ABSTRACT

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                            ACID BIOSYNTHESIS IN MARINE
                            BACTERIA


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Members of the *Roseobacter* clade of alphaproteobacteria are among the most abundant and ecologically relevant marine bacteria. The antibiotic tropodithietic acid (TDA) produced by roseobacters is hypothesized to be a critical component of the roseobacter-phytoplankton symbiosis. TDA production is influenced by environmental conditions. Specifically, in the lab, TDA activity is highest when bacteria are cultured in standing liquid nutrient broth, whereas cells produce negligible TDA in nutrient broth with shaking. Random mutagenesis was used to construct loss-of-function mutants defective in TDA activity (Tda⁻). Twelve genes were identified as required for Tda activity. Six *tda* genes, *tdaA-F*, are physically linked and are carried on pSTM3, a ca. 130-kb plasmid, while the remaining 6 genes are located on the sequenced genome. Genetic and molecular biological evidence

demonstrates that *tdaA* and *tdaB* form a bicistronic message, *tdaCDE* are part of a separate operon, and *tdaF* is likely a part of a third operon.

The expression of *tdaAB* is constitutive, whereas *tdaCDE* and *tdaF* mRNA are regulated, showing significantly increased levels when cells are grown in standing liquid broth compared to shaking liquid culturing. Expression of *tdaCDE* is lost in Tda⁻ strains, but could be restored– *tdaA* and *tdaH* failed to respond – by placing wild-type Tda⁺ strains in close proximity or by adding exogenous TDA to the mutant. These results indicate that TDA acts as an autoinducer of its own synthesis and suggest that roseobacters may use TDA as a quorum signal.

Next, I focused attention on the only known regulatory protein, TdaA, involved in *tda* expression. Disruption of *tdaA* results in loss of *tdaCDE* expression, and expression of *tdaA* in an *Escherichia coli* background is sufficient to transcribe *tdaCDE*. Transcriptional activation of the *tdaC* promoter by TdaA is supported by data from electrophoretic mobility shift assays (EMSA) showing that purified TdaA protein binds specifically to a fragment of DNA containing the *tdaC* promoter. These results support a hypothesis that TdaA is a positive transcriptional regulator of *tdaCDE* gene expression whose function requires binding to the *tdaC* promoter region. One of the mechanisms used by TM1040 to regulate TDA biosynthesis is therefore through TdaA regulator.

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MARINE BACTERIA


By


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# Dedication

To my family, especially…

       to my wife, Airong Yu, for  her patience and understanding;

       to my daughter, Ava Geng, for her lovely smile;

       to Dad and Mom for all the encouragement and support for higher education;

       to my sister for all the caring;

       to grandpa and grandma for encouragement.

# Acknowledgements

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# Chapter 1:  Introduction

## 1.1  Prokaryotic microbes and phytoplankton in the marine ecosystem

Phytoplankton are responsible for one half of global marine primary production (49). It is conventionally viewed that primary production was transferred in a linear chain through the foodweb from small to large animals, and microorganisms were thought to be insignificant. However, modern investigations show that bacteria and archaea are important in the marine carbon cycle, due to not only their abundance but also their physiological diversity and high metabolic activities (6). Marine phytoplankton are organic matter producers. Up to half of their primary production is converted into dissolved organic matter (DOM) (**FIG. 1.1.**), which is exclusively assimilated by prokaryotic microorganisms (37). Phytoplankton exude dissolved organic carbon (DOC) and dissolved organic nitrogen (DON), which fuel the growth of heterotrophic bacteria. The pathway that DOC is reintroduced to higher level organisms via bacterial biomass is termed microbial loop (8), enforcing the notion that bacteria are closely coupled to marine phytoplankton.

Phytoplankton do not live reclusively but are coinhabited by prokaryotes. Bacteria live in the phycosphere (6), a region influenced by and immediately surrounding phytoplankton. The density of bacteria in the phycosphere is remarkably higher than those in the sea water within the phycosphere (40). In the course of

diatom bloom observations, marine alphaproteobacteria come to dominate the bacterial population in the post bloom phase when size of bacterial colonized fraction
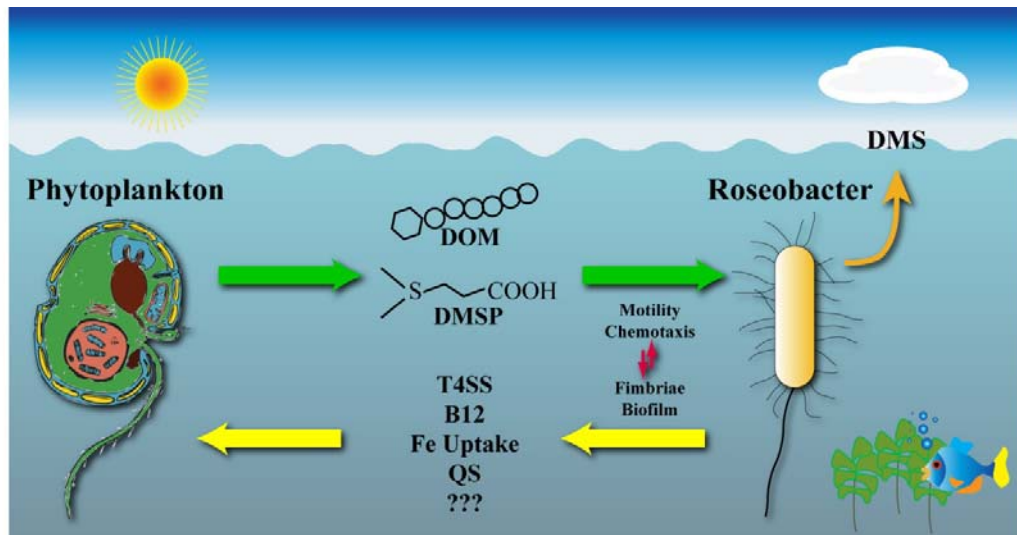


**FIG. 1.1. A model of the molecular mechanisms involved in the symbiosis between roseobacters and phytoplankton.** Motile phase roseobacters use chemotaxis behavior to sense and swim towards phytoplankton to form initial attachment to the algal surface. Once close to the surface of the algal cell, a currently unknown molecular 'switch' is turned on resulting in a transition of the bacteria to a sessile life phase whose phenotype includes loss of flagella, formation of fimbrial adhesins and subsequent biofilm development. Roseobacters attached to a phytoplankton cell have immediate access to dissolved organic matter (DOM) (chemical structure is only for illustration) and dimethylsulfoniopropionate (DMSP) (chemical structure is shown above), which may be used in the synthesis of one or more biologically active molecules, such as antibiotic tropodithietic acid (TDA), that act to prevent algicidal bacterial species from harming the phytoplankton and enhance

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to roseobacter symbiosis by limiting competition from other species. In addition to the potential benefit from producing TDA, the roseobacter biofilm may also provide necessary nutrients, e.g., vitamin B12 (cobalamin), iron-binding siderophores, and et.al, to the alga. Many of these exchanges and interactions may be mediated through a *vir*-gene-mediated Type 4 Secretion System (T4SS) and/or quorum signaling through known and (currently) unknown chemicals.

shifts from small <1.0 μm size towards >1.0 μm (130). Although close coupling between bacteria and phytoplankton is common, the molecular mechanisms behind marine bacteria-phytoplankton interactions are poorly understood. Research on bacteria and phytoplankton interactions will help to reveal new insights on the processes involved in the marine ecosystem.

## 1.2    Sulfur cycle and dimethylsulfoniopropionate (DMSP)

In the complex global sulfur cycle, marine phytoplankton synthesize the sulfur- containing compound DMSP (**FIG. 1.2.**), which accounts for $\geq$ 50% of the sulfur flux (154). DMSP is ubiquitously synthesized by marine phytoplankton, including prymnesiophytes and dinoflagellates, and coastal vascular plants in which it functions as an organic osmolyte (165) and as an antioxidant (148). In particular, algal blooms on the time scale of weeks make large amounts of DMSP. For example in prymnesiophytes and dinoflagellates, intracellular DMSP concentrations can reach up to 100-400 mM (159). The principal organisms responsible for catabolism of DMSP are bacteria. This is significant, since it is a preferred source of reduced sulfur despite DMSP being nearly $10^7$-fold less abundant than sulfate in seawater (89, 125). There are two possible pathways for the breakdown of DMSP: the lyase or cleavage pathway via DddD gene leads to the production of dimethyl sulfide (DMS) (**FIG. 1.2.**), a volatile gas (31, 95, 152, 166) and the demethylation/demethiolation pathway via DmdA through which sulfur contained in DMSP is retained by the bacteria and not released as DMS (76). Within the atmosphere, DMS is readily oxidized to sulfate aerosols that increase the abundance of cloud condensation nuclei leading to greater backscattering and cloudiness (10), counteracting the effects of global
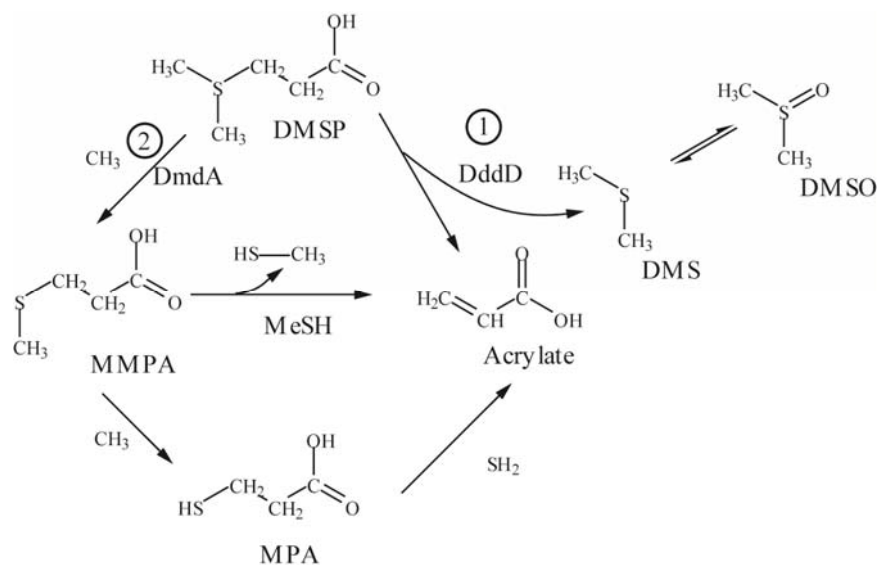
**FIG. 1.2. Pathways for DMSP catabolism**. It shows the demethylation pathway (reaction 1) being catabolized by DmdA protein and Lyase pathway derived from DddD protein resulting in DMS (reaction 2). 3-methymercaptopropionate (MMPA) may be further demethylated to 3-mercaptopropionate (MPA) or demethiolated to acrylate.

warming (10, 62). It has been postulated that cloud formation and the backscattering of sunlight may theoretically affect primary productivity in the ocean by reducing mean light intensity and temperature (10). While the bacterial DMSP lyase pathway is the only pathway yielding DMS, factors that impact on bacterial growth, physiology, and the bacterial interaction with the phytoplankton seem to modulate DMSP dynamics and further adjust DMS flux, determining whether the sulfur is retained within the marine microbial food web or removed from it as volatile DMS (22, 88). So, the symbiosis between marine bacteria and phytoplankton may play a pivotal role in regulating global climate on Earth.

## 1.3    The *Roseobacter* clade

Bacteria phylogenetically related to the *Roseobacter* clade of alphaproteobacteria are the second most abundant species of bacteria in the ocean, after SAR11 '*Pelagibacter ubique*' (22). Since 1991 when they were first detected, the *Roseobacter* clade species have drawn much interest because of their widespread distribution in the marine environment. The term "*Roseobacter* clade" refers to bacteria with >89% 16S rRNA identity, comprising 38 affiliated genera and 41 phylogenetic lineages (22).

Members of the *Roseobacter* clade are found to be associated with higher eukaryotes, for instance scallop larvae (131), macro-algae (127), rotifers (20), and dinoflagellates (110). For example the first two roseobacters, *Roseobacter denitrificans* and *Roseobacter litoralis*, were isolated from the surfaces of green seaweeds and not from seawater (140). An analysis of the bacteria associated with dinoflagellates of the genus *Alexandrium* showed these phytoplankton harbors many

roseobacters in common despite isolation from different geographic origins (82). Furthermore, cultures of *Alexandrium* (74, 82), *Scrippsiella trocholidea* (74), and *Gymnodinium catenatum* dinoflagellates show a high level of association with members of the *Roseobacter* clade (64).

Roseobacters are also distributed widely in diverse environments. They are found in coastal and open ocean environments, salt marsh ecosystems, salt lakes, coral reefs, within marine sponges, marine sediments, scallop larvae, sea grasses, and coastal biofilms (22). Marine *Roseobacter* was also found to be associated with sponges (42). Accordingly, bacteria possess diverse and flexible metabolic capabilities, including aerobic anoxygenic photosynthesis (140), polyhydroxybutyrate metabolism (34), catabolism of organic sulfur compound DMSP (59), and the synthesis of biologically active chemicals, such as antibiotic tropodithietic acid (TDA) (16). Accumulating evidence points roseobacters as potentially important participants in DMSP catabolism (61, 154). In the open ocean, the *Roseobacter* clade has been found to be the most abundant bacteria associated with blooms of high-DMSP-producing phytoplankton (61, 154). Howard *et al.* discovered that DmdA, an important enzyme in DMSP demethylation, is widely found in *Roseobacter* clade bacteria, suggesting roseobacters dominate demethylation in coastal regions and algal blooms (75). Roseobacterial catabolism of DMSP produced by phytoplankton underscores that investigation of roseobacter and phytoplankton interaction will enhance our understanding of global scale sulfur cycle.

## 1.4    Symbiosis between roseobacters and dinoflagellates

Outbreaks of dinoflagellates known as harmful algal blooms (HABs) have affected many countries over the past decades (68). The heterotrophic dinoflagellate *Pfiesteria piscicida*, or more likely mixotrophic dinoflagellate *Karlodinium micrum*,(86), is responsible for massive fish kills and human illness, the latter of which has been nebulously called "possible estuary associated syndrome (PEAS)". The syndrome includes learning and memory problem, skin lesions, and acute respiratory and eye irritation The life cycle of *P. piscicida* (99) was originally reported as complex with 24 stages (26). It has been revised and simplified to that it consist of asexual division and sexual reproduction producing dinospores, gametes, zygotes, and cysts (99).

The Belas laboratory has analyzed the bacterial community associated with laboratory microcosms of *P. piscicida* (1, 111). The microcosm contains *P. piscicida*, its prey algae (*Rhodomonas*, CCMP768), and the community of bacteria derived from Chesapeake Bay (Maryland, USA), where the original dinoflagellate sample was obtained in 1998. The bacterial community inhabiting this microcosm is a diverse group of over 30 species numerically dominated by roseobacters. Roseobacter clade bacteria are key members of this community occurring in numbers over 50% of the total bacterial assemblage (1). One species, *Silicibacter* sp. TM1040 (referred to as TM1040 hereafter) is notable as it exists in such close physical association with the dinoflagellates that it forms a biofilm on their surface. Not only does TM1040 develop a biofilm on the surface of *P. piscicida*, but studies also have shown that axenic dinoflagellate cultures, i.e., those lacking bacteria, failed to grow and
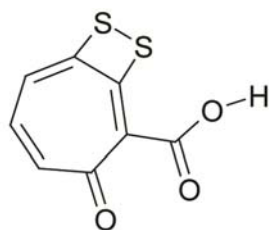
tropodithietic acid

**FIG. 1.3 Tropodithietic acid structure** (TDA)

ultimately died. Surprisingly, adding back TM1040 or mixed bacterial assemblages containing bacteria physiologically like TM1040 restores the growth of *P. piscicida* (1, 110). TM1040 cells sense and are attracted to *P. piscicida* homogenates, as well as DMSP and amino acids (112). TM1040 cells can also attach to the surface of the zoospore and develop a biofilm (1). Here the cells obtain the sulfur and/or carbon source DMSP supplied by *P. piscicida* (109). Thus, TM1040 and *P. piscicida* demonstrate a mutualistic symbiotic relationship. The TM1040:*Pfiesteria* association is the only known "obligate" association between a dinoflagellate and a culturable bacterium, and fits the definition of a symbiosis as 'the close, permanent relationship between two or more different organisms' (117).

The genome of TM1040 has been annotated (113), and a large array of genetic tools have been developed to probe the molecular mechanisms that aid this roseobacter in its symbiosis with its phytoplanktonic host. Thus, while TM1040 is ecologically extremely relevant, it is also an excellent choice for laboratory studies. Using the TM1040:*P. piscicida* symbiosis as a model for other roseobacter-phytoplankton symbioses, the following mechanisms are likely to participate in initiating and maintaining the symbiosis.

## 1.5    Molecular mechanisms underlying roseobacter-phytoplankton symbioses

### 1.5.1    Motility and chemotaxis to phytoplankton

Chemotaxis behavior allows motile bacteria to swim toward an attractant and away from toxic conditions, providing bacteria with a competitive advantage for

nutrition in natural environments (155). More than half (16 out of 28) of the annotated roseobacter genomes harbor homologs to known chemotaxis genes and diverse chemoreceptor proteins that are required for binding different ligands (143). The presence of multiple cytoplasmic chemotaxis genes reflects the metabolic versatility of the roseobacters, and are undoubtedly used as part of chemotactic behaviors to allow these bacteria to swim towards nutrient sources (**FIG. 1.1.**), such as an algal cell (6).

It is demonstrated that bacterial motility is an important factor in the symbiosis (110). Three transposon-insertion TM1040 mutants with motility defects were analyzed for their ability to form biofilms on and support the growth of *P. piscicida*. Mutation of *flaA* (a novel flagellar gene whose function is unknown) or *cckA* results in the loss of flagella (Fla⁻) and non-motile cells (Mot⁻), while CtrA⁻ cells possess flagella, but have reduced motility due to increased cell length. CckA and CtrA constitute a two-component master regulator system where CckA is a histidine kinase to phosphorylate CtrA and positively regulates CtrA activity (80). All three flagellar mutants were defective in attaching to and forming biofilms on the dinoflagellate, and the growth of the dinoflagellates was reduced compared to symbiosis with wild-type cells (110). Thus, motility and subsequent biofilm development are linked to the physiological requirement of the dinoflagellate for the bacteria.

The TM1040 genome is replete with genes with the potential to encode over 20 methyl-accepting chemotaxis membrane transducers proteins (MCPs; also known as chemoreceptors) and multiple copies of cytoplasmic chemotaxis (Che) proteins

(143), suggesting that TM1040 is proficient in sensing and responding to chemical attractants. TM1040 responds specifically to heat-labile compounds found in dinoflagellate homogenates, while homogenates from *Rhodomonas* or culturable heterotrophic bacteria isolated from *P. piscicida* do not elicit a similar response (112).

While TM1040 responds positively to sugars, with sucrose, *N*-acetylglucosamine (NAG), galactose, glucose, and maltose, and to tricarboxylic acid cycle (TCA or Krebs cycle) intermediate compounds, ex., citrate and fumarate (112), TM1040 also responds strongly to DMSP and metabolic byproducts of DMSP metabolism, and is attracted to all amino acids, with the greatest response observed to methionine. These results suggest that TM1040's physiology, motility, and chemotaxis machinery is adapted to ensure that the bacteria are able to find the dinoflagellate in oligotrophic seawater and initiate the symbiosis through sensing, response, and swimming to the dinoflagellate surface.

## 1.5.2  Biofilm formation on phytoplankton

As described earlier, marine bacteria often cluster around phytoplankton for metabolic requirements, thereby increasing the density of the bacterial population beyond what is typical for open ocean waters (15). As they come closer to the surface of the phytoplankton, various molecular mechanisms that promote bacterial attachment are activated thereby allowing the bacteria to attach to the surface of their host.

Bacterial attachment to surfaces is a complex phenomenon involving numerous molecular mechanisms that are often species-dependent; however, there is strong evidence that extracellular appendages, such as fimbriae or pili, are frequently

used by bacteria to stick to surfaces (**FIG. 1.1.**) (160). The genome of many species of roseobacters harbor key homologs to the *Caulobacter crescentus cpaBCEF* genes, which play important roles in the biofilm formation in this alphaproteobacterium (45). Additionally, open reading frames (ORFs) with homology to *Actinobacillus actinomycetemcomitans* TadB and TadC and flp fimbrial proteins are also present (113). Tad proteins form fimbriae that cause the cells to autoaggregate, while *flp* fimbriae are often involved in tight adherence of bacteria to eukaryotic cells (84). It is possible that the homologs in TM1040 function similarly in its colonization of surfaces on phytoplankton, although experimental evidence is lacking to support this hypothesis.

It should be noted that bacteria-like particles or structures have been observed within phytoplankton, suggesting that some bacteria may exist as intracellular symbionts in these unicellular eukaryotes. Endocytoplasmic and endonuclear bacteria have been observed by electron microscopy in cells of *Gymnodinium splendens* and *Glenodinium foliaceum* (141), and *Gyrodinium instriathum* (13). Rigorous tests to confirm the presence of viable and physiologically relevant intracellular bacteria have not been reported, and a degree of caution should prevail when interpreting these findings.

### 1.5.3   Acquisition of nutrients derived from phytoplankton

There is an obvious benefit for a bacterium to attach to an algal cell: it obtains a readily available source of organic matter from the eukaryote, the bacterium is in close proximity to other bacteria, which permits more effective transfer of genes and response to chemical inducers, and forming a biofilm on the alga makes it more

difficult for bacteriovores to prey on the bacterium (**FIG. 1.1.**). Of these benefits, the first – utilization of phytoplankton-derived nutrients – has been extensively studied. Members of *Roseobacter* clade are also capable of utilizing complex organic polymers, such as cellulose and lignin (62). This is ecologically important since coastal runoff distributes much plant-derived cellulose and lignin from coastal marshes into the nearby ocean. Therefore degradation of these materials by roseobacters could contribute a significant source of carbon to coastal waters (62). Genomic analyses have shown that roseobacters contain numerous pathways for degradation of aromatic hydrocarbons, which may include phytoplankton-derived molecules that function as antioxidants, toxins, and predator deterrents (113).

### 1.5.4 The effect of bacteria on the physiology of phytoplankton

The interaction between bacteria and phytoplankton may be complex within the phycosphere, and it is probable that multiple bacterial mechanisms may come into play that benefit the host cell. Bacteria help phytoplankton regenerate inorganic nutrients (44) and often provide essential compounds that the microalgae themselves cannot synthesize. A powerful example of this is Vitamin B12, cobalamin**,** which is required but not synthesized by many phytoplankton, and instead provided by the bacterial community associated with the unicellular alga (38). Bacteria associated with phytoplankton also help the microalgae acquire iron (**FIG. 1.1.**) through the synthesis of bacterial siderophores that increase iron solubility (145), thus conferring to the algae an advantage under iron-limiting conditions such as are found in the open ocean. These studies show that micronutrients acquired by phytoplankton through

bacterial metabolism benefit the alga and are an important facet in the symbiosis between phytoplankton and bacteria.

While the previous example provides substantial empirical evidence of the benefits to the phytoplankton, there are many other examples of how bacteria positively affect the physiology of marine unicellular algae. For example, 14 strains of bacteria taxonomically related to the *Roseobacter* lineage were isolated during blooms of *Alexandrium tamarense* (a red-tide dinoflagellate) and shown to have beneficial effects on the growth of the dinoflagellates (48, 70).

### 1.5.5   Vir (Type IV Secretion) system

Genomic analyses have also hinted that roseobacters may be able to interact with their eukaryotic microalgal hosts through cell-to-cell direct transfer of molecules. One of the many surprises that has come from analyses of roseobacter genomes is that half of the roseobacter species with annotated genomes harbor a putative Type IV secretion system (T4SS) (113, 143). T4SS is an important secretion mechanism in many Gram negative bacteria (163). For example, *Agrobacterium tumefaciens*, another alphaproteobacterium, uses a set of *vir* genes products to transfer DNA (referred to as T-DNA) from the bacterium into plant cells, and thereby initiating the formation of crown galls in terrestrial dicot plants. A similar set of *vir* genes is found in TM1040 (and other roseobacters). The TM1040 "*vir*" genes have homology to *virD2* and *virD4*, which encode for the relaxase and coupling proteins (providing the energetics for export of DNA), and *virB1-11* (excluding *virB7*), which encode the inner membrane channel and pilus structure of the transfer machinery (35). The symbiosis between TM1040 and dinoflagellate *P. piscicida* tempts

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speculation that Vir proteins enable roseobacters to transfer DNA or proteins directly to phytoplankton cells (1, 109, 110). While this has yet to be experimentally proven, roseobacters have been found inside of galls formed on the red macroalgal genus *Prionitis* (4). These galls are morphologically similar to crown galls formed by *A. tumefaciens*, hinting that the roseobacter T4SS may have a similar function to the *vir* system of *Agrobacterium*.

## 1.5.6  Quorum signaling

Quorum signaling (QS) is a form of population-density-dependent chemical communication used by bacteria to control cellular functions through excreted small molecules that interact directly to regulate the expression of sets of genes within certain bacterial species (27). QS is widespread among bacteria and has been shown to be involved in pathogenesis, *e.g.*, in the virulence of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients (14)*,* and symbioses, such as the symbiosis between the bioluminescent marine bacterium *Vibrio fischeri* and *Euprymna scolopes*, a squid (52, 150). Many other species of marine bacteria produce QS molecules, particularly acylated homoserine lactones (AHLs), including many roseobacters (18, 150). AHLs have been detected in roseobacters obtained from samples of marine snow (63) and elsewhere, emphasizing the importance of AHL QS as one of the most common chemical communication mechanisms in roseobacters (52).

However, not all members of the *Roseobacter* lineage possess the capacity to synthesize AHLs. It has been shown that TM1040 lacks genes encoding known QS systems and does not produce the most common AHL molecules (18, 113), yet some

of the phenotypes of this species, such as biofilm development, rosette formation, a multicellular star-like aggregation where cells stick by their poles (22), and TDA biosynthesis, appear to be population-density-dependent, suggesting that molecules other than canonical AHLs may be used by TM1040.

Although QS has been studied primarily as a prokaryote-to-prokaryote signal system, eukaryote hosts have also developed strategies that modulate QS. For example, research has shown that higher plants produce furanone and AHL-like chemicals that mimic AHL compounds and affect specific bacteria (100). Conversely, the production of bacterial QS chemicals can also affect the behavior of higher plants, such that bacterial biofilms producing AHLs cause planktonic zoospores of *Enteromorpha* to settle down on or near those same bacteria (83). Although yet to be experimentally proven, it is likely that QS controls many bacterial behaviors that are essential for roseobacter-phytoplankton symbioses, including formation of biofilms and production of biologically active compounds, that ultimately influence the initiation and establishment of the symbiosis.

### 1.5.7 Tropodithietic acid (TDA)

Several reports have highlighted that members of the *Roseobacter* clade produce numerous molecules whose biological activity is of potential importance to marine phytoplankton (18, 119). Specifically, the production of TDA, which cooccurs with the production of brown pigment, is a common feature of certain roseobacters (18). It has been reported that TDA is produced by the following roseobacters: *Phaeobacter* 27-4 (20), *Roseobacter* sp.T5 (16), *P. gallaeciensis* BS107 (131), and TM1040 (57). TDA is a tautomer of thiotropocin (65), a compound that has been

isolated from a marine *Agrobacterium* and *Caulobacter* PK 654, both alphaproteobacteria, and most likely members of *Roseobacter* clade that were misidentified in these earlier studies (162). Recently, 11 roseobacters isolated from a turbot farm were shown to produce TDA (122). These findings suggest that TDA synthesis is possessed by many roseobacters underscoring its potential physiological linkage to roseobacters.

TDA is a novel dual-sulfur tropolone antibiotic that is synthesized by an oxidative ring expansion of phenylacetic acid derived from the shikimate-chorismate pathway (28). Liang (98) reported that phenylalanine and histidine significantly enhanced production of TDA in *Phaeobacter* T5. TDA kills many species of bacteria while causing little or no inhibition of TM1040 or other roseobacters (18). TDA may also influence higher eukaryote organisms. For example, diatom growth is inhibited by high levels of 1 mM TDA where control DMSO does not have effect in tested laboratory experiment(16). Thiotropocin inhibits red tide phytoplankton at concentration of 1 µg/ml (equal to 4.7 µM) (162). Turbot larvae disease is prevented by the presence of TDA producing *Phaeobacter* 27-4 (72). Extractions of *P. gallaeciensis* BS107 cultures produce a probiotic effect on scallop larvae (131).

## 1.6    TM1040: a biphasic life style bacterium

Many species in the *Roseobacter* clade have a biphasic "swim-or-stick" lifestyle, e.g., these bacteria have an apparent life cycle that includes the formation of either motile cells that swim via one or more flagella and respond to attractant and repellent molecules via chemotaxis behavior (112), or sessile cells that lack flagella

and readily attach to surfaces (18, 57). It is highly likely that the dual lifestyle of roseobacters greatly influences and may control their symbiosis with phytoplankton.

TM1040 exhibits signs of a biphasic lifestyle consisting of motile and sessile phases (**FIG. 1.1.**) (18). The choice of culturing method greatly influences the physiology of the bacteria and alters the composition of the population. Vigorous shaking cultures are composed of small, flagellated cells that are highly motile and chemotactic (1, 57). The motile phase population produces little TDA and pigment, has few rosettes and many motile cells (19). TM1040 uses the motile phase to swim towards its dinoflagellate host, responding to chemical cues emitted by the dinoflagellate, to ultimately to allow the bacteria to move into close proximity to the dinoflagellate surface.

In contrast, when TM1040 is grown in standing liquid culture, the cells are found in biofilms or attached to one another as "rosettes", and the bacteria produce a yellow-brown pigment, as well as TDA (18). High TDA activity is correlated with an overall change in the physiology of the bacteria that includes the production of a yellow-brown pigment, in addition to formation of rosettes and biofilms, and the loss of flagella and motility (20). The sessile phase of life may aid TM1040 in adapting to life on the surface of the dinoflagellate. During the sessile phase, we hypothesize that the bacteria lose their flagella and increase expression of the genes required for attachment (fimbrial adhesins) and TDA.

## 1.7    The Big Question

Members of the *Roseobacter* clade live closely with phytoplankton, and they metabolize DMSP, which is produced in high amounts by dinoflagellates. Thus, the

symbiosis between roseobacters like TM1040 and phytoplankton such as *P. piscicida* has a huge impact on the marine primary production, nutrient cycling, and the sulfur cycle. The model for the symbiosis of TM1040 between *P. piscicida* involves multiple steps. These steps are (1) bacteria respond to dinoflagellate molecules; (2) they swim via chemotaxis into close proximity of the dinoflagellate; (3) bacteria attach and form a biofilm on the dinoflagellate; (4) bacteria sense themselves attached to dinoflagellate; (5) bacteria increase TDA production from DMSP; and (6) dinoflagellates gain growth benefits from TDA. What are the molecular and genetic mechanisms used by roseobacters that allow them to establish and maintain their symbiosis with phytoplankton and thereby enhance survival in the marine environment?

# Chapter 2:   Genetic Dissection of Tropodithietic Acid

# Biosynthesis by Marine Roseobacters

## 2.1   Summary

The symbiotic association between the roseobacter TM1040 and the dinoflagellate *P. piscicida* involves bacterial chemotaxis to dinoflagellate-produced DMSP, DMSP demethylation, and ultimately formation of a biofilm on the surface of the host. Biofilm formation is coincident with the production of an antibiotic and a yellow-brown pigment. In this report, we demonstrate that the antibiotic is a sulfur-containing compound, tropodithietic acid (TDA). Using random transposon insertion mutagenesis, 12 genes were identified as critical for TDA biosynthesis by the bacteria, and mutation in any one of these results in loss of antibiotic activity (Tda⁻) and pigment production. Unexpectedly, six of the genes, referred to as *tdaA-F*, could not be found on the annotated TM1040 genome and were instead located on a previously unidentified plasmid (ca. 130 kB; pSTM3) that exhibited a low frequency of spontaneous loss. Homologs of *tdaA* and *tdaB* from TM1040 were identified by mutagenesis in another TDA-producing roseobacter, *Phaeobacter* 27-4, which also possesses two large plasmids (ca. 60 and ca. 70 kb, respectively), and *tda* genes were found by DNA:DNA hybridization in 88 % of a diverse collection of 9 roseobacters with known antibiotic activity. These data suggest that roseobacters may employ a common pathway for TDA biosynthesis that involves plasmid-encoded proteins. Using metagenomic library databases and a bioinformatics approach, differences in the biogeographical distribution between the critical TDA synthesis genes were

observed. The implications of these results with respect to roseobacter survival and the interaction between TM1040 and its dinoflagellate host are discussed.

## 2.2    Introduction

Bacteria of the *Roseobacter* clade of marine alphaproteobacteria and out as some of the most critical players in the oceanic sulfur cycle due to the ability of several genera to degrade dimethylsulfoniopropionate (DMSP) (114, 156). While roseobacters are wide-spread throughout the marine ecosystem, their abundance is significantly correlated with DMSP-producing algae, especially prymnesiophytes and dinoflagellates, such as *Prorocentrum*, *Alexandrium,* and *Pfiesteria* species (1, 93).

Our laboratory has been studying the interaction of a roseobacter, TM1040, and *P. piscicida* (1, 109, 110, 112, 113). TM1040 was originally isolated from a laboratory microcosm culture of the heterotrophic DMSP-producing dinoflagellate *P. piscicida* (109). Marine algae are the major producers of DMSP in the marine environment (53) while bacteria, and specifically members of the *Roseobacter* clade, are largely responsible for DMSP catabolism (156). TM1040 degrades DMSP via a demethylation pathway producing 3-methylmercaptopropionate (MMPA) as a major breakdown product (109). The bacteria respond via chemotaxis to dinoflagellate homogenates, and are specifically attracted to DMSP, methionine, and valine (112). Experimental evidence has shown that TM1040 motility is important in the initial phases of the symbiosis (110). Once the bacteria are in close proximity to their host, TM1040 forms a biofilm on the surface of the dinoflagellate (1, 18, 110). Thus, the symbiosis may be divided into two parts: one that involves chemotaxis and motility, and a second step in which a biofilm predominates.

It has recently been reported that specific phenotypes, e.g., the ability to produce antibacterial compounds and biofilm formation, may give members of the *Roseobacter* clade a selective advantage, and help to explain the dominance of members of this clade in association with marine algae (18). Specifically, the production of an antibiotic activity is commonly observed in roseobacters and is hypothesized to provide an advantage when colonizing phytoplanktonic hosts, such as dinoflagellates (18).

A sulfur-containing antibiotic compound TDA has been isolated and chemically characterized from *Phaeobacter* 27-4 (referred to as 27-4 for the remainder) (20) and *Roseobacter* T5 (16). The chemical backbone of TDA (shown in **FIG. 1.3.**) is a seven member aromatic tropolone ring, which is highly significant as tropolone derivatives, notably hydroxylated forms, are widely seen as medically important sources of antibacterial, antifungal, antiviral, and antiparasitic agents (24, 115, 116, 134). Components of the biosynthetic pathway leading to the production of thiotropocin, another tropothione derivative closely related to TDA, have been described by Cane et al. (28) who suggested that thiotropocin is synthesized from shikimate by an oxidative ring expansion of phenylacetic acid.

In this chapter, I used both genomic and genetic techniques to uncover the genes and proteins required for TDA synthesis in TM1040 and 27-4 as models for the *Roseobacter* clade. In the process of locating these genes, I discovered a megaplasmid critical for TDA biosynthesis that is part of the TM1040 genome but escaped sequencing.

## 2.3    Materials and Methods

### 2.3.1    Bacteria and media

The strains used in this study are listed in **Table 2.1.**. TM1040, Phaeobacter 27-4 and *Vibrio anguillarum* 90-11-287 were grown and maintained in 2216 marine broth or 2216 agar as recommended by the manufacturer (BD Biosciences, Franklin Lakes, NJ). A marine basal minimal medium (MBM; per liter: 8.47g Tris HCl, 0.37 g of $NH_4Cl$, 0.0022 g of $K_2HPO_4$, 11.6 g NaCl, 6 g $MgSO_4$, 0.75 g KCl, 1.47 g $CaCl_2 \cdot 2H_2O$, 2.5 mg FeEDTA; pH 7.6, 1 ml of RPMI-1640 vitamins [Sigma R7256]) was used for determining carbon and sulfur requirements. Sole carbon sources were added at a final concentration of 1 g/l. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth (5) or on LB agar containing 1.5% Bacto Agar (Becton Dickinson, Franklin Lakes, N.J.). As appropriate, kanamycin was used at 120 μg per ml for *Roseobacter* strains and 50 μg per ml for *E. coli*.

