot (Systat Software) and R Studio (The R Project for Statistical Computing). Statistical significance was determined between treatments at each sample time point at 0, 24, 48, and 72 h. One-way ANOVA, with all assumptions met, was performed for time points 0 h and 72 h. Kruskal-Wallis ANOVA of Ranks was completed for the non-parametric timepoints 24 h and 48 h. Tukeys HSD test was used for post hoc pairwise comparison of treatments. Power analysis was performed to determine that the triplication was the sufficient replication to give ~100% power to detect statistical significance with no type I or type II error.

2.3 Results

2.3.1 *P. inhibens* can inhibit *R. italica* growth on solid media in a diffusion-dependent manner

The sensitivity of *R. italica* to TDA producing *P. inhibens* was tested on hMB agar solid media. *P. inhibens* wild type had dark brown color, which correlates to TDA production (Geng and Belas 2010; Berger *et al.* 2011). A clear zone of inhibition formed after 24 h incubation upon plating *P. inhibens* wild type liquid culture onto *R. italica*-inoculated hMB agar plates, while Δ*tdaB* colonies were white in color and had no zone of inhibition on the same agar plates (Figure 2-1). *R. italica* liquid culture was also plated to test for self-inhibition, and there was no zone of inhibition and the colonies were white in color which is the same color as the *R. italica*-inoculated agar (Figure 2-1).

2.3.2 *R. italica* is a better primary colonizer than *P. inhibens* on host *E. huxleyi*

To further investigate the sensitivity of *R. italica* to TDA-producing *P. inhibens* in L1-Si media with *E. huxleyi*, the two bacteria were inoculated at the same time to the algal host, thus established a triculture system. The population density of each bacterial strain in the tri-culture was compared to that of the bacteria co-cultured separately with *E. huxleyi* (Figure 2-2). All experiments were set up at the same time, run in parallel, and sampled every 24 h. There were bacteria, identifiable with SYBR Green I stain, associating with *E. huxleyi* in all co-cultures and tri-cultures after 24 h of incubation (Figure 2-3). Surprisingly, *R. italica* outcompeted *P. inhibens* in terms of having a higher cell density. The *R. italica* population density in co-culture with *E. huxleyi* and in tri-culture with *P.inhibens* and *E. huxleyi* both increased from 10^3 cfu/mL to 10^6 cfu/mL,

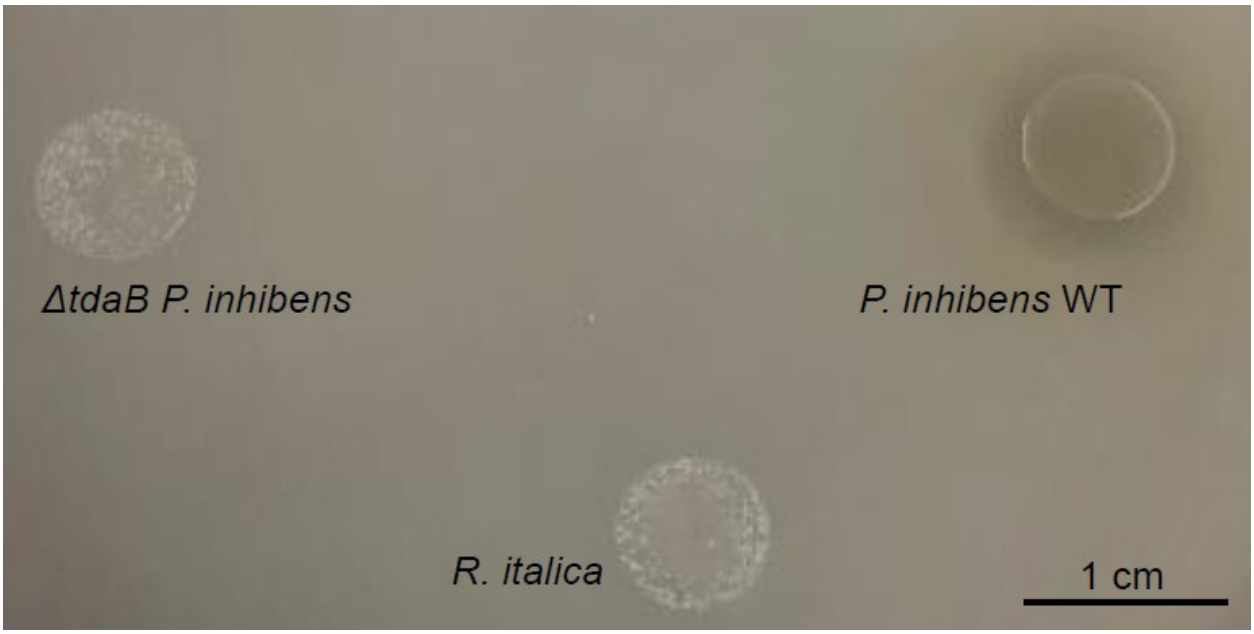

Figure 2-1: *Phaeobacter inhibens* production of tropodithietic acid (TDA) inhibits *Ruegeria italica*. *R. italica, P. inhibens* wild type, and *ΔtdaB* were plated on the solid hMB agar media that is inoculated with. *R. italica* before agar started to solidify. Each colony was grown from a drop of 20 µL liquid culture of each strain. Plates were incubated for 24 h at 30 °C. A zone of clearance was observed around *P. inhibens* wild type brown color colony. Colonies of *R. italica* and *ΔtdaB P. inhibens* were white, and no zone of clearance was formed. The scale bar represents 1 cm.

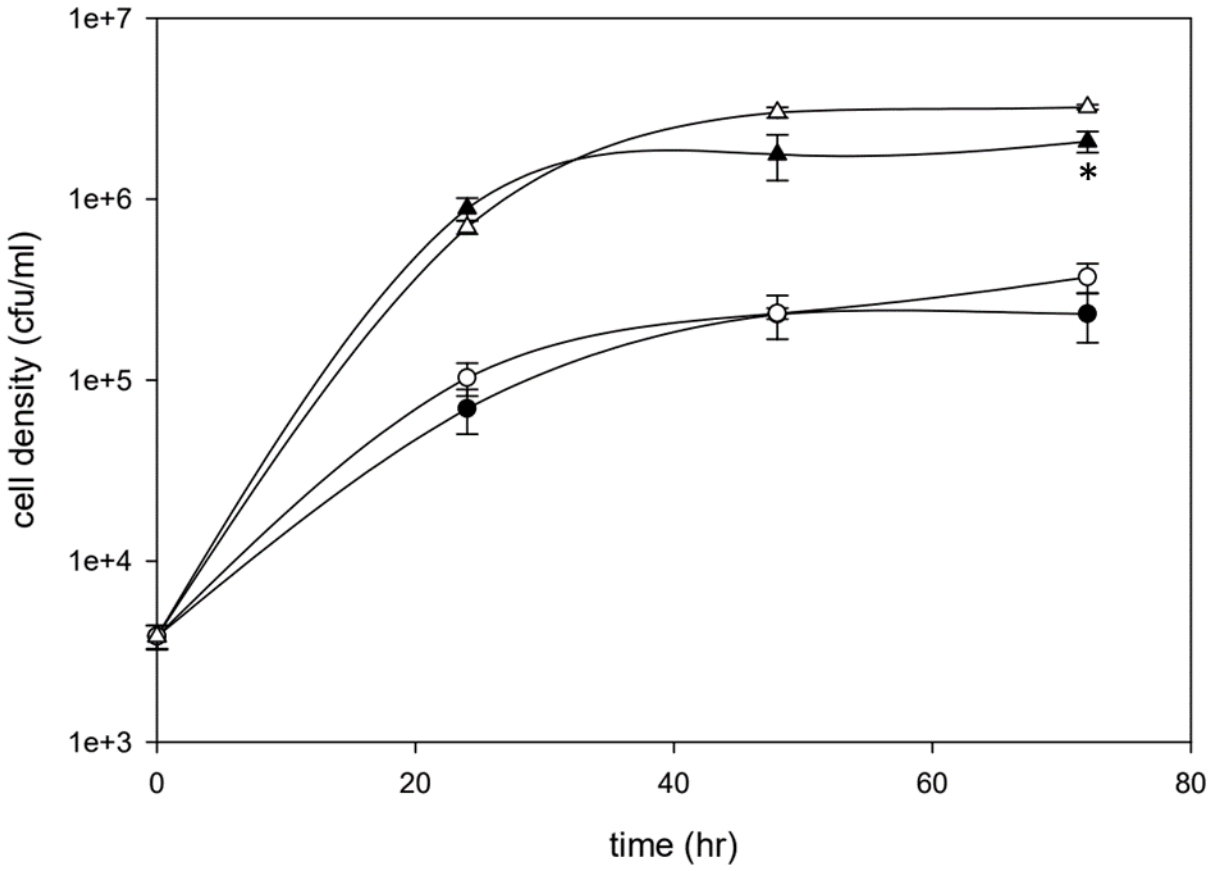

Figure 2-2: Competing *Phaeobacter inhibens* and *Ruegeria italica* as primary colonizers of *Emiliania huxleyi*. *E. huxleyi* (10^5 cells/mL) was cultured with *P. inhibens* (10^3 cells/mL), with or without *R. italica* (10^3 cells/mL), monitored over 72 h to determine the interactions between the two bacteria. Bacterial counts (cfu/mL) were performed on *P. inhibens* and *R. italica* cultured in with *E. huxleyi* in L1-Si media. Data for *P. inhibens* is shown as circles while data for *R. italica* is shown as triangles. The black circle (*P. inhibens*) and black triangle (*R. italica*) are bacterial counts of the tri-culture experiment. The white circle and white triangle are bacterial counts of the co-culture experiments. The population density of *R. italica* in tri-cultures is significantly lower than its density in co-cultures ($P < 0.001$, one-way ANOVA). Bars on either side of the mean represents standard error. All experiments were performed with triplicate sacrificial sampling for each time point.

