Exploring the Ecological Implications of Tropodithietic Acid Production in Phaeobacter piscinae

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Exploring the Ecological Implications of Tropodithietic Acid Production in *Phaeobacter piscinae*

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Contents

Preface

This thesis describes the work of my PhD carried out in the Center for Microbial Secondary Metabolites (CeMiSt) in the Department of Biotechnology and Biomedicine at the Technical University of Denmark. Lone Gram, Sheng-Da Zhang, and Eva Sonnenschein supervised the project. Additionally, part of the work was carried out during an external research stay from September to December, 2022, at University of York, Department of Biology, under the supervision of Dr. Paul CM Fogg and Dr. Pavol Bárdy.

The project was funded by the Danish National Research Fund (Dansk Grundforskningsfond, grant no. DNRF137). Funding for expenses related to the external stay was received from Augustinus Fonden, William Demant Fonden, and IDA- og Berg-Nielsens Fond.

The work described in this thesis was carried out between the 1st of April, 2020, and the 31st of March, 2023 and has resulted in the publication of two articles and another two articles in preparation.

Laura Louise Lindqvist

Kgs. Lyngby, March 2023

Acknowledgements

What is a PhD dissertation if not an opportunity to practice the Oscar speech, I will never give. First and foremost, I want to thank Lone for providing me with the opportunity to do this PhD and for your support the past three years. To my co-supervisors Eva and Shengda, a million thanks for your support during this project, and, most importantly, for your friendship. I owe all three of you a big thanks for shaping me into the scientist I am today. A big thank you also goes out to my external stay supervisors, Paul and Pavol, as well as the rest of the people I met during my time at University of York. An extra special thanks to Paul for always having time for a '10 minute meeting', which not once lasted only 10 minutes (I'm sorry, but not *that* sorry). Also a thank you to all the people I've co-authored with, your contributions have been invaluable.

A huge thanks goes out to all the past and present members of the BEB group and CeMiSt who I have had the immense pleasure of sharing my daily life with. Thanks to Scott for endless chemistry support (but mostly for letting me dog-sit Freya). Thanks to Mikael, Morten, and Yannick for not only scientific discussions, but even more so for all the fun times and personal talks. Mathilde, Pernille, and Katrine, thank you for always being there to share joys and sorrows over who knows how many cups of coffee of varying quality in 202. To Nathalie, thanks for engaging in a billion different side projects during our PhD years – between diving with sea lions, vegetable farming, and (attempts at) beaver spotting, you made sure daily life was always just a tad more fun, which, as we know, is my primary focus. These three years had never been the same without any of you.

Last, but most certainly not least, I want to thank my family and friends for talking to me about everything BUT microbiology. To my partner, Jonas, whose calm and patience I admire and can only aspire to, thank you for being my best friend and sharing your life with me for the past 7 years. I am beyond excited to see what life holds for us. And to my parents, Steen and Anette, for always allowing me to go my own way (almost) without objections and for being my biggest cheerleaders. It is truly hard to thank you enough.

Summary

Microbial secondary metabolites are small, diffusible molecules which, as opposed to primary metabolites, are not required for growth and proliferation. Instead, they play important roles in how microorganisms respond to and interact with their environment by acting as antibiotics, nutrient scavengers, and signals, to name a few. However, we still have a relatively limited understanding of the ecological implications of secondary metabolite production, as we have primarily studied them in relation to their industrial applications. The purpose of this PhD thesis was to investigate the ecological role(s) of secondary metabolites using the multifunctional secondary metabolite tropodithietic acid (TDA), produced by members of the marine *Roseobacter* group, as a case study.

In **Manuscript II**, we constructed a scarless deletion mutant devoid of TDA production and found that TDA-production drastically changes the physiology of the producing strain, including global changes in transcriptome, proteome, and proteome. Phenotypically, removing TDA production led to increased motility and decreased cell length. Disruption of TDA production also caused the expression of three putative horizontal gene transfer (HGT) systems; a type IV secretion system, a prophage, and a gene transfer agent (GTA). HGT is generally believed to promote adaptation to novel niches, indicating that TDA production affects adaptation potential of the producer. We proposed a model where TDA acts as a coordinator of colonization and adaptation: Once a TDA-producing population has established itself in a new niche, the accumulation of TDA serves as a signal of successful colonization, prompting a shift to a sessile lifestyle. This would reduce motility and the rate of HGT, while filamentous cells could form the foundation of a biofilm. Furthermore, the antibiotic properties of TDA may inhibit invading competing microorganisms, providing an additional role for TDA. Thus, TDA production may benefit the producer in a number of ways, highlighting the broader ecological importance of secondary metabolites.

GTAs are virus-like particles which packages and transfer random pieces of host DNA and thus, can facilitate HGT in a bacterial population. However, GTA release happens at the expense of the donor cell, which lyses to release the particles. GTA activation is therefore

governed by an intricate regulatory network. Given the observation that abolishing TDA production caused GTA activation, we in **Manuscript III** set out to investigate the regulatory network behind this repression. Based on bioinformatic analysis, we propose that TDA interacts with the quorum sensing (QS) response regulator PgaR to repress expression of GafA, a direct GTA activator, ultimately resulting in repression of GTA activation. Furthermore, TDA may also affect GTA activation through interaction with the CtrA phosphorelay system through an uncharacterized mechanism. These results are the first report of GTA regulation by an endogenously produced secondary metabolite and contribute to the growing understanding of GTA regulation.

A major challenge in microbial secondary metabolite research is the chemical detection of these compounds. As an alternative to direct chemical detection of TDA, we developed a transcriptional and a translational *tdaC* reporter fusion to investigate TDA production dynamics in **Manuscript IV**. These systems permit further downstream studies into the model proposed in **Manuscript II** by studying how TDA production proceeds during colonization and which factors influence TDA production.

The results presented in this thesis contributes to the growing evidence of the importance that secondary metabolites have in modulating bacterial interactions with other organisms and their environment. Furthermore, I conclude that unravelling the ecological role of secondary metabolites requires a holistic and exploratory approach, which may ultimately lead us to novel functions and roles for these compounds.

Resumé

Mikrobielle sekundære metabolitter er små diffusible molekyler, som, i modsætning til primære metabolitter, ikke er påkrævede for vækst og celledeling. I stedet spiller de en vigtig rolle i, hvordan mikroorganismer responderer på og interagerer med deres omkringliggende miljø ved at virke som antibiotika, bidrage til opsamling af næringsstoffer og som signaler, for at nævne nogle få funktioner. Vi ved dog stadig relativt lidt om de økologiske implikationer ved at producere sekundære metabolitter, da vi primært har studeret dem med henblik på deres industrielle anvendelser. Formålet med denne PhD afhandling var derfor at undersøge sekundære metabolitters økologiske rolle ved at bruge den multifunktionelle sekundære metabolit, tropodithietic acid (TDA), produceret af medlemmer af den marine *Roseobacter* gruppe, som et case study.

I **Manuskript II** konstruerede vi en deletions-mutant, der var ude af stand til at producere TDA, og fandt, at dette drastisk ændrede fysiologien af den producerende stamme, inklusiv globale ændringer i transkriptomet, proteomet og metabolomet. Fænotypisk ledte ophøret af TDA produktion til øget motilitet og mindsket celle-længde. Fraværet af TDA produktion ledte også til øget ekspression af tre potentielle systemer for horisontal genoverførsel (HGO); et Type IV sekretionssystem, en profag og en gene transfer agent (GTA). HGO menes generelt at fremme tilpasning til nye nicher, hvilket ultimativt indikerer, at TDA produktion påvirker tilpasningspotentialet af producenten. Vi foreslår en model, hvori TDA koordinerer kolonisering og tilpasning: Når en TDA-producerende population har etableret sig selv i en ny niche, signalerer akkumuleringen af TDA, at koloniseringen har været succesfuld, hvilket leder til et skift til en stationær livsstil. Herved reduceres motiliteten og raten af HGO, mens de filamentøse celler danner basen for en biofilm. Derudover inhiberer TDA konkurrerende mikroorganismer ved at virke som et antibiotika, og der er herved flere roller for TDA. TDA produktion kan derfor være en fordel for producenten på mere end én måde, hvilket fremhæver den bredere økologiske relevans for sekundære metabolitter.

GTAer er virus-lignende partikler, der pakker og overfører tilfældige stykker af værtens DNA og derved kan facilitere HGO i en bakteriepopulation. Frigivelsen af GTAer sker dog på

bekostning af donor-cellen, som undergår lysering for at frigive partiklerne. GTA aktivering er derfor stramt reguleret. Ud fra vores observation af, at fraværet af TDA produktion ledte til GTA aktivering, undersøgte vi i **Manuskript III,** hvad det regulatoriske netværk bag dette var. Baseret på bioinformatiske analyser foreslår vi, at TDA interagerer med quorum sensing (QS) regulatoren PgaR for at hæmme udtrykkelsen af GafA, en direkte GTA aktivator, hvilket ultimativt ledder til hæmmelse af GTA aktivering. Derover kan TDA muligvis påvirke GTA aktivering gennem interaktion med CtrA phosphorelay systemet via en ukarakteriseret mekanisme. Disse resultater udgør den første rapport af GTA regulering vha. en endogent produceret sekundær metabolit og bidrager til den voksende forståelse af GTA regulering.

En stor udfordring inden for forskning af mikrobielle sekundære metabolitter er den kemiske detektion af disse. Som et alternativ til direkte kemisk detektion af TDA udviklede vi en transkriptionel og en translationel *tdaC* rapporter-fusion for at undersøge TDA produktions dynamikker i **Manuskript IV**. Disse systemer muliggør yderligere studier af den foreslåede model fra **Manuskript II** ved at undersøge hvordan TDA produktion er koordineret under en koloniseringsproces.

Resultaterne fra denne afhandling bidrager til den voksende mængde beviser, der indikerer, hvorledes sekundære metabolitter modulerer bakteriers interaktion med andre organismer og deres miljø. Derudover konkluderer jeg, at vi, for at forstå den økologiske rolle af sekundære metabolitter, må benytte os af en holistisk og eksplorativ tankegang, hvilket ultimativt kan lede os til nye roller og funktioner for disse kemiske forbindelser.

Articles

This thesis is based on work from the following manuscripts:

Manuscript I: Henriksen, N. N. S. E.*, **Lindqvist, L. L.***, Wibowo, M., Sonnenschein, E. C., Bentzon-Tilia, M., & Gram, L. 2022. Role is in the eye of the beholder - the multiple functions of the antibacterial compound tropodithietic acid produced by marine *Rhodobacteraceae*. FEMS Microbiol Rev 46:1-15.
**These authors contributed equally.*

Manuscript II: **Lindqvist, L. L.,** Jarmusch, S. A., Sonnenschein, E. C., Strube, M. L., Kim, J., Nielsen, M. W., Kempen, P. J., Schoof, E. M., Zhang, S-D., & Gram L. 2023. Tropodithietic acid, a multifunctional antimicrobial, facilitates adaption and colonization of the producer, *Phaeobacter piscinae*. mSphere 8:1-15.

Manuscript III: **Lindqvist, L. L.,** Fogg, P. C. M, Zhang, S-D., & Gram, L. Tropodithietic acid production represses the production of a novel gene transfer agent in *Phaeobacter* spp.
In preparation, March 2023

Manuscript IV (in preparation): **Lindqvist, L. L.**, Zhang, S-D., Melchiorsen, J., & Gram L. Monitoring production of tropodithietic acid using fluorescent gene reporter fusions,
In preparation, March 2023

During my PhD, I also contributed to the following articles not included in the thesis:

Zhang, S. D., **Lindqvist, L. L.**, Isbrandt, T., Borre, I. L., Wibowo, M., Nielsen, M. W., Ding, L., Larsen, T. O., & Gram, L. 2022. Solonamides, a Group of Cyclodepsipeptides, Influence Motility in the Native Producer *Photobacterium galatheae* S2753. Appl Environ Microbiol 88:1-16

Zhang, S. D., Isbrandt, T., **Lindqvist, L. L.**, Larsen, T. O., & Gram, L. 2021. Holomycin, an antibiotic secondary metabolite, is required for biofilm formation of the native producer *Photobacterium galatheae* S2753. Appl Environ Microbiol 87:1-15

Abbreviations

AHL	N-Acyl homoserine lactone
AI	Autoinducer
BGC	Biosynthetic gene cluster
c-di-GMP	Cyclic dimeric guanosinmonophosphate
CCR	Carbon catabolite repression
DMSP	Dimethylsulfoniopropionate
GTA	Gene transfer agent
H-NS	Histone-like nucleoid-structuring protein
HGT	Horizontal gene transfer
NRP	Non-ribosomal peptide
NRPS	Non-ribosomal peptide synthetase
PAA	Phenylacetic acid
PK	Polyketide
PKS	Polyketide synthase
PSTF	Pathway specific transcription factor
RNAP	RNA polymerase
T4SS	Type IV secretion system
TDA	Tropodithietic acid
QS	Quorum sensing

List of figures

Chapter 1:
Introduction

In spite of their deceptively simple nature, bacteria wield incredible power in shaping our world. Bacteria constitute the earliest form of life, with fossil records dating as far back as 3.7 billion years ago (1). That we and other organisms are even able to inhabit Earth is also attributed to bacteria; around 2.4 billion years ago, the cyanobacteria evolved and facilitated the rise of atmospheric O_2 (2). Quite literally, we owe our life to bacteria. Not only are they one of the earliest lifeforms, they are also, by a large margin, the most diverse (3). Consequently, they display tremendous metabolic diversity, and this metabolic activity is detrimental for global biochemical cycling of nutrients (4).

Ever since Alexander Fleming discovered the penicillin-mediated inhibition of bacteria in what would probably become the World's most famous petri dish (5), humans have explored and exploited the potential of secondary metabolites, small molecules produced by a plethora of organisms, including, but not limited to, bacteria, fungi, plants, and archaea (6–9). In this thesis, I will focus on secondary metabolites produced by bacteria and, to a lesser extent, fungi. The wide range of bioactivities exhibited by these compounds has warranted a massive interest in their applications, and today microbial secondary metabolites are used for a variety of purposes, including as therapeutics, food additives, and for crop protection (10). Clearly, human-kind have benefitted tremendously from Nature's chemical ingenuity, and it is hard to imagine life without it.

However, microbial secondary metabolites have changed not only our lives, but also the lives of the organisms that produce them. Until recently though, secondary metabolites were perceived as dispensable as they are not necessary for growth and proliferation of the producing organism. This could not be further from the truth; secondary metabolites provide the producer with a plethora of advantages by acting as antibiotics, nutrient scavenging

molecules, and signals, to name a few (11, 12). Thus, secondary metabolite production is a key strategy for microbial survival and adaptation to a range of ecological niches. Our previous lack of interest in the ecology of secondary metabolite production also means that there is still a lot to uncover about these compounds. In the search for novel secondary metabolites, we often employ assays looking for specific bioactivities, highlighting the need for exploratory studies if we want to uncover their lesser-known functions and roles. This may in turn provide us with novel applications for these compounds, whilst providing us with novel insight into how microorganisms interact and how this affects our World.

Tropodithietic acid (TDA) is a secondary metabolite produced by several members of the *Roseobacter* group, including *Phaeobacter* spp., *Tritonibacter* spp. (previously *Ruegeria* spp.), and *Pseudovibrio* spp (13). Interest in this compound was originally fueled by the discovery that producing bacteria had potential as aquaculture probiotics due to the broad-spectrum antibiotic properties of TDA, proposed to be due to disruption of the proton motive force (14, 15). Aside from this though, TDA also exhibits iron chelating properties (16) and is proposed to act as a quorum sensing (QS) signal, providing several potential ecological roles for TDA (13, 17). This makes TDA an excellent example for exploring the versatile roles of secondary metabolites.

The purpose of this PhD project is to investigate the ecological implication of bacterial secondary metabolite production using the antibiotic secondary metabolite TDA as a case study.

To address this aim, we set out to unravel the ecological implications of TDA production for the producing strain, here *Phaeobacter piscinae* S26, in **Manuscript II.** We constructed and compared a TDA-deficient *P. piscinae* to its wild type (WT) counterpart through a series of physiological comparisons, coupled with a comparison of the global transcriptome,

proteome, and metabolome. We found that TDA production represses the activation of a previously uncharacterized gene transfer agent (GTA). **Manuscript III** follows up on an observation, investing how TDA production mediates this repression. In **Manuscript II**, we propose that TDA acts as a colonization signal, coordinating adaptation to novel niches. To further investigate this idea, we developed two genetic reporter fusion to track *tdaC* expression in **Manuscript IV** to use as a proxy for investigating the dynamics of TDA production, with a focus on host colonization processes.

Manuscript I is a review of TDA, providing an in-depth presentation of TDA producers, biosynthesis, regulation, and function with a focus on the ecological significance of the compound. For the theoretical part of this thesis, I will therefore stick to an overall description of secondary metabolism to set the context for evaluating the ecological role of TDA. **Chapter 2** provides a general overview of what secondary metabolites are and how they are synthesized, with a focus on the class of tropones, which TDA belongs to. In **Chapter 3**, I discuss which factors regulate secondary metabolite production and how we can track secondary metabolism to better understand the dynamics of secondary metabolite production. **Chapter 4** covers some of the functions and potential ecological roles that secondary metabolites have. Following this, in **Chapter 5** I give an introduction to GTAs in *Rhodobacteraceae* to set the context for **Manuscript II** and **III,** where I propose that TDA acts as a regulator of GTA activation. Lastly, in **Chapter 6** I draw in theory from the previous chapters to ultimately evaluate the ecological implications of TDA production and expand to include other multifaceted secondary metabolites.

Chapter 2:
An introduction to microbial secondary metabolism

Traditionally, metabolites are classified as either primary or secondary. Primary metabolites are predominantly produced during exponential growth and include compounds such as nucleotides, vitamins, and amino acids. These metabolites are highly conserved, even across kingdoms, and are directly involved in proliferation, growth, and development of the producing organism. Secondary metabolites, on the other hand, are often lineage specific and are not necessary for normal growth and development, at least in laboratory settings (18). Instead, they play important roles in how the organism interacts with its environment which I will return to in **Chapter 4.** In microorganisms, the production of secondary metabolites was initially believed to be confined to stationary growth; however, this likely reflects limitations in detection methods, as later studies have demonstrated that some are also produced during exponential phase (19). Another key difference between primary and secondary metabolites is the cellular localization; primary metabolites are charged, preventing diffusion through the cell membrane and are therefore commonly located inside of the cell. On the other hand, secondary metabolites are typically uncharged and non-polar and can therefore readily diffuse between cells, although some are also actively transported (20).

The term 'secondary metabolites' was originally coined in 1891 by Nobel laureate Albrecht Kossel (21) and has since been the most common term for these compounds. Whilst the term 'secondary' seem to imply that these compounds are dispensable, they are integral to how microorganisms interact with their environment: Some act as nutrient scavengers, providing the bacteria with an advantage in nutrient-poor environments (11). Others act as signaling molecules, coordinating responses to changing environments, and some also act as

antibiotics, warding off potential competitors (12). Another observation disputing their dispensability is the fact that some bacteria dedicate as much as 15% of their genome to secondary metabolism (22, 23). Clearly, these compounds play a vital role in microbial lives.

Because of this, the nomenclature surrounding these molecules have long been a topic of debate in the so-called natural product community. As stated, the term 'secondary' suggests a hierarchy of importance between primary and secondary metabolism. Instead the term 'specialized metabolites' is now sometimes used as an alternative used to avoid minimizing the importance of these compounds (24). Another commonly used term is 'natural products', reflecting the drug potential of many of these compounds (25). However, as secondary metabolites is still the most widely used name, this is the term that will be used throughout this thesis.

In this chapter, I will give a brief introduction to secondary metabolites and their biosynthesis with a focus on the class of tropones to which the compound TDA belongs.

2.1. Biosynthesis of secondary metabolites

The biosynthesis of secondary metabolites incorporates precursors from primary metabolism, such as amino acids and cofactors, and as such, primary and secondary metabolism are interweaved (26, 27). However, whilst primary metabolism is well-mapped out, we are still lacking a holistic overview of secondary metabolism. As opposed to primary metabolism, which is tightly interconnected, secondary metabolite pathways function in parallel with little interplay between pathways due to an assembly-line structure of many of these pathways where intermediates are passed directly between the enzymes with little diffusion involved (28). The resulting end products also differ substantially: Primary metabolism usually converges into specific end-products, whilst secondary metabolite pathways are often promiscuous and result in a range of analogs (29). This has been described as primary metabolism being target-oriented whilst secondary metabolism is diversity-oriented (29), reflected in the extensive chemical variety of secondary metabolites.

In **Manuscript I** we reviewed the biosynthesis of TDA which exemplifies several of these distinctions of secondary metabolism (**Figure 1**). The biosynthesis of TDA is a particular interesting case of how products of primary metabolism can be shunted towards secondary metabolism: The precursors for this pathway arise from phenylacetic acid (PAA) catabolism, and here, a homologue of the enzyme PaaZ, which is involved in primary metabolism, shunts intermediates from primary metabolism towards secondary metabolism and biosynthesis of TDA (30). TDA biosynthesis is also a good example of pathway promiscuity since the pathway gives rise to a series of analogues as seen in **Manuscript II.** Except for the non-antibiotic methyl troposulfenin (31), these analogues have yet to be structurally characterized**.** A set of well-characterized tropolactones, the roseobacticides, also rely on the *tda* and *paa* biosynthetic machinery (32) demonstrating how structurally and functionally distinct products may arise from the same pathway.

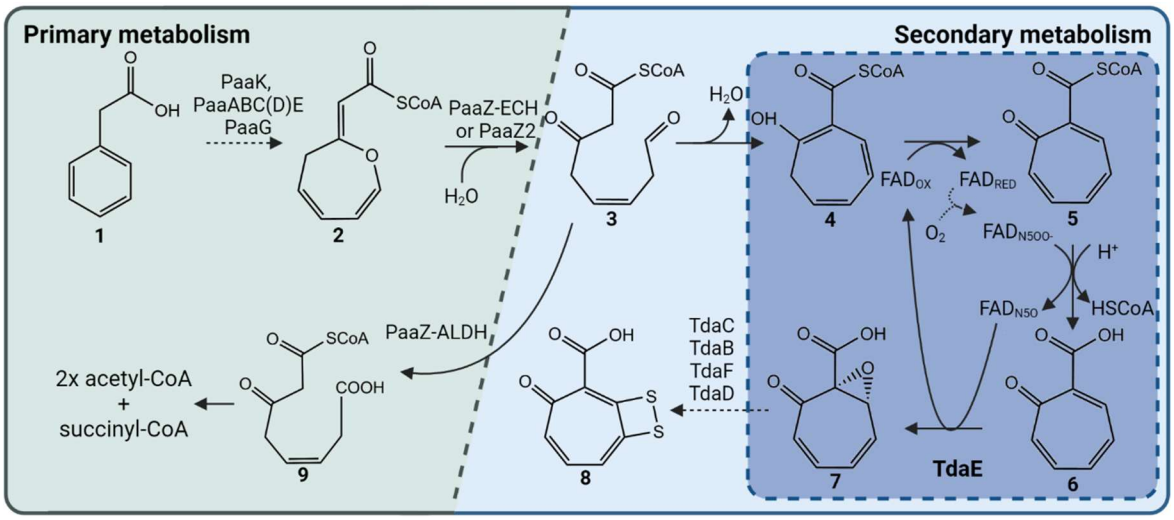

Figure 1. Schematic representation of TDA biosynthesis and the link between primary metabolism (green) and secondary metabolism (blue). The carbon backbone of TDA arises from the PAA catabolon, and a homologue of PaaZ, PaaZ2, shunts the production of **3** towards TDA biosynthesis. TdaE connects primary and secondary metabolism through dehydrogenation, oxygenolysis, and ring epoxidation to form **7**, which is converted to TDA (**8**) by TdaBCFD. Figure reproduced from **Manuscript I** with permission (Copyright © 2022, FEMS Microbiol Rev).

2.2. Biosynthetic gene clusters

A key trait of secondary metabolites is that their metabolic machinery is often encoded together in biosynthetic gene clusters (BGCs, **Figure 2**) (33). For canonical BGCs, as a minimum they encode a synthase or synthetase, defining the chemical class of the resulting compound, as well as a set of tailoring enzymes which modify the compound e.g. through methylation, epimerization, or oxidation (6). In addition to this, a BGC may also contain cluster-specific transcription factors, which regulate expression of the BGC, genes conferring resistance to the compound produced by the BGC, transport genes, and genes without any known function (6, 34).

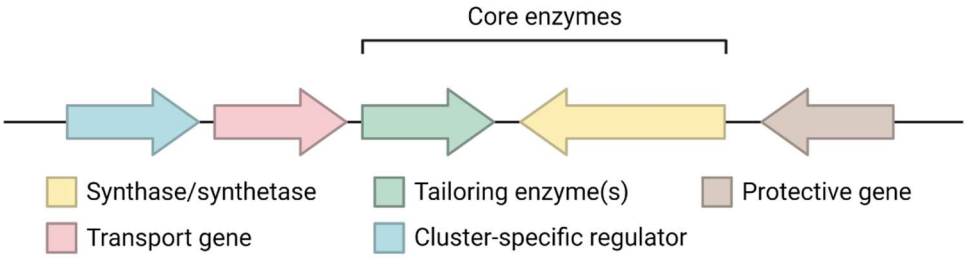

Figure 2. Structure of canonical BGCs. Created with Biorender.com

2.2.1. Canonical pathways: Non-ribosomal peptides, polyketides, and terpenes

Major classes of secondary metabolites produced by canonical pathways include non-ribosomal peptides (NRPs), polyketides (PKs), and terpenes. I will here give a brief intro to these classes.

NRPs are synthesized by non-ribosomal peptide synthetases (NRPSs), large modular enzymes where amino acid building blocks are added and modified at each unit (28). A module consists, as a minimum, of a phosphopantetheinylated peptidyl carrier protein, to which the growing peptide-chain is bound, an adenylation domain which selects and activates the amino acid building block, and a condensation domain which forms the peptide-bond (28). Additionally, the module may contain tailoring enzymes, which may perform reactions such as epimerizations or oxidations (28). The final NRP is released by a terminal thioesterase domain (28).

PKs are short chain carboxylic acids synthesized from acyl-coA thioester units by polyketide synthases (PKSs), which bear resemblance of fatty acid synthases (35). PKSs consist of an acyl-transferase, which selects, activates and loads the starter-unit onto the acyl-carrier protein, which the growing chain is attached to, as well as a ketosynthase, which catalyzes the condensation of the incoming unit. Additional tailoring enzymes such as ketoreductases, dehydratases, and enoylreductases contribute to structural diversity within this class by additional modifications (28).

Lastly, terpenes consist of multiple isoprene units and constitute the largest and most diverse class of secondary metabolites (36). As opposed to PKSs and NRPSs which share somewhat the same modular assembly-logic, isoprenes are joined to form the core structure by a single enzyme, terpene cyclase (36). The resulting product is a linear polyene with branching methyl-groups, which can be further modified e.g. by the addition of alternative building blocks such as sugars, and amino acids (36).

The architecture of some of these BGCs facilitate the prediction of the resulting compounds based on the organization and specificity of the encoded enzymes. By predicting the specificity of the selective domains as well as the presence of any tailoring domains, using software such as antiSMASH or MIBiG, we can identify novel secondary metabolites based solely on sequence information (37, 38). However, not all secondary metabolites are encoded by canonical BGCs, making their detection and structural prediction more challenging (39).

2.2.2. Non-canonical pathways: Tropones

An example of a non-canonical class of secondary metabolites are the tropones, non-benzene aromatic compounds (**1, Figure 3**). A distinctive feature of this class of secondary metabolites is the origin of their carbon backbone, which arises from primary metabolism through degradation of PAA (40). Tropones are produced by both bacteria, fungi, and plants, and upwards of 16% of all publicly available bacterial genomes encode the machinery for PAA degradation and hence, principally the machinery for tropone biosynthesis (40).

Several tropones have pharmaceutically relevant bioactivities: Tropolone derivatives (**2, Figure 3**), tropones with a hydroxy-group in position 2 of the aromatic ring, exhibit both antiviral, anticancer, antimicrobial, insecticidal, and inflammatory bioactivities (41, 42). Tropone derivatives also play important roles in nature: For example, *Burkholderia plantarii* causes seedling blight in rice where tropolone acts as a virulence factor to promote this infestation (43).

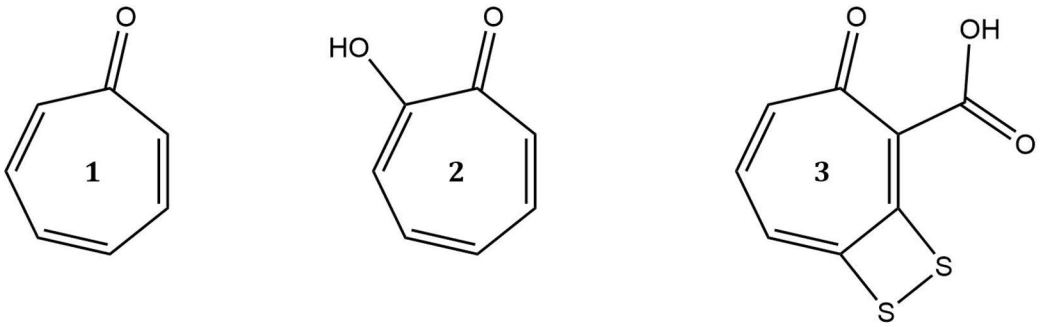

Figure 3. Structure of tropone natural products. 1. Tropone. 2. Tropolone. 3. Tropodithietic acid.

Another example of a both clinically and ecologically relevant tropone derivative is the sulfur-containing TDA (**3, Figure 3**), reviewed in **Manuscript I**, which is both a potent antibiotic and has shown anticancer activities (15). Thiotropocin, the tautomer of TDA, was first isolated from a soil *Pseudomonas* spp. in 1984 (44, 45) and TDA itself was since detected in the marine bacterium *Phaeobacter inhibens* (46). Since then, TDA production has been reported for a number of members of the *Roseobacter* group, including strains of the genera *Phaeobacter*, *Tritonibacter*, and *Pseudovibrio* (13). The *Roseobacter* group is predominantly marine and the group is often associated with microalgal blooms where they may constitute up to 30% of the bacterial population (47, 48).

TDA is proposed to act as an electroneutral proton antiporter leading to disruption of the proton motive force (**Figure 4**), explaining its antibiotic properties (15). Extracellular protons are bound by the carboxyl group and transported across the cell membrane, where the proton is exchanged for a metal ion in the cytosol (15). This metal ion is then transported back to the extracellular space, which leads to a disruption of the transmembrane potential whilst the membrane potential is conserved (15). Additionally, TDA can chelate iron weakly (16) and is proposed to act as a QS signal (49) which led us to speculate in **Manuscript I** that TDA has several ecological roles, summarized in **Figure 4**. I will return to this multifunctionality and the ecological implications of this in **Chapter 6**.

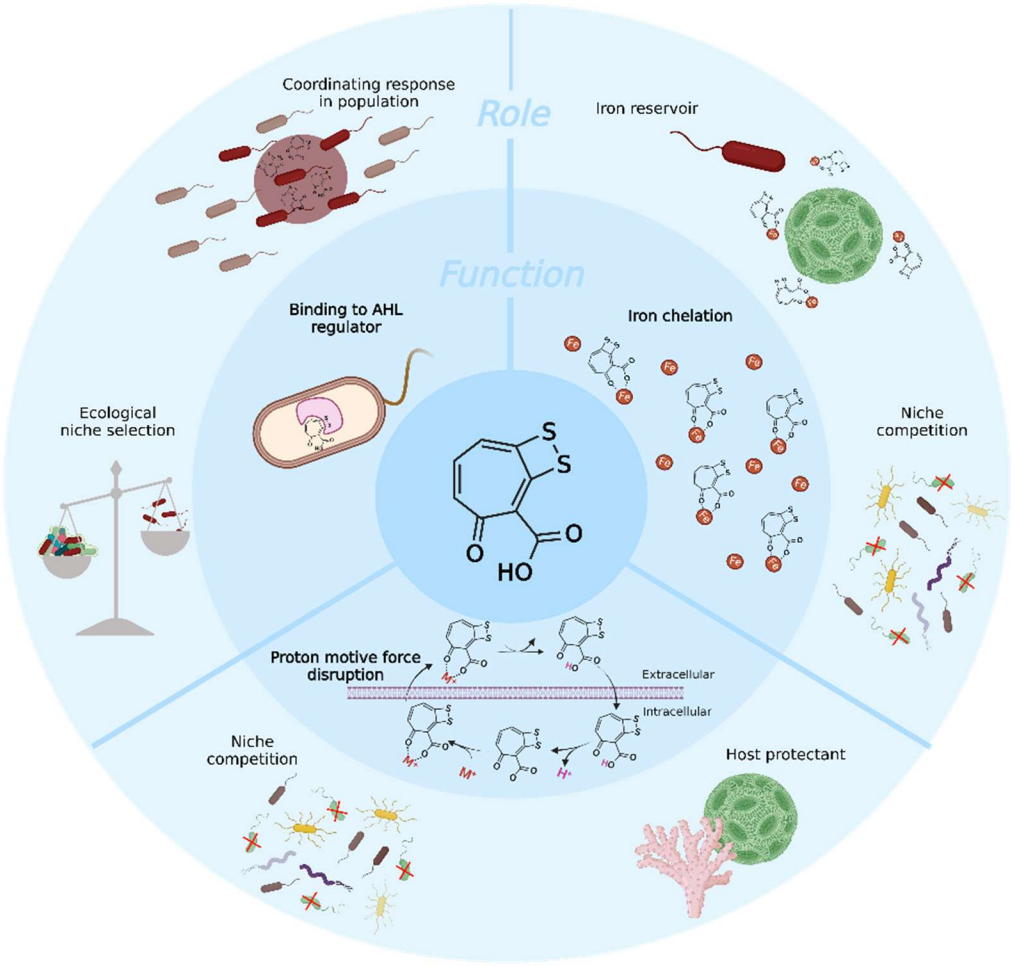

Figure 4. Overview of TDA functions and the proposed ecological roles of these functions. Figure reproduced from **Manuscript I** with permission (Copyright © 2022, FEMS Microbiol Rev).