### 2.3.2    Characterization of antibiotic

Bacterial culture was centrifuged and cell-free supernatant, i.e. spent medium, was either injected directly (up to 10 μl) or purified by mixed phase anion-exchange reversed phase mini column chromatography on Oasis MAX columns as previously described (20). Tropodithietic acid was analyzed by reverse phase liquid chromatography (LC) on an Agilent 1100 HPLC system equipped with a diode array detector (DAD). Separation was conducted using a Phenomenex (Torrance, CA) Curosil PFP column (15 cm, 2 mm, 3 μm) using a water-acetonitrile gradient system. Both solvents contained 200 μl of trifluoroacetic acid/liter and started at 35%

acetonitrile, increasing linearly to 60% in 6 min. A wavelength of 304 ± 4 nm was used for detection. LC-DAD with online high-resolution mass spectrometry using positive and negative electrospray was used for validation of the TDA detection as previously described (20).

### 2.3.3   Transposon mutagenesis and Tda⁻ screening

Electrocompetent roseobacter strains were prepared following the method described by Garg et al. (54) as modified by Miller and Belas (110). Random transposon insertion libraries were constructed in TM1040 and 27-4 using the EZ-Tn*5*<R6K*γori*/KAN-2>Tnp Transposome™ Kit (Epicentre, Madison, WI). Strains were spread onto 2216 plates containing kanamycin and incubated for 1 day at 30 °C. Individual Kan[r] transposon insertion strains were transferred to 7x7 arrays on 2216 marine agar plus kanamycin to facilitate further screening. To screen for loss-of-function, antibiotic-negative (Tda⁻) mutants, a modification of the method described by Bruhn et al (20) was used. Bacteria were replicated, as a 7x7 array, to a lawn of *Vibrio anguillarum* strain 90-11-287 (18, 20), and incubated at 20°C for 24 h, after which a zone of clearing indicative of antibiotic production was measured and compared to the parental strain (TM1040 or 27-4). For purposes of this study, Tda⁻ is defined as a strain lacking a detectable zone of clearing on *V. anguillarum*. Strains determined to be Tda⁻ by the modified well-diffusion assay were further tested by incubation at 30°C for 48 h in 2216 marine broth without shaking. Bacteria were removed by filtering through a 0.22 μm MCE membrane (Millex, Millipore, Bedford, MA) and the antibacterial activity of the supernatant measured using the *V. anguillarum* well diffusion assay, as described by Bruhn et al. (18, 20).

### 2.3.4  Sole carbon and sulfur source growth

Bacterial utilization of sole carbon sources was determined by measuring growth in MBM broth that was modified by replacing glycerol with the carbon source to be tested. Carbon compounds tested with concentration 1 g/l included amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine); sugars (arabinose, fructose, galactose, glucose, lactose, maltose, mannose, N-acetylglucosamine, ribose, sucrose, xylose); tricarboxylic acid cycle (TCA) intermediates (citrate, fumurate, succinate); as well as phenylacetic acid and sodium phenylpyruvate.

Sulfur utilization was tested by growth in MBM containing different 100 mM sulfur sources: DMSP, cysteine, methionine, sodium sulfate, and sodium sulfite.

### 2.3.5  Bioinformatics analysis

Approximately 1 μg of genomic DNA isolated from the candidate mutant was digested with *Nco*I, self-religated with T4 DNA ligase, and electroporated into DH5α (λpir). Following selection for kanamycin resistance, Kan$^r$ colonies were picked and the plasmid isolated for bidirectional sequencing with transposon-specific primers as recommended by the supplier (Epicentre, Madison, WI). Nucleotide sequence thus obtained was analyzed by BLAST analyses using DNA-DNA homology searches

**Table 2.1. Bacterial strains and plasmids used in Chapter 2.**

| Strain/plasmid | Genotype/phenotype | Source or reference |
|---|---|---|
| *Escherichia Coli* | | |
| DH5α | F⁻ *endA1 hsdR17 ($r_K^-$ $m_K^-$) supE44 thi-1 recA1 gyrA96 relA1Φ80dlacZΔM15* | (135) |
| DH5α(λpir) | DH5α transduced with λ*pir* | (69, 91) |
| EC100D *pir+* | F⁻ *mcrA D(mrr-hsdRMS-mcrBC) f80dlacZDM15 DlacX74 recA1 endA1 araD139 D(ara, leu)7697 galU galK l– rpsL nupG pir+*(DHFR). | Epicentre™ |
| Roseobacters | | |
| TM1040 | Wild type, antibacterial activity | (109) |
| Mutants derived from TM1040 | | |
| *Silicibacter* sp. TM1040 SM | None pigment and tda spontaneous strain | This study |
| HG1005 | *paaK*::EZ-Tn5,Kan | This study |
| HG1015 | *tdaB*::EZ-Tn5,Kan | This study |
| HG1050 | *tdaF*::EZ-Tn5,Kan | This study |
| HG1056 | *paaJ*::EZ-Tn5,Kan | This study |
| HG1080 | *tdaC*::EZ-Tn5,Kan | This study |
| HG1110 | *tdaD*::EZ-Tn5,Kan | This study |
| HG1213 | *malY*::EZ-Tn5,Kan | This study |
| HG1220 | *cysI*::EZ-Tn5,Kan | This study |

| | | |
|---|---|---|
| HG1244 | *tdaH*::EZ-Tn5,Kan | This study |
| HG1265 | *tdaE*::EZ-Tn5,Kan | This study |
| HG1299 | *paaI*::EZ-Tn5,Kan | This study |
| HG1310 | *tda*A::EZ-Tn5,Kan | This study |
| *Phaeobacter* sp. 27-4 | Wild type, antibacterial activity | (18, 72) |
| Mutants derived form 27-4 | | |
| JBB1001 | *tdaB*::EZ-Tn5,Kan | This study |
| JBB1003 | *tdbC*::EZ-Tn5,Kan | This study |
| JBB1005 | *traI*::EZ-Tn5,Kan | This study |
| JBB1006 | *clpX*::EZ-Tn5,Kan | This study |
| JBB1007 | *tdbF*::EZ-Tn5,Kan | This study |
| JBB1009 | *tdbA*::EZ-Tn5,Kan | This study |
| JBB1011 | *tdbD*::EZ-Tn5,Kan | This study |
| JBB1029 | *tdbE*::EZ-Tn5,Kan | This study |
| JBB1030 | *tda*A::EZ-Tn5,Kan | This study |
| JBB1044 | *metF*::EZ-Tn5,Kan | |
| JBB1045 | *tdbB*::EZ-Tn5,Kan | |
| Other Roseobacters | | |
| *Roseobacter algicola* 51442 | Wild type, none antibacterial activity | (18, 93) |
| *Roseobacter denitrificans* 33942 | Wild type, none antibacterial activity | (18, 140) |

| | | |
|---|---|---|
| *Roseobacter litoralis* 49566 | Wild type, none antibacterial activity | (18, 140) |
| *Roseobacter* sp. TM1038 | Wild type, antibacterial activity | (18, 109) |
| *Roseobacter* sp. TM1039 | Wild type, antibacterial activity | (18, 109) |
| *Roseovarius* sp. ISM | Wild type, antibacterial activity | (18) |
| *Roseovarius* sp. TM1035 | Wild type, antibacterial activity | (18, 109) |
| *Roseovarius* sp. TM1042 | Wild type, antibacterial activity | (18, 109) |
| *Ruegeria pomeroyi* DSS-3 | Wild type, antibacterial activity | (18, 58) |
| *Sulfitobacter* sp.1921 | Wild type, none antibacterial activity | (18) |
| *Sulfitobacter* sp. EE36 | Wild type, antibacterial activity | (18, 21) |
| *Sulfitobacter* sp. SE62 | Wild type, none antibacterial activity | (18, 23) |
| | | |
| *Vibrio anguillarum* 90-11-287 | Wild type, Susceptible to tropodithietic acid | (18, 142) |

Plasmid

| | | |
|---|---|---|
| *pSTM3* | Harboring *tda* genes | This study |
| *pSTM3-1265* | *pSTM3* carrying a Tn5 insertion in *tdaE*, derived from HG1265 | This study |
| *pSTM3-1265n* | *Nco*I digested rescue clone plasmid from HG1265, 6.4kb | This study |

against the TM1040 genome (Accession numbers: NC_008044，NC_008043，NC_008042). The genes identified are listed in **Table 2.2** for TM1040 and **Table 2.4.** for 27-4. Signature amino acid domains in the deduced amino acid sequence of the respective ORFs were identified using BLASTP (2), Pfam (50), SMART (96), and the Conserved Domains Database (CDD; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Homologs in roseobacters were identified using BLASTP analysis of Roseobase (http://www.roseobase.org/) and Gordon and Betty Moore Foundation Marine Microbial Genome databases (https://research.venterinstitute.org/moore/) with respective predicted protein sequence as the query sequence and a maximum E value of 1E-30. Homologs in the Global Ocean Sampling Expedition metagenomic libraries (http://camera.calit2.net/index) (133) were identified by BLASTP analysis using a cutoff E value 1E-20.

### 2.3.6 DNA extraction and separation

Chromosomal DNA was extracted from bacterial cells by routine methods (5) or by the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). Plasmid DNA was prepared by the alkaline lysis method (5), digested with *Nco*I (New England Biolabs, Beverly, MA), and the resulting restriction fragments were separated by agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer.

Pulsed Field Gel Electrophoresis (PFGE) was performed using a CHEF DR-III clamped homogeneous electric field system (Bio-Rad, Richmond, Calif.) with a 1% agarose gel, a 3- to 15-s pulse ramp, an electrophoresis rate of 6.0 V/cm with an included angle of 120° at a constant temperature of 14°C, and a run time of 26 h. Gels

were stained with ethidium bromide (EB) and visualized with a Typhoon 9410 (Amersham Biosciences, Piscataway, N.J.).

### 2.3.7   PCR amplification

Multiplex PCR amplification was used to screen for the presence of *tda* genes in Tda⁻ mutants. A 716-bp sequence internal to *tdaE* was amplified using primers 5'-CAGATGATGGTGCCAAAGGACTAT-3' and 5'-GGTCAGTTTCTTCTGCACATACTGG-3', while (in the same reaction), an internal 401-bp fragment of *flaA* (accession number: CP000377, locus tag: TM1040_2952) was also amplified using primers 5'-TTGCAGTATCCAATGGTCGTG-3' and 5'-TGAATTGCGTCAGAGTTTGCC-3' as a control. Standard PCR amplification conditions were 100 μM dNTP each, 0.2 μM of each primer, 1 U Taq DNA polymerase (New England Biolabs, Beverly, MA) in 1× reaction buffer (New England BioLabs) with an initial denaturing step at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min each, annealing at 55°C for 30 s, and an elongation at 72°C for 1 min.

To detect the *tdaA-E* locus, PCR amplification was conducted with a forward primer complementary to *tdaA* (5'-CGCTTTCCGGAACTGGAGAT-3') and a reverse primer complementary to *tdaE* (5'-GGCTGCCGTATAGTTTCAGCA-3') using the. Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) and the PCR program conditions and cycle parameters as described by the supplier.

### 2.3.8  DNA hybridization

DNA:DNA hybridization by Southern 'slot' blot (5) was used to detect the presence of *tda* genes in other roseobacters. The roseobacter strains used were: *Phaeobacter* strain 27-4, *Roseobacter algicola* ATCC 51442, *Roseobacter denitrificans* ATCC 33942, *Roseobacter litoralis* ATCC 49566, *Roseobacter* sp. strain TM1038, *Roseobacter* sp. strain TM1039, *Roseovarius* sp. strain TM1035, *Roseovarius* sp. strain TM1042, *Roseovarius* strain ISM, *Ruegeria pomeroyi* DSS-3, TM1040, *Sulfitobacter* strain EE36, *Sulfitobacter* strain 1921, *Sulfitobacter* strain SE62, and *Vibrio anguillarum* 90-11-287. Following extraction, 100 ng of total genomic DNA purified from each strain was spotted onto a positively charged nylon membrane (Roche applied science). The DNA was cross-linked to the membrane with ultraviolet light using a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA), followed by prehybridization of the membrane at 25°C for 30 min, using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche applied science) as described by the manufacturer. The membrane was incubated at 25 °C overnight with a double-stranded DNA probe prepared by *Hin*dIII digestion of a plasmid bearing *tdaA* cloned from strain HG1310 that was labeled with digoxigenin- dUTP using random priming as recommended by the manufacture (Roche applied science). Unbound labeled DNA was removed from the membrane by 2 ×5 min in 2×SSC, 0.1% SDS followed by 2 ×15 min in 0.2×SSC, 0.1% SDS (5). In the southern blot, the membrane was prehybridized for 30 min in the same buffer to which was added a *tdaD* gene probe, and the probe allowed to hybridize overnight at 42°C. The blots were washed under high stringency conditions following the manufacturer's protocol

(Roche applied science) and exposed to Lumi-film chemiluminescent detection film (Roche applied science) for subsequent detection of the hybridization signal.

## 2.4 Results

### 2.4.1 TM1040 produces the sulfur-containing antibiotic tropodithietic acid

Bruhn et al. showed that TM1040 produces an extracellular broad spectrum antibacterial compound capable of inhibiting or killing many bacteria (18). It was found that greater antibacterial activity occurred when the bacteria were grown in a nutrient broth culture under static conditions. The clearing zone used to assess antibiotic production was 11 mm greater in samples tested from cultures that were not shaken compared to those that were (**FIG. 2.1**). Under static conditions, TM1040 cells attached to one another forming rosettes and produced a distinct yellow-brown pigment. These phenotypes are consistent with those described for 27-4 (20) and other roseobacters (18). Nonpigmented colonies were sometimes seen after TM1040 was incubated on nutrient agar, and subsequent analysis revealed that these white spontaneous mutants also had lost antibacterial activity.

TM1040 produces an antibiotic and shares phenotypic traits with other roseobacters, notably 27-4, whose antibiotic has been identified as TDA (20). To compare the antibacterial compound produced by TM1040 to TDA, cell-free supernatants from TM1040 and 27-4 were analyzed by HPLC using previously described methods (20). A peak from TM1040 had the same retention time as the
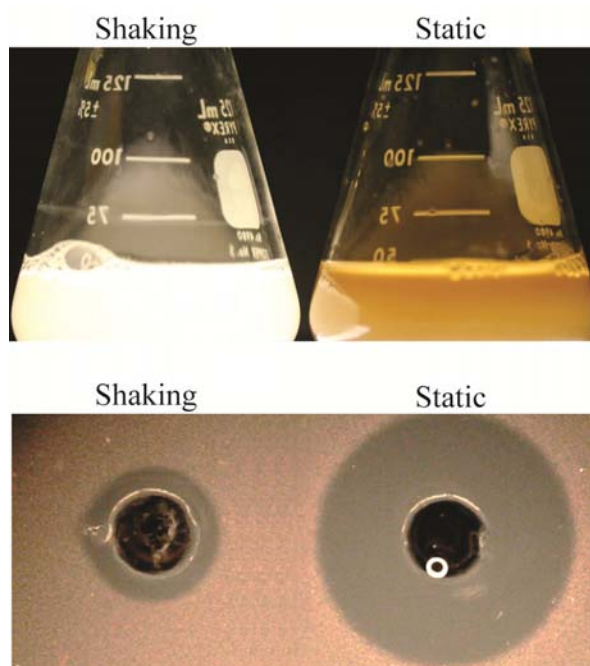
**FIG. 2.1.** *Silicibacter* **sp. strain TM1040 produces a yellow-brown pigment and has an antibacterial activity**, which was tested by a well diffusion assay using *V. anguillarum* as the target organism (Materials and Methods), under 30°C static conditions. In contrast, pigment and antibacterial compound is very low under 30°C shaking conditions indicating the close correlation between their production.

TDA peak from 27-4 (**FIG. 2.2**, 4.2 min). The UV spectra corresponding to both peaks were the same as the published spectrum of TDA (**FIG. 2.2**, insets) (20, 98). Mass spectroscopy analysis of this compound from TM1040 was also consistent with the conclusion that TDA is the antibacterial metabolite produced by TM1040 (data not shown).

### 2.4.2 Identification of genes involved in the synthesis of TDA

With the exception of some genes involved in shikimate and phenylacetate metabolism (113), analysis of the genome sequence of TM1040 does not suggest genes likely to participate in the biosynthesis and regulation of TDA. To detect such genes, a random-insertion transposon bank of 11,284 Kan$^r$ colonies was generated in TM1040 and screened for the Tda$^-$ phenotype that indicates the loss of antibiotic production. Approximately 0.7% of the transposon mutants (81 of 11,284) were defective in both TDA synthesis and pigment formation. To help identify the genetic basis of the phenotype, TM1040 DNA adjacent to each side of the transposon was sequenced in all 81 of the Tda$^-$ mutants. Surprisingly, the transposon-associated sequences from 32 or nearly 40% of the Tda$^-$ mutants did not match DNA sequence in the annotated TM1040 genome (113). However, the newly identified *tdaA-E* sequences overlapped and were assembled into one large contiguous DNA fragment of 4.5 kb (**Table 2.2** and **FIG. 2.3A**). These genes are not part of the original annotation of the genome, suggesting that this DNA
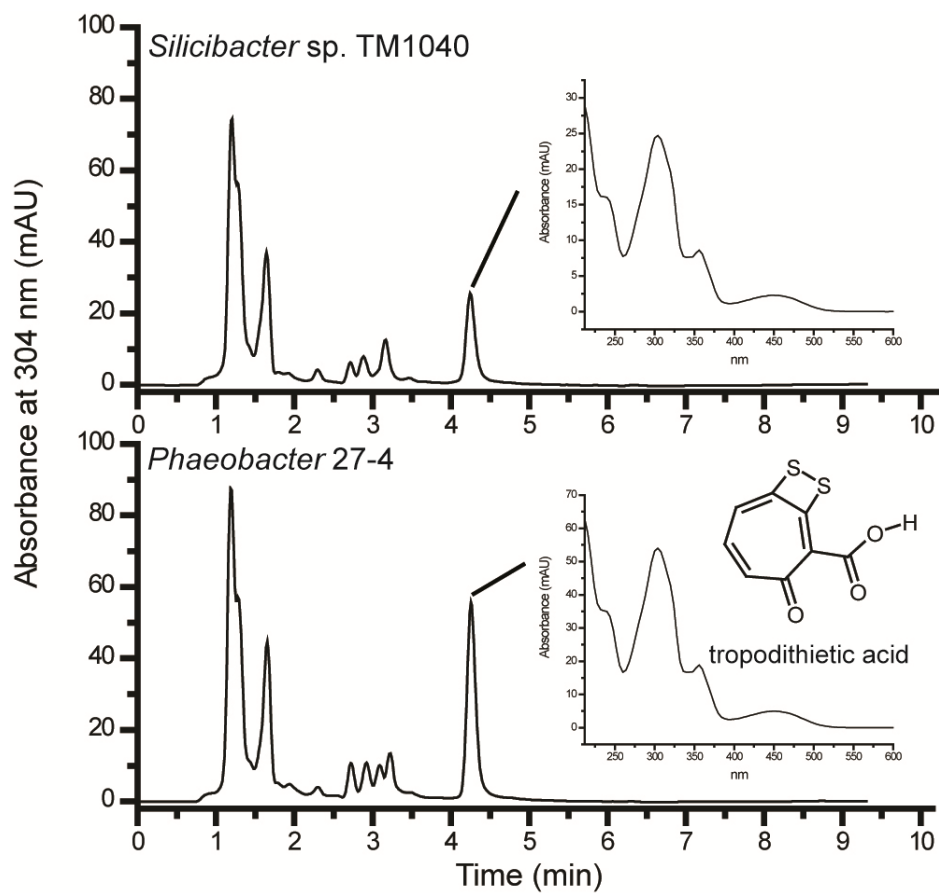
**FIG. 2.2. Tropodithietic acid. C18 reverse phase HPLC chromatograms of ethyl acetate extracts from TM1040 and *Phaeobacter* 27-4**. Insets show the UV spectra of the HPLC TDA peak corresponding to the antibiotic activity. The peak with a retention time 4.2 min is TDA.

**Table 2.2. TM1040 genes and encoded proteins required for the regulation and synthesis of tropodithietic acid.**

| Gene Number | GenBank Assession Number | Gene Designation | Putative Function | Best Hit Ortholog / E score |
|---|---|---|---|---|
| **Ring Precursors, Oxidation, and Expansion** | | | | |
| TM1040_3728 | CP000376 | *paaK* | Phenylacetate oxidoreductase | *Roseobacter* sp. MED193 phenylacetic acid degradation oxidoreductase PaaK / 8e-161 |
| TM1040_3726 | CP000376 | *paaI* | Phenylacetate oxygenase | *Roseobacter* sp. MED193 phenylacetic acid degradation protein PaaI / 4e-110 |
| TM1040_3727 | CP000376 | *paaJ* | Phenylacetate oxygenase | *Roseobacter* sp. MED193 phenylacetic acid degradation protein PaaJ / 2e-69 |
| EF139203 | EF139203 | *tdaD* | 4-hydroxybenzoyl-CoA thioesterase | *Paracoccus denitrificans* PD1222 conserved hypothetical protein / 2e-45 |
| EF139204 | EF139204 | *tdaE* | Acyl-CoA dehydrogenase | *Paracoccus denitrificans* PD1222 acyl-CoA dehydrogenase / 9e-120 |
| EF139201 | EF139201 | *tdaB* | β-etherase, glutathione S transferase | *Paracoccus denitrificans* PD1222 putative β-etherase (β-aryl ether cleaving enzyme) protein / 6e-56 |
| EF130202 | EF130202 | *tdaC* | Prephenate dehydratase | *Paracoccus denitrificans* PD1222 hypothetical protein / 2e-45 |
| **Sulfur Metabolism and Addition** | | | | |
| TM1040_2581 | CP000377 | *malY* | β-C-S lyase (cystathionase); amino transferase | *Roseobacter* sp. MED193 aminotransferase, classes I and II / 0.0 |
| TM1040_0961 | CP000377 | *tdaH* | Sulfite oxidase domain protein | *Sulfitobacter* sp. NAS-14.1 hypothetical protein / 7e-34 |
| TM1040_1758 | CP000377 | *cysI* | Sulfite reductase | *Roseobacter* sp. MED193 sulfite reductase / 0.0 |
| **Co-enzyme A Metabolism** | | | | |

| EF139205 | EF139205 | *tdaF* | Phosphopantothenoylcysteine decarboxylase | *Paracoccus denitrificans* PD1222 flavoprotein / 2e-55 |
|---|---|---|---|---|
| **Regulatory Mechanism** | | | | |
| EF139200 | EF139200 | *tdaA* | LysR substrate binding domain protein | *Paracoccus denitrificans* PD1222 regulatory protein, LysR:LysR, substrate-binding / 1e-29 |
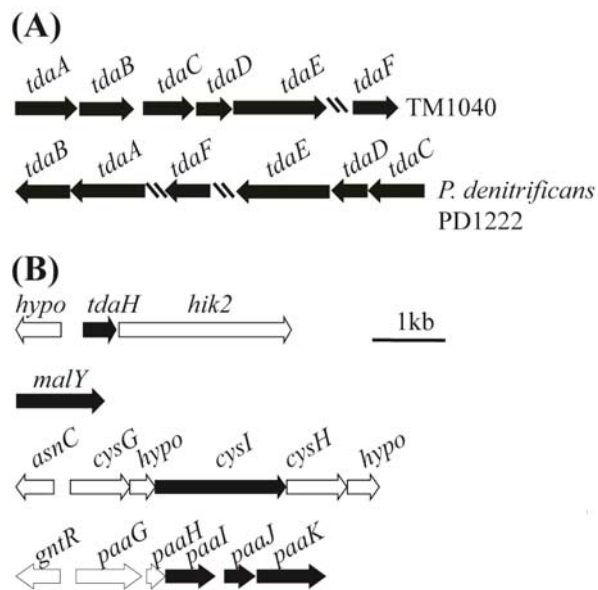
**FIG. 2.3. Genes required for synthesis of TDA in TM1040.** The black boxes indicate the ORF interrupted by the transposon. Arrows indicate ORFs transcriptional orientations, hatch marks indicate a break in the region, and the relative distance is indicated by the 1-kb marker. (A) *tdaA~tdaF* genes reside on a plasmid, with their closest homologs found on the chromosome of *P. denitrificans* PD1222. An intergenic space of 54 bp separates TM1040 *tdaA* and *tdaB*, and 345 bp separate *tdaB* from *tdaC-E*, which overlap each other by one bp, and >10 kb separate *tdaF* from *tdaE*. (B) The remainder of the genes involved in TDA biosynthesis are located either on the chromosome (*tdaH*, *malY*, and *cysI*) or, in the case of the genes involved in phenylacetate catabolism (*paaIJK*), on plasmid pSTM1.

may have been lost from the sequenced variant of TM1040. Below we present a thorough analysis of these "orphan" genes that were later found to be involved in TDA biosynthesis and to reside on a 130-kb plasmid.

DNA adjacent to the transposon in 49 Tda⁻ mutants matched the available genome sequence. In these strains we assessed the presence of *tdaE* with PCR and could not detect an amplification product in 43 of the 49 mutants. The loss of plasmid-borne *tdaA-F* might cause their Tda⁻ phenotype, and this type of loss might also account for low-frequency spontaneous loss of TDA synthesis (estimated $< 10^{-5}$ cells). These mutants were not investigated further.

In three of the six mutants that retained *tdaE*, the transposons disrupted putative genes encoding phenylacetate catabolism, *paaI*, *paaJ*, and *paaK* (**FIG. 2.2B**). Their deduced amino acid sequences were similar to homologs in other roseobacters (**Table 2.2**). In other bacteria, *paaGHIJK* encodes a ring-hydroxylating complex of proteins that is responsible for the first step in the aerobic catabolism of phenylacetate involving coenzyme A (CoA) activation (102, 120), producing 1, 2-dihydrophenylacetate- CoA (47, 79). The loss of TDA synthesis from disruption of the *paa* genes supports the biochemical evidence of phenylacetate metabolism in thiotropocin synthesis published in 1992 by Cane et al. (28).

Mutants with defects in phenylacetate metabolism were also unable to grow on phenylalanine, phenylacetic acid, tryptophan, sodium phenylpyruvate, or phenylbutyrate as a sole carbon source (**Table 2.3**). This result is consistent with the hypothesis that *paaIJK* of TM1040 function in the phenylacetate catabolism pathway similarly to other bacteria (113).

TDA is a disulfide-modified tropolone compound, indicating that sulfur metabolism must be involved in TDA synthesis. This idea is supported by the identification of three Tda⁻ mutants (**Table 2.2**), each with a transposon inserted in a gene whose product is involved in sulfur metabolism: *cysI*, *malY*, and an ORF (*tdaH*) with homology to sulfite oxidase (**Table 2.2**). The identification of these genes suggests that sulfur from reductive sulfur pathways is used and incorporated into TDA, which was tested by observing the growth of the sulfur metabolism mutants on a minimal medium containing a sole sulfur source (see Materials and Methods). The results are shown in **FIG. 2.4**. The *cysI* mutant grew when provided complex sulfur sources or cysteine and was unable to utilize DMSP, $SO_3^{2-}$, $SO_4^{2-}$, or methionine. The addition of cysteine to the medium resulted in enhanced growth of the *cysI* mutant, as well as increased synthesis of TDA (**FIG. 2.4**).

**Table 2.3. Sole carbon source tested for TM1040 and TDA⁻ mutants.**

| Gene | Cys | Trp | Phe | Phenylacetic acid | Sodium phenylpyruvate | Sodium phenylbutyrate | 2216 | Other extra Amino acid [b] |
|------|-----|-----|-----|-------------------|-----------------------|------------------------|------|------------|
| WT | +[a] | + | + | + | + | + | + | + |
| *paaI* | + | - | - | - | - | - | + | + |
| *paaJ* | + | - | - | - | - | - | + | + |
| *paaK* | + | - | - | - | - | - | + | + |
| *tdaA* | + | + | + | + | + | + | + | + |
| *tdaB* | + | + | + | + | + | + | + | + |
| *tdaC* | + | + | + | + | + | + | + | + |
| *tdaD* | + | + | + | + | + | + | + | + |
| *tdaE* | + | + | + | + | + | + | + | + |
| *tdaF* | + | + | + | + | + | + | + | + |
| *cysI* | + | - | - | - | - | - | + | - |
| *malY* | + | + | + | + | + | + | + | + |
| *tdaH* | + | + | + | + | + | + | + | + |

[a] + indicates growth; - indicates no growth.
[b] as described in Materials and Methods.
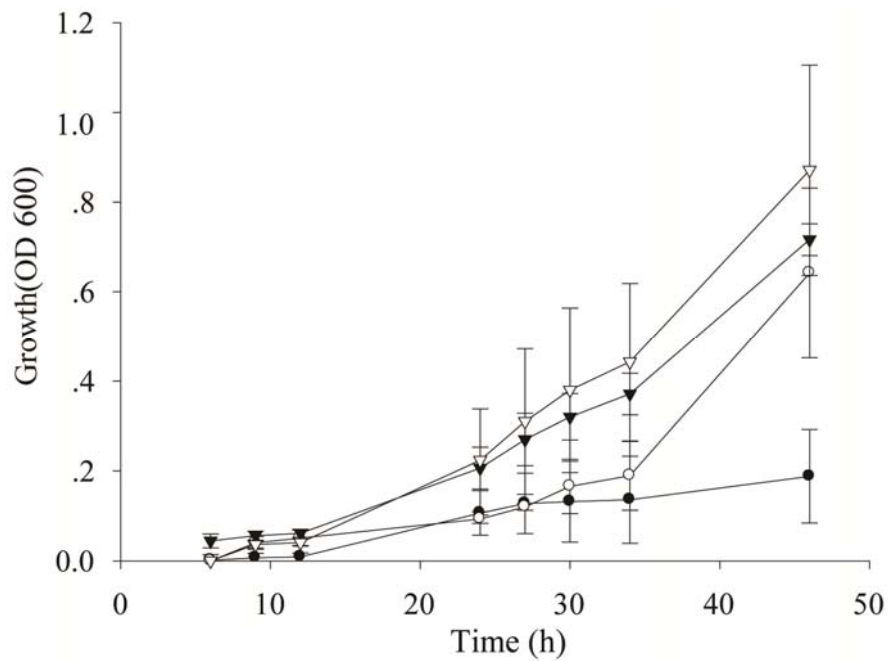
| strain | SO$_4$ | SO$_3$ | Met | Cys | DMSP |
|--------|--------|--------|-----|-----|------|
| *cysI* growth | − | − | − | + | − |
| WT growth | + | + | + | + | + |
| *cysI* TDA | − | − | − | + | − |
| WT TDA | + | + | + | + | − |

**FIG. 2.4. Growth and TDA synthesis are affected by mutations in *cysI*.** TM1040 (inverted triangles) and a *cysI* mutant (HG1220, *cysI*::EZ-Tn*5*; circles) were grown in minimal medium lacking sulfate and containing either methionine (closed symbols) or cysteine (open symbols), and growth was measured optically at 600 nm. Unlike the wild-type, the CysI⁻ mutant cannot grow on methionine, but does utilize cysteine.

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Measurement of antibiotic activity indicates that the *cysI* defect also affects TDA synthesis, which is corrected by the addition of cysteine to the medium but not by the addition of methionine, DMSP, sulfite, or sulfate.

### 2.4.3   TDA biosynthesis genes resides on a 130 kb plasmid

A bioinformatic analysis was done on TdaA-F to help elucidate the potential function of these proteins (**Table 2.2**). Interestingly, these proteins share their strongest homology with a similar set of proteins encoded by chromosome 1 of *Paracoccus denitrificans* PD1222 (accession no. NC_008686), a nonmotile alphaproteobacterium first isolated from soil by Beijerinck (7). TdaA (**Table 2.2**) has homology with LysR regulatory proteins, possessing a helix-turn-helix DNA-binding domain and a LysR substrate-binding domain (167). TdaA was the only putative regulatory protein detected in the present study, perhaps indicating that it is the sole regulator of TDA synthesis. The remaining ORFs encode putative enzymes. TdaB contains a glutathione S-transferase (GST) domain and belongs to the bacterial GST protein family (**Table 2.2**). TdaC has an amino acid domain with homology to prephenate dehydratase (PheA), an enzyme involved in the conversion of chorismate to prephenate, a step in the pathway leading to phenylacetate synthesis (161).

The involvement of CoA metabolism, addition, or modification is evident from the functional domains on TdaD and TdaE. TdaD is predicted to be a member of the thioesterase superfamily of acyl-CoA thioesterases (**Table 2.2**) (12), TdaE encodes a putative acyl-CoA dehydrogenase (ACAD) (90), and TdaF has homology to aldehyde dehydrogenase (92).

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The secondary evidence suggests that *tdaA-F* reside on a "cryptic" plasmid that may be spontaneously lost. To develop a means to test the hypothesis, we used three strains, TM1040, a spontaneous Tda⁻ nonpigmented strain of TM1040 (TM1040SM), and HG1265 (*tdaE*::EZ-Tn*5*) (**FIG. 2.5A** and **Table 2.1**), along with a PCR amplification using primers for *tdaA-E*, predicted to generate a 3.8-kp product from wild-type DNA. As shown in **FIG. 2.5B**, PCR amplification of wild-type DNA gave the predicted 3.8-kb band, a 5.7-kp product when *tdaE*::EZ-Tn*5* DNA was used as a template, and no product when the DNA from the SM strain was amplified, indicating that the SM strain had lost the *tdaA-E* locus.

Total DNA from TM1040, TM1040SM, and HG1265 (*tdaE*::EZ-Tn*5*) was separated by PFGE. As observed in **FIG. 2.5C**, all three strains had high-molecular-weight DNA, presumably a mixture of chromosomal and pSTM1, and a band or bands at ca. 130 kb, corresponding to the size of pSTM2 (132 kb) (113). Close inspection of this region and comparison between the SM DNA lane (middle, **FIG. 2.5C**) and either the TM1040 or *tdaE*::EZ-Tn*5* DNA (left and right lanes, respectively) shows that the SM band is thinner than either TM1040 or *tdaE*:Tn, hinting that SM DNA is missing a DNA species in this size range that overlaps with pSTM2. Repeated attempts to change PFGE conditions failed to resolve this region further. To overcome this limitation, a Southern blot (**FIG. 2.5D**) using a *tdaE* DNA probe was performed on the gel shown in **FIG. 2.5C**, and the results confirmed that the SM DNA, while possessing a 130-kb band, fails to hybridize to *tdaE*. In contrast, both wild-type DNA and *tdaE*:Tn DNA hybridizes to the expected band (ca. 130 kb). This confirms the loss of tda DNA in SM and adds evidence supporting the idea that
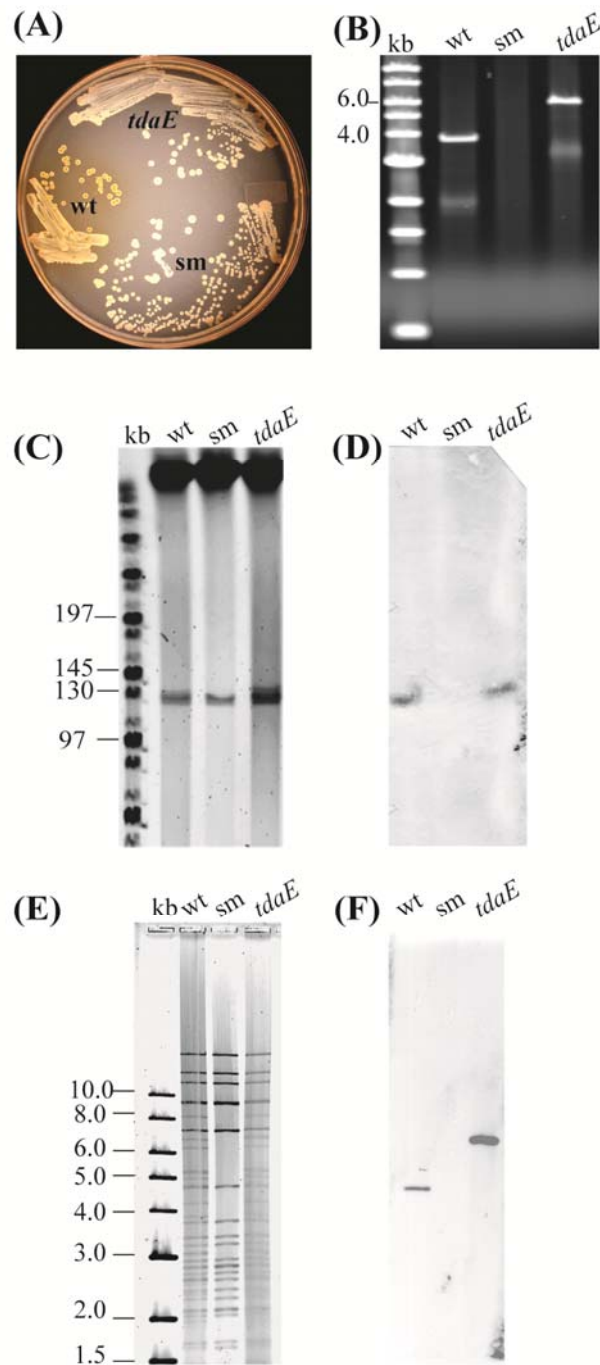
**FIG. 2.5. TM1040 *tda* genes reside on a plasmid that undergoes a low frequency spontaneous loss.** (A) Pigment synthesis. TM1040 (wt) produces a yellow-brown extracellular pigment that is correlated with TDA synthesis. In contrast, a *tdaE*::EZ-

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Tn*5* mutant (strain HG1265) and a spontaneous mutant (sm; TM1040SM) are nonpigmented and have lost the ability to produce both TDA and pigment. (B) Spontaneous loss of pigment and antibiotic activity results from a loss of *tda* genes. PCR amplification of *tdaE* results in a band from wild-type (wt) and *tdaE*::EZ-Tn*5*DNA, respectively, with the additional 2 kb in size of the *tdaE*::EZ-Tn*5* product resulting from insertion of the transposon. No product was amplified from the spontaneous nonpigmented mutant (sm). (C) PFGE separation of total DNA obtained from TM1040 (wt), the spontaneous nonpigmented mutant (sm), and the *tdaE*::EZ-Tn*5* mutant. (D) Southern blot hybridization of the PFGE gel to labeled *tdaE* DNA. (E) *NcoI* digestion of plasmid DNA isolated from TM1040 (wt), the spontaneous nonpigmented mutant (sm), and HG1265 (*tdaE*::EZ-Tn*5*), respectively. (F) Southern blot hybridization of *NcoI* digested plasmid DNA to *tdaE*.

the missing *tda* DNA is on a plasmid. It does not rule out the (unlikely) possibility that *tda* genes reside on pSTM2 and are somehow deleted from that known molecule.

To resolve the issue, we isolated plasmids from each of the three strains (TM1040, TM1040SM, and HG1265) and subjected each mixture to *Nco*I digestion (**FIG. 2.5E**), chosen because an in silico *Nco*I digestion of pSTM2 provided a recognizable pattern of DNA fragments. As can be seen in **FIG. 2.5E**, the TM1040SM DNA digest had fewer bands than wild type DNA or DNA from *tdaE*:Tn. This would be expected if the TM1040SM strain lost a large plasmid. Consistent with the hypothesis, Southern blotting showed that a *tdaE* probe hybridized to a 4.5-kb fragment in wild-type plasmid DNA and to a 6.4-kb fragment from plasmids isolated from the *tdaE*:Tn strain (**FIG. 2.5E**).

The EZ:Tn transposon contains a kanamycin resistance gene, as well as the *ori*R6K origin of replication, permitting replication in permissive hosts carrying the *pir* gene (91). Thus, the plasmid from *tdaE*:Tn was used to transform *E. coli* EC100D (**Table 2.1**) with a subsequent selection for kanamycin resistance (see Materials and Methods). This transformation was successful despite a very low transformation efficiency, resulting in 7 CFU per µg of mixed plasmid DNA, and provides strong evidence for the existence of a ~130-kb plasmid harboring *tda* genes. This new plasmid is called pSTM3.

Twelve random colonies were chosen from the transformation with pSTM3, and the *Nco*I digestion pattern of each was compared. Four common restriction digestion patterns emerged from this analysis (see **FIG. 2.6.**). Although each plasmid was PCR positive for the *tda* genes (data not shown) and the set of four shared many

common bands, they had remarkably different patterns, indicating that deletion and/or

rearrangements had occurred during or after the transfer of pSTM3 to *E. coli*. The

reason and molecular mechanism underlying these band pattern differences is not

known; however, the sum of the results indicates that TM1040 harbors an ~130-kb
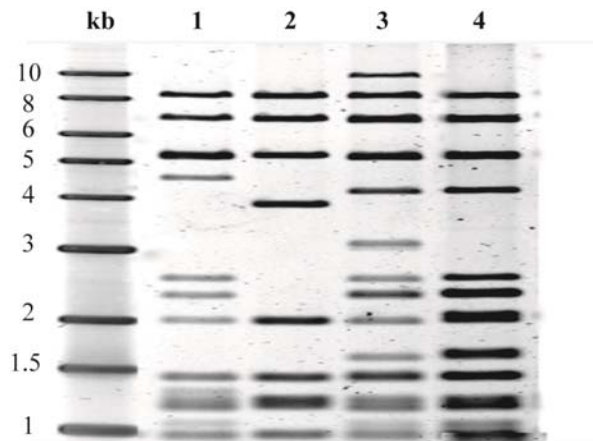
**FIG. 2.6.** *Nco*I **digested plasmid extraction from four strains of** *E. coli* **EC100D**

*pir+* that was transformed by DNA from plasmid extraction of HG1265. Ladder

marker was 1 kb DNA ladder (NEB biolabs).

plasmid, pSTM3, that is essential for TDA and pigment biosynthesis and that may be spontaneously lost in laboratory culture.

### 2.4.4  Distribution of *tda* genes in other *Roseobacter* spp.

We used EZ-Tn5 to construct a 6,321-member library in 27-4 and screened these mutants for the Tda⁻ phenotype. A total of 37 Tda⁻ mutants were found, 12 of which were analyzed further. Two of the twelve ORFs mutated were similar to TdaA (identity 38%) and TdaB (identity 55%) from TM1040 (**Table 2.4**), suggesting that these two roseobacter types share a common TDA biosynthesis and regulation scheme. The remaining nine genes were not identified as important to TDA synthesis in TM1040 and had various degrees of homology to genes in the annotated TM1040 genome but, unlike TM1040, were not part of the phenylacetate or reductive sulfur pathways. The one exception was 27-4 *metF* (**Table 2.4**), which may possibly be involved in sulfur metabolism (139).

We also used DNA-DNA hybridization to measure the hybridization of a *tdaA-F* gene probe to DNA from 14 *Roseobacter* clade species (**FIG. 2.7**). The *tda* probe hybridized to eight of the nine species of roseobacters that have been reported to produce antibacterial activity (**FIG. 2.7**), with the ninth, *Ruegeria pomeroyi* DSS-3, showing a low amount of hybridization. Three of six non-antibiotic-producing roseobacters also positively hybridized to the *tda* DNA. This may have resulted from a strain that has very low *tda* expression and antibiotic activity below the detection limits of the well diffusion assay or from spurious hybridization to non-*tda* DNA. The *tda* probe did not hybridize with DNA from *V. anguillarum*, implying that the second possibility is the less likely scenario.

**Table 2.4.** *Phaeobacter* 27-4 genes and encoded proteins required for the regulation and synthesis of tropodithietic acid.

| Mutant Number | GenBank Assession Number | Gene Designation | Function | Best Hit Ortholog / E score |
|---|---|---|---|---|
| **Ring Precursors, Oxidation, and Expansion** | | | | |
| JBB1001 / JBB1030 | EF139212 | *tdaB* | β-etherase, glutathione S transferase | *Sinorhizobium meliloti* putative β-etherase (β-aryl ether cleaving enzyme / 4e-52 |
| | | | | |
| **Sulfur Metabolism and Addition** | | | | |
| JBB1044 | EF139218 | *metF* | 5-methyltetrahydrofolate--homocysteine S-methyltransferase | *Silicibacter* sp. TM1040 MetF protein / 2e-77 |
| | | | | |
| **Co-enzyme A Metabolism** | | | | |
| JBB1009 | EF139215 | *tdbA* | D-β-hydroxybutyrate dehydrogenase | *Roseovarius* sp. 217 D-β-hydroxybutyrate dehydrogenase / 2e-32 |
| JBB1045 | EF139216 | *tdbB* | Phosphate acetyltransferase | *Roseobacter* sp. MED193 phosphate acetyltransferase / 8e-81 |
| | | | | |
| **Transport: Import and Export** | | | | |
| JBB1003 | EF139213 | *tdbC* | Lytic transglycosylase, peptidase C14 | *Roseobacter* sp. MED193 hypothetical protein / 6e-85 |
| JBB1005 | EF139221 | *traI* | TraI, Type IV (Vir-like) secretion | *Rhodobacter sphaeroides* 2.4.1 TraI / 5e-58 |
| JBB1011 | EF139222 | *tdbD* | Type I secretion target repeat protein | *Roseobacter* sp. MED193 type I secretion target repeat protein / 8e-54 |
| JBB1029 | EF139216 | *tdbE* | Oligopeptide/dipeptide ABC transporter | *Silicibacter* sp. TM1040 binding-protein-dependent transport systems inner membrane |

| | | | | component / 6e-124 |
|---|---|---|---|---|
| | | | | |
| **Regulatory Mechanism** | | | | |
| JBB1006 | EF139220 | *clpX* | ATP-dependent Clp protease | *Silicibacter* sp. TM1040 ATP-binding subunit ClpX / 1e-47 |
| JBB1007 | EF139214 | *tdbF* | Ribonuclease D | *Roseobacter* sp. MED193 ribonuclease D / 6e-49 |
| JBB1030 | EF139217 | *tdaA* | LysR substrate binding domain protein | *Paracoccus denitrificans* PD1222 regulatory protein, LysR:LysR, substrate-binding / 3e-51 |
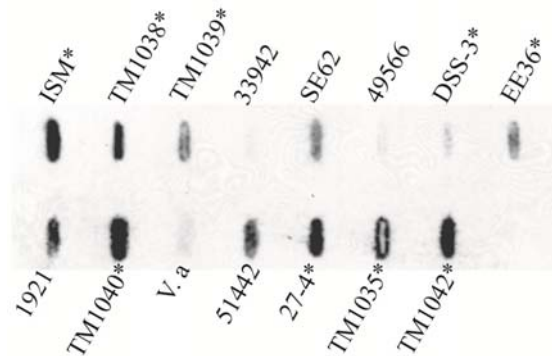
**FIG. 2.7. DNA from other Roseobacter species hybridizes to *tda* DNA.** Total DNA was extracted from 13 roseobacters, TM1040, and a non-roseobacter control species (*V. anguillarum*) and used in a slot blot hybridization with labeled *tda* DNA. Positive hybridization was strongly correlated with measurable antibiotic activity (indicated by an asterisk). The strains used were as follows: ISM: *Roseovarius* strain ISM; TM1038: *Roseobacter* sp. strain TM1038; TM1039: *Roseobacter* sp. strain TM1039; 33942: *Roseobacter denitrificans* ATCC 33942; SE62: *Sulfitobacter* strain SE62; 49566:*Roseobacter litoralis* ATCC 49566; DSS-3: *Ruegeria pomeroyi* DSS-3; EE36: *Sulfitobacter* strain EE36; 1921: *Sulfitobacter* strain 1921; TM1040: *Silicibacter* sp. strain TM1040; V. a: *Vibrio anguillarum*; 51442: *Roseobacter algicola* ATCC 51442; 27-4: *Phaeobacter* strain 27-4; TM1035: *Roseovarius* sp. strain TM1035; and, TM1042: *Roseovarius* sp. strain TM1042.

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### 2.4.5 Distribution of *tda* genes in the environment

The marine genome and metagenomic databases were searched for sequences with homology to one of the twelve genes (**Table 2.2**) required for TDA synthesis by TM1040. Although homologs to the proteins involved in phenylacetate and reductive sulfur metabolism were quite commonly found within the 14 selected roseobacter genomes (*Jannaschia* sp. strain CCS1, *Ruegeria pomeroyi* DSS-3, *Sulfitobacter* sp. strain EE-36, *Sulfitobacter* sp. strain NAS-14.1, *Sagittula stellata* E-37, *Rhodobacterales bacterium* HTCC2654, *Roseobacter* sp. strain MED193, *Roseovarius nubinhibens* ISM, *Loktanella vestfoldensis* SKA53, *Oceanicola batsensis* HTCC2597, *Oceanicola granulosus* HTCC2516, *Roseovarius* sp. strain 217, *Roseovarius* sp. strain HTCC2601, and *Roseovarius* sp. strain TM1035) in *Roseobase* (http://www.roseobase.org) and the Gordon and Betty Moore Foundation Marine Microbial Genome databases (https://research.venterinstitute.org/moore), close homologs of TdaA-F were absent (at a BLASTP E value cutoff of 1E-30). Although the reason for the absence of homologs is not known, it is possible, although unlikely, that all 14 roseobacters do not produce TDA, produce an antibacterial activity that involves another compound, or lost their *tda* plasmid. The last possibility is most likely to have resulted from laboratory culturing; therefore, we searched for Tda homologs in the GOS metagenomic database (http://camera.calit2.net) (133) that should contain abundant uncultivated roseobacter DNA.

The data gathered from searching the GOS database are shown graphically in **FIG. 2.8**, where a circle and its relative size indicates the presence and abundance
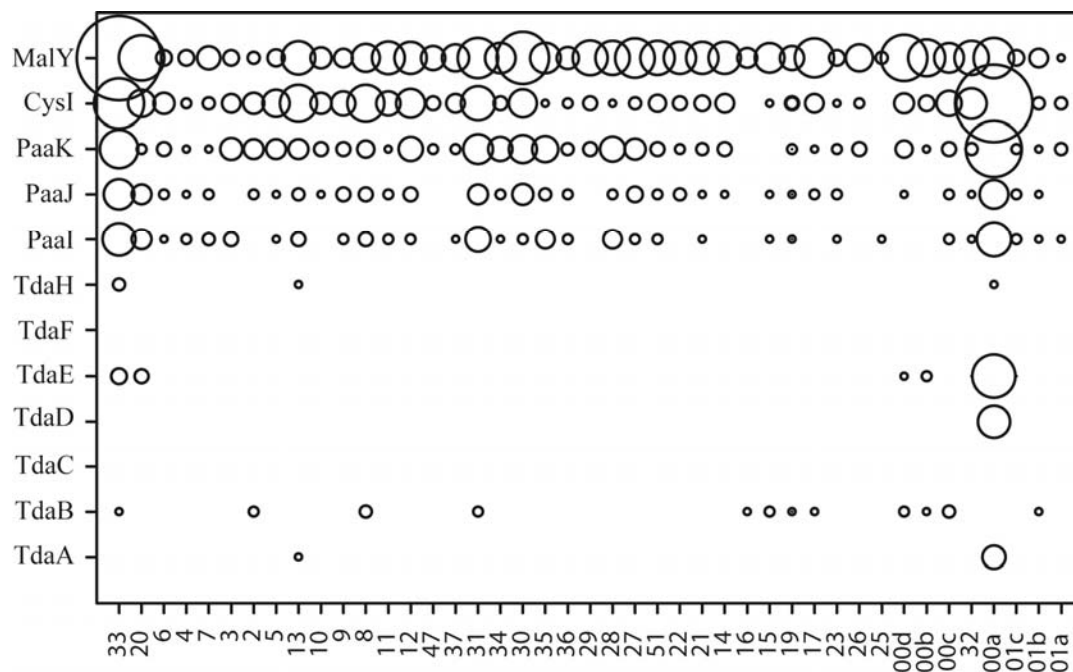
**FIG. 2.8. Presence and relative abundance of each of the Tda proteins identified in TM1040 (rows) in the GOS metagenomic database.** Relative abundance is indicated by the size of the circle. GOS sample numbers are indicated on the horizontal axis.

(respectively) of a given protein. As observed with the roseobacter genomes, phenylacetate and reductive sulfur metabolism proteins were readily found at numerous sites, with the greatest abundance of PaaIJK and CysI at site GS00a, a Sargasso Sea sample (31°32_6_N, 63°35_42_W). Positive Tda protein "hits" were also recorded in a hypersaline pond sample (GS033) and a sample obtained from Lake Gatun, Panama Canal (**FIG. 2.8**). In no sample did we find hits to all 12 proteins involved in TDA biosynthesis.

## 2.5    Discussion

It is not surprising members of the *Roseobacter* clade, whose genomes reveal a great potential for the synthesis of bioactive molecules (22, 107), produce TDA. Many marine bacteria produce an antibiotic activity (101, 113), and there are numerous reports of antibacterial activity from roseobacters, including a compound that produces a probiotic effect on scallop larvae (131, 132) and is antagonistic to gammaproteobacterium strains (131), as well as a compound that is antagonistic to fish larval bacterial pathogens (72, 73). From my data, it is likely that much of the antibiotic activity seen in roseobacters may be due to plasmid-borne *tda* genes.

TDA activity and biosynthesis depends on culture conditions and the physiology of TM1040. Bruhn et al. (18) have shown that TDA activity is significantly enhanced when TM1040 is cultured in a static nutrient broth, a condition that accentuates rosette and biofilm formation, as well as the synthesis of TDA and pigment. We have divided the symbiosis into two phases: the motile phase in which TM1040 cells actively respond to dinoflagellate-derived molecules by swimming toward the host, and the sessile phase, whereupon having located the zoospore, the

bacteria cease motility and form rosettes and a biofilm on the surface of the dinoflagellate (1, 110, 112). Thus, there is a direct correlation between rosette and biofilm formation, pigment production, and TDA biosynthesis, all of which may affect the symbiosis.

There is a direct link between the spontaneous appearance of nonpigmented Tda⁻ colonies and the loss of pSTM3 of TM1040. More than 40 of the mutants initially screened as Tda⁻ were ultimately found to have lost pSTM3. This suggests that pSTM3 is lost at a relatively low frequency during laboratory cultivation of TM1040. Instability of the Tda⁻ phenotype is not unique to TM1040. The appearance of spontaneous nonpigmented Tda⁻ mutants or variants has been observed in other roseobacters, including 27-4 (18) and *Roseobacter gallaeciensis* T5 (16). The simplest explanation for the cause of these spontaneous mutants is a loss of a plasmid carrying one or more critical genes required for TDA synthesis. Indeed, 27-4 possesses at least two plasmids of ca. 60 and 70 kb, respectively (data not shown). I speculate that one or both of these plasmids may be involved in the TDA biosynthesis of 27-4, and *tdaA* and *tdaB*, identified by transposon insertion mutagenesis in 27-4 Tda⁻ mutants, reside on one of these plasmids (data not shown). It is also worth noting that the transformation of *E. coli* with pSTM3 resulted in instability of the plasmid and the apparent loss or rearrangement of plasmid DNA sequences when in the foreign host (see **FIG. 2.6**). Preliminary data indicate that pSTM3 harbors a *repC* that is distinct from the *repC* found on pSTM2, which further supports the existence of pSTM3 as a discrete DNA molecule, separate from pSTM2.

One of the unexpected results from my study is the paucity of Tda homologs in the genomes of other sequenced roseobacters. There are several possible explanations why Tda homologs may be difficult to find, but the strongest lines of evidence support the idea that *tda* genes and Tda proteins have poorly conserved sequences, which is highlighted when TdaA (38% identity) and TdaB (55% identity) from 27-4 are compared to the same proteins from TM1040. Evidence of poorly conserved gene sequences is also apparent in other data in the present study. The Southern slot blot shown in **FIG. 2.7** was done under low-stringency hybridization and produced several weak positive signals. These weak positives are likely caused by poor DNA-DNA homology, further supporting the idea that the *tda* gene (and Tda protein) sequences are poorly conserved among the roseobacters. The choice of algorithm parameters used in BLAST searches that may also preclude finding genes or proteins with poor sequence conservation to the Tda target. For example, the amino acid sequence divergence between Tda proteins of TM1040 and other roseobacters could result in BLASTP E values greater than our chosen cutoff (1E-20 or less). Indeed, when higher E values are used, more Tda homologs are found in the roseobacter genome database. Despite the difficulties in finding Tda homologs, many of the roseobacters used in the present study have been shown by Bruhn et al. (18) to produce an antibiotic activity that is correlated with rosette and biofilm formation and coincides with the production of a yellow-brown pigment, phenotypes associated with TDA synthesis in both TM1040 and 27-4.

Tda homologs were differentially distributed in the GOS metagenomics data set. The two metagenomic samples that showed relatively good Tda homolog hits

were from a site in the Sargasso Sea and a hypersaline pond, respectively. It is interesting that DMSP is thought to be used by algae as an osmolyte that protects the cells against changes in salinity (165). Although our results suggest that DMSP is not used as a sole sulfur source in the biosynthesis of TDA, the correlation between salinity, DMSP, and the presence of Tda homologs makes for a tantalizing hypothesis. However, the apparent differential distribution of *tda* genes in the GOS metagenomic data set cannot be confirmed unless the sequencing coverage at each site is also considered. Further, if the genes are indeed distributed differentially, the selection may be on other characteristics of the organisms that carry them, not necessarily these genes.

To the best of my knowledge, this is the first report describing the genes and proteins required for TDA synthesis by roseobacters and highlighting the occurrence of *tda* genes on a previously unknown plasmid (pSTM3) of TM1040. Although this report answers numerous questions about TDA genetics, it has also opened new and exciting avenues for discovery. For example, underscoring and extending earlier biochemical studies (28), my data, specifically the identification of *paaIJK* and *tdaC* (prephenate dehydratase), indicate that TDA biosynthesis originates from the shikimate pathway and proceeds through phenylacetate. The results also emphasize a role for phenylacetate-CoA and CoA metabolism as vital to TDA production and suggest that the reductive sulfur pathway moving through CysH and CysI is critical for TDA activity.

The biosynthesis of TDA is predicted to have several beneficial effects on TM1040-dinoflagellate symbiosis. TDA may benefit the dinoflagellate by acting as a

probiotic with antibacterial activity, whose action prevents the growth and colonization of bacteria on the surface of the dinoflagellate that could potentially harm the zoospore. In turn, the antibacterial activity of TDA may enhance the growth of TM1040 cells attached to the zoospore by warding off other biofilm-forming bacteria that compete with TM1040 for space on the surface of and nutrients from *P. piscicida*. Although DMSP appears not to be a primary source of the sulfur atoms for TDA, it is probable that one or more non-DMSP sulfur-containing metabolites produced by the dinoflagellate are used by TM1040 in the biosynthesis of TDA.

# Chapter 3: Expression of Tropodithietic Acid (TDA) Biosynthesis is Controlled by a Novel Autoinducer

## 3.1 Summary

The interactions between marine prokaryotic and eukaryotic microorganisms are crucial to many biological and biogeochemical processes in the oceans. Often the interactions are mutualistic, as in the symbiosis between phytoplankton, e.g., the dinoflagellate *P. piscicida* and TM1040, a member of the *Roseobacter* taxonomic lineage. It is hypothesized that an important component of this symbiosis is bacterial production of TDA, a biologically active tropolone compound whose synthesis requires the expression of *tdaABCDEF* (*tdaA-F*), as well as six additional genes (*cysI*, *malY*, *paaIJK*, and *tdaH*). The factors controlling *tda* gene expression are not known, although growth in laboratory standing liquid cultures drastically increases TDA levels. In this chapter, I measured the transcription of *tda* genes to gain a greater understanding of the factors controlling their expression. While the expression of *tdaAB* was constitutive, *tdaCDE* and *tdaF* mRNA increased significantly (3.7- and 17.4-fold, respectively) when cells were grown in standing liquid broth compared to their levels with shaking liquid culturing. No transcription of *tdaC* was detected when a *tdaCp::lacZ* transcriptional fusion was placed in 11 of the 12 Tda⁻ mutant backgrounds, with *cysI* being the sole exception. The expression of *tdaC* could be restored to 9 of the remaining 11 Tda⁻ mutants – *tdaA* and *tdaH* failed to respond – by placing wild-type (Tda⁺) strains in close proximity or by supplying exogenous TDA to the mutant, suggesting that TDA induces *tda* gene expression. These results

indicate that TDA acts as an autoinducer of its own synthesis and suggest that roseobacters may use TDA as a quorum signal.

## 3.2    Introduction

Microorganisms play crucial roles in most of the biogeochemical cycles in the oceans due to their high abundance and potential metabolic activity (6). This is especially true of marine bacteria that form symbiotic associations with phytoplankton, such as dinoflagellates. These mutualistic interactions often benefit both partners by promoting carbon, nitrogen, and sulfur exchanges and cycling (36). Members of the *Roseobacter* taxonomic clade of marine alphaproteobacteria are the most abundant bacteria in coastal waters, the second most abundant bacteria in open ocean seawater (60), and some of the most frequently sampled bacteria associated with blooms of phytoplankton that produce a reduced sulfur-rich compound called DMSP (61, 154). Roseobacters preferentially metabolize DMSP rather than sulfate despite the latter being nearly $10^7$-fold more abundant in sea water (89). Roseobacterial metabolism of DMSP occurs through either a demethylation pathway by which bacteria retain sulfur from DMSP, or a lyase or cleavage pathway wherein DMSP is converted to DMS whose production affects cloud formation and global climate (76).

The Belas laboratory have previously reported on a laboratory model symbiosis between a roseobacter, TM1040, and *P. piscicida*, a marine heterotrophic, DMSP-producing dinoflagellate (1, 11, 57, 109, 110, 112). TM1040 forms an obligate symbiosis with *P. piscicida* such that the dinoflagellates have an absolute requirement for TM1040 or physiologically similar bacteria. TM1040 develops a

close physical association with the dinoflagellates and forms a biofilm on the surface of its host (1, 110).

TM1040, like many other roseobacters, has a biphasic "swim-or-stick" lifestyle. In its swim phase, TM1040 populations are dominated by single motile cells propelled by one to four flagella whose movements are controlled by chemotaxis behavior (112). In contrast, when TM1040 enters its 'stick' or sessile phase, cells lose their flagella and adhere to one another forming rosettes, and at the same time begin the formation of biofilms on animate and inanimate surfaces (20).

Roseobacters exploit the swim-or-stick dual life style to initiate and maintain symbiosis with their algal hosts. Previous reports have shown that TM1040 is attracted to DMSP and other chemicals emitted by the dinoflagellates (112), drawing the bacteria into close proximity to their host. We hypothesize that, once within range, roseobacters switch to the sessile phase strategy, which involves formation of a biofilm and the production of a novel tropolone antibiotic, tropodithietic acid (TDA) (**FIG. 3.1A**) (57). The switch to the sessile life helps the bacteria establish a close interaction with the alga and may benefit the host through production of an antibiotic preventing other algal pathogenic bacteria from harming the dinoflagellate (18, 57).

TDA production is common in many marine roseobacters, especially in the subgroup composed of the genera *Phaeobacter*, *Silicibacter*, and *Ruegeria* that are commonly associated with algae (16, 20, 57, 122). The presence of TDA in many roseobacters highlights this physiological trait among subgroups of roseobacters. It also suggests that TDA is likely to be specific to roseobacters (or closely allied

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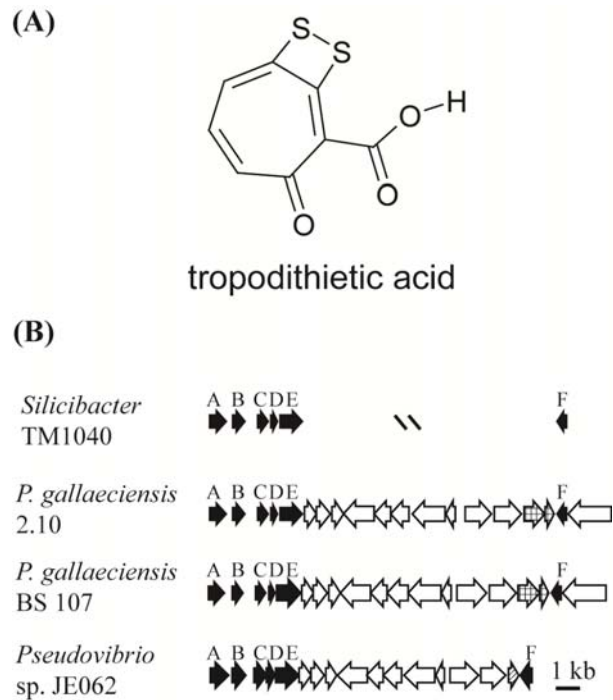
**FIG. 3.1. (A) The chemical structure of TDA. Biologically relevant moieties on TDA include the seven-carbon tropolone ring and the disulfide bond. (B) Organization of the *tdaA-tdaF* locus in TM1040, *P. gallaeciensis* 2.10, *P. gallaeciensis* BS 107, and *Pseudovibrio* sp. JE062**. Genes empirically determined to be important for TDA biosynthesis are showing in black and reside on a ca. 130 kb plasmid, called pSTM3 in TM1040. An additional six genes required for TDA biosynthesis (*cysI, malY, paaIJK*, and *tdaH*) are located elsewhere on the TM1040 genome (57). The hatch marks in the TM1040 *tda* locus indicate an area for which nucleotide sequence data is lacking (57).

alphaproteobacteria), and may confer specific survival and adaptation advantages to these bacteria.

Twelve essential genes for TDA biosynthesis have been identified by transposon insertion mutagenesis (57). These homologs include *paaIJK* (TM3726, TM3727, and TM3728, respectively), encoding proteins required phenylacetate catabolism, homologs of *cysI* (TM1758), *malY* (TM2581), and a gene, *tdaH* (TM0961), which encodes a molybdopterin-binding domain which may be a sulfite oxidase. The latter three ORFs are believed to be involved in adding sulfur atoms to the molecular precursors of TDA. Additionally, six genes, *tdaABCDEF* (*tdaA-F*), were found on a large ca. 130-kb plasmid called pSTM3 (57). Defects in any one of these 12 genes results in a loss-of-function Tda⁻ phenotype (57). Based on their orientation and proximity, it is believed that *tdaA* and *tdaB* form an operon, that *tdaCDE* are likely on a separate operon, and *tdaF* is in a third putative operon (**FIG. 3.1B**) (57). Indeed, our RT-PCR results support the idea that *tdaA* and *tdaB* form an operon (Chapter 4). Of the 12 essential genes required for TDA biosynthesis, *tdaA* encodes the only potential regulatory protein possessing a helix-turn-helix DNA-binding domain and a LysR substrate-binding domain, suggesting that TdaA may regulate TDA biosynthesis at the transcriptional level (57).

TDA production is influenced by the culture and environmental conditions (18, 57). Specifically, TDA activity is highest when bacteria are cultured in standing liquid nutrient broth, whereas cells produces negligible TDA in nutrient broth with shaking (57). Little is known about what component(s) of standing growth trigger TDA biosynthesis. However, Liang reported that addition of phenylalanine and

histidine significantly increased production of TDA in *Phaeobacter* T5 (98), suggesting that these amino acids may induce TDA synthesis. This is supported by our previous findings showing that TM1040 is attracted to amino acids, DMSP, and dinoflagellate cell homogenates (112), implicating a metabolic requirement for these substances, so it is likely that some of these compounds may influence TDA production. There has been speculation that expression of TDA may be controlled by acyl homoserine lactone (AHL) quorum sensing (63, 107). However, TM1040 lacks AHL synthesis genes and genes involved in the function of other known autoinducer molecules (113), and does not produce known AHL molecules (18), thus TM1040 is unlikely to use AHL signaling for this purpose.

The goal of the current research was to determine what environmental and molecular factors are required for expression of *tda* gene transcription. The results suggest that TDA is a density-dependent autoinducer controlling the expression of the key *tda* genes, *tdaCDE* and *tdaF*, and that TDA may be used as a quorum sensing signal among a subgroup of the ubiquitous marine *Roseobacter* clade.

## 3.3    Materials and Methods

### 3.3.1   Bacteria and media

The strains and plasmids used in this study are listed in **Table 3.1**. Tda⁻ transposon mutant strains were derived from TM1040 by EZ-Tn5 mutagenesis (57), and maintained on Difco 2216 marine broth or 2216 agar as

**Table 3.1. Bacterial strains and plasmids used in Chapter 3.**

| Strain/plasmid | Genotype/phenotype[a] | Source or reference |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | F⁻ *endA1 hsdR17 ($r_K^-$ $m_K^-$) supE44 thi-1 recA1 gyrA96 relA1Φ80dlacZΔM15* | (136) |
| S17-1 *λpir* | *thi pro recA hsd*(r⁻ m⁺) RP4-2 Tc::Mu-Km::Tn7 Sm *λpir* | (39) |
| Roseobacters | | |
| *Silicibacter* sp. TM1040 | Wild type | (109) |
| Mutants derived from TM1040 | | |
| HG1005 | *paaK* (TM3728) ::EZ-Tn5, Kan, | (57) |
| HG1015 | *tdaB*::EZ-Tn5, Kan | (57) |
| HG1050 | *tdaF*::EZ-Tn5, Kan | (57) |
| HG1056 | *paaJ* (TM3727)::EZ-Tn5, Kan | (57) |
| HG1080 | *tdaC*::EZ-Tn5, Kan | (57) |
| HG1110 | *tdaD*::EZ-Tn5, Kan | (57) |
| HG1213 | *malY* (TM2581)::EZ-Tn5, Kan | (57) |
| HG1220 | *cysI* (TM1758)::EZ-Tn5, Kan | (57) |
| HG1244 | *tdaH* (TM0961)::EZ-Tn5, Kan | (57) |
| HG1265 | *tdaE*::EZ-Tn5, Kan | (57) |

| | | |
|---|---|---|
| HG1299 | *paaI* (TM3726)::EZ-Tn*5*, Kan | (57) |
| HG1310 | *tdaA*::EZ-Tn*5*, Kan | (57) |
| Other *Roseobacter*s | | |
| *Phaeobacter* sp. 27-4 | Wild type, antibacterial activity | (20) |
| *Marinovum algicola* ATCC 51442 | Wild type, no antibacterial activity | (18, 93) |
| *Roseobacter litoralis* ATCC 49566 | Wild type, no antibacterial activity | (18, 140) |
| *Roseovarius* sp. TM1035 | Wild type, antibacterial activity | (18, 109) |
| *Roseovarius* sp. TM1042 | Wild type, antibacterial activity | (18, 109) |
| *Ruegeria pomeroyi* DSS-3 | Wild type, antibacterial activity | (18, 58) |
| *Sulfitobacter* sp. EE36 | Wild type, antibacterial activity | (18, 21) |
| HG6036 | *Silicibacter* isolate from a *Karlodinium veneficum* culture | This study |
| HG6037 | *Ruegeria pelagia*- or *Ruegeria mobilis*-like isolate from a *Karlodinium veneficum* culture | This study |
| HG6038 | *Ruegeria pelagia*- or *Ruegeria mobilis*-like isolate from a *Karlodinium veneficum* culture | This study |
| HG6039 | *Ruegeria pelagia*- or *Ruegeria mobilis*-like isolate from a *Karlodinium veneficum* culture | This study |
| *Pseudovibrio* sp. JE005 | Bacterial symbiont of marine sponges | (46) |
| *Pseudovibrio* sp. JE008 | Bacterial symbiont of marine sponges | (46) |

| | | |
|---|---|---|
| *Pseudovibrio* sp. JE021 | Bacterial symbiont of marine sponges | (46) |
| *Pseudovibrio* sp. JE061 | Bacterial symbiont of marine sponges | (46) |
| *Pseudovibrio* sp. JE062 | Bacterial symbiont of marine sponges | (46) |
| *Pseudovibrio* sp. JE0626 | Bacterial symbiont of marine sponges | (46) |
| Plasmids | | |
| pEA103 | *cdhA'*::*lacZ*, Ori pC2A, Amp, Pur$^r$, Ori R6K | (3) |
| pRK415 | Conjugative expression shuttle vector for *Silicibacter* sp.TM1040 and *E. coli*; $P_{lac}$; Tet | (85) |
| pHG1011 | 363-bp upstream of *tdaC* fused in front of *lacZ* in pRK415 | This study |

$^a$ Kan, kanamycin resistance; Sm, streptomycin resistance; Tet, tetracycline resistance; Amp, ampicillin resistance.

recommended by the manufacturer (BD Biosciences, Franklin Lakes, NJ). A marine basal minimal medium (MBM; per liter: 8.47g Tris HCl, 0.37 g of $NH_4Cl$, 22 mg of $K_2HPO_4$, 20 g sea salts, 2.5 mg FeEDTA; pH 7.6, 1 ml of RPMI-1640 vitamins [Sigma Aldrich, St. Louis, MO]) with 27 mM sulfate was used to test the ability of the bacteria to utilize different sulfur sources for TDA production. Glycerol was added as carbon source at a final concentration of 1 ml per l. Sulfur sources, 3-methiopropionate (MMPA), cysteine, DMSP, methionine, sulfate, and taurine, were respectively added to minimal broth at a final concentration of 200 μM. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar containing 1.5% Bacto Agar (Becton Dickinson, Franklin Lakes, N.J.) (5). As appropriate, kanamycin was used at 120 μg per ml for *Roseobacter* strains and 50 μg per ml for *E. coli*. For mating between *E. coli* and TM1040, HIASW10 (25 g of Difco Heart Infusion broth plus 10 g of Instant Ocean sea salts [Aquarium Systems, Mentor, OH] per liter) was used supplemented with tetracycline at a final concentration of 15 μg per ml (5).

### 3.3.2  Construction of plasmids

A transcriptional fusion between the *tdaC* promoter (*tdaCp*) and a promoterless copy of *lacZ*, encoding β-galactosidase, was constructed using overlap extension PCR (71) and subsequent ligation to the broad-host-range vector pRK415 (85) to generate plasmid pHG1011 (*tdaCp*::*lacZ*). Briefly, a 363-bp region 5' to the start codon of *tdaC* was amplified using primers tdaC-F and tdaC-R (**Table 3.2**) that added a *Pst*I site. The *lacZ* gene from pEA103 (3) was amplified by PCR to include a *Hin*dIII site at the 3' end using primers lacZ-F and lacZ-R. The products were mixed

**Table 3.2. Oligonucleotides used in Chapter 3.**

| Primer | Sequence (5' to 3') |
| --- | --- |
| qPCR tdaA forward | 5'-TCTTTCCTGCATCTGGTCTC-3' |
| qPCR tdaA reverse | 5'-AAGGTAGATGCCGAAACTGGC-3' |
| qPCR tdaC forward | 5'-TTCTGAGCGTGGTGGCCTA-3' |
| qPCR tdaC reverse | 5'-CGCATAGATCCGCTGCATC-3' |
| qPCR tdaF forward | 5'-GATCTTCTTGCGGTGCTGC-3' |
| qPCR tdaF reverse | 5'-AAACACCTCATACCCGTCGG-3' |
| qPCR cysI forward | 5'-TCAGTTCATGGGCCGTAAGTT-3' |
| qPCR cysI reverse | 5'-TCGATGTAGGGCAGGAGGTC-3' |
| qPCR paaK forward | 5' -TTCATGTGCTGGAAACAGATGC-3' |
| qPCR paaK reverse | 5'-CGAACTTGATCTGCTCATCCG-3' |
| qPCR malY forward | 5'-TCTATCACGCTTTTGCCAAGG-3' |
| qPCR malY reverse | 5'-CAACTTGCGTGAGCTCGTCTT-3' |
| qPCR rpoD forward | 5'-GATGACGAAGCCAAGTACCAGC-3' |
| qPCR rpoD reverse | 5'-CACGGTAAGCTTCGACGAATTC-3' |
| tdaC-F | 5'-AGTACTGCAGAAGCCTGAGCTGAATCTGCG-3' |
| tdaC-R | 5'-ACCGGATCCAGAGCCCATTGCGTTGGTCTCTGGGAC-3' |
| lacZ-F | 5'-GTCCCAGAGACCAACGCAATGGGCTCTGGATCCGGT-3' |
| lacZ-R | 5'-TGATAAGCTTCAATGGTGATGGTGATGATGAC-3' |
| tdaH'-forward | 5'-AAATGCAGATCAGGTCCATGAA-3' |
| tdaH'-reverse | 5'-GTCGGAATTTCCACTGCGTAG-3' |
| tdaI'-forward | 5'-GTCTTGCAGCTTTCGCGACT-3' |

tdaI'-reverse          5'-GCGCGTTTCCGATGAGATT-3'

and used as templates for a second round of PCR, using primers tdaC-F and lacZ-R
(**Table 3.2**). The resulting PCR product was digested with *Pst*I and *Hin*dIII, ligated
into pRK415, and this new plasmid, pHG1011, was then transferred to *E. coli* DH5α
by electroporation. Following analysis to confirm its efficacy, pHG1011 was moved
to *E. coli* S17-1 (λ*pir*), which was used in biparental mating with TM1040 and its
derivatives, as previously described (110). Exconjugants were selected using
kanamycin and tetracycline on HIASW10 agar, and subsequent resistant colonies
were screened to confirm the presence of pHG1011.

### 3.3.3 RNA preparation and real time PCR

Total RNA was extracted using an RNeasy purification kit (Qiagen, Valencia,
CA) according to the manufacturer. cDNA was synthesized from RNA with a
QuantiTect reverse transcription kit (Qiagen). Control reaction mixtures lacking
reverse transcriptase were included to confirm the absence of contaminating genomic
DNA. Oligonucleotide primers (**Table 3.2**) were designed to specific genes using
Primer Express 3.0 (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR)
amplification was performed using SYBRGreen and the respective forward and
reverse primer pairs as listed in **Table 3.2**. PCR Master Mix (Applied Biosystems)
with the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at
95°C, and 1 min at 62°C, using a 7500 fast real-time PCR system (Applied
Biosystems). All samples were normalized relative to expression of *rpoD* (TM2141),
encoding a putative sigma70 factor. Relative expression values represent the mean ±
standard deviation (S.D.) of triplicate samples from three independent experiments.

The expression of *tdaC* and *tdaF* in TM1040 was measured by qPCR after TDA induction using a bacterial culture grown in 2216 broth to late exponential phase (optical density at 595 nm [$OD_{595}$] of 0.6). Purified TDA was individually added to triplicate samples of these bacteria to a final concentration of 50 μM, and the incubation continued using standing broth culturing for 3 h at 30°C, in parallel with uninduced samples in triplicate. Cells were harvested by centrifugation before total RNA extraction, cDNA synthesis, and qPCR analysis as previously mentioned.

### 3.3.4 *tdaC* promoter activity assays using *tdaCp::lacZ*

Expression of *tdaCp*::*lacZ* was measured as β-galactosidase activity using the Miller assay (108). An overnight culture of pHG1011 in TM1040 (TM1040/pHG1011) was diluted 1:100 in 250 ml of 2216 marine broth containing tetracycline and incubated at 30°C under either standing and shaking conditions. At intervals during growth, cell samples were collected by centrifugation and the pellets placed at -20°C until the time of measurement. For enzyme measurements, pellets were thawed by resuspension in 1 ml of 0.85% NaCl immediately before the Miller assay.

The *tdaCp*::*lacZ* transcriptional fusion plasmid in TM1040 (TM1040/pHG1011) was also used to assess the ability of various chemicals to induce the expression of *tdaC* as follows. Bacteria were incubated in 2216 marine broth at 30°C under shaking condition to $OD_{595}$ of 0.6, at which time an aliquot of a sterile concentrated stock of the tested chemical was added to a final concentration of 50 μM. The compounds tested included: 5-dihydroxy-3-oxo-1,4,6-cycloheptatriene-1-carboxylic acid (cycloheptatriene), cysteine, DMSP, glucose, histidine, methionine,

phenylacetate, phenylalanine, shikimate, succinate, TDA, tropolone, and tropone. Stock solutions of TDA and cycloheptatriene were dissolved in methanol to a concentration of 50 mM. In preliminary experiments, I found that methanol does not induce *tdaC* expression. The remainder of the chemicals were prepared as stock solutions of 100 mM in 50 mM Tris (pH 7.5). DMSP was synthesized from acrylate and dimethylsulfide, extracted, and purified as previously described (30). MMPA was synthesized by alkaline hydrolysis of its methyl ester, methyl-3-(methylthio)propionate (Aldrich, Milwaukee, Wis.) (81). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO). None of these compounds caused a significantly change in pH of the medium. The incubation continued for 3 h at 30°C under standing conditions. After exposure to the respective chemical, bacteria were pelleted by centrifugation, washed once with 1 ml 0.85% NaCl, and β-galactosidase activity was measured and expressed in Miller units (108). Expression levels and the degree of induction were analyzed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test at a 95% confidence interval, using GraphPad Prism software (GraphPad Software, San Diego, CA).

The activity of the *tdaCp* promoter in different genetic backgrounds was measured by moving the transcriptional fusion (*tdaCp*::*lacZ*) into various Tda⁻ mutants and assessing β-galactosidase activity by cleavage of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) added to the medium at a final concentration of 60 μg per ml. Briefly, 100 μl of an overnight culture of pHG1011 in strain HG1299 (*paaI*::EZ-Tn5) was mixed into molten 45°C 2216 marine agar containing 30 μg tetracycline per ml and X-Gal (60 μg per ml). Once gelled, 5 μl of each chemical (5

mM stock concentration) was pipetted on the surface of the solidified agar medium. The level of *tdaC* promoter activity was determined by qualitative assessment of the resulting blue color due to cleavage of the X-Gal substrate after 3 days of incubation at 30°C.

Cross-feeding experiments between Tda⁻ mutants and TM1040 were performed on 2216 agar with X-Gal, using overnight broths of mutant strains harboring the reporter plasmid pHG1011 (*tdaCp::lacZ*), which were inoculated, respectively, as single streaks perpendicular to TM1040. The bacteria were further incubated for 2 days at 30°C, after which β-galactosidase activity was assessed by eye and recorded by digital photography.

### 3.3.5   Cross-feeding assay among marine roseobacters

Similarly, 2216 agar containing pHG1011 in strain HG1299 (*paaI*::EZ-Tn5) plus antibiotic and X-Gal was prepared and allowed to gel. After the medium solidified, a 10 μl sample from a 3-day, standing 2216 broth culture of one of several *Roseobacter*-clade or *Pseudovibrio* species was species (**Table 3.1**) was pipetted onto the surface of the plates, and incubated for 3 days at 30 °C. β-galactosidase activity resulting from the expression of *lacZ* was assessed as described for the previous cross-feeding experiment.

### 3.3.6   TDA purification

Bacteria were incubated in 2216 broth for 4 days under standing culture conditions, followed by centrifugation (10,000 ×g) to remove the cells. The cell-free spent medium was adjusted to pH 2.2 with hydrochloric acid. Ethyl acetate was added

to the acidified medium, evaporated, redissolved in water-acentonitrile (19:1) solution, and further fractioned by column chromatography on Oasis MAX columns (Waters, Milford, MA) as previously described (57). The TDA-rich fraction was applied to preparative Luna 5U C18(2) 250×10.0 mm high-performance liquid chromatography (HPLC) column (Phenomenex, Torrance, CA) at a flow rate of 5 ml per min under isocratic conditions, using a water-acetonitrile gradient 30% (0.02% trifluoroacetic acid in water) and 70% (60% acentonitrile, 0.02% trifluoroacetic acid). TDA was detected at a wavelength of 304 ± 4 nm as previous described (20, 57). An Agilent fraction auto-collector G1364A (Agilent, Santa Clara, CA) was used to automatically collect the TDA peak, and purified TDA was confirmed by analytic HPLC (57).

For rapid analyses, TDA was measured directly from standing broth cultures as follows. After 120-h incubation in standing 2216 liquid culture conditions, at which time TDA production had leveled off, the medium was adjusted to pH 2.2. The cells were removed by centrifugation (10,000 ×g) and filtration through a 0.22-μm-pore-size membrane (mixed-cellulose-ester membrane; Millex; Millipore, Bedford, MA), and the cell-free liquid retained. A 20-μl sample of the cell-free spent medium was applied to a Curosil PFP 15 x 2 mm, 3 μm particle size HPLC column (Phenomenex, Torrance, CA) using the same method as described for purification of TDA. The amount of TDA was determined from a standard curve derived from known amounts of pure TDA.

### 3.3.7 DNA extraction, separation, and preparation

Total DNA was extracted from roseobacter cells using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). Plasmid DNA was prepared by the alkaline lysis method (5). DNA was separated by agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer and stained with either ethidium bromide or SYBR Gold (Molecular Probes, Eugene, OR), and scanned with a Typhoon 9410 (Amersham Biosciences, Piscataway, NJ) using standard methods.

### 3.3.8 DNA sequence analysis and taxonomic analysis of roseobacter isolates

Homologs of *tda* genes in other, non-TM1040 roseobacters were found by BLASTP analysis (2) of the Roseobase (http://www.roseobase.org/), Gordon and Betty Moore Foundation Marine Microbial Genome (https://research.venterinstitute.org/moore/), and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) databases, using a maximum cutoff E value of 1E-30, as previously used (57).

The 16S rRNA gene from each roseobacter isolate was amplified by PCR using primers 27f and 1492r (94). Standard PCR amplification conditions were 100 μM dNTP each, 0.2 μM of each primer, 1 U Taq DNA polymerase (New England Biolabs, Beverly, MA) in 1× reaction buffer (New England BioLabs) with an initial denaturing step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min each, annealing at 58°C for 30 s, and an elongation at 72°C for 1.5 min. Sequences were compared to the available databases using BLASTN (2) to determine approximate phylogenetic affiliations to the genus level.

## 3.4 Results

### 3.4.1 Genetic organization of *tda* genes in marine roseobacters

I have previously reported in Chapter 2 on the identification of 12 genes required for TDA biosynthesis by TM1040 and *Phaeobacter* 27-4 (57). As shown in **FIG. 3.1B**, the same set of *tda* genes is also found in other marine isolates including the roseobacters *Phaeobacter gallaeciensis* BS107 from the scallop *Pecten maximus* (131) and *Phaeobacter gallaeciensis* sp. 2.10 from the macroalga *Ulva australis* (126), as well as *Pseudovibrio* sp. JE062, a sponge isolate in the same *Rhodobacterales* order as the roseobacters (46). The organization of *tdaA-F* in *P. gallaeciensis* sp. BS107, *P. gallaeciensis* sp. 2.10, and *Pseudovibrio* sp. JE062 is identical to that of TM1040 (57). Slight differences occur in the adjacent non-*tda* genes, which encode proteins whose amino acid domains suggest they may function as membrane proteins involved in molecular transport (data not shown). The discovery of *tda* genes in other members of the *Rhodobacterales* not only broadens the list of TDA-producing bacteria, but also highlights the conservation of *tda* genes at the genetic level among various species.

### 3.4.2 Kinetics of TDA production under shaking and standing conditions

TM1040 produces a yellow brown pigment that is correlated with the synthesis of TDA, both of which are produced in much higher amounts in standing nutrient broth cultures than in shaking broth cultures (57). A time course of TDA
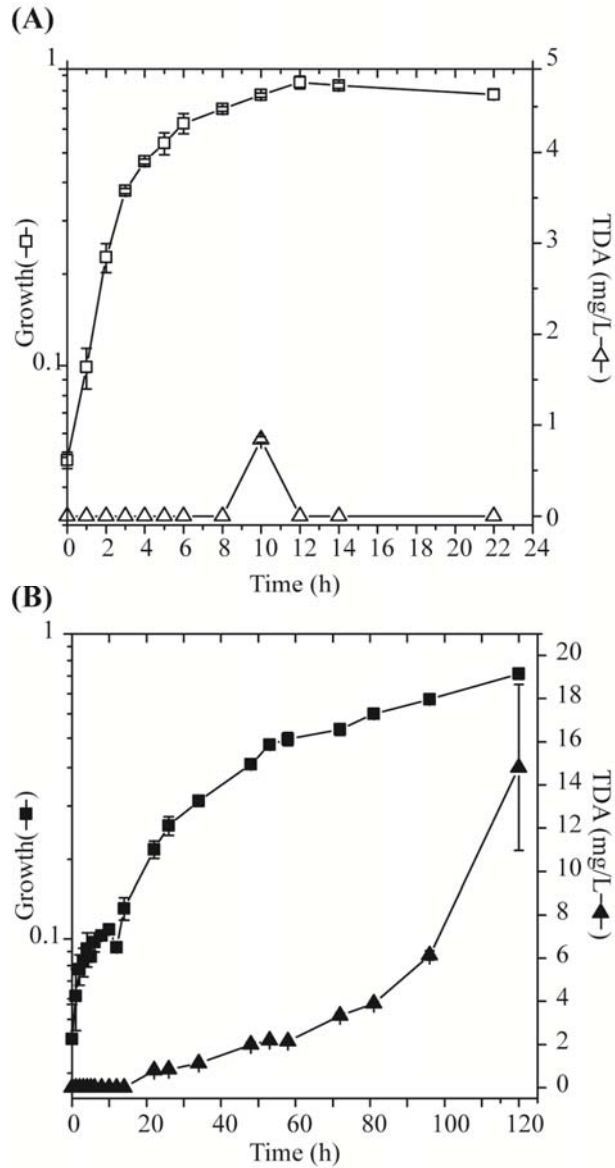
**FIG. 3.2. TDA biosynthesis and bacterial growth of TM1040 are influenced by culture conditions.** Bacterial growth rate and TDA production were compared over a time course when bacteria were grown in static liquid nutrient broth or in the same broth but with vigorous shaking. Bacterial growth is shown as $OD_{595}$ (left axis; square symbols), while TDA production is measured in mg per liter (right axis; triangle

symbols). (A) Incubation with shaking ($\square$ = growth and $\Delta$ = TDA concentration). (B)

Incubation in standing liquid broth ($\blacksquare$ = growth and $\blacktriangle$ = TDA concentration). Error

bars indicate standard deviation (SD) (n=3).

production under these two growth conditions is shown in **FIG. 3.2**. In shaking liquid broth, with the bacteria doubling every 40 min (**FIG. 3.2A**), TDA is not synthesized, except for a brief period at ca. 10 h post-inoculation. In contrast to when grown in shaking conditions, the doubling time in standing culture conditions increases significantly to ca. 12 h (**FIG. 3.2B**). The concentration of TDA in standing culture exponentially increased starting in the early exponential phase of growth (20 h), with TDA production increasing rapidly as the cells enter stationary phase. The striking differences in bacterial growth rate and TDA production kinetics between shaking and standing culture conditions indicate that TM1040 has an acute response to environmental conditions (as reflected in a laboratory culture flask) that manifests itself in changes to the bacterium's behavior and physiology which are especially evident in the production of TDA.

### 3.4.3  Effect of bacterial sulfur sources on TDA production

TDA contains two sulfur atoms (**FIG. 3.1A**), and its synthesis is thus probably influenced by the amount and type of sulfur compounds provided to the cells from the surrounding environment. This was tested by comparing the synthesis of TDA when cells were grown under standing culture conditions in a sulfur-limited basal marine broth with glycerol as a carbon source to which various sulfur sources were added, including 3-methiopropionate (MMPA), cysteine, DMSP, methionine, sulfate, and taurine (**FIG. 3.3**). Although addition of DMSP and methionine significantly increased the growth of TM1040, only DMSP increased the amount of TDA produced. As shown in **FIG. 3.3**, 200 μM DMSP increased the concentration of TDA
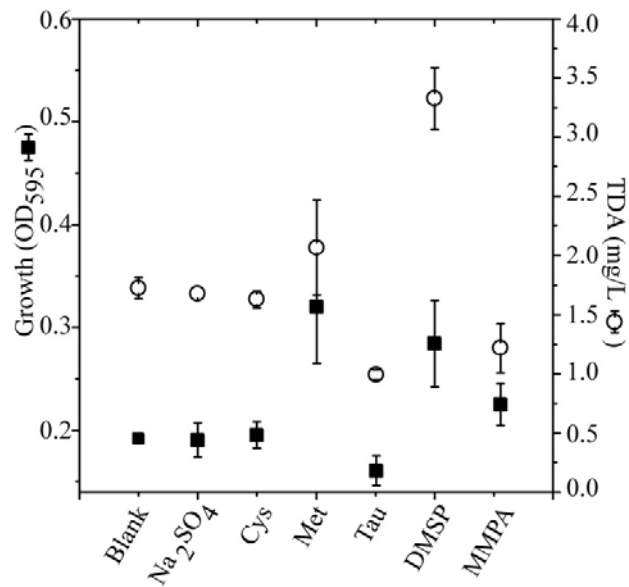
**FIG. 3.3. The source of sulfur provided the bacteria affects TDA production.**

Cells were incubated for 72 h under standing culture conditions in a minimal medium containing a 'subsistence' level (27 mM) of sulfate to which 200 μM of either Na$_2$SO$_4$, cysteine (Cys), taurine (Tau), 3-methiolpropionate (MMPA), DMSP, or methionine (Met) was added. Bacterial growth was measured as the OD$_{595}$, and the concentration of TDA is shown as mg per liter. Error bars indicate standard deviation (SD) (n = 3).

by 2 times relative to its concentration in the control. These results suggest that DMSP is a preferred source of sulfur used by TM1040 for the synthesis of TDA. These results also hint that DMSP may function as an inducer of *tda* gene transcription, a hypothesis that I tested subsequently.

### 3.4.4 Expression of *tdaC* and *tdaF* is modulated by environmental cues

Since TDA production is influenced by culture conditions and the source of sulfur provided the bacteria, I tested a hypothesis that the increase in TDA was due to induction of *tda* gene expression. Quantitative reverse transcription PCR (qPCR) was used to measure mRNA from *tdaA*, *tdaC*, *tdaF*, *paaK*, *cysI,* and *malY* (respectively), as these genes were either the first gene in a putative operon or monocistronic [**FIG. 3.1B** and (57)]. Measurements of these transcripts (**FIG. 3.4**) show that *tdaC* and *tdaF* are up-regulated, respectively, 3.7-fold (19.01 vs. 5.07) and 17.4-fold (47.75 vs. 2.75) when cells are grown in standing culture conditions, compared with values obtained from shaking cultures. On the other hand, transcription of *tdaA*, *paaK*, *cysI*, and *malY* was not affected by the type of culturing used (**FIG. 3.4**). Constitutive expression of these four genes is not surprising, as *tdaA* is thought to encode a transcriptional regulator, many of which are constitutively expressed, while the function of PaaK, CysI, and MalY (respectively) is required for fundamental core physiological processes and are not specific to TDA biosynthesis.

| Gene | Shaking[a] | Standing |
|------|-----------|----------|
| *tdaA* | $1.90 \pm 0.07$ | $0.65 \pm 0.03$ |
| *tdaC* | $5.07 \pm 0.53$ | $19.01 \pm 0.54$ |
| *tdaF* | $2.75 \pm 0.27$ | $47.75 \pm 15.25$ |
| *paaK* | $5.46 \pm 0.68$ | $1.50 \pm 0.06$ |
| *cysI* | $0.67 \pm 0.03$ | $1.03 \pm 0.04$ |
| *malY* | $0.79 \pm 0.03$ | $0.89 \pm 0.03$ |

[a] Mean ± Standard Deviation
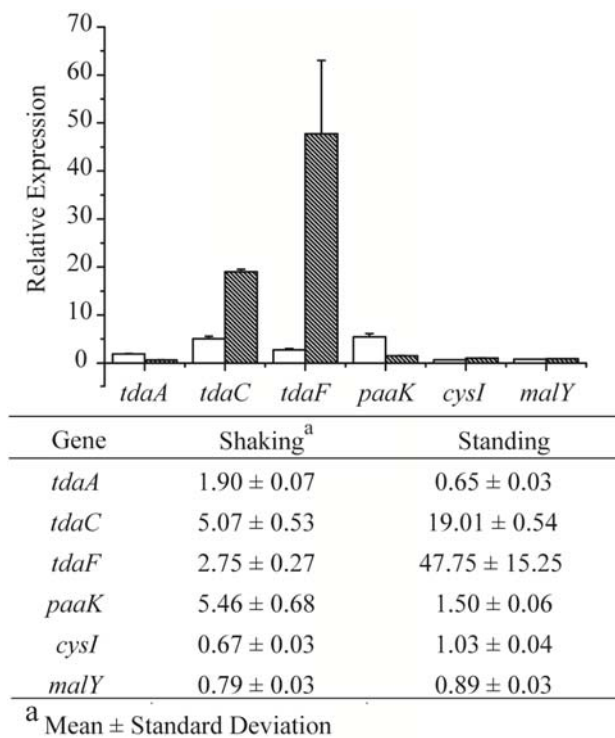
**FIG. 3.4. Relative level of *tda* gene transcription in cells grown under shaking vs. standing liquid culture conditions.** Transcription is shown as relative expression of each target gene compared to *rpoD* and was measured by quantitative PCR (qPCR), as described in Materials and Methods. Open (white) bars represent expression in shaking culture and hashed (solid) bars indicate target gene transcription in standing culture conditions. Error bars indicate standard deviation (SD) (n=3). The data from which the bar graph were derived are shown in the table beneath the graph.

### 3.4.5  Kinetics of *tdaC* expression

As illustrated in **FIG. 3.1B**, *tdaC* forms an operon with *tdaD* and -*E*, and the 364-bp segment of DNA 5' to the *tdaC* start codon is likely to contains a regulatory region and transcriptional promoter site. To determine if this region contains regulatory elements needed for *tdaC* expression and to monitor the activity of *tdaC* more conveniently, a plasmid bearing a transcriptional fusion between the 364-bp *tdaC* upstream DNA and a promoterless *lacZ* gene, *tdaCp*::*lacZ*, was constructed and moved into TM1040. The level of *tdaC* activity, as measured by β-galactosidase activity (Miller units), in standing broth conditions was compared to activity found in cells obtained from shaking broth cultivation. A representative data set from four separate experiments with five replicates each is shown in **FIG. 3.5**. The activity of *tdaC* as measured using the *tdaCp*::*lacZ* transcriptional fusion plasmid is equivalent to the qPCR results (**FIG. 3.4**) and shows that expression of *tdaC* is higher in standing broth culture conditions (**FIG. 3.5**) than in shaking condition, where little to no expression was observed (**FIG. 3.5**). In contrast, when bacteria are grown in standing broth cultures, the expression of *tdaC* reproducibly commences after a lag phase of ca. 10 h, and then rapidly increases over the next 40 h, reaching a maximum at approx. 50 h (**FIG. 3.5**). These data confirm that transcription of *tdaC* is induced during incubation of the bacteria in standing liquid conditions, but not in shaking broth cultures. The 10-h lag prior to the onset of the increase in *tdaC* expression may suggest that an inducing factor accumulates in the standing broth as the cells grow. I hypothesize that the unknown inducer could be a chemical, a
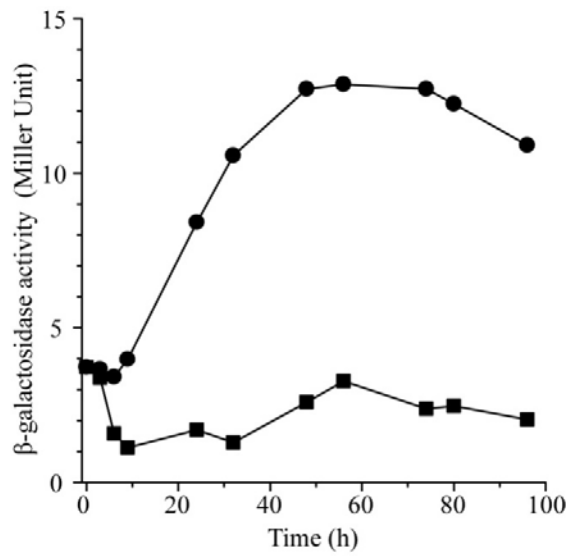
**FIG. 3.5. Kinetics of *tdaC* expression during standing (●) vs. shaking (■) liquid culture conditions**. Expression of *tdaC* was measured as β-galactosidase activity (Miller units) produced from a plasmid harboring a *tdaCp*::*lacZ* transcriptional fusion. Shown is a representative data set from four independent experiments each with five replicates.

change in cellular physiology, or change in exterior environment around the cells, i.e., increased anaerobicity, during incubation in a standing nutrient broth.

### 3.4.6  Expression of *tdaC* in Tda- mutant backgrounds

My initial experiments to understand more about what genes modulate the expression of *tdaC* focused on *tdaA*, since it encodes the sole (putative) regulatory protein required for TDA synthesis (57). This was accomplished by moving the *tdaCp*::*lacZ* plasmid into the *tdaA* strain, as well as into the other 11 Tda⁻ mutants (**Table 3.1**). I predicted that the *tdaA* (HG1310; *tdaA*::EZ-Tn*5*) mutant would be the only genetic background in which *tdaC* was not expressed, as the other *tda* genes encode proteins presumed to be required for TDA synthesis and are not thought to be involved in the transcriptional regulation of *tda* genes. As showed in the **FIG. 3.6A**, when *tdaCp*::*lacZ* is in a wild-type background, β-galactosidase (represented by a blue colony in **FIG. 3.6**) was produced. In agreement with the predictions, the *tdaC* transcriptional fusion was not expressed in a *tdaA* genetic background. Unexpectedly, β-galactosidase was also not detected when *tdaCp*::*lacZ* was placed in any of the *tda*-defective backgrounds (*tdaBCDEF*, *tdaH*, *malY*, and *paaIJK*), with one exception: *cysI* (HG1220; *cysI*::EZ-Tn*5*) (**FIG. 3.6A**). These results show that defects affecting TDA synthesis, as well as *tda* transcription, result in loss of *tdaC* expression. One interpretation of these results is that TDA itself, or a late-stage chemical intermediate in TDA biosynthesis, is required for *tda* gene expression. Thus, TDA may be acting as an autoinducer of its own synthesis.

If this is true, then why did the *cysI* mutation (HG1220; *cysI*::EZ-Tn5), which we originally characterized as a TDA loss-of-function mutant (57), still allows the
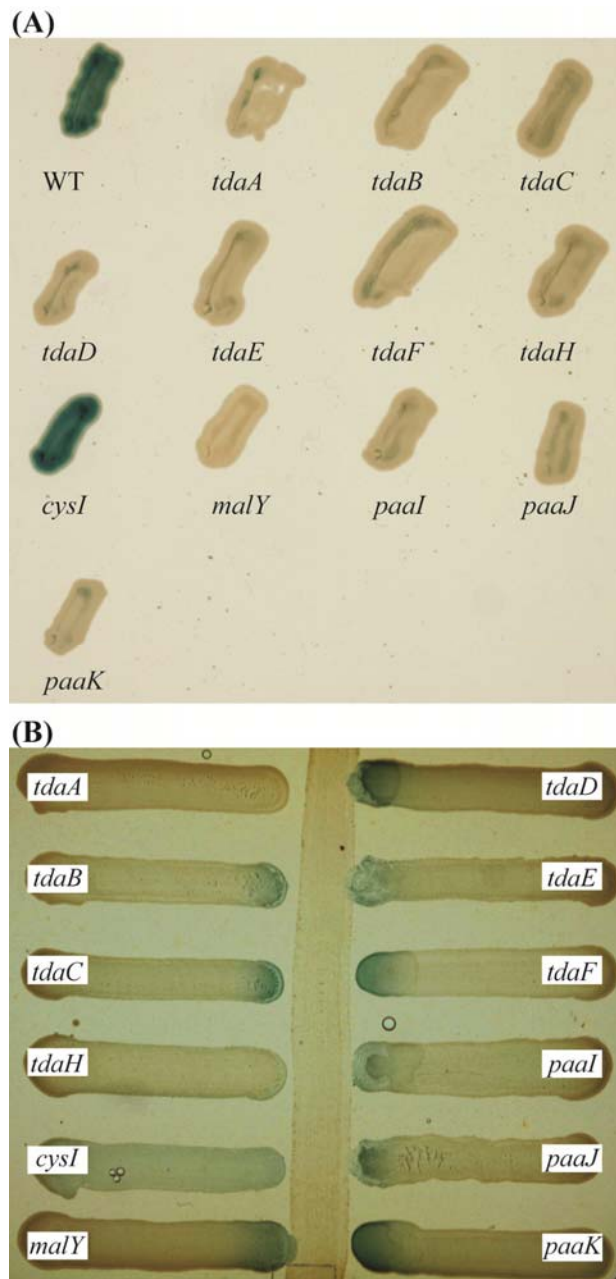
**FIG. 3.6. (A) Expression of *tdaC* in different Tda⁻ mutant backgrounds.** A

plasmid-borne copy of a *tdaCp*::*lacZ* transcriptional fusion was placed into wild-type

and each of the 12 Tda⁻ mutant strains, after which β-galactosidase activity was

qualitatively assessed by the blue resulting from cleavage of X-Gal contained within

the nutrient agar. (B) **Cross-feeding of the 12 Tda⁻ mutants** harboring the

*tdaCp*::*lacZ* reporter fusion by wild-type cells. TM1040 (wild-type cells) was

inoculated as a solid vertical line in the center of the agar, and each mutant streaked

perpendicular to TM1040. A blue color indicates cleavage of X-Gal and expression of

*tdaC*.

expression of *tdaC*? I rechecked HG1220 for production of TDA by concentrating a large volume of spent cell-free supernatant from the strain and analyzed the compounds it contained by HPLC. The chromatogram of the concentrated extract from HG1220 had a distinct peak migrating at the same time and with the same UV spectrum as the TDA peak obtained from wild-type supernatant (**FIG. 3.7**). This peak was collected and shown to have antibacterial activity (data not shown). Therefore, the *cysI* mutant produces a trace amount of TDA that can be detected by the *tdaCp*::*lacZ* reporter plasmid resulting in the LacZ$^+$ phenotype of HG1220.

To provide further evidence that TDA is required for the expression of *tdaC*, a cross-feeding experiment was performed in which each of the 12 Tda$^-$ mutant strains harboring the *tdaCp*::*lacZ* reporter plasmid was streaked perpendicular to the wild-type on medium contain X-Gal (**FIG. 3.6B**). As shown in **FIG. 3.6B**, when in close proximity to TM1040, β-galactosidase was produced by 10 of 12 Tda$^-$ mutants, supporting the hypothesis that TDA produced from wild-type cells can cross-feed Tda$^-$ strains. The most reasonable explanation for the failure of the *tdaA* mutant to produce β-galactosidase is a loss of regulation of *tdaC* expression due to the defect in the LysR-like regulatory protein encoded by the gene. On the other hand, the failure of the *tdaH* mutant to express *tdaCp*::*lacZ* was unexpected. Possible reasons for the *tdaH* phenotype are offered in the Discussion section of this chapter.
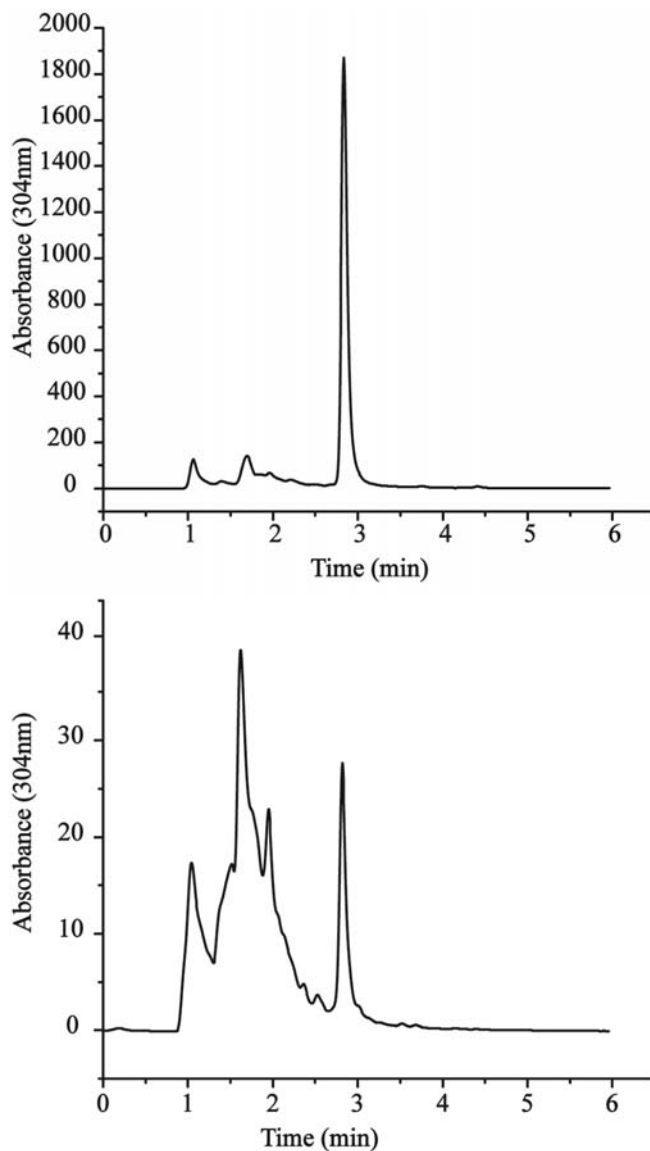
**FIG. 3.7. Identification of TDA in concentrated extracts from the *cysI* (HG1220) mutant.** TM1040 (wild type) and HG1220 (*cysI*::EZ-Tn*5*) were inoculated respectively in 2 l of marine broth and cultured under standing culture conditions for 3 days. After incubation, the cells were removed by centrifugation and membrane filtration, and the spent cell-free supernatant from each strains (wild-type and *cysI*) were extracted with ethyl acetate, separated on an Oasis MAX column (materials and

methods), lyophilized, redissolved in 400 μl methanol, and finally separated by HPLC

and chemically analyzed by UV spectrum composition. The major peak (retention

time 2.82 min) in the upper chromatogram from wild-type extracts and a

corresponding peak in the extract from the *cysI⁻* mutant (lower chromatogram) are

TDA.

### 3.4.7   TDA induces *tdaC* and *tdaF* expression

Expression of *tdaC* was also measured when pure TDA and related compounds were added exogenously to pHG1011 (*tdaCp*::*lacZ*) in TM1040 (as described in Materials and Methods). As can be seen in **Table 3.3**, a ca. 3-fold increase in *lacZ* expression (compared to the lacZ expression in a water-only control) was observed when cells harboring the *tdaCp*::*lacZ* reporter plasmid were exposed to HPLC-purified TDA. Unlike TDA, which demonstrated the strongest induction, other compounds, such as cysteine, methionine, DMSP, phenylacetate, and cycloheptatriene, elicited slight increases in β-galactosidase above background. The *tdaC* reporter showed no detectable response when chemicals containing a tropone ring, e.g., tropone and tropolone, were provided to the cells. ANOVA analysis confirmed that only induction by TDA was statistically significant ($p < 0.05$), and the results for all of the other tested compounds were statistically equivalent to the water control. The induction of *tdaC* transcription by TDA is dose dependent (**FIG. 3.8A**). I also measured the effect of exogenous TDA on the transcription of *tdaC* and *tdaF* using qPCR. As shown in **FIG. 3.8B**, the expression of *tdaC* and *tdaF* increased respectively 7.74- and 9.34-fold relative to the uninduced control following exposure to TDA. These results indicated that induction of *tdaC* and *tdaF* transcription is specific to TDA.

**Table 3.3. Induction of *tdaC* expression.**

| Chemical[a] | β-Galactosidase Activity[b] |
|---|---|
| Cycloheptatriene | 3.86±0.82 |
| Cysteine | 2.95±1.23 |
| DMSP | 3.26±1.64 |
| Glucose | 2.61±0.32 |
| Histidine | 2.13±1.43 |
| Methionine | 2.92±0.15 |
| Phenylacetate | 3.23±1.42 |
| Phenylalanine | 2.58±0.42 |
| Shikimate | 2.57±0.18 |
| Succinate | 2.6±0.33 |
| TDA | 6.80±1.76 |
| Tropolone | 1.72±0.13 |
| Tropone | 2.10±0.30 |
| Water | 2.29±0.54 |

[a] Chemicals were amended to final concentration of 50 μM in the tested 2216 marine broth media.

[b] β-Galactosidase activity is calculated as previously described (108). Value is shown as mean ± standard deviation (SD) (n=3) .

**(A)**

y-axis: β-galactosidase activity (Miller Unit)

x-axis: TDA (μM): 0, 25, 50, 75

**(B)**

|      | Average | S.D. |
|------|---------|------|
| *tdaC* | 7.74    | 0.53 |
| *tdaF* | 9.34    | 0.49 |
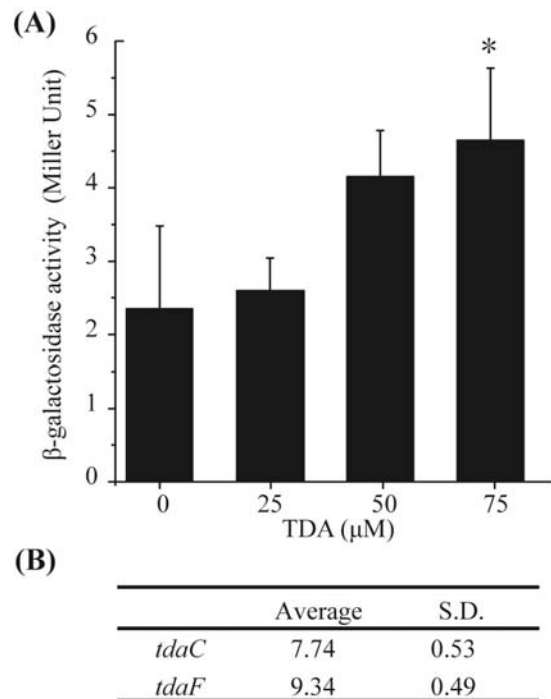
**FIG. 3.8. Pure TDA induces a concentration-dependent expression of *tdaC* and *tdaF* transcription.** (A) Transcription of *tdaC* measured as β-galactosidase activity (Miller units) produced from the *tdaCp*::*lacZ* transcriptional fusion in TM1040 3 h post exposure to TDA (from left to right 0, 25 μM, 50 μM, and 75 μM TDA). Error bars indicate standard deviation (n=3). The asterisk indicates statistically significant difference from the control (ANOVA; $p < 0.05$). (B) Relative abundance of *tdaC* and *tdaF* mRNA after 50 μM TDA induction relative to an uninduced control sample, as measured by qPCR (n=3). S.D., standard deviation.

### 3.4.8 Taxonomically distinct roseobacters induce *tdaC* expression in TM1040

The results thus far obtained suggest that extracellular TDA either actively or passively transported into a recipient cell where, at some unknown threshold concentration, it induces expression of *tdaC* and *tdaF*. These data led us to predict that other TDA-producing species of the *Roseobacter* clade would also able to induce *tda* gene expression in TM1040. Supernatants from several different *Roseobacter* clade and *Pseudovibrio* species were spotted on the surface of nutrient agar containing X-Gal, which had been seeded with strain HG1299 (*paaI*::EZ-Tn5) harboring the *tdaCp*::*lacZ* reporter plasmid. HG1299 was chosen because it is a loss-of-function Tda⁻ mutant that does not make TDA, and so, *tdaCp*::*lacZ* has no detectable activity in this background unless provided with TDA. As shown in **FIG. 3.9**, a positive response, i.e., *tdaC* transcription, was observed from species of *Phaeobacter*, *Pseudovibrio*, *Ruegeria,* and *Silicibacter*; strains of last two genera were obtained as random bacterial isolates from a culture of the dinoflagellate *Karlodinium veneficum*. On the other hand, *Roseobacter algicola*, *Roseobacter litoralis*, two species of *Roseovarius*, *Ruegeria pomeroyi*, and two species of *Sulfitobacter* failed to induce *tdaCp*::*lacZ* transcription (**FIG. 3.9**), suggesting that these species do not produce TDA. The production of TDA from each of the roseobacters was confirmed by purification of the compound from cell-free supernatants and HPLC analysis (data not shown). Taken together these results emphasize a role of TDA as an extracellular cross-genera roseobacterial (auto)inducer of *tda* gene expression.
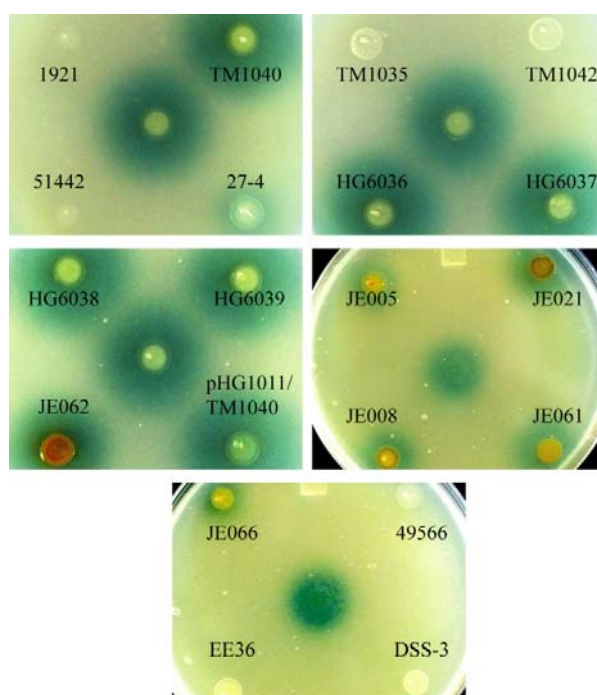
**FIG. 3.9. Marine *Rhodobacterales* species induce *tdaCp*::*lacZ* expression in TM1040.** 10 μl of an individual 3 day standing culture was spotted on agar that contained a strain harboring the *tdaCp::lacZ* transcriptional fusion plus X-Gal. TM1040 was inoculated in the center on each plate as a positive control. Starting at the upper left image and moving to the right and down through the set of five images, the colony labels are (clockwise): 1921, *Sulfitobacter* 1921; TM1040 ; 27-4, *Phaeobacter* 27-4; 51442, *Marinovum algicola* ATCC 51442; TM1035, *Roseovarius* sp. TM1035; TM1042, *Roseovarius* sp. TM1042; HG6037, *Ruegeria pelagia/mobilis;* HG6036, *Silicibacter* sp.; HG6038, *Ruegeria pelagia/mobilis;* HG6039, *Ruegeria pelagia/mobilis*; TM1040/pHG1011, *tdaCp::lacZ* in TM1040; JE062, *Pseudovibrio* sp. JE062; JE005, *Pseudovibrio* sp. JE005; JE021, *Pseudovibrio* sp. JE021; JE061, *Pseudovibrio* sp. JE061; JE008, *Pseudovibrio* sp. JE008; JE066, *Pseudovibrio* sp.

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JE066; 49566, *Roseobacter litoralis* ATCC 49566; DSS-3, *Ruegeria pomeroyi* DSS-3; and, EE36, *Sulfitobacter* EE36.

## 3.5    Discussion

The ability of bacteria such as TM1040 to alter their physiological state and change their behavior through sensing and response to environmental signals provides a significant adaptive advantage, especially during the transition these cells make in going from free-living bacteria to those engage in a symbiotic association with their host phytoplankton. I refer to this change as the 'swim-or-stick' switch (11), and the processes controlling it are critical for the initiation and maintenance of this symbiosis. In the present study, I explored some of those processes controlling a hallmark of the 'stick' or sessile phase, namely regulation of the biosynthesis of TDA. My results suggest that TDA expression is modulated by environmental and cultivation conditions, i.e., growth of the bacteria in static liquid nutrient broth cultures increases transcription of *tda* genes (specifically *tdaCDE* and *tdaF*), with commensurate increase in TDA biosynthesis. Unexpectedly, I also found that TDA itself is required for maximal expression of *tda* genes and that non-TM1040 *Roseobacter* clade and *Pseudovibrio* species producing TDA can cross-feed and elicit an increase in *tda* gene expression from TM1040. The latter suggests that TDA, which has antibiotic activity against many non-roseobacter marine bacteria (16, 19, 20, 57), also functions as a chemical signal or quorum sensing autoinducer molecule among several marine *Rhodobacterales* species. The use of TDA as an autoinducer in the marine bacteria has profound implications to their survival and adaptation, to the symbioses many of them engage in, and to marine ecosystems. The results and their implications are discussed next.

The genes encoding TDA biosynthesis are widely distributed in a broad range of marine *Rhodobacterales* species (**FIG. 3.1B**). Conservation of *tda* genetic information in this group suggests that these ubiquitous marine bacteria exploit the biochemical activities of TDA to enhance their adaptation and survival in certain niches, such as on the surface of host phytoplankton cells.

This and previous studies (20, 57) have shown significantly enhanced TDA production when bacteria are grown in standing liquid broth cultures (**FIG. 3.2**). The qPCR transcription data presented in this report builds upon the earlier findings and show that transcription of the *tdaC* operon and *tdaF* is much higher when cells are incubated in standing liquid cultures (compared to shaking culturing), whereas the expression of other genes previously shown (57) to be required for TDA synthesis, i.e., *tdaAB*, *cysI*, *malY*, and *paaIJK*, remains unchanged (**FIG. 3.4**). While the data do not provide first hand evidence, it is likely that the expression of the latter set of seven genes remains unchanged due to the respective role these gene products (CysI, MalY, and PaaIJK) have in essential metabolic pathways, or, in the case of *tdaA*, in controlling the transcription of *tdaCDE* and *tdaF*.

Why does culturing in standing nutrient broth induce *tda* transcription? The evidence thus far accumulated emphasizes that the standing culture environment positively affects TDA activity and therefore must allow TDA to reach threshold levels in order for the chemical to induce *tda* expression. Bruhn et al (20) have shown that the half-life of TDA activity is lessened by exposure to oxygen and elevated temperature. Therefore, one possible reason for the increase in *tda* gene expression in standing liquid culture may be related to the instability of TDA in shaking cultures,

where higher oxygen tension is predicted to reduce or destroy the activity of TDA. In turn, the oxygenated medium would prevent TDA from accumulating to a threshold concentration required to induce *tda* gene expression. I believe this is a very likely scenario.

It is also possible that *tda* expression is a byproduct of a change in the physiology of sessile phase cells, which are more prevalent in standing broth cultures than shaking cultures (20, 57). We do not know if TDA induces biofilm and rosette formation in TM1040 or conversely something about the physiological change in the sessile cells induces TDA biosynthesis. Transcriptomic analyses comparing TDA-induced to uninduced cells, looking for changes in the expression of genes involved in biofilm formation, such as those encoding fimbrial adhesins or capsular polysaccharides, would be one way to test whether TDA induces biofilm formation. The alternative hypothesis stating that a physiological change in sessile cell induces TDA expression may be tested by comparing *tda* gene expression in motile vs. sessile cells. It is of great interest to us to test the second hypothesis, since its validity has direct implications to the physiological changes that roseobacters undergo as they attach to the surface of their phytoplankton host and initiate the symbiosis. In this case, biofilm-dependent increases in *tda* gene expression may allow the building up of TDA near the bacteria and its host, which may have the consequence of serving to thwart other non-roseobacters from forming biofilms on the host. This would offer a competitive advantage to TM1040 and other physiologically similar roseobacters in maintaining their biofilm on and symbiosis with the phytoplankton host. While speculative, induction of *tda* genes by sessile TM1040 cells may be envisaged to

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encourage the attachment of other roseobacters on the surface of the phytoplankton, which may benefit both the mixed roseobacter biofilm as well as the phytoplankton host.

In contrast to the observed difference in *tda* expression during cell growth in standing vs. shaking liquid culture conditions, the type of sulfur compound provided the cells did not significantly affect transcription of *tdaCDE* or *tdaF*, although addition of DMSP did result in a marked increase in TDA (**FIG. 3.3**). DMSP, which contains two sulfur atoms per molecule, is produced abundantly by many phytoplankton (31, 165) including the dinoflagellate host, *P. piscicida*, used in our laboratory (109). DMSP is efficiently metabolized by TM1040 and other roseobacters as a source of carbon and sulfur (76, 109, 147). Moreover, since DMSP is a strong chemoattractant of TM1040 (112), it provides the bacteria with an important signal directing their swimming behavior towards the host phytoplankton (112) and the surface that is ultimately colonized. Thus, the bacteria use DMSP as a cue to locating the dinoflagellates and in the synthesis of TDA, yet DMSP does not act as an inducer of transcription of *tda* genes.

Several lines of evidence support a hypothesis that TDA acts in a density-dependent manner as an autoinducer of *tda* expression. First, the maximum concentration of TDA is achieved late during the growth of bacteria in standing nutrient broth cultures at the time of highest cell density (**FIG. 3.2**). Second, expression of *tdaC* transcription mirrors TDA concentration and peaks late in the growth of TM1040 in standing broths (**FIG. 3.2** and **FIG. 3.5**). Third, time course experiments of *tdaC* expression during standing liquid culturing also show a

reproducible lag in *tdaC* expression post-inoculation into a new medium (**FIG. 3.5**, closed circles, 0 to 10 h). The *tdaC* expression curve is similar to expression curves of other molecules known to function as quorum signals (41, 157, 158). This is especially significant as previous work has shown that the genome of TM1040 does not harbor homologs to any known quorum signal genes (113) nor do these bacteria produce N-acyl homoserine lactones or other common quorum signalling molecules (18).

The fourth line of evidence supporting a role of TDA as a density-dependent autoinducer comes from the series of cross-feeding experiments (**FIG. 3.6** and **FIG. 3.8**). When *tdaCp*::*lacZ* on a replicating plasmid was placed in each of the 12 Tda⁻ mutants originally described in Geng et al. (57), *tdaC* was not expressed in any of the Tda⁻ backgrounds, except *cysI* (**FIG. 3.6A**). This was puzzling, as this mutant (HG1220, *cysI*::EZ-Tn5) has been reported by us to be defective in TDA synthesis (57). However, we discovered that the mutation in *cysI* (*cysI*::EZ-Tn5) is not a true loss-of-function mutation, rather the *cysI* strain produces extremely low amounts of TDA, which could only be detected once a large volume of supernatant was extracted and concentrated from HG1220. Therefore, the results from the *cysI* background are due to presence of trace amount of TDA, and emphasize the sensitivity of the *tdaC* transcriptional fusion reporter plasmid in detecting TDA.

In addition to supporting a role for TDA as a density-dependent autoinducer, the results from cross-feeding experiments between wild-type cells and each of the 12 mutant strains harboring the *tdaC* transcriptional fusion (**FIG. 3.6B**) also produced two interesting results. First, a mutation in *tdaA* was unable to respond (no β-

galactosidase activity was produced from the *tdaCp*::*lacZ* transcriptional fusion) to extracellular molecules from wild-type, presumably because these cells do not respond to TDA. TdaA is predicted to be a transcriptional regulatory protein and a member of the LysR family (57), many of which require binding of a cognate small molecule ligand for optimal activation of transcription of the target genes they control (137). We hypothesize that TdaA regulation of *tda* expression requires the binding of TDA (the molecule) for maximal activity, and this hypothesis is tested in the next chapter.

The second interesting outcome of the cross-feeding experiments came from the *tdaH* mutant (HG1244, *tdaH*::EZ-Tn*5*), which also failed to show induction of the *tdaC* reporter fusion in the presence of TDA. This was unexpected as we have previously shown that the *tdaH* mutation gives rise to a loss-of-function Tda⁻ phenotype, and we have identified TdaH as a putative oxidoreductase, molybdopterin-binding domain protein or sulfite oxidase, and therefore presumed it to have a role in the synthesis of TDA (57). It is possible that TdaH is involved in chemically modifying TDA into an active intracellular form, but there are no data to support or reject this hypothesis. However, I re-evaluated the *tdaH* mutation and discovered that the transposon had inserted 10 bp from the 3' end of *tdaH*, and speculate that a nearly full-length TdaH protein could be produced. Furthermore, *tdaH* is the first gene (TM0961) in what is most likely an operon in which the second gene, TM0962, encodes a putative hybrid histidine kinase with PAS (168), PAC (168), HisKA (146), HATPase (146), REC (146), and HPT domains (**FIG. 3.10**).

This suggested that the transposon may have polar effects on a gene (TM0962) downstream of *tdaH*.

I used RT-PCR to test this hypothesis, the results of which are shown in **FIG. 3.10.B**. In wild-type bacteria, *tdaH* and *tdaI* mRNA are present, while strain HG1244 produces *tdaH* mRNA but does not transcribe *tdaI*. These data substantiate the idea that *tdaH* and *tdaI* form an operon, and show that the transposon insertion in *tdaH* has negative polar effects on the transcription of *tdaI*. Thus, an alternate hypothesis to explain the phenotype of the "*tdaH* mutant" is it is due to loss of function of the hybrid histidine kinase, TdaI. This is certainly tantalizing, as one can imagine how such a protein could sense redox, energy, or other environmental conditions via its PAS-PAC domains (and heme pocket) and relay this signal via a phosphorylation cascade to control gene expression during symbiosis.

Bacteria use quorum sensing to gauge the density of surrounding prokaryotic populations (158). Quorum sensing may occur between cells of the same species or between diverse groups of bacteria. Many species of bacteria use quorum sensing to coordinate their gene expression and certain behaviors according to the local density of their population. In this respect, TDA functions as a quorum sensing autoinducer of *tda* gene expression among the various TDA-producing marine *Rhodobacterales*. This is supported by the data in **FIG. 3.9** showing that TDA produced by non-TM1040 roseobacters and *Pseudovibrio* species is sensed and induces *tdaC* transcription in recipient TM1040 cells. The genera inducing $tdaC_{TM1040}$ expression include species of *Phaeobacter*, *Pseudovibrio*, and *Ruegeria* (**FIG. 3.9**). Our taxonomic analysis did not provide sufficient data to bring identification to the
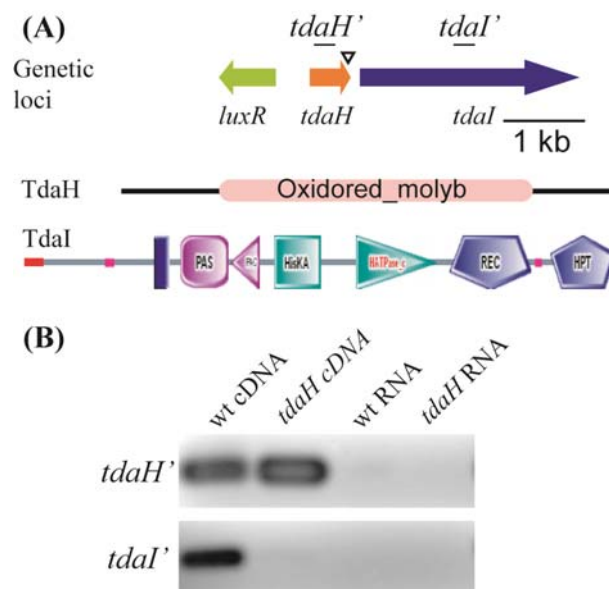
**FIG. 3.10. (A) Organization of *tdaH* locus**. The *tdaH* ORF is upstream, in the same

orientation, and in close proximity to a second ORF, referred to as *tdaI*, suggesting

that the two genes may comprise an operon. An ORF with homology to a LuxR

response regulator is also present near the *tdaH* locus, but this gene is transcribed in

the opposite direction from *tdaH* and is ca. 350 bp from the 5' start of *tdaH*. TdaH

contains a molybdopterin binding domain with homology to sulfite oxidases, while

TdaI contains PAS, PAC, HisKA, HATPase_c and HPT domains typically found in

hybrid histidine kinases. **(B) The transposon insertion in *tdaH* has negative polar**

**effects on the transcription of *tdaI***. RT-PCR was used to test the hypothesis that the

mutation in *tdaH* resulted in loss of *tdaI* mRNA. Total RNA was purified from wild-

type (TM1040) and *tdaH* mutant (HG1244, *tdaH*::EZ-Tn*5*) strains, converted into

cDNA, which was used in *tdaH*-specific (primer tdaH') and *tdaI*-specific (primer

tdaI') reactions. The DNA products of these reactions were analyzed by agarose gel

electrophoresis. The results show that wild-type cells express both *tdaH* and *tdaI*

(first lane), while HG1244 (*tdaH*::EZ-Tn*5*) transcribes *tdaH* but not *tdaI* (lane 2).

Lanes 3 and 4 are controls. These data confirm that the transposon in HG1244 located

in *tdaH* results in the loss of transcription of *tdaI*.

species level, but it was sufficient to conclude that none of these bacteria is a strain of TM1040, whose affiliation with the genus *Ruegeria* is currently being questioned (164).

Thus, the results support a hypothesis that TDA serves as a quorum sensing autoinducer that may be used as a cross-species/cross-genus communication signal among subgroups of marine *Rhodobacterales*, especially species in the *Roseobacter* clade. This has profound implications for the adaptation and survival of this clade of marine bacteria, especially in their symbioses. Extrapolating from what I have learned in the laboratory back to the ocean environment, one may imagine scenarios where TDA may be used as a chemical signal produced by initial roseobacterial colonizers but intended for new recruits telling them that life on the surface is good, come on down, and join the biofilm. Cooperative synthesis of TDA may also be beneficial to mixed species assemblages of roseobacters which, through combined induction of *tda* genes and TDA biosynthesis, may be able to produce concentrations of the antibiotic far beyond the capabilities of a single species. Finally, TDA may have activity that transcends roseobacters and includes the phytoplankton host as well. While this is speculative, such inter-kingdom signaling has been reported (77).

# Chapter 4:   TdaA Regulates Tropodithietic Acid Synthesis by Binding to the *tdaC* Promoter Region

## 4.1   Summary

Development of the symbiosis between the marine bacterium TM1040 and its unicellular eukaryotic host, *P. piscicida*, is a dynamic process that involves a bacterial biphasic swim-or-stick lifestyle. In the stick life phase, the bacteria produce the antibiotic tropodithietic acid (TDA), encoded by *tdaABCDEF*, which also acts as a quorum signal to induce *tda* gene expression. Little is known about how TDA induces the expression of *tda* genes or the molecular mechanisms that underlie the process. In this report, I focused attention on the only known regulatory protein, TdaA, involved in *tda* expression. Reverse transcription PCR (RT-PCR) confirmed that *tdaA* and *tdaB* constitute an operon*,* while *tdaCDE,* located adjacent to *tdaAB*, comprise a second operon. Disruption of *tdaA* results in loss of *tdaCDE* expression, and expression of *tdaA* in an *Escherichia coli* background is sufficient to transcribe *tdaCDE*. Transcriptional activation of the *tdaC* promoter by TdaA is supported by data from electrophoretic mobility shift assays (EMSA) showing that purified TdaA protein binds specifically to a fragment of DNA containing the *tdaC* promoter. Despite TDA acting as an autoinducer of its own synthesis, the affinity of TdaA binding to *tdaC* DNA is unaffected by the presence of TDA indicating that TDA is not directly

required for TdaA to bind to *tdaC* DNA. These results support a hypothesis that TdaA is a positive transcriptional regulator of *tdaCDE* gene expression whose function requires binding to the *tdaC* promoter region.

## 4.2 Introduction

TM1040 is a gram negative bacterium in the alphaproteobacteria that participates in a symbiosis with the marine dinoflagellate *P. piscicida* (1, 11, 57, 109, 110, 112). TM1040, as well as other marine bacteria in the genera *Phaeobacter*, *Silicibacter*, *Ruegeria*, and *Pseudovibrio,* produces an antibiotic called tropodithietic acid (TDA) (18, 122) that is both an inducer of its own biosynthesis and a chemical signaling molecule in certain marine bacteria (55).

Genetic and biochemical evidence suggest that TDA is produced by a ring oxidation expansion of phenylacetate with further modification involving the addition of sulfur atoms that requires at least twelve genes (28, 57, 149). Among these genes, six of them, *tdaA-F* (**FIG. 3.1A**), are conserved among TDA-producing bacteria and are specifically involved in TDA biosynthesis (55). Genomic analyses suggest that *tdaA* and *-B* form an operon, while *tdaC*, *-D*, and *-E* are part of a second operon adjacent to the first. *tdaF* is physically separated from the other *tda* genes and most likely a part of a third independent operon (57).

Environmental and chemical factors affecting the expression of *tda* genes have been reported (55), as described in Chapter 3 of my dissertation. Expression of *tda* genes, as well as the production of TDA, is higher in standing nutrient broth cultures than in shaking cultures (18, 55, 57). In shaking liquid cultures, TM1040 populations are dominated by single motile cells, termed the motile phase, which swim via chemotaxis using two to four flagella (112). In contrast, when in standing broth cultures, the population is dominated by nonmotile cells that attach to one another forming rosettes, or that form biofilms on biotic and abiotic surfaces (20). This is referred to as the sessile phase. Cells in the sessile phase have strikingly higher levels of TDA and *tda* gene expression than motile phase cells (18). Furthermore, TDA induces the transcription of *tda* genes in TM1040, as well as their homologs in taxonomically related marine bacteria (55). This has led to a hypothesis suggesting that recipient bacteria may sense TDA produced by neighboring cells and transduce this signal to change their physiology, lifestyle, and behavior, which could profoundly and beneficially affect the symbiosis (56).

Although the signal transduction circuit underlying TDA regulation has not been defined, genetic studies show that TdaA, a LysR type transcriptional regulator (LTTR), is required for *tda* gene transcription, and defects in *tdaA* result in a loss of the ability to produce and respond to TDA (57). TdaA possesses a helix-turn-helix (HTH) DNA binding motif near its N terminal end and a LysR substrate-binding

domain at C terminal in common with other LTTR family proteins (57). The LTTR

family represents the most abundant class of transcriptional regulators in bacteria

(137). LTTRs can act as either activators or repressors of transcription by binding to

the upstream region of genes they regulate (137). LTTR binding sites include two

dissimilar loci: the regulatory binding site (RBS) and the activator binding site

(ABS). The RBS site is typically located -55 bp relative to the transcriptional

initiation site, with some variant sites locating up to -218 bp or down to +350 bp

(105). Among different LTTRs, RBSs share a consensus DNA binding motif (T-$N_{11}$-

A) within a small region of dyad symmetry (105). The ABS, located between the

RBS and the target promoter, does not have a conserved sequence (105). Classical

LTTRs bind to both RBS and ABS in the promoter region of their cognate target

genes to control transcription (105), and such regulation is often modulated by

specific ligands that interact with their respective LTTRs (137). Binding of a ligand to

an LTTR can alter the binding sites (25), change LTTR-DNA binding affinity (144),

or induce LTTR-dependent DNA bending (105), thereby modulating target gene

transcription.

Here I report on the molecular mechanisms related to TDA regulation, with a

specific focus on understanding how TdaA regulates expression of *tda* genes. Genetic

complementation analysis shows that TdaA is essential for the transcription of

*tdaCDE* operon, in either TM1040 or in *E. coli*. Electrophoretic mobility shift assays

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(EMSAs) indicate that TdaA binds to the *tdaC* promoter, providing evidence that

TdaA activates the expression of *tda* genes by directly binding to their promoters.

## 4.3    Materials and Methods

### 4.3.1    Bacteria and media

TM1040 and the transposon mutants derived from it (**Table 4.1.**) (57) were

maintained at 30 °C on Difco 2216 marine broth or 2216 agar (BD Biosciences,

Franklin Lakes, NJ) as previously described (110). *E. coli* strains were grown in

Luria-Bertani (LB) broth or on LB agar containing 1.5% Bacto Agar (BD

Biosciences, Franklin Lakes, NJ) (5). HIASW10 (25 g of Difco Heart Infusion broth

[BD Biosciences, Franklin Lakes, NJ] plus 10 g of Instant Ocean sea salts [Aquarium

Systems, Mentor, OH] per liter) was used for matings between *E. coli* and TM1040.

Kanamycin was used at 120 µg per ml for TM1040 derived strains and 50 µg per ml

for *E. coli*. Tetracycline was supplemented at 15 µg per ml for TM1040 in HIASW10

and *E. coli* in LB (5).

An overnight culture of TM1040 derivative cells carrying the *lacZ* gene were

placed to 2216 marine agar containing X-Gal (60 µg per ml), tetracycline (30 µg per

ml), gentamicin (30 µg per ml), and isopropyl-β-D-thiogalactopyranoside (IPTG)

(100 µM). The presence of galactosidase activity in TM1040 derivatives was

**Table 4.1. Bacterial strains and plasmids in Chapter 4.**

| Strain/plasmid | Genotype/phenotype[a] | Source or reference |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | F⁻ *endA1 hsdR17 (r_K⁻ m_K⁻) supE44 thi-1 recA1 gyrA96 relA1Φ80dlacZΔM15* | (136) |
| Rosetta (DE3) | F⁻ ompT hsdS_B(r_B⁻ m_B⁻) gal dcm (DE3) pRARE (Cam^R) | Novagen |
| S17-1 λ*pir* | *thi pro recA hsd*(r⁻ m⁺) RP4-2 Tc::Mu-Km::Tn7 Sm λ*pir* | (39) |
| *Silicibacter* sp. TM1040 | Wild type | (109) |
| | | |
| Derivatives from TM1040 | | |
| HG1015 | *tdaB*::EZ-Tn5, Kan | (57) |
| HG1080 | *tdaC*::EZ-Tn5, Kan | (57) |
| HG1310 | *tdaA*::EZ-Tn5, Kan | (57) |
| Plasmids | | |
| pRK415 | Conjugative expression shuttle vector for *Silicibacter* sp.TM1040 and *E. coli*; P_lac; | (85) |

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| | Tet | |
|---|---|---|
| pSRKGm | pBBR1MCS-5-derived broad-host-range expression vector containing lac promoter and lacIq, lacZα+, Gm | (87) |
| pET21a | Overexpression vector, Amp | Novagen |
| pHG1011 | 363-bp upstream of *tdaC* fused in front of *lacZ* in pRK415, Tet | (55) |
| pHG1012 | pSRKGm::*tdaA⁻B⁺B⁺*, IPTG induces frameshift mutant of *tdaA* and intact *tdaB*, Gm | This study |
| pHG1014 | pSRKGm::*tdaA⁺B⁺*, IPTG induces complete *tdaAB* operon, Gm | This study |
| pHG1015 | pET21::*tdaA*, inducible *tdaA* expression with 6His fused at C terminus | This study |

[a] Kan, kanamycin resistance; Sm, streptomycin resistance; Tet, tetracycline resistance;

Amp, ampicillin resistance; Gm, gentamicin resistance.

determined by blue color after 3 days 30 °C incubation. *E. coli* derivate cells carrying the *lacZ* gene were tested in LB agar plates containing X-Gal (60 μg per ml), tetracycline (15 μg per ml), gentamicin (30 μg per ml), and IPTG 100 μM after overnight incubation at 37 °C.

## 4.3.2    Plasmid constructions

PCR with oligonucleotide primers TdaA-His FP and TdaA-His RP (**Table 4.2**) was used to amplify *tdaA* from total genomic DNA. The PCR product was cloned into the expression vector pET-21a(+) (Novagen, Madison, WI) via *Nde*I and *Xho*I restriction sites to construct a plasmid, pHG1015, with a C-terminal 6x-histidine tag fused to TdaA (TdaA-6His). C-terminal fusion was chosen to minimize any effect the His-tag might have on activity of TdaA, as this placement of the His-tag has been used successfully with other LTTRs (25, 103). The cloned region was sequenced to confirm its correctness and the plasmid was then transformed into *E. coli* Rosetta (DE3) (Novagen).

Plasmid pHG1012 and pHG1014 were constructed using plasmid pSRKGm (87) to express either a truncated (pHG1012) or an intact copy of the *tdaAB* operon (pHG1014) from an IPTG-inducible promoter (*lac$_P$*) as follows. pHG1012 was constructed by ligating a PCR fragment, generated using Taq DNA polymerase (New England Biolabs, Beverly, MA) and primers *tdaAB* forward and *tdaAB* reverse (**Table 4.2**), into the *Nde*I and *Hind*III sites on pSRKGm (87). This PCR generated a single base pair deletion at nucleotide 664 relative to the start codon of *tdaA*, resulting pHG1012 bearing a frameshift mutation in *tdaA* leading to a truncated TdaA protein that is missing 55 amino acids from the C-terminus of the protein. pHG1014

**Table 4.2. Oligonucleotides used in Chapter 4.**

| Primers | Sequence (5' to 3') |
|---|---|
| *tdaAB* forward | 5'-AGTACATATGGACATTCAACAGCTAAGAGTCTT-3' |
| *tdaAB* reverse | 5'-CATCAAGCTTGCAGATTCAGCTCAGGCTTAGC-3' |
| *tdaA* RTforward | 5'-AGCGACACCTCTGATCTGACG-3' |
| *tdaA* RTreverse | 5'-TCATCTACCGCGCGAATAGA-3' |
| *tdaB* RTforward | 5'-GCCTCTGTGGCAAAGACGATAT-3' |
| *tdaB* RTreverse | 5'-GACACGCCAAAACGCTCTTC-3' |
| *tdaC* RTforward | 5'-CTGGCACCAACTGTGAAAAGG-3' |
| *tdaC* RTreverse | 5'-CTCAAGTGCCGCAATGCTG -3' |
| *tdaD* RTforward | 5'-CATTTGAGACCCGGATCGAA-3' |
| *tdaD* RTreverse | 5'-GGTATCGCCGTAATAGGCCTG-3' |
| *tdaE* RTforward | 5'-CCTTTGTGCTCAACGGGAAG-3' |
| *tdaE* RTreverse | 5'-AGCGGCACATTCTCAAACG-3' |
| GeneRacer RNA | 5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3' |
| GeneRacer 5' primer | 5'-CGACTGGAGCACGAGGACACTGA-3' |
| *tdaC* RACE RT | 5'-CCAGAAGACCCAGATGCGCGATATG -3' |
| *tdaC* RACE RTa | 5'-CGGTCTTCATGATGAACACA-3' |
| *tdaC* emsaforward | 5'- GTTGGTTTTGGGGCATTTACG-3' |
| *tdaC* emsareverse | 5'-GTCCATTCAAGAGTCCTCCAATG -3' |
| TdaA-His FP | 5'-AGTACATATGGACATTCAACAGCTAAGAGTCTT-3' |

TdaA-His RP          5'- CATCCTCGAGCGCCAATTGCAACAAGGAG-3'

was constructed following the same procedure as described for pHG1012 except that PfuUltra (Stratagene, La Jolla, CA) polymerase was used for this PCR. In both cases, the nucleotide sequence of the resulting clone was confirmed by sequencing.

### 4.3.3 RNA preparation and reverse transcription-PCR (RT-PCR)

Total RNA was extracted using a RiboPure-Bacteria purification kit (Ambion, Austin, TX) according to the manufacturer's protocol. cDNA was synthesized from RNA with a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) following manufacturer's directions. Control reaction mixtures lacking reverse transcriptase were included to confirm the absence of contaminating genomic DNA. The PCR was performed under conditions as described previously (55) with gene specific primers (**Table 4.2**).

### 4.3.4 Rapid amplification of 5' complementary DNA ends (5'-RACE)

5'-RACE was performed using a GeneRacer kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with slight modifications. Briefly, 0.5 U tobacco acid pyrophosphatase (TAP) was used to convert RNA 5'-triphosphates to monophosphates at 37°C for 60 min, after which the reaction was stopped by phenol-chloroform extraction. RNA was precipitated using ethanol-sodium acetate, dissolved in water, ligated with GeneRacer RNA oligonucleotide (**Table 4.2**) at 37°C for 1 h with 5 U of T4 RNA ligase. After extraction and ethanol precipitation, the treated RNA was reverse transcribed (RT) with 2 pmol of reverse transcription *tdaC* primer *tdaC*RACERT (**Table 4.2**). The RT reaction was then treated with RNase H at 37°C for 20 min, and 1 μl of the RT reaction product was used for PCR amplification with

*tdaC* specific PCR primer *tdaC* RACE RTa and GeneRacer 5' primer (**Table 4.2**). Cycling conditions were as follows: 95°C for 3 min; 28 cycles of 95°C for 40 s, 68°C for 40 s, and 72°C for 1 min; 72°C for 8 min. PCR products were separated on an agarose gel and cloned into pCR4-TOPO vector with a TOPO TA cloning kit for sequencing (Invitrogen). Sequence alignments were conducted using Clustal W (151). The putative promoter of *tdaC* was identified using the Neural Network program (http://www.fruitfly.org/seq_tools/promoter.html) (128). I searched for the LTTR consensus binding site, T-n11-A, within an interrupted dyadic sequence using the pattern search tool at http://www.cmbl.uga.edu/software/patloc.html.

### 4.3.5 Overexpression and purification of TdaA

An overnight culture of *E. coli* Rosetta cells containing plasmid pHG1015 was diluted 1:100 in 1000 ml LB broth with ampicillin, and incubated at 37 °C under shaking condition until an $OD_{600}$ of 0.4 was achieved. The bacteria were placed on ice for 10 min to cool, after which, the culture was induced with 1 mM IPTG at 20 °C. After overnight incubation, cells were pelleted by centrifugation at 6000 g for 10 min at 4 °C. Pellets were resuspended in 20 ml of binding buffer A [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 10% (v/v) glycerol, 5 mM imidazole] and then lysed using a French press at 4 °C. Lysed cells were centrifuged at 15,000 g for 15 min, and the supernatant was loaded onto a His-Trap column (GE Healthcare, Piscataway, NJ) which had been equilibrated with buffer A. The column was washed extensively with buffer A to remove unbound proteins, and bound proteins were eluted from the column with a 100 to 500 mM imidazole gradient at a flow rate of 1 ml/min. Purified protein fractions were pooled and dialyzed overnight in 10 KDa molecular weight

cutoff dialysis tubing (Pierce) against 1 l of buffer (20 mM Tris-HCl, 500 mM NaCl, and 10% glycerol) at 4 °C.

### 4.3.6 Electrophoretic mobility shift assay (EMSA)

A 225-bp segment of DNA from -182 to +43 relative to the transcriptional initiation site of *tdaC* was amplified by PCR using 5' biotin labeled primers *tdaC* emsaforward and *tdaC* emsareverse (**Table 4.2**). The PCR product was purified after separation on an agarose gel (Gel Extraction Kit, QIAGEN, Valencia, CA). Reaction mixtures contained 5 nM of biotin-labeled DNA fragments with varying amounts of purified TdaA protein in binding buffer [20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, Tween 20, 0.2 % (w/v), and 30mM KCl]. Binding reactions were incubated for 10 min on ice followed by 20 min at room temperature. Samples were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel (Biorad, Hercules, CA) in 0.5X Tris-borate-EDTA (TBE; 0.44 M Tris base, 0.44 M boric acid, and 0.01 M EDTA [pH 7.6]) which had been pre-run for 1 h. Samples were loaded onto the gel and separated by electrophoresis at 80 V for 2 h at 4°C. Biotin-labeled DNAs in the gel were transferred to a nylon membrane (Roche Applied Science, Indianapolis, IN) at 300 mA for 1 hr using Trans-Blot (CellBiorad, Hercules, CA), immobilized onto the membrane by UV cross-linking using UV Stratalinker 2400 (Stratagene), and then detected with a LightShift chemiluminescence EMSA kit (Pierce Thermo Fisher Scientific, Rockford, IL).

## 4.4   Results

### 4.4.1   Operon structure of *tda* genes

Previously, I showed that *tdaABCDE* and *-F* are essential for TDA biosynthesis (57), that they are found among members of the *Roseobacter* clade (55), and that their genomic organization is conserved as shown in **FIG. 4.1A**. Based on their organization, it appears that *tdaAB* and *tdaCDE* constitute separate operons. To test this hypothesis, primers were designed so that RT-PCR using combinations of *tdaA* and *tdaB*, *tdaB* and *tdaC*, *tdaC* and *tdaE* primers would cross boundaries between the genes, as shown in **FIG. 4.1A**. The presence of amplified PCR products from *tdaA*-*tdaB* (**FIG. 4.1B**) indicates that *tdaA* and *tdaB* are transcribed on the same mRNA, while a separate mRNA contains *tdaC*, *tdaD*, and *tdaE*. No PCR product was produced when primers to *tdaB* and *tdaC* were used, indicating that *tdaAB* and *tdaCDE* reside in separate operons.

### 4.4.2   Analysis of the *tdaC* promoter

Based on the RT-PCR results and bioinformatic data, the promoter of *tdaC* is located within the 363 bp intergenic region between *tdaB* and *tdaC*. However, an analysis of this locus failed to identify a known promoter consensus sequence. Therefore, to help find the location of the promoter, I determined the transcription
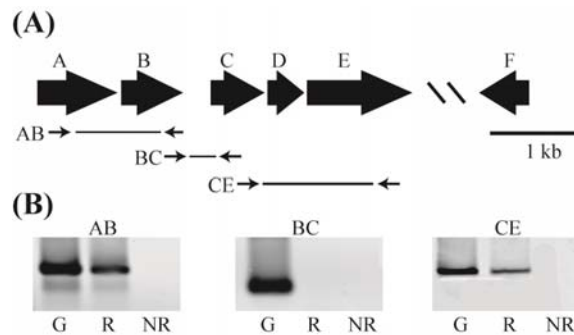
**FIG. 4.1. (A) The *tdaA-F* genes of *Silicibacter* sp. TM1040.** Large black arrows indicate relative size and the direction of transcription of each gene, and the double hash mark indicates that an unknown amount of DNA separates *tdaE* from *tdaF*. Oligonucleotide primers used in RT-PCR to determine transcriptional units are shown as thin arrows under the genes. **(B) RT-PCR results showing that *tdaA* and -B are cotranscribed as an operon and *tdaC, -D, and -E* form a second operon.** cDNA was reverse transcribed (R) from total RNA. Total genomic DNA (G) and no reverse transcriptase (NR) served as respective positive and negative controls.

initiation site of *tdaC* with 5'-RACE using RNA extracted from a standing culture, where *tdaCDE* is highly transcribed (55). The sequence of the 5'-RACE mapped the transcription initiation site to an A located at -100 bp relative to the start codon of *tdaC* (**FIG. 4.2, FIG. 4.3**). A putative promoter sequence is present 33 bp 5' of the transcription initiation site. The -10 element, TATCCG, and -35 sequence, TGAACA, of the putative promoter share sequence similarities to the *E. coli* $\sigma^{70}$ -10 (TATAAT) and -35 consensus sequence (TTGACA) respectively (**FIG. 4.3A**), and are separated by 14-base-pairs (**FIG. 4.3A**).

Since *tdaA* likely encodes a LTTR protein (57), I searched for the LTTR consensus binding sequence, T-$N_{11}$-A, within an interrupted dyadic sequence (137), near the start of transcription initiation and putative promoter of *tdaC*. The region upstream of the putative *tdaC* promoter contains two possible LTTR binding sites: site 1, TG<u>T</u>C-$N_9$-G<u>A</u>TA (underlined characters indicate the conserved T/A sites in the LTTR binding sequence), and site 2, <u>T</u>CAG-$N_5$-CCG<u>A</u> (**FIG. 4.3B**). The presence of two putative LTTR binding sites is consistent with the hypothesis that TdaA regulates transcription of *tdaC* by binding to nucleotides near the *tdaC* promoter.

The databases contain homologs to *tdaC* and *tdaF* from other roseobacters, and the promoter regions of these genes are hypothesized to be similar to that of *tdaC* from TM1040 (55). An alignment of six of these sequences including *tdaC* is shown in **FIG. 4.3B** and shows that both sites 1 and 2 sequences (respectively) are conserved among the different roseobacter homologs of *tdaC* and *tdaF*. Moreover, sites 1 and 2 share a consensus T(C/T)-$N_9$-GA motif in a dyadic region for all aligned DNA sequences except for *tdaF* of *Pseudovibrio* sp. JE062 (062C, in **FIG. 4.3B**).
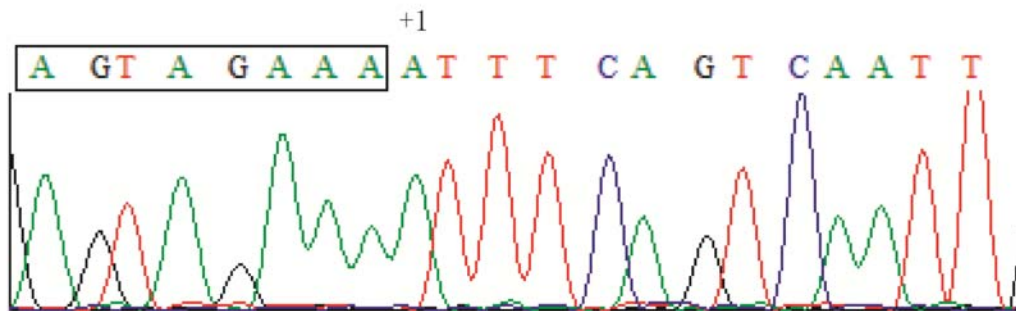
**FIG. 4.2. Sections of a representative DNA chromatogram from the 5'-RACE.**

DNA sequence analysis indicated that the GeneRacer RNA (rectangle) was ligated to

*tdaC* mRNA, mapping the transcription initiation site to the A indicated by "+1".

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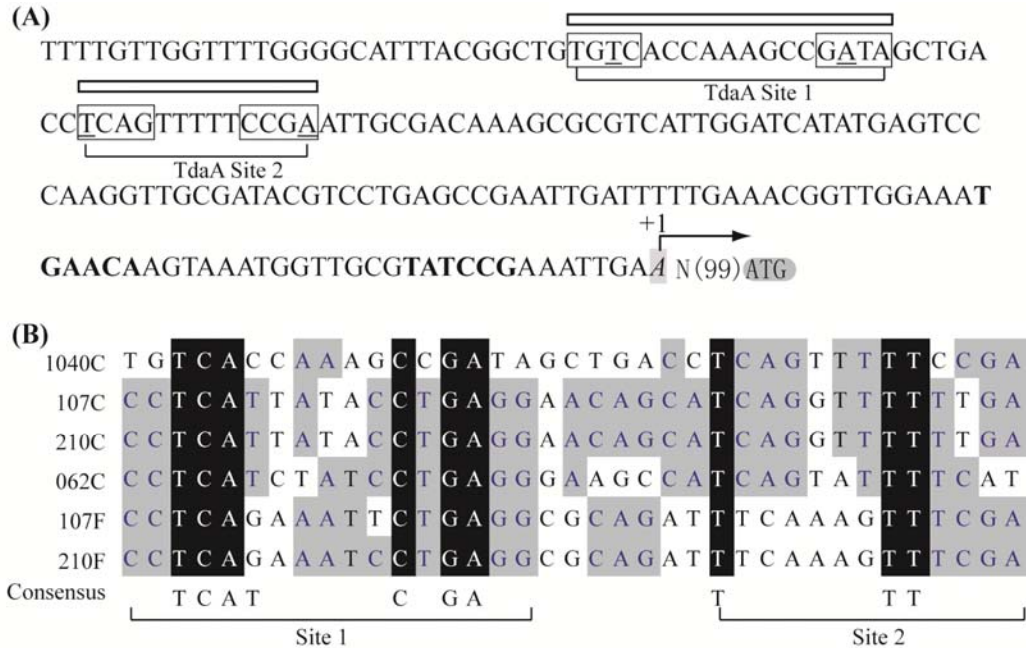
**FIG. 4.3. The 363 bp intergenic region between *tdaB* and *tdaC*. (A) The intergenic region contains two LysR binding sites,** T-N$_{11}$-A within an area of interrupted dyad symmetry (rectangles). The transcription initiation site (+1) as determined by 5'-RACE, the *tdaC* start codon (ATG), and the putative *tdaC* promoter -10 and -35 sequences (bold letters) are indicated. **(B) TdaA binding sites 1 and 2 are conserved in the regulatory regions of genes involved in TDA biosynthesis.** Clustal W alignment of TdaA nucleotide binding sites 1 and 2 in the upstream regulatory region of *tdaC* from TM1040 (1040C), *P. gallaeciensis* BS107 *tdaC* (107C), *P. gallaeciensis* 2.10 *tdaC* (210C), *Pseudovibrio* sp. JE062 *tdaC* (062C), TM1040 *tdaF* (1040F), *P. gallaeciensis* BS107 *tdaF* (107F), and *P. gallaeciensis* 2.10 *tdaF* (210F) are shown from top to bottom in the alignment. The resulting consensus sequence is highlighted in black, whereas gray letters denote partial matches.

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The sequence homology of LysR-binding sequences in the regulatory region of these *tda* genes from taxonomically distinct roseobacters suggests that they share a common mechanism to control transcription that involves TdaA or a homolog of the protein.

### 4.4.3  TdaA regulates *tdaCDE* transcription

Since *tdaAB* forms an operon, insertion of a transposon in *tdaA* is likely to have a polar effect on the transcription of *tdaB*, which encodes a putative bacterial glutathione S-transferase (55). To circumvent this difficulty, a wild type *tdaAB* operon containing sequence from the start codon of *tdaA* to the stop codon of *tdaB* ($tdaA^+B^+$) was constructed such that the expression of this operon was under the control of a *lac* promoter (*lac$_P$*; Materials and Methods), i.e. expression of these genes could be induced by addition of IPTG. A second plasmid bearing the *tdaAB* operon with a mutation in *tdaA* ($tdaA^-B^+$) was also constructed using the same IPTG-induction scheme. This resulted in two plasmids: pHG1014 (*lac$_P$*::$tdaA^+B^+$) and pHG1012 (*lac$_P$*::$tdaA^-B^+$) (Materials and Methods). These plasmids were individually moved into (1) a *tdaA* transposon insertion strain (HG1310; *tdaA*::EZ-Tn*5*), (2) a *tdaB* transposon insertion strain (HG1015, *tdaB*::EZ-Tn*5*), and (3) a *tdaC* transposon insertion strain (HG1080, *tdaC*::EZ-Tn*5*). Each of these strains also carried a plasmid (pHG1011; *tdaCp::lacZ*) bearing a transcriptional fusion between the promoter region of *tdaC* and a promoterless *lacZ* that we used previously to measure expression of *tdaC* (55). **FIG. 4.4** shows the results produced by each of these strains when β-galactosidase (*tdaC* expression) and pigment production (coincident phenotype associated with TDA biosynthesis) were measured. When either
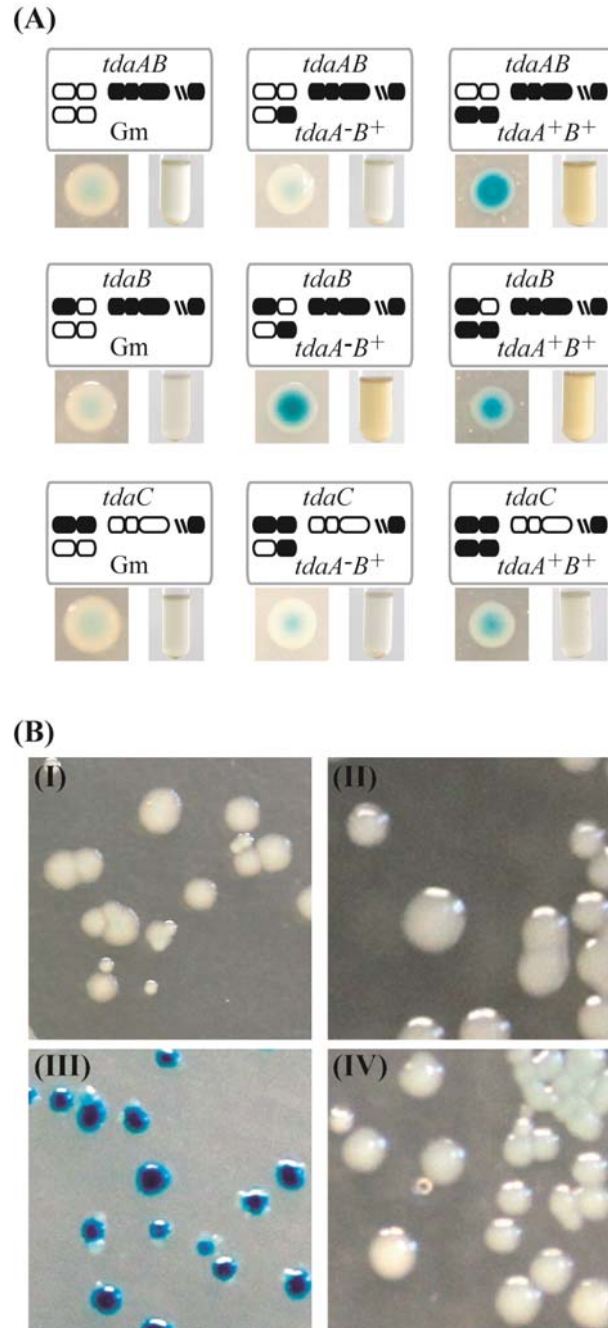
**FIG. 4.4. (A) Upper three panels: Expression of *tdaC* requires intact *tdaA*.** A *tdaCp::lacZ* reporter plasmid was placed into HG1310 (*tdaA*::EZ-Tn*5*; TdaA⁻ TdaB⁻). Into this strain were placed a plasmid control (pSRKGm; labeled Gm), a plasmid containing *tdaA⁻ tdaB⁺*(*lacP*::*tdaA⁻B⁺*; labeled *tdaA⁻B⁺*), or a plasmid containing *tdaA⁺ tdaB⁺* (*lacP*:*tdaA⁺B⁺*; labeled *tdaA⁺B⁺*). Expression of *tdaC* is indicated by a

blue colony resulting from the cleavage of X-Gal by β-galactosidase (lower left image in each panel). TDA production was assessed by measuring the amount of brown pigment produced by the culture (lower right image in each panel) whose synthesis is correlated with TDA production (16, 20, 57). In a *tdaAB* background, expression of *tdaC* is dependent on the presence of *tdaA* (compare upper middle to upper right panels). Controls: Middle three panels are the same set of plasmids in a *tdaB* background and demonstrate that cells with wild-type *tdaA*$^+$ *tdaB*$^+$ genes have *tdaCp::lacZ* expression and TDA production, while the lower three panels show the lack of *tdaC* expression when the same set of plasmids is placed in a *tdaC* strain that does not synthesize TDA and therefore has little or no *tdaCp::lacZ* expression. **(B) Expression of *tdaC* is *tdaA*-dependent in *E. coli*.** The same three plasmids as used in (A) were placed in an *E. coli* DH5α background. Panels: I = pSRKGm; II = *lac*$_P$::*tdaA*$^-$*B*$^+$; III = *lac*$_P$::*tdaA*$^+$*B*$^+$; and IV = no plasmid control.

pHG1012 (*lac$_P$::tdaA$^-$B$^+$*) or pHG1014 (*lac$_P$::tdaA$^+$B$^+$*) was placed in the *tdaC* mutant, there was little or no detectable β-galactosidase activity, i.e., there was a dramatic loss of *tdaC* expression. This was expected as previous studies have shown that transcription of *tdaC* is dependent on the presence of TDA, and defects in *tdaC* result in a loss of TDA synthesis (55). Strains with a defect either in *tdaB* (**FIG. 4.4A**; middle row) or in both *tdaA* and *tdaB* (**FIG. 4.4A**; top row) could be complemented with plasmids bearing the respective wild-type gene(s) when IPTG was added to the culture. Complementation also resulted in the production of pigment (**FIG. 4.4A**; lower right image in each panel) and synthesis of TDA (data not shown). However, strains without a wild-type copy of *tdaA* failed to express *tdaC* and lacked detectable β-galactosidase activity as illustrated in **FIG. 4.4**, top row middle column. These results demonstrate the requirement for both TdaA and TDA in the transcription of *tdaC*, and suggest that TdaA positively controls *tda* gene expression.

I next asked if TdaA in the absence of TDA, a known inducer of *tda* gene expression (55), affects *tdaC* transcription. This was tested by transforming a heterologous (non-TDA-producing) bacterium, *E. coli*, with a plasmid expressing TdaA (pHG1014, *lac$_P$::tdaA$^+$B$^+$*) and a second plasmid (pHG1011, *tdaCp::lacZ*) harboring a *tdaCp::lacZ* transcriptional fusion, as used in the previous experiment. When *tdaA* expression was induced by IPTG, the *E. coli* cells produced β-galactosidase and turned visibly blue, indicating expression of *tdaC* had occurred (**FIG. 4.4BIII**). The controls, pSRKGm without insert (**FIG. 4.4B I**), cells alone (**FIG. 4.4B IV**), or cells lacking a wild-type copy of *tdaA* (pHG1012, *lac$_P$::tdaA$^-$B$^+$*) (**FIG. 4.4B II**), were LacZ$^-$. These results confirm that TdaA is required for

transcription of *tdaC*, and surprisingly demonstrates that transcription of *tdaC* in *E. coli* does not require TDA.

### 4.4.4   TdaA binds to DNA near the promoter of *tdaC*

The results from the previous experiments, combined with bioinformatics about the domain architecture of TdaA (57), suggest that the molecular mechanism by which TdaA up-regulates *tdaC* expression occurs via TdaA binding to DNA near the putative *tdaC* promoter. This hypothesis was tested in an *in vitro* EMSA using purified TdaA protein (**FIG. 4.5**) and a DNA fragment bearing the *tdaC* promoter region. As shown in **FIG. 4.6A**, increasing amount of TdaA reduced the mobility of *tdaC* DNA in a concentration-dependent manner. The shift in *tdaC* mobility was unaffected by addition of either a nonspecific protein (BSA; **FIG. 4.6B**), nonspecific DNA (herring sperm DNA; **FIG. 4.6C**), or purified TDA (**FIG. 4.6D**). However, an excess of unlabelled *tdaC* DNA added to the reaction abolished the observed shifts (**FIG. 4.6E**). These results give further credence to the idea that TdaA binds to the promoter of *tdaC* causing an increase in the transcription of *tda* genes, and further supports previous results in *E. coli* that TDA is not required for this process.
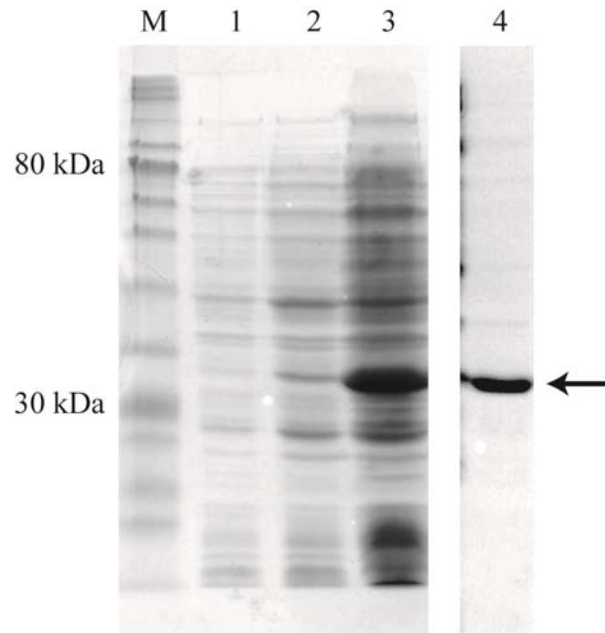
**FIG. 4.5. Overexpression and purification of TdaA protein.** (A) SDS-PAGE of soluble fraction of whole-cell lysate in uninduced (lane 1), induced 37°C for 2 hr (lane 2), induced 20°C for 20 hr (lane 3) and affinity-purified TdaA-6His protein (lane 4). Electrophoretic mobility of protein standards is loaded in lane M.
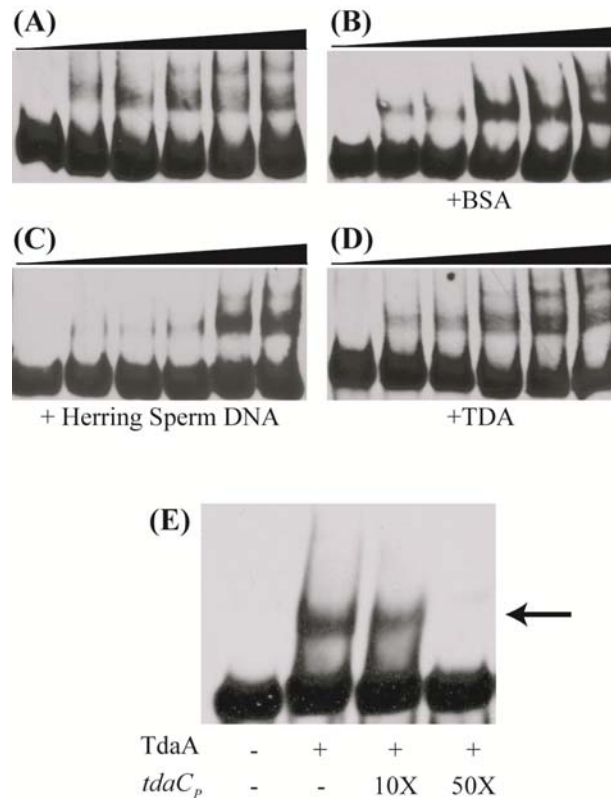
**FIG. 4.6. Electrophoretic mobility shift assays of TdaA binding to DNA containing the promoter of *tdaC*.** (A) Increasing concentration of TdaA (0, 10, 25, 50, 100, and 200 nM, from left to right lane in each gel) causes a shift in multiple *tdaC* DNA bands. Addition of BSA (panel B), or addition of non-specific competitor DNA (herring sperm DNA; panel C), or addition of 500 nM HPLC-purified TDA (panel D) do not affect the shift in *tdaC* DNA. (E). Addition of cold, unlabeled DNA containing the promoter of *tdaC* results in a reduction in the amount of shifted, labeled *tdaC* DNA. These results show that TdaA binds to tdaC promoter DNA specifically and the binding does not require TDA as a co-effector ligand.

## 4.5   Discussion

TDA has antibiotic activity against many non-roseobacters, and the compound is also an inducer of *tda* gene expression (55). Thus, appropriate sensing and response to TDA are likely important factors in the survival and adaptation of roseobacters in the marine ecosystem. The results obtained in the present study support a model whereby one of the mechanisms used by TM1040 to regulate TDA biosynthesis is through TdaA binding to DNA sequences near the promoter region of target genes and thereby acting as an activator of *tda* gene expression.

Since TDA synthesis and *tda* gene expression are maximally expressed in stationary phase (55), I expected to find promoter sequences commonly associated with starvation or stationary phase. Therefore, I were surprised to find a match to a $\sigma^{70}$ (RpoD) promoter in the regulatory region of *tdaC*. The putative *tdaC* promoter is not a perfect match to an *E. coli* $\sigma^{70}$ consensus sequence, with only a 50% match of the *tdaC* promoter -10 element and a 66 % match of -35 element to the respective elements of the *E. coli* $\sigma^{70}$ promoter. It is not unusual to see this degree of mismatch to a promoter consensus sequence at both sites (17), rather the differences suggest that the *tdaC* promoter sequence is optimized for TDA biosynthesis and the physiological requirements the cell needs to make this compound. Moreover, $\sigma^{70}$ promoters also orchestrate the transition from exponential to stationary phase growth often with the help of other regulatory elements (67) and are also involved in the production of antibiotics, such as observed in *Pseudomonas fluorescens* CHA0 (138), giving further support to this finding.

LTTRs are known to bind to target-regulatory promoter regions, often occupying several binding sites (153), such as RBSs, which have conserved T-$N_{11}$-A motifs in regions of interrupted dyad symmetry (137). Our analysis of the promoter of *tdaC* suggests that the best matches to the LTTR RBS conserved binding motif are TGTC-$N_9$-GATA (site 1) centered at -146 and TCAG-$N_5$-CCGA (site 2) centered at -126 relative to the transcription initiation site (**FIG. 4.3**). The location of these putative TdaA binding sites in the *tdaC* promoter region is unusual, since LTTRs binding sites are typically centered at -55 (105); however, atypical LTTR binding sites have also been identified in the promoter regions of other genes and can be located from -218 bp to +350 bp in various LysR regulators (105). Despite their unusual position, sites 1 and 2 of *tdaC* are found at the same respective positions in the promoter region of several genes from other TDA-producing marine bacteria (**FIG. 4.3B**). The conservation of these sequences within the regulatory regions of TDA-synthesis genes enforces a belief that they play a role in *tda* gene expression and suggests that they are most likely sites for TdaA binding.

The results from our DNA binding assays (EMSAs) show a TdaA-concentration-dependent decrease in the mobility of a *tdaC* promoter fragment (**FIG. 4.6**), indicating that TdaA binds to this DNA. Interestingly, increasing the concentration of TdaA also resulted in a reproducible increase in the number of shifted DNA bands in the EMSA. There are several plausible explanations for the occurrence of these multiple shifted bands. First, our analysis suggests that the *tdaC* promoter region contains more than one TdaA binding site. If the sites had different affinities for TdaA, one would expect a concentration-dependent change in the

number of shifted bands, which is what is observed. Second, different oligomers of TdaA may binds to *tdaC*$_P$ DNA in a concentration-dependent manner. For example, hypothetically dimers of TdaA may have greater affinity to bind the DNA than do tetramers of the protein, the commonly active form of most LTTRs (105). Third, extra shifted bands could be caused by changes in bending of *tdaC* DNA. This has been observed in the binding of other LTTRs (105).

Despite its role as an autoinducer of *tda* gene expression (55), several lines of evidence presented here support the hypothesis that TDA is not necessary for TdaA-dependent expression of *tdaC*. For example, a *tdaCp::lacZ* reporter produces a slightly blue colony on X-Gal-containing medium when plasmid-borne *tdaA* is induced by IPTG even in a *tdaC* mutant background in which no TDA is produced (**FIG. 4.4**). TDA also does not change the binding of TdaA to *tdaC* promoter DNA, as is seen in the EMSA results (**FIG. 4.6**). Furthermore, TDA is not required for TdaA-dependent expression of *tdaC* in *E. coli*. Thus, the simplest explanation for these results is that TDA is not essential for TdaA-dependent *tdaC* expression. Yet, our previous report demonstrating that TDA acts as an autoinducer (55), coupled with the results herein (**FIG. 4.4**) showing that β-galactosidase activity from a strain containing wild-type *tdaC*$^+$ is enhanced when compared to a strain lacking TDA (*tdaC*$^-$), clearly show that TDA enhances *tdaC* expression. How can this contradiction be resolved? One simple explanation is that IPTG-induction of *tdaA*$^+$ results in a physiologically excessive amount of TdaA, and at such high levels, TdaA activates *tdaC* expression in the absence of TDA. Such enhancement of transcription caused by high levels of an LTTR has been reported *Neisseria meningitides* CrgA (78) and *E.*

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*coli* NhaR (124). Similarly, it is possible that *E. coli* produces a TDA-like molecule that may compensate for the lack of TDA, but I have not detected such a molecule or activity. Thus, it appears that induction of *tda* gene expression by TDA occurs through a path or mechanism that does not directly involve TdaA, perhaps via the activities of TdaI, a hybrid histidine kinase (55) or other regulator, such as FlaC (11).

In conclusion, the results underscore the importance of TdaA as a regulator of *tda* genes, whose activity requires protein binding to *tda* promoter DNA. I speculate that TdaA is relatively low in the hierarchy of proteins controlling *tda* gene expression, with other regulatory proteins, such as FlaC and CtrA (11) situated above TdaA and perhaps controlling *tdaA* expression. A fuller appreciation of this regulatory hierarchy and how it interacts to affect the swim-to-stick lifestyle switch, is certain to lead to better understanding of the events and processes required for initiation, development, and final dissolution of the symbiosis between marine roseobacters and their phytoplankton hosts.

# Chapter 5:   The effects of TDA on the growth of algae

## 5. 1   Summary

Members of the *Roseobacter* clade of marine bacteria are abundant and often associated with phytoplankton that cause harmful algal blooms (HABs). The roseobacter TM1040 and the heterotrophic dinoflagellate *P. piscicida* (CCMP 1830) form a mutualistic symbiosis, e.g., *P. piscicida* has an obligate requirement for TM1040. TM1040 cells form a biofilm on the surface of *P. piscicida* where they obtain sulfur and carbon from 3'- dimethylsulfoniopropionate (DMSP), an abundant organic sulfur compound produced by dinoflagellate and other HAB species. TM1040 and other roseobacters utilize DMSP to produce the sulfur-containing compound tropodithietic acid (TDA) that has antibiotic and quorum sensing activity. TDA is hypothesized to be a critical component of the roseobacter-phytoplankton symbiosis. Addition of TDA to a culture of *P. piscicida* increased the final biomass of the dinoflagellate. Surprisingly, TDA addition also increased the biomass of *Rhodomonas* sp. (CCMP 768), the cryptophyte prey alga consumed by *P. piscicida*. Adding back defined mutants of TM1040 or TM1040 strains with IPTG-inducible *tda* expression confirmed the correlation between TDA level and *P. piscicida* and *Rhodomonas* biomass. The results suggest that TDA production by roseobacters benefits mixotrophic and heterotrophic dinoflagellates indirectly through physiological effects that increase the biomass of the cryptophyte prey that these

dinoflagellates consume. The possible impact TDA may have on the dynamics of HAB communities is discussed.

## 5. 2    TDA increases *P. piscicida* biomass in the culture

I hypothesized that TDA may benefit the symbiosis between TM1040 and *P. piscicida* due to the potential multiple functions of TDA mentioned in the Introduction. Therefore, I prepared semi-purified TDA, Fraction 3, which is in methanol after column purification from TM1040 broth. Fraction 3 contained TDA (ca. 80 % of total mixture) but also had other minor compounds, as shown in HPLC chromatogram **FIG. 5.1.**

Next, I added semi-purified TDA (Fraction 3, F3) into a culture of *P. piscicida* CCMP 1830 containing zoospores, the native microbiota, and *Rhodomonas sp.* CCMP 768. *P. piscicida* was fed with axenic *Rhodomonas* to a final density of *P. piscicida* ca. 2500 cells/ml and ca. *Rhodomonas* 12,500 cells/ml (5:1) with a light-dark cycle of 14 h light and 10 h dark. TDA was added to final concentration ca. 2.5 μM, and an equivalent volume of methanol (2 μl) solvent was added to a 5 ml culture as a control. On days 1, 4, 6, 9 and 12, 100 μl cultures was removed and *P. piscicida* and *Rhodomonas* were stained with Lugol's  acid solution, and the density (cells/ml) of each was determined using a hemacytometer (Corning) (110). The experiments were repeated four times, and one represented set of experiment results is shown in **FIG. 5.2.** All showed that the addition of semi-purified TDA fraction increased 50 %, the final cell density of *P. piscicida* in the tested date (day 4, 6, 9 and day 12) significantly ($p < 0.05$), compared to *P. piscicida* culture without semi-purified TDA addition.
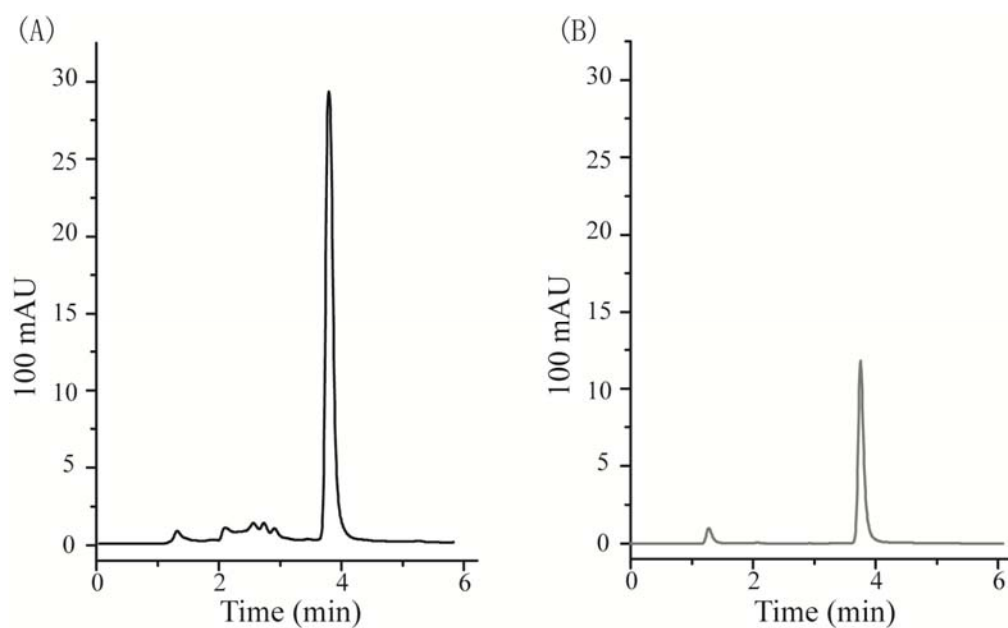
**FIG. 5.1. Comparison of HPLC chromatography of fraction 3 (A) and HPLC purified TDA (B) indicated that the resulting purified TDA was devoid of impurities. The major peak in (A) and the only peak in (B), after injection peak which is 1.6 minn (B), to TDA peak with retention time 3.8 min.**
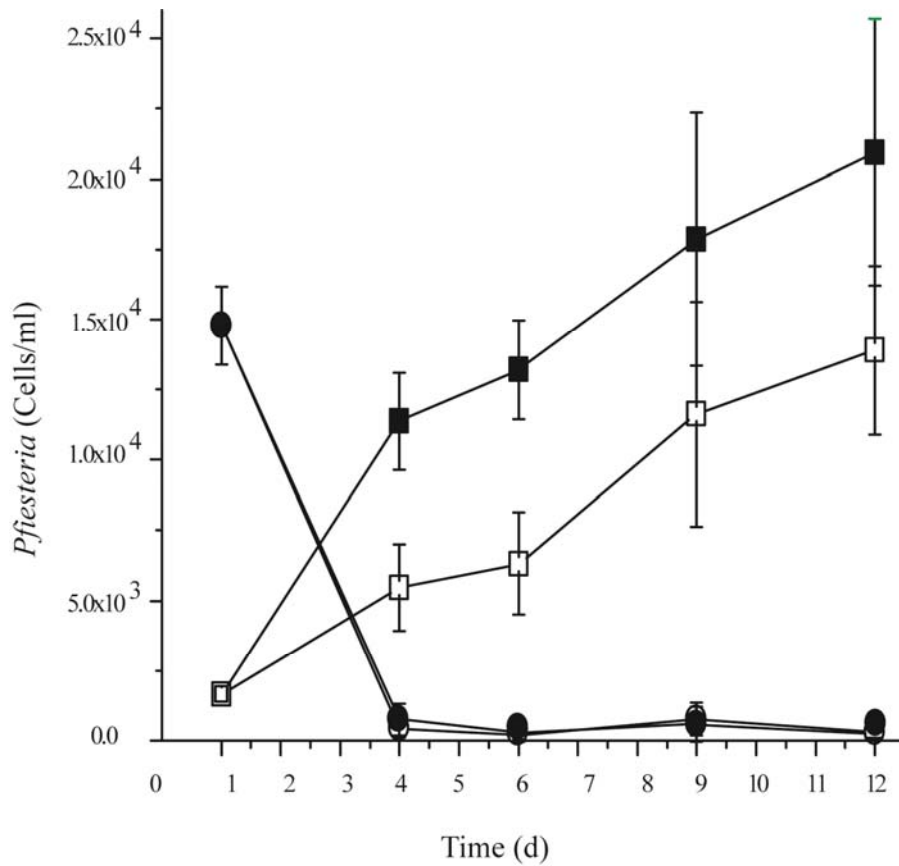
**FIG. 5.2. Semi-purified TDA effect on the *P. piscicida* growth.** *P. piscicida* (■, □) 2500 cell/ml was fed with *Rhodomonas* (●, ○) 125,000 cell/ml (5:1) in the presence (■, ●) or absence (□, ○) of fraction 3 (semi-purified TDA). The graph is constructed by plotting the mean (±S.D.) of cell density over the 12 day period. It suggested that addition of fraction 3 solution increase *P. piscicida* cell densities in the observed days.

## 5. 3  TDA increases *Rhodomonas* biomass in the culture

*P. piscicida* is a heterotroph and consumes *Rhodomonas*, thus I reasoned that an increase of the prey numbers may have caused the observed increase in the number of *P. piscicida*. This hypothesis was tested by measuring the cell density of the prey algae when fraction 3 was added. As shown in **FIG. 5.3**, higher *Rhodomonas* growth was achieved in cultures to which semi-purified TDA was added when compared to cultures without TDA (**FIG. 5.3.**).

As a control for the previous experiment, I tested the hypothesis that the addition of fraction 3 from a loss-of-function Tda⁻ mutant would not change the growth of *Rhodomonas.* Three strains were used: TM1040, HG1213/pRK415 (*malY*::EZ-Tn*5* harboring plasmid pRK415), and HG1213/pHG1005 (*malY* mutation with pRK415 harboring a wild-type copy of *malY*⁺). To confirm that pHG1005 complemented the *malY* defect in HG1213, the antibacterial activity of spent medium filtrate (0.22 μm MCE membrane) was measured using the well diffusion assay with *Vibrio anguillarum*, as described by Bruhn *et.al* (18, 20). Production of pigment, which is correlated with TDA production, was measured spectroscopically at $OD_{398}$. The complement strain (HG1213/pHG1005) showed pigment production and regained TDA production (**FIG. 5.4.**), which was confirmed by HPLC analysis (data not shown).
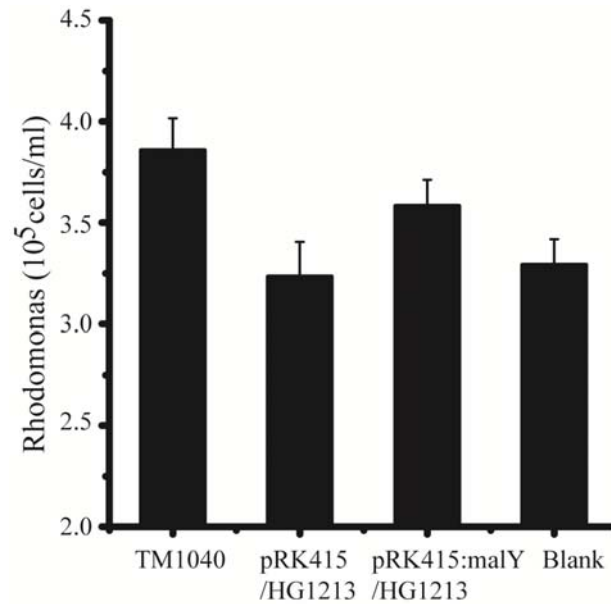
**FIG. 5.3. The effect of fraction 3 from different strains on *Rhodomonas* growth.** Initial *Rhodomonas* 12,500 cell/ml culture was amended with different fraction 3 from TM1040, HG1213/pRK415, HG1213/pRK415::*malY* and blank control with equivalent solvent methanol (2 μl) in 5 ml *Rhodomonas* culture. *Rhodomonas* cell numbers were recorded after 3 days. Statistical analysis showed that solution from TM1040 is significantly higher than blank control or than HG1213/pRK415 solution ($p<0.05$). However, the different *Rhodomonas* cell numbers resulting from HG1213/pRK415::*malY* and from pRK415/HG1213 were insignificant ($p>0.05$).

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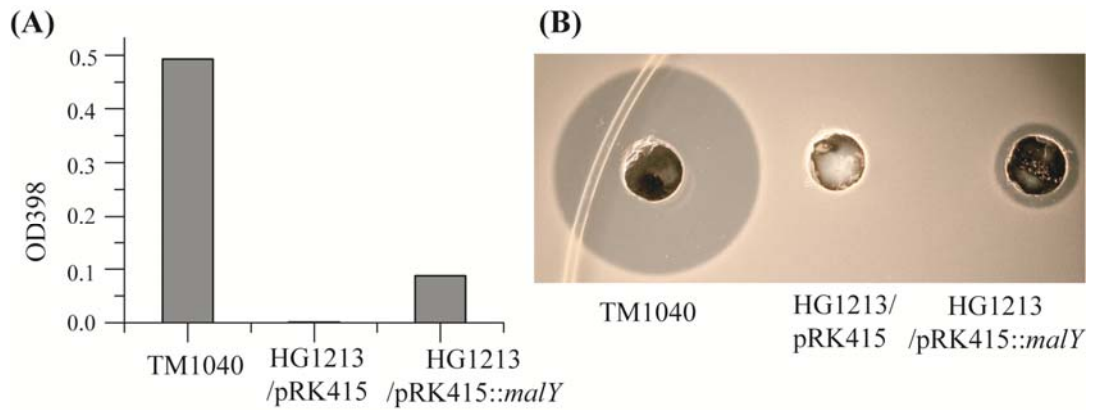
**FIG. 5.4. Pigment production (OD$_{398}$) and antibacterial activity in TM1040 (wt) and its derivatives.** HG1213 (*malY*::EZ-Tn*5*) is TM1040 with a transposon (EZ::Tn5) insertion in the *malY* gene. HG1213/pRK415 is HG1213 mutant with a plasmid pRK415. HG1213/pHG1005is HG1213 with malY on pRK415. It showed that *in trans* copy of *malY* complement HG1213 host partial pigment and antibacterial activity.

Fraction 3 was obtained from each of the three strains and added to *Rhodomonas* culture (**FIG. 5.3**) to a final concentration of 2.5 µM TDA for TM1040 or equivalent amount for the other two strains. The growth of the algae was measured by hemacytometer. As shown in **FIG. 5.3**, addition of extracted fraction 3 from TM1040 significantly increased *Rhodomonas* growth by ca. 20 % compared to fraction 3 from HG1213/pRK415, $P=0.04$) or a blank control (Blank, $P=0.03$). Higher yield of the algae was observed when fraction 3 from HG1213 mutant containing plasmid pHG1005 in which *malY* was cloned to pRK415 was added to the culture; however, this increase was not statistically significant ($P=0.09$).

The direct effect of TDA on algal growth was measured by addition of HPLC-purified TDA to the *Rhodomonas* culture. After TDA addition, the culture was grown for an additional 3 days after which the density of the algae was measured using a hemacytometer. The results, shown in **FIG. 5.5**, indicate that growth of *Rhodomonas* increased with increasing concentration of TDA up to 0.45 µM, reaching a plateau at 2.26 µM TDA. Higher concentrations of TDA, i.e., 11 µM, resulted in a decrease in algal cell numbers (**FIG. 5.5.**), presumably due to changes in concurrent unfavorable environmental or physiological conditions. I reason that such high concentration of TDA is unlikely to be found in marine niches. Usually, marine bacterial density is $10^5 \sim 10^6$ cells/ml in marine dinoflagellate culture. Assuming that the level of TDA production in the laboratory, i.e. ca. 80 µM TDA for cell density of $10^9$ cells/ml, is the same as in environment, I would expect the concentration of TDA to be at most in the range of 0.1 µM~1 µM in the environment, a concentration that augments the
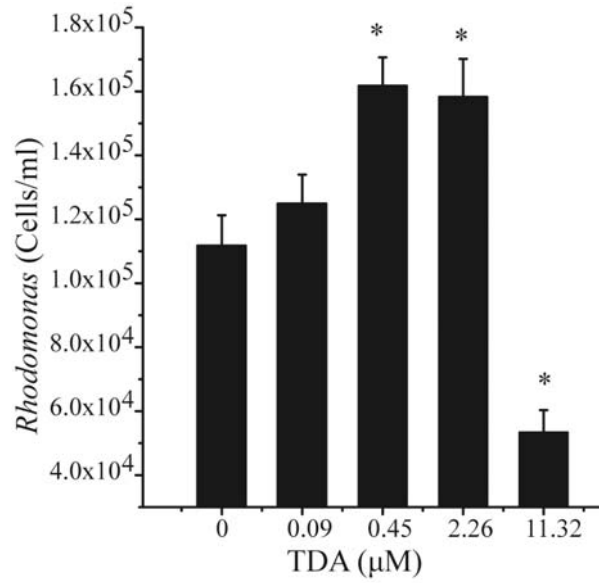
**FIG. 5.5. The *Rhodomonas* cell biomass response to purified TDA is dose dependent.** *Rhodomonas* 12500 cell/ml culture was applied with various amounts of TDA added. The data represent the *Rhodomonas* cell numbers mean±standard deviation (SD) after 3 days light: dark photo period in culture room. Asterisks indicate that the mean of that sample is significantly different from the mean of no TDA addition control (*P*<0.05, ANOVA).

growth of algae. Taken together, these data show that algae respond to the addition of TDA by increased cell density.

## 5. 4   Discussion

The results suggest that TDA positively affects the growth of *Rhodomonas* algae, increasing the final biomass of phytoplankton by approximate 60%. Stimulation of algal growth by TDA may influence harmful algal bloom (HAB) dynamics, and thus could be important to HAB ecology. It highlights the importance of TDA synthesis and regulation in bacteria symbioses with DMSP-producing phytoplankton.

One of the unexpected results presented in this chapter is that TDA enhancement on *P. piscicida* growth only occurs between day 0 and day 4 (**FIG. 5.2**). There are several possible reasons for this unusual phenomenon. For example, prolonged TDA exposure to strong light could disrupt its activity. It is also likely that TDA allocated to *P. piscicida* per cell declined due to 20 fold algal cells at day 4 relative to day 1. The decrease of TDA per cells likely lowered TDA concentration to below the effective value that is needed for enhancing algal growth.

While data here demonstrated that pure TDA enhances *P. piscicida* biomass, I have not addressed the question of what is the physiologically relevant range of TDA to the algae culture. Future experiments could extend this investigation by using the inducible TDA$^+$ strain to produce the antibiotic. TDA addition could be controlled by varying the IPTG inducer concentration, so that TDA concentration can be tested over a physiologically relevant range. So, this experiment could allow TDA to be "administered" directly to the dinoflagellate by the TM1040 biofilm on its surface,

thereby increasing the TDA delivery efficiency. It would be important to measure dynamics of bacterial TDA gene expression *in situ* during the development of symbiosis. This question could be tested using the *tdaC* transcriptional fusion plasmid that provides output via fluorescence. This reporter strain could read out activity of the *tdaC$_p$* promoter, indicating TDA gene expression *in situ* in bacteria. The successful completion of this future research will improve understanding of bacteria-algae interaction at the molecular level.

# Chapter 6: Discussion

TDA is an antibiotic and signaling chemical whose properties may be a critical component of the roseobacter-phytoplankton symbiosis. Using a combination of genetic and molecular biology approaches, I investigated the molecular mechanisms controlling TDA synthesis, with an ultimate goal to better understand the molecular mechanisms used by TM1040 in its symbiosis with phytoplankton. In the process of completing this research, I identified the genes required for TDA synthesis, determined the physiological, environmental, and genetic factors that modulate expression of *tda* genes, and characterized the molecular mechanisms of *tda* genes regulation. These results increase our understanding of the signal transduction and regulatory mechanisms used by roseobacters, and improve our knowledge of the interactions between roseobacters and phytoplankton.

## 6.1    *tda* genes and their potential products

### 6.1.1    TDA Biosynthetic pathway model

The backbone of TDA is a seven member aromatic tropolone ring, which is highly significant because tropolone derivatives, notably hydroxylated forms, are widely seen as medically important sources of antibacterial, antifungal, antiviral, and antiparasitic agents (24, 115, 116, 134). Significantly, TDA essential  genes include phenylacetic acid (PAA) and sulfur metabolism, which confirms the hypothesis from biochemical precursor feeding experiments that TDA is synthesized from oxidative ring expansion of phenylacetic acid (28, 149); specifically the identification of

*paaIJK* and *tdaC* (prephenate dehydratase), indicate that TDA biosynthesis originates from the shikimate pathway and proceeds through phenylacetate (**FIG. 6.1**). The results also emphasize phenylacetate-CoA and CoA metabolism are vital to TDA production and offer a suggestion that TdaB-F are involved in a ring expansion reaction which converts PAA-CoA to the seven-member tropolone ring (step 8 in **FIG. 6.1**). TdaB, a homolog of glutathione S-transferase, may function in the addition of sulfur to the nascent TDA molecule, but its role (as are the roles of all these proteins) is merely speculative at this point. Chemical synthesis of tropolone and derivatives can be difficult, making natural sources of tropolone precursors often the preferred choice as starting material for the synthesis of new tropolone antibiotics.

As the synthesis pathway illustrated in **FIG. 6.1**, I reasoned that disruption of a structure gene, e.g. *tdaB*, will likely result in accumulation of upstream intermediates that is predicted to be novel compound. Thus, several of the mutants obtained in this study may be useful for the development of bacterial sources of medically important tropolone compounds and a suite of new antimicrobial agents based around TDA.

### 6.1.2 pSTM3 plasmid

The *tdaA-F* genes are carried on a plasmid, pSTM3, which can be spontaneously lost. The occurrence of TDA encoding plasmid similar topSTM3 like plasmids in other roseobacters, hints that pSTM3 also may be transferred to other marine bacteria, perhaps other roseobacters. TM1040 has many ways to achieve horizontal gene transfer, including the presence of several prophage genomes in the bacterium's
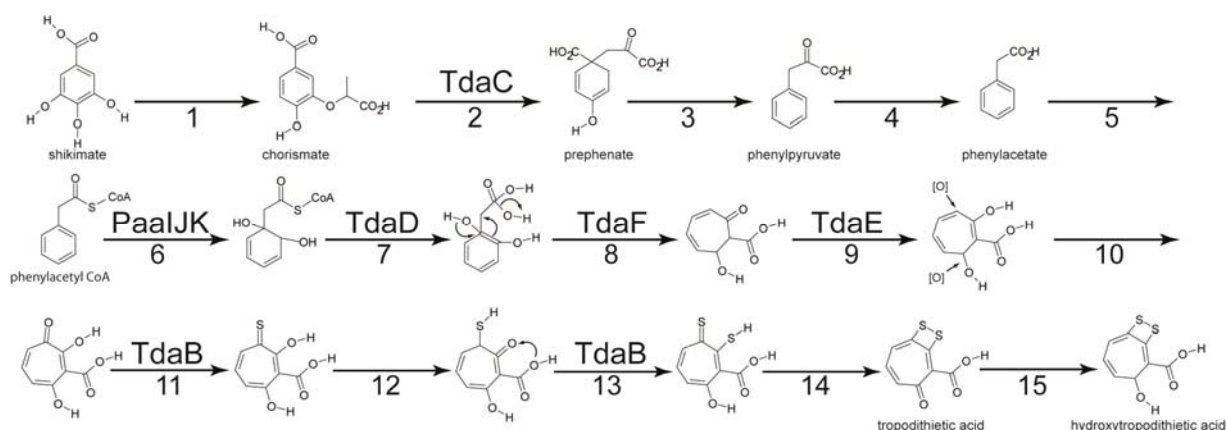
**FIG. 6.1. Working model for the molecular genetics of the TDA biosynthetic pathway.** The suggested pathway involved phenylacetate derivation from shikimate-chorismate and degradation pathway providing precursors (step 1~6) and an core oxidative ring-expansion pathway forming the seven carbon tropone skeleton (step7~10) followed by sulfur-oxygen exchange (step11~15),consistent with the proposed TDA synthesis based on chemical labeling studies in *Pseudomonas* CB-104, where TDA is produced by ring expansion of phenylacetic acid, and final oxidation to generate a 4-hydroxytropolone carboxylic acid (28), which could be the yellow-brown pigment. The protein assignment was based on predicted functions.

genome, one of which is homologous to the gene transfer agent of other alphaproteobacteria (GTA) (32), and many of genes on pSTM2 (113) are homologs of the *vir* system of *Agrobacterium tumefaciens* (35). Partial sequence of pSTM3 in TM1040 indicates that pSTM3 harbors a replication *repC* gene upstream of *tdaA*. RepC along with RepAB is required for plasmid replication and maintenance (104). Similarly, the *A. tumefaciens* Ti plasmid, transferred by Vir Type IV secretion, requires RepABC (33) hinting that a similar mechanism may allow pSTM3 transfer to other roseobacters. Plasmids similar to pSTM3, such as pSymA of *Sinorhizobium meliloti* (9) and the Ti plasmid (35) which are also from the *repABC* plasmid family (29), are important for the proper interaction of those bacteria and their respective hosts, arguing that TM1040 pSTM3 and pSTM2 may serve to enhance the TM1040-dinoflagellate symbiosis.

*tda* genes, which are an indication of the presence of the pSTM3-likeplasmid in the environment, were searched in metagenomic database. The occurrence of homologous Tda proteins in the CAMERA metagenomic library were fund to be low presumably due to low sequence depth. This paucity of *tda* genes in the environment makes it difficult to interpret what is the distribution and abundance of *tda* genes in the environment. Yet, answers to this issue may be obtained through the use of *tda* genes as probes on a global collection of roseobacters. For example, *tda* genes are found in several marine bacteria, e.g. *P. gallaeciensis* BS107*, P. gallaeciensis* 2.10, *Pseudovibrio* sp. JE062 and *Phaeobacter* 27-4. Furthermore, the occurrence of TDA in other bacteria underscores its potential biological roles for roseobacters.

Significantly, the genetic organization of *tda* genes is conserved among those bacteria, embracing the idea that the biosynthesis and regulation of TDA could be similar based on known TDA producing bacterial genome. *tda* genes may also find use in studies addressing the composition and structure of planktonic marine communities, and in understanding sulfur cycling in the marine environment. My sequence analysis of TDA-encoding plasmid in other marine bacteria, e.g. *P. gallaeciensis* BS107*, P. gallaeciensis* 2.10, revealed plasmids are filled with numerous transport genes, suggesting that they may help cells in transportation of certain substrate, like TDA, or plasmid pSTM3 or protein. Instability of pSTM3 is apparent in lab culture. However, if pSTM3 plasmid were to be maintained in laboratory culture, transposon mutants in the accessory genes in the plasmid pSTM3 could be selected for maintaining the pSTM3 plasmid.

## 6.2    Regulation of *tda* genes

### 6.2.1    Cultural and environmental conditions

I observed that the choice of culturing method greatly influenced the physiology of the bacteria and altered the composition of the population. This lifestyle-dependent regulation argues for accessory controlling mechanisms in bacteria to integrate environmental cues into TDA-sensing circuits. In doing so, bacteria presumably fine tune their gene expression into and out of the TDA controlled cell physiology. Bacteria could presumably experience less oxygen and higher stress in standing culture than cells in shaking culture.

### 6.2.2 Genetic elements

This is the first genetic investigation to discover genes essential for TDA biosynthesis. These include *paaIJK*, genes for phenylacetate catabolism, homologs of *cysI*, *malY*, and *tdaH* for sulfur metabolisms. Six other genes, *tdaA-F*, are conserved and are specifically involved in TDA biosynthesis. Transcription of the *tdaCDE* operon and *tdaF* is much higher in standing liquid cultures (compared to shaking culturing), whereas the expression of other genes essential for TDA synthesis, i.e., *tdaAB*, *cysI*, *malY*, and *paaIJK*, remains unchanged. Thus, it is likely that the expression of the latter set of seven *tda* genes remains unchanged due to the respective role these gene products (CysI, MalY, and PaaIJK) have in essential metabolic pathways, or, in the case of *tdaA*, in controlling the transcription of *tdaCDE* and *tdaF*.

### 6.2.3 Sulfur sources: DMSP

DMSP is synthesized by algae, probably as an osmolyte, antioxidant or chemical signal (165). TM1040 is a potentially important DMSP consumer in the phycosphere, where DMSP is synthesized from methionine and can reach up to 400 mM in algae **(109)**. Consistently, my data show that 200 μM DMSP enhances bacterial growth and TDA production in artificial sea water. Therefore, concentration of DMSP for TM1040 growth and TDA production is feasible within the phycosphere. While TM1040 catabolizes DMSP by the demethylation pathway for metabolism purpose **(109)**, DMSP does not act as an inducer of transcription of *tda* genes, suggesting that DMSP could be used by TM1040 readily **(109)** and thus likely help TDA production by providing sulfur source efficiently. It is reasonable to

envision that TDA is prevalently produced around DMSP enriched niches, *i.e.* the phycosphere of phytoplankton.

## 6.3 Molecular mechanisms of regulation of *tda*

### 6.3.1 TdaA: a positive regulator controlling *tda* gene expression

TdaA is a positive transcriptional regulator of *tdaCDE* gene expression by binding to the *tdaC* promoter region. Purified TdaA bound specifically to the $tdaC_p$ promoter DNA probe in the absence of TDA. My data demonstrate that TDA acts as an autoinducer. TdaA activation on *tdaC* transcription does not require TDA as a ligand. My results, however, have shown that TdaA is sufficient to activate $tdaC_p$ promoter in TDA-independent manner in *E. coli* DH5α. It is possible that *E. coli* produces a TDA-like molecule that may compensate for the lack of TDA, but I have not detected such a molecule or activity. More likely, molecular components and genetic setting in *E. coli* are different from TM1040. This is possibly that a TdaA responsive repressor co-regulates *tda* gene expression. This hypothesis could be tested by the construction of truncations, deletions and/or mutations within the 5' end of the 363 bp region upstream of *tdaC* that was fused to *lacZ* in the reporter construct. The test could identify a construct that retained the positive activity in *tdaC⁻* mutant, indicating *tdaC* expression independent on TDA production. Alternatively, or in addition, potentially a plasmid library of TM1040 genes could be added to the *E. coli* reporter system such that a repressor could be identified in *E. coli*.

### 6.3.2 Proposed model of *tda* regulation

Here I uncovered key elements of a regulatory circuit controlling TDA sensing as shown in the proposed model in **FIG. 6.2.** In the following section, I compare and contrast my model of TDA signaling with canonical quorum-sensing (QS) systems. I outline core molecular components in the TDA signal transduction cascade, and explore molecular mechanisms which TM1040 may use in relative to classical QS sensing systems.

First, this circuit also involves cytoplasmic regulators for gene expression. The key player is a cytoplasmic regulator known as TdaA. However, TDA seams does not directly interact with this cytoplasmic regulator TdaA, which is different from classical AHL regulation in Gram-negative bacterial QS system, where AHL is sensed by the cytoplasmic receptor and AHL-bound LuxR regulates gene expression by binding to promoter regions of target genes (118). It is possible that TDA is sensed by membrane-bound histidine kinase receptors results in phosphorylation of a cytoplasmic cognate response regulator, as shown in QS system in Gram positive bacteria as well as in *Vibrio harveyi* (118).

Second, TDA signal relaying is involved in TdaHI proteins, which are encoded on the *tdaHI* operon. TdaH is predicted to be a protein similar to an oxidoreductase and TdaI with N-terminal signal sensor PAS PAC domains is predicted be a hybrid histidine kinase, in which a histidine kinase combines with additional response regulators (REC domain) or histidine-containing phosphotransfer (HPT) domain (121). PAS PAC domains could allow TdaI to sense redox, energy, or

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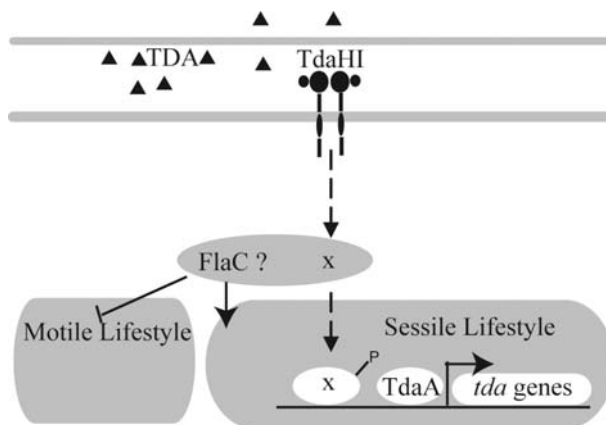
**FIG. 6.2. Working regulatory model for *tdaA* and *tdaCDE*.** The diagram displays the regulatory regions of *tdaCDE* and potential regulatory factors unveiled in the present and previous studies (11, 55). Dashed lines indicate a possible indirect effect. TdaA regulator binds and directly regulates the expression of *tdaCDE*. TDA is synthesized and transported out of cell into environment. Then, TDA is either sensed by TdaHI, conveying signal to response regulator by phosphorelay signal cascade and thereby regulating interaction of TdaA-*tdaC$_p$*, or modified by TdaHI into active form TDA* as a ligand that binds and modulates binding of TdaA to *tdaC$_p$*. Expression of *tdaCDE* may be also influenced by overall bacterial life styles, i.e. single vs. sessile rosette cells, choice of which is controlled by response regulators like FlaC. Signal sensing by *tdaHI* are tentative and not based on experimental evidence.

other environmental conditions and relay this signal to control gene expression (128). Loss of TDA induction in *tdaHI* mutant combined with bioinformatics argue for the postulation that TdaHI could likely be a protein complex for sensing TDA or TdaI could sense signals involved in sessile phase that are essential for *tda* genes expression. Since TdaH protein belongs to an oxidoreductase family, it is possible that TdaH is involved in chemically modifying TDA into an active intracellular form, TDA*. It is also possible membrane-bound histidine kinase TdaI may sense autoinducer signal and relay this signal via phosphorylation of a response regulator into cells (118), as AI-2 is sensed by LuxPQ in *V. harveyi* (118). This kind of signal transduction occurs in a way similar to AI-2 autoinducer, which is sensed by the periplasmic protein LuxP in complex with the LuxQ histidine kinase (118).

Third, TDA production also appears to be influenced by bacterial lifestyle, showing that high TDA production coincides with abundance of sessile cells in standing culture (18). TDA production, hence TDA induction, primary occurs in standing culture where sessile cell dominate. That the quorum sensing signal depends on cell phase is not uncommon, like AI-2 system in *E. coli* (97), production of which is not only regulated by AI-2 signal but also regulated by glucose and cAMP/CRP (100).

Central to the molecular mechanisms underlying these processes are implicated with expression and activities of transcriptional regulators. Swim cells are governed by genetic circuits that involve at least four regulatory proteins: *flaB*, *flaC*, *flaD* (11), and *ctrA* (110). FlaB is hypothesized to be a histidine phosphotransferase; FlaC, a response regulator phosphorylated by an unknown histidine kinase; FlaD, a

MarR like regulator; and CtrA is a homologue of the master regulator CtrA of the bacterium *Caulobacter crescentus* (11). While *flaB*, *flaD*, or *ctrA* cells lose motility and are biased towards sessile phase, *flaC* cells are biased towards a motile phase and away from the sessile phase, suggesting it could modulate the switch of swim/stick biphasic life style (11). FlaC⁻ cells also produce less pigment and less TDA, suggesting FlaC could have an influence on TDA production or *tda* gene expression (11). *flaC* (TM0051) is a monocistronic response regulator gene. FlaC is hypothesized to require phosphorylation to be active (11). FlaC~P could upregulated the genes required for sessile phase like *tda* genes or biofilm genes and represses those required for motility such as genes for motility or flagella synthesis (**FIG. 6.2**).While the cognate histidine kinase that phosphorylates FlaC is unknown, TdaI a hybrid histidine kinase with N-terminal PAS PAC domains is the first choice to test the hypothesis that TdaI donates phosphor to FlaC, because it is the only known histidine kinase for TDA production and regulation. Taken together, TM1040 could employ a two-component signal transduction in the TDA circuit. The model suggests that, if either TdaHI receives TDA, or TdaI may sense redox, energy, or other environmental conditions via its PAS-PAC domains. , TdaI autophosphorylates itself and transfers the phosphor to phosphorylate FlaC. Phosphorylated FlaC could bind to *tdaC* promoter region independent of TdaA to switch cells towards the sessile phase, which allows TdaA to activate *tda* genes expression. ,. While hundreds of two-component systems have been investigated, little of known about mechanisms of sensor kinase binding with chemical ligands (118). Further confirmation and characterization of TDA-TdaHI interaction could present an important novel system

for understanding sensor kinase-chemical ligand interaction since TDA is a novel chemical containing a novel tropolone ring.

## 6.4    TDA implication in roseobacter-phytoplankton symbiosis

### 6.4.1    TDA production in TM1040's sessile lifestyle

TM1040 demonstrates a biphasic lifestyle consisting of a motile phase and a sessile phase. Data in this dissertation, however, focus on the bacterial sessile phase, where the cells are found attached to one another as "rosettes" or in biofilms on the surface of phytoplankton. By living closely with phytoplankton in the sessile phase, TM1040 benefits from enriched nutrients provided by the phytoplankton. Production of TDA in sessile cells is likely timed to ensure the cells to administer TDA at an appropriate time, i.e. TDA is only produced after bacteria settle down to the phytoplankton. By doing so, bacteria do not need to waste nutrition and energy in producing TDA when they are far way from host in motile life style. TM1040's abilities to attach to dinoflagellate cells , and to form biofilm on the surface of dinoflagellate (110), enables cells to live in proximity to *P. piscicida*, thus efficiently delivering TDA to dinoflagellate and microbiota. Furthermore, since TDA kills many species of marine pathogens, while does not inhibit roseobacters (18), it may confer roseobacters an advantage in symbiosis with phytoplankton by reducing competitors of roseobacters or inhibiting pathogens of dinoflagellate. The production of the antibiotic TDA may explain the dominance of roseobacters within marine algal microbiota (40, 156).

**6.4.2   TDA chemical communication in the symbiosis**

High concentration of bacteria clustered around phytoplankton raises the possibility of chemical communication mechanisms among the interactions in microbiota as well as in bacteria-phytoplankton. Chemical communication, in the form of antibiotic inhibition or gene regulation, could allow the system to structure and regulate microbial composition, architecture and physiology of the microbial community around the phytoplankton by choosing certain inhabited bacterial species, activating specific metabolism pathways, and/or switch on toxic/antibiotic production. Indeed, antibiotic phenazine regulates gene expression in *Pseudomonoas aeruginosa* and could be important for the development and maintenance of diverse microbial communities containing *P*. aeruginosa (43).

The data here suggest that TDA is a novel QS signal, a common chemical used by bacteria in cell-to-cell communication. TDA cross-talking between different marine bacteria suggests that the establishment of the symbiosis could be an active process, in which chemical signals allow the system to choose and coordinate bacterial species to form symbiosis with phytoplankton. TDA may also act as a chemical signal to other TDA-receptive roseobacters telling them that conditions are favorable and therefore it's time to enter sessile phase.

This finding is significant to research in microbial ecology because it highlights a novel chemical signal in marine bacteria that worth investigating. While the biochemical interactions in microbiota remains elusive, TDA, as a chemical signal, should enable us to more specifically interrogate the biological and chemical characteristics of the microbiota associated with phytoplankton. A question could be

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addressed such as: What is the abundance and dynamics of TDA producing bacteria associated with HABs compared to those living with normal phytoplankton.

### 6.4.3 Symbiosis specificity

The development of symbiosis involves interdomain signaling to allow for specificity in the selection of both partners (106). Specifically, an eukaryote host elicits bacteria to produce a specific chemical, which in turn interacts with the host to induces symbiotic development. For example, flavonoids produced from plants induce bacteria to produce Nod factor, which in turn initiate nodule development of *Rhizobium*-legume (129). Perhaps, DMSP production in algae and TDA synthesis in roseobacteria (DMSP-TDA bioactive coupling) confer the advantage in specificity for both partner for sampling marine microbes to establish symbiosis. Some phytoplankton-bacteria interactions might be species specific, like differences of bacterial community in two marine diatoms (*Thalassiosira rotula* and *Skeletonema costatum*) (66). And perhaps, the relationship of phytoplankton-bacteria coupling may be of much diversity that allows for potentially enormous novel bioactive chemicals, *e.g.* novel antibiotics, which function specifically in different environmental conditions. Thus, while we investigated interactive processes at the level of individual microorganisms, to understand the biogeochemical processes at a global scale, the need for the integration of different chemical processes should provide the basis for understanding the interactions among biotic and abiotic environments.

## 6.5    Future work

In line with the notion of *tda* gene regulation, it would be interesting to investigate *tda* gene expression in response to marine phytoplankton. With discovered *tda* genes and their associated promoter regions, it should be feasible to construct target *tda* genes fused with fluorescence protein gene reporter. By doing so, it would be possible to explore bacterial *tda* gene expression, *in situ* in response to presence of phytoplankton and perturbation of environment conditions, without intrusion of the assemblage. It is conceivable that high level of gene expression might arise due to bacteria living nearby phytoplankton.

Broadly speaking, while host-symbiont symbiosis composed of normal microbiota, the outcome of the interaction, e.g. beneficial or as pathogenic relationship, are determined by different underlying processes, as shown *Staphylococcus aureus* in human skin microbiota (51). So, it stresses to study mechanisms that lead to abnormal scenario, for example, chemical communications between bacteria-phytoplankton that result in the formation of harmful algae blooms.

Although *tda* gene regulation model is proposed, future research in characterizing the signal cascade is required to further test the model. For example, transposon insertion in *tdaH* gene disrupts TDA production and TDA induction. This observation could be due to either i) loss of the TdaH, which could modify cytoplasmic TDA and convert it into a suitable ligand (TDA*) for TdaA (**FIG. 3.10, FIG. 6.2)**; ii) the transposon insertion in *tdaH* may have polar effects on *tdaI*, which is responsible for sensing TDA; or iii) both of them are responsible for the TDA-phenotype. These possibilities could be tested for change of phenotypes in TM1040,

e.g. TDA production and *tda* gene expression, by separately construction of nonpolar null mutations in *tdaH* and *tdaI* using pK18mobsacB (123). If i) is true where *tdaH* is essential for TDA, *tda* mutant is expected to produce TDA and be able to have TDA induction phenotype; if ii) is the case where *tdaI* is important, inframe deletion of *tdaH* will not disrupt on TDA production and induction; If iii) both of *tdaH* and *tdaI* are important, neither *tdaH* nor *tdaI* will not produce TDA and will lose TDA induction. For purposes of discussion, I will cover all above mentioned scenario to discuss experiments identifying protein functionality. If TdaH is required for the chemical modification of cytoplasmic TDA, the *tdaH* mutant should be not have modified TDA* compared to wild type TM1040. This hypothesis could be tested by comparing TDA like chemicals from extraction of wild type and *tdaH* mutant, if *tdaI* is responsible for TDA induction, there is likely a *tdaI* point mutant that maintains kinase activity independent of TDA. The test is to identify a construct that retained the positive activity in *tdaI* mutant, indicating *tdaC* expression independent on TDA production. A plasmid library of *tdaI* gene generated by random mutagenesis via error-prone PCR could be added to the *tdaI* deletion mutant with a *tdaCp::lacZ* reporter system. The goal is to identify the *tdaI* complementation mutant that constitutively expresses *tdaC* in shaking and standing conditions. Alternatively, a set of plasmids individually containing deletions of *tdaI* lacking DNA coding for each of the functional domains of TdaI (PAS, PAC, HisKA, HATPase, REC, and HPT) could be move to *tdaI* deletion mutant. Expression of *tdaCp::lacZ* in each construction will be assessed in shaking and standing culture conditions. Results of this could help to characterize the respective function of each domain.

## 6.6    Conclusion

Despite striking advances made in the characterization and assessment of microbes living with phytoplankton, in most cases, the molecular mechanisms underlying the interactions of both partners remain unclear. The symbiosis of the bacterium TM1040 and the dinoflagellate *P. piscicida* has become an important model to study the interactions between marine bacteria and phytoplankton at the genetic and molecular level. Several key features of TM1040 have important implications for the bacteria-phytoplankton symbiosis. They include: motility and biofilm formation, chemotaxis and metabolism of DMSP, and TDA production.

I focused on a hallmark feature in sessile phase: TDA synthesis. I have discovered the biosynthetic genes and operons involved in TDA biosynthesis through genomic and genetic methods, revealed that TDA autoinduces its gene expression, and further delineated molecular mechanisms for *tda* gene regulation. The result imply that the symbiotic roseobacteria, being specialized to attach to phytoplankton, can coordinate behavior in the population scale using known or novel QS signals to initiate and establish symbiosis of bacteria-phytoplankton. This study improves our understanding of the signal transduction and genetic regulatory mechanisms used by roseobacters. The data also help to shed light on molecular mechanisms on how TM1040 cells initiate and establish a beneficial and obligate symbiosis with marine phytoplankton whose activities are important to the marine environment. In a broad sense, mechanisms underlying the bacteria-phytoplankton interactions could help to

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explain the dynamics of harmful algal bloom dynamics, marine nutrient cycling, sulfur and carbon cycling.

# Appendix A: Media and Solutions

## A.1. Media

### A.1.1 Luria Bertani (LB) Broth

| | |
|---|---|
| Tryptone | 10 g |
| NaCl | 5 g |
| Yeast Extract | 5 g |

H$_2$O 1000 ml

Autoclave at 120°C for 20 min

### A.1.2 Luria Bertani (LB) Agar

| | |
|---|---|
| Tryptone | 10 g |
| NaCl | 5 g |
| Yeast Extract | 5 g |
| Bacto Agar | 15 g |
| H$_2$O | 1000 ml |

Autoclave

### A.1.3 Marine Broth 2216

| | |
|---|---|
| Marine Broth | 37.4 g |
| H$_2$O | 1000 ml |

Autoclave

### A.1.4 Marine Agar 2216

| | |
|---|---|
| Marine Broth | 37.4 g |
| Bacto Agar | 15 g |

H₂O                          1000 ml

Autoclave

### A.1.5    Heart Infusion Artificial Sea Water (HIASW) Broth

Heart infusion powder        25 g

Instant Ocean sea salts      15 g

H₂O                          1000 ml

Autoclave

### A.1.6    Heart Infusion Artificial Sea Water (HIASW) Agar

Heart infusion powder        25 g

Instant Ocean sea salts      15 g

Bacto Agar                   15 g

H₂O                          1000 ml

Autoclave

### A.1.7    Heart Infusion Artificial Sea Water 10 ppt (HIASW10) broth

Heart infusion              25 g

Sea salts (Sigma)           10 g

H₂O                         1000 ml

Autoclave

### A.1.8    Heart Infusion Artificial Sea Water 10 ppt (HIASW10) agar plates

Heart infusion              25 g

Sea salts (Sigma)           10 g

Bacto agar                  15 g

H₂O                         1000 ml

Autoclave

## A.1.9    A marine basal minimal medium

| | |
|---|---|
| Tris HCl | 8.47g |
| $NH_4Cl$ | 0.37 g |
| $K_2HPO_4$ | 0.0022 g |
| NaCl | 11.6 g, |
| $MgSO_4$ | 6g, |
| KCl | 0.75 g |
| $CaCl_2 \cdot 2H_2O$ | 1.47 g |
| FeEDTA | 2.5 mg |
| RPMI-1640 vitamins [Sigma R7256]) | 1 ml |
| Sole carbon sources | 1 g |

pH 7.6, Autoclave

## A.1.10    SOC medium

| | |
|---|---|
| | 250 ml |
| Tryptone Peptone | 5 g |
| Yeast extract | 1.25 g |
| NaCl | 0.15 g |
| KCl | 0.05 g |
| MgCl2 | 0.51 g |
| MgSO4 | 2.5 ml |
| 20% glucose (add after autoclaving) | 10 ml |

## A.2. Solutions

### A.2.1    1 mg/ml Ethidium Bromide 50 ml

Ethidium Bromide                                50 mg

Wrap bottle in aluminum foil.

### A.2.2    SDS Running 5X Gel Buffer

|  | 5 l | 2 l |
|---|---|---|
| Tris | 150 g | 60 g |
| SDS | 25 g | 10 g |
| Glycine (electrophoresis grade) | 720 g | 288 g |

### A.2.3    20X SSC

|  | 1 l | 2 l |
|---|---|---|
| Sodium Chloride | 175.32 g | 350.64 g |
| Sodium Citrate | 88.23 g | 176.46 g |

pH to 7.0

### A.2.4    50X TAE

|  | 1 l |
|---|---|
| Tris | 242 g |
| Glacial Acetic Acid | 57.1 ml |
| 0.5M EDTA | 100 ml |

### A.2.5    10X TBE

|  | 1 l | 2 l |
|---|---|---|
| Tris | 108.8 g | 217.6 g |
| Boric Acid | 55.0 g | 110.0 g |
| 0.5M EDTA | 40 ml | 80 ml |

### A.2.6    10X TE Buffer        1 l

| | |
|---|---|
| 1M Tris-Cl, pH 7.5 | 100 ml |
| 0.5M EDTA pH 8.0 | 20 ml |

Filter sterilize.

| | |
|---|---|
| **A.2.7  Qiagen Buffer P1** | <u>107 ml</u> |
| 1M Tris, pH 8.0 | 5 ml |
| 0.5M EDTA, pH 8.0 | 2 ml |
| $H_2O$ | 100 ml |
| Autoclave | |
| Rnase A (add after autoclaving) | 0.0107g |
| Store at 4° C | |
| **A.2.8  Qiagen Buffer P2** | <u>100 ml</u> |
| NaOH | 0.8 g |
| SDS | 1.0 g |
| **A.2.9  Qiagen Buffer P3** | <u>100 ml</u> |
| Potassium Acetate | 25.03 g |
| pH to 4.8 (use 12N) | |
| Autoclave | |
| **A.2.10  1M Glucose** | <u>100 ml</u> |
| Glucose | 18 g |
| **A.2.11  30% Acrylamide/Bis-Acrylamide (37.5:1)** | |
| | <u>500 ml</u> |
| Acrylamide | 150 g |
| Bis-acrylamide | 4 g |

## A.2.12    10% SDS-PAGE, Denaturing, for Proteins

|  | Lower Layer | 3% Stack |
|---|---|---|
| 40% Acrylamide/Bis (29:1) | 2.5 ml | 500 µl |
| 4X Lower Buffer | 2.5 ml | 1.25 ml |
| dH$_2$O | 4.9 ml | 3.2 ml |
| Polymerize with: | | |
| Temed | 10 µl | 20 µl |
| 10% APS | 50 µl | 20 µl |

Makes 2 mini gels.

## A.2.13    10% (w/v) Ammonium Persulfate (APS)

|  | 500 ml |
|---|---|
| Ammonium persulfate | 50 g |

Make 50 ml aliquots.  Store at -20°C.

## A.2.14    Chloroform/ Isoamyl Alcohol Solution

|  | 100 ml |
|---|---|
| Chloroform | 96 ml |
| Isoamyl Alcohol | 4 ml |

## A.2.15    0.1% DEPC-Treated H$_2$O    1 l

| dH$_2$O | 1000 ml |
|---|---|
| Diethyl Pyrocarbonate (DEPC) | 1 ml |

Shake well for several minutes.

Stir overnight with cap loosened at 37°C and autoclave.

## A.2.16    10 mM dNTP Dilution

| | |
|---|---|
| *Use 100mM dNTP Set | 2.5 ml |
| GTP | 250 μl |
| ATP | 250 μl |
| TTP | 250 μl |
| CTP | 250 μl |
| dH$_2$O | 1.5 ml |

Combine dNTPs.  Divide into two tubes of 500 μl each.  Add 750 μl water to each tube.  Make 50 μl aliquots and store at -20°C.

| | |
|---|---|
| **A.2.17  500 mM EDTA, pH 8.0** | <u>500 ml</u> |
| EDTA Disodium Salt Dihydrate | 93.05 g |

Heat to dissolve.  pH to 8.0

**EDTA does not dissolve until nearly pH 8.0

| | |
|---|---|
| **A.2.1  200 μg/ml IPTG,** | <u>10 ml</u> |
| <u>IPTG</u> | 0.002 g |
| Sigma H$_2$O | 10 ml |

| | |
|---|---|
| **A.2.2  Lysozyme, 50 mg/ml** | <u>10 ml</u> |
| Lysozyme | 0.5 g |

Store at -20°C.

### A.2.3    Phenol/ Chloroform/ Isoamyl Alcohol Solution

|  | 100 ml |
|---|---|
| Phenol (heat until liquid) | 50 ml |
| Chloroform | 48 ml |
| Isoamyl alcohol | 2 ml |

### A.2.4    5× Protein loading dye

| | |
|---|---|
| 1M Tris-HCl pH 6.8 | 1.56 ml |
| Glycerol | 2.5 ml |
| 20% SDS | 2.5 ml |
| 2-β mercaptoethanol | 1.25 ml |
| Bromphenol blue | 5 mg |

H2O 2.19 ml

### A.2.5    10% (w/v) SDS                500 ml

| | |
|---|---|
| SDS | 50 g |

### A.2.6    20 mg/ml X-Gal                5 ml

| | |
|---|---|
| X-Gal | 0.100 g |
| Dimethylformamide (use glass pipette) | 5 ml |

Make ~500 μl aliquots.  Store  at -20° C

# Appendix B: Protocols

**TOPO TA Cloning**

**<u>Reagents</u>**

1.     Fresh PCR product (confirmed by agarose gel electrophoresis)

2.     Dilute salt solution

3.     10 µl salt solution (provided with kit)

4.     30 µl dH2O

5.     Sterile dH2O

6.     TOPO vector

**<u>Reaction (held on ice until start)</u>**

2 ul fresh PCR product

1 ul dilute salt solution

2 ul sterile dH$_2$O

1 ul TOPO vector

total 6 µl

1.     mix gently incubate at room temp for 5 min (for <1 kb PCR

products) to 30 min for larger fragments

2.     place on ice and electrotransform

**B.2       Electroporation**

Note: 1. it may be advantageous to clean-up the DNA per our protocol before electroporation, as the TOPO reaction contains a fair amount of salt that can cause arcing. 2. held on ice; all reagents ice-cold as per Belas Electroporation Protocol)

1.    Add 2 ul TOPO cloning reaction into a vial of One Shot Electrocompetent *E. coli* (supplied with kit)

2.    mix gently (do not pipet up and down)

3.    Transfer to ice-cold cuvette

4.    Electroporate as per SOP

5.    Add SOC (or SOB) and incubate as per SOP

6.    Plate on either Amp or Kan agar containing 40 μg/ml X-Gal


**B.3**    **TM1040 electrocompetent cells**

1.    Inoculate single colony of TM1040 into 50 ml HIASW broth in 125 flask at 30 °C overnight with shaking.

2.    1% inoculation the culture into 250 ml HIASW broth in 1l flask and incubate at 30°C with shaking until OD600 is 0.6.

3.    Immediately place cells in ice for 30 min.

4.    Harvest cells by centrifugation at 2,600 g (6,000 RPM) for 10 min at 0 °C.

5.    Repeat step 4, and wash the cells four times in 200 ml ice-cold sterile water

6.    Resuspend cells in 1 ml 10% glycerol.

7.   The cells can be directly used for electroporation or store at -80°C
until ready to use.

**B.4   Transposon mutagenesis**

1.   Add 25 ng of EZ-Tn5 transposome (Epitcentre, Madison,
Wisconsin) into TM1040 electrocompetent cells.

2.   Incubate on ice for 30 min.

3.   Transfer the mixture to pre-chilled 0.2 cm Electroporation
cuvette an electroporate and electrotransform under the condition of 5
Kv per cm, 400 ohms and 25 μF using Bio-Rad Gene Pulser (Bio-Rad,
Hercules, California).

4.   Add 1 ml HIASW, pre-warmed 30°C, to the transformation
mixture. Continue incubation at 30oC with shaking for 2 h.

5.   Spread 100 μl of culture on HIASW agar plates plus
Kanamycin (120μg/ml). Incubate at 30°C for 2 days.

6.   Pick the Kanamycin resistant colonies onto a 7-by-7 array on
2216 agar plates containing Kanamycin 120μg/ml (Kan120) and
incubate at 30oC ~2 days.

7.    Replicate clonces7-by-7 array to Tda- screening plate, and
incubate at 30°C for 2 days.

**B.5   Well diffusion assay of TDA production**

1.   TM1040 culture was incubated at 30°C at standing for 3 days.

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2.   Pellet down cells and transfer the supernatant to a new tube, put on ice until use.

3.   Autoclave 0.5 g agar in 37 ml water. Place flask at 44°C to keep warm.

4.   Sequentially add 5 μl of 1M $CaCl_2$, 0.1 ml 1M $MgSO_4$, 10 ml 5xM9 salts, 1 ml 20% glucose, 1.5 ml 10% casamino acids, and finally 50 μl of overnight culture of V. anguillarum

5.   Immediately pour into large petri dish (150x15 mm).

6.   After solidification, punch wells in the agar with 200 μl pipette tip.

7.   Add 60 μl of spent medium to wells, and incubate at room temperature for 2-3 days until a inhibition zone is observed.

**B.6   Southern blot**

**DNA Transfer (Day 1)**

1.   Run the PFGE gel according to its protocol

2.   Dye the gel with 1.0 μg/ml EtBr for exactly 30 min with constant agitation

3.   Immediately UV irradiates the gel, using the UV linker chamber, with 80mJ/cm$^2$for nicking.

4. soak gel in a clean glass dish containing ~10 gel volumes of 0.25M HCl. Shake slowly on a platform shaker for 30min at room temperature

5. Pour off the HCl and rinse the gel with distilled water. Add~ 10vol denaturizing solutions and shake as before for 20 min. Replace with fresh denaturation solution and shake for a further 20min

6. Pour off the denaturation solution and rinse the gel with distilled water. Add ~ 10vol neutralization solution, shake as before for 20 min, then replace with fresh neutralization solution and shake for a further 20min.

7. setup the transfer following C.P.M.B (current protocols of molecular biology) page 2.9.3

**DNA Fixation (Day 2)**

1. remove the membrane gently, lay it side up, make the location of the wells and orientation marker on the membrane

2. place the membrane on 3MM paper soaked with 10*SSC

3. UV-crosslink the membrane by stratagene UV-3000

4. rinse the membrane briefly in ddH2O

**Hybridization (Day 2)**

1.   Pre-heat an appropriate volume of DIG Easy Hyb to room temperature, prehybridize filter for 30 min with gentle agitation in the Biorad hybridization container

2.   Denatured the probe stored at -20°C  at 68 °C for 10 min

3.   Pour off prehybridization solution and add probe mixture to membrane

4.   Incubate o/n with gentle agitation


**Washing (Day 3)**

1.   1 wash 2*5 min in ample 2*SSC, 0.1% SDS at 15~25C under constant agitation

2.   2 wash 2*15min in 0.2*SSC, 0.1% SDS at room temperature under constant agitation


**Immunological detection (Day 3)**

3.   Follow the Roche instruction starter KitII page18


**Visualization (Day 3/4)**

1.   Prewarm CSPD Ready-to use to RT

2.   Transfer membrane face up to transparent film, add 1ml CSPD to, and IMMEDIATELY cover the membrane with the second folder to spread the substrate EVENLY and WITHOUT air bubbles over the membrane.

3.  Do not use plastic wrap to cover blot; use hybridization bags, acetate sheet protectors, or two sheets of transparent film

4.  Incubate at RT 5min

5.  Squeeze out the excess liquid and seal the edges of the bag by hand

6.  Put it to the cassette, incubate 37C 10min to activate the activity

7.  In the dark room, expose to X-ray film

8.  First exposure to film should be 15-30 minutes. Examine film, and then make other exposures based on initial result.

**B.7      High Pressure Liquid Chromatography (HPLC)**

**Sample Preparation: Semipurify TDA**

1.  TM1040 was grown in 500 ml 2216 marine broth medium in a 1 liter Erlenmeyer flask at 30C temperature for 4 days.

2.  The cells were removed by centrifugation (10,000 x g for 10 min), JA14 rotor 8,000 rpm/min for 10 min.

3.  The pH of the supernatant was adjusted in the beaker to 3.0 by acetic acid measure by pH meter.

4.  Extract with 3 times 150 ml ethyl acetate acidified with 0.1% formic acid (FA) in 1l reparatory funnel after mixing thoroughly and put static at least 1hrs.

5. Transfer the organic phase to a 250 ml round bottle flask and evaporated to dryness under nitrogen flow.

6. The dry ethyl acetate extract was redissolved in $3 \times 3$ ml acetonitrile (ACN)-water (1:19) containing 1% FA

7. Sequentially apply to two 60 mg Oasis MAX columns (Waters, Milford, MA) which had previously been sequentially conditioned with 4 ml methanol (HPLC grade) and 3 ml ACN-water (1:19) containing 1 % FA.

8. After loading the samples by gravity the columns were washed with 4 ml PBS buffer (pH 7).

9. Then 3.5 ml ACN-water (1:1) was passed through the column and collected (fraction 1)

10. 3.5 ml ACN- water (9:1)(fraction 2)

11. 3.5 ml ACN-water (1:1) with 2% FA (fraction 3) stored in brown and green cap bottle

12. 3.5 ml ACN-water (9:1) with 2% FA (fraction 4) stored in brown and green cap bottle

**13.** The solvents were then removed in vacuo on a SpeedVac (ThermoSavant, Holbrook, NY).

**Running Parameters:**

1. Turn on the HPLC machine and log into Windows.

2. (Analytical HPLC) Set up running method and data analysis following instruction in the help menu and following parameters:

Column Flow = 0.5 ml/min; Column= Curosil PFP 15 cm, 2 mm, 3 µm column, Phenomenex (Torrance, CA); Solvent A = 35% ACN . 200 µl/l trifluoroacetic acid; Solvent B = 60% ACN. 200 µl/l trifluoroacetic acid; Running solution changes linearly from A to B in 6 min. Diode Array Detector (DAD) acquire wave length at 304 nm. Column compartment temperature = 30.0°C. TDA peak is expected to be at approximately 4.2 minutes.

3.  (Preparative HPLC). The TDA-rich fraction was applied to preparative Luna 5U C18(2) 250×10.0 mm high-performance liquid chromatography (HPLC) column (Phenomenex, Torrance, CA) at a flow rate of 5 ml per min under isocratic conditions, using a water-acetonitrile gradient 30% (0.02% trifluoroacetic acid in water) and 70% (60% acentonitrile, 0.02% trifluoroacetic acid). Injection volume 100 ul. Auto fraction collector was set up as: upslope, 9 units/s; down slop, 5 unit/s; cut off 200 mA. Max peak duration: 1 min.

4.  When the run is finished, the HPLC is cleaned by running 80% ACN through the machine for 20 mins when the column pressure decreases and stabilize to lower level.

**B.8** *tdaC* **5'-RACE assay**

1.  **Day 1**: streak TM1040 to 2216, 30C, O/N

2.  **Day 2**: Inoculate single clone of TM1040 to 2216 liquid broth, 30C, O/N, shaking

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3. **Day 3**: 1 % inoculates to 50 ml 2216 liquid broth in 125 ml PYREX flask, 30C, static, for 3 days.

4. **Day 6**: Collect 2 ml out, and extract total RNA with RiboPure-Bacteria purification kit (Ambion, Austin, TX).

5. **Day 7**:

5.1 Decap mRNA:5'-triphosphates were converted to monophosphates by treatment of 5 μg total RNA with 0.5 U tobacco acid pyrophosphatase (TAP) at 37°C for 60 min (Page 8).

5.2 Phenol extraction and Precipitation: The reaction was stopped by phenol-chloroform extraction followed by ethanol-sodium acetate precipitation (Page 9).

6. **Day 8**:

6.1 Ligation: Following GeneRacer kit (Invitrogen, Carlsbad, CA), precipitated RNA was redissolved in water, mixed with 250 ng of GeneRacer RNA oligonucleotide (5'-CGA CUG GAG CAC GAG GAC ACU GAC AUG GAC UGA AGG AGU AGA AA-3'), heat denatured at 65°C for 5 min, quick-chilled on ice, and centrifuge briefly. GeneRacer RNA oligonucleotide was ligated at 37°C for 1 h with 5 U of T4 RNA ligase.

6.2 Phenol-chloroform extraction and ethanol precipitation

6.3 Reverse Transcription: the RNA was reverse transcribed at 45°C for 60 min with 2 pmol of reverse transcriptase (RTa) primer specific for tdaC (5'-CGGTCTTCATGATGAACACA-3') and 200 U

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of SuperScript III reverse transcriptase according to the manufacturer's instructions. After inactivation at 70°C for 15 min, the RT reaction was treated with 2 U of RNase H at 37°C for 20 min.

6.4 PCR amplification, touch down experiment with temperature starting from 72 °C: 1 μl of the RT reaction product was used for PCR amplification with 10 pmol of PCR primer specific for tdaC (5'-CCAGAAGACCCAGATGCGCGATATG -3') and 20 pmol of the GeneRacer 5' primer (5'-CGA CTG GAG CAC GAG GAC ACT GA-3'). Cycling conditions were as follows: 95°C for 3 min; 28 cycles of 95°C for 40 s, 68°C for 40 s, and 72°C for 1 min; 72°C for 8 min. PCR products were separated on a 2% agarose gel

7. **Day 9**:

7.1 TA cloning: the band of interest was excised, purified with a GenElute agarose spin column, and cloned into pCR4-TOPO vector (Invitrogen) with a TOPO TA cloning kit for sequencing (Invitrogen).

7.2 Plasmid extraction and sequence.


**B.9      Electrophoretic mobility shift assays  (EMSA)**

1. **PCR**: tdaCp probe for EMSA was prepared by PCR with biotin labeled primers (synthesized by invitrogen) landing respectively 5'- of tdaCp intergenic region with 5'- GTTGGTTTTGGGGCATTTACG-3'and 3'- of tdaCp intergenic region with 5'-GTCCATTCAAGAGTCCTCCAATG-3'. PCR product size will be

225 bp. Gel purified PCR product with Gel purification kit (cat. 28704, Qiagen).

2.  **Binding assay**: TdaA and probe were mixed, incubated at ice 10 min followed by room temperature for 20 min. The reaction components contain: TdaA-His 0-300 nM, biotin labeled probe 5 nM from step 1, and 1x EMSA Binding Buffer [20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM (NH4)2 S04, 1 mM DTT, Tween 20, 0.2 % (w/v), 30mM KCl].

3.  For TDA addition, TDA final concentration is 500 nM for all tested samples with various amount of TdaA protein. 240 nM TdaA without TDA is used as control

4.  For non-specific DNA competition experiment, Sheared Herring Sperm DNA is used at final concentration of 10 ng/ul, equivalent to 1000 times amount of tested probe. 240 nM TdaA without HS DNA  is used as control

5.  Additional BSA is used at final concentration 240 nM nM for all tested samples with various amount of TdaA protein. 240 nM TdaA without BSA DNA  is used as control

6.  **Electrophoresis**

1)  Commercial 5% precast gel from Biorad (cat. 161-1181, Biorad)

2)  The binding reaction is mixed with 1/10 volume of 10 X loading dye before loading to gel with Hamilton syringe. Note: 10X

loading Dye + glycerol stock solution: 10 mM Tris, 1 mM EDTA, 50% vol/vol glycerol, 0.001% wt/vol bromophenol blue, 0.001% wt/vol xylene cyanol FF. running at 0.5 X TBE

3) Load all rxn to gel & run at 150V in the cold room at 4°C to keep from overheating the gel. The gel is run at Mini-Protean Tetra Cell apparatus (cat. 165-8000, Biorad)

4) Stop electrophoresis when orange dye reaches end of gel (or just run it off; orange dye runs at ~50bp of DNA).

**7. Transfer**

1) Cut 4 Whatman blotting papers & a nylon membrane (cat. 11209272001, Roche applied science) to appropriate size of gel & pre-soak all but one Whatman in 0.5x TBE buffer.

2) Carefully disassemble the gel plates so that the gel adheres to one of the two plates, and place the dry Whatman paper over the gel.

3) Carefully remove the gel/Whatman layer (the gel should adhere to the paper & come off the plate relatively easily) & place in 0.5x TBE buffer.

4) Place the nylon membrane over the opposite side of the gel, and sandwich the Whatman papers, gel & membrane on a semi-dry transfer apparatus; ensure liberal soaking of the entire sandwich with 0.5x TE buffer.

5) ORDER (top-to-bottom): 2 Whatman > gel > membrane > two Whatman > bottom half of the transfer apparatus.

6)   Roll out any bubbles in the sandwich with a clean disposable pipette.

7)   Transfer for 1 hr at 300 mA of constant current at Mini Trans-Blot Cell (Cat, 170-3930, Biorad)

**8.   Detection**

1)   the labeled probe will be detected by an streptavidin-horseradish peroxidase in Chemiluminescent Nucleic Acid Detection Module (cat. 89880 , Pierce)

2)   To block membrane, add 16 ml Blocking Buffer (Pierce) and incubate for 15 minutes with gentle shaking.

3)   Prepare conjugate/blocking buffer solution by adding 50 μl of the Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 16 ml Blocking Buffer (1:300 dilution).

4)   Decant blocking buffer from the membrane and add 16 ml of the conjugate/blocking solution. Incubate membrane in the conjugate/blocking buffer solution for 15 minutes with gentle shaking.

5)   Transfer membrane to a new container and rinse briefly with 20 ml of 1X wash solution (Pierce).

6)   Wash membrane four times for 5 minutes each in 20 ml of 1X wash solution with gentle shaking.

7)   Transfer membrane to a new container and add 30 ml of Substrate Equilibration Buffer (Pierce). Incubate membrane for 5 minutes with gentle shaking.

8) Remove membrane from the Substrate Equilibration Buffer and carefully blot an edge of the membrane on a paper towel to remove excess buffer.

9) Pour the Substrate Working Solution onto the membrane so that it completely covers the surface.

10) Remove membrane from the Working Solution and blot an edge of the membrane on a paper towel for 2-5 seconds to remove excess buffer. Do not allow the membrane to become dry.

11) Wrap the moist membrane in plastic wrap, avoiding bubbles and wrinkles.

12) Place the membrane in a film cassette and expose to X-ray film for 10s-5 minutes. The film was chosen because it has over 10 times sensitivity than Imager.

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167. **Zaim, J., and A. M. Kierzek.** 2003. The structure of full-length LysR-type transcriptional regulators. Modeling of the full-length OxyR transcription factor dimer. Nuc. Acids. Res. **31:**1444-1454.

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# Curriculum Vitae

## Haifeng Geng

## Education

| | |
|---|---|
| 2005- 4.2011 | Ph.D., in Environmental Molecular Biology/Biotechnology, MEES<br>**University of Maryland, College Park (UMCP)** |
| 2000-2003 | M.S., in Biochemistry and Molecular Biology<br>**Shanghai Jiao Tong University (SJTU), China** |
| 1994-1998 | B.S., in Marine Biology<br>**Ocean University of Qingdao (OUC), China** |

## Research Experience

2005- 2010        **Research Assistant**

*Doctoral research*

Research advisor: Dr. Robert Belas

Center of Marine Biotechnology

University of Maryland Biotechnology Institute

**Genetic Analysis of Tropodithietic Acid Biosynthesis in Marine Bacteria**

- Generated transposon library in *Silicibacter* sp. TM1040
- Setup a set of genetic manipulation tools in *Silicibacter* sp. TM1040, which could be applicable to other marine roseobacters
- Combined microbial genetics, molecular biological techniques, HPLC-MS to identify genes required for TDA synthesis
- Determined the expression of *tda* genes, suggesting that TDA could be a novel autoinducer
- Characterized TdaA regulator and its regulation in signaling cascade
- Identification and characterization of bacteria living with marine algal *Karlodinium veneficum* culture
- Measured effects of TDA on phytoplankton, underscoring chemical roles in marine microbial ecosystem

2003-2005        **Research Scientist**

*Working Research*

Shanghai Fudan-Yueda Bio-tech Co., Ltd, Shanghai

***In-vitro* diagnostics for microorganisms**

- Developed assays for sensitive quantification of Hepatitis

B virus, Hepatitis C virus, SARS coronavirus
- Developed assays for detection of lamivudine-resistance mutant of Hepatitis B virus

2000-2003 **Research Assistant**
*Master research*
Research advisor: Dr. Yuquan Xu
Department of Biochemistry and Molecular Biology
Shanghai Jiao Tong University
**Biosynthesis and regulation of Phenazine-1-carboxylic Acid (PCA) in *Pseudomonas* sp. M18**
- Optimized fermentation process for PCA production
- Disrupted *rsmA* gene in *Pseudomonas* sp. M18
- Tested effects of *rsmA* mutation on PCA production

## Honors and Awards
| | |
|---|---|
| 2009 | **Rita Colwell** UMBI Graduate Fellowship |
| 2009 | **ASM** Student Travel Grant Award for 109th General Meeting |
| 2008 | **COMB** Graduate Student Research Fellowship |
| 2000-2003 | **SJTU** Graduate Student Research Fellowship |
| 1994-1998 | **OUC** Excellent undergraduate Fellowship |
| 1993 | Third Prize for **National Physics Competition**, China |

## Professional Membership
| | |
|---|---|
| 2008- | American Society for Microbiology |
| 2009- | American Association for the Advancement of Science |

## Publications

1. **Geng, H., and Belas, R.** "The LysR-Type Transcriptional Regulator TdaA Is Required for the Expression of the *tda* Genes in *Silicibacter* sp. TM1040." In preparation.
2. **Geng, H., and Belas, R.** 2010. Expression of tropodithietic Acid biosynthesis is controlled by a novel autoinducer. J. Bacteriol. **192:**4377-4387.
3. **Geng, H., and Belas, R.** 2010. Molecular mechanisms underlying roseobacter-phytoplankton symbioses. Curr. Opin. Biotechnol. **21:**1-7.
4. **Geng, H., Bruhn, J. B., Nielsen, K. F., Gram, L., and Belas, R.** 2008. Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters. Appl. Environ. Microbiol. **74:**1535-1545. (**Featured on cover photograph**).
5. **Geng, H., Hua, B., Wang, H., Cao, Y., Sun, Y., and Yu, A.** 2006. Dual-probe assay for detection of lamivudine-resistance hepatitis B virus by real-time PCR. J. Virol. Methods. **132:**25-31.
6. **Yu, A., Geng, H., and Zhou, X.** 2006. Quantify single nucleotide polymorphism (SNP) ratio in pooled DNA based on normalized fluorescence real-time PCR. BMC genomics **7:**143.

7. **Zhang, X., Wang, S., Geng, H., Ge, Y., Huang, X., Hu, H., and Xu, Y.** 2004. Differential Regulation of *rsmA* Gene on Biosynthesis of Pyoluteorin and Phenazine-1-carboxylic Acid in *Pseudomonas* sp. M18 World. J. Microbiol. Biotechnol. **21:**883-889.

8. **Zhu, D., Xu, W., Geng, H., Zhang, X., and Xu, Y.** 2003. Gene Cloning of *rpoD* and its Impact on Biosynthesis of Antibiotics in fluorescent *Pseudomonas* M18. Acta Microbiologica Sinica **43:**315-323.

## Presentation and Posters

1. **Geng, H., and R. Belas.** 2011. Expression of Tropodithietic Acid Biosynthesis Is Controlled by a Novel Autoinducer, BLAST Meeting, New Orleans, LA.

2. **Geng, H., and R. Belas.** 2010. Regulation of tropodithietic acid (TDA) biosynthesis: TDA is a clade-specific quorum signaling molecule, Sensory Transduction in Microorganims Gordon Research Conference, Ventura Beach,CA.

3. **Geng, H., and R. Belas.** 2009. A ubiquitous chemical from marine bacteria increases biomass of alga, 109th Annu. Meeting Amer. Soc. for Microbiol., Philadelphia, PA.

4. **Geng, H., and R. Belas.** 2007. Genetic analysis of antibiotic synthesis in *Silicibacter* sp. TM1040, BLAST IX Meeting, Laughlin, Nevada.

## Academic Service

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| 2008-2009 | Graduate student representative in UMBI Senator Committee |
| 2009 | Organization of Wageningen University and UMBI Graduate Student Research Day |

## Teaching Experience:

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|---|---|
| 2009 | Mentor for summer intern Monica. N.Williams (high school science teacher) |
| 2007 | Mentor for summer intern David McGaffin (high school science teacher) |
| 2006 | Mentor for summer intern Preston Miller (UM College Park) |