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Expression of Tropodithietic Acid Biosynthesis Is Controlled by a Novel Autoinducer▽†

Haifeng Geng and Robert Belas*

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, Maryland 21202

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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 *Pfiesteria piscicida* and *Silicibacter* sp. TM1040, a member of the *Roseobacter* taxonomic lineage. It is hypothesized that an important component of this symbiosis is bacterial production of tropodithietic acid (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 report, we 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.

Microorganisms play crucial roles in most of the biogeochemical cycles in the oceans due to their high abundance and potential metabolic activity (5). 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 (14). 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 (20), and some of the most frequently sampled bacteria associated with blooms of phytoplankton that produce a reduced sulfur-rich compound called dimethylsulfoniopropionate (DMSP) (21, 46). Roseobacters preferentially metabolize DMSP rather than sulfate despite the latter being nearly 10^7-fold more abundant in seawater (28). 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 dimethylsulfide (DMS), whose production affects cloud formation and global climate (24).

We have previously reported on a laboratory model symbiosis between a roseobacter, *Silicibacter* sp. TM1040 (hereinafter referred as TM1040), and *Pfiesteria piscicida*, a marine heterotrophic, DMSP-producing dinoflagellate (1, 6, 18, 34–36). 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, 35).

TM1040, like many other roseobacters, has a biphasic "swim-or-stick" lifestyle. In the swim phase, TM1040 populations are dominated by single motile cells propelled by one to four flagella whose movements are controlled by chemotaxis behavior (36). 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 (10).

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 (36), drawing the bacteria into close proximity to their host. We hypothesize that, once within range, roseobacters switch to the sessile-phase strategy, which involves the formation of a biofilm and the production of a novel tropolone antibiotic, tropodithietic acid (TDA) (Fig. 1A) (18). The switch to the sessile life helps the bacteria establish a close interaction with the alga and may benefit the host through the production of an antibiotic, preventing other algal pathogenic bacteria from harming the dinoflagellate (8, 18).

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 (7, 10, 18, 38). The presence of TDA in many roseobacters highlights this physiological trait among sub-

* Corresponding author. Mailing address: Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202. Phone: (410) 234-8876. Fax: (410) 234-8896. E-mail: belas@umbi.umd.edu.

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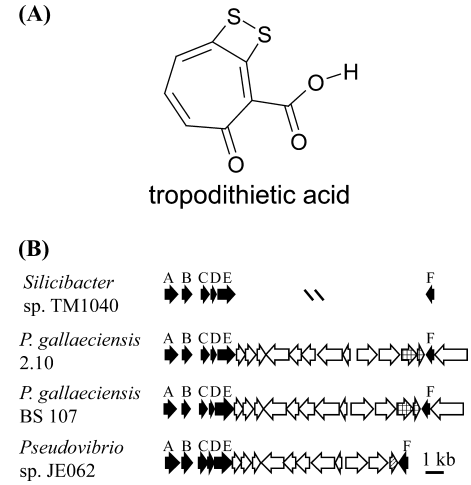


FIG. 1. (A) The chemical structure of TDA. Biologically relevant moieties on TDA include the seven-carbon tropolone ring and the disulfide bond. (B) Organization of the *tdaA-tdaF* locus in *Silicibacter* sp. TM1040, *P. gallaeciensis* 2.10, *P. gallaeciensis* BS 107, and *Pseudovibrio* sp. JE062. Genes empirically determined to be important for TDA biosynthesis are shown 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 (18). The hatch marks in the TM1040 *tda* locus indicate an area for which nucleotide sequence data are lacking (18).

groups of roseobacters. It also suggests that TDA is likely to be specific to roseobacters (or closely allied 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 (18). These homologs include *paaIJK* (TM3726, TM3727, and TM3728, respectively), encoding proteins required for phenylacetate catabolism, and homologs of *cysI* (TM1758), *malY* (TM2581), and *tdaH* (TM0961), which encodes a molybdopterin-binding domain which may be a sulfite oxidase. The latter three open reading frames 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 (18). Defects in any one of these 12 genes result in a loss-of-function Tda$^-$ phenotype (18). 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 that *tdaF* is in a third putative operon (Fig. 1B) (18). Indeed, our preliminary reverse transcription (RT)-PCR results support the idea that *tdaA* and *tdaB* form an operon (unpublished data). 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 (18).

TDA production is influenced by culture and environmental conditions (8, 18). Specifically, TDA activity is highest when bacteria are cultured in standing liquid nutrient broth, whereas cells produce negligible amounts of TDA in nutrient broth with shaking (18). Little is known about what component(s) of standing growth trigger TDA biosynthesis; however, Liang reported that the addition of phenylalanine and histidine significantly increased the production of TDA in *Phaeobacter* sp. strain T5 (31), suggesting that these amino acids may induce TDA synthesis. This is supported by our previous findings showing that strain TM1040 is attracted to amino acids, DMSP, and dinoflagellate cell homogenates (36), 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 the expression of TDA may be controlled by acyl homoserine lactone (AHL) quorum sensing (22, 32). However, TM1040 lacks AHL synthesis genes and genes involved in the function of other known autoinducer molecules (37) and does not produce known AHL molecules (8); 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 the 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.

MATERIALS AND METHODS

Bacteria and media. The strains and plasmids used in this study are listed in Table 1. Tda$^-$ transposon mutant strains were derived from *Silicibacter* sp. TM1040 by EZ-Tn*5* mutagenesis (18) and maintained on Difco 2216 marine broth or 2216 agar as recommended by the manufacturer (BD Biosciences, Franklin Lakes, NJ). A marine basal minimal medium (MBM; per liter, 8.47 g Tris HCl, 0.37 g NH_4Cl, 22 mg K_2HPO_4, 20 g sea salts, 2.5 mg Fe-EDTA, pH 7.6, 1 ml 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 a carbon source at a final concentration of 1 ml per liter. Sulfur sources, including 3-methiopropionate (MMPA), cysteine, DMSP, methionine, sulfate, and taurine, were added to minimal broth at a final concentration of 200 μM. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar containing 1.5% Bacto agar (Becton Dickinson, Franklin Lakes, NJ) (4). As appropriate, kanamycin was used at 120 μg per ml for *Roseobacter* strains and 50 μg per ml for *E. coli*. For matings 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) supplemented with tetracycline at a final concentration of 15 μg per ml was used (4).

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 (23) and subsequent ligation to the broad-host-range vector pRK415 (27) 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 (see Table S1 in the supplemental material) that added a PstI site. The *lacZ* gene from pEA103 (3) was amplified by PCR to include a HindIII site at the 3′ end using primers lacZ-F and lacZ-R. The products were mixed and used as templates for a second round of PCR, using primers tdaC-F and lacZ-R (see Table S1 in the supplemental material). The resulting PCR product was digested with PstI and HindIII and 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 (35). Exconjugants were selected using kanamycin and tetracycline on HIASW10 agar, and the subsequent resistant colonies were screened to confirm the presence of pHG1011.

RNA preparation and real time PCR. Total RNA was extracted using an RNeasy purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 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 (see Table S1 in the supplemental material) were designed to amplify specific genes using Primer Express 3.0 (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) amplification was performed using SYBRGreen

TABLE 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype[a]	Source or reference
Escherichia coli strains		
DH5α	F$^-$ *endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 recA1 gyrA96 relA1* ϕ80d*lacZ*ΔM15	41
S17-1 λ*pir*	*thi pro recA hsd*(r^- m^+) RP4-2 Tc::Mu-Km::Tn*7* Sm λ*pir*	15
Roseobacter strains		
Silicibacter sp. TM1040	Wild type	34
Mutants derived from TM1040		
HG1005	*paaK* (TM3728)::EZ-Tn*5* Kan	18
HG1015	*tdaB*::EZ-Tn*5* Kan	18
HG1050	*tdaF*::EZ-Tn*5* Kan	18
HG1056	*paaJ* (TM3727)::EZ-Tn*5* Kan	18
HG1080	*tdaC*::EZ-Tn*5* Kan	18
HG1110	*tdaD*::EZ-Tn*5* Kan	18
HG1213	*malY* (TM2581)::EZ-Tn*5* Kan	18
HG1220	*cysI* (TM1758)::EZ-Tn*5* Kan	18
HG1244	*tdaH* (TM0961)::EZ-Tn*5* Kan	18
HG1265	*tdaE*::EZ-Tn*5* Kan	18
HG1299	*paaI* (TM3726)::EZ-Tn*5* Kan	18
HG1310	*tdaA*::EZ-Tn*5* Kan	18
Other roseobacters		
Phaeobacter sp. 27-4	Wild type, antibacterial activity	10
Marinovum algicola ATCC 51442	Wild type, no antibacterial activity	8, 29
Roseobacter litoralis ATCC 49566	Wild type, no antibacterial activity	8, 43
Roseovarius sp. TM1035	Wild type, antibacterial activity	8, 34
Roseovarius sp. TM1042	Wild type, antibacterial activity	8, 34
Silicibacter pomeroyi DSS-3	Wild type, antibacterial activity	8, 19
Sulfitobacter sp. EE36	Wild type, antibacterial activity	8, 11
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	17
Pseudovibrio sp. JE008	Bacterial symbiont of marine sponges	17
Pseudovibrio sp. JE021	Bacterial symbiont of marine sponges	17
Pseudovibrio sp. JE061	Bacterial symbiont of marine sponges	17
Pseudovibrio sp. JE062	Bacterial symbiont of marine sponges	17
Pseudovibrio sp. JE0626	Bacterial symbiont of marine sponges	17
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	27
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; Pur^r, puromycin resistance.

and the respective forward and reverse primer pairs as listed in Table S1 in the supplemental material. PCR master mix (Applied Biosystems) was used with a 7500 fast real-time PCR system (Applied Biosystems) under 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. The results for all samples were normalized relative to the level of expression of *rpoD* (TM2141), encoding a putative sigma70 factor. The relative expression values represent the means ± standard deviations of the results 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.

***tdaC* promoter activity assays using *tdaCp*::*lacZ*.** The expression of *tdaCp*::*lacZ* was measured as β-galactosidase activity using the Miller assay (33). 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 or 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 conditions to an 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, we 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 (12). MMPA was synthesized by alkaline hydrolysis of its methyl ester, methyl-3-(methylthio)propionate (Aldrich, Milwaukee, WI) (26). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO). None of these compounds caused a significant change in the pH of the medium, based on prior experience. The incubation continued for 3 h at 30°C under standing conditions. After exposure to the respective chemical, bacteria were pelleted by centrifugation and washed once with 1 ml 0.85% NaCl, and the β-galactosidase activity was measured and expressed in Miller units (33). The 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), which was 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-Tn*5*) 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 onto 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.

Cross-feeding assay among marine roseobacters. Similarly, 2216 agar containing pHG1011 in strain HG1299 (*paaI*::EZ-Tn*5*) 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 (Table 1) was pipetted onto the surface of the plates and incubated for 3 days at 30°C. The β-galactosidase activity resulting from the expression of *lacZ* was assessed as described for the previous cross-feeding experiment.

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 and the medium was evaporated, redissolved in a water/acetonitrile (19:1) solution, and further fractioned by column chromatography on Oasis MAX columns (Waters, Milford, MA) as previously described (18). The TDA-rich fraction was applied to a preparative Luna 5u C18(2) 250- by 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 of 30% (0.02% trifluoroacetic acid in water) and 70% (60% acetonitrile, 0.02% trifluoroacetic acid). TDA was detected at a wavelength of 304 ± 4 nm (mean ± standard deviation) as previous described (10, 18). An Agilent G1364A fraction autocollector (Agilent, Santa Clara, CA) was used to automatically collect the TDA peak, and purified TDA was confirmed by analytic HPLC (18).

For rapid analyses, TDA was measured directly from standing broth cultures as follows. After 120 h of 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 (Millex mixed-cellulose-ester membrane; 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- by 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.

DNA extraction, separation, and preparation. Total DNA was extracted from roseobacter cells using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). Plasmid DNA was prepared by the alkaline lysis method (4). DNA was separated by agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer, 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.

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 used previously (18).

The 16S rRNA gene from each roseobacter isolate was amplified by PCR using primers 27f and 1492r (30). The standard PCR amplification conditions were 100 μM each deoxynucleoside triphosphate, 0.2 μM each primer, and 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 step at 72°C for 1.5 min. The sequences were compared to the available databases using BLASTN (2) to determine approximate phylogenetic affiliations to the genus level.

RESULTS

Genetic organization of *tda* genes in marine roseobacters. We have previously reported on the identification of 12 genes required for TDA biosynthesis by TM1040 and *Phaeobacter* sp. 27–4 (18). As shown in Fig. 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* (40) and *Phaeobacter gallaeciensis* 2.10 from the macroalga *Ulva australis* (39), as well as *Pseudovibrio* sp. JE062, a sponge isolate in the same *Rhodobacterales* order as the roseobacters (17). The organization of *tdaA-F* in *P. gallaeciensis* sp. BS107, *P. gallaeciensis* 2.10, and *Pseudovibrio* sp. JE062 is identical to that of TM1040 (18). Slight differences occur in the adjacent non-*tda* genes, which encode proteins whose amino acid domains suggest that 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.

Kinetics of TDA production under shaking and standing culture conditions. TM1040 produces a yellow brown pigment which is correlated with the synthesis of TDA, both of which are produced in much larger amounts in standing nutrient broth cultures than in shaking broth cultures (18). A time course of TDA production under these two growth conditions is shown in Fig. 2. In shaking liquid broth, with the bacteria doubling every 40 min (Fig. 2A), TDA is not synthesized, except for a brief period at ca. 10 h postinoculation. In contrast to growth in shaking conditions, the doubling time in standing culture conditions increases significantly, to ca. 12 h (Fig. 2B). The concentration of TDA in standing culture increased exponentially starting in the early exponential phase of growth (20 h), with TDA production increasing rapidly as the cells entered 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.

Effects of bacterial sulfur sources on TDA production. TDA contains two sulfur atoms (Fig. 1A), and its synthesis is thus probably influenced by the amount and type of sulfur compounds provided to the cells from the surrounding environ-

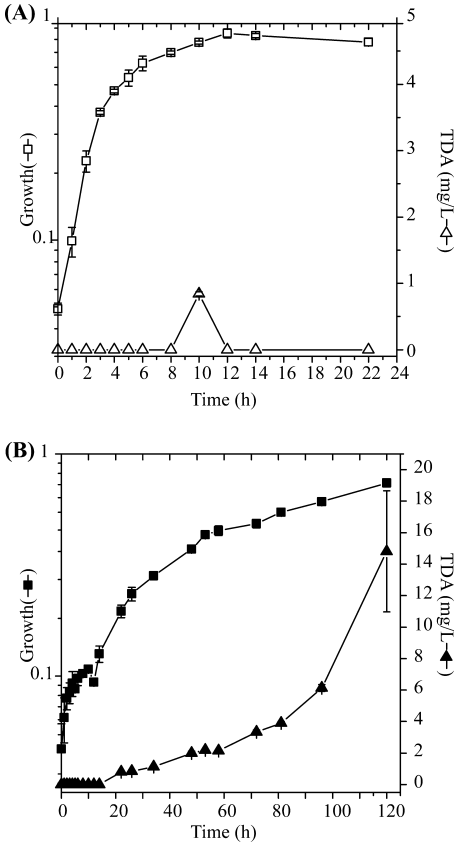


FIG. 2. TDA biosynthesis and bacterial growth of TM1040 are influenced by culture conditions. The bacterial growth rate and TDA production were compared over a time course when bacteria were grown in static liquid nutrient broth or in the same broth but with vigorous shaking. Bacterial growth is shown as the OD_{595}, while TDA production is measured in mg per liter. (A) Incubation with shaking. (B) Incubation in standing liquid broth. Error bars indicate standard deviations ($n = 3$).

ment. 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). Although the addition of DMSP and methionine significantly increased the growth of TM1040, only DMSP increased the amount of TDA produced. As shown in Fig. 3, 200 μM DMSP increased the concentration of TDA 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 we tested subsequently.

The expression of *tdaC* and *tdaF* is modulated by environmental cues. Since TDA production is influence by culture conditions and the source of sulfur provided to the bacteria, we tested a hypothesis that the increase in TDA was due to the 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. 1B) (18). Measurements of these transcripts (Fig. 4) show that *tdaC* and *tdaF* are upregulated, respectively, 3.7-fold (19.01 versus 5.07) and 17.4-fold (47.75 versus 2.75) when cells are grown in standing culture conditions compared with the values obtained from shaking cultures. On the other hand, the transcription of *tdaA*, *paaK*, *cysI*, and *malY* was not affected by the type of culturing used (Fig. 4). Constitutive expression of these four genes is not surprising, as *tdaA* is thought to encode a transcriptional regulator, many of