Chapter 3:
Factors influencing secondary metabolism

Coordinating microbial metabolism is key to responding to changes in environment and as such, successful survival of microorganisms are highly dependent on such coordination. Since primary metabolism is required for growth and proliferation, these pathways are commonly highly active. Contrastingly, many secondary metabolites are not detected during standard laboratory cultivation. This became apparent with the rise of sequence-based genome mining, where far more BGCs could be identified than were known to correspond to products (50). This observation sparked an interest in identifying triggers of secondary metabolite production in a search for novel bioactive compounds.

Secondary metabolism is under both pleiotropic and pathway-specific control. As previously mentioned, pathway-specific transcription factors (PSTFs) are often, but not always, encoded in a BGC. BGCs vary greatly in size, and there appears to be some correlation between the number of enzymes encoded in a BGC and the presence of a PSTF. In *Aspergillus* spp. for example, BGCs with more than five enzymes encoded are more likely to encode a PSTF (51). This is proposed to ensure co-regulation of multiple genes that would be hard to achieve through global regulatory networks (51). However, PSTFs are often targeted by global regulators: An example of this is found in *Streptomyces coelicolor*, where the actinorhodin BGC encodes the PSTF actII-ORF4 which is affected by numerous global regulators (52), including DasR which regulates metabolism of N-acetylglucosamine (53). Thus, regulation of secondary metabolism is often coordinated with a number of other physiological changes.

The reasons for the tight multi-level regulation of secondary metabolism is likely found in the specificity of the roles they play. Production of secondary metabolites can be a costly affair, as it directs energy and intermediates away from primary metabolism (54–56), and,

hence, growth and proliferation. This accentuates the need to only produce secondary metabolites when necessary. As a result, it is common for bacteria to incorporate both biotic and abiotic cues in the decision of when to activate production of a given secondary metabolite. Identifying triggers of secondary metabolism may shine a light on the ecology of these compounds. At least in some cases, regulation of secondary metabolite production appears to reflect an ecological function of the resulting compound; for example, competition with the bacterium *Ralstonia solanacearum* increases the production of the antibacterial compound bikaverin in *Fusarium* spp, suggesting that bikaverin aids in fungal protection against bacterial competition (57).

In this chapter, I will cover the environmental and microbial signals that commonly affect secondary metabolite production, including examples of this. The pathways underlying the responses to these cues will not be covered as this is beyond the scope of this thesis, but rather emphasis will be put on what the different cues tell us about the ecology of secondary metabolites. Lastly, I will cover the means by which we can track secondary metabolite production in our quest to understand their regulation.

3.1. Environmental factors

Unsurprisingly, environmental factors such as nutrients, pH, and temperature affect secondary metabolism (6). For example, in fungi the transcription factor PacC controls the expression of several secondary metabolite BGCs in response to changes in pH (58–60). A classic example of nutrient-regulated secondary metabolites are siderophores, which are iron-chelating compounds which are typically produced during iron-limitation to give the producer preferential access to the available iron (61).

Secondary metabolism is also affected by the availability of key nutrients such as nitrogen, phosphate, and carbon (52, 62). Most commonly, secondary metabolism is upregulated in response to depletion of these nutrients consistent with secondary metabolite production being predominantly associated with stationary phase.

One of the most well-studied examples of nutrient-mediated regulation is carbon catabolite repression (CCR). CCR is an important regulatory phenomenon ensuring the utilization of the preferential carbon-source by an organism if numerous sources are present. Optimal carbon source utilization is a major factor in optimizing growth rate, and, thus, in optimizing competitive fitness of an organism (63). The regulatory effects of choice of carbon source can be wide-reaching: In the fungi *Schizosaccharomyces pombe*, cultivation on glycerol resulted in differential expression of ~73% of all genes compared to cultivation on glucose or sucrose (64), and secondary metabolite production is also commonly affected by CCR (65). In marine *Vibrionaceae*, the polysaccharide chitin affects the secondary metabolome (66, 67). Chitin is the most abundant polymer in the marine environment, commonly found in the exoskeleton of crustaceans (68). In marine *Vibrionaceae*, the genes for chitin catabolism are well-conserved (69), indicating that chitin is an important nutrient for this family. This hints that some of the nutrient-specific responses in secondary metabolite production may be linked to niche specificity.

A similar example of a niche-specific nutrient which effects secondary metabolism is the sulphur dimethylsulfoniopropionate (DMSP), an algal osmolyte. Members of the *Roseobacter* group are often associated with algal blooms, and this group of bacteria play a central role in DMSP cycling (70). As reviewed in **Manuscript I**, some roseobacters produce the antibiotic TDA, and it has been demonstrated that DMSP increases TDA production in *Tritonibacter mobilis* as compared to other sulfur sources (71). Additionally, DMSP acts as a chemoattractant for *T. mobilis* (72), and the coupling between DMSP and TDA production could therefore indicate a lifestyle specific requirement for TDA. I will return to this in **Chapter 6**.

3.2. Microbial interactions influence secondary metabolite production

Secondary metabolite production can also be affected by interactions with other microorganisms. Co-cultivation is a common approach to the induction of secondary

metabolism within bioprospecting (73–75), highlighting that secondary metabolites are often produced in response to signals from other organisms. Microbial interactions may broadly be categorized as either competitive or cooperative (76), and both types of interactions may affect secondary metabolism.

An example of competitive interactions is the induction of secondary metabolites by sub-inhibitory concentrations of a wide array of antibiotic secondary metabolites. A study of the malleilactone cluster in *Burkholderia thailandensis* found that, in a screening of 640 small molecule elicitors, the nine most potent inducers were all antibiotics (77). This tendency has been reported in studies of small molecule elicitors of secondary metabolism in other microorganisms and the induction often affects several BGCs (19, 78, 79). The adjacent ecological interpretation of this phenomenon is that it reflects the need to 'fight back' against competing ecological neighbors.

On the other hand, a classical example of cooperative behavior is QS. QS is a mechanism of intercellular communication that bacteria use to coordinate the behavior of a group of cells based on the accumulation of a cell-cell signal, referred to as an autoinducer (AI). A QS system consists of an AI synthase, an AI, and a receptor which responds to the AI and in turn regulates a set of genes (80). These systems are commonly used to control behaviors such as motility, biofilm formation, and secondary metabolite production (81–83).

In **Manuscript I**, we reviewed how the production of TDA is regulated by the LuxIR-like PgaIR system in *P. inhibens* (81). Here, the accumulation of AI leads to induction of TDA biosynthesis, and furthermore, TDA itself may act as an AI in the presence of PgaR leading to autoinduction (81). This is likely the case in other *Phaeobacter* spp. as well: In **Manuscript II**, we saw that abolishment of TDA production in *P. piscinae* led to a decrease in transcripts and proteins of the TDA BGC, indicating that autoinduction also takes place in this strain.

QS is generally believed to have arisen to control processes that are only beneficial if undertaken as a group. In light of this, QS-mediated regulation of secondary metabolites

would indicate that whatever their function, a concerted production is necessary to achieve the ecological benefit of producing a given secondary metabolite. For example, in line with the idea of antibiotic secondary metabolites being excreted as weapons, reaching a high enough concentration of excreted compound to achieve even localized inhibition would likely require a coordinated effort. However, non-antagonistic roles may also require coordination: Some secondary metabolites, e.g. rhamnolipids and surfactins (84, 85), aid in motility and biofilm formation, and both of these are processes where community coordination is key. I will return to the ecological roles of secondary metabolites in **Chapter 4**.

3.3. Biofilms and spatial control of secondary metabolism

In multicellular organisms, such as plants and some fungi, there are spatial variations in secondary metabolite production. For example, in the filamentous fungi *Aspergillus terreus*, the secondary metabolite aspulvinone E is converted to different products depending on tissue localization; in the hyphae, it is converted to aspulvinones and in conidia to melanins (86). Intuitively, this makes sense for complicated multicellular organisms, where cell differentiation is a key process.

However, in spite of their seemingly simple, single-celled nature, it has long been recognized that bacteria also live in complex communities where coordinated multicellular behavior and spatial organization is of utmost importance (87, 88). Concordantly, spatial organization may affect the secondary metabolome of a microbial community (89). One form of multicellular bacterial behavior is the formation of biofilms, which has been defined by the International union of Pure and Applied Chemistry as “aggregates of microorganisms in which cells are frequently embedded in a self- produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface” (57). In the marine environment, surface-associated bacteria more likely to display antagonistic activities (90, 91), and as reviewed in **Manuscript I,** TDA-producers are also predominantly surface-

associated (92). In line with this observation, many secondary metabolites are upregulated during cultivation in biofilms compared to planktonic growth (93, 94). Thus, biofilms are a great environment for studying spatial expression of secondary metabolites.

Bacillus subtilis is one of the most well-studied biofilm formers which differentiates into at several distinct cell types, including motile, sporulating, matrix producing, and cannibal cells to name a few (95). As part of this, van Gestel and colleagues demonstrated that during *B. subtilis* sliding motility, two distinct populations arose; an 'inner' population of matrix-producers, whilst, at the outer edge of the spreading colony, a population produced the secondary metabolite surfactin (96). The presence of surfactin-producers at the outer edge of the colony allowed the inner population of matrix-producing cells to form bundles and spread outwards from the colony center (96).

These spatial differences in secondary metabolite production may serve several purposes; first of all, it may serve as a strategy for division of labor (97). It has also been proposed that having at least some cells maintain a certain level of secondary metabolite production could enable more rapid adaptation to abiotic or biotic changes as opposed to completely turning off production (98). Lastly, this type of regulation may reflect a spatio-specific requirement of the produced compounds, perhaps indicative of their ecological role. In the before mentioned case of differential spatial production of surfactin in *B. subtilis*, surfactin is commonly known to reduce surface tension, which is proposed to facilitate movement (85, 99, 100). The direct observation that surfactin is primarily produced at the edge of a spreading colony strengthens this ecological interpretation.

3.4. Tracking secondary metabolism

When studying secondary metabolite production, the ability to track secondary metabolites is crucial to accurately pinpoint production dynamics. Unfortunately, detection also seems to be one of the biggest obstacles of secondary metabolite research, although developments

in chemical detection has taken us a long way, with the sensitivity of these methods increasing rapidly.

The most direct way of tracking secondary metabolism is through detection of the compounds themselves. Today, techniques such as mass spectrometry imaging (MSI) even allows us to visualize the distribution of metabolites *in situ*, bringing us one step closer to studying 'natural' samples (101, 102). A more indirect approach is to track BGC expression, e.g. through the use of proteomics, RNA-seq, or reverse transcription quantitative real-time PCR (RT-qPCR) (77, 98, 103). Depending on the research question, one or more of these methods may be more suitable than the others.

In **Manuscript IV**, we set out to develop a system for tracking TDA production in *P. piscinae in situ* and over time, whilst also allowing for high-throughput studies. An (indirect) approach that incorporates all of these criteria is the use of reporter-fusions, fluorescent tags used to measure expression of one or more of the core genes in the biosynthetic pathway of the compound of interest. This approach has been used in numerous studies as a proxy to follow secondary metabolite production (98, 104, 105). We therefore constructed both a plasmid-borne, translational P*tdaC-gfp* reporter fusion as well as a transcriptional *tdaC-mCherry* reporter fusion. Whilst the *tdaC-mCherry* reporter could only be observed on colony-level, the P*tdaC-gfp* reporter fusion allowed for detection of *tdaC* expression on single cell level (**Figure 5A and B**).

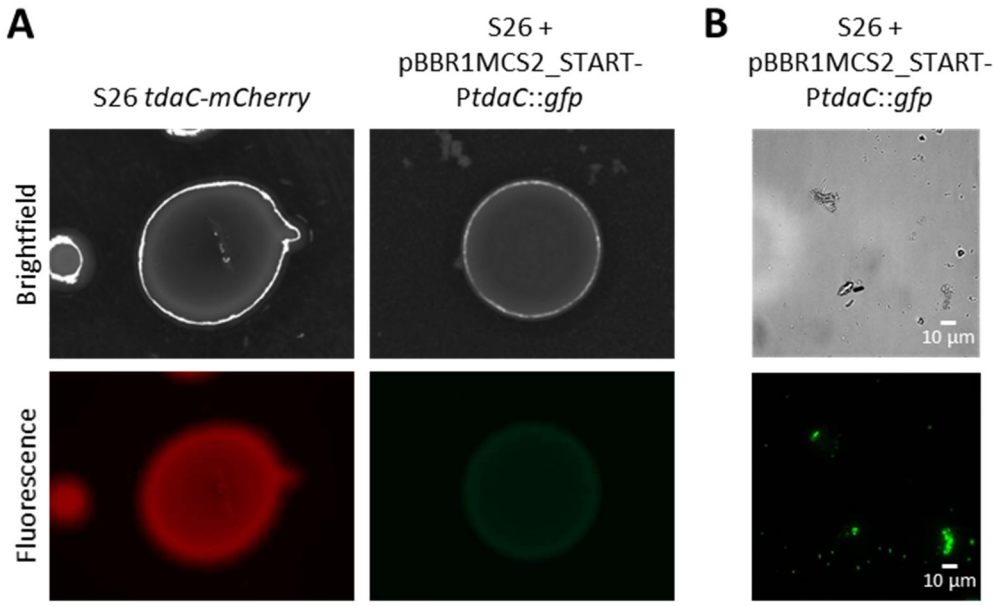

Figure 5. Imaging of transcriptional and translational *tdaC* reporter-fusions. A. Colony-level fluorescence of a translational and transcriptional reporter, S26 *tdaC-mCherry* and S26 carrying pBBR1MCS2_START-P*tdaC*::*gfp.* **B.** Single-cell fluorescence of the translational reporter, S26 carrying pBBR1MCS2_START-P*tdaC*::*gfp.* Figure adapted from **Manuscript IV.** Negative controls are not shown in this adaptation.

When deciding on an approach for tracking secondary metabolism, it is important to keep in mind that regulation of secondary metabolite production happens at multiple levels. First of all, expression of the BGC machinery may be controlled transcriptionally, e.g. by global- and cluster-encoded regulators (6). Post-transcriptional, translational, and post-translational regulation also affects production of the BGC-encoded biosynthetic machinery. Additionally, factors independent of the biosynthetic machinery also affects secondary metabolism, such as precursor and energy influx also play a part in determining whether and how much of a secondary metabolite is produced (56). These intricacies ultimately mean that one can only be certain that a secondary metabolite is present if the compound is chemically detected. However, chemical detection is not always sensitive enough to detect low levels of secondary

metabolites, exemplified by the fact that secondary metabolites are rarely detected in nature (106, 107), and chemical detection is often both costly and tedious. The use of indirect methods for tracking secondary metabolite production is hence still justified, but the results should be interpreted with caution.

Chapter 4:
Functions and ecological roles of secondary metabolites

Darwins 'survival-of-the-fittest' holds true in the bacterial world as well; bacteria live and thrive in environments where it is hard to imagine anything can survive. From deep inside the crust of the earth to the ice of the Antarctic, they have successfully adapted to extremities. The bacterial life is also often a social one, as they live side by side with other organisms in dynamic communities. Taken together, this demands an exceptional degree of metabolic flexibility.

One way of coping with stressful conditions is through the production of secondary metabolites. The structural versatility of these compounds is vast, and this is reflected in their function. Secondary metabolites are especially important in interactions, whether it be with the environment, other microorganisms, or even with higher organisms. In this chapter, I will cover some of the ecological roles secondary metabolites may play to highlight the versatility and importance of these compounds.

A long-running debate within the microbial secondary metabolite field is whether these compounds, especially the ones exhibiting antimicrobial activities, are indeed meant as 'weapons of mass destruction' (108, 109). I will briefly outline this discussion and bring in examples of different roles for secondary metabolites. In this context, I will also differentiate between effects caused by exogenous and endogenous secondary metabolite exposure. I will especially focus on the latter as we, in **Manuscript II**, investigated the effect of endogenous TDA production on the physiology of the producing strain. I will bring in data from this and **Manuscript III** in an attempt to add to the debate of the ecological role of secondary metabolites. As in **Manuscript I**, I will distinguish between the terms 'function' and 'role',

with ‘function’ referring to a direct effect and the more holistic term ‘role’ referring to the possible ecological significance of the compounds.

4.1. Antibiotic secondary metabolites: Weapons of mass destruction or molecules of communication?

Whilst it is relatively easy to observe the antibiotic effect of microbial secondary metabolites in the laboratory, a harder endeavor is to infer the true ecological role of these compounds in natural microbial systems. Since we have mostly studied these compounds in controlled laboratory environments, this has likely limited our understanding of their broader natural roles. Some of these compounds also exhibit more than one function, making it even harder to conclude on their purpose. Additionally, secondary metabolite research has often had an anthropocentric focus, and as such, our interpretation of their natural roles often relies on the purposes for which we use them. An obvious example of this are antibiotic secondary metabolites, which were for a long time believed to be produced solely to kill other bacteria.

A key argument against antibiotic secondary metabolites being primarily molecules of antagonism is that they are rarely detected in clinically relevant doses in nature, leading to speculations of whether they in the environment accumulate to inhibiting concentrations (109, 110). However, even at doses below the minimal inhibitory concentration, antibiotics may still have a measurable effect on growth of target microorganisms (111). Concentrations where this effect is large enough to cause a selective pressure for resistant mutants are referred to as the minimum selective concentration (111). Additionally, it is important to remember that the conditions under which we traditionally study antibiotic exposure are rarely reminiscent of naturally occurring conditions: Hence, it is possible that secondary metabolites may reach antagonistic concentrations locally, and this is backed by the observation that antibiotic resistance genes are more prevalent in nature than the genes encoding for antibiotic production (112).

One of the observations that sparked a revival in the interest in the ecological role of microbial secondary metabolites is the, by now common, observation that sub-inhibitory concentrations of exogenous antibiotic secondary metabolites causes considerable changes in gene expression profiles in bacteria exposed to the antibiotic compounds (12). This instead lead to the proposition that their primary function is perhaps as signaling molecules rather than as 'weapons of mass destruction' (108, 109).

However, it can be argued these changes in gene expression profiles may be categorized as a stress response rather than a signal, making the exposed bacteria prepared for incoming competition. This concept is referred to as competition sensing (113). Competition sensing is divided into two categories; interference competition where direct damage is inflicted on the cell, and exploitative competition where one organism depletes the resources of another (114, 115). The type of competition is reflected in the ensuing stress response. For example, antibiotic secondary metabolites cause direct damage to the cell and thus, are a type of interference competition. Biofilm formation is often affected by antibiotic secondary metabolite exposure (12, 116), and this is a common strategy for coping with interference competition, as biofilms increase the tolerance of the population towards antibiotics (117). Thus, this would still point to antibiotic secondary metabolites being primarily weapons, with responses having developed as a defense system.

Studies often focus on the effect of exogenous antibiotic secondary metabolites, but when searching for the ecological role of a compound, a lot can be learned by studying effects of endogenous secondary metabolite production as well. Similar to exogenous antibiotic exposure, endogenous antibiotics can also alter global gene expression profiles. In **Manuscript II**, we found that abolishing the production of the antibiotic secondary metabolite TDA causes global changes in both transcriptome and proteome of the producing strain (**Figure 6A-C**). In the following paragraphs, I will examine examples of secondary metabolite functions, with an emphasis on endogenous secondary metabolite functions, in

an attempt to broaden the perception of the ecological roles that microbial secondary metabolites may play.

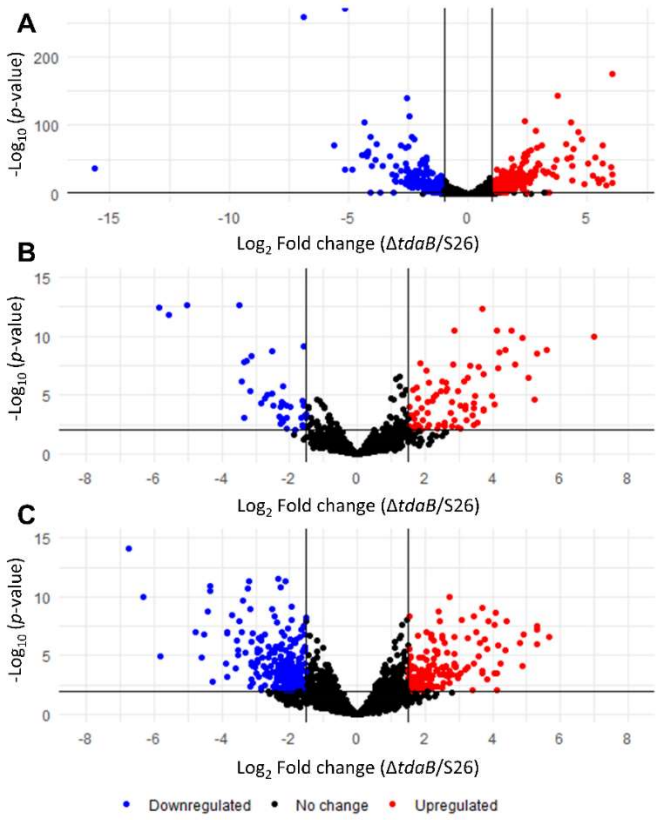

Figure 6. Global changes in proteome and transcriptome in a comparison of *P. piscinae* S26 WT and a TDA-deficient mutant, Δ*tdaB*. A. Transcriptome data. **B.** Proteome data, celullar fraction. **C.** Proteome data, supernatant fraction. Horizontal and vertical lines indicate significance tresholds. Blue: downregulated, red: upregulated, black: no difference. Genes were defined as significant at p > 0.05 and log2FC > 1. Proteins were defined as significant at p > 0.01 and log2FC > 1.5. Figure and parts of the figure legend was reproduced from **Manuscript II** with permission (Copyright © 2023, mSphere).

4.2. Secondary metabolite-mediated antagonism

One way of dealing with a crowded environment is by antagonizing other organisms. The secondary metabolites in charge of this are perhaps the most well-studied ones, since these

are the one commonly used for clinical purposes as antibiotics. Antibiotic secondary metabolites target a plethora of cellular functions, including bacterial protein synthesis (e.g. tetracyclines and aminoglycosides), cell wall biosynthesis (e.g. β-lactams), and DNA replication (e.g. quinolones) (118–121). In a study of pairwise interactions between 67 bacterial species of different environmental origin, antagonism was more prevalent between phylogenetically and metabolically similar bacteria (122), concurrent with the Darwinian theory of competition-relatedness that competition is increased between close relatives due to overlap in ecological niches (123). Antagonism mediated by antibiotic secondary metabolites follow this idea as well: In a study of a bacterial soil-derived mock community, secondary metabolite-producing *B. subtilis* suppressed members of the closely related genera *Lysinibacillus* and *Viridibacillus* (124). Similarly, addition of a TDA producing *P. inhibens* to an algal community suppressed the growth of a native *Phaeobacter* strain (125).

4.3. Nutrient acquisition: Metal chelation by secondary metabolites

Nutrients are often sparse in the microbial world, and one way of combatting this is to produce secondary metabolites that provides the organism preferential access to the available nutrients. One nutrient which is often sparsely available is iron (126). The most common strategy for iron acquisition in the microbial world is the production of siderophores (127). Siderophores are structurally diverse secondary metabolites which sequester iron from the environment and deliver it back to the bacteria through specific receptors (61). This process was likely evolved as a competitive trait against other microorganisms lacking the required receptors (128).

Siderophores are classically upregulated in response to low iron concentrations (129), although there are examples of iron-chelating compounds which do not follow this pattern. One such compound is TDA, which D'Alvise and colleagues reported was a weak chelator of iron (16). However, in contrast to classical siderophores, TDA was only detected during cultivation in iron-rich media (16). Whilst siderophores are most commonly associated with

iron chelation, some can also bind other metals such as manganese, zinc, and copper (61). In **Manuscript II**, we found a significant downregulation of genes involved in zinc acquisition upon abolishment of TDA production in *P. piscinae*. Specifically, we saw a downregulation of *znuA* and *znuC*, encoding parts of the ABC transporter ZnuABC, and *zinT*, which encodes a periplasmic Zn^{2+}-binding protein which in some bacteria work in conjunction with ZnuA (130, 131) (**Figure 7**). Like iron, zinc is an essential micronutrient with structural and/or catalytic functions in many bacterial proteins (132). This apparent coupling between TDA production and zinc-acquisition could perhaps indicate that TDA is necessary for the chelation of zinc or other metals rather than iron, although further studies are necessary to investigate this idea.

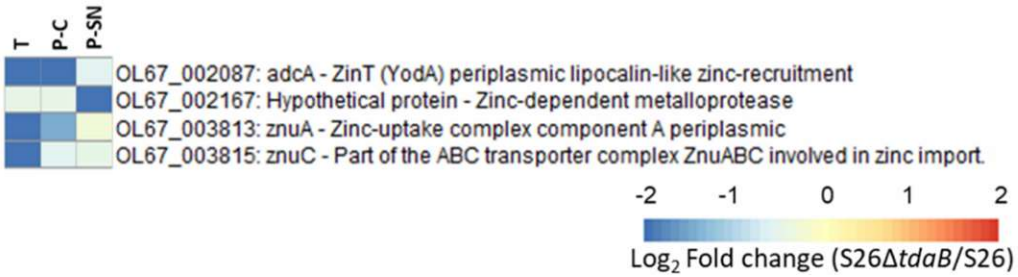

Figure 7. Changes in zinc acquisition systems in a TDA-deficient mutant. Results were obtained from a comparison of the transcriptome and proteome of *P. piscinae* S26 WT and a TDA-deficient mutant, Δ*tdaB*. T: Transcriptome, P-C: Proteome, cellular fraction, P-SN: Proteome, supernatant fraction. Figure reproduced from **Manuscript II** with permission (Copyright © 2023, mSphere).

4.4. Effects of secondary metabolites on biofilm and motility

As mentioned in **Section 3.3**, secondary metabolite production is often associated with biofilm formation. Not only is the production of microbial secondary metabolites often induced in biofilms (93), but secondary metabolites themselves may also play important

roles in the formation of these structures (133, 134). Furthermore, secondary metabolites may affect bacterial motility which impacts biofilm formation and -dispersion.

A good example of an antibiotic secondary metabolite with several functions is surfactin, produced by *Bacillus* spp. (135). Surfactin is an NRPS that exists in several isomeric forms and can cause cell lysis by penetration of the lipid bilayer membranes of microorganisms (135, 136). In addition to being implicated in antagonism, surfactin reduces surface-tension, promoting both swarming and sliding motility, which in turn also promotes dispersal of biofilms (85, 99, 100). In *Pseudomonas* spp., the rhamnolipids have a function similar to that of surfactin in reducing surface tension and thus promoting swarming (137). Rhamnolipid also promotes biofilm dispersal and is important for maintaining biofilm structure (138, 139).

As opposed to the previous examples, where the ability to produce a secondary metabolite has a direct effect on motility and biofilm formation, these phenotypes are also commonly affected indirectly by secondary metabolite-mediated changes in gene expression. These changes are often seen as a response to sub-inhibitory levels of exogenous antibiotic secondary metabolites, as discussed in **Section 4.1**. For example, an increase in biofilm formation was seen in *Pseudomonas aeruginosa* in response to the structurally different antibiotics ciprofloxacin, tetracycline, and tobramycin (12). Additionally, swimming and swarming was induced by tobramycin but reduced in the presence of ciprofloxacin (12).

Endogenous secondary metabolites may also have similar effects on biofilm and motility through modulation of gene expression. In **Manuscript II**, we found that a TDA-deficient mutant was more motile compared to wild-type in a soft agar assay (**Figure 8A**), accompanied by an increase in flagellar transcripts and proteins. Although no clear difference in biofilm formation was observed between the strains, we did see changes in cell morphology in the absence of TDA production; whilst the wild-type strain over time became filamentous, TDA-deficient mutant cells remained short (**Figure 8B**). Cellular shape in turn

affects localization within a biofilm (140), and thus, TDA production may still potentially impact biofilm structure.

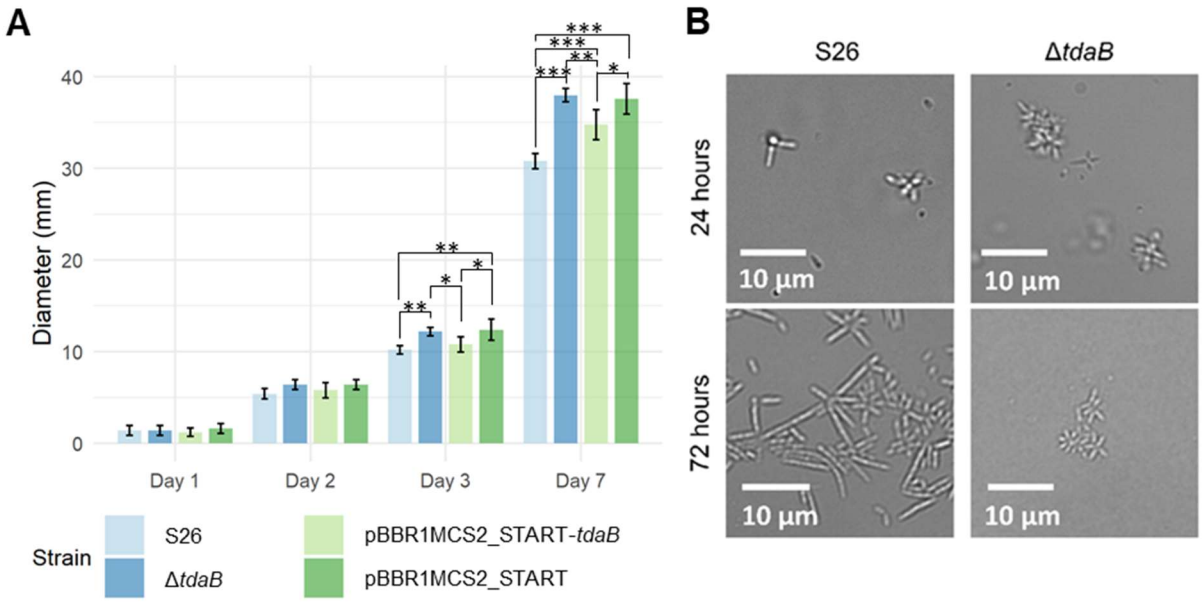

Figure 8. Comparison of motility and cell morphology of *P. piscinae* S26 WT and a TDA-deficient mutant, Δ*tdaB*. A. Motility in a soft-agar assay of S26 WT and Δ*tdaB*, as well as genetic complements. *: $P > 0.05$, **: $P > 0.01$, ***: $P > 0.001$. Error bars indicate standard deviation. **B.** Cell morphology of S26 WT and Δ*tdaB* at 24 and 72 hrs. Figures were adapted from **Manuscript II** with permission (Copyright © 2023, mSphere).

4.5. Secondary metabolites as quorum sensing signals

One of the most common 'side-effects' of antibiotic secondary metabolite exposure is global changes in gene expression (12, 141). As mentioned, this prompted the idea that these molecules were, in fact, signals rather than weapons. A common bacterial type of signaling is QS, a cell density-dependent signaling system. For this part, it is important to note that there is a difference between those secondary metabolites which indirectly *affect* QS and those that are *involved* in QS. For example, indole interferes with folding of the QS regulator

AqsR in *Acinetobacter oleivorans*, thus indirectly affecting QS signaling (142). However, in this paragraph I will focus on secondary metabolites which directly engage in QS.

QS was first discovered in *Vibrio fischeri* and *Vibrio harveyi*, where the LuxIR QS circuit induces light emission at high cell densities (143). A QS circuit consists, as a minimum, of an AI synthase and a corresponding response regulator, but some bacteria may only carry an AI synthase or a response regulator (144, 145). The AI accumulates with increasing cell densities until a critical threshold is reached, where after the response regulator-AI complex activates the expression of a range of target genes. QS controls group-behaviors such as motility (49), biofilm formation (83, 146, 147), competence (148), and, coincidentally secondary metabolite production (81, 147), among other things. How these phenotypes respond to QS is highly variable, likely reflecting differences in ecological niches and how the phenotypes benefit the thereto pertaining lifestyle (149).

In Gram-negative bacteria, acyl homoserine lactones (AHLs) are the most common AIs (150), whilst Gram-positive bacteria typically use oligopeptides (151). However, secondary metabolites may also be used as AIs: 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) is a secondary metabolite produced by *Pseudomonas* spp. which acts as an AI (152). PQS interacts with the response regulator PqsR to control the expression of a number of genes, including the PQS biosynthesis genes and genes involved in virulence (153). Another example is found in *P. inhibens*, where deletion of the PgaI AI synthase (Δ*pgaI*) or PgaR response regulator (Δ*pgaR*) results in global changes in QS-controlled gene expression (49). These changes were restored by addition of the antibiotic secondary metabolite TDA in Δ*pgaI*, but not in Δ*pgaR*, indicating that TDA can function as an alternative AI for PgaR (49).