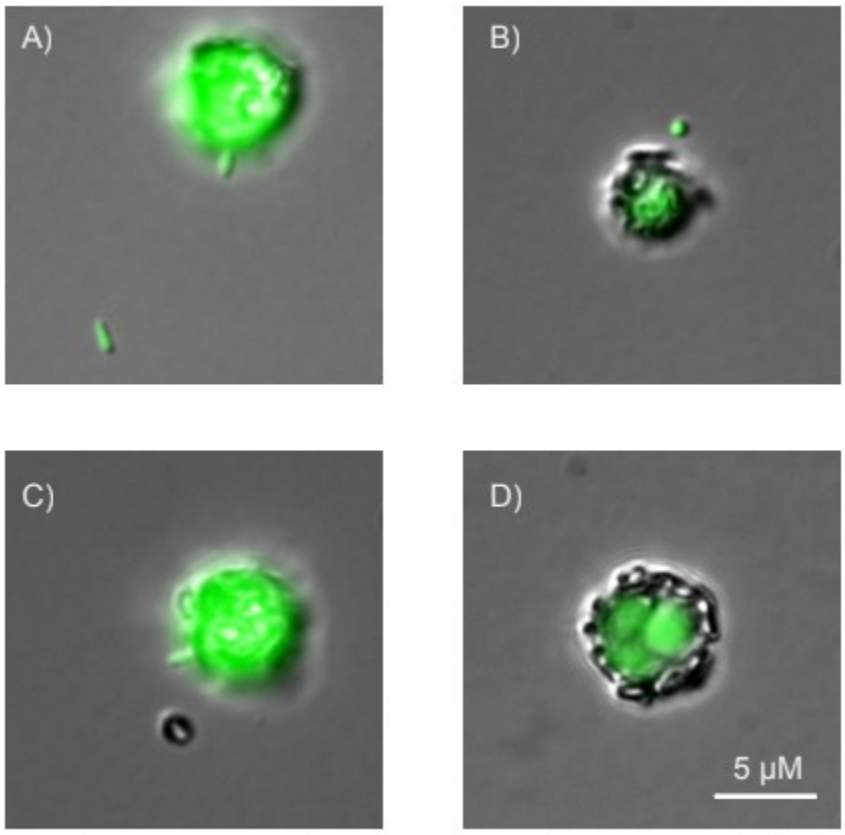

Figure 2-3: Cell associations among *Emiliania huxleyi, Phaeobacter inhibens,* and *Ruegeria italica* were observed via DIC and Sybr Green microscopy. After 24h of inoculation of *R. italica* and/or *P. inhibens* to algal host *Emiliania huxleyi*, Sybr Green fluorescence stain was used to show the DNA content of the algal and bacterial cells **A)** co-culture of *E. huxleyi* and *R. italica*. **B)** co-culture of *E. huxleyi* and *P. inhibens* **C)** tri-culture of *E. huxleyi* with *P. inhibens* and *R. italica*. **D)** *E. huxleyi* axenic culture control. The scale bar represents 5 µM. No morphological changes of *E. huxleyi* caused by associating bacteria were observed.

while *P. inhibens* density only increased from 10^3 cfu/mL to 10^5 cfu/mL in these two scenarios (Figure 2-2, Table S-1). The presence of *P. inhibens* did have a statistically significant impact on the population density of *R. italica* by a reduction of 35% ($P < 0.001$, one-way ANOVA) when both bacteria were inoculated together at the same time with *E. huxleyi* (Figure 2-2, Table S-1). There was no significant effect of *R. italica* on *P. inhibens* population density ($P > 0.05$, one-way ANOVA).

The higher cell density of *R. italica* compared with *P. inhibens* was unexpected given the latter's production of the potent antibacterial compound TDA, which was effective on solid media (Figure 2-1). To verify if *P.inhibens* TDA production in liquid culture was insufficient to inhibit *R. italica* growth, the inoculum of *P. inhibens* was increased to 10^4 cfu/mL, one order of magnitude higher than *R. italica*. At 72 h, *R. italica* (10^6 cfu/mL) still had a higher cell density than *P. inhibens* (10^5 cfu/mL) (Figure 2-4, Table S-2). The *R. italica* population was always more abundant than *P. inhibens* at early stationary phase.

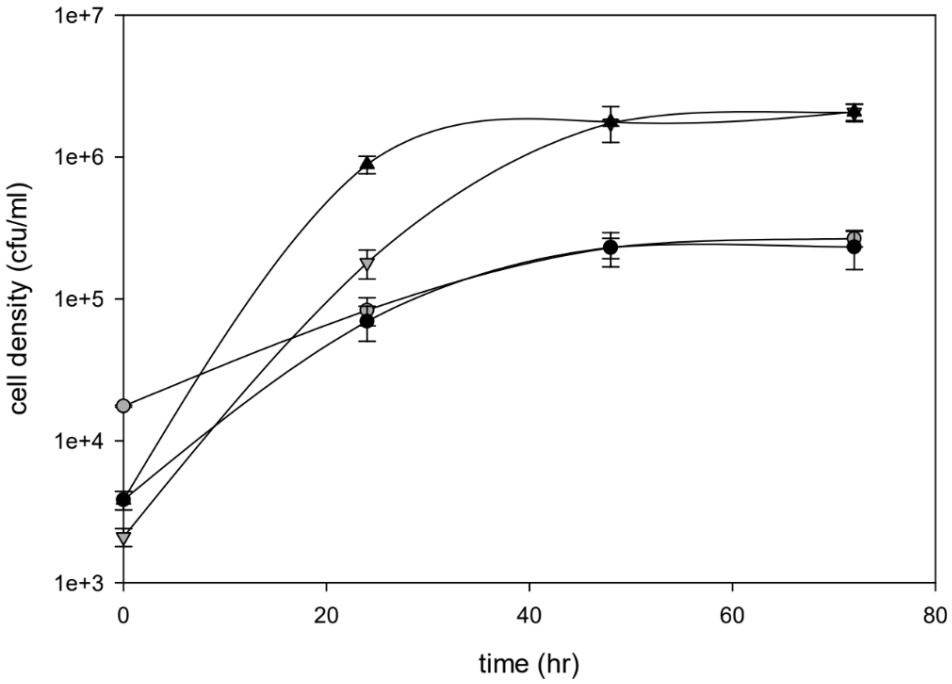

Figure 2-4: Cell density has no effect on *Phaeobacter inhibens* success when competing with *Ruegeria italica* as primary colonizers of *Emiliania huxleyi*. *P. inhibens* was cultured with *R. italica* at different amount of inoculum together with *Emiliania huxleyi* and monitored daily over 72 h. Bacterial counts (cfu/mL) were performed on *P. inhibens* and *R. italica* cultured in L1-SI and tri-cultured with *E. huxleyi*. Data for *P. inhibens* is shown as circles while data for *R. italica* is shown as triangles. Black circle and triangle are bacterial counts in tri-culture experiments where *P.inhibens* and *R. italica* were inoculated at the same concentration (10^3 cfu/mL). Grey circle and triangle are bacterial counts in tri-culture experiments where *P. inhibens* and *R. italica* were inoculated at the same time with the cell density of *P. inhibens* (10^4 cfu/mL) ten times the density of *R. italica* (10^3 cfu/mL). Bars on either side of the mean represents standard error. All experiments were performed with triplicate sacrificial sampling for each time point.

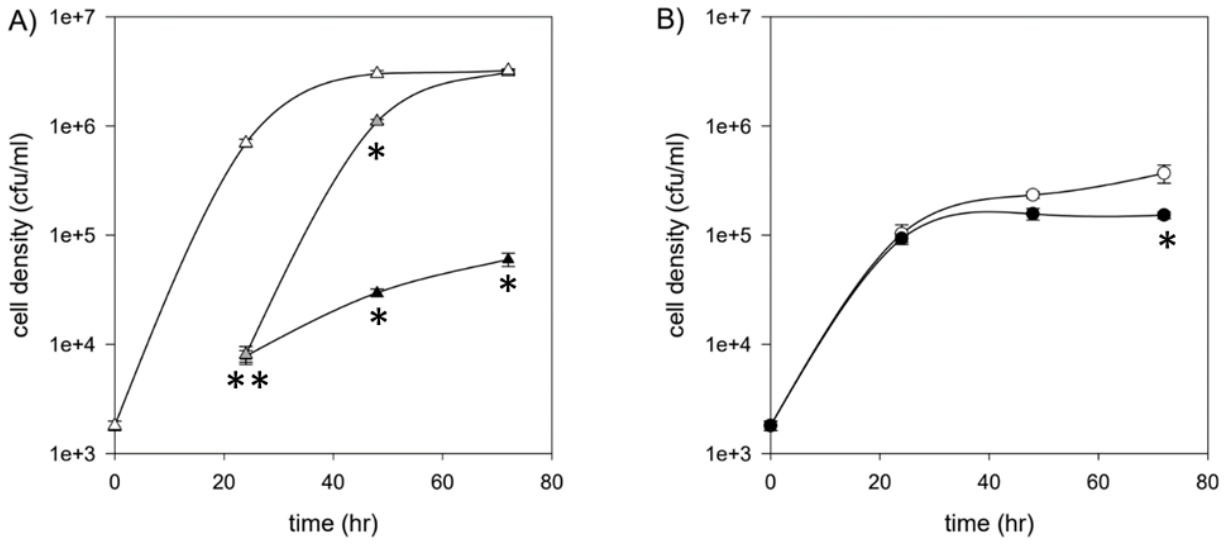

Figure 2-5: Primary colonizer *Phaeobacter inhibens* inhibits secondary colonization of *Ruegeria italica*. To determine the effect of *P. inhibens* inhibition of the pathogen *R. italica* in secondary colonization, *P. inhibens* was inoculated (10^3 cfu/mL) as the primary colonizer (T = 0 h) of the host, *Emiliania huxleyi* in all experiments, and *R. italica* was inoculated (10^3 cfu/mL) as both the primary (T = 0 h) and the secondary colonizer (T = 24 h). The cell density of *R. italica* is represented by triangles (A), while the density of *P. inhibens* is depicted circles (B). White triangles depict *R. italica* inoculated at T = 0 h to *E. huxleyi* as co-culture. Grey triangles depict *R. italica* inoculated at T = 24 h to *E. huxleyi* as co-culture. Black triangles depict *R. italica* inoculated at T = 24 h to *E. huxleyi* and *P. inhibens* as tri-culture. Black circles depict *P. inhibens* in tri-culture, while white circle depict *P. inhibens* in co-culture. Bars on either side of the mean represents standard error. An asterisk indicates population densities statistically significant different from each other, as determined using one-way ANOVA. All experiments were performed with triplicate sacrificial sampling for each time point.

2.3.3 *P. inhibens* inhibits the secondary colonization of *R. italica*

To test the hypothesis that *R. italica*'s higher cell density than *P. inhibens* was due to it being a better primary colonizer, another tri-culture experiment was conducted with *P. inhibens* inoculated first as a primary colonizer and *R. italica* being subsequently added after 24 h as a secondary colonizer. As shown in Figure 2-4A, the co-cultures of *R. italica* and *E. huxleyi*, inoculated at 0 h and 24 h, reached the same order of magnitude concentration (10^6 cfu/mL) at 72 h (stationary phase), suggesting no effect of delayed inoculation of *R. italica* on the final population density in the absence of *P. inhibens*. However, there was a significant 98% reduction in *R. italica*'s population density (Figure S-1) when it is added as a secondary colonizer to the primary colonizer, *P. inhibens* ($P < 0.001$, one-way ANOVA) (Figure 2-5A, Table S-3). The population density of *P.inhibens* co-cultured with *E. huxleyi* (no *R. italica*) was significantly higher when this bacterium was tri-cultured with *E. huxleyi* and *R. italica* (with delayed inoculation) ($P < 0.001$, one-way ANOVA) (Figure 2-5B, Table S-3). This suggests a mutual inhibitory interaction between *R. italica* and *P. inhibens*, which is a form of interspecific competition (Begon, Harper and Townsend 2006).

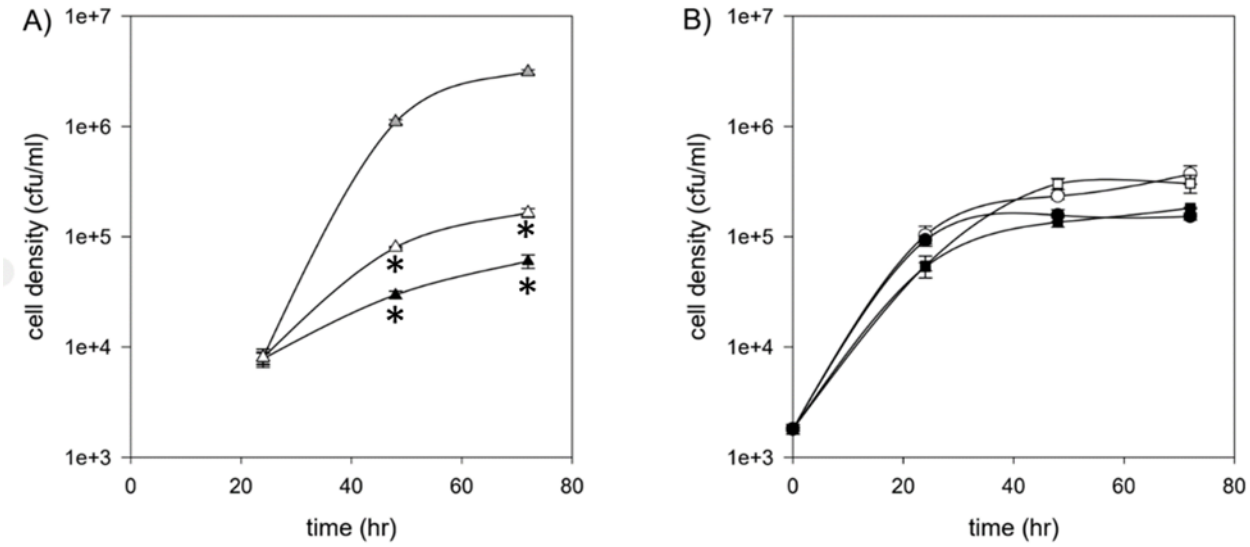

Figure 2-6: Role of tropodithietic acid (TDA) in the inhibition of secondary colonizer by *Phaeobacter inhibens*. *P. inhibens* was inoculated (10^3 cfu/mL) as the primary colonizer (T = 0 h) of the host, *Emiliania huxleyi*, and *R. italica* was inoculated (10^3 cfu/mL) as the secondary colonizer (T = 24 h) in all experiments. To determine the role of tropodithietic acid (TDA) in *P. inhibens* inhibition of the pathogen *R. italica*, *P. inhibens* wild type (wt) and *P. inhibens* deficient in TDA production (*ΔtdaB)* were compared for their ability to reduce secondary colonization of *R. italica*. The cell density of *R. italica* is represented by triangles (A), while the density of *P. inhibens* wt is depicted circles and that of *ΔtdaB P. inhibens* as squares (B). Grey triangles depict *R. italica* with only *E. huxleyi*. White triangles depict *R. italica* with *E. huxleyi* and *ΔtdaB P. inhibens*. Black triangles depict *R. italica* with *E. huxleyi* and *P. inhibens* wt. *P. inhibens* wt and *P. inhibens ΔtdaB* co-cultured with *E. huxleyi* are depicted by white triangles. In panel (B), white shapes depict co-cultures while black shapes depict tri-cultures. Bars on either side of the mean represents standard error. An asterisk indicates population densities statistically significant different from each other, as determined using one-way ANOVA. All experiments were performed with triplicate sacrificial sampling for each time point.

2.3.4 TDA plays a part in *P. inhibens* inhibition of *R. italica*'s secondary colonization

A *P. inhibens* Δ*tdaB* mutant, deficient in the production of TDA, was used to characterize the role of this antibacterial in inhibiting *R. italica*'s secondary colonization. Compared to the *R. italica* population density in tri-culture with *P. inhibens* wild type (wt), there was a significant increase of *R. italica* population density by 64% (Figure S-2) in tri-culture with the Δ*tdaB* mutant ($P < 0.001$, one-way ANOVA), which suggests TDA plays a role in the inhibitory effect on *R. italica* (Figure 2-6A, Table S-4). However, this population density was still one order of magnitude lower than the co-culture of *R. italica* and *E. huxleyi*, which suggests that the effect of TDA is partial in the whole inhibition provided by *P. inhibens* (Figure 2-6A, Table S-4). The population densities of Δ*tdaB* mutant were not significantly different from the wild type in either co-culture control with *E. huxleyi* or tri-culture with *E. huxleyi* and *R. italica* ($P > 0.05$, one-way ANOVA), which suggest that there is no effect from the absence of TDA production on the *P. inhibens* population density itself (Figure 2-6B, Table S-4).

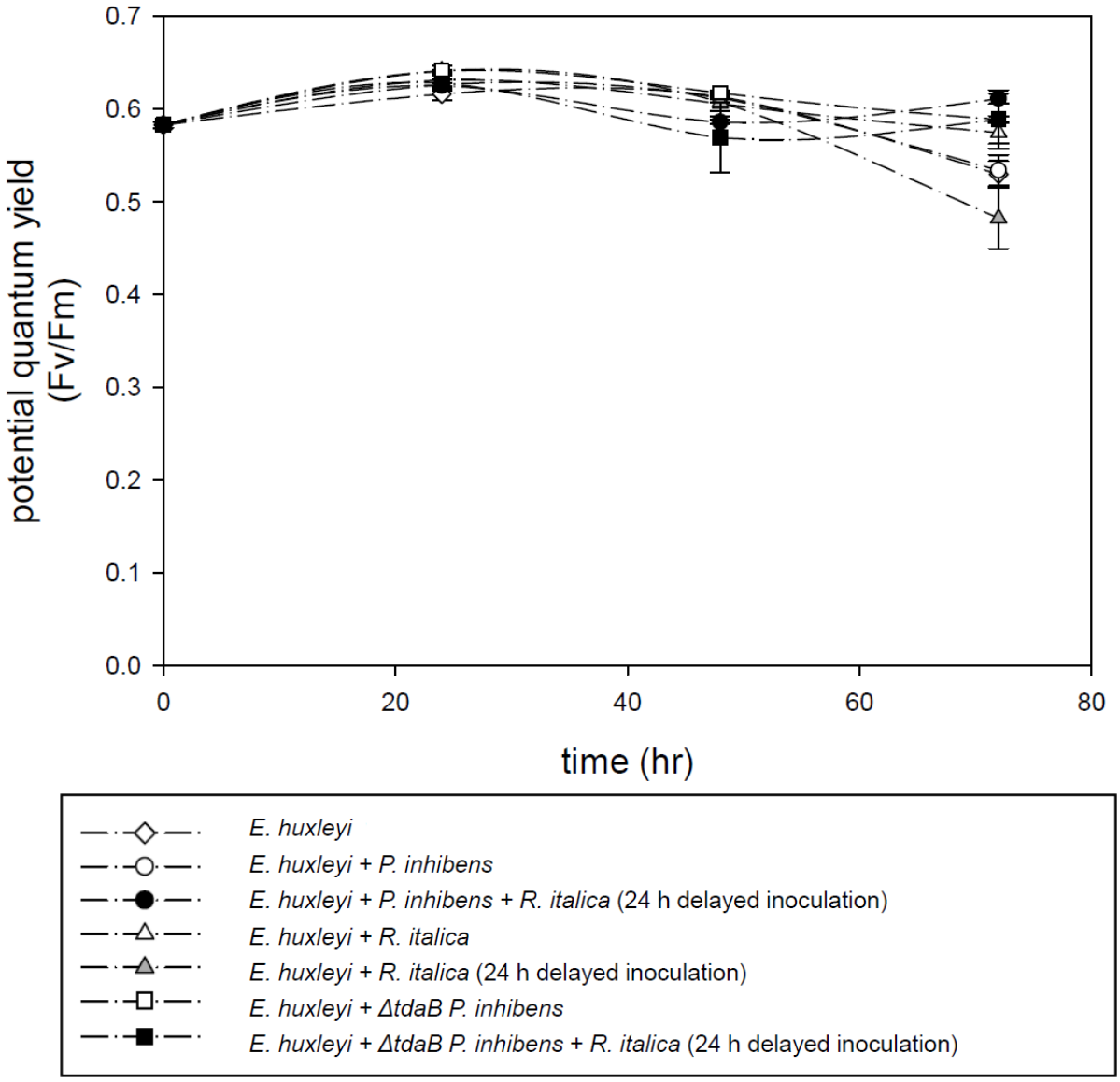

Figure 2-7: No change in the efficiency of Photosystem II (PSII) of *Emiliania huxleyi* were caused by bacterial colonization. The efficiency of PSII of the algal host *E. huxleyi* was monitored throughout all experiments by measuring the potential quantum yield. A dark adaption period of 3 min was applied to each sample, after which a fluorescence reading was taken as F_o. A saturating pulse was applied and another fluorescence reading (F_m) was taken to calculate PSII potential quantum yield, (F_m – F_o)/F_m. Bars on either side of the mean represents standard error. All experiments were performed with triplicate sacrificial sampling for each time point.

2.3.5 The algal host maintains its health in interactions with *R. italica* and *P. inhibens*

Previous experiments have shown that *P. inhibens* and *R. italica* kill *E. huxleyi* in the co-culture experimental model used in this study (Labeeuw *et al.* 2016; Mayers *et al.* 2016; Bramucci *et al.* 2018). To make sure that the *P. inhibens* and *R. italica* were not in their pathogenic phase of interaction with *E. huxleyi*, I monitored the efficiency of PSII of the algal host throughout experiments by measuring the potential quantum yield. Throughout the experiments, no algal death was observed in any cultures. All cultures were compared to a control culture of axenic *E. huxleyi* grown by itself. There was no significant difference in PSII efficiency found between cultures of algae with and without bacteria ($P > 0.05$, one-way ANOVA).

The *E. huxleyi* control, co-cultured of *R. italica* or *P. inhibens*, and tri-cultures of *R. italica*, *P. inhibens* and *E. huxleyi*, were observed under a microscope. In all cultures of *E. huxleyi* cells appeared healthy, with coccoliths surrounding the cells and an intact nucleus, stained by SYBR Green I (Figure 2-3), which is consistent with PSII efficiency data (Figure 2-7).

2.4 Discussion

2.4.1 Primary and secondary colonization of *E. huxleyi* by two roseobacter pathogens

When both *R. italica* and *P. inhibens* were introduced as the primary colonizer of *E. huxleyi*, the population density of *R. italica* was significantly reduced, while the density of *P. inhibens* was not affected by the *R. italica* (Figure 2-2). Their interaction dynamics fit the description of amensalism, where one organism is inhibited while the other is unaffected (Fredrickson and Stephanopoulos 1981; Begon, Harper and Townsend 2006). However, we did observe a decrease in the average of the cfu counts of *P. inhibens*, with relatively large standard error (Figure 2-2, Table S-1). It was surprising that the decrease in *P. inhibens* population density was not statistically significant (Figure 2-2), as this suggests that they do not compete for habitat nor nutrients from the medium or host. It has been shown that antagonistic interactions are critical for the structure of a community, but amensalism is rare (Aguilar, Vanegas and Klotz 2011; Long *et al.* 2013; Weimer 2015), especially in marine environments (Maybruck 2000).

The decrease in *R. italica* population density was expected, as *P. inhibens* is known to produce several kinds of secondary metabolites, including TDA, an antibacterial compound against many marine bacteria and human pathogens (Porsby *et al.* 2011). This study also demonstrates that TDA production is critical for inhibition of *R. italica* growth on hMB agar plates in our preliminary experiment (Figure 2-1). Further data suggest that *P. inhibens* is more effective against secondary colonization of *R. italica* rather than when competing with *R. italica* for primary colonization. While there was a significant decrease of population density of *R. italica* caused by *P. inhibens*

when both bacteria were primary colonizers, *R. italica* was still the most abundant bacterium in the tri-culture system during primary colonization (Figure 2-1). Simply increasing the inoculum of *P. inibens* by ten folds did not enhance its inhibition of *R. italica* (Figure 2-2).

Both bacteria were shown in previous studies to be efficient colonizers of seaweeds, *P. inhibens* on the green macroalgal host *Ulva australis* (Rao, Webb and Kjelleberg 2006) and *R. italica* on the red macroalga, *Delisea pulchra* (Case *et al.* 2011) and *E. huxleyi* (Mayers *et al.* 2016). However, this study is the first demonstration of the dynamics between the two bacterial colonizers on *E. huxleyi*. *P. inhibens* is known to produce antimicrobial molecules effective on many marine bacteria, while *R. italica* shows a higher population densityand production of RTX toxin potentially affecting the growth of its competitors in the environment (Gardiner *et al.* 2017). It was hypothesized that *R. italica* dominance over *P. inhibens* was likely due to an advantage in primary colonization of *E. huxleyi* instead of a higher growth ability in the L1-Si media, because it has been shown that *P. inhibens* actually grows better in L1-Si media at 18 °C than *R. italica* without the algal host (Mayers *et al.* 2016; Bramucci *et al.* 2018). While *P. inhibens* reached to 10^5 cfu/ml at the maximum cell density by day 3 (Bramucci *et al.* 2018), the population density of *R. italica* declined rapidly below the acceptable range for cfu counts by day 2 without the presence of algal host (Mayers *et al.* 2016).

P. inhibens had a higher cell density than *R. italica* only when *R. italica* was introduced as a secondary colonizer (i.e. 24 h later than *P. inhibens*) (Figure 2-4A). *P. inhibens* is a good colonizer of algal hosts, and has a great biofilm forming ability comparing to other common algae-associated bacteria (Rao, Webb and Kjelleberg 2006;

Thole *et al.* 2012). As a primary colonizer, *P. inhibens* has time to colonize the host and potentially produce TDA before secondary colonizers are introduced, creating a chemical defense protecting the host from invaders, which led to the significant reduction by 98% (Figure S-1) in *R. italica* population density in tri-cultures compared to the *R. italica* density in *R. italica-E. huxleyi* co-culture (Figure 2-5A). In contrast, when *R. italica* is a secondary colonizer, there is also a significant reduction in the population density of *P. inhibens* compare to that of *P. inhibens-E. huxleyi* co-culture (Figure 2-5B). In this scenario, the two species interaction is competitive as both species are negatively affected by the other (Begon, Harper and Townsend 2006; Gram, Melchiorsen and Bruhn 2010).

The substantial difference observed between the effectiveness of inhibition by *P. inhibens* between primary and secondary colonization was not surprising. A genomic study has revealed that the structure of bacterial community can be highly dynamic by comparing the ribosomal DNA sequences collected at 24- and 72- incubations from bacterial colonized surfaces (Dang and Lovell 2000). Early surface colonization by bacteria usually mediated by a series of recruitment processes in order. The primary colonization by *P. inhibens* can be essential for maximizing its inhibition ability against *R. italica*. Synergistic and/or competitive interactions among the early colonizers would result in recruitment and/or loss of other bacteria, thus shape the structure of a matured colonizing bacteria community (Davey and O'toole 2000; Burmølle *et al.* 2006). Similar examples have also been reported in microorganism interactions. For instance, ants *Tapinoma sessile* and *Wasmannia auropunctata* were found that they only colonize the giant leather-fern, *Acrostichum danaeifolium*, when the fern is infested by moth larvae

because of its production of domatia as attractants for ants (Mehltreter, Rojas and Palacios-Rios 2003). In another example of plant host colonization, fungus *Piriformospora indica* can trigger both local and systemic host response to inhibit subsequent secondary colonization on distal roots of the host (Pedrotti, Mueller and Waller 2013).

2.4.2 Factors mediating the interaction between two bacterial pathogens of *E. huxleyi*

A model to illustrate factors involved in the antagonistic relationship between *P. inhibens* and *R. italica* is proposed which include chemical defense, and other potential fitness traits such as nutrients scavenge ability, and motility, and gene regulation by quorum sensing (QS) (Figure 2-8). This study is the first to test the function of TDA in the interactions among marine bacterial pathogens and a microalgal host, *E. huxleyi*, in seawater-based media, which is much closer to their natural environment. It was revealed that there was a significant increase in *R. italica* population density by 64% in the presence of Δ*tdaB P.inhibens* relative to the wild type (Figure 2-6A, Figure S-2). This demonstrates that TDA constitutes part of the defense mechanism of *P. inhibens* for *E. huxleyi*, its host.

The competition between *P. inhibens* and other bacteria (*Pseudoalteromonas tunicata*) isolated from the same host (*Ulva lactuca*) is largely controlled by TDA as *P. inhibens* can clear all biofilms formed by marine bacteria tested (Rao, Webb and Kjelleberg 2005) which is consistent with TDA inhibition determined using the agar plate diffusion assay (Brinkhoff *et al.* 2004; Bruhn *et al.* 2005; Rabe *et al.* 2014). In a recent

study, the susceptibility of different bacteria to purified TDA was also tested in LB broth, which is a nutrient rich media for bacteria culturing (Porsby et al., 2011). The minimal inhibitory concentration of TDA for *Escherichia coli* under these conditions is 1000 times lower than common antibiotics like ampicillin and penicillin (Kojima & Nikaido, 2013; Porsby et al., 2011). Considering that antibiotic production is energetically expensive (Garbeva *et al.* 2011; Will *et al.* 2017), the cost of making TDA was investigated by comparing the population density of Δ*tdaB* mutant and wild type *P. inhibens*. Here we defined fitness as the population density reached by the bacterium. There was no significant increase in population density in Δ*tdaB P.inhibens* relative to the wild type, suggesting low or no cost in TDA production, in terms of cell density, for *P. inhibens* under these growth conditions (Figure 2-6B). Overall, the results show that TDA is a factor chemically modulating the interactions between *P.inhibens* and *R. italica* on the host, *E. huxleyi*.

However, the loss of TDA production in Δ*tdaB P.inhibens* does not lead to a complete loss of inhibition on *R. italica* population. As the results have shown, there was still a significant reduction in the density of *R. italica* with the presence Δ*tdaB P.inhibens* relative to *R. italica* inoculated without either *P.inhibens* wild type or mutant (Figure 2-6A). In addition to the antimicrobial activity of TDA, It was demonstrated in a recent study that shows TDA in sub-inhibitory concentration (1.5 μM) can function as a signaling molecule, thus controlling the expressions of genes which are regulated by a typical QS gene regulation system (Beyersmann *et al.* 2017). As the previous study has shown, genes involved in motility and surface colonization are down-regulated by TDA-mediated QS in *P. inhibens* wild type (Beyersmann *et al.* 2017).

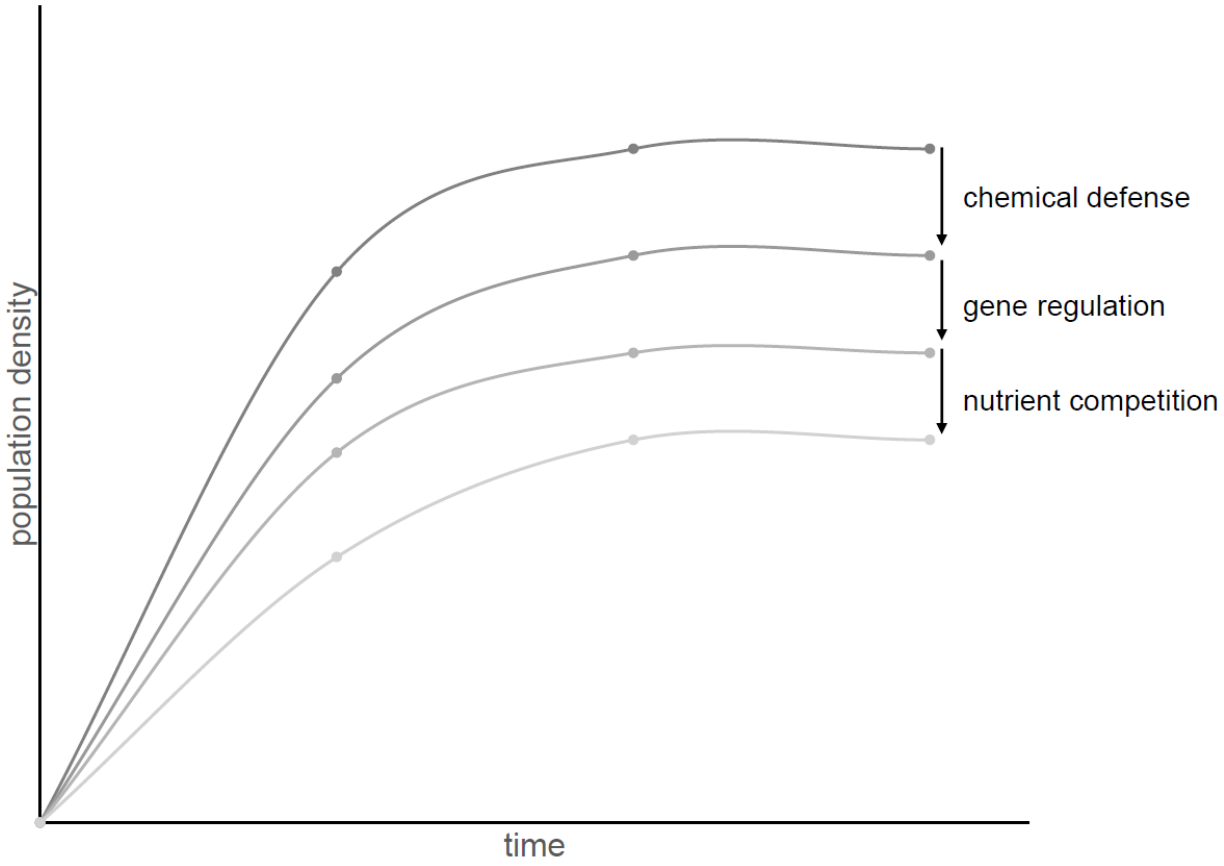

Figure 2-8: Model of the antagonistic interactions between *P. inhibens* and *R. italica*. The fitness of *R. italica* is represented by population density. All arrows are showing the inhibitory factors of *P. inhibens* forming an antagonistic interaction with *R. italica*. Inhibitory factors suggested in this model including: chemical defense (direct antimicrobial activity from secondary metabolites produced by *P. inhibens*); gene regulation (*P. inhibens* produce signaling molecules that regulate expression of genes involved in success in motility and surface colonization); nutrient competition (*P. inhibens* potentially compete with *R. italica* for nutrients in limited media).

Motility is an important fitness trait that enables organisms to reposition in response to changing nutrients, physical conditions, and provide mechanisms to avoid competitive pressure from other organisms (Hibbing *et al.* 2010). Motility also plays an essential role in early colonization, as bacteria with greater motility could be more efficient at swimming towards algae by chemotaxis for early cell attachment, biofilm formation, and subsequent symbiosis (Miller and Belas 2006; Bruhn, Gram and Belas 2007; Seymour *et al.* 2010). Therefore, the deficiency in TDA production shall remove the down-regulation and increase motility and the ability of surface colonization of Δ*tdaB P.inhibens*, thus mediate an antagonistic effect on the secondary colonizer *R. italica*.

Another potential fitness traits mediating antagonistic interactions can be nutrient scavenge ability because nutrients availability is a central factor mediating microbial competition (Hibbing *et al.* 2010). It has been shown that bacterial density is linearly related to the initial concentration of limiting nutrient in defined medium (Monod 1949). In mixed cultures, the predominance of different species co-existing in the same habitat is determined by the nutrient availability, individual demand, and consumption rate of nutrients (Tilman 1977). In natural marine environments, algal surfaces are usually nutrient limited, which are heterogeneously distributed (Rao, Webb and Kjelleberg 2006).

Both *P. inhibens* and *R. italica* are isolated from algae from marine environments (Rao, Webb and Kjelleberg 2006; Case *et al.* 2011), and it has been shown that both can be pathogenic to *E. huxleyi* in late colonization stages around day 12 (Seyedsayamdost *et al.* 2011b; Labeeuw *et al.* 2016; Mayers *et al.* 2016; Segev *et al.* 2016; Bramucci *et al.* 2018). Experiments conducted in this study are only within 72 hrs

of the colonization on *E. huxleyi*, however, the results suggested that both *R. italica* (secondary colonizer) and *P. inhibens* (primary colonizer) already negatively affected the population density of each other in tri-culture experiment during early colonization (Figure 3B). Therefore, it is likely that ecological niches of the two bacteria overlap and they compete for habitat, or space, and nutrients on the surface of the algal host.

2.4.3 Relevance of the research

The antagonistic effect by *P. inhibens* poses interests for aquaculture, and *P. inhibens* was proposed as a probiotic for rainbow trout, because of its ability in improving the survival rate of fish larvae and the killing and inhibition of fish pathogens via TDA production (Porsby *et al.* 2011). The use of TDA producing *P. inhibens* as probiotics also potentially expands to agriculture and medicine as they are inferred to deter human pathogens including *Salmonella Typhimurium* and *Staphylococcus aureus* (Porsby et al. 2011). In addition to the antimicrobial activity, TDA produced by *P. inhibens* is also an effective anticancer agent towards several cancer cell lines (Wilson et al. 2016).

In natural marine habitats, roseobacters are ubiquitous and rapid colonizers (Dang and Lovell 2000). *E. huxleyi* and the roseobacter symbionts, potentially including *P. inhibens* and *R. italica*, may co-exist as one ecological unit as an holobiont, and the synergistic and/or competitive interactions among the colonizing bacteria will structure the mature microbial community. The dynamic interactions within the holobiont can allow itself to adapt to the changing environment effectively (Rosenberg *et al.* 2007). This study contributes to understanding how chemical ecology is mediating those

dynamic interactions through revealing the role of TDA as both antimicrobial compounds and signaling molecules in the antagonistic interactions between *P. inhibens* and *R. italica*. Investigating the interactions among marine microorganisms, which are commonly chemically mediated, may potentially leads to the discovery of novel natural products that have multiple interesting functions.

2.4.4 Conclusions

In our multi-species culture experiments, *R. italica* performed better as a primary colonizer of *E. huxleyi* than *P. inhibens*. However, *P. inhibens* was most successful at inhibiting the secondary colonization of algal pathogen *R. italica*, which is in part due to the production of TDA that acts as a chemical defense for the host, *E. huxleyi*. Moreover, it was shown that the role of TDA as an antimicrobial molecule in the defense mechanism is only part of *P. inhibens* success at inhibiting secondary colonization, as the population size of *R. italica* is still reduced with the TDA deficient mutant, Δ*tdaB P. inhibens*. The role of TDA as a signaling molecule may also play a part in the inhibition by *P. inhibens* against *R.* italica, and other fitness traits including nutrients scavenge ability may also contributes to shaping the antagonistic interaction between the two roseobacters.

Chapter 3 Discussion

3.1 General Discussion

Sophisticated ecological interactions exist among marine microorganisms. The significant impact of bacteria on algal hosts physiology, such as growth dynamics, has been recognized in recent studies, and the ecological interactions among those algal and bacterial symbionts can be mediated by novel chemicals (Grossart et al. 2005; Mayers et al. 2016; Bramucci et al. 2018). This thesis investigated the interactions among *Emiliania huxleyi*, the most abundant calcifying microalgae, and two representative bacterial symbionts, *Phaeobacter inhibens* and *Ruegeria italica,* from the *Roseobacter* clade. Both were previously shown to be able to be virulence to *Emiliania huxleyi* when the alga enters senescence (*P. inhibens*) or is cultured in elevated temperatures (*R. italica*) (Seyedsayamdost *et al.* 2011b; Mayers *et al.* 2016; Bramucci *et al.* 2018). In these previous studies, the interactions between each bacterial symbiont and *E. huxleyi* were separately investigated in co-culture systems (Mayers *et al.* 2016; Bramucci *et al.* 2018). My research is the first to establish a tri-culture system with the two bacteria and *E. huxleyi*, allowing the study of the interactions between two bacteria during early colonization of their algal host.

My results show that once *P. inhibens* primary colonization is established on *E. huxleyi*, it is able to provide chemical defense against secondary colonization of *R. italica* (Chapter 2). The aggressive colonizing ability of *P. inhibens* was previously demonstrated in a multi-species biofilm study, where *P. inhibens* was introduced both as primary and secondary colonizer to the green algal host *Ulva australis* (Rao, Webb and Kjelleberg 2006). It was speculated that the advantages of *P. inhibens* in multi-

species colonization were due to the production of antimicrobial compound tropodithietic acid (TDA) (Rao, Webb and Kjelleberg 2005, 2006). This hypothesis was tested here by removing the ability of *P.* inhibens to produce TDA through deletion of the *tdaB* gene. The inhibitory effect of the mutant on secondary colonization of its host was significantly reduced, demonstrating a direct link between TDA production and host defense (Chapter 2).

The concentration of TDA was not measured in the experiment, but it would be interesting to know if the TDA produced in *P. inhibens* was above minimal inhibitory concentration for *R.italica*. In addition to the antimicrobial activity of TDA, it was demonstrated in previous studies that this compound can act, in low concentrations, as a signaling molecule autoinducing its own production and regulating other genes likely involved in the antagonistic interactions with other bacteria (Beyersmann *et al.* 2017). As our data show, *ΔtdaB P.inhibens* can also cause a significant reduction in *R. italica* density in the tri-culture experiment (Chapter 2). Due to the loss of TDA signaling, there can be an upregulation of genes responsible for motility and surface colonization in *ΔtdaB P. inhibens*, thus give advantages to *ΔtdaB P. inhibens* in the antagonistic interaction with the secondary colonizer *R. italica* (Beyersmann *et al.* 2017).

3.2 Marine pathogens

Pathogens are biological agents that cause disease. As humans have lived in association with the ocean for centuries, there has been a great focus on human marine pathogens, such as *Vibrio cholerae* O1, responsible for epidemic and pandemic diarrhea outbreaks (Blake 1994). In addition, many human communities developed in coastal regions because of their importance as a source of food from aquaculture.

Common fish pathogens like *Vibrio harveyi* (causing eye disease in turbo eel) and *Moritella viscosa* (causing skin lesions in Atlantic salmon) have therefore received a lot of attention (Liu and Lee 1999; Karlsen *et al.* 2014). However, pathogens in marine habitats which are less directly associated with human activities, are not as well acknowledged, but yet have phenomenal impacts on natural ecosystems, which can eventually cause problems for human. For instance, coral reefs contain high biodiversity, even comparable to habitats like the rain forest (Knowlton *et al.* 2010). They also provide physical protection to coastlines and islands from erosion by tides (Porter, J.W. and Tougas 2001). Over the past decades, more than 30% of coral reefs have been destroyed by coral diseases, such as bleaching caused by *Vibrio shiloi* (Kushmaro *et al.* 2001). The infection and bleaching caused by *V. shiloi* was temperature dependent, linked to elevated seawater temperatures (Kushmaro *et al.* 1998).

Other natural systems, such as massive blooms of *E. huxleyi,* also play important ecological roles. Because of their ability for photosynthesis and their high abundance, *E. huxleyi* are important primary producers in the world oceans. The coccolith bearing cell types can fix a tremendous amount of carbon dioxide from the environments to form coccoliths, which eventually sink down to the bottom of the ocean, leading to carbon removal from the global carbon cycle (Paasche 2001). The formation and disappearance of the algal blooms was previously thought to be simultaneous, however, it was found that both marine bacteria and virus can kill the bloom via programmed cell death and bleaching (Bidle *et al.* 2007; Seyedsayamdost *et al.* 2011b; Bidle and Kwityn 2012; Mayers *et al.* 2016; Bramucci *et al.* 2018). It was suggested that *P. inhibens* and *R. italica* can alter the life history of *E. huxleyi* because of their selective pathogenesis

on different algal cell types, thus further change the composition of the cell types within the algal bloom (Mayers *et al.* 2016; Bramucci *et al.* 2018). Although an algal host is typically associated with multiple bacterial symbionts and pathogens (Rao, Webb and Kjelleberg 2005), both of the previous studies were focusing on a single pathogen of *E. huxleyi* at one time, while this thesis studies the interactions between multiple pathogens, and may aid in understanding how bacteria community interacting with natural *E. huxleyi* blooms.

In multi-species culture experiments, we focused on the early colonization stage of the bacteria, which gave us insights on bacteria-bacteria competition over colonizing host within the first 72 h of culturing. It would be also interesting to see how the pathogens affect algal physiology as time passes into late stages as algae senescence. Based on previous studies (Labeeuw, Bramucci and Case 2017; Bramucci *et al.* 2018), *P. inhibens* starts to cause bleaching and lower the photosystem health of *E. huxleyi* after day 12. The degrading of the algal host could release contained nutrients which is also beneficial for *R. italica*. Therefore, growth in both populations of *P. inhibens* and *R. italica* would be expected.

3.3 Chemical defense and its role in shaping interspecific interactions

Chemical defense in bacterial community can play important roles in shaping interspecific ecological interactions. *Pseudomonas* is a well-known soilborne plant-pathogenic bacteria because of the antimicrobial metabolites and pathogenic virulence factors produced by the organisms (Raaijmakers and Mazzola 2012). *Pseudomonas syringae* produce syringomycin mediating pathogenesis on plant host by causing

necrosis of plant cells (Scholz-Schroeder *et al.* 2001). Meanwhile, *Pseudomonas* species are quite favored by predatory soil fauna and protists (Weekers *et al.* 1993; Raaijmakers and Mazzola 2012). To maintain the pathogenic interactions with plants, *Pseudomonas* produce another type of bioactive molecules, lipopeptides, disrupting membrane integrity of gazing protists (De Souza *et al.* 2003). Another secondary metabolite, 2,4-diacetylphloroglucinol, produced by different *Pseudomonas* species, shows antibiotic activities to bacteria, fungus, and also plants (Haas and Défago 2005). Those bioactive molecules produced by *Pseudomonas* provide a great chemical defense against its predators and competitors, thus maintain stable pathogenic interactions with the plant hosts.

Similarly, bioactive molecules involved in chemical defense in marine environments also play an important role in the algal-bacterial interactions. In addition to the role of a pathogen, *P. inhibens* was proposed to have a mutualistic phase of interactions with the host *E. huxleyi* (Seyedsayamdost *et al.* 2011b; Wang, Gallant and Seyedsayamdost 2016). *E. huxleyi* provides nutrients, such as the sulfur-containing compound DMSP, to *P. inhibens*, while the latter can provide chemical defense due to the production of a novel antimicrobial compound TDA (Wang, Gallant and Seyedsayamdost 2016). As my results have shown, TDA procuring *P. inhibens* inhibiting marine pathogen *R. italica* upon colonizing on *E. huxleyi*. The role of TDA in maintaining symbiosis with the algal host was also previously observed in green algal *Ulva lactuca* (Rao, Webb and Kjelleberg 2005). The antimicrobial activity of TDA gives *P. inhibens* an advantage over other bacterial symbionts isolated from *U. lactuca*, thus

grant high fitness to *P. inhibens* competing with other members from the multispecies bacterial community associated with *U. lactuca* (Rao, Webb and Kjelleberg 2005).

It was previously shown that TDA producing *P. inhibens* also kill or inhibit marine fish pathogens such as *Vibrio anguillarum* that causes diseases to rainbow trout, and improve the survival rate of fish larvae, which make *P. inhibens* and other TDA producing roseobacters a great interest in aquaculture for probiotic uses (Porsby *et al.* 2011). In addition to marine pathogens, TDA was shown to have antimicrobial activity against human pathogens including *Salmonella Typhimurium* and *Staphylococcus aureus*, which are outside of the marine environment (Porsby *et al.* 2011). Haliangicin is another example of antibiotic isolated from marine bacteria, *Haliangium lutem* (Kasanah and Hamann 2004). This novel compound has shown antimicrobial activity against filamentous fungi and oomycetes (Kasanah and Hamann 2004). It is believed that marine environment is the new reservoir for drug development and natural product discovery.

3.4 Conclusion

In this thesis, the interactions within natural communities were studied in conjunction with laboratory models consisted of algal host *E. huxleyi* and roseobacters *P. inhibens* and *R. italica*. The laboratory settings give us defined approaches to reveal the direct contribution of TDA to the chemical defense provided by *P. inhibens* for inhibiting the secondary colonization of *R. italica* on *E. huxleyi.* While using single bacterial culture as one model organism is straightforward for investigating on the antibiotic activity of a novel molecule, the multi-species culturing system is closer to the natural marine habitats where multiple bacteria symbionts colonize the same algal host. This thesis has

set an example for studying complex multi-species interactions among bacteria during the colonization process of the algal hosts *E. huxleyi*. Understanding the role of antibiotics play in the multi-species interactions can help further understand the functions of antibiotics in aquaculture and even medical treatment usages. In addition, the complexity of the chemical-mediated interactions within dynamic algal-bacterial community gives a huge potential for natural product discovery.

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Appendix

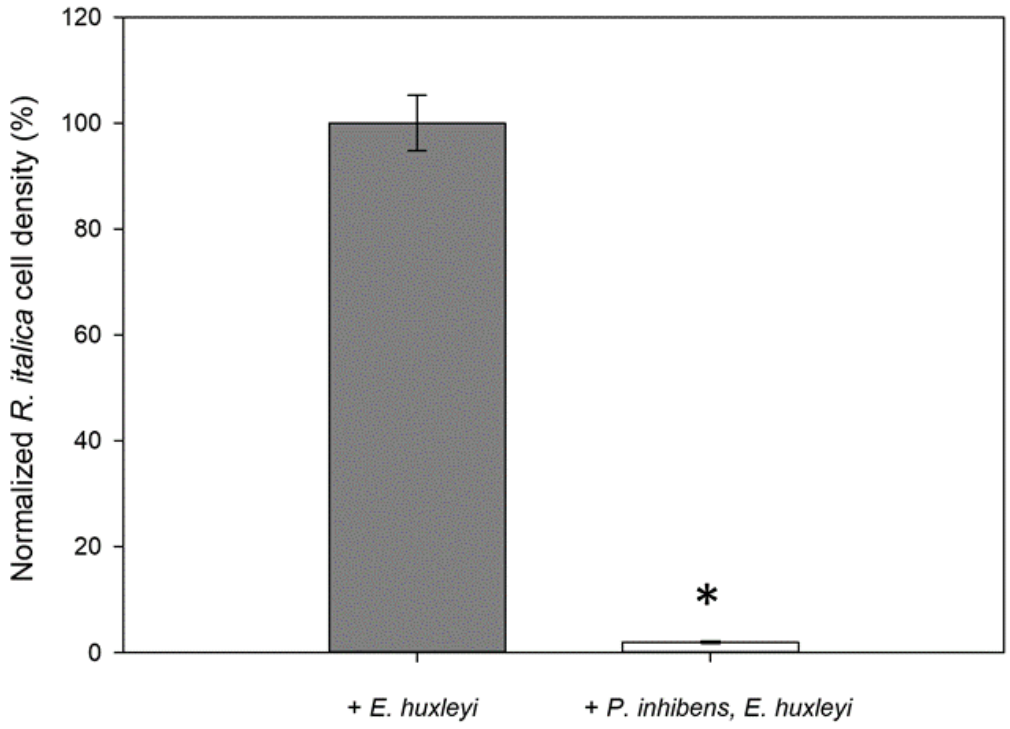

Figure S-1: Percentage reduction in *R. italica* population density caused by *P. inhibens* during secondary colonization. *R. italica* was inoculated as the secondary colonizer in all experiments. Grey bar on the left represent the *R. italica* density at 72 h in co-culture with *E. huxleyi* alone as control (100%), while the white bar on the right the *R. italica* density at 72 h in triculture with both *E. huxleyi* and *P. inhibens*, which was inoculated (10^3 cfu/mL) as the primary colonizer (T = 0 h). An asterisk indicates the 98% reduction in density was statistically significant as determined using one-way ANOVA. Bars on either side of the mean represents standard error. All experiments were performed with triplicate sacrificial sampling.

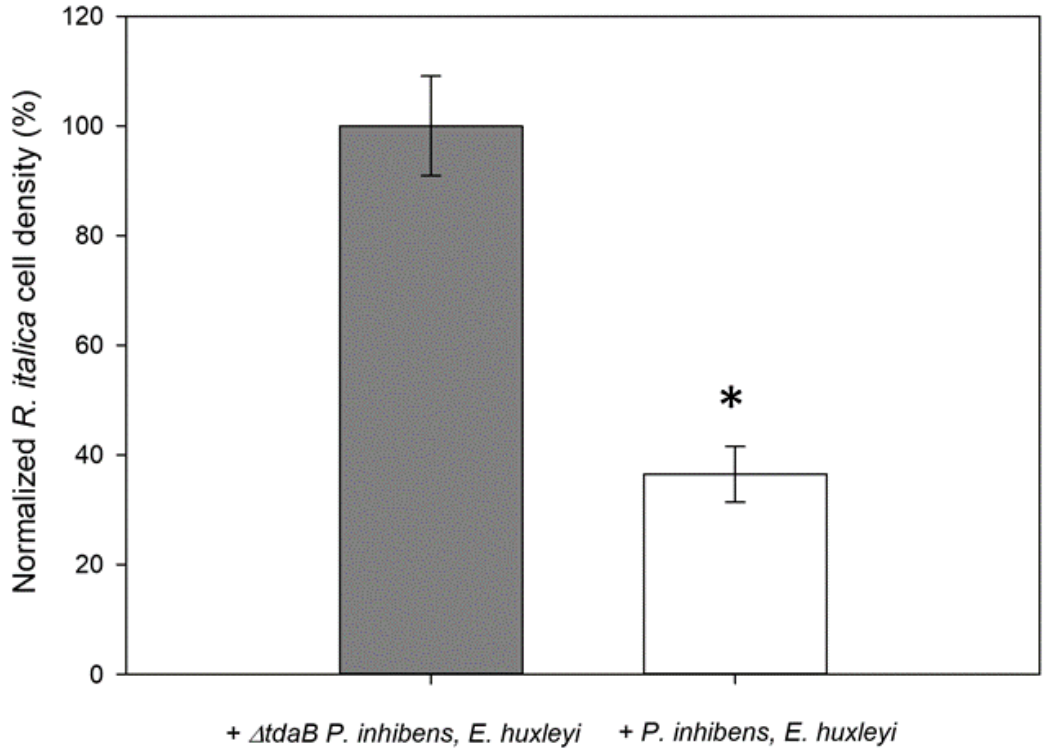

Figure S-2: Percentage reduction in *R. italica* population density caused by TDA production of *P. inhibens* during secondary colonization. *R. italica* was inoculated as the secondary colonizer (T = 24 h) in all experiments. Grey bar on the left represent the *R. italica* density at 72 h in tri-culture with *E. huxleyi* and *ΔtdaB P. inhibens* as control (100%), while the white bar on the right the *R. italica* density at 72 h in triculture with *E. huxleyi* and *P. inhibens* wild type. An asterisk indicates the 64% reduction in density was statistically significant as determined using one-way ANOVA. Bars on either side of the mean represents standard error. All experiments were performed with triplicate sacrificial sampling.

Table S-1: Bacterial cell counts (cfu/ml ± SE) for Figure 2-2

Measurement	Experimental condition	0h	24h	48h	72h
R. italica	control	3.8E+03 ± 5.7E+02	7.0E+05 ± 5.8E+04	3.0E+06 ± 2.0E+05	3.2E+06 ± 1.0E+05
R. italica+	+ *P. inhibens*	3.8E+03 ± 5.7E+02	8.9E+05 ± 1.3E+05	1.8E+06 ± 5.0E+05	2.1E+06 ± 2.8E+05
P. inhibens	control	3.8E+03 ± 5.7E+02	1.0E+05 ± 2.1E+04	2.3E+05 ± 1.6E+04	3.7E+05 ± 7.0E+04
P. inhibens+	+ *R. italica*	3.8E+03 ± 5.7E+02	7.0E+04 ± 1.9E+04	2.3E+05 ± 6.3E+04	2.3E+05 ± 7.1E+04

+ -tri-culture experiment

Table S-2: Bacterial cell counts (cfu/ml ± SE) for Figure 2-4

Measurement	Experimental condition	0h	24h	48h	72h
R. italica+	+ *P. inhibens*	3.8E+03 ± 5.7E+02	8.9E+05 ± 1.3E+05	1.8E+06 ± 5.0E+05	2.1E+06 ± 2.8E+05
R. italica+	+ *P. inhibens* (10X)	2.1E+03 ± 3.1E+02	1.8E+05 ± 4.2E+04	1.7E+06 ± 8.1E+04	2.1E+06 ± 2.9E+05
P. inhibens+	+ *R. italica*	3.8E+03 ± 5.7E+02	7.0E+04 ± 1.9E+04	2.3E+05 ± 6.3E+04	2.3E+05 ± 7.1E+04
P. inhibens (10X) +	+ *R. italica*	1.8E+04 ± 3.0E+02	8.3E+04 ± 1.9E+04	2.3E+05 ± 3.8E+04	2.7E+05 ± 3.3E+04

+ -tri-culture experiment

Table S-3: Bacterial cell counts (cfu/ml ± SE) for Figure 2-5

Measurement	Experimental condition	0h	24h	48h	72h
R. italica	control	1.8E+03 ± 1.8E+02	7.0E+05 ± 5.8E+04	3.0E+06 ± 2.0E+05	3.2E+06 ± 1.2E+05
R. italica	24 h delay	N. A.	8.1E+03 ± 1.5E+03	1.1E+06 ± 5.0E+04	3.1E+06 ± 1.6E+05
R. italica 2°+	+ *P. inhibens*	N. A.	7.8E+03 ± 9.7E+02	3.0E+04 ± 2.3E+03	6.0E+04 ± 8.4E+03
P. inhibens	control	1.8E+03 ± 1.8E+02	1.0E+05 ± 2.1E+04	2.3E+05 ± 1.6E+04	3.7E+05 ± 8.2E+04
P. inhibens +	+ *R. italica*	1.8E+03 ± 1.8E+02	9.3E+04 ± 7.4E+03	1.6E+05 ± 1.9E+04	1.5E+05 ± 8.8E+03

2°-inoculated as a secondary colonizer; + -tri-culture experiment

Table S-4: Bacterial cell counts (cfu/ml ± SE) for Figure 2-6

Measurement	Experimental condition	0h	24h	48h	72h
R. italica	24 h delay	N. A.	8.1E+03 ± 1.5E+03	1.1E+06 ± 5.0E+04	3.1E+06 ± 1.6E+05
R. italica 2°+	+ *ΔtdaB P. inhibens*	N. A.	8.0E+03 ± 1.0E+03	8.0E+04 ± 1.4E+03	1.6E+05 ± 1.5E+04
R. italica 2°+	+ *P. inhibens*	N. A.	7.8E+03 ± 9.7E+02	3.0E+04 ± 2.3E+03	6.0E+04 ± 8.4E+03
P. inhibens	control	1.8E+03 ± 1.8E+02	1.0E+05 ± 2.1E+04	2.3E+05 ± 1.6E+04	3.7E+05 ± 8.2E+04
P. inhibens +	+ *R. italica*	1.8E+03 ± 1.8E+02	9.3E+04 ± 7.4E+03	1.6E+05 ± 1.9E+04	1.5E+05 ± 8.8E+03
ΔtdaB P. inhibens	control	1.8E+03 ± 1.8E+02	5.5E+04 ± 1.2E+04	3.0E+05 ± 3.5E+04	3.0E+05 ± 5.6E+04
ΔtdaB P. inhibens +	+ *R. italica*	1.8E+03 ± 1.8E+02	5.3E+04 ± 4.6E+03	1.4E+05 ± 1.3E+04	1.8E+05 ± 6.8E+03

2°-inoculated as a secondary colonizer; + -tri-culture experiment

Table S-5: Growth rates calculated with mean bacterial cell counts for Figure 2-2

Measurement	Experimental condition	Growth rate α(tn1-tn2)
R. italica	control	α(t0-t24) = 5.21 α(t24-t48) = 1.46 α(t48-t72) = 0.07
R. italica +	+ *P. inhibens*	α(t0-t24) = 5.45 α(t24-t48) = 0.69 α(t48-t72) = 0.17
P. inhibens	control	α(t0-t24) = 3.29 α(t24-t48) = 0.82 α(t48-t72) = 0.46
P. inhibens +	+ *R. italica*	α(t0-t24) = 2.90 α(t24-t48) = 1.20 α(t48-t72) = 0.01

+ -tri-culture experiment; Growth rate α(tn1-tn2) = ln(tn1/tn2)

Table S-6: Growth rates calculated with mean bacterial cell counts for Figure 2-4

Measurement	Experimental condition	Growth rate α(tn1-tn2)
R. italica	+ *P. inhibens*	α(t0-t24) = 5.45 α(t24-t48) = 0.69 α(t48-t72) = 0.17
R. italica+	+ *P. inhibens* (10X)	α(t0-t24) = 4.45 α(t24-t48) = 2.27 α(t48-t72) = 0.17
P. inhibens	+ *R. italica*	α(t0-t24) = 2.90 α(t24-t48) = 1.20 α(t48-t72) = 0.01
P. inhibens (10X) +	+ *R. italica*	α(t0-t24) = 1.56 α(t24-t48) = 1.02 α(t48-t72) = 0.15

+ -tri-culture experiment; Growth rate α(tn1-tn2) = ln(tn1/tn2)

Table S-7: Growth rates calculated with mean bacterial cell counts for Figure 2-5

Measurement	Experimental condition	Growth rate α(tn1-tn2)
R. italica	control	α(t0-t24) = 5.94 α(t24-t48) = 1.49 α(t48-t72) = 0.07
R. italica	24 h delay	α(t0-t24) = 4.88 α(t24-t48) = 1.04
R. italica 2°+	+ *P. inhibens*	α(t0-t24) = 1.43 α(t24-t48) = 0.70
P. inhibens	control	α(t0-t24) = 4.30 α(t24-t48) = 0.59 α(t48-t72) = 0.46
P. inhibens +	+ *R. italica*	α(t0-t24) = 3.95 α(t24-t48) = 0.52 α(t48-t72) = -0.03

2°-inoculated as a secondary colonizer; + -tri-culture experiment;

Growth rate α(tn1-tn2) = ln(tn1/tn2)

Table S-8: Growth rates calculated with mean bacterial cell counts for Figure 2-6

Measurement	Experimental condition	Growth rate α(tn1-tn2)
R. italica	24 h delay	α(t0-t24) = 4.88 α(t24-t48) = 1.04
R. italica 2°+	+ *ΔtdaB P. inhibens*	α(t0-t24) = 2.41 α(t24-t48) = 0.72
R. italica 2°+	+ *P. inhibens*	α(t0-t24) = 1.43 α(t24-t48) = 0.70
P. inhibens	control	α(t0-t24) = 4.30 α(t24-t48) = 0.59 α(t48-t72) = 0.46
P. inhibens +	+ *R. italica*	α(t0-t24) = 3.95 α(t24-t48) = 0.52 α(t48-t72) = -0.03
ΔtdaB P. inhibens	control	α(t0-t24) = 3.53 α(t24-t48) = 1.71 α(t48-t72) = 0.01
ΔtdaB P. inhibens +	+ *R. italica*	α(t0-t24) = 3.51 α(t24-t48) = 0.79 α(t48-t72) = 0.44

2°-inoculated as a secondary colonizer; + -tri-culture experiment;

Growth rate α(tn1-tn2) = ln(tn1/tn2)