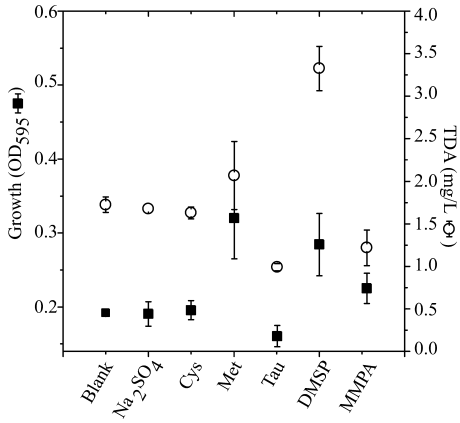
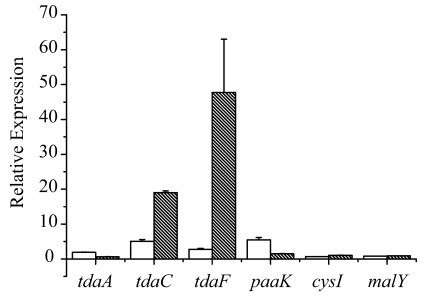


FIG. 3. The source of sulfur available to the bacteria affects TDA production. Cells were incubated for 72 h under standing culture conditions in a minimal medium containing a "subsistence" level (27 mM) of sulfate to which 200 μM of either Na_2SO_4, cysteine (Cys), taurine (Tau), 3-methiolpropionate (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 deviations ($n = 3$).



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

[a] Mean ± Standard Deviation

FIG. 4. Relative levels of *tda* gene transcription in cells grown under shaking versus standing liquid culture conditions. Transcription is shown as the relative expression of each target gene compared to the expression of *rpoD* and was measured by quantitative PCR (qPCR), as described in Materials and Methods. Open bars represent expression in shaking culture, and hashed bars indicate target gene transcription in standing culture conditions. Error bars indicate standard deviations (SD) ($n = 3$). The data from which the bar graph was derived are shown in the table beneath the graph.

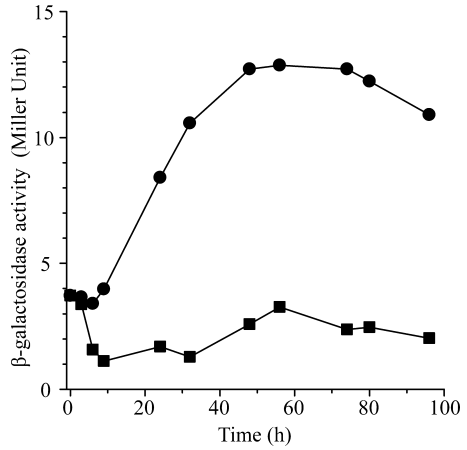


FIG. 5. Kinetics of *tdaC* expression during standing (●) versus shaking (■) liquid culture conditions. The expression of *tdaC* was measured as β-galactosidase activity (Miller units) produced from a plasmid harboring a *tdaCp*::*lacZ* transcriptional fusion. Shown is a representative data set from four independent experiments, each with five replicates.

which are constitutively expressed, while the functions of PaaK, CysI, and MalY (respectively) are required for fundamental core physiological processes and are not specific to TDA biosynthesis.

Kinetics of *tdaC* expression. As illustrated in Fig. 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 contain 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 of DNA upstream from *tdaC* 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 the level of 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. 5. The activity of *tdaC* as measured using the *tdaCp*::*lacZ* transcriptional fusion plasmid is equivalent to the qPCR results (Fig. 4) and shows that the expression of *tdaC* is higher in standing broth culture conditions (Fig. 5, circles) than in shaking conditions, where little to no expression was observed (Fig. 5, squares). 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 approximately 50 h (Fig. 5, circles). These data confirm that the 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. We hypothesize that the unknown inducer could be a chemical, a change in cellular physiology, or a change in the exterior environment around the cells, i.e., increased anaerobicity, during incubation in a standing nutrient broth.

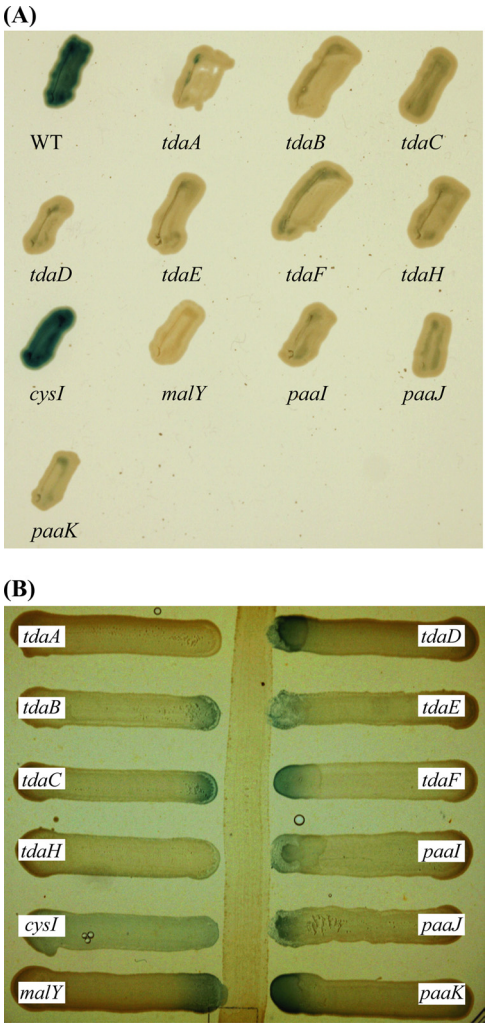


FIG. 6. (A) Expression of *tdaC* in different Tda$^-$ mutant backgrounds. A plasmid-borne copy of a *tdaCp*::*lacZ* transcriptional fusion was placed into the wild type and each of the 12 Tda$^-$ mutant strains, after which β-galactosidase activity was qualitatively assessed by the blue resulting from 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 the expression of *tdaC*.

Expression of *tdaC* in Tda$^-$ mutant backgrounds. Our 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 (18). This was accomplished by moving the *tdaCp*::*lacZ* plasmid into the *tdaA* strain, as well as into the other 11 Tda$^-$ mutants (Table 1). We predicted that the *tdaA* mutant (HG1310; *tdaA*::EZ-Tn*5*) 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 shown in Fig. 6A, when *tdaCp*::*lacZ* was in a wild-type background, β-galactosidase (represented by a blue color in the colonies in Fig. 6) was produced. In agree-

TABLE 2. 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 a final concentration of 50 μM in the tested 2216 marine broth medium.

[b] β-Galactosidase activity is calculated as previously described (33).Values are shown as means ± standard deviations.

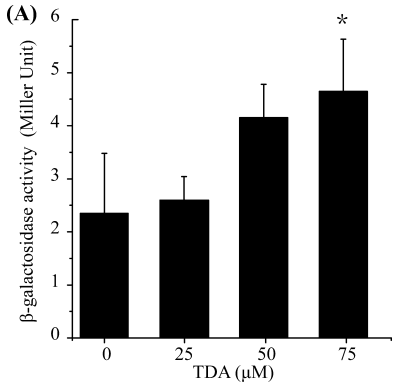
ment with the predictions, the *tdaC* transcriptional fusion was not expressed in a *tdaA* genetic background. Unexpectedly, β-galactosidase was also not detected when *tdaCp*::*lacZ* was placed in any of the *tda*-defective backgrounds (*tdaBCDEF*, *tdaH*, *malY*, and *paaIJK*), with one exception: the *cysI* mutant (HG1220; *cysI*::EZ-Tn*5*) (Fig. 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 mutant with the *cysI* mutation (HG1220; *cysI*::EZ-Tn*5*), which we originally characterized as a TDA loss-of-function mutant (18), still allow the expression of *tdaC*? We rechecked HG1220 for the 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 (see Fig. S1 in the supplemental material). 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 containing X-Gal (Fig. 6B). As shown by the results in Fig. 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-defective 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 Discussion.

TDA induces *tdaC* and *tdaF* expression. The 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 2, 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 the background level. 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 compounds tested were statistically equivalent to the results for the water control. The induction of *tdaC* transcription by TDA is dose dependent (Fig. 7A). We also measured the effect of exogenous TDA on the transcription of *tdaC* and *tdaF* using qPCR. As shown in Fig. 7B, the expression of *tdaC* and *tdaF* increased, respectively, 7.74- and 9.34-fold relative to their levels of expression in the uninduced control following exposure to TDA. These results indicate that the induction of *tdaC* and *tdaF* transcription are specific to TDA.

Taxonomically distinct roseobacters induce *tdaC* expression in TM1040. The results thus far obtained suggest that extracellular TDA is either actively or passively transported into a recipient cell where, at some unknown threshold concentration, it induces the expression of *tdaC* and *tdaF*. These data led



(B)

	Average	S.D.
tdaC	7.74	0.53
tdaF	9.34	0.49

FIG. 7. Pure TDA induces 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 after exposure to indicated concentrations of TDA. Error bars indicate standard deviations ($n = 3$). The asterisk indicates a statistically significant difference from the control (ANOVA; $P < 0.05$). (B) Relative abundance of *tdaC* and *tdaF* mRNAs after induction with 50 μM TDA relative to their abundance in an uninduced control sample, as measured by qPCR ($n = 3$). S.D., standard deviation.

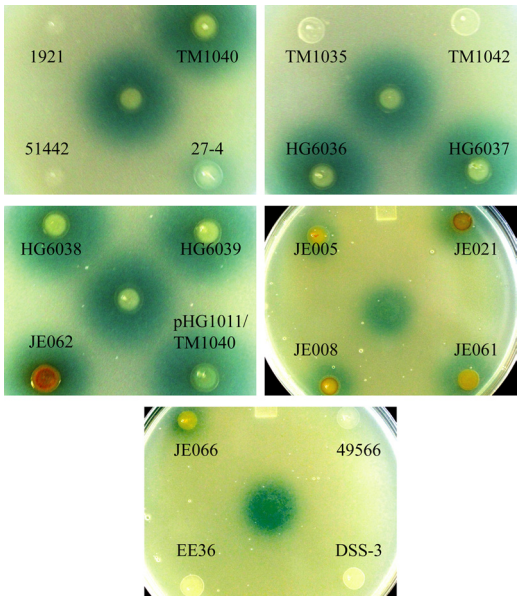


FIG. 8. Marine *Rhodobacterales* species induce the expression of *tdaCp*::*lacZ* of TM1040. A 10-μl amount of an individual 3-day standing culture was spotted on agar that contained a strain harboring the *tdaC-lacZ* transcriptional fusion plus X-Gal. TM1040 was inoculated in the center on each plate 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 as follows (clockwise): 1921, *Sulfitobacter* 1921; TM1040, *Silicibacter* sp. 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*; pHG1011/TM1040, *tdaC-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. JE066; 49566, *Roseobacter litoralis* ATCC 49566; DSS-3, *Silicibacter pomeroyi* DSS-3; EE36, *Sulfitobacter* EE36.

us to predict that other TDA-producing species of the *Roseobacter* clade would also be able to induce *tda* gene expression in TM1040.

Supernatants from several different *Roseobacter* clade and *Pseudovibrio* species were spotted onto the surface of nutrient agar containing X-Gal which had been seeded with strain HG1299 (*paaI*::EZ-Tn*5*) 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. 8, a positive response, i.e., *tdaC* transcription, was observed from species of *Phaeobacter*, *Pseudovibrio*, *Ruegeria*, and *Silicibacter*; strains of the 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*, *Silicibacter pomeroyi*, and two species of *Sulfitobacter* failed to induce *tdaCp*::*lacZ* transcription (Fig. 8), 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-genus roseobacterial (auto)inducer of *tda* gene expression.

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 engaging in a symbiotic association with their host phytoplankton. We refer to this change as the "swim-or-stick" switch (6), and the processes controlling it are critical for the initiation and maintenance of this symbiosis. In the present study, we explored some of the processes controlling a hallmark of the "stick," or sessile, phase, namely, the regulation of the biosynthesis of TDA. Our 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 the transcription of *tda* genes (specifically, *tdaCDE* and *tdaF*), with a commensurate increase in TDA biosynthesis. Unexpectedly, we also found that TDA itself is required for the 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 nonroseobacter marine bacteria (7, 9, 10, 18), 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 for their survival and adaptation, for the symbioses many of them engage in, and for 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. 1B). The 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 (10, 18) have shown significantly enhanced TDA production when bacteria are grown in standing liquid broth cultures (Fig. 2). The qPCR transcription data presented in this report build 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 levels of other genes previously shown (18) to be required for TDA synthesis, i.e., *tdaAB*, *cysI*, *malY*, and *paaIJK*, remain unchanged (Fig. 4). While the data do not provide firsthand evidence, it is likely that the expression of the latter set of seven genes remains unchanged due to the respective roles 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. (10) 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. We believe this is a very likely scenario.

It is also possible that *tda* expression is a by-product of a change in the physiology of sessile-phase cells, which are more prevalent in standing broth cultures than in shaking cultures (10, 18). We do not know if TDA induces biofilm and rosette formation in TM1040 or, conversely, if something about the physiological change in the sessile cells induces TDA biosynthesis. Transcriptomic analyses comparing TDA-induced to uninduced cells to look 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 cells induces TDA expression, may be tested by comparing *tda* gene expression in motile and sessile cells. It is of great interest to us to test the second hypothesis, since its validity has direct implications for 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 buildup of TDA near the bacteria and its host, which may have the consequence of serving to thwart other, nonroseobacter bacteria 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 this is speculative, the induction of *tda* genes by sessile TM1040 cells may be envisaged to encourage the attachment of other roseobacters on the surface of the phytoplankton, which may benefit both the mixed roseobacter biofilm and the phytoplankton host. Experiments to test these hypotheses are under way in our laboratory.

In contrast to the observed difference in *tda* expression during cell growth in standing versus shaking liquid culture conditions, the type of sulfur compound provided the cells did not significantly affect the transcription of *tdaCDE* or *tdaF*, although the addition of DMSP did result in a marked increase in TDA (Fig. 3). DMSP, which contains two sulfur atoms per molecule, is produced abundantly by many phytoplankton species (13, 50), including the dinoflagellate host, *P. piscicida*, used in our laboratory (34). DMSP is efficiently metabolized by TM1040 and other roseobacters as a source of carbon and sulfur (24, 34, 45). Moreover, since DMSP is a strong chemoattractant of TM1040 (36), it provides the bacteria with an important signal directing their swimming behavior toward the host phytoplankton (36) 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. 2). Second, the expression of *tdaC* transcription mirrors the TDA concentration and peaks late in the growth of TM1040 in standing broths (Fig. 2 and 5). Third, time course experiments of *tdaC* expression during standing liquid culturing also show a reproducible lag in *tdaC* expression after inoculation into a new medium (Fig. 5, closed circles, 0 to 10 h). The *tdaC* expression curve is similar to the expression curves of other molecules known to function as quorum signals (16, 47, 48). This is especially significant as previous work has shown that the genome of TM1040 does not harbor homologs to any known quorum signal genes (37), nor do these bacteria produce *N*-acyl homoserine lactones or other common quorum-signaling molecules (8).

The fourth line of evidence supporting a role of TDA as a density-dependent autoinducer comes from the series of cross-feeding experiments (Fig. 6 and 8). When *tdaCp*::*lacZ* on a replicating plasmid was placed in each of the 12 Tda$^-$ mutants originally described in Geng et al. (18), *tdaC* was not expressed in any of the Tda$^-$ backgrounds except the *cysI* mutant (Fig. 6A). This was puzzling, as this mutant (HG1220) has been reported by us to be defective in TDA synthesis (18). However, we discovered that the mutation in *cysI* (*cysI*::EZ-Tn*5*) is not a true loss-of-function mutation; rather, the CysI$^-$ strain produces extremely small 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 the presence of trace amounts 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. 6B) also produced two interesting results. First, a mutant with a mutation in *tdaA* was unable to respond (no β-galactosidase activity was produced from the *tdaCp*::*lacZ* transcriptional fusion) to extracellular molecules from the 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 (18), many of which require the binding of a cognate small-molecule ligand for optimal activation of the transcription of the target genes they control (42). We hypothesize that TdaA regulation of *tda* expression requires the binding of TDA (the molecule) for maximal activity. Experiments are currently being performed in our laboratory to test this hypothesis.

The second interesting outcome of the cross-feeding experiments came from the *tdaH* mutant (HG1244), 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 (18). 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, we recently reevaluated the *tdaH* mutation and discovered that the transposon had inserted 10 bp from the 3′ end

of *tdaH*, and we 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 (51), PAC (51), HisKA (44), HATPase (44), REC (44), and HPT domains (see Fig. S2 in the supplemental material). This suggests that the transposon may have polar effects on a gene (TM0962) downstream from *tdaH*.

We have used RT-PCR to test this hypothesis, the results of which are shown in Fig. S2B in the supplemental material. In wild-type bacteria, *tdaH* and *tdaI* mRNAs 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's 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 that 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 (48). 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. 8 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* expression in TM1040 include species of *Phaeobacter*, *Pseudovibrio*, and *Ruegeria* (Fig. 8). Our taxonomic analysis did not provide sufficient data to bring identification to the species level, but it was sufficient to conclude that none of these bacteria is a strain of *Silicibacter* sp. TM1040, whose affiliation with the genus *Ruegeria* is currently being questioned (49).

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 we have learned in the laboratory back to the ocean environment, we 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 the roseobacters and includes the phytoplankton host as well. While this is speculative, such interkingdom signaling has been reported (25).

ACKNOWLEDGMENTS

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