QS is not only limited to intra-species communication; inter-species and even inter-kingdom communication is also facilitated by these systems (154). An interesting case of this is the effect of bacterial QS on the lysis/lysogeny switch of bacterial prophages (145). In *Vibrio anguillarum*, QS repress induction of a ϕH20-like prophage at high cell densities through

control of the VanT transcription factor (155). Similar to this, in **Manuscript II** we saw an upregulation of a prophage-encoding region when production of the secondary metabolite TDA, which is proposed to act as an AI (49), was abolished in *P. piscinae* (**Figure 9**). This could reflect a role for TDA as an inter-kingdom AI, resulting in prophage induction when TDA accumulates at high cell densities.

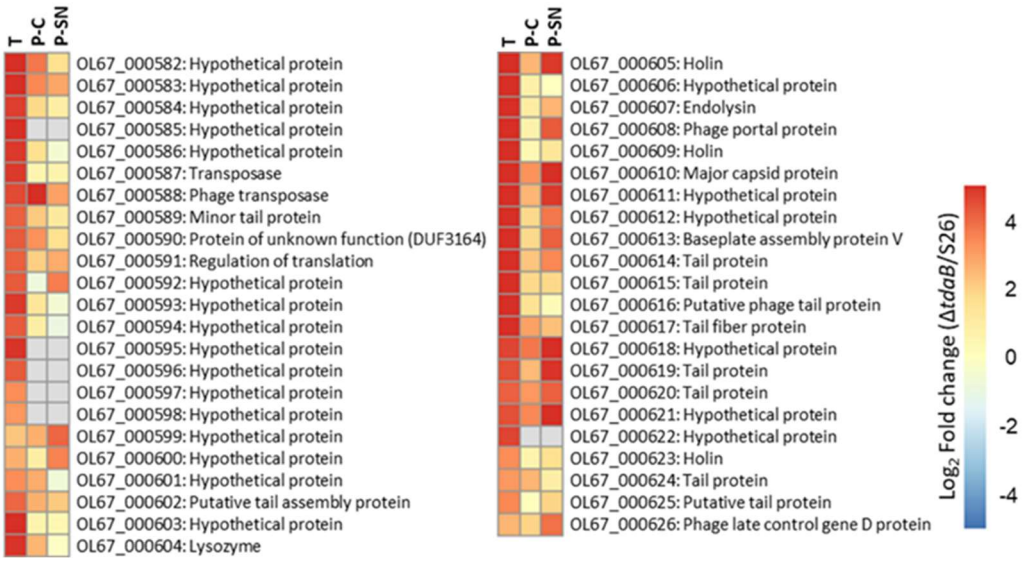

Figure 9. Upregulation of a prophage-region in a TDA-deficient *P. piscinae* S26 *ΔtdaB*, compared to WT. Results were obtained from a comparison of the transcriptome and proteome of *P. piscinae* S26 WT and a TDA-deficient mutant, Δ*tdaB*. T: Transcriptome, P-C: Proteome, cellular fraction, P-SN: Proteome, supernatant fraction. Figure adapted from **Manuscript II** with permission (Copyright © 2023, mSphere).

By now, it should be clear that secondary metabolites exhibit a variety of functions which may affect how their producers interact with their environment. Additionally, some secondary metabolites exhibit more than one potential function, complicating the interpretation of their ecological role and I will return to this in **Chapter 6**.

Chapter 5:
Gene Transfer Agents: Role and regulation in *Rhodobacteraceae*

Traditionally, we are taught about three modes of horizontal gene transfer (HGT): transformation, transduction, and conjugation. However, as it seems to be with most biology, this simplification does not hold entirely true. A fourth mediator of HGT, first observed in *Rhodobacter capsulatus* in 1974 (156), are GTAs, small phage-like particles mediating HGT in a mechanism akin to generalized transduction combined with elements from natural transformation (157).

In **Manuscript II**, we found that abolishment of TDA production led to an increase of proteins and transcripts of a region encoding a putative GTA, an observation which went on to be the focus of **Manuscript III**. To understand and discuss the ecological implications of this observation, this chapter will give an introduction to GTAs, their proposed ecological function, and how they are regulated. As most of the research on the *Rhodobacteraceae* GTA has been carried out in *R. capsulatus*, the contents of the following chapter are based on studies in this organism unless otherwise stated.

R. capsulatus is an Alphaproteobacteria belonging to the Rhodobacteraceae family. Genetically distinct GTAs have also been identified in other bacteria and even in archaea (158–161). Despite their shared ability to transfer host DNA in viral particles, these GTAs are believed to be a result of convergent evolution (162). For simplicity I will limit my focus to GTAs related to the archetypical *R. capsulatus* GTA, as the S26 GTA belongs in this group of GTAs.

5.1. Structure and function of gene transfer agents

GTAs resemble phages but has a lower packaging capacity that typically vary between 4 to 8.3 kb, which is not sufficient to package its own machinery (163–165). Instead, the particles contain (semi-)random pieces of host DNA, and this distinguishes the GTA from the typical phage lifecycle where host DNA is only sporadically packaged (163, 166, 167). Whilst one study has reported that GTAs from *Roseovarius nubinhibens* and *T. mobilis* can transfer DNA to a variety of other bacterial species (168), other studies of the *R. capsulatus* GTA indicate that that this system only performs intra-species DNA transfer (169).

5.1.1. Mechanism of GTA-mediated gene transfer

As mentioned in the beginning of the chapter, GTA-mediated HGT combines aspects of transduction and natural transformation, illustrated in **Figure 10.** First, the GTA particle is assembled from proteins transcribed from the core GTA cluster as well as several auxiliary gene loci (170, 171). A part of the host genome is then targeted and packaged into the particle, likely through headful packaging where the capacity of the viral head determines the size of the cargo DNA (166, 172). The particles are then released through cell lysis, mediated by holin and endolysin (173, 174). Once released, the GTA particles adsorb to the recipient through initial attachment via the GTA head spikes (175). The tail fibers of the GTA is proposed to interact with a receptor on the outer membrane of the recipient cell, facilitating correct orientation of the GTA (175). One strand of the dsDNA is then injected into the recipient cell, where the ssDNA is either degraded or integrated into the host chromosome through homologous recombination (157). This process is mediated by proteins homologous to those involved in natural transformation in other bacteria, DprA, ComEC, ComF, and ComM (157).

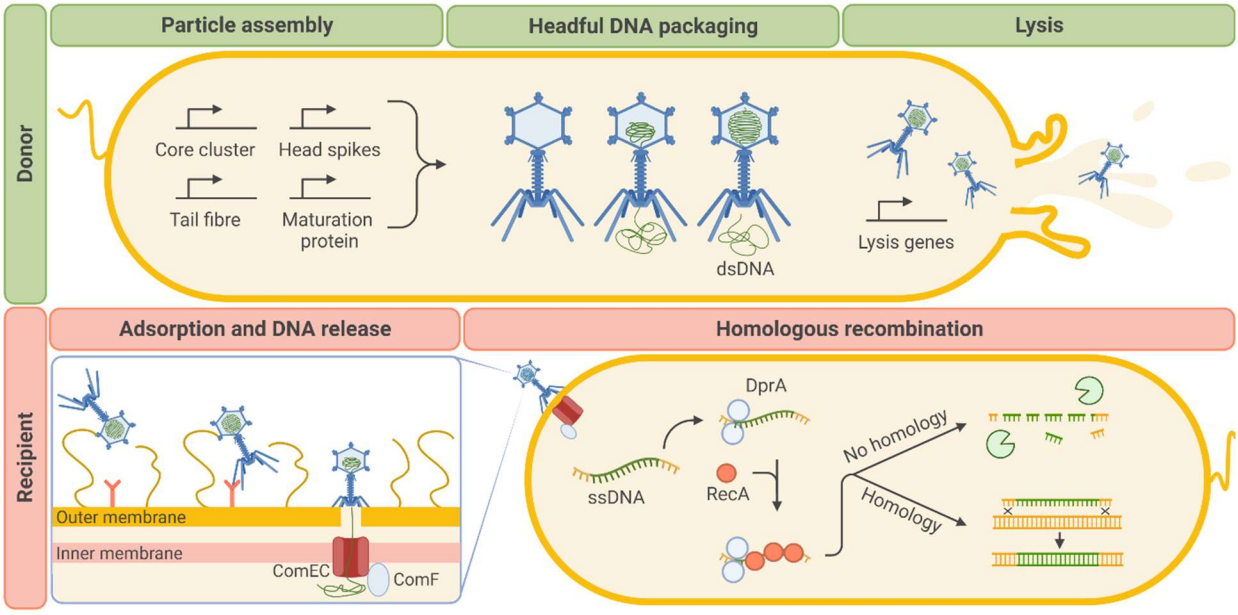

Figure 10. **Overview of GTA-mediated gene transfer**. Parts of the figure were inspired by Bárdy *et al.* 2020 (175) and Brimacombe *et al.* 2015 (157). Figure made using Biorender.com

In *D. shibae*, packaging is not fully random, with overrepresentation of some chromosomal regions, and the authors proposed that chromatin structure might play a role in accessibility of the host DNA for packaging (167). In **Manuscript II**, we found that the upregulation of GTA genes coincides with the downregulation of a putative heat-stable nucleoid structure (H-NS), which, similarly to eukaryotic histones, structure chromatin structure and hereby control gene expression (176). It is possible that this H-NS plays a role in controlling the GTAs access to the host genome and, as a result, in DNA packaging.

2.1. Structure and genome organization of GTAs

The genetic organization of GTAs appear highly conserved within the Rhodobacterales and the core gene cluster consists of three modules with a total of 15 genes: A DNA packaging module, a head morphogenesis module, and a tail morphogenesis module (**Figure 11A**). This conservation allowed us to identify a putative GTA in *P. piscinae* in **Manuscript II**. In addition to this cluster, several other genes necessary for successful GTA release are spread through-

out the genome, including lytic genes (166, 173, 174), head spike genes (171, 177), and tail fiber genes (171). Likewise, the genes required for GTA recipience also reside in different loci spread through-out the genome (157, 178), and so are regulators of both GTA release and recipience and regulatory genes (170, 171, 179–184). In **Manuscript III**, we were able to identify homologues of majority of the multi-locus GTA machinery in *P. piscinae*, supporting the presence of a likely functional GTA in this strain.

Evolutionarily, GTAs likely arose from a *Siphoviridae* prophage which lost genes for replication and integration into and excision from the host DNA (185). GTAs therefore structurally resemble *Siphoviridae*, forming tailed particles (164). A characteristic of the GTA particles is the oblate capsid with a length of 38 nm, with the tail axis being slightly shortened (175). Furthermore, the capsid is decorated with 70 Å long head spikes, which as previously discussed facilitate adhesion to recipient cells (175, 177). The GTA tails are 49 nm long, ending in tail fibers which are believed to bind receptors on the surface of the recipient cell (171, 175).

In **Manuscript II**, we visualized capsid particles from *P. piscinae* using transmission electron microscopy (**Figure 11B**). These capsid particles show strong structural resemblance to the GTAs; the capsid is approximately 40 nm in diameter, with a slight elongation along one axis (**Figure 11B**). This supports the notion that *P. piscinae* produces a GTA particle. We were, however, not able to observe any tails and the capsids did not appear to contain DNA. This could of course reflect that the structure of the S26 GTA differs from that of the *R. capsulatus* GTA. However, in **Manuscript III**, we identified homologues of the tail genes, and a more likely explanation is therefore an issue of sample preparation, as previous studies have shown that GTAs are sensitive to various purification methods (165).

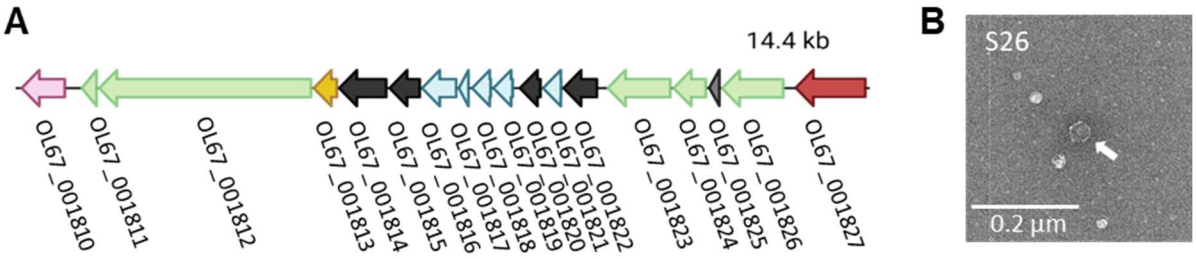

Figure 11. **Genomic and structural characteristics of a GTA from *P. piscinae* S26**. **A.** Structure of the head-tail cluster of the *P. piscinae* S26 GTA, color-coded according to function: Pink, serine O-acetyl transferase (*cysE*); green, portal, capsid protease and major capsid proteins; yellow, lytic enzyme; black, unknown/hypothetical protein; blue, tail associated proteins; red, terminase large subunit. **B.** Transmission electron microscopy image of a GTA particle from *P. piscinae* S26, highlighted with an arrow. Particle is approximately 40 nm in diameter and slightly elongated along one axis. Figures adapted from **Manuscript II** with permission (Copyright © 2023, mSphere).

5.2. Conservation and ecological role of GTAs

GTAs are widely conserved within the Alphaproteobacteria (186–188) but the reason for this conservation is somewhat debated. As opposed to their phage ancestors, GTAs are seemingly not able to transmit their own "genome", for a number of reasons: Firstly, the packaging capacity of the characterized GTAs is not sufficient to package the GTA "genome" (163–165) and in some species there is even a bias *against* packaging of the core gene cluster (166, 167). Additionally, several genes vital for GTA production are located outside of the core cluster (171). Taken together, this means that the maintenance of GTAs in the Alphaproteobacteria is not the result of self-transmission (189). Instead, it is assumed that GTAs must provide a significant ecological advantage to warrant their maintenance.

5.2.1. Diversification and adaptation

Due to their ability to package and transfer DNA between cells, GTAs have been proposed to be an effective mechanism for HGT between related bacteria in a microbial community, hereby accelerating adaptation to stressful conditions or novel niches (187, 190). Genomic analysis have identified several horizontally acquired elements in *Phaeobacter* spp. of a size

consistent with the limited DNA packaging capacity of GTAs, prompting the conclusion that GTAs are important drives of diversification and niche adaptation (191). Based on this theory, we proposed in **Manuscript II** that GTA production is controlled by TDA production as part of a colonization- and adaptation response.

However, mathematical modelling and computer simulations indicated that the population-level benefits of GTA-mediated HGT could not alone overcome the cost of lysis associated with GTA release, suggesting that there has to be another reason for their preservation (189). As an alternative explanation, the authors of this study proposed that GTAs could act as a 'vaccine system' against novel phages (189). If an infected cell could package part of the incoming phage DNA and transfer this to other cells in the population, this could trigger immunity against the incoming phage, restricting the effect of the phage-attack on the population (189). However, no experimental evidence has been presented to support this idea yet. In **Manuscript II**, we observed an upregulation of an encoded prophage in a TDA-deficient mutant concurrent with upregulation of a GTA. However, the prophage was only upregulated in part of the replicates, whereas GTA was always upregulated, indicating that GTA activation cannot be activated solely by the induction of the prophage. However, it would still be interesting to investigate if there is a regulatory coupling between these two observations.

5.2.2. Stationary phase stress

Early on, GTA release was observed to be correlated with growth phase, with GTA release being associated with stationary phase growth (192). In line with this, carbon limitation leads to an increase in GTA production, which was attributed to depletion of amino acids and linked to the stringent response (193). In another study knock-out of the GTA genes lead to fitness decreases during growth on different carbon sources, providing another link between carbon utilization and GTA production (188), and perhaps indicating a role of GTAs in managing stationary phase stress.

A recent study in *Caulobacter crescentus* proposed that GTAs may be important players in DNA repair, acting as templates for homologous recombination-based repair (163). The study demonstrated that GTAs promoted survival after exposure to several types of DNA-damaging agents, and that DNA from GTAs can be used to repair double-stranded DNA breaks on the chromosome (163). This in turn lead to an increase in survival during long term incubation (163). This survival extended beyond the producing cells, demonstrating that GTAs could be viewed as 'public goods' not only for the producer, but also for other nearby bacteria with the capability to take up the released GTAs (163). This theory follows the idea that the advantage in HGT lies in the ability to restore genes that have been deleted or mutated, preventing the accumulation of deleterious mutations in a population referred to as 'Muller's ratchet' (194, 195).

In line with this theory, another study found that GTA-mediated gene transfer was increased in the presence of sub-inhibitory levels of DNA gyrase inhibitors (196). DNA gyrase promotes negative supercoiling (underwinding) during DNA replication and transcription, affecting gene expression patterns (197). The effect of DNA gyrase inhibitors on GTA-mediated gene transfer was found to be a result of an effect on the recipient rather than an increase in GTA particles (196). The recipient-specific effect aligns well with the role of GTAs in DNA repair hypothesis, as the damaged cells would be the ones in need of DNA repair template.

5.3. Regulation of GTA release

While the conservation of GTAs indicates an important ecological role, GTA release comes at a high cost because donor cells must be lysed to release the particles (173). Hence, it is not surprising that the process of GTA-mediated gene transfer is governed by several regulatory systems. GTA release is restricted to only a subpopulation amounting to 0,15-3% of the population (166, 173). In contrast, the systems ensuring the capability to receive GTAs do not appear to be restricted to a subpopulation (198). It remains a mystery how exactly the

subpopulation effect is achieved, highlighting that there is still a lot to be discovered when it comes to GTA regulation.

GTA release is intricately weaved into the host regulatory systems, which in turn respond to a number of biotic and abiotic factors. In **Manuscript II** and **III** we found an upregulation of genes involved in GTA-mediated HGT upon abolishment of TDA biosynthesis, indicating a secondary metabolite-mediated regulation of GTA release in *P. piscinae* (**Figure 12**). In the next paragraphs I will highlight selected regulatory systems relevant to the data presented in **Manuscript II** and **III**.

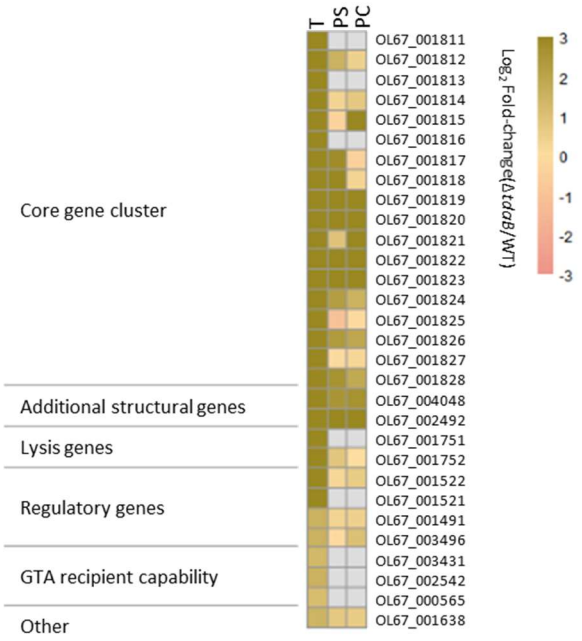

Figure 12. Log_2(fold change) of gene/protein abundance in Δ*tdaB* relative to WT of gene homologues associated with GTA maturation, release, regulation, and recipient capability. Samples were included in the heatmap if there was significant differential abundance of transcripts/proteins in one of the three comparisons (transcriptome (T), cell proteome (PC), or supernatant proteome (PS)). Figure and figure legend reproduced from **Manuscript III**.

5.3.1. GafA

The first direct activator of GTA release, GafA, was identified in *R. capsulatus* (179) and this protein is found throughout the *Rhodobacterales* (171). GafA induces expression of the GTA core genes as well as several auxiliary genes through direct interaction with the RNA polymerase omega subunit, likely by altering promoter specificity of the RNA polymerase (RNAP) holoenzyme (199). It contains two DNA-binding domains (DBD) located at distal ends of the protein, tied together by a central protein-binding domain, proposed to facilitate RNAP interaction (199). GafA is also an autoinducer of *gafA* expression (179), and whilst full-length *gafA* is required for GTA activation, only the C-terminal domain is required for *gafA* autoinduction (199).

In **Manuscript III**, we identified a homologue of GafA in *P. piscinae*. Re-examining the data from **Manuscript II**, this GafA homologue was found to be upregulated in the TDA-deficient mutant, concurrent with the observation of upregulation of GTA genes in this mutant, leading us to speculate that TDA represses GTA release through repression of *gafA*.

5.3.2. The CckA-ChpT-CtrA phosphorelay system

At the core of GTA regulation is the CckA-ChpT-CtrA phosphorelay system, which controls a number of other phenotypes as well, including motility, pilus formation, and cell shape, to name a few (200, 201). The CckA-ChpT-CtrA phosphorelay system is a regulatory system widespread in the Alphaproteobacteria (202), but the regulatory role differs somewhat between strains (200). The system was first discovered in *C. crescentus* and is most well-understood within this bacterium, where it acts as a master regulator of the cell cycle and is essential for viability (203, 204). On the other hand, CtrA is not required for viability in e.g. *T. mobilis* and *R. capsulatus* (170, 205).

The regulatory cascade is initiated by autophosphorylation of CckA, which subsequently phosphorylates ChpT, which again phosphorylates the response regulator CtrA (206). Several inputs are integrated to control the proceeding of this cascade; notably DivL, a

kinase-deficient sensor kinase homologue, is required for optimal phosphorylation of CckA, which ultimately affects the phosphorylation state of CtrA (179, 184).

The CckA-ChpT-CtrA phosphorelay system affects virtually all aspects of GTA-mediated gene transfer. Both CtrA abundance and phosphorylation state plays a role: CtrA is required for transcription of the head-tail cluster, as well as several auxiliary structural genes, through induction of the previously discussed *gafA* (170, 179, 181, 200). Furthermore, CtrA is also required for recipience and ensuing integration into the genome (157, 198). On the other hand, phosphorylated CtrA, CtrA~P, promotes cell lysis and subsequent GTA release (174, 181, 184, 206). Westbye and colleagues proposed that these observations reflected a growth phase dependent response; in early phase growth, CtrA is unphosphorylated and thus promotes expression of the GTA head-tail cluster (184). During late phase growth, CtrA~P accumulates, resulting in particle maturation and release through lysis (179, 184). This type of regulation would also ensure that lysis, and thereto pertaining cell death, does not occur until GTA particles are ready for release.

Revisiting data from **Manuscript II** in **Manuscript III**, we found that both CtrA and DivL transcripts were more abundant in a TDA-deficient mutant, indicating that TDA production represses CtrA abundance and potentially also phosphorylation of CtrA. This may in turn explain the observed repression of both GTA production, release, and receipt by TDA production in *P. piscinae* S26. This is also concurrent with the observations in **Manuscript II**, where we showed that several other CtrA-related phenotypes, such as motility, a type IV secretion system (T4SS), and cell morphology, are affected by the abolishment of TDA production.

A number of other inputs are integrated into CckA-ChpT-CtrA phosphorelay system, including cyclic dimeric guanosinmonophosphate (c-di-GMP) (206–208), the SOS response (183), and QS (199, 209–211). Wang and colleagues suggested that integration of QS and the CtrA phosphorelay system may be a common occurrence in the marine *Roseobacter* group

as CtrA binding sites are often present in the promoter region of encoded QS systems in this group (201).

5.3.3. Quorum sensing

QS is an integral part of regulating GTA-mediated gene transfer at all levels; from GTA particle maturation to recipient ability (178, 182, 198, 199). The first QS-system linked to GTA regulation was the LuxIR-like system GtaIR (182). The proposed mechanism for this system is that GtaR, which contains a DNA-binding motif binds the promoter of the target genes, repressing expression (199). GtaI, an AHL synthase, synthesizes mainly long-chain AHLs, which are proposed to interact with the bound GtaR, relieving the sequestered promoters, hereby allowing transcription (199). GtaR binds to and represses GafA, leading to repression of all downstream targets of GafA (179), outlined in **Section 5.3.1**. Furthermore, GtaIR negatively regulates recipience through repression of the polysaccharide capsule necessary for recipient ability (178).

In *Dinoroseobacter shibae*, expression of the head-tail cluster is controlled through QS. Here, several LuxIR-like systems are involved (210); $LuxIR_{1,2}$ which are located on the chromosome, and an orphan $LuxI_3$ located on a 86-kb plasmid (212). These QS systems are hierarchically regulated with $LuxI_1$ controlling the expression of $LuxI_{2\text{-}3}$ (213). This regulation happens through an intricately weaved interplay with the CtrA phosphorelay system. Here, $LuxI_1$ induces expression of both *cckA*, *chpT*, and *ctrA* (201, 213), and CtrA in turn controls expression of $luxIR_2$ and $luxI_3$ (201, 213). Additionally, $LuxI_2$ has been proposed to restrict GTA release by repression of a *gafA* homologue (210).

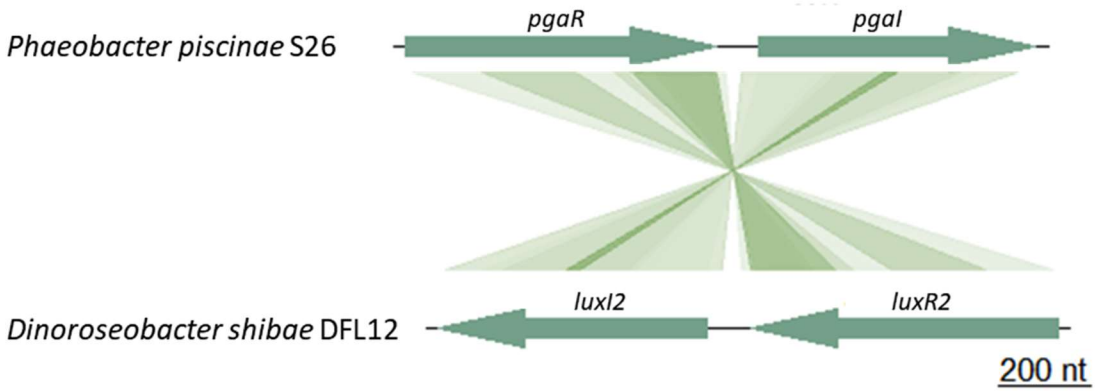

Figure 13. **Genomic comparison of the *pgaRI* system from *P. piscinae* S26 and the *luxIR2* system from *D. shibae* DFL12**. Darker hues indicate higher degree of amino acid similarity. Figure and figure legend reproduced from **Manuscript III**

In **Manuscript III,** we found that the PgaRI system of S26 is homologous to the *gafA*-repressing LuxRI$_2$ system of *D. shibae* (**Figure 13**). As reviewed in **Manuscript I**, TDA can act as a QS signal through the PgaRI system in related *P. inhibens* (49). This led us to hypothesize that TDA interacts with PgaR to repress the expression of GafA, hereby preventing GTA release. However, the previous model for LuxIR$_2$ in *D. shibae* does not explain the effect of TDA production on CtrA abundance and phosphorylation, discussed in **Section 5.3.2**, as LuxIR$_2$ only targets GafA in this model (210). This indicates that a secondary regulatory route for TDA is responsible for this or, alternatively, that the regulatory circuits differ between *D. shibae* and *P. piscinae*. The proposed regulatory pathway of TDA in GTA regulation is outlined in **Figure 14.**

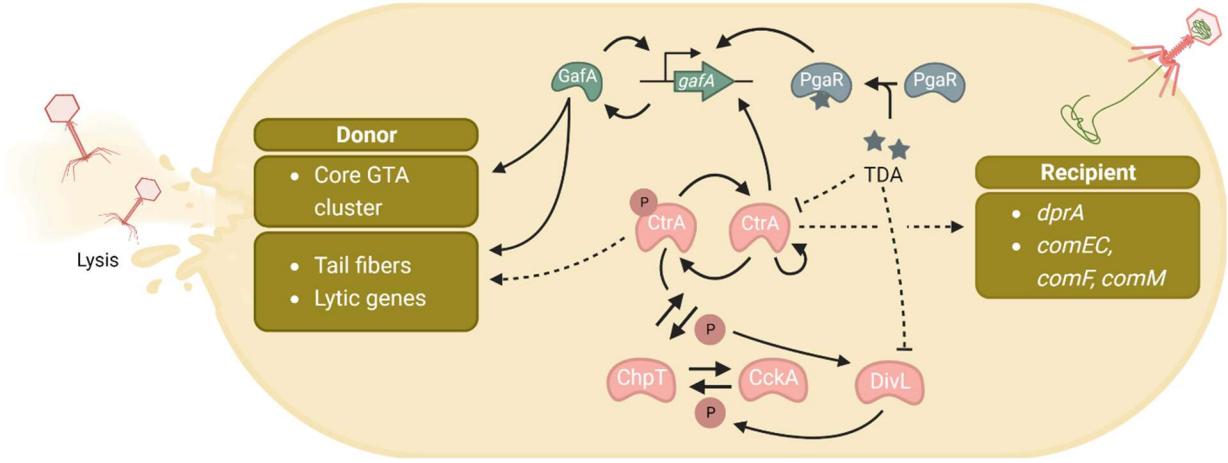

Figure 14. **Proposed pathway of TDA-mediated effect on GTA release in *P. piscinae*** S26. Figure reproduced from **Manuscript III**. Figure made using Biorender.com

TDA production is linked to biofilm formation and attachment (104, 214), and QS, which TDA is proposed to partake in (49), coordinates cell density-dependent behaviors. In the marine environment, where many GTA producing roseobacters reside, the constant flow of water is likely to affect GTA dispersal. To avoid wasting resources on GTA production, which as we have learned is a costly affair, GTA-mediated HGT has been proposed to be most efficient in high-cell-density environments (187) and an interveawed relationship between GTA regulation and biofilm has been proposed (180). The regulatory link between GTA activation and TDA production would support this intertwining. Our results add to the growing insight into the regulatory network governing GTA activation, which may ultimately broaden our understanding of their role in the environment.

Chapter 6:
A case of multifunctionality: Tropodithietic acid

As if deciphering ecological roles of secondary metabolites was not difficult enough as is, some compounds exhibit a plethora of functions and thus, a number of potential ecological roles. This is the case with TDA, which can disrupt the proton motive force (15), chelate iron (16), and act as an AI (49), as reviewed in **Manuscript I** and summarized previously in **Figure 4, Chapter 2**. In this final chapter, I will collect observations from the studies included in this thesis in an attempt to unravel the ecological implications of TDA production, thus returning to the original aim of this thesis.

6.1. TDA as a surface colonization signal

In **Manuscript II**, we set out to define the ecological role of TDA in the producing bacterium, *P. piscinae* S26. Through a series of characterization experiments, including a global survey of changes in the transcriptome, proteome, and metabolome in a TDA-deficient mutant compared to WT, we saw a number of changes; when TDA production was abolished, motility increased, and cells became shorter. Although we could not detect any changes in biofilm quantity, cellular shape affects biofilm structure (140). Furthermore, we saw an increase in transcripts and proteins of a prophage, a T4SS, and a GTA. These three systems may all mediate HGT (168, 215–217) and HGT is believed to be a major driver of niche adaptation (218, 219).

Phaeobacter spp. generally exist as surface colonizers (191, 220), and based on our observations, we proposed that the secondary metabolite TDA acts as a colonization signal, coordinating a phenotypic switch to a sessile lifestyle, as summarized in **Figure 15**. When the cells are planktonic, TDA levels are low and cells are highly motile, allowing them to travel to new niches. During the early stages of colonization TDA levels are still low,

permitting HGT through transduction, conjugation, and via GTAs. As TDA accumulates, this inhibits susceptible neighboring bacteria, whilst reducing the motility and promoting filamentous cell morphology. The filamentous cells form the base of a biofilm (140) and potentially also prevents predation by grazers (221).

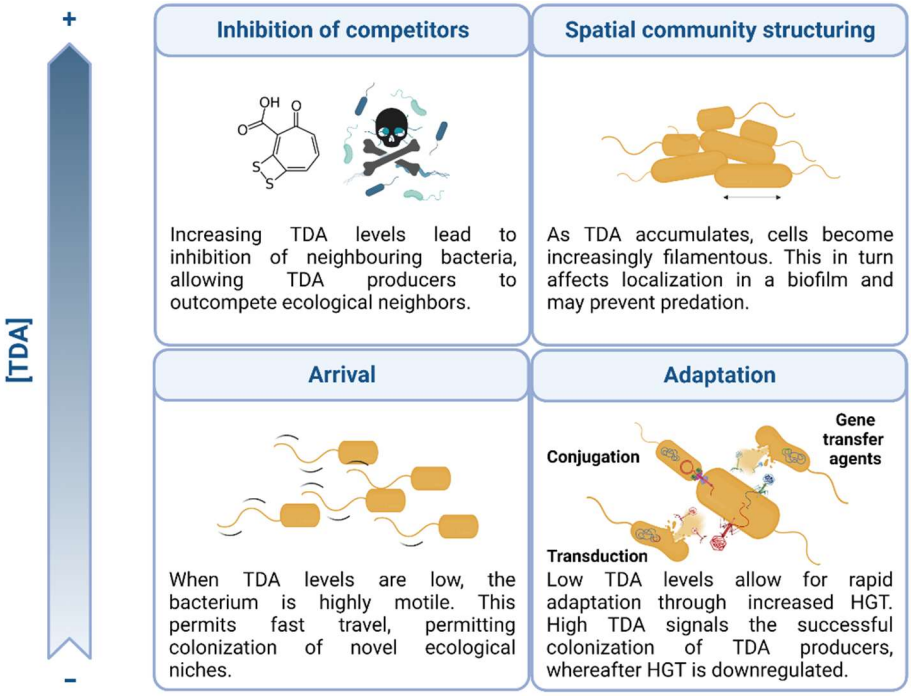

Figure 15. Proposed role of TDA as a signal for coordinating surface colonization and adaptation. Figure made using Biorender.com.

6.2. TDA as a mediator and signal in algal-bacteria symbiosis

Members of the *Roseobacter* group are frequently associated with algae (48, 92, 222), and have been proposed to be involved in symbiotic relationships with microalgae (223–226). TDA has been implicated in the aptly named 'Jekyll-and-Hyde'-relationship between the microalgae *Emiliana huxleyi* and the TDA-producing *P. inhibens* illustrated in **Figure 16** (223). During healthy algal growth, *E. huxleyi* provides *P. inhibens* with DMSP and a surface to form biofilm on (223). In turn, *P. inhibens* produces the algal growth promoter PAA and

the antibiotic TDA, protecting *E. huxleyi* against pathogens (223). When *E. huxleyi* starts to senesce it produces the algal breakdown product *p*-coumaric acid and this stimulates production of the algaecidal roseobacticides by *P. inhibens*, hereby switching the relationship from mutualistic to antagonistic (223).

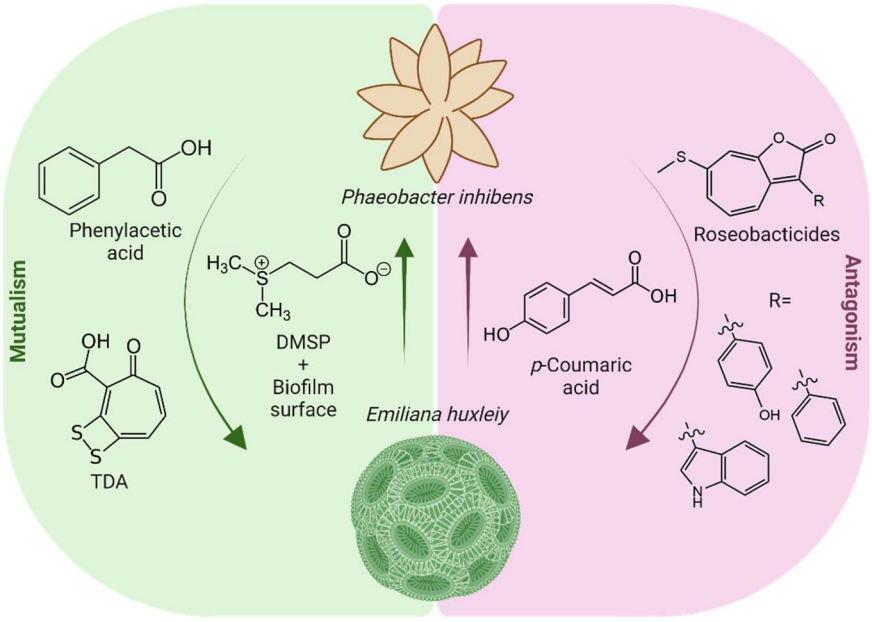

Figure 16. **Model for the proposed interaction between *P. inhibens* and *E. huxleyi***. During the mutualistic phase, *E. huxleyi* provides *P. inhibens* with DMSP and a biofilm surface, whilst *P. inhibens* stimulates algal growth through the production of phenylacetic acid, which simultaneously producing the antibiotic TDA which protects the algae against fast-growing pathogens. As *E. huxleyi* starts to senesce, the algal breakdown product *p*-coumaric acid prompts *P. inhibens* to produce the potent algaecides, roseobacticides. This figure was inspired by Seyedsayamdost *et al.* 2011 (223) and created using Biorender.com.

D. shibae is part of a similar Jekyll-and-Hyde relationship with the dinoflagellate *Prorocentrum minimum* (226). A study by Wang and colleagues characterized the transcriptomic response of *D. shibae* during the switch from mutualism to early pathogenic phase, and from early to late pathogenic phase (225). Here they found that genes encoding

QS systems, flagella, and the CtrA phosphorelay system was upregulated during early pathogenic phase, but downregulated later on in the late pathogenic phase (225), indicating that these phenotypes affect the bacteria-algae interaction. Furthermore, the study demonstrated that disruption of flagellar synthesis or the CtrA phosphorelay system in *D. shibae* led to a faster decline of *P. minimum* during cocultivation, demonstrating that these systems are important for establishing the mutualism between *D. shibae* and *P. minimum* (225). Similarly, flagellar motility is required for interaction of the TDA-producing *T. mobilis* with the dinoflagellate *Pfiesteria piscicida* (205). Furthermore, at least some roseobacters possess homologues of a chemotaxis system (227), which has been implicated in chemotaxis towards algal metabolites, including DMSP, of the TDA-producing *T. mobilis* (72).

Another system potentially involved in the algal-roseobacter symbiosis are the T4SS conserved in many roseobacters (227–229). T4SSs mediate the transport of macromolecules, such as DNA and proteins, into both prokaryotic and eukaryotic cells through conjugation (230), and may hence mediate inter-kingdom interactions. It has therefore been speculated that T4SSs may also play a role in the interaction between roseobacters and algae.

In **Manuscript II** and **III**, we noted an upregulation of both flagellar motility, components of a chemotaxis system, and the CtrA phosphorelay system upon abolishment of TDA biosynthesis, resembling the response seen by Wang and colleagues (225) during early pathogenic phase in the *D. shibae-P. minimum* symbiosis. Following the 'Jekyll-and-Hyde' model, this could indicate that abolishment of TDA production steers the bacteria towards the pathogenic relationship. We also observed an upregulation of a T4SS upon abolishment of TDA production, providing another potential link between TDA production and the roseobacter-algae symbiosis. These results ultimately indicate that TDA functions not only as an actor in this symbiosis, but also as a regulator of this lifestyle switch from friendly to hostile. Further investigations into TDA production dynamics during these lifestyle switches

could be undertaken using the fluorescent *tdaC*-reporter systems developed in **Manuscript IV** and discussed in **Section 3.4**.

6.3. Piecing the puzzle together: Regulation of TDA biosynthesis

In **Chapter 3**, I discussed how factors affecting secondary metabolite production may support interpretations of the ecological roles of a given compound. A review of the factors governing TDA production can be found in **Manuscript I** and here, I will highlight some of the regulatory inputs that feed into the ecological models proposed in the previous sections.

As with many other secondary metabolites, there are several indicators of an association between biofilm and TDA induction: The first indications of this link arose from the observation that stagnant cultivation induced TDA-production of *Tritonibacter* spp., (231). Furthermore, expression of the TDA BGC is triggered upon attachment to a surface and induced by c-di-GMP in *T. mobilis* (104). C-di-GMP is proposed to be a universal secondary messenger in bacteria, controlling the switch between planktonic and sessile living (232). Thus, there is a clear correlation between biofilm and TDA production in *T. mobilis*. However, with few exceptions, *Phaeobacter* spp. produce TDA during both stagnant (mimicking biofilm) and shaken growth (231, 233), pointing to species-specific differences in regulation of TDA production. However, in *Phaeobacter* spp., TDA production is regulated by the PgaIR QS system (81), demonstrating a link between high population densities, as found within biofilms, and TDA production. Relating this back to the model for the role of TDA presented in **Section 6.1,** this reinforces the idea that TDA production is triggered upon successful colonization of a surface as part of a switch to a sessile lifestyle.

As covered in **Section 6.3**, TDA-producers are frequently associated with algae and incorporate the algal sulfur compound DMSP into their metabolism (234). In *T. mobilis*, DMSP promotes TDA production (71), corroborating the idea that TDA production plays a significant role in the symbiosis between algae and TDA-producing roseobacters.

6.4. Stress or signal?

A question that remains unanswered is *how* TDA production causes these changes. As previously mentioned, three separate functions have been proposed for TDA: iron chelation (16), disruption of the proton motive force (15), and QS (49), and it is therefore adjacent to consider whether any of these functions can be linked to our observed phenotypic changes.

As demonstrated in **Manuscript II**, abolishment of TDA biosynthesis led to global changes in gene expression and physiology. Although we can no reject the possibility that this is an internal stress response to TDA production, these changes resemble those observed by Beyersmann and colleagues upon disruption of the QS system PgaIR (49). This study also demonstrated that TDA can act as an alternative AI for this system in the absence of the AHL synthase, PgaI (49). Thus, it seems adjacent to conclude that our observations in **Manuscript II** are likely caused by TDA's AI properties, rather than a result of a stress response. Interestingly, the PgaIR QS system was also proposed to promote the switch from planktonic to sessile living in the non-TDA producer Rhodobacterales sp. Y4I (147), supporting our hypothesis that TDA interacts with this system as an AI to coordinate this lifestyle switch.

Systems for QS are ubiquitous within the *Roseobacter* group (235–237), highlighting the importance of QS-mediated regulation within this group. The AHLs employed vary greatly in chain-length and structure, and the AHLs appear to overlap between species (238). This has led to the intriguing idea that interspecies communication through QS may be a common occurrence for roseobacters, resulting in gene expression changes as a response to total AHL density in a community rather than to species-specific AHL accumulation (215). Furthermore, given the frequent host-association of roseobacters, there has been speculations about these AHL systems acting as inter-kingdom signals (238) as seen in e.g. rhizobia-plant interactions (239). Thus, the potential implications of TDA as an alternate AI are major for the lifestyle of the producers.

6.5. Beyond TDA: Lessons from other multifaceted secondary metabolites

TDA is not the only multifunctional secondary metabolite. Another excellent example of this is the antibiotic NRPS surfactin, produced by *Bacillus* spp. As mentioned in **Section 4.4**, surfactin promotes motility by reducing surface tension (85, 99, 100), but this is not the only known function. Surfactin promotes the release of extracellular DNA through lysis (240). eDNA is required for HGT through transformation, and intriguingly, the *srfA* operon encodes the machinery for surfactin biosynthesis, as well as a small out-of-frame gene, *comS*, involved in natural competence development (241). Coupling these two observations, it seems adjacent to assume a link between surfactin production and regulation of HGT. Surfactin can also act as a QS signal by forming pores on the cell membrane leading to K^+ leakage from the cytoplasm which signals the activation of KinC, a membrane histidine kinase, ultimately leading to phosphorylation of Spo0A (242, 243), which controls differentiation of a population in regards to behaviors such as sporulation and biofilm formation (244–246). In the plant-colonizer *Bacillus velezensis,* surfactin is stimulated by plant root exudates, and surfactin also promotes colonization of plants (247, 248). Thus, like we proposed for TDA, surfactin appears to coordinate and participate in several different processes which may affect niche colonization, host interaction, and adaptation potential, corroborating that secondary metabolites may possess more than one function and role.

As previously discussed in **Section 4.1**, dosage is a key factor when it comes to secondary metabolite function. However, other factors also affect function. A classic example of this is in the case of the phenazines, a colorful, redox-active group of secondary metabolites produced by many species of soil *Actinobacteria* and *Proteobacteria* (249). The activity of these compounds may be both beneficial and antagonistic, depending on their redox state. When oxidized, the phenazines are believed to create reactive oxygen species, which inhibit fungi and other bacteria (250, 251). On the other hand, reduced phenazines are proposed to aid in the solubilization of hard-to-access nutrients such as iron and phosphorus (252), and this idea is strengthened by the observation that P-limitation induces phenazine production

in *Pseudomonas* (253). These liberalized nutrients would in this case be available to all organisms, providing a community-level benefit of phenazines. The redox state of the phenazines may be affected by the oxygen-level of the surrounding environment, and this example hence highlights why it is necessary to investigate secondary metabolites under different conditions to truly appreciate their versatility.

Clearly, context matters. This extends beyond abiotic conditions; interactions with other organisms, even across kingdoms, also aids in the interpretation of the ecological role of secondary metabolites. Attine ants grow fungal cultivars, typically of the *Leucocoprinus* genus, which they use to feed on (254). However, invading species of parasitic fungi belonging to the genus *Escovopsis* threaten this mutualism. To combat this, a bacterial symbiont, *Pseudonocardia* spp., produces secondary metabolites which selectively inhibit the parasitic *Escovopsis* (255, 256). A long evolutionary history supports this quadripartite symbiosis (257), and emphasizes how secondary metabolites may be part of complex interactions and ecosystems in Nature.

Unfortunately, observing the role of secondary metabolites in natural settings is a daunting task, as the complexity of natural systems warrants little control. On the other hand, laboratory based cultivation of simple systems, such as mono- or dual cultures, permits a higher degree of manipulation, but loses the context. Ultimately, this means that there is no 'catch-all' approach to elucidating the ecological role of secondary metabolites. However, by combining observations and approaches, we can expand our insights into the complex field of chemical ecology. Ultimately though, these examples highlight that secondary metabolites exhibit activities that appear to have far more wide-reaching roles than just as 'weapons of mass destruction'.

Chapter 7:
Conclusion and perspectives

Secondary metabolites are a diverse class of compounds with a range of bioactivities, including, for some, antibiotic activity. Antibiotic secondary metabolites were traditionally perceived as weapons, produced to inhibit competing bacteria. However, evidence is accumulating that secondary metabolites play far more diverse roles in how the producing organisms interact with their surroundings. In this thesis, we explored the ecological role of the multifunctional secondary metabolite TDA, reviewed in **Manuscript I**, in *P. piscinae*. TDA is a secondary metabolite produced by members of the *Roseobacter* group, for which several potential functions exist. First of all, TDA is a potent antibiotic with broad spectrum effects against a range of other bacteria. Additionally, TDA can also chelate iron in a siderophore-resembling manner, at is has also been proposed to act as a QS signal. Hence, TDA is an excellent case study for investigating the potential ecological role for multifunctional secondary metabolites.

In **Manuscript II**, we investigated the effect of endogenous TDA production in the producing strain using an exploratory approach. Our results demonstrate that abolishing TDA production causes significant physiological changes in *P. piscinae*, including changes in cell morphology, motility, as well as global changes in the transcriptome, proteome, and metabolome. We also found an up-regulation of several potential HGT systems when TDA production was abolished, such as a T4SS, a prophage, and a GTA, which may reflect an effect of TDA on the adaptation potential of *P. piscinae*. Motility and cell morphology both affect biofilm formation and –structure, and the effects on these phenotypes may therefore affect colonization dynamics. Additionally, HGT is proposed to be a key driver for niche adaptation. Based on this, we propose that TDA acts as a signal to coordinate colonization and adaptation to novel niches. We also conclude that this is most likely due to the QS properties of TDA,

rather than a general stress response, but further studies are necessary to confirm the mode of action behind our observed changes.

GTAs are viral particles encoded in the genomes of many *Rhodobacteraceae* containing random pieces of host DNA, which are released through lysis of the donor cells. The ecological role of GTAs Regulation of GTA activity is tightly coupled to the regulatory systems of the host. In **Manuscript III**, we set out to characterize the TDA-mediated repression of a putative GTA in *P. piscinae* to uncover the underlying regulatory pathway. Through bioinformatics analysis, we found that TDA likely interacts with the LuxR-like PgaR response regulator as an AI to repress GafA, which directly activates GTA release in other GTA producers. Furthermore, we found that TDA production affects expression and potentially phosphorylation state of CtrA, a master regulator of cell cycle control, which controls GTA release in other roseobacters. Aside from GTA release, the CtrA phosphorelay system is also linked to motility, T4SS, and cell morphology in other *Rhodobacteraceae*, providing a potential regulatory link between the phenotypes observed in **Manuscript II**. This is, to my knowledge, the first endogenous secondary metabolite proposed to affect GTA activation in the *Rhodobacteraceae*. Further experimental evidence is necessary to confirm the proposed pathway. Furthermore, it would be interesting to investigate whether TDA-mediated regulation of GTA activation is a common occurrence in other TDA-producers.

Secondary metabolite production is regulated by both biotic and abiotic cues, and tracking spatiotemporal dynamics of secondary metabolites may provide valuable clues as to their ecological role. To elaborate on our previous assumption of TDA playing a role in colonization, in **Manuscript IV** we developed a fluorescent reporter-system for tracking expression of *tdaC*, a key gene within TDA biosynthesis. These systems can ultimately facilitate downstream studies of *tdaC* expression dynamics during colonization to further investigate the role of TDA in this process. In the future, these systems can be applied to study TDA production dynamics. As reviewed in **Manuscript I**, triggers of TDA production

differs between producers: Whilst this has been hypothesized to reflect different environmental niches of the producers, it highlights that it may not be possible to extrapolate between species and that further studies are necessary to fully unravel the regulatory systems governing TDA production.

Ultimately, the work carried out in this thesis show that secondary metabolites can have profound effects on physiology of the producing bacteria. Whilst the debate of whether antibiotic secondary metabolites are signals or weapons may not reach a conclusion anytime soon, our results clearly show that secondary metabolites play an important part in shaping microbial lives. Pinpointing the true ecological role of secondary metabolites is often an educated guess at best. Ultimately, there is often a trade-off between complexity and controllability when choosing experimental strategies. However, simplified artificial conditions, such as the ones used for the work of this thesis, are more controllable than natural systems, thus serving as an excellent starting point for studying more complex contexts. If we truly want to understand the ecological role of secondary metabolites a holistic approach must be employed, combining the observations of several experimental fields; from the mechanistic understandings of molecular biology all the way to large-scale studies of natural microbial communities. All of these approaches provide pieces of the puzzle in our quest to understand the importance of secondary metabolites. It is also important to remember that we are often biased in our interpretations. In the case of antibiotic secondary metabolites being perceived mainly as weapons, this was largely due to the fact that this was the function we originally isolated secondary metabolites based on. The observations presented in this thesis, particularly in **Manuscript II** and **III**, highlight why exploratory research is a key stone for broadening the context in which we view scientific observations in. Finally, our understanding of the ecological roles of secondary metabolites will continue to expand as we study them, providing us with even more insight in how to speak the language of bacteria and maybe even new ideas on how to employ them for our own benefit.

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Research articles

Manuscript I

Role is in the eye of the beholder - the multiple functions of the antibacterial compound tropodithietic acid produced by marine *Rhodobacteraceae*

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Review Article

Role is in the eye of the beholder—the multiple functions of the antibacterial compound tropodithietic acid produced by marine *Rhodobacteraceae*

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One sentence summary: Review of the multiple roles and functions of the secondary metabolite, tropodithietic acid, produced by some marine *Rhodobacteraceae*.
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Abstract

Many microbial secondary metabolites have been studied for decades primarily because of their antimicrobial properties. However, several of these metabolites also possess nonantimicrobial functions, both influencing the physiology of the producer and their ecological neighbors. An example of a versatile bacterial secondary metabolite with multiple functions is the tropone derivative tropodithietic acid (TDA). TDA is a broad-spectrum antimicrobial compound produced by several members of the *Rhodobacteraceae* family, a major marine bacterial lineage, within the genera *Phaeobacter*, *Tritonibacter*, and *Pseudovibrio*. The production of TDA is governed by the mode of growth and influenced by the availability of nutrient sources. The antibacterial effect of TDA is caused by disruption of the proton motive force of target microorganisms and, potentially, by its iron-chelating properties. TDA also acts as a signaling molecule, affecting gene expression in other bacteria, and altering phenotypic traits such as motility, biofilm formation, and antibiotic production in the producer. In microbial communities, TDA-producing bacteria cause a reduction of the relative abundance of closely related species and some fast-growing heterotrophic bacteria. Here, we summarize the current understanding of the chemical ecology of TDA, including the environmental niches of TDA-producing bacteria, and the molecular mechanisms governing the function and regulation of TDA.

Keywords: antimicrobials, secondary metabolites, *Rhodobacteraceae*, tropodithietic acid, marine microbiomes

Introduction

Since the discovery of penicillin by Alexander Flemming in 1929 (Fleming 1929), humanity has benefited from its antimicrobial effects and an array of similar bioactive compounds produced by microorganisms. These molecules, commonly referred to as secondary metabolites, have been studied for decades because of their antimicrobial properties and their use as anti-infective agents in the clinic. It has been the perception that the antibiotic properties also define their predominant function and role in natural microbial niches—as weapons to kill off competitors (Traxler and Kolter 2015). However, sublethal concentrations of antibiotics can influence gene expression in exposed microorganisms and result in changes in phenotypes such as biofilm formation or motility (Goh *et al.* 2002; Linares *et al.* 2006; Straight, Willey and Kolter 2006; Liu *et al.* 2013). These effects have predominantly been observed in bacteria exposed to 'external' antimicrobials, but it is not known if these effects are caused indirectly by induction of a stress response or by a direct effect of the compounds on cellular targets (Romero *et al.* 2011; Foster and Bell 2012; Cornforth and Foster 2013; Yoon and Nodwell 2014; Dittmann *et al.* 2019a; Li *et al.* 2021). Some antimicrobial secondary metabolites can also modulate gene expression in the producer itself, acting as signaling molecules mediating quorum sensing (QS; Romero *et al.* 2011; Beyersmann *et al.* 2017). Other secondary metabolites have nonantibiotic functions serving as iron scavengers (siderophores), in predator defense, as antivirulence compounds, or as promoters of horizontal gene transfer (Mansson *et al.* 2011; Seyedsayamdost *et al.* 2011b; Briand *et al.* 2016; Zhang et al. 2016, 2021; Danevčič *et al.* 2021). Thus, this challenges the original perception of antibiotic secondary metabolites being predominantly involved in direct interference competition between microbes, whilst not being required for growth and metabolism (Linares *et al.* 2006; Yim, Huimi Wang and Davies 2007; Davies 2013; Pishchany and Kolter 2020). In contrast, it has been argued that the physiological effects at subinhibitory concentrations serve to prime the recipient for competition to come, supporting the natural role of these compounds in interference competition (Foster and Bell 2012; Cornforth and Foster 2013; Abrudan *et al.* 2015). Despite the different perceptions of the predominant role of antimicrobial secondary metabolites, if a single role exists, one concept may not overrule the other (Firn and Jones 2000). However, it is evident that the physiological and ecological function of microbial secondary metabolites should be re-examined in the producing organism and in natural systems in an ecological context. Fortunately, the '–omics' era has facilitated global studies of complex microbial communities, also in the presence of a host organism. In addition, the development of

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in situ chemical detection of metabolites, for instance by mass spectrometry imaging, allows direct, high resolution analyses of secondary metabolites not hampered by bulk extraction, and enabling analysis of spatial metabolomes (Moree *et al.* 2012; Geier *et al.* 2020).

An example of an antimicrobial secondary metabolite with multiple functions and roles is the redox active metabolite pyocyanin, produced by *Pseudomonas aeruginosa*. It serves as an antimicrobial, but also as a respiratory pigment and a quorum-related molecule (Hernandez and Newman 2001). Similarly, in *Streptomyces coelicolor*, the antimicrobial red pigment prodigiosin can induce programmed cell death in subpopulations, and thereby provide nutrients to the surviving kin, suggesting an additional ecological role besides being an antibiotic (Tenconi *et al.* 2018). Expanding our holistic understanding of secondary metabolites demands in-depth studies of their facets, from chemistry to ecology. Here, we present a case study of tropodithietic acid (TDA; Fig. 1), a sulfur-containing tropone derivative with several functions.

TDA can act as antimicrobial and is produced by marine members of the *Rhodobacteraceae* family (class of Alphaproteobacteria; Brinkhoff *et al.* 2004; Bruhn *et al.* 2005; Geng *et al.* 2008; Harrington *et al.* 2014; Sonnenschein *et al.* 2017a, 2018; Duan *et al.* 2020). In addition to the antimicrobial properties of TDA, the compound exhibits other activities as a signaling molecule, anticancer agent, and weak iron chelator (Geng and Belas 2010; Wichmann *et al.* 2015; D'Alvise *et al.* 2016; Wilson *et al.* 2016; Beyersmann *et al.* 2017). TDA-producing bacteria have been detected in oceanic metagenomic data sets (Segev *et al.* 2016; Sonnenschein *et al.* 2017a) and have also been isolated from marine aquaculture and oceanic environments (Hjelm *et al.* 2004a; Lauzon *et al.* 2008; Porsby, Nielsen and Gram 2008; Grotkjær *et al.* 2016b). They can inhibit, or kill, fish pathogenic bacteria when co-cultivated in aquaculture live feed (microalgae, *Artemia*, rotifers, and copepods) or fish larvae, sparking a commercial interest in TDA-producing bacteria as aquaculture probiotics (D'Alvise *et al.* 2012; Grotkjær *et al.* 2016a; Dittmann *et al.* 2017; Rasmussen *et al.* 2018; Sonnenschein *et al.* 2021).

The purpose of this review is to provide an overview of the different functions of TDA, thus also addressing its possible broader physiological and ecological roles, with perspectives to other secondary metabolites. We focus on TDA-producing bacteria belonging to the marine *Rhodobacteraceae* family, but it should be noted that TDA-producing bacteria outside of this group have been isolated (Kintaka *et al.* 1984; Tsubotani *et al.* 1984; Kawano *et al.* 1997). The term 'function' will cover direct molecular or chemical responses attributed to TDA, whilst 'roles' will comprise the more holistic possible ecological effects of the compound (Fig. 1).

Given the potential broader functions of antimicrobial secondary metabolites, their definition and terminology have been debated (Bérdy 2005; Price-Whelan, Dietrich and Newman 2006; Davies 2013; Chevrette *et al.* 2020). 'Secondary metabolites' was introduced by the Nobel Prize laureate Albrecht Kossel in 1891 (Kossel 1891), and adopted by the botanist Friedrich Czapek, who in the 1920s coined the term 'secondary modifications' in work on plant nitrogen metabolism (Czapek 1922), with the purpose of distinguishing the compounds from growth-related primary metabolites. More recently, the term 'specialized metabolites' has gained traction emphasizing functions broader than merely secondary (Price-Whelan, Dietrich and Newman 2006; Davies 2013). We will, however, use the term 'secondary metabolites' in this review as this remains the term most commonly used.

TDA-producing bacteria and their environmental niches

The tautomer of TDA, thiotropocin, was discovered in 1984 in a *Pseudomonas* species isolated from soil (Kintaka *et al.* 1984; Tsubotani *et al.* 1984), and TDA was later detected in a marine bacterium, *Roseobacter gallaeciensis* (now: *Phaeobacter inhibens*; Brinkhoff *et al.* 2004). Since then, TDA has only been detected in a subset of members belonging to the *Rhodobacteraceae* family. This includes strains belonging to the three genera *Phaeobacter* (formerly *Roseobacter*; Ruiz-Ponte *et al.* 1998; Brinkhoff *et al.* 2004; Martens *et al.* 2006; Porsby, Nielsen and Gram 2008; Geng and Belas 2010; Berger *et al.* 2011; Breider *et al.* 2014; Sonnenschein *et al.* 2017b), *Tritonibacter* (formerly *Epibacterium*, *Ruegeria*, or *Silicibacter*; Hjelm *et al.* 2004a; Bruhn, Gram and Belas 2007; Muramatsu *et al.* 2007; Geng *et al.* 2008), and *Pseudovibrio* (Enticknap *et al.* 2006; Geng and Belas 2010; Penesyan *et al.* 2011; Bondarev *et al.* 2013; Harrington *et al.* 2014)

TDA is produced by strains belonging to four of the six described *Phaeobacter* species (Sonnenschein *et al.* 2018), namely *Phaeobacter gallaeciencis* (Martens *et al.* 2006), *P. inhibens* (Martens *et al.* 2006), *Phaeobacter piscinae* (Sonnenschein *et al.* 2017b), and *Phaeobacter porticola* (Breider *et al.* 2017). Production of TDA has so far not been detected in the species *Phaeobacter italicus* (Wirth and Whitman 2018) and the proposed species *Phaeobacter marinintestinus* (Lee *et al.* 2015), nor has the biosynthetic gene cluster of TDA been detected through genome mining (NCBI accession numbers NZ_VOGO01000001.1 (*P. marinintestinus* UB-M7) and FOOZ00000000.1 (*P. italicus* DSM26436)) using antiSMASH 5.0 in these species (Blin *et al.* 2019). Several strains of *Tritonibacter mobilis* (formerly *Epibacterium mobilis*) produce TDA, while strains belonging to other species in the genera, e.g. *Tritonibacter* scottomollicae (formerly Epibacterium scottomollicae), do not (Geng *et al.* 2008; Wang and Seyedsayamdost 2017; Sonnenschein *et al.* 2017a). In the *Pseudovibrio* genus, TDA-producing strains (unclassified at species level) have repeatedly been isolated (Enticknap *et al.* 2006; Geng and Belas 2010; Penesyan *et al.* 2011; Bondarev *et al.* 2013; Harrington *et al.* 2014) with *Pseudovibrio ascidiaceiola* being the closest relative to the TDA-producing *Pseudovibrio* isolates (Penesyan *et al.* 2011). However, a number of *Pseudovibrio* strains do not harbor the *tda* genes, suggesting that TDA production is not a widely distributed trait within this genus (Crowley *et al.* 2014; Romano 2018).

Since TDA production is not a conserved trait within the three genera, and since *Phaeobacter*, *Tritonibacter*, and *Pseudovibrio* are not close phylogenetic neighbors within the *Rhodobacteraceae* family, this could suggest that TDA genes and the ability to produce the compound have been distributed by horizontal gene transfer (Sonnenschein *et al.* 2018). However, short-term, noncompetitive biofilm cultivation of *P. inhibens* 2.10 induced single nucleotide polymorphisms in genes responsible for TDA production, leading to TDA deficiency (Majzoub *et al.* 2021). This points toward strong selection for the loss of TDA production in *P. inhibens*, presenting another possible explanation for the nonconserved pattern of TDA genes observed within the genera. To this day, the evolutionary history of TDA production is not fully understood, but would be important for unravelling the ecological role(s) of TDA. For most secondary metabolites the evolutionary route responsible for the chemical diversification remains poorly understood. Horizontal gene transfer has been identified to be an integral driver of secondary metabolite evolution (Fischbach, Walsh and Clardy 2008; Medema *et al.* 2014), but it is evident that vertical inheritance also influences the evolution of secondary metabolite

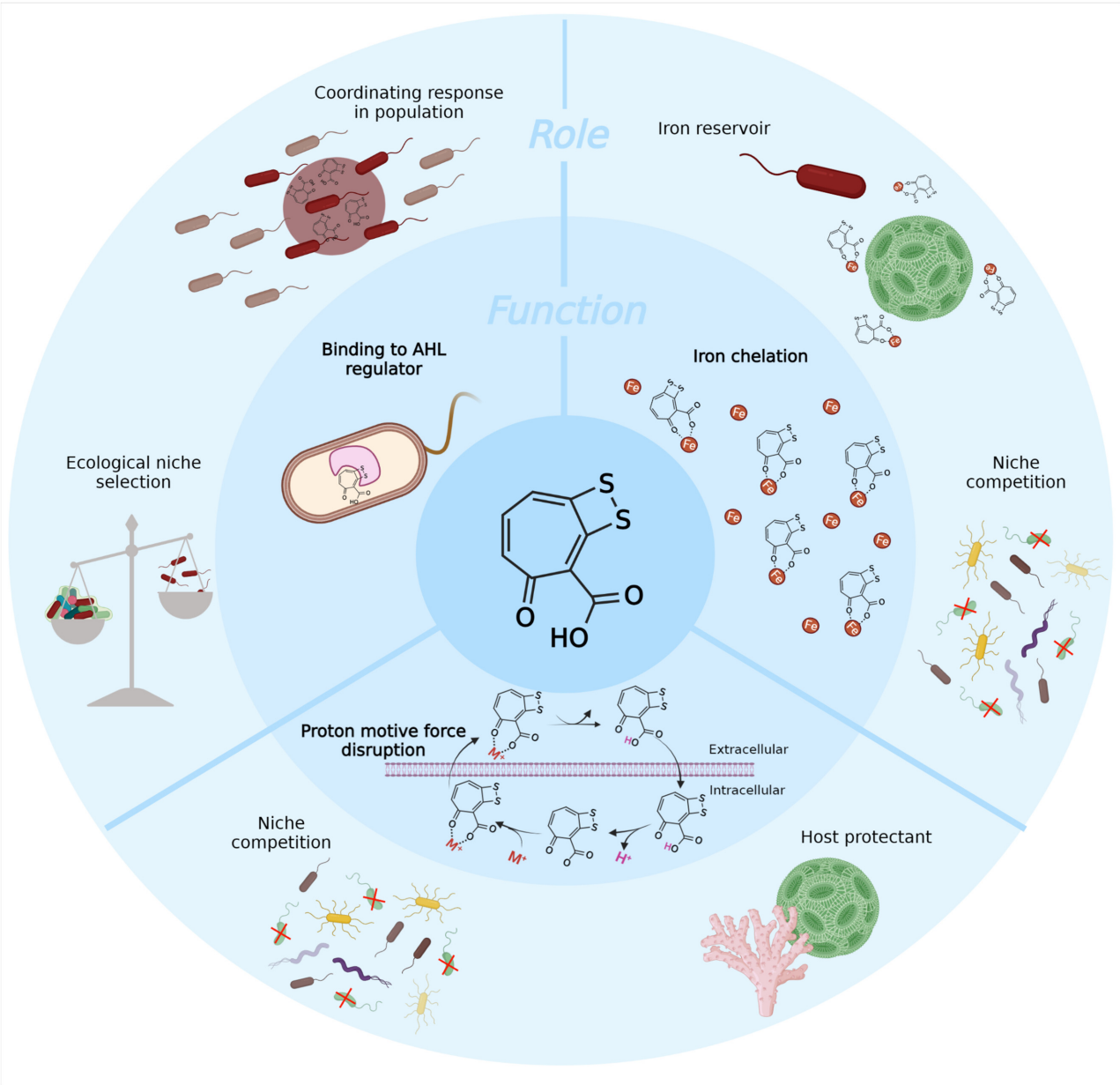

Figure 1. Proposal for three functions of TDA and potential ecological roles. Bold text indicate functions. Nonbold text indicate potential ecological roles. Created with Biorender.com.

gene clusters (Lind *et al.* 2017; Adamek *et al.* 2018; Chase *et al.* 2021; Undabarrena *et al.* 2021). Genetic diversification of the salinosporamides A biosynthetic gene cluster, found in the marine *Salinispora* genus, had direct consequences on the secondary metabolite production (Chase *et al.* 2021). This highlights that long-term evolutionary processes can lead to genetic and chemical diversification of secondary metabolites within closely related species, potentially generating new chemical diversity.

The genera *Phaeobacter* and *Tritonibacter* belong to the *Roseobacter* group ('roseobacters'), which is a paraphyletic subgroup within the family *Rhodobacteraceae* (Simon *et al.* 2017). The *Roseobacter* group contains multiple branching clades (Newton *et al.* 2010; Simon *et al.* 2017), where clade 1 contains strains that produce TDA (Brinkhoff *et al.* 2004; Newton *et al.* 2010). In the environment, the abundance of roseobacters is highest near the surface of temperate coastal waters and polar oceans (Buchan, González and Moran 2005), globally averaging 3.8% of bacterial populations (Wietz *et al.* 2010). The abundance of roseobacters correlates positively with chlorophyll *a* concentrations (Wietz *et al.* 2010), and roseobacters can constitute as much as 30% of the bacterial population in microalgal blooms (González and Moran 1997; Gonzalez *et al.* 2000; West *et al.* 2008).

In global marine metagenomic data, the *Phaeobacter* genus represents approximately 0.03% of all bacterial sequences in surface waters (Sunagawa *et al.* 2015; Sonnenschein *et al.* 2021), and while *Phaeobacter* species have not been isolated from open ocean water (Gram, Melchiorsen and Bruhn 2010), they have frequently been recovered from aquaculture facilities (Hjelm *et al.* 2004a; Porsby, Nielsen and Gram 2008; Grotkjær *et al.* 2016b) and solid surfaces in harbors (Ruiz-Ponte *et al.* 1998; Bernbom *et al.* 2011; Gram *et al.* 2015; Breider *et al.* 2017). This is in concordance with comparative genomic analyses of the *Phaeobacter* genus suggesting an

adaptation to a surface-associated lifestyle (Dang and Lovell 2000; Hjelm *et al.* 2004a; Rao, Webb and Kjelleberg 2006; Porsby, Nielsen and Gram 2008; Thole *et al.* 2012; Gram *et al.* 2015; Freese *et al.* 2017). Antagonistic interactions are believed to be more frequent in particle- and between surface-associated bacteria due to the high cell densities in structured microenvironments (Long and Azam 2001; Gram, Melchiorsen and Bruhn 2010). This could indicate that production of TDA provides a competitive advantage for the producer in surface colonization. TDA-producing *Tritonibacter* strains have also been isolated from aquaculture environments (Buchan, González and Moran 2005; Porsby, Nielsen and Gram 2008; Alsmark *et al.* 2013), as well from open ocean waters (Gram, Melchiorsen and Bruhn 2010; Sonnenschein *et al.* 2017a). The *Tritonibacter* genus represents 0.2% of the bacterial population in the surface ocean, and is thus more abundant in oceanic surface waters than *Phaeobacter* (Sunagawa *et al.* 2015). However, like *Phaeobacter*, *Tritonibacter* is adapted to a surface-attached lifestyle and occurs rather in the particle-associated than the free-living fraction of seawater (Sonnenschein *et al.* 2017a). Consequently, it has been proposed that *T. mobilis* could be the open-water equivalent to the coastal, macrosurface-attached *Phaeobacter*.

TDA-producing bacteria are also found in association with a range of marine eukaryotes, such as zooplankton (Freese *et al.*), sponges (Harrington *et al.* 2014), molluscs (Ruiz-Ponte *et al.* 1999; Prado *et al.* 2009), and algae (Rao, Webb and Kjelleberg 2006; Nappi, Soldi and Egan 2019) and it has been suggested that up to one-third of TDA-producing bacteria are host-associated (Nappi, Soldi and Egan 2019). This is specifically seen with TDA-producing *Pseudovibrio* strains that are present on, and genetically adapted to, a symbiotic lifestyle with marine invertebrates such as corals and macroalgae (Enticknap *et al.* 2006; Penesyan *et al.* 2011; Bondarev *et al.* 2013; Crowley *et al.* 2014; Raina *et al.* 2016; Romano 2018). *Pseudovibrio* represent 0.04% of all bacteria in oceanic surface waters (Sunagawa *et al.* 2015) and TDA-producing *Pseudovibrio* species harbor genes associated with a free-living lifestyle (Enticknap *et al.* 2006; Bondarev *et al.* 2013)

Like other members of the *Rhodobacteraceae* family, TDA-producing bacteria are characterized by a high versatility of metabolic pathways (Newton *et al.* 2010; Bondarev *et al.* 2013; Zech *et al.* 2013; Sonnenschein *et al.* 2017a). TDA-producing *Phaeobacter* and *Tritonibacter* can catabolize several algal osmolytes, such as dimethylsulfoniopropionate (DMSP; Miller and Belas 2004; Newton *et al.* 2010; Thole *et al.* 2012), which has been suggested to act as a chemo-attractant for TDA-producing bacteria (Miller and Belas 2004; Miller *et al.* 2004) capable of utilizing DMSP in their primary metabolism (Curson *et al.* 2011). Furthermore, DMSP may provide sulfur to be incorporated into TDA (Geng *et al.* 2008), and TDA-producing bacteria, thus benefit from living in association with microalgae and corals (Raina *et al.* 2009, 2016; Harrington *et al.* 2014; Segev *et al.* 2016). However, other studies have not been able to detect the incorporation of DMSP sulfur in TDA in either *P. inhibens* DSM17395 or in *P. gallaeciensis* DSM26640, and instead suggested that cysteine serves as the sulfur precursor (Dickschat *et al.* 2017).

Biosynthesis, tautomers, and analogues of TDA

TDA is a sulfur-containing tropone derivative (Fig. 2). It can tautomerize into two other known sulfur-containing tropones; thiotropocin and troposulfenin (Fig. 2). The structures of these compounds are characterized by an aromatic cyclohepta-2,4,6-trienone moiety. Their biosynthesis draws on the central carbon and sulfur metabolism, as well as a range of enzymes encoded by a cluster of dedicated so-called *tda* genes (Fig. 3A; Duan *et al.* 2020). For an in-depth review of the chemistry underlying the biosynthesis of TDA as well as other tropones, we refer to the review by Duan et al. (2020).

The basic skeleton of TDA and other tropone natural products arise from phenylacetic acid (**1**, PAA) catabolism (Fig. 3A), where PaaK, PaaABC(D)E (*E. coli* nomenclature, also referred to as PaaGHI(J)K in *Pseudomonas putida*), PaaG, as well as PaaZ are required to form a highly reactive intermediate, 3-oxo-5,6-dehydrosuberyl-CoA semialdehyde (**3**, Fig. 3B, Teufel *et al.* 2010, 2011; Berger *et al.* 2012; Brock, Nikolay and Dickschat 2014). Interestingly, in TDA-producers, a homologue of PaaZ, PaaZ2, is encoded on a megaplasmid along with the core enzymes of TDA biosynthesis, TdaABCDEF (Fig. 3B; Brock, Nikolay and Dickschat 2014). PaaZ contains two domains; a C-terminal enoyl-CoA hydratase (ECH) and a N-terminal aldehyde dehydrogenase (ALDH) domain (Brock, Nikolay and Dickschat 2014). In PaaZ2, only the C-terminal ECH is conserved (Brock, Nikolay and Dickschat 2014). In the absence of the ADHL domain, the intermediate **3** cyclizes to a seven-membered ring 2-hydroxycyclohepta-1,4,6-triene-1-formyl-CoA (**4**), the proposed universal tropone precursor, via a spontaneous intramolecular condensation (Teufel *et al.* 2011). A recent study pointed to the acyl-CoA dehydrogenase-like flavoenzyme TdaE being the linker between primary metabolism and TDA biosynthesis (Duan *et al.* 2021). TdaE converts the PAA catabolism shunt product **4** and carries out a series of reactions, including dehydrogenation, CoA-ester oxygenolysis, and ring epoxidation to form **7** (Duan *et al.* 2021), which then presumably to be converted by TdaF, TdaB, and PatB to form TDA (**5**; Duan *et al.* 2021). This biosynthetic pathway therefore serves as an example where the bacteria 'direct' primary metabolism toward secondary metabolism.

Different analogues of TDA have been detected from TDA-producing bacteria (Choudhary *et al.* 2018; Phippen *et al.* 2019). The first analogue was tentatively characterized as TDA-methyl ester, was isolated from a TDA-producing *Pseudovibrio* sp. (Choudhary *et al.* 2018; Fig. 2). Methyl-troposulfenin, an S-methylated congener of TDA, was identified as a natural analogue of TDA from a TDA-producing *P. inhibens* (Phippen *et al.* 2019; Fig. 2). Notably, the antimicrobial activity of methyl-troposulfenin is lower than that of TDA (Phippen *et al.* 2019). A comparison of the MS fragmentation patterns of methyl-troposulfenin (Phippen *et al.* 2019) and the proposed TDA-methyl ester (Choudhary *et al.* 2018) show a very high level of similarity (Choudhary *et al.* 2018; Phippen *et al.* 2019), and due to the lack of NMR experiments in the characterization of TDA-methyl ester, it cannot be ruled out that TDA-methyl ester is a misassignment of methyl-troposulfenin. For other secondary metabolites, the production of analogues may serve to broaden the chemical (antimicrobial) repertoire of the producing organism or may serve as a detoxification and self-protection of the producer (Li *et al.* 2013; Gallagher *et al.* 2017).

Total synthesis of TDA has not been reported thus far. However, owing to its bioactivity, a series of TDA inspired analogues have been synthesized to elucidate structure activity relationship (Rabe *et al.* 2014). One particular synthetic analogue, tropone-2-carboxylic acid (Fig. 2B), a nonsulfur variant of TDA, had a stronger antibacterial activity against *Staphylococcus aureus* and *Vibrio anguillarum* than TDA itself, suggesting that the sulfur atoms are not necessary for the antimicrobial effect of TDA (Rabe *et al.* 2014).

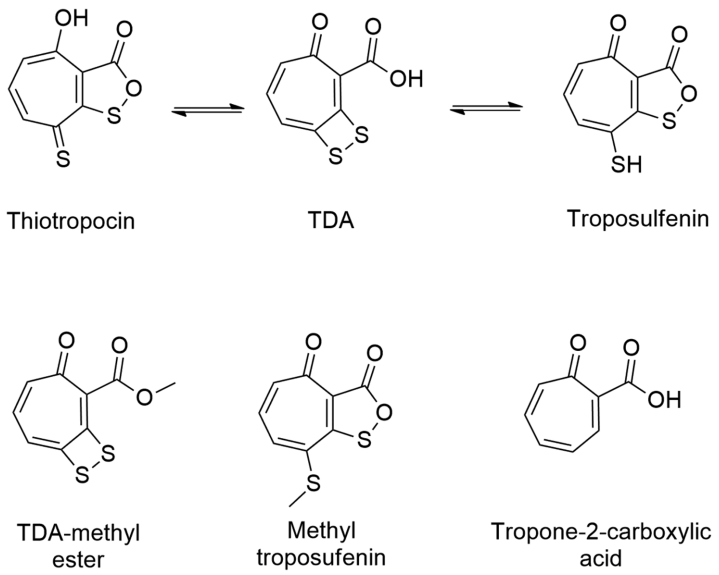

Figure 2. TDA and its tautomers (upper panel) and analogues (lower panel). Created with ChemDraw Professional (PerkinElmer Informatics).

Factors influencing TDA production

As previously mentioned, TDA biosynthesis draws on central carbon metabolism as well as sulfur metabolism (Geng *et al.* 2008). The cascading production of TDA across different metabolic processes may explain why TDA production is influenced by a multitude of factors including mode of growth, nutrients, and cell density.

Mode of growth

When *P. piscinae* 24–7 is cultured in Marine Broth (MB), stagnant as compared to shaken conditions increase brown pigmentation (Bruhn *et al.* 2005), which is a TDA–iron complex, and thus a proxy for TDA production (D'Alvise *et al.* 2016). Similarly, TDA production by *Tritonibacter* is facilitated by stagnant growth conditions, whereas several *Phaeobacter* strains also produce TDA in aerated cultures (Bruhn *et al.* 2005, 2007; Porsby, Nielsen and Gram 2008; Belas *et al.* 2009; Geng and Belas 2010; Berger *et al.* 2011; D'Alvise *et al.* 2014).

A distinct phenotype associated with stagnant growth of TDA-producing bacteria is the formation of a thick biofilm at the liquid–air interface (Bruhn, Gram and Belas 2007; Gram, Melchiorsen and Bruhn 2010) and the appearance of the brown TDA–iron complex in this biofilm. This observation has prompted the hypothesis that TDA production and biofilm formation could be linked (Bruhn *et al.* 2005). In *Tritonibacter* sp. TM1040, deficiency of the swimming regulator *flaC* is associated with a shift toward the motile phase, a reduction in biofilm formation as well as decreased antibiotic activity (Belas *et al.* 2009). The secondary messenger cyclic dimeric guanosin-monophosphate (c-di-GMP), is likely involved in the interconnection of these phenotypic traits as increased production of c-di-GMP induces both biofilm formation and TDA production in *T. mobilis* F1926 (D'Alvise *et al.* 2014). However, attachment, biofilm formation, and TDA biosynthesis are not universally linked across TDA-producing genera (Prol García *et al.* 2014; Zhao *et al.* 2016; Majzoub *et al.* 2018). While stagnant growth conditions facilitate biofilm formation and TDA production in many *Tritonibacter* strains, this is not the case in most *Phaeobacter* species. Collectively, these observations support the notion that *Phaeobacter* and *Tritonibacter* occupy separate niches (Sonnenschein *et al.* 2017a), where *Tritonibacter* species predominantly reside in open waters, and requires a tighter regulation of TDA production as not to waste metabolic energy during dispersed planktonic growth.

Nutrients

Variation of carbon, nitrogen, sulfur, phosphorus, or iron sources affect production of TDA. In *P. inhibens* DSM17395, production of TDA increases dramatically when phenylalanine is used as the primary carbon source instead of glucose, and more TDA is produced in general, when aromatic compounds are utilized as a carbon source (Berger *et al.* 2012). As the backbone of TDA originates from the phenylacetic acid catabolon, this increase in TDA production is likely a result of increased precursor availability (Berger *et al.* 2012; Brock, Nikolay and Dickschat 2014).

TDA is a sulfur-containing compound and in *Tritonibacter* sp. TM1040, growth on DMSP is associated with an increase in TDA concentrations as compared to growth on sulfate-containing substrates (Geng and Belas 2010). DMSP is produced by phytoplankton and roseobacters preferentially metabolize DMSP over the more readily available sulfate (Kiene *et al.* 1999), likely pointing to a niche-specific adaptation. In *Pseudovibrio* sp. FO-BEG1, phosphate limitation induces TDA production but this is likely attributed to a global change in sulfur metabolism rather than a specific phosphate effect (Romano *et al.* 2015).

In laboratory cultures, production of bioactive TDA is dependent on iron in concentrations that far exceed those observed in natural marine systems (D'Alvise *et al.* 2016). Despite this, *Phaeobacter* exhibits TDA-dependent antagonism against vibrios in low-iron artificial seawater as well as in systems mimicking multitrophic level seawater systems (D'Alvise *et al.* 2010, 2012). A nonantibacterial (inactive) form of TDA ('pre-TDA') is produced when iron is not available, and this 'pre-TDA' can be converted to TDA by acidification (D'Alvise *et al.* 2016). Thus, the biosynthesis of TDA does not appear to be regulated by iron at the transcriptional level, yet bioactive TDA only forms in its presence. This is in contrast to other iron-chelating secondary metabolites, siderophores, which are typically upregulated in the absence of iron. The weak iron-chelating properties of TDA indicate that iron sequestering is not its main function, but potentially relate to its mode of action (Wilson *et al.* 2016), or to symbiosis where the TDA–iron complex

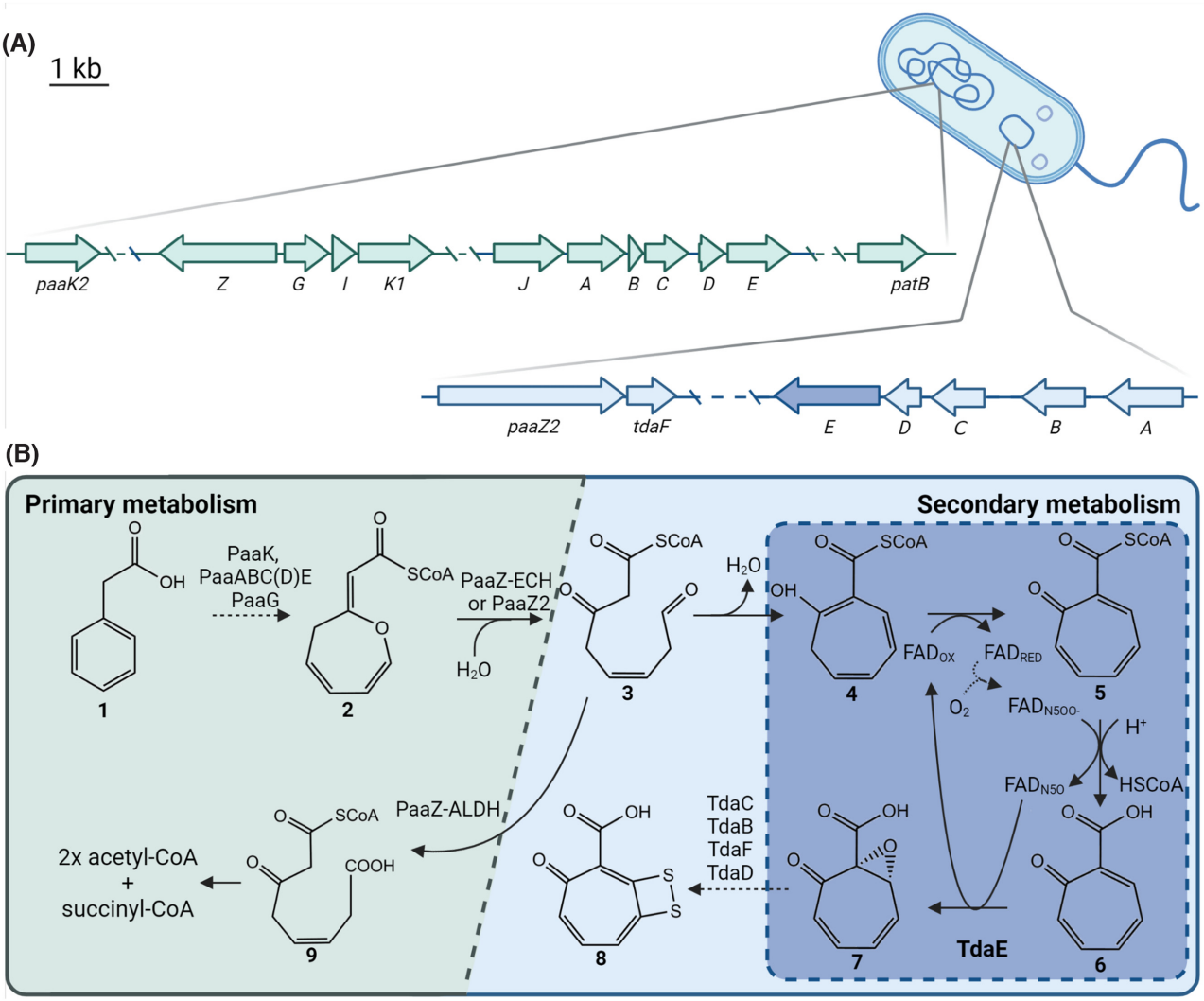

Figure 3. TDA biosynthesis and genes responsible for TDA production. **(A)** Biosynthetic genes involved in TDA biosynthesis in *P. inhibens* DSM17395. *paaABCDEIJK1K2* and *patB* (in green) are located on the chromosome, whilst *tdaABCDEF* and *paaZ2* (in blue) are located on a 262-kb megaplasmid. **(B)** TDA biosynthesis draws on primary metabolism for formation of the carbon backbone. Phenylacetic acid, **1**, is converted to **2** by PaaK, PaaABC(D)E, and PaaG. PaaZ-ECH or PaaZ2 catalyzes hydrolytic ring cleavage to form **3**, which is then either converted to **9** by the ALDH domain of PaaZ or spontaneously cyclized to **4.** TdaE then connects primary and secondary metabolism through a series of reactions: dehydrogenation to **5**, CoA-ester oxygenolysis to **6**, and ring epoxidation to form **7**. TdaBCDF subsequently forms TDA, **8**. Stipled lines indicate multiple reactions taking place. Created with ChemDraw Professional (PerkinElmer Informatics) and Biorender.com.

could serve as an iron reservoir (D'Alvise *et al.* 2016), similarly to what has been proposed for vibrioferrin produced by *Marinobacter* (Amin *et al.* 2009; Yarimizu *et al.* 2019).

Quorum sensing and autoinduction

TDA production is measurable in late exponential or early stationary growth phase (Geng and Belas 2010; Berger *et al.* 2011; Harrington *et al.* 2014; Romano *et al.* 2015), which is correlated with high cell densities. Since many roseobacters also produce the QS molecules acyl-homoserine lactones (AHLs), it has been suggested that TDA production is QS regulated (Gram *et al.* 2002; Bruhn *et al.* 2005, 2006, 2007; Martens *et al.* 2007; Berger *et al.* 2011; Zan *et al.* 2014). Transposon insertions in either the AHL synthase gene, *pgaI*, or the gene encoding the response regulator, *pgaR*, of *P. inhibens* DSM17395 results in a reduction in TDA production (Berger *et al.* 2011). However, the effect is temporary, indicating that TDA biosynthesis is not fully dependent on this QS system (Prol García, D'Alvise and Gram 2013). Furthermore, the effect is reversible through supplementation with phenylalanine, suggesting a hierarchical regulation of TDA biosynthesis (Berger *et al.* 2012). AHLs are not universally involved in the regulation of TDA production since *Tritonibacter* sp. TM1040 does not produce any known AHLs and no AHL synthase genes have been identified in their genomes (Sonnenschein *et al.* 2017a). Other compounds than AHLs may be involved in QS, and using a *tdaCp::lacZ* reporter plasmid in several TDA-deficient *Tritonibacter* sp. TM1040 strains, it was discovered that *tdaC* was not expressed in the absence of TDA (Geng and Belas 2010). Subsequently, *tdaC* expression was restored by cross-feeding with the wildtype, demonstrating that TDA acts as an autoinducer of its own biosynthesis in a manner similar to AHL signaling TDA (Geng and Belas 2010). Addition of both the AHL produced by *pgaI*, 3-OH-C10-homoserine lactone (HSL), and TDA restored TDA production in the *pgaI*$^-$ mutant, but not in the *pgaR*$^-$ mutant, indicating that TDA could potentially act as an autoinducer through the same response regulator as 3-OH-C10-HSL (Berger *et al.* 2011). This provides a possible link between autoin-

duction and AHL-mediated regulation of TDA biosynthesis (Berger *et al.* 2011).

QS regulation of TDA production has only been demonstrated in the strains TM1040 and DSM17395. Adding TDA to a final concentration of 1 μM to the TDA-producing *Pseudovibrio* sp. W74 did not result in earlier onset or higher production of TDA, indicating that autoinduction did not take place (Harrington *et al.* 2014; D'Alvise *et al.* 2016). However, since W74 produces TDA, TDA would also be present in the control cultures and testing TDA addition to a TDA-deficient mutant of W74 would be required to determine any possible QS function.

The exact regulatory network controlling TDA biosynthesis has not been elucidated, however, using a *tdaCp::lacZ* reporter fusion, it has been demonstrated that TdaA is necessary for the transcriptional activation of *tdaC* expression in *Tritonibacter* sp. TM1040, independent of TDA (Geng and Belas 2011). TdaA also acts as a transcriptional activator in *P. inhibens* DSM17395, where expression of *tdaB, tdaE,* and *tdaF* is strongly downregulated in *tdaA*$^{-}$ mutants (Berger *et al.* 2011). A putative binding site of TdaA near the *tdaC* promoter has been identified in *Tritonibacter* sp. TM1040, and binding of TdaA to the *tdaC* promoter has been confirmed using an electrophoretic mobility shift assay (Geng and Belas 2011). Understanding the regulatory switches governing TDA biosynthesis, and how these differ between genera, may provide an important clue as to the ecological role of this compound.

Effect of TDA on the producing bacteria

Deletion of the 262-kb megaplasmid encoding the last part of the TDA biosynthetic pathway leads to an increase in growth rate and yield, demonstrating a significant burden of this plasmid (Trautwein *et al.* 2016; Wünsch *et al.* 2020). Similarly, transposon insertions in any of the *tdaA, tdaB, tdaC,* or *tdaE* genes on the plasmid result in TDA-deficient mutants exhibiting increased growth rates and yields (Will *et al.* 2017). The negative effects of TDA on the producer is, however, not due to the metabolic cost associated with TDA biosynthesis as supplementation with TDA-containing supernatant reverts the growth of the TDA-deficient mutant to wildtype levels (Will *et al.* 2017) indicating an autoantibacterial activity. Thus, as TDA production impairs growth of the producer, the compound must represent a significant ecological advantage in natural marine systems.

The fact that TDA interacts with AHL response regulators in *Tritonibacter* sp. TM1040 has major implications for global gene expression in the producing organism (Berger *et al.* 2011; Beyersmann *et al.* 2017). With the exception of 15 genes, TDA regulates the same genetic circuitry as the AHL 3-OH-C10-HSL in *P. inhibens* DSM17395, including genes involved in chemotaxis, motility, attachment, and biofilm integrity (Beyersmann *et al.* 2017). In effect, the QS signaling molecule and TDA in conjunction likely facilitate a swim-and-stick lifestyle through the induction of biofilm dispersion. While similar mechanisms have been observed for *P. inhibens* 2.10 (Majzoub *et al.* 2018), the evident connection between TDA and the biofilm mode of growth is likely different among producers with different niche adaptations, and more studies are necessary to fully resolve the effect of TDA on the producing organism. Several other antimicrobial secondary metabolites are known to function as QS signals in the producing strain, e.g. surfactin in *Bacillus subtilis* and *Bacillus amyloliquefaciens* (López *et al.* 2009; Chen *et al.* 2020), and pyocyanine in *P. aeruginosa* (Dietrich *et al.* 2006). Similarly to TDA, these compounds also affect biofilm formation and motility (Das and Manefield 2012). These molecules vary widely in chemical structure and are produced by bacteria that are taxonomically distant; hence, it seems plausible that this may be a somewhat common function of antimicrobial secondary metabolites which is likely facilitated by many different mechanisms.

TDA-mediated inhibition of other microorganisms

TDA can inhibit or kill a wide range of Gram-positive and Gram-negative bacteria, including both fish and human pathogens (Ruiz-Ponte *et al.* 1998, 1999; Hjelm *et al.* 2004a; Planas *et al.* 2006; Porsby, Nielsen and Gram 2008; Prado *et al.* 2009; Porsby *et al.* 2011; Porsby and Gram 2016; Zhao *et al.* 2016; Grotkjær *et al.* 2016b; Sonnenschein *et al.* 2021). Also eukaryotes such as the fungal pathogens *Rizoctonia solani*, *Candida albicans*, and some microalgal strains of the genera *Chlorella* and *Scenedesmus* are negatively affected by TDA (Kintaka *et al.* 1984; Liang 2003). Pure TDA can be lethal to some mammalian cell lines, including neuronal and cancer cells, and it has been suggested that TDA may lead to disruption of the mitochondrial membrane potential and activation of oxidative stress responses (Wichmann *et al.* 2015). The algal compound DMSP is to some extent protective against TDA-induced cytotoxicity, as preincubation with DMSP of mammalian neural cells exerted protective effects against TDA, potentially due to DMSP acting as an antioxidant (Wichmann *et al.* 2016). This could suggest that DMSP could have a role in the interplay between marine eukaryotes and TDA-producing bacteria, thus acting as a protectant against TDA, although this is highly speculative and requires further studies (Duan *et al.* 2020). Despite the negative effects of TDA on eukaryotic cells, no adverse effect of TDA producers has so far been observed on higher organisms (Sonnenschein *et al.* 2021) such as microalgae, *Artemia*, rotifers, or nauplii (D'Alvise *et al.* 2012; Prol García, D'Alvise and Gram 2013; Rasmussen *et al.* 2018, 2019; Sonnenschein *et al.* 2018). In fact, TDA producers may be important symbionts for microalgae and corals, and TDA has been proposed to act as algal and coral protectant (Seyedsayamdost *et al.* 2011b; Raina *et al.* 2016). Some TDA-producers, i.e. *P. inhibens*, *P. galleciensis,* and *P. piscinae*, also produce the algicidal troponoids roseobacticides (Seyedsayamdost *et al.* 2011a; Sonnenschein *et al.* 2018). These secondary metabolites are synthesized in response to *p*-coumaric acid, a potential senescence signal produced by algae (Seyedsayamdost *et al.* 2011a). In *P. inhibens* DSM17395, TDA and roseobacticides share parts of the same biosynthetic pathway, and the metabolites probably share the same precursor (Wang, Gallant and Seyedsayamdost 2016). This, thus, challenges the one-cluster–one-compound paradigm. These findings highlight that secondary metabolites may also be involved in beneficial interkingdom cross-talk. A well-studied example of this is found in *Bacillus*–plant interactions, where production of the secondary metabolite surfactin is stimulated in response to plant host cues and in turn triggers plant immunity against pathogens in *Bacillus*–plant interactions (Hoff *et al.* 2021).

In the proposed mechanism of antibacterial action, TDA disrupts the proton motive force by binding extracellular protons to the carboxyl group and transporting them across the cell membrane to the cytosol (Wilson *et al.* 2016). Here, the proton is exchanged for a metal ion, i.e. transported back to the extracellular space (Wilson *et al.* 2016). This destroys the transmembrane proton gradient whilst maintaining the membrane potential, making TDA an electroneutral proton antiporter (Wilson *et al.* 2016). In concordance with this, exposure of a *Vibrio vulnificus* to sublethal concentrations of pure TDA lead to upregulation of genes involved

in iron-uptake, oxidative stress, and regeneration of the cell envelope (Dittmann *et al.* 2019a).

The antibacterial effect of TDA against fish pathogenic bacteria has been extensively studied, due to the interest in using TDA-producing bacteria as probiotics in marine aquaculture (Ruiz-Ponte *et al.* 1998, 1999; Bruhn, Gram and Belas 2007; D'Alvise *et al.* 2012; Porsby and Gram 2016; Zhao *et al.* 2016; Grotkjær *et al.* 2016b; Rasmussen *et al.* 2018, 2019; Dittmann *et al.* 2019a; Ringø 2020). TDA-producing *Phaeobacter* and *Tritonibacter* are antibacterial against *Vibrio* species, such as *V. anguillarum*, *V. vulnificus*, *and Vibrio coralliilyticus* (D'Alvise *et al.* 2010; Porsby and Gram 2016; Zhao *et al.* 2016). Extracts from TDA-producing *Pseudovibrio* sp. P12 strongly inhibited the growth of *V. coralliilyticus* and *Vibrio owensii*, two coral pathogens causing white syndrome in Scleractinian corals (Raina *et al.* 2016). Growth of *V. coralliilyticus* is not suppressed by common coral-associated bacteria and the bacterium exhibits antibiotic resistance to a wide range of commercial antibiotics, greater than that of other vibrios such as *V. vulnificus* (Shnit-Orland and Kushmaro 2009; Rypien, Ward and Azam 2010; Vizcaino *et al.* 2010), emphasizing the antibiotic potential of TDA. TDA also inhibits other fish pathogens such as *Aeromonas* and *Tenacibaculum* spp. (Porsby and Gram 2016; Grotkjær *et al.* 2016b; Tesdorpf *et al.* 2022).

The antagonistic effect of TDA-producing strains has primarily been determined using agar-based assays (Brinkhoff *et al.* 2004; Hjelm *et al.* 2004a; Bruhn *et al.* 2005; Rao *et al.* 2007; Porsby, Nielsen and Gram 2008; Prado *et al.* 2009) and different broth and/or biofilm-based co-culture setups (Hjelm *et al.* 2004b; Porsby, Nielsen and Gram 2008; Prado *et al.* 2009; Grotkjær *et al.* 2016b). Exposure of a *V. anguillarum* to surface-attached *Phaeobacter* or *Tritonibacter* resulted in significant reduction or complete elimination of *V. anguillarum* (D'Alvise *et al.* 2010). Furthermore, *P. inhibens* DSM17395 successfully inhibited *V. vulnificus* in co-culture experiments, keeping it at inoculum level, whereas monocultures of *V. vulnificus* were 1000-fold higher (Porsby and Gram 2016). TDA is most likely responsible for this inhibition, since TDA-negative mutants did not inhibit *V. anguillarum* (D'Alvise *et al.* 2010).

The activity of TDA-producing roseobacters against vibrios has also been assessed in more complex, nonaxenic, and microcosm experiments including marine organisms of multiple trophic levels. As pathogenic *Vibrio* strains may enter the aquaculture unit via live feed, many experiments have been conducted in cultures of microalgae, rotifers, brine shrimps, and copepods (D'Alvise *et al.* 2012; Prol García, D'Alvise and Gram 2013; Porsby and Gram 2016; Grotkjær *et al.* 2016a; Rasmussen *et al.* 2018). In these laboratory experiments, TDA-producing *Phaeobacter* can reduce the number of vibrios and other fast-growing heterotrophic bacteria (D'Alvise *et al.* 2012; Grotkjær *et al.* 2016a; Rasmussen *et al.* 2018, 2019).

Resistance and tolerance to TDA

Phaeobacter inhibens DSM17395 carries three genes, *tdaR123*, conferring its self-resistance to TDA (Wilson *et al.* 2016). *tdaR1* and *tdaR2* are predicted to encode transmembrane proteins, while *tdaR3* is predicted to encode a γ-glutamyl-cyclotransferase, which in *Escherichia coli* is involved in cation–proton exchange (Wilson *et al.* 2016). All three genes are co-located to the TDA gene cluster on the megaplasmid, which to this day has not proven transmissible or to encode transmission genes (Petersen *et al.* 2013). Sensitivity to TDA in *E. coli* is reduced when the *tdaR123* genes are transferred and heterologously expressed (Wilson *et al.* 2016). However, the *tdaR123* genes have not been found in bacteria not producing TDA, and it has not been possible to develop resistance to TDA in target bacteria *in vitro* (Porsby *et al.* 2011; Rasmussen *et al.* 2016). Different *in vitro* approaches have been used to induce mutations or adaptations conferring TDA resistance in the non-TDA-producer species *P. aeruginosa*, *S. aureus*, *Salmonella typhimurium*, and *E. coli*, which are all species susceptible to TDA (Porsby *et al.* 2011). Using adaptive laboratory evolution experiments, *V. anguillarum* strains capable of tolerating two times the minimum inhibitory concentration were evolved, however, the tolerance was transient and vanished after one passage in medium free of TDA (Rasmussen *et al.* 2016). The difficulty in developing TDA resistance or tolerance could suggest that TDA has multiple additional targets beside the disruption of the proton motive force. Since only a few TDA resistant bacteria have been found, and resistance is difficult to develop, the use of TDA as an antibiotic and of TDA producers as probiotics will not add to the risk of antimicrobial resistance (Sonnenschein *et al.* 2021).

TDA tolerance has been observed in natural microbial communities containing indigenous TDA-producing *Pseudovibrio*. Here, 126 out of 136 isolated non-TDA producing bacteria were tolerant to TDA (Harrington *et al.* 2014). Among the TDA-tolerant isolates were *Psychrobacter*, *Alteromonas*, *Salinibacter*, *Alcanivorax*, *Flavobacterium*, and *Micrococcus* strains, whilst TDA-sensitive isolates included *Staphylococcus*, *Idiomarina*, and *Rhodococcus* strains (Harrington *et al.* 2014). The mechanisms enabling this tolerance are not known.

Influence of TDA or TDA producers on marine microbial communities

The potential use of TDA-producing bacteria as probiotics in marine aquaculture has prompted studies determining how TDA or TDA-producing bacteria affect natural marine microbiomes. The possible effect of TDA or bacteria producing TDA on taxonomic composition of natural microbiomes has been determined, typically by 16S rRNA gene amplicon sequencing (Table 1).

Adding pure TDA to the microalgae *Nannochloropsis salina* colonized by a seawater microbiome caused a decrease in relative abundance of bacteria belonging to *Rhodobacteraceae, Flavobacteriia*, and *Alteromonadaceae* after 24 h, while bacteria of unclassified families within the Alteromonadales order increased in relative abundance (Geng *et al.* 2016). The changes were more rapidly seen in communities exposed to high concentrations (500 nM as opposed to 21 nM) of TDA, indicating a dose-dependent effect of TDA. The addition of TDA accelerated the development of the microbial community that after 3 h had a composition similar to the one reached in the nontreated community after 24 h (Geng *et al.* 2016). It should be noted that the concentration of TDA found in natural systems is not known. Addition of pure TDA to the microalgae *Tetraselmis suecica* could be detected to a lower limit of 50 nM, but TDA was not detectable when TDA-producing bacteria were cultured in the system (D'Alvise *et al.* 2012). Thus, it remains uncertain if the concentrations used in the *N. salina* study (Geng *et al.* 2016) were representative of the TDA concentrations in natural communities. Subsequent studies have typically studied changes in the bacterial community in the presence of TDA-producing bacteria.

The TDA-producing *P. inhibens* DSM17395 was added to the microbiome of the marine microalgae *Emiliana huxleyi* in concentrations reflecting the *in situ* abundances of roseobacters during algal blooms (Amin *et al.* 2015; Segev *et al.* 2016; Sonnenschein *et al.* 2018; Dittmann *et al.* 2019b). The addition of 10^6 CFU/ml of DSM17395 caused a decrease in the relative abundance of bacteria belonging to Rhodobactereales, with *Loktanella* and *Marivita*

Table 1. Model systems used to study the effect of TDA-producing bacteria and TDA on microbial communities.

In vivo model	TDA dose	Controls	Duration (days)	Bacteria that increase	Bacteria that decrease	Reference
Nannochloropsis salina (microalgae)	Pure TDA (31–500 nM)	Untreated (glucose)	0–1	Alteromonadales (Unclassified families)	*Alteromonadaceae*, Flavobacteriia, *Rhodobacteraceae*	Geng *et al.* (2016)
Emiliania huxleyi (microalgae)	*P. inhibens* DSM17395 (10^4 and 10^6 cells/ml)	Untreated (medium)	0–4	*Colwellia* sp., *Winogradskyella* sp., *Neptuniibacter* sp. (absent in controls)	*Vibrio* sp., *Pseudoalteromonas* sp., Alteromonadales *Marivita*	Dittmann *et al.* (2019b)
Ostrea edulis (oyster)	*P. inhibens* DSM17395 (10^4 and 10^6 cells/ml)	Untreated (medium)	0–4	*Mycoplasma* sp.	*Vibrionaceae*, *Mycoplasma* sp., *Pseudoalteromonas* sp., *Shewanella* sp.	Dittmann *et al.* (2019b)
Tetraselmis suecica (microalgae)	*P. inhibens* DSM17395 (10^6 cells/ml)	Untreated (medium)	0–4			Dittmann *et al.* (2020)
Acartia tonsa (copepod)	*P. inhibens* DSM17395 (10^6 cells/ml)	Untreated (medium)	0–4		*Rhodobacteraceae: Ruegeria*, *Celeribacter*, *Pseudophaeobacter*	Dittmann *et al.* (2020)
Scophthalmus maximus (turbot larvae)	*P. inhibens* DSM17395 (10^6 cells/ml)	Untreated (medium)	0–4		*Rhodobacteraceae: Ruegeria*, *Celeribacter*, *Pseudophaeobacter*	Dittmann *et al.* (2020)
Thalassiosira rotula (microalgae)	*P. inhibens* 2.10	TDA-deficient mutant	0–8		*Rhodobacteraceae: Sulfitobacter*, *Phaeobacter*, *Pelagicola*, *Loktanella*	Majzoub *et al.* (2019)

being the most affected genera (Dittmann *et al.* 2019b). This is similar to the changes observed in the *N. salina* study, where pure TDA caused a decrease in relative abundance of *Rhodobacteraceae* (Geng *et al.* 2016). When adding DSM17395 to the European flat oyster, *Ostrea edulis*, the relative abundance of the Vibrionales decreased markedly as compared to untreated microbiomes. In the *E. huxleyi* microbiome, individual amplicon sequence variants (ASVs) assigned as *Vibrio* sp. also decreased when treated with DSM17395, although no changes were seen at the order level. Also, the relative abundance of Alteromonadales in the *O. edulis* microbiome was higher in the DSM17395-treated microbiomes (up to 70%) compared to the untreated microbiomes (up to 47%). However, specific ASVs assigned as *Pseudoalteromonas* sp. (belonging to the Alteromonadales) decreased upon the addition of DSM17395, which was also observed in the *E. huxleyi* microbiome treated with DSM17395. Once again, this is similar to the *N. salina* study using pure TDA, where both increases and decreases were found for bacteria belonging to the Alteromonadales when treated with TDA (Geng *et al.* 2016).

The addition of DSM17395 to three common aquaculture livefeeds—the microalgae (*T. suecica*), copepod nauplii (*Acartia tonsa*), and turbot eggs/larvae (*Scophthalmus maximus*)—caused a decrease in relative abundance of closely related taxa particularly of the Rhodobacterales in the microbiomes (Dittmann *et al.* 2020). Specifically, species of *Ruegeria*, *Celeribacter*, and *Pseudophaeobacter* decreased in relative abundance. However, in contrast to the *E. huxleyi* microbiome (Dittmann *et al.* 2019b), bacteria belonging to the Vibrionales and Alteromonadales were not affected by DSM17395 in any of the microbiomes, despite the *S. maximus* microbiome having a high relative abundance of ASVs assigned as *Vibrio* sp. This is surprising since several studies have demonstrated an anti-*Vibrio* effect of TDA-producing *Phaeobacter* compared to a TDA-deficient mutants (D'Alvise *et al.* 2010, 2012), indicating that the effect of DSM17395 toward vibrios depend on the commensal microbiome composition or TDA being species-specific. Future studies should include a TDA-deficient mutant to specifically address the role of TDA in the microbiome development. Such a comparison of the effect of TDA-producing *P. inhibens* 2.10 and its TDA-deficient mutant was conducted on the microbiome assembly of the microalgae *Thalassiosira rotula* (Majzoub *et al.* 2019). Strain 2.10 demonstrated strain-specific effects in the microbiome, in concordance with the previous microbiome studies (Geng *et al.* 2016; Majzoub *et al.* 2019; Dittmann *et al.* 2019b, 2020). Furthermore, closely related strains belonging to the *Sulfitobacter*, *Phaeobacter*, *Pelagicola*, and *Loktanella* genera were reduced or eliminated by the TDA-producing wildtype but not by the TDA-deficient mutant (Majzoub *et al.* 2019).

Overall, the addition of a TDA-producing *P. inhibens* has only minor effects on the taxonomic composition of marine microbial communities. Changes due to the presence of TDA-producing *P. inhibens* strains or TDA appear to be species, if not strain-specific, particularly decreasing the relative abundance of closely related taxa. This is in line with the competition-relatedness concept (Russel *et al.* 2017), where closely related species more often compete for the same metabolic and environmental niches. Niche competition has been suggested to be one of the evolutionary explanations for the selection of antimicrobial compounds—a concept known as competition sensing (Cornforth and Foster 2013). In several studies (Geng *et al.* 2016; Dittmann *et al.* 2019b), the relative abundance of bacteria belonging to the fast-growing heterotrophs of Vibrionales or Alteromonadales were affected by the addition of TDA or the TDA-producing strains, in particular the genus *Pseudoalteromonas*, which generally decreased in relative abundance (Geng *et al.* 2016; Dittmann *et al.* 2019b). Since TDA can act as an antiporter (Wilson *et al.* 2016), the compound may be particularly effective in antagonizing fast-growing bacteria depending on a high metabolic turnover. Several species of *Pseudoalteromonas* are potent secondary metabolite producers (Paulsen *et al.* 2019), and it has also been suggested that TDA-producing bacteria antagonize specifically potent secondary metabolite producing bacteria found in the same ecological niches (Lutz *et al.* 2016; Dittmann *et al.* 2019b), potentially due to competition sensing (Cornforth and Foster 2013). In fact, the ability of *P. inhibens* to produce TDA has been suggested to be maintained by interspecies competition with *Pseudoalteromonas tunicata* in biofilms (Lutz *et al.* 2016; Majzoub *et al.* 2018). However, the specific mechanism driving this pattern between TDA and *Pseudoalteromonas* species is not fully explored.

Conclusions

TDA is a molecule with multiple functions: antibiotic (disruption of the proton motive force in target bacteria), QS signal, and iron chelation (Fig. 1). The (weak) extracellular iron chelation by TDA could indicate that TDA also can act as an iron provider (reservoir) for other organisms, similar to other weak iron chelators, such as vibrioferrin, suggested to promote bacterial–algal mutualism (Amin *et al.* 2009; Yarimizu *et al.* 2019).

In microbial communities, TDA has predominantly been studied as an antimicrobial compound. It reduces the relative abundance of bacteria closely related to the TDA producer, and sometimes fast-growing, potential secondary metabolite producers, such as vibrios and members of the *Pseudoalteromonas* genus. These observations point toward TDA playing a role in niche competition. The natural concentrations of TDA are not known and we speculate that most of the studies addressing the (antimicrobial) effect of TDA, or its producer are using concentrations of the compound or producer higher than the natural levels. Thus, there is a need for tractable model systems that reflect the natural environment in order to study secondary metabolism and community dynamics (Pessotti, Hansen and Traxler 2018; Gralka *et al.* 2020).

The effects of TDA on gene expression patterns in TDA-producing bacteria indicate that TDA can serve as a QS signal, affecting biofilm formation and motility in the TDA producer. A putative receptor, *pgaR*, is also present in *P. inhibens*, but more studies are necessary to fully understand the molecular mechanism by which TDA regulates gene expression. TDA production may be part of adaptation to a surface-associated lifestyle, possibly in association with eukaryotic host organisms. TDA production in *Tritonibacter* occurs primarily during stagnant growth, whilst in *Phaeobacter,* TDA is also produced in aerated cultures, perhaps reflecting a tighter regulation of TDA biosynthesis in the open water *Tritonibacter* than in its coastal relative *Phaeobacter*.

In conclusion, TDA is indeed an antimicrobial secondary metabolite and as such serves multiple ecological roles such as an algal or coral protectant. However, TDA has other less explored functions being involved in QS regulation. Other antimicrobial secondary metabolites, such as surfactin, also serve multiple functions and this may be the case for several other secondary metabolites. Most studies of antimicrobial secondary metabolites has predominantly been motivated by their antibacterial activity and their effects studied on pure cultures of bacteria, mainly pathogens, using concentrations that are likely higher than those found in natural settings. To fully unravel the roles of antimicrobial secondary metabolites can and may play in natural commu-

nities, we must study the producing organisms and the compound *in situ* in natural systems.

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Manuscript II

Tropodithietic acid, a multifunctional antimicrobial, facilitates adaption and colonization of the producer, *Phaeobacter piscinae*

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RESEARCH ARTICLE

Tropodithietic Acid, a Multifunctional Antimicrobial, Facilitates Adaption and Colonization of the Producer, *Phaeobacter piscinae*

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ABSTRACT In the marine environment, surface-associated bacteria often produce an array of antimicrobial secondary metabolites, which have predominantly been perceived as competition molecules. However, they may also affect other hallmarks of surface-associated living, such as motility and biofilm formation. Here, we investigate the ecological significance of an antibiotic secondary metabolite, tropodithietic acid (TDA), in the producing bacterium, *Phaeobacter piscinae* S26. We constructed a markerless in-frame deletion mutant deficient in TDA biosynthesis, S26Δ*tdaB*. Molecular networking demonstrated that other chemical sulfur-containing features, likely related to TDA, were also altered in the secondary metabolome. We found several changes in the physiology of the TDA-deficient mutant, Δ*tdaB*, compared to the wild type. Growth of the two strains was similar; however, Δ*tdaB* cells were shorter and more motile. Transcriptome and proteome profiling revealed an increase in gene expression and protein abundance related to a type IV secretion system, and to a prophage, and a gene transfer agent in Δ*tdaB*. All these systems may contribute to horizontal gene transfer (HGT), which may facilitate adaptation to novel niches. We speculate that once a TDA-producing population has been established in a new niche, the accumulation of TDA acts as a signal of successful colonization, prompting a switch to a sessile lifestyle. This would lead to a decrease in motility and the rate of HGT, while filamentous cells could form the base of a biofilm. In addition, the antibiotic properties of TDA may inhibit invading competing microorganisms. This points to a role of TDA in coordinating colonization and adaptation.

IMPORTANCE Despite the broad clinical usage of microbial secondary metabolites with antibiotic activity, little is known about their role in natural microbiomes. Here, we studied the effect of production of the antibiotic tropodithietic acid (TDA) on the producing strain, *Phaeobacter piscinae* S26, a member of the *Roseobacter* group. We show that TDA affects several phenotypes of the producing strain, including motility, cell morphology, metal metabolism, and three horizontal gene transfer systems: a prophage, a type IV secretion system, and a gene transfer agent. Together, this indicates that TDA participates in coordinating the colonization process of the producer. TDA is thus an example of a multifunctional secondary metabolite that can mediate complex interactions in microbial communities. This work broadens our understanding of the ecological role that secondary metabolites have in microbial community dynamics.

KEYWORDS biofilm, motility, *Phaeobacter*, secondary metabolites, tropodithietic acid, prophage, gene transfer agent, horizontal gene transfer, niche colonization

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Microorganisms often attach to and colonize biotic and abiotic surfaces, and such biofilms are believed to be the predominant microbial lifeform (1). In biofilms, multiple microbial species coexist in structured environments that allow for close interactions, including exchanging metabolites (2) and genetic material (3, 4). Additionally, surface-associated bacteria are more prolific producers of antimicrobial secondary metabolites (5–7) than their planktonic counterparts. These antimicrobial secondary metabolites are believed to provide a significant advantage to the producing microorganism by inhibiting competing microorganisms (8), in line with the classical perception that antimicrobial secondary metabolites predominantly act as competition molecules (5, 6). This perception has been challenged by the observation that many secondary metabolites engage in nonantibiotic activities, e.g., as signaling or nutrient-scavenging molecules (7, 9). Some of these nonantibiotic activities also affect hallmarks of surface-associated living, and traits such as biofilm formation and motility are often affected by sublethal concentrations of exogenous antibiotics (10–13).

Although it has been demonstrated that exogenous antibiotics affect bacterial phenotypes, only very few studies have explored how antibiotic production affects the producing organism itself. Recently, Zhang et al. (2021) showed that *Photobacterium galatheae*, which produces the antibiotic holomycin, is reduced in its biofilm-forming capacity when holomycin biosynthesis is abolished (14). Also, the lipopeptide surfactant facilitates horizontal gene transfer (HGT) in *Bacillus subtilis* by promoting cell lysis and DNA release (15) and may thereby facilitate niche adaptation (16, 17). Thus, production of molecules with antibiotic activity can affect the producing organism in previously unforeseen ways, some of which may provide more than one advantage to the organisms, for instance, during surface colonization. This indicates that there may be more than one reason secondary metabolite production prevails in the surface-associated microorganisms.

Tropodithietic acid (TDA) is a secondary metabolite produced by members of the globally occurring marine *Roseobacter* group (18–23), including the *Phaeobacter* genus, which exhibits strong adaptations for a surface-associated lifestyle (24, 25). In the closely related genus, *Tritonibacter*, TDA production and biofilm formation appear directly coupled since TDA is predominantly produced during stagnant (nonshaken) biofilm growth rather than during aerated planktonic growth (19, 26, 27). As an antibiotic, TDA targets both Gram-positive and Gram-negative bacteria through a proposed mode of action in which the proton motive force is disrupted (28). Introducing TDA and TDA-producing microorganisms into microbiomes reduces the abundance of closely related species and fast-growing secondary metabolite producers such as vibrios (29–32), indicating that TDA could arbitrate niche competition. In addition to its antibiotic properties, TDA is a weak chelator of iron, perhaps indicating a role as an iron reservoir (33). In *Phaeobacter inhibens*, exogenous TDA can substitute for N-3-hydroxydecanoylhomoserine lactone, a quorum sensing signal molecule, resulting in analogous changes in biofilm formation, motility, and antibiotic production (34–37). TDA production may be important in algal-*Phaeobacter* symbiosis as it has been suggested to protect the algae from bacterial pathogens (38, 39). TDA thus serves as a model molecule of a bacterial antibiotic secondary metabolite with multiple functions (21).

The purpose of the present study was to investigate the broader ecological roles of TDA in a producing bacterium, *Phaeobacter piscinae* strain S26, focusing on its possible involvement in surface colonization. To this end, we constructed a scarless TDA-deficient mutant, S26Δ*tdaB*, and compared the physiology of S26 wild type (WT) and mutant through a series of phenotypical assays, as well as global comparisons of the transcriptome, metabolome, and proteome. TDA serves as a case study for gaining a more holistic understanding of the ecological role of antibiotic secondary metabolites.

RESULTS

Deletion of *tdaB* abolishes TDA production and affects the expression of several TDA biosynthetic genes and proteins. We generated a TDA-deficient mutant, Δ*tdaB*, by scarless deletion of the core biosynthetic gene *tdaB* of S26 WT (Fig. S1A and

S1B). *tdaB* encodes a putative β-etherase/glutathione *S*-transferase, which is proposed to catalyze the addition of an *S*-thiocysteine to a CoA ester (40) in the biosynthetic pathway of TDA. To confirm the abolishment of TDA biosynthesis, Δ*tdaB* and WT were grown to stationary phase in marine broth (MB), and secondary metabolites were extracted and analyzed by HPLC-DAD-HRMS. TDA was detected in extracts of WT but not Δ*tdaB* (Fig. S1C). No precursors of TDA were found in WT or in Δ*tdaB*. TDA production of the mutant was partially restored upon genetic complementation with pBBR1MCS2_START-*tdaB* (Fig. S1C). The absence of TDA biosynthesis furthermore resulted in abolishment of antibiotic activity against *V. anguillarum* (Fig. S1D). TDA is an autoinducer of its own synthesis in some, but not all, TDA producing bacteria (23, 36), and we therefore inspected the expression of the remaining genes and proteins in the biosynthetic pathway (Fig. S1E). Genes *paaZ2*, *paaK2*, and *tdaF* were significantly less abundant in the mutant transcriptome, and the relative abundance of five proteins related to TDA biosynthesis was significantly lower in the Δ*tdaB* proteomic samples, including TdaC, TdaD, TdaR3, and Paaz2 in the culture supernatant proteome and TdaF in the cellular fraction.

Global transcriptomic and proteomic comparison of WT and the TDA-abolished mutant Δ*tdaB*. The genome of S26 contains 4,077 open reading frames. Of the 4,004 genes detected (98.2% of all predicted genes) in the stationary-phase transcriptome, 519 were differentially expressed ($P < 0.05$, log2FC > 1, Fig. S2A) between WT and Δ*tdaB*. Of the 2,655 proteins detected from the proteome (65% of total predicted), 126 were differentially produced between WT and Δ*tdaB* ($P < 0.01$, $\log_2$ fold change > 1.5) in the cellular fraction (Fig. S2B), and 403 in the supernatant fraction (Fig. S2C). A list of log2FC and *P*-values can be found for both gene expression and protein abundances in Supplemental Data Set 1.

We then sought to link these transcriptomic and proteomic changes to the physiological changes. Following annotation with PROKKA, 1,903 proteins remained annotated as hypothetical proteins; further functional annotation using eggNOG-mapper reduced this number to 1,720 hypothetical proteins (Supplemental Data Set 1). Genes/proteins were divided into Clusters of Orthologous Groups (COGs) using eggNOG (Fig. S3). A large percentage yielded no hits (9.36%) or were categorized as unknown function (30.87%). Otherwise, the most affected COG group across all three sampling types was amino acid metabolism and transport which is also the most represented group across the genome, comprising 8.74% of all genes. In the transcriptome and supernatant proteome, a large fraction of downregulated genes/proteins in Δ*tdaB* compared to WT belonged to the energy production and conversion COG group.

Deletion of *tdaB* alters the secondary metabolome of S26. Extracts of WT and Δ*tdaB* cultures were analyzed for changes in the secondary metabolome through LC-MS at two time points, 17 h (exponential phase) and 72 h (stationary phase). A principal-component analysis (PCA) showed marked convergence of WT and Δ*tdaB* at the exponential phase, whereas significant divergence was observed at the stationary phase (Fig. 1A). This divergence between WT and Δ*tdaB* cannot only be traced to the onset of significant TDA production (Fig. 1B and Fig. S4A) but many unknown features also drive this divergence (Fig. 1B and Supplemental Fig. S4A). All of this considered, the Δ*tdaB* mutant affects TDA-related metabolites as well as the secondary metabolome (typically masses above >300 Da [41]) beyond TDA).

We used feature-based molecular networking, Ion Identity molecular networking, and SIRIUS formula prediction to further explore the differences between WT and Δ*tdaB* (Fig. 2). Multiple small molecular families (groupings of related features) were observed only in WT samples at the stationary phase, including TDA and methyl troposulfenin (Fig. S4B). Additional features were observed within the same samples, possibly indicating a relationship to TDA production in the stationary phase. Ion identity molecular networking further indicated that many of these molecular families contain the monomeric unit and its accompanying dimer for both TDA and the other features (Fig. 2). To confirm the suspected relationship of these additional features to TDA, formula prediction was undertaken using SIRIUS. Overall, features *m/z* 338.1244 and *m/z* 238.0387 (Fig. 2) were only present in the stationary growth WT samples and contained sulfur in their predicted formulae (Supplemental Data Set 1, sheet "Formula predictions_SIRIUS_WT").

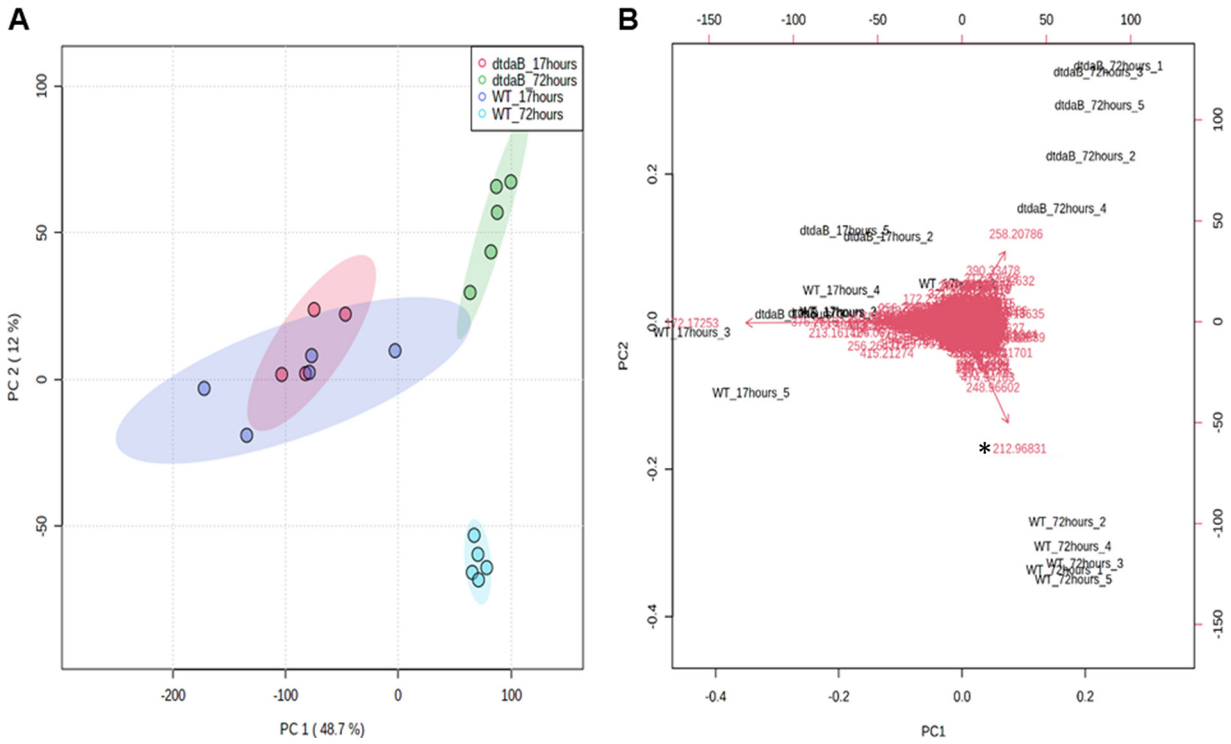

FIG 1 (A) Principal component analysis of metabolome of *Phaeobacter piscinae* S26 (WT) and Δ*tdaB* (dtdaB) mutant grown for 17 and 72 h (quintuplet replicates). Data were normalized to account for variable differences. Instrument and media blanks were included in this analysis but excluded from the PCA plot. (B) PCA biplot where samples (black) and features (red) are cocorrelated. Coordinate location of features corresponds to which samples they are found in. TDA (*m/z* 212.96831, indicated with a '*') is located furthest from the origin and located in the same quadrant as S26-72 h samples.

Cell morphology is altered in Δ*tdaB*. The doubling times of WT and Δ*tdaB* were similar, being 1.04 ± 0.18 h and 0.93 ± 0.05 h (Student's *t* test, $P > 0.05$, calculated based on log[CFU/mL] at 7 and 24 h), respectively (Fig. 3A). Both strains grew to a maximum cell density of 10^8 CFU/mL, demonstrating that TDA production does not hinder nor enhance the growth of *Phaeobacter piscinae* S26. WT cells became elongated over time, with some cells reaching ~10 μM (Fig. 3B). Cell length varied and was on average 2.7 ± 2.2 μm after 3 days. Additionally, WT cells formed star-shaped rosettes. While Δ*tdaB* cells also formed rosettes, cell length was significantly reduced (Student's *t* test, $P < 0.001$) to an average of 1.6 ± 0.3 μm with the longest observed cells reaching ~2 μM (Fig. 3B). Genetic complementation did not restore cell length after 3 days of incubation (data not shown); however, after 7 days of incubation genetically complemented cells reached an average cell length of 2.6 ± 1.0 μm while cells carrying an empty vector were significantly shorter (Student's *t* test, $P < 0.001$), reaching an average cell length of 1.7 ± 0.6 μm (Fig. S5A). Similar cell morphology alterations were also observed in cultures of another TDA-producing bacterium, *P. inhibens* DSM17395, and its TDA-deficient mutant DSM17395 *tdaB*::*gmR* (Fig. S5B). We hypothesized these morphological changes may stem from changes in cell cycle control and therefore examined genes encoding proteins of the COG group D: Cell cycle control (Fig. 3C), but no obvious changes were observed as only one cell cycle-associated protein was significantly downregulated across all samples, OL67_000906, a hypothetical protein containing an EF-hand domain pair. We also investigated homologs of the CckA-ChpT-CckA phosphorelay system, as this has been linked to cell differentiation in other *Rhodobacteraceae* (42, 43) and found a significant upregulation of *ctrA* ($\log_2$[FC]: 1.6, $P < 0.001$) in the transcriptome (Fig. 3D).

Motility of Δ*tdaB* is increased compared to WT. Since TDA can act as a QS-signaling molecule and previous studies have reported an effect of TDA production on biofilm formation and motility (34), we compared the capability of WT and mutant to

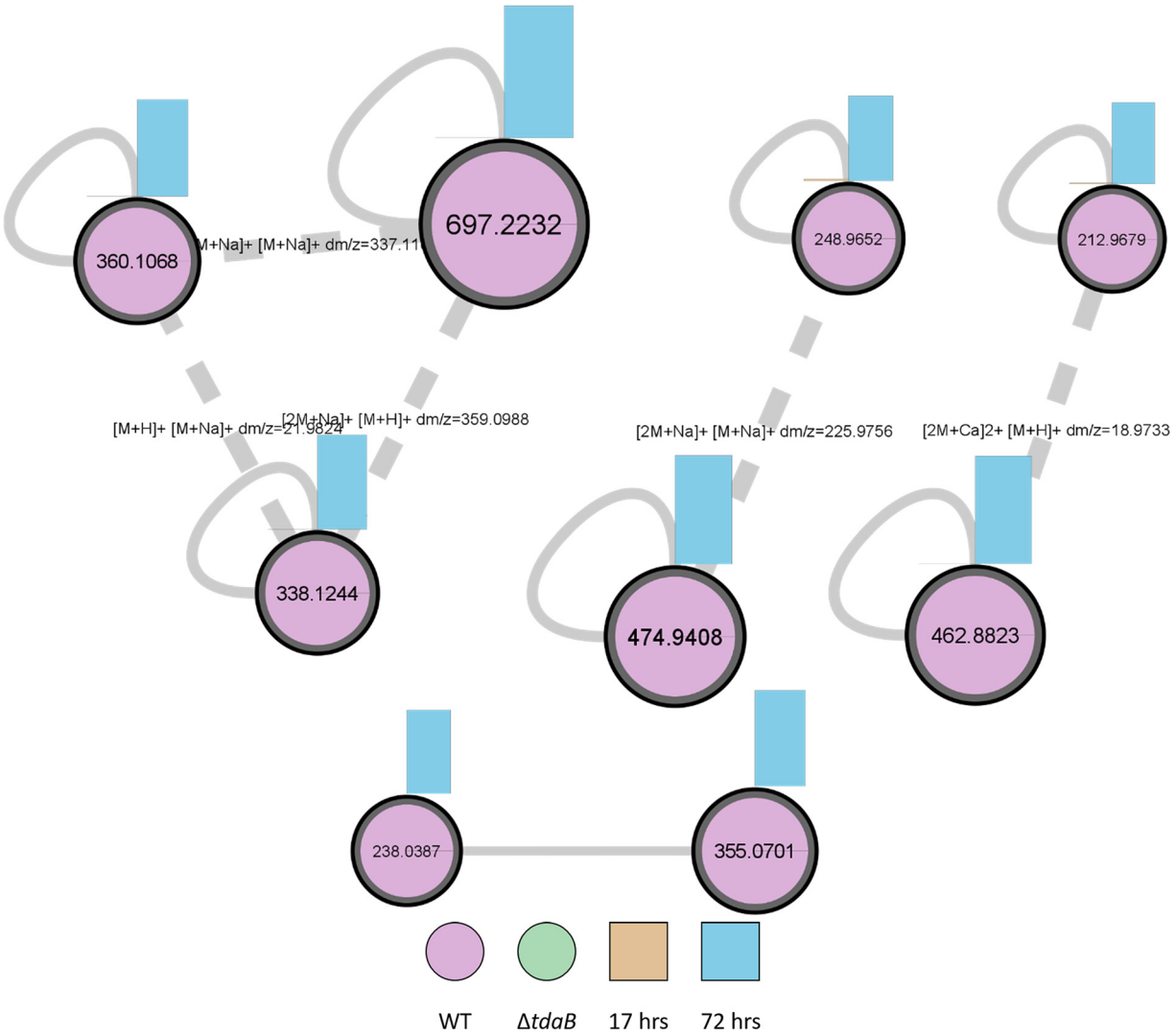

FIG 2 Molecular families corresponding to TDA (*m/z* 212.96), methyl-TDA (*m/z* 248.96) and a potential new TDA-biosynthesis derived feature (*m/z* 338.1244 and *m/z* 238.0387). Nodes are distinguished by presence in *Phaeobacter piscinae* S26 WT (pink) or Δ*tdaB* mutant (green). The bar graphs on top of each node corresponds to the time point tested (17 h (orange) or 72 h (blue)). Ion Identity Molecular Network allowed for connections to be made between the data (dotted edges), allowing for the annotation of TDA-related adducts and dimers. Traditional molecular networking represents these features as singletons and does not allow for these connections to be annotated easily.

form biofilm in a crystal violet assay biofilm after one, two, and 3 days (Fig. 3E). At day one, WT formed significantly less biofilm than Δ*tdaB*, and this difference was partially eliminated upon genetic complementation. However, on day two and three, no significant difference in biofilm formation was recorded between WT and Δ*tdaB* (Fig. 3E). The mutant Δ*tdaB* spread significantly faster than WT on soft agar after 3 days (Fig. 4A), and the diameter of the motility rings formed by Δ*tdaB* increased to 123.4 ± 2.3% of those formed by WT after 1 week. Reintroducing the *tdaB* gene to the mutant partially reduced the swimming of Δ*tdaB* to that of the WT, whereas introducing an empty control vector did not (Fig. 4A). Several genes or proteins involved in motility (COG: N, Fig. 4B), were upregulated in the mutant, including a region encoding the flagellar machinery (OL67_003516-3551). Three chemotaxis-related genes/proteins were also significantly upregulated in Δ*tdaB* in at least one comparison out of three (transcriptome, cell-, or SN proteome), i.e., a chemotaxis response regulator protein CheB (OL67_001709) and two methyl-accepting chemotaxis proteins OL67_000958 and OL67_002412.

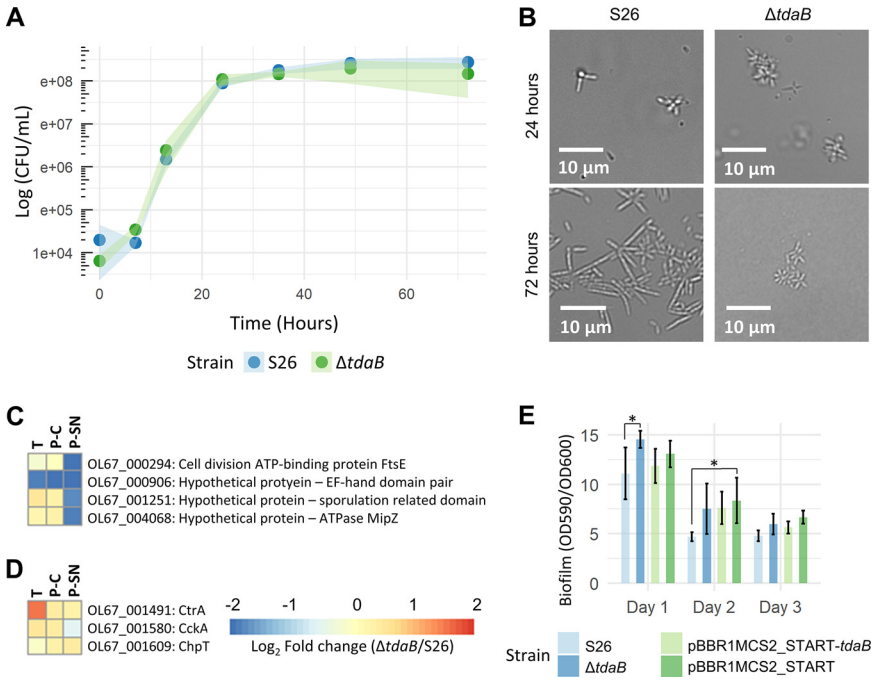

FIG 3 (A) Growth (log_{10}[CFU/mL]) of *Phaeobacter piscinae* S26 WT and Δ*tdaB* over 72 h. Plot shows mean with standard deviation. (B) Cell morphology of S26 WT and Δ*tdaB* after 24 (end log-phase) and 72 h (stationary phase). (C) Log_2(fold change) of proteins and genes belonging to COG D: Cell cycle control that were differentially expressed in at least one comparison out of three (transcriptome, cell-, or SN proteome) in Δ*tdaB* relative to S26 WT. T: Transcriptome. P-C: Proteome, cellular fraction. P-SN: Proteome, supernatant fraction. Same colorbar is used for both C and D. (D) Log_2(fold change) of the *cckA-chpT-ctrA* phosphorelay system in Δ*tdaB* relative to S26 WT. (E) Biofilm formation of S26 WT, Δ*tdaB*, and genetic complements after one, two, and 3 days as assessed by a crystal violet assay. Statistical comparisons for each day were made through an ANOVA followed by a Tukey's test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate standard deviation.

Metal metabolism is affected in Δ*tdaB*. TDA is a weak iron-chelator (33), so we investigated whether iron-acquisition systems were affected in the transcriptome or proteome of Δ*tdaB*. The TonB system is involved in siderophore transportation (44); fittingly, the transcription of two predicted *tonB* genes, OL67_002475 and OL67_003315, were upregulated in the transcriptome of Δ*tdaB* compared to WT (Fig. S5C). In contrast, a transcript encoding a FurB-homologue (OL67_003754) that is part of the ferric uptake regulator family was less abundant in the mutant transcriptome. No evidence of metal chelation was observed in the metabolome. In the transcriptomic comparison, the most differentially expressed gene is OL67_002087, which is downregulated in the mutant and predicted to encode a homologue of the metal-binding protein ZinT (Fig. S5D). Because ZinT aids in zinc acquisition in *Salmonella enterica* during zinc shortage in conjunction with the ZnuABC system (45), we searched for any other changes in zinc metabolism. In the transcriptome, two genes (OL67_003813 and OL67_003815), encoding homologues of ZnuA and ZnuC, respectively, were downregulated in the mutant. Although not significantly downregulated in accordance with the set thresholds, OL67_002813 saw a (log_2[FC]: 1.4, *p*: 0.03) lower relative abundance in the mutant cell proteome compared to WT.

Several horizontal gene transfer (HGT) systems were highly expressed in Δ*tdaB*. Both the transcriptome and proteome revealed a significant and systematic increase in expression of a region spanning from OL67_003884 to OL67_003920 (Fig. 5A) in Δ*tdaB* compared to WT. Although the region mainly encodes hypothetical proteins, several plasmid-located genes were predicted to encode proteins of a type IV secretion system (T4SS); OL67_002884, OL67_003913, OL67_003915, and OL67_003918 showed homology to proteins from the *icm* conjugal transfer system found in *Legionella* spp. (46). Notably, OL67_000327, predicted to encode a domain of a histone-like nucleoid-

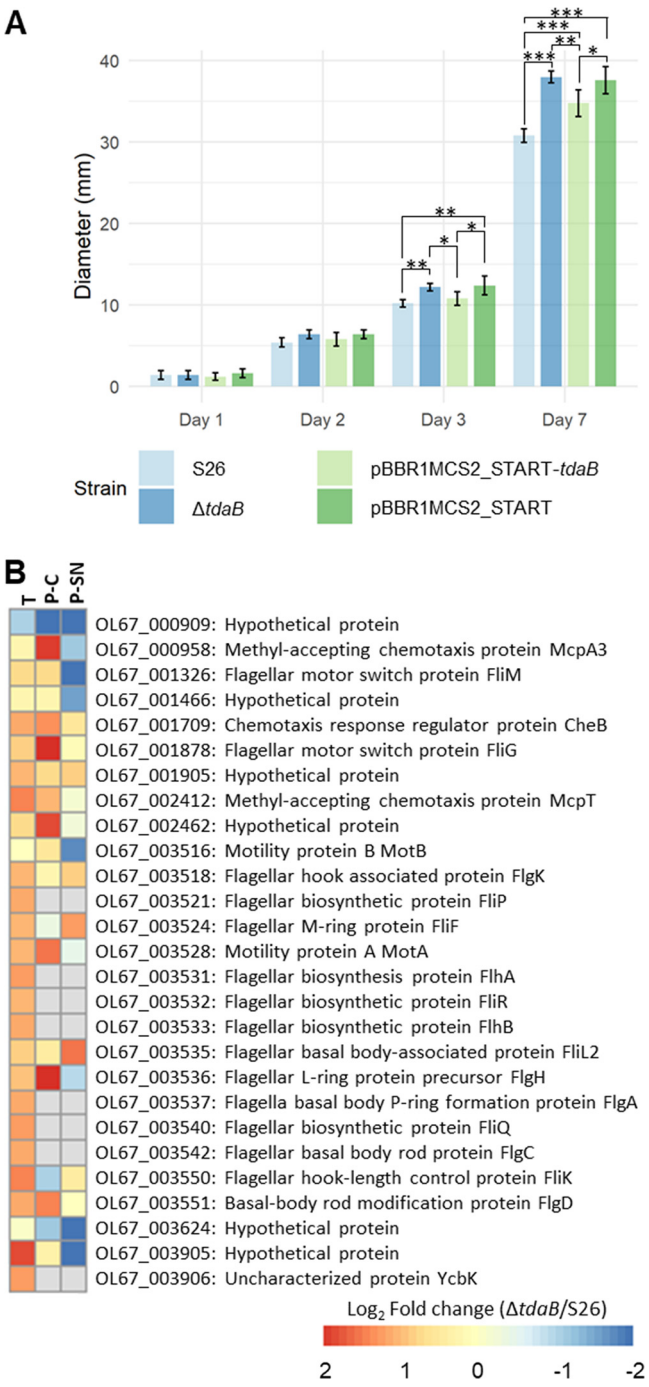

FIG 4 (A) Swimming motility of *Phaeobacter piscinae* S26 WT, Δ*tdaB*, and genetic complements. Statistical comparisons for each day were made through an ANOVA followed by a Tukey's test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate standard deviation. (B) Log_2(fold change) of proteins and genes belonging to COG: N (Cell motility) differentially expressed in at least one comparison out of three (transcriptome, cell-, or SN proteome) in Δ*tdaB* relative to S26 WT. T: Transcriptome. P-C: Proteome, cellular fraction. P-SN: Proteome, supernatant fraction.

structuring (H-NS) protein HvrA, was the single most significantly downregulated protein (sorted by *P*-values) in the mutant for both cellular and supernatant proteome fractions (Supplemental Data Set 1). Histone-like nucleoid-structuring (H-NS) proteins are proposed to aid in regulation and physical integration following HGT (47).

Two other regions, OL67_000581:626 (Fig. 5B) and OL67_001810:27 (Fig. 6B), were also highly expressed/produced in the transcriptome and proteome of Δ*tdaB* (up to

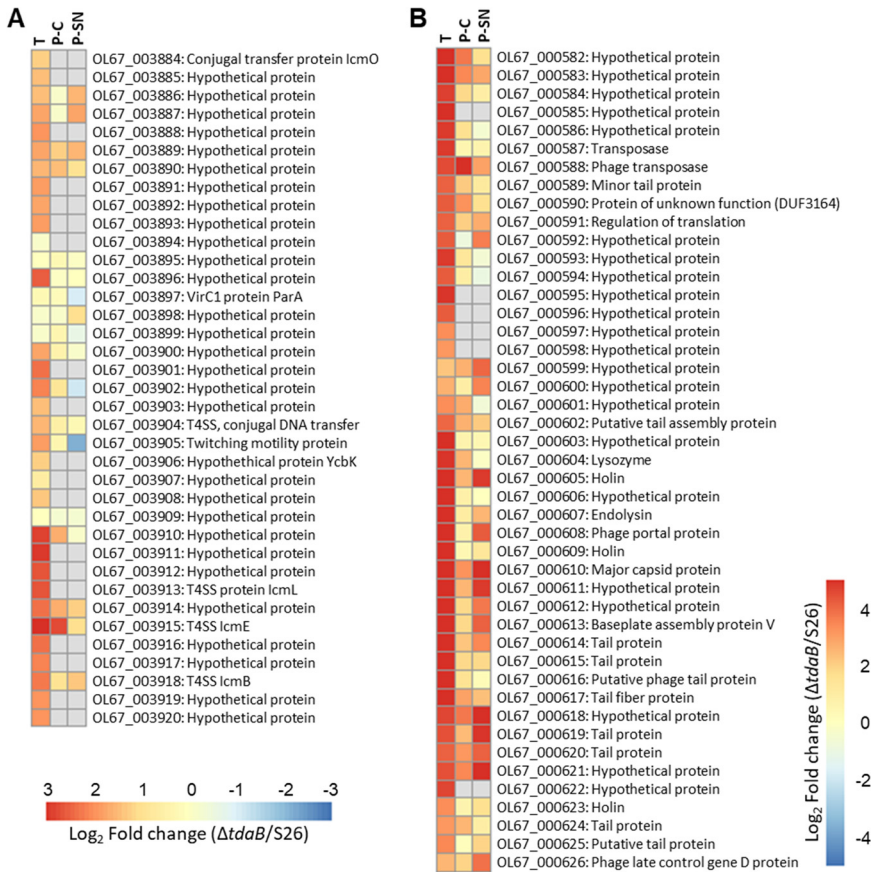

FIG 5 (A) Log_2(fold change) of a region encoding a putative T4SS in *Phaeobacter piscinae* Δ*tdaB* relative to S26 WT. T: Transcriptome. P-C: Proteome, cellular fraction. P-SN: Proteome, supernatant fraction. (B) Log_2(fold change) of a region encoding a putative prophage in Δ*tdaB* relative to S26 WT.

40-fold) compared to WT. Both regions contained phage-associated proteins and were identified as a complete phage 1 and an incomplete phage 3, respectively, by PHAge Search Tool Enhanced Release (PHASTER, Table 1) (48, 49). Phage 1 (Table S1) consists of 46 proteins in total, with six proteins matching the *Escherichia coli* phage vB_EcoM-ep3. The differential expression of this region was not consistent across Δ*tdaB* replicates; in the transcriptome, this was observed in one out of three replicates, while in the proteome, three out of five replicates showed upregulation of the region (Fig. S6). Inspection of phage 3 revealed a genetic structure resembling a gene transfer agent (GTA, Fig. 6A and Table S1), as reviewed by Paul, 2008 (50). In contrast to phage 1, the GTA region was highly expressed/produced in all replicates of both transcriptome and proteome (Fig. 6B). PHASTER also detected two other prophages (Table 1), phage 2 and 4, which was not induced in the mutant cultures. Prophage- and GTA-release can be part of a stress response, and we therefore looked for changes in genes/proteins involved in the SOS response, but no differences were observed.

To confirm the induction and subsequent release of prophages and/or GTAs, supernatants were visualized using transmission electron microscopy. Capsids were identified in both WT and Δ*tdaB* samples, indicating that TDA biosynthesis does not completely abolish phage and/or GTA release (Fig. 6C).

DISCUSSION

Members of the *Phaeobacter* genus are predominantly surface-associated, and several species produce TDA, an antimicrobial secondary metabolite that provides the producers a competitive advantage by inhibiting competing bacteria in an ecological niche (24, 51). TDA may also act as a QS signal, affecting global gene expression (34).

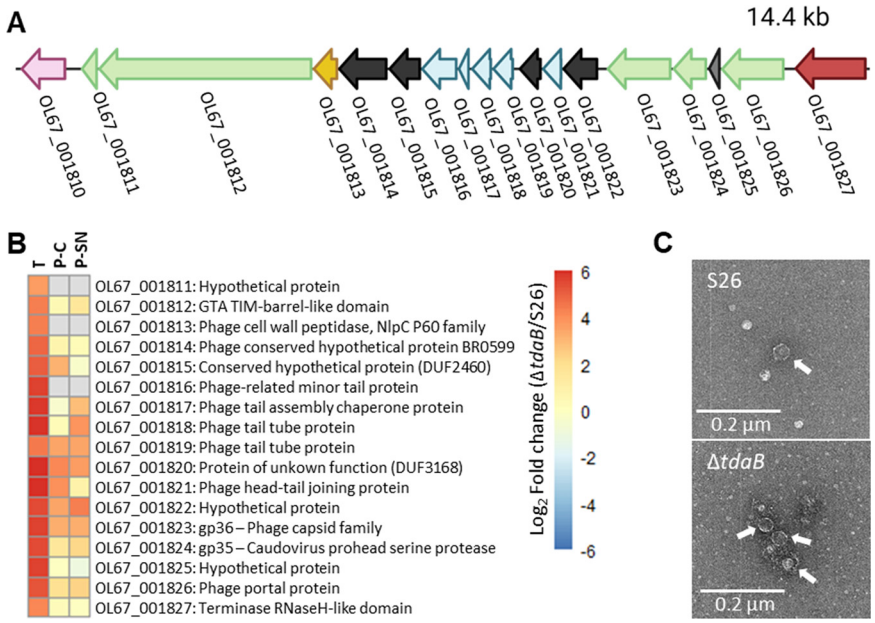

FIG 6 (A) Genomic arrangement of the predicted GTA in *Phaeobacter piscinae* S26. Pink, serine O-acetyltransferase (*cysE*); green, portal, capsid protease and major capsid proteins; yellow, lytic enzyme; black, unknown/hypothetical protein; blue, tail associated proteins; red, terminase large subunit. (B) Log_2(fold change) of a region encoding a putative GTA in Δ*tdaB* relative to S26 WT. T: Transcriptome. P-C: Proteome, cellular fraction. P-SN: Proteome, supernatant fraction. (C) Transmission electron microscopy images of phage capsids (indicated by white arrows) in S26 WT and Δ*tdaB* supernatant.

Here, we demonstrated that TDA biosynthesis, or rather the lack thereof, significantly changes the physiology of the producing strain, including several phenotypes associated with colonization of and adaption to novel niches.

Our results indicate that the secondary metabolism associated with TDA production drives significant changes in the producing organism, and that additional TDA-related metabolites proposed to derive from the same biosynthetic pathway (52), such as methyl-troposulfenin, show a similar reduction in the mutant. We also observed potential new chemical analogs that may derive from the TDA biosynthetic pathway. This confirms the previously observed promiscuity of the TDA biosynthetic pathway (53), resulting in the production of additional secondary metabolites beyond TDA. Such pathway promiscuity may result in analogs with individual functions as is, e.g., the case with surfactants where differences in fatty acid chain length dictate the regulatory outcome (54). Whether this could also be the case for the TDA biosynthetic pathway requires further studies.

Previous studies have reported that TDA-deficient mutants formed more biofilm and were hyper-motile compared to the WT and have proposed that this was linked to quorum sensing circuit(s) (34, 35, 55). We, similarly, observed an increase in motility of the mutant strains, further corroborated by an upregulation of flagellar proteins. We saw an initial increase in biofilm formation by Δ*tdaB* compared to WT, but this difference subsided after 2 days. However, the marked difference in cell morphology between WT and mutant could affect spatial localization in biofilms (56), but further studies are necessary to investigate these dynamics.

Several of our results point to a role of TDA production in repressing HGT, as proteins and genes belonging to a prophage, a GTA, and a T4SS located on one of the plasmids in S26 were highly expressed in the TDA-deficient mutant. Plasmid-located T4SS frequently mediate conjugation (57, 58), and prophages can similarly promote HGT through generalized transduction (59). GTAs are phage-like particles carrying pieces of host DNA found in the genomes of almost all marine *Rhodobacteraceae,* and these also mediate HGT (60–63). In *Phaeobacter* spp., which possess a high metabolic

TABLE 1 PHASTER prediction. Four potential phage regions were identified in *Phaeobacter piscinae* S26 using PHASTER

Number	Region length	Completeness	Score	Number total proteins	Region position	Genome ID range	Most similar phage[a]	GC%
1	31.3 Kb	Intact	150	46	626399-657712	OL67_000581-626	PHAGE_Escher_vB_EcoM_ECO1230_10_NC_027995(6)	59.70%
2	44.2 Kb	Intact	150	38	692249-736465	OL67_000659-719	PHAGE_Escher_vB_EcoM_ECO1230_10_NC_027995(17)	58.71%
3	15.6 Kb	Incomplete	20	18	1889854-1905516	OL67_001810-1827	PHAGE_Roseob_1_NC_015466(4)	64.97%
4	30.6 Kb	Incomplete	50	43	2056836-2087483	OL67_001976-2018	PHAGE_Rhodov_vB_RhkS_P1_NC_031059(13)	59.75%

[a]The phage(s) with the highest number of proteins most similar to those in the region.

and ecological versatility (18, 64), GTAs are believed to be important drivers of diversification and niche adaptation (25). Hence, the impact of TDA production on these HGT systems may ultimately impact the genetic diversity of a population and, consequently, adaption to novel niches upon colonization, contributing to the widespread success of the *Roseobacter* group (65).

An obvious question for future studies is how TDA mediates these observed changes. As mentioned, TDA can act as a QS signal (32). QS-regulated flagellar motility (34) has been demonstrated in other *Phaeobacter* spp. and in other closely related *Rhodobacteraceae*, and QS has been linked to cell morphology changes (42, 66), T4SS expression (67), and GTA-release (42, 68–71). In several of these taxonomically close relatives, these phenotypical changes are mediated through the CtrA phosporelay system, which integrates QS inputs (42, 72, 73). We speculate that TDA may be incorporated as a QS signal in a similar system, as this would provide a unified explanation of the observed phenotypical changes, although further experimental evidence would be necessary to confirm this. Interestingly, this signaling cascade is involved in symbiont-relationships in two *Tritonibacter* spp., which can also be TDA-producing roseobacters (43, 73). It was recently suggested that up to one-third of TDA-producers are host-associated (74), and several studies have suggested a role for TDA in symbiotic interactions (38). In *T. mobilis*, TDA production increases in the presence of dimethylsulfoniopropionate (DMSP), an algal osmolyte (36), suggesting that TDA production is triggered in the presence of host-surfaces suitable for colonization.

Our results clearly show that TDA biosynthesis considerably changes the physiology of the producing organism. We suggest that TDA is not only an asset as part of a switch to a sessile lifestyle but is an integral signal controlling this switch. We, therefore, propose a model wherein TDA coordinates colonization behaviors, potentially through a QS-like mechanism. Planktonic cells will experience very low levels of TDA, leading to increased motility, allowing them to travel fast to novel niches and initiate colonization. During early colonization, high rates of HGT, mediated by conjugation, generalized transduction, and GTAs, are permitted by low TDA levels, which would allow for adaption to novel niches. Since prophage- and GTA-release comes with the trade-off cell lysis, tight control of this process is necessary to prevent a total collapse of the population: As the density of TDA producers increase, the accumulation of TDA halts the rate of HGT as a signal of successful adaptation and colonization. Simultaneously, the antimicrobial properties of TDA may ward off competing ecological neighbors, and TDA-mediated changes in motility and cell length may impact biofilm development, although the relationship between TDA production and biofilm remains somewhat ambiguous. Given the surface- and host-associated lifestyle of *Phaeobacter* spp., these results indicate that TDA may provide the producer with a competitive advantage in surface colonization in several ways. Hence, TDA becomes yet another example of the versatile nature of secondary metabolites and contributes to the building evidence that microbial secondary metabolites are more than "weapons of mass destruction."

MATERIALS AND METHODS

Strains and culture conditions. A list of strains used in this study can be found in Table S2. The *Phaeobacter* strains were cultured on Marine Agar (MA, BD Biosciences), in Marine Broth (MB, BD Biosciences), or Instant Ocean medium with Casamino Acids and glucose (IOCG, [51]) containing 30 g/L Instant ocean salts, (IO, Aquarium Systems, Inc.), 3 g/L HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 3 g/L Bacto Casamino Acids (BD Biosciences), and 2 g/L glucose adjusting pH was to 6.8–7.0. Cultures were grown at 25°C with shaking at 200 rpm, unless otherwise indicated.

Escherichia coli strains (Table S2) were routinely cultured in LB broth (BD Biosciences) or LB agar (BD Biosciences) during conjugation. *E. coli* WM3064 used for electroporation was cultured in low salt LB broth (1 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) or agar (1% wt/vol bacteriology grade agar) supplemented with 0.3 mM diaminopimelic acid (DAP). *E. coli* strains were grown at 37°C with shaking at 200 rpm. When required, antibiotics were added to the media in the following concentrations: 10 μg/mL (liquid) or 15 μg/mL (agar plates) chloramphenicol; 100 μg/mL (liquid) or 200 μg/mL (agar plates) ampicillin; 50 μg/mL (liquid and agar plates) kanamycin for *E. coli* strains; and 200 μg/mL (liquid and agar plates) kanamycin for *P. piscinae* mutant strains. For assays assessing the physiology of the strains, antibiotics were not added.

Genome sequencing. Genomic DNA was extracted using the Promega Wizard Genomic DNA purification kit (Promega) and Qiagen Genomic-tip 20/G. MinION sequencing (Oxford Nanopore Technologies) was performed using the Rapid Barcoding kit (SQK-RBK004) and the Flow Cell Priming kit (EXP-FLP002) following the protocol version RBK_9054_v2_revM_14Aug2019. Nanopore reads were demultiplexed using EPI2me (Oxford Nanopore Technologies). Adaptors were removed using porechop v0.2.4 (75). Oxford Nanopore MinION and Illumina MiSeq reads (obtained from a previous draft genome assembly project (NCBI accession number GCA_000826835.1) (76) were assembled using Unicycler v0.4.7 (77). The assembly was annotated using prokka v1.14.6 (78) and eggNOG-mapper V5.0 (79). To distinguish the hybrid assembly from the initial Illumina-based draft version of the genome on NCBI, the locus tags of the new annotation were adjusted to OL67XXXX.

Construction and complementation of a scarless deletion mutant. A list of plasmids used in the study can be found in Table S2. The suicide plasmid pDM4-D-*tdaB* was constructed and transferred to S26 by conjugation (Supplemental Materials 1). A two-step homologous recombination procedure based on sucrose-counterselection was used to generate the markerless *tdaB* gene in-frame deletion mutant, Δ*tdaB*. Genotype of mutants were verified with diagnostic PCR (Fig. S1B) and sequencing (Macrogen Europe) using oligoes in Table S2. For complementation, pBBR1MCS2_START-*tdaB* and the backbone vector pBBR1MCS2_START (80) were transferred to Δ*tdaB* through conjugation. Methods for DNA manipulation, plasmid construction and conjugation can be found in Supplemental Text S1.

Global metabolome, transcriptome, and proteome experimental setup. Strains were grown in MB and diluted 10,000-fold and grown stagnant in 5 mL IOCG in TPP6-well tissue culture plates (Merck) at 25°C. Plates were incubated in a humidity chamber to prevent desiccation. Samples were taken after 17 (exponential phase) and 72 h (stationary phase) for metabolome analysis, and after 72 h for transcriptomics and proteomics. Experiments were carried out with five biological replicates for metabolomics and proteomics, and in three biological replicates for transcriptomics. CFU determination and microscopy was performed for each sample. Transcriptomics, proteomics, and metabolomics workflows can be found in Supplemental Text S1.

Antibiotic inhibition assays. *Vibrio anguillarum* 90-11-287 (81) and the *Phaeobacter* strains were grown 3 days stagnant at 25°C in MB and *Vibrio* embedded in IO agar (30 g/L IO, 3.3 g/L Casamino Acids, 1% bacterial grade agar in dH_2O). Sterile filtered (Minisart 0.2 μm filter (Merck) supernatant of *Phaeobacter* cultures were added to wells punched into the *Vibrio* agar and inhibition zones recorded after 3 days of incubation at 25°C.

Growth kinetics. *Phaeobacter* strains were grown in MB and diluted to OD_{600} = 0.00001 in IOCG and incubated at 25°C stagnant in TPP6-well tissue culture plates (Merck) with 5 mL media per well. The experiment was done in biological triplicates and growth followed by colony counts that were log transformed and plotted using ggplot2 (82) in Rstudio.

Cell elongation assays. *Phaeobacter* strains were grown in MB and diluted 10,000-fold and grown in IOCG in TPP6-well tissue culture plates (Merck) at 25°C in biological triplicates. Plates were incubated in a humidity chamber to prevent desiccation. For chemical complementation, samples were supplemented with 50% sterile-filtered WT or mutant supernatant of 3-day-old cultures grown in IOCG stagnant at 25°C. For genetic complementations, strains were grown in IOCG with 200 μg/mL kanamycin and incubated in the humidity chamber, stagnant at 25°C for 7 days. Cell morphology was assessed through brightfield microscopy using a Nikon ECLIPSE Ti2 inverted microscope at ×60 magnification. Cell length measurements were carried out using ImageJ (83). For each replicate, three pictures were taken and five random cells within each picture were measured for statistical comparisons.

Swimming motility assays. Swimming motility assays were performed in IOCG soft agar (0.3% wt/vol bacteriology grade agar). For supernatant complementation, plates were supplemented with 50% sterile-filtered supernatant of a 3 day old culture grown in IOCG stagnant at 25°C. Single colonies of strains to be tested were inoculated in MB and incubated stagnant for 2 days. A sterile needle was dipped in the tested cultures and used to inoculate the agar. Five biological replicates were included for each strain. For statistical comparison, a one way analysis of variance (ANOVA) followed by a Tukey's test with a 95% confidence interval was carried out.

Biofilm formation. Biofilm formation was assessed using a modified protocol based on Jensen et al. (2007), Djordevic et al. (2002), and O'Toole and Kolter (1998) (84–86). Briefly, precultures were diluted

1,000-fold in IOCG and incubated in 96-well plates (ThermoFisher Scientific). MilliQ was added to border wells to prevent desiccation. Prior to staining, cell density was measured as OD_{600} in a SpectraMax i3 with 10 s orbital shaking prior to measurement. Wells were then washed with sterile MilliQ water and 125 μL crystal violet (CV, 1% wt/vol in sterile MilliQ) was added to each well and left for 15 min. CV was then removed, wells were washed three times with MilliQ, and the plate was set to dry in a flow bench for 15 min. 200 μL 96% EtOH was subsequently added to each well and left to sit for 30 min biofilm, and CV intensity was then measured at OD_{590} in a SpectraMax i3. Five biological replicates were used for each strain. For statistical comparison, a one way analysis of variance (ANOVA) followed by a Tukey's test with a 95% confidence interval was carried out.

Phage purification, transmission electron microscopy, and molecular detection. Samples for transmission electron microscopy (TEM) were prepared according to Dragoš et al. 2021 (87). Bacterial supernatant was sterile filtered and pH adjusted to 7. PEG-solution (20% PEG-8000 [VWR International A/S], 116 g/L NaCl) was added in a 1:4 ratio, followed by incubation overnight at 4°C. After incubation, samples were centrifuged for 60 min at 18.500 g. Supernatant was then discarded and the pellet was resuspended in SM buffer (5.8 g/L NaCl, 0.96 g/L $MgSO_4$, 6 g/L Tris-HCl, pH 7.5) to 1% of the initial volume. Five μL of purified phage sample were placed on freshly glow discharged Formvar coated 200 mesh nickel TEM grids (EMS Diasum) and allowed to adsorb for 5 min. Excess solution was wicked away with filter paper and the grids were subsequently rinsed 3 times on droplets of MilliQ water before being sequentially stained on droplets of 2% uranyl acetate in water for 10 s, 1 s, and 30 s, respectively. The excess staining solution was wicked away and the grids were dried in ambient conditions prior to imaging. Micrographs were acquired using a Tecnai T12 BioTwin TEM (Thermo Fisher Scientific) equipped with a Orius CCD camera (Gatan).

Data availability. The new version of the now closed *Phaeobacter piscinae* S26 genome with one chromosome and four plasmids is accessible on NCBI under the accession number CP080275-CP080279 (BioProject PRJNA266107). Metabolomics data were deposited and made publicly available in MassIVE at the following: MSV000089868. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD035448. Bacterial strains are available upon request. RNAseq data have been uploaded to the Sequence Read Archive at NCBI under BioProject PRJNA859106.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

DATA SET S1, XLSX file, 1.2 MB.
TEXT S1, DOCX file, 0.1 MB.
FIG S1, TIF file, 1.3 MB.
FIG S2, TIF file, 2.3 MB.
FIG S3, TIF file, 2.2 MB.
FIG S4, TIF file, 2.3 MB.
FIG S5, TIF file, 2.6 MB.
FIG S6, TIF file, 2.3 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.03 MB.

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L.L.L., S.-D.Z., E.C.S., and L.G. conceived and designed experiments; L.L.L. performed the biological experiments and data analysis; J.K. performed transcriptomics on the samples; S.A.J. performed the chemical analysis; M.W.N. supervised proteomics sample preparation and carried out the proteomics MS analysis; E.M.S. supervised analysis of the proteomics data; M.L.S. processed the transcriptomics data and supervised the analysis of the data; P.J.K. performed transmission electron microscopy imaging; L.L.L.,

S.A.J., S.-D.Z., E.C.S., L.G., M.W.N., E.M.S., and P.J.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Manuscript III

Tropodithietic acid production represses the production of a novel gene transfer agent in *Phaeobacter* spp.

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In preparation

Tropodithietic acid production represses the production of a novel gene transfer agent in *Phaeobacter* spp.

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Abstract

Horizontal gene transfer (HGT) is an important mediator of bacterial evolution. In *Rhodobacteraceae*, gene transfer agents (GTAs) are believed to be the main driver of horizontal gene transfer. GTAs are viral particles mediating horizontal gene transfer, which are proposed to play a vital role in diversification and niche adaptation in *Rhodobacteraceae*. We have found that in *Phaeobacter piscinae* S26, the production of the antibiotic compound tropodithietic (TDA) acid represses the expression and production of GTA genes and proteins. The purpose of the present study was first of all to investigate the effect of TDA production on HGT. Secondly, we aimed to characterize the regulatory mechanisms behind TDA-mediated GTA repression. We were unable to develop a functional assay for HGT detection using spontaneous rifampicin resistant (Rif^R) strains. Through RT-qPCR we found that the previously observed upregulation of the encoded GTA in a TDA-deficient strain was abolished in the Rif^R strain, likely explaining the difficulties in establishing a functional HGT assay. Further experiments are thus necessary to confirm the effect of TDA-production on HGT. Through bioinformatic analysis, we identified homologs of 38 out of 42 genes of the multi-locus GTA genome, including a homologue of GafA, a direct activator of GTA release. which is repressed by TDA production. In *Dinoroseobacter shibae*, GafA is repressed by the $LuxIR_2$ quorum sensing system, and we found that this system is homologous to the PgaIR system of *Phaeobacter*, which TDA is proposed to act as an autoinducer of. Based on these data, we propose a regulatory system where TDA interacts with PgaR to repress GafA, leading to a repression of GTA activation. Furthermore, TDA production may affect CtrA abundance and phosphorylation state, which may also affect GTA activation, through a mechanism not understood yet.

Introduction

Horizontal gene transfer (HGT) allows for the rapid acquisition of new traits in a bacterial population, a process which is generally proposed to promote adaptation to the environment (1, 2). Three main modes of HGT are usually recognized: Transduction, transformation, and conjugation. A fourth HGT route involves so-called gene transfer agents (GTAs), which are virus-like particles resembling bacteriophages that promiscuously pieces of host DNA into the transducing particle (3–5), thereby facilitating HGT between cells in a manner that combines elements of transduction and natural transformation (6). The first and most well-studied GTA was identified in *Rhodobacter capsulatus* in the 1970s (7–9), and since then several genetically distinct GTAs have been identified in both bacteria and archaea (10–13).

GTAs are believed to arise from defect prophages that were subsequently brought under the control of host regulatory systems (14). Whilst HGT may accelerate adaption and hence be beneficial in a bacterial population, GTA release comes with a high cost since the donor cells undergo lysis to release the particles (15) Hence, it is not surprising that this process is tightly controlled by multiple core regulatory systems, including the SOS response system (16), c-di-GMP (17, 18), the CckA-ChpT-CtrA phosphorelay system (14, 19, 20), and quorum sensing (QS) (21–24). Central to GTA regulation in the *Rhodobacterales* is *gafA*, the first direct activator of gene transfer agent production and release to be identified (25). GafA integrates inputs from several of the aforementioned regulatory systems (25, 26) and consists of two distal DNA-binding domains linked by a protein-binding domain, which is proposed to facilitate interaction with the Rpo-ω subunit of RNA polymerase (RNAP) (27). Whilst the full GafA protein is required for GTA activation in collaboration with RNAP, only the C-terminal DNA-binding domain is required for *gafA* autoinduction, independently of RNAP (27). This split function of *gafA* is corroborated by the observation that in *Hyphomicrobiales* and *Caulobacter crescentus*, *gafA* is split into two parts corresponding to the N- and C-terminal domain, respectively (5, 27).

Rhodobacter capsulatus is a member of the *Rhodobacterales* order, and homologs of the core GTA cluster are widely distributed within this Order (28, 29). The marine *Phaeobacter* genus belongs to this Order and GTA genes are ubiquitous within the genera (30). Here, GTAs have been proposed as a major contributor to diversification and niche adaptation by promoting

HGT (30). However, the wider ecological role of GTAs is disputed; aside from mediating HGT, GTAs have been shown to aid in DNA repair (5) and they have also been proposed to act as a defense against phage-attack, transmitting phage DNA into uninfected cells, hereby activating viral defense systems in uninfected cells (31).

In a previous study, we observed that production of the antibiotic secondary metabolite tropodithietic acid (TDA) repressed expression of a region encoded a previously uncharacterized GTA in *Phaeobacter piscinae* S26 (32), providing a new potential mode of GTA regulation. *P. piscinae* is a member of the marine *Roseobacter* group, a globally occurring group of bacteria (33). TDA, produced by several members of this group, is a potent multifunctional antibiotic which is also proposed to act as a QS autoinducer, interacting with the LuxR-like PgaR response regulator (34, 35) The purpose of the present study was to examine the effect of TDA production on HGT, and characterize a previously undescribed GTA produced by *P. piscinae* S26. We also investigated the regulatory network underlying the TDA-mediated repression of GTA release.

Results

Development of a horizontal gene transfer assay (preliminary data). To expand on our previous observation that GTA genes were upregulated in a TDA deficient mutant, *P. piscinae* S26Δ*tdaB*, we sought to investigate whether this was also reflected by an increase in HGT events. We initially tested whether addition of cell-free supernatant from a spontaneous rifampicin resistant Rif^R donor to a rifampicin sensitive S26 recipient could mediate HGT (**Figure 1A**). However, no Rif^R colonies were observed using supernatant from neither S26 Rif^R nor S26Δ*tdaB* Rif^R (data not shown), possibly indicating that GTA production was below the detection limit of the assay. As a result, an alternative coculture HGT assay was pursued. Here, spontaneous Rif^R mutants of both S26 wildtype (WT) and Δ*tdaB* were co-cultivated with spontaneous streptomycin resistant S26 $Strep^R$ and the appearance of double-resistant colonies were used as a proxy for HGT events (**Figure 1B**). To account for spontaneous double resistance, both recipient and donor strains were also incubated individually. After three days, significantly more double resistant colonies were recorded when S26 $\underline{Strep^R}$ was cocultured with Δ*tdaB* Rif^R compared to coculture with S26 Rif^R or incubation of any of the strains in monoculture (**Figure 1D**). We then expanded this setup to include trans-

complemented *ΔtdaB* strains, carrying either pBBR1MCS2_START-*tdaB* or an empty vector control. To determine whether the observed HGT was mediated by the GTA, we also included Δ*tdaB* ΔGTA, where the major capsid protein (OL67_001823) of the gene transfer agent was replaced with a gentamicin resistance cassette in the S26Δ*tdaB* background. However, when the experiment was repeated, we were unable to replicate these data and saw no notable sign of HGT in any of the coculture setups (data not shown).

We speculated that GTA release from Δ*tdaB* could be repressed in coculture due to presence of TDA produced by S26 StrepR. However, if our hypothesis that this strain produces an increased amount of GTAs is correct, using Δ*tdaB* StrepR as a recipient in a coculture setup would result in two-way transfer of genetic material when cocultured. To avoid this, we employed a membrane-separated coculture system (**Figure 1C**) with Δ*tdaB* (without any resistance markers) as recipient. However, although some Rif^R colonies were isolated from all wells, except those either without donor or with Δ*tdaB* ΔGTA as donor, these differences were not significant (**Figure 1E**).

Development of an RT-qPCR based assay for measuring expression of GTA genes (preliminary data). As we were unable to establish a functional gene transfer assay, we developed a set of primers for RT-qPCR quantification of the GTA major capsid protein, OL67_001823, as an alternative approach for quantifying effects on GTA gene expression. Two primer sets were employed (**Table 1**); rpoB Fw/Rv, targeting the housekeeping gene *rpoB* encoding the RNA polymerase β subunit, and OL67_001823 Fw/Rv, targeting the gene encoding the predicted GTA major capsid protein. Standard curves were produced for all primer sets, resulting in efficiencies of 94% (rpoB Fw/Rv), and 88% (OL67_001823 Fw/Rv).

We used this RT-qPCR setup to measure expression of OL67_001823 in S26 Rif^R and Δ*tdaB* Rif^R as an indirect measure of GTA production. Contradicting our previous observations with rifampicin sensitive strains (32), we did not find any difference in OL67_001823 expression between S26 Rif^R and Δ*tdaB* Rif^R after 72 h incubation, with relative (OL67_001823/rpoB) Cq values of 1.04±0.02 and 1.01±0,05, respectively. The raw data, including melting curves confirming the presence of only one product in each reaction, can be found in **Supplemental Figure 1**. The only difference between the strains used for the HGT/qPCR assays and those

used in our previous article (32), is that the former have been subjected to selection for rifampicin resistance. Based on this, we hypothesize that the GTA overproduction phenotype may be so deleterious to the strain that successive rounds of selection results in the downregulation of GTA release.

4 kB DNA band could be identified from RNA extractions. RNA was extracted from the rifampicin sensitive strains in preparation for a second round of RT-qPCR (results not ready). Prior to DNase treatment, a band of approx. 4 kB was present in samples from Δ*tdaB* but not from WT samples (**Figure 2**). This corresponds to the expected packaging capacity of the encoded GTA, as most other *Roseobacter* GTAs package around 4.5 kB of dsDNA (36, 37). The band disappeared after DNase treatment (data not shown), confirming that the band was indeed DNA rather than RNA.

Identification of homologues of the multi-locus GTA "genome". In our previous studies (32), we noted the upregulation of the structural GTA cluster in S26Δ*tdaB*, wherein TDA production was abolished (32). In addition to the gene cluster encoding majority of GTA genes, several other genes have been implicated in the production and release of the archetypical RcGTA. These genes exist throughout the chromosome, and include genes necessary for lysis (3, 15, 38), additional structural genes (38, 39), regulatory genes (14, 20, 21, 25, 27, 39) and related biosynthesis genes, such as those involved in capsule production, which is necessary for the reception of GTAs (22). Since these genes are integral to GTA-mediated gene transfer in *R. capsulatus*, we searched for homologues of these genes in S26. A list of identified homologues as well as genes we were unable to identify homologues for can be found in **Table 2.** Out of the 42 *R. capsulatus* genes, we were able to identify homologues of 38 of these. We were unable to identify homologues of rcc01079, rcc01080, rcc01682, rcc01932, and rcc01081. Rcc01682, encoding the small terminase protein TerS (40), is the first gene of the core GTA gene cluster (14), and whilst a homologue was not identified in the initial search, visual inspection of the genome revealed a gene (OL67_001827) located in the same genomic region in S26 as in *R. capsulatus* sharing 33.33% amino acid sequence identity with rcc01682 and this gene was therefore included as homologue. rcc01079 and rcc01080 encode GhsA and GhsB, respectively, which are linked to head spike formation on the RcGTA capsule (41). *R. capsulatus* cells are characterized by

the presence of a polysaccharide capsule which is proposed to act as a receptor for GTA adsorption, and both rcc01932 and rcc01081 are required for formation of this capsule (22).

After identifying putative homologues, we examined our previous transcriptomic and proteomic comparisons of a TDA-deficient mutant, Δ*tdaB*, compared to WT to look for differentially abundant transcripts/proteins (32). Transcripts were considered significantly different if $p > 0.05$ and the $\log_2$(Fold-change) (log2FC) > 1, whilst proteins were considered significantly different if $p > 0.01$ and log2FC > 1.5. Out of 38 identified homologues, 30 genes/proteins were significantly more abundant in Δ*tdaB* compared to WT (**Figure 3**). These results show that upregulation is not limited to the core gene cluster but rather is a genome-wide increase in abundance of transcripts/proteins involved in GTA-mediated gene transfer.

We previously noted the upregulation of a CtrA homologue (OL67_001491) in Δ*tdaB* (32). CtrA is part of a phosphorelay system along with CckA, ChpT, and DivL. Mutations in any of these result in reductions in GTA activity (14, 19, 20, 38). Whilst no differential expression was observed for ChpT and CckA homologues, a DivL homologue (OL67_003496) is significantly upregulated in the Δ*tdaB* transcriptome (**Figure 3**).

S26 possesses a homologue of the GTA activator GafA. Notably, OL67_001522 transcripts were significantly more abundant in Δ*tdaB*, encoding a homologue of GafA (**Figure 3**). GafA was identified as a direct activator of gene transfer agent release in *R. capsulatus* (25) and homologues of this gene is found throughout the *Rhodobacterales* order (27, 39). A *gafA* gene homologue (OL67_001522) was identified in S26 with a 70% sequence similarity on amino acid level. A Clustalω alignment of the S26 *gafA* homologue and *gafA* from *R. capsulatus* SB1003 was made for comparison (**Figure 4**). The highest sequence similarity was found at the C-terminal of GafA, which contains a sigma factor-like DNA binding domain (25). Further inspection of the alignment revealed that the predicted DNA binding domain in the N-terminal of GafA (39) is also well-conserved in S26.

Homology between QS systems from S26 and DFL12/ Regulatory pathway behind GTA activation, bioinformatics. In *Dinoroseobacter shibae* DFL12, a *Rhodobacteraceae* which also produces a functional GTA, several LuxIR-like systems are integrated into the CtrA

phosphorelay system to control GTA release (26). Here, $LuxI_2$, encoding an AHL synthase, represses GafA, leading to repression of GTA release (4). TDA has been proposed to interact with the LuxIR-like system PgaIR, interacting with the PgaR regulator in the same manner as the AHL produced by PgaI (34). We therefore speculated whether the the TDA-mediated repression of GafA could be linked to this quorum sensing system. A BLAST comparison of *pgaIR* (OL67_000332, OL67_000331) and *luxIR2* (Dshi_2851, Dshi_2852) showed a high degree of amino acid-level homology between the systems (**Figure 5**), indicating that these likely share a conserved function in the two.

On-going experiments

Investigation of the TDA regulatory pathway: Deletion of *gafA* (preliminary data). To confirm that GafA is indeed an activator of GTA release in S26, we constructed a suicide plasmid targeting *gafA* for deletion in S26. We were not able to finalize the *gafA* deletion mutant in time for finalization of this thesis.

Generation of a *gafA* overexpressor strain (preliminary data). In order to prove that the GTA of S26 is indeed functional in gene transfer, we set out to construct a GTA-overexpressor strain in a Rif^R background to avoid additional rounds of selection after promoting GTA release. For this, we constructed a plasmid for overexpression of *gafA* under control of an IPTG-inducible promoter, pCMF66T-P*tac-gafA*. We were not able to finalize the construction of this over-expresser strain in time for the finalization of this thesis.

Discussion and conclusion

Based on our observations in a previous study (32), we speculated that TDA production represses GTA-mediated HGT. In spite of a clear increase in abundance of GTA transcripts and proteins in a TDA–deficient mutant, Δ*tdaB*, compared to WT we were not able to reliably detect an increase in HGT events. Likely, this is because a mutation have occurred in either of the strains during rifampicin selection, affecting GTA expression, as our RT-qPCR data were not able to detect any difference in expression of the GTA major capsid protein (OL67_001823) between Δ*tdaB* Rif^R and S26 Rif^R, contradicting the results obtained when comparing non- Rif^R strains (32). Given the unexpected RT-qPCR data, it will be necessary to test the strains used in the initial study to confirm the differential expression of the GTA core

genes in Δ*tdaB* compared to S26. In *R. capsulatus*, overexpression of *gafA* results in a 57-fold increase in GTA-mediated gene transfer (25). We initiated the construction of an inducible *gafA* over-expresser strain. Once this strain is constructed, we will hopefully be able to use this to confirm the functionality of the previously uncharacterized S26 GTA.

As opposed to phages, the genes for GTA production, regulation, and recipience are spread through-out the genome (39). We were able to identify homologues of majority of these genes; additionally, majority of these genes were also upregulated in response to deletion of *tdaB*. Furthermore, we were able to identify a band of approx. 4 kB during RNA extraction, which was attributed to DNA contamination, and this band was only present in Δ*tdaB* mutant samples. This band size corresponds to the packaging capacity of other characterized GTAs (4, 9, 36, 42), indicating that the S26 GTA can likely package DNA. Whilst GTA DNA packaging is considered to be mostly random, some species exhibit preferential packaging of some regions (4) or bias against the core structural genes (3). In the future, it would be interesting to investigate whether GTA DNA packaging exhibitis any biases in S26.

GafA is a direct activator of GTA release found across the *Rhodobacterales* (25, 39). Its role as a direct activator has been confirmed for several GTA producers, including the TDA-producer *Tritonibacter mobilis* (previously *Ruegeria mobilis*) (25). We identified a homologue of *gafA* in S26, which was upregulated upon abolishment of TDA biosynthesis. TDA is a multi-functional secondary metabolite proposed to double as a QS signal, interacting with the LuxIR-like system PgaIR (34), which we here show is homologous to the LuxIR2 system of *D. shibae*. This system has been shown to repress *gafA* expression (26). Based on these results, we propose that TDA interacts with the PgaR response regulator to inhibit *gafA* expression, affecting all downstream targets of GafA, which includes the GTA head-tail cluster, several distantly related structural genes, and lytic genes (25).

However, it appears that TDA also interacts with other regulatory systems linked to GTA release. Abolishment of TDA-production leads to an increase in both DivL and CtrA transcripts. These proteins are part of a phosphorelay system, together with CckA and ChpT, which is widespread within the α-proteobacteria (43). CckA phosphorylates ChpT, which again phosphorylates the response regulator CtrA (44). DivL promotes kinase activity of

CckA and as such also affects the phosphorylation state of CtrA (19). Both CtrA abundance and phosphorylation state are important for GTA functionality; CtrA is required for transcription of the head-tail cluster and additional structural genes in GTA donor cells (14, 20, 25, 45), whilst in recipient cells, CtrA is required for receipt of the GTA DNA and subsequent integration into the genome (6, 46). On the other hand, phosphorylated CtrA promotes cell lysis (19, 20, 38, 44). Based on our data, TDA production decreases CtrA abundance, but the repression of DivL indicates that CtrA phosphorylation state may also be altered in response to TDA production. Whether this interaction is also mediated through the PgaR QS regulator is hard to predict, but this would then mean that the regulatory circuit is different to the model proposed for *D. shibae*, as the PgaIR homologues, LuxIR2, only interact with GafA in this model (26). As CtrA activates *gafA* expression (ref), and an alternative explanation for our observed data is that TDA interacts only with CtrA, as this would also have downstream effects on *gafA* in alignment with our results.

We propose a model where TDA acts as a QS signal through the PgaR response regulator, repressing expression of *gafA* (**Figure 6**). Since GafA is a key activator of the core GTA cluster as well as the lytic operon required for cell lysis and GTA release (25), this results in the repression of GTA release by TDA. In addition, TDA also represses the master regulator CtrA and DivL, a PAS domain protein promoting the phosphorylation of CckA (19), leading to reduced expression of several genes required for GTA maturation, release, and recipience.

Methods and Materials

Strains and culture conditions. All strains used in the study can be found in **Table 3**. *Phaeobacter* strains were routinely cultured on Marine Agar (MA 2216, BD Biosciences) or in Marine Broth (MB 2216, BD Biosciences) at 25°C, with shaking at 200 rpm for liquid cultures, unless otherwise indicated. Instant Ocean medium with casamino acids and glucose (IOCG, (10)) was prepared using 30 g/L Instant ocean salts, (IO, Aquarium Systems, Inc.), 3 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 3 g/L BactoTM Casamino Acids (BD Biosciences), and 2 g/L glucose adjusting pH was to 6.8-7.0. For RT-qPCR and gene transfer assays, strains were cultivated in IOCG and incubated stagnant for three days at 25°C. *Escherichia coli* strains were routinely cultured in LB broth (BD Biosciences) or LB agar (BD Biosciences) during conjugation. E. coli WM3064 used for electroporation was cultured in low salt LB broth (1 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) or agar (1% w/v bacteriology grade agar) supplemented with 0.3 mM diaminopimelic acid (DAP). *E. coli* strains were grown at 37°C with shaking.

DNA manipulation. Genomic DNA extractions were performed using NucleoSpin® Tissue kit (Macherey-Nagel, Fisher Scientific). A list of plasmids used in the study can be found in **Table 4**, and primers are listed in **Table 1**. Primers were synthesized by Integrated DNA Technologies (Belgium). PCR amplification was performed using either Q5® High-Fidelity 2X Master Mix (NEB, Bionordika, Denmark) for fragments used for cloning, or TEMPase Hot Start 2X Master Mix K (Ampliqon, VWR) for diagnostic PCRs. PCR products were purified using the GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare). Plasmids were extracted using The Monarch® Plasmid Miniprep Kit (NEB, Bionordika).

Plasmid construction. All plasmids were designed in ApE - A plasmid Editor (v2.0) (47) and assembled through direct RecET-mediated cloning as described by Wang et al. 2016 (48). For deletion of OL67_001823, homology arms of ~500 bp flanking the targeted region were amplified using 5' arm Fw/Rv and 3' arm Fw/Rv . An antibiotic resistance marker gene was amplified using gmR Fw/Rv and introduced between the homology arms through overlap PCR. For construction of the pCMF66T-P*tac-gafA* expression plasmid, the promoter region of *gafA* was replaced with P*tac* through PCR amplification with the P*tac* sequence added as

a primer tail. The recombinant plasmids were verified by restriction digestion and sequencing (Macrogen Europe).

Bacterial conjugation. Plasmids were transferred to *Phaeobacter* strains using bacterial conjugation with *E. coli* WM3064 as donor, as described by Lindqvist *et al.* 2023 (32). Briefly, plasmids were first transferred to donor through electroporation. Over night (ON) cultures of plasmid-carrying donors were diluted 100-fold and grown to exponential phase, whilst recipients were grown stagnant ON to early stationary phase prior to conjugation. Donors were collected through centrifugation and washed with LB + DAP (0.3 mM) to remove antibiotics. Recipients were added in a 1:3 ratio, and the conjugation mixture was again washed once with LB + DAP (0.3 mM) to remove endogenous antibiotics produced by the recipient strains. Cells were pelleted, resuspended in approx. 40 µL LB + DAP (0.3 mM) and mated on sterile 0.2 µM pore-sized mixed cellulose esters (MCE) membranes (Merck) on LBA + DAP plates for 3-4 hours at 30°C. After mating, cells were recovered for 1 hr in MB at 25°C with shaking. Cells were then plated on selective media and incubated until colonies appeared. For double homologous crossover, 1st crossover transconjugants were subsequently subjected to counter-selection on ½YTSS agar (30 g/L Sigma Sea Salts, 3 g/L yeast extract, 3 g/L tryptone) with 10% sucrose.

RT-qPCR. RNA was extracted and DNase treated as described previously (32). RNA was converted to cDNA using the SuperScript® IV Reverse Transcriptase (ThermoFisher Scientific) according to manufacturer's protocol. Standard curves were generated for each primer-set using 10-fold dilutions of genomic DNA from S26.

Bioinformatic analysis and data visualization. Homology searches were performed using NCBI BLAST with standard settings (49). Protein alignments were visualized using the R package GenoPlotR (50) and Clustal Omega (51, 52) with default parameters.

Transcriptomic and proteomic data was pulled from the datasets described in Lindqvist *et al.* 2023 (32). Data was visualized in R using Pheatmap (v.1.0.12, https://CRAN.R-project.org/package=pheatmap), ggplot2 (53), and the Wes Anderson color palette (https://github.com/karthik/wesanderson).

Gene transfer assays. Supernatant assays were performed as described by Tomasch et al. 2018 (4). In brief, 500 µL supernatant from the donor strain was sterile-filtered through a 0.45 µM filter and mixed with 100 µL recipient strain in an Eppendorf tube. The mixture was incubated stagnant for 1 hr, followed by 4 hr incubation with shaking. 900 µL MB was then added and the mixture was incubated ON with shaking. All incubation steps were performed at 25°C. Following ON incubation, cells were spun down and plated on MA + 100 µg/mL rifampicin.

For coculture assays, spontaneous antibiotic resistant mutants were selected for on MA containing 100µg/mL streptomycin or rifampicin. Precultures were 1000-fold diluted and incubated individually and in mixed cultures in 5 mL IOCG and incubated stagnant at 25°C for three days in 30 mL culture tubes. Following incubation, cells were concentrated 5-fold and 100 µL was plated on MA plates containing either 100µg/mL streptomycin, 100µg/mL rifampicin or both for CFU enumeration.

For membrane-separated coculture assays, 5 mL IOCG was added to each well in a 24 well plate with Thincert® Cell Culture inserts dividing each well into two compartments. Strains where inoculated from precultures in a 2000-fold dilution, with the donor strains added to the inner well and recipient strains added to outer well. Plates were incubated at 25°C in a humidity chamber with a wet papertowel to conserve humidity. When sampling, the content of the outer well was homogenized by pipetting up and down with a 1000 µL pipette. 1 mL was then taken from each well, spun down, and 800 µL supernatant was removed. The remaining 200 µL was homogenized and 100 µL was plated on MA + 100 µg/mL rifampicin.

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533 ## Tables and figures

534 **Table 1.** Primers used in this study.

Primer	Sequence (5'-3')	Notes
pDM4 Fw	GAACATTGCCGGTGATTGCCCGTCAGTAGCTGAACAGGAG	Primers for construction of pDM4-*gta*::*gmR*
pDM4 Rv	TCGAGTGATGCCTGATAGGGTTATCCGCTCACAATTCCAC	
5' arm Fw	TGGAATTGTGAGCGGATAACCCTATCAGGCATCACTCGAC	
5' arm Rv	GCGTAACATCTGTCTGTCCTTTGGGTCG	
gmR Fw	AGACAGATGTTACGCAGCAGCAACG	
gmR Rv	CAAGCGTCAGTGGCGGTACTTGGGTC	
3' arm Fw	CGCCACTGACGCTTGGACCGATGG	
3' arm Rv	TCCTGTTCAGCTACTGACGGGCAATCACCGGCAATGTTCC	
Check P1	CGTTTCGGTGAGGATCTGAC	Diagnostic primers for checking homologous crossovers
Check P2	CTGATGTTGGGAGTAGGTGG	
Check P3	TGCAAGCAGATTACGGTGAC	
Check P4	ATAGTAATGCGCCGCCAG	
CmR Fw	GGCATTTCAGTCAGTTGCTC	
CmR Rv	CCATCACAAACGGCATGATG	
OL67_001823 Fw	CACTGTGACCGAAACATCCA	Primers for RT-qPCR
OL67_001823 Rv	GTCAAAGGCACTGTCATCCA	
rpoB Fw	AAACGGGCATCCAGAGCA	
rpoB Rv	AGATCGAGCGTGAAGAAGT	
Ptac-gafA Fw	AGAGTTGACAATTAATCATCGGCTCGTATAATGCAGTACGGTGAATTGTGTCTG	Primers for construction of pCMF66T-P*tac-gafA*
Ptac-gafA Rv	GAGCTCGGTACCCGGGGATCAACGCGTTCAGCCAATCAAC	
pCMF66T Fw	GAGTTGATTGGCTGAACGCGTTGATCCCCGGGTACCGAGC	
pCMF66T Rv	CAGACACAATTCACCGTACTGCATTATACGAGCCGATGATTAATTGTCAACTCTAGAGTCGACCTGCAG	
Seq P1	GTTAGCTCACTCATTAGGCAC	Sequencing primers for pCMF66T-P*tac-gafA*
Seq P2	AGGCGGTTGAGGTTCTGAC	
Seq P3	ATCTCGCGCAATCGACTG	

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Table 2. Homologs of S26 genes involved in GTA release in SB1003 identified by a BLASTp homology search. *Identified by manual inspection of the genome.

Function	Locus tag		Gene	Reference
	SB1003	S26		
Core gene cluster	rcc01699	OL67_001811		Sherlock and Fogg 2022 (54)
	rcc01698	OL67_001812		
	rcc01697	OL67_001813		
	rcc01696	OL67_001814		
	rcc01695	OL67_001815		
	rcc01694	OL67_001816		
	rcc01693	OL67_001817		
	rcc01692	OL67_001818		
	rcc01691	OL67_001819		Lang and Beatty 2000 (14)
	rcc01690	OL67_001820		
	rcc01689	OL67_001821		
	rcc01688	OL67_001822		
	rcc01687	OL67_001823		
	rcc01686	OL67_001824		
	rcc01685	OL67_001825		
	rcc01684	OL67_001826		
	rcc01683	OL67_001827		
	rcc01682	OL67_001828*		
Additional structural genes	rcc00171	OL67_004048		Hynes *et al.* 2016 (39)
	rcc01079	na	*ghsA*	Westbye *et al.* 2016 (41); Hynes *et al.* 2016 (39)
	rcc01080	na	*ghsB*	
	rcc02623	OL67_002492		Hynes *et al.* 2016 (39)
Lysis genes	rcc00555	OL67_001751		Hynes *et al.* 2012 (3)
	rcc00556	OL67_001752		Westbye *et al.* 2013 (38)
Regulatory genes	rcc01865	OL67_001522	*gafA*	Hynes *et al.* 2016 (39); Fogg 2019 (25)
	rcc01866	OL67_001521		Hynes *et al.* 2016 (39)
	rcc01700	OL67_001810	*cysE1*	Sherlock and Fogg 2022 (54)
	rcc01749	OL67_001580	*cckA*	Lang and Beatty 2000 (14)
	rcc03000	OL67_001609	*chpT*	Mercer *et al.* 2012 (20)
	rcc01663	OL67_001491	*ctrA*	Lang and Beatty 2000 (14)
	rcc00328	OL67_000677	*gtaR*	Schaefer *et al.* 2002 (21)
	rcc00329	OL67_000676	*gtaI*	
	rcc02361	OL67_001743	*lexA*	Kuchinski et al 2016 (16)
	rcc00042	OL67_003496	*divL*	Westbye *et al.* 2018 (19)
GTA recipient capability	rcc02362	OL67_001744	*comEC*	Brimacombe et al. 2015 (6)
	rcc00197	OL67_003431	*comF*	
	rcc00460	OL67_002542	*comM*	
	rcc03098	OL67_000565	*dprA*	

	rcc01932	na	Brimacombe *et al.* 2013 (22)
	rcc01081	na	
Other	rcc01849	OL67_001638	Mercer et al. 2010 (45)
	rcc00537	OL67_002365	

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544 **Table 3.** Strains used in this study.

Strain	Genotype/Phenotype	Reference
Phaeobacter piscinae S26		Grotkjær *et al*. 2016 (55)
Phaeobacter piscinae S26Δ*tdaB*	Δ*tdaB*	Lindqvist *et al.* 2022
Phaeobacter piscinae S26 Rif^R	Rif^R	This study
Phaeobacter piscinae S26*ΔtdaB* Rif^R	Δ*tdaB*, Rif^R	This study
Phaeobacter piscinae S26 Strep^R	Strep^R	This study
Phaeobacter piscinae S26*ΔtdaB Δ*GTA Rif^R	Δ*tdaB*, OL67_001823::*gm^R* Rif^R	This study
Escherichia coli WM3064	thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD)567 ΔdapA1341::[erm pir]	Strain developed by William Metcalf at UIUC
Escherichia coli GBdir-*pir116*	F$^-$ Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMluI)::pir-116	Wang *et al*. (2016) (48)

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547 **Table 4.** Plasmids used in this study.

Plasmid	Features	Reference
pJET1.2	pMB1 origin of replication, Amp^R, P_{lacUV5}, *eco47IR*, T7 promoter	ThermoFisher Scientific
pDM4	*sacB*. cm^R. R6Kγ origin.	Milton *et al.* (1996) (56)
pDM4-gm^R::gta	pDM4 backbone carrying homology arms and $genta^R$ marker targeting the major capsid protein, OL67_001823.	This study
pCMF66T	oriV. Kan^R	
pCMF66T-*Ptac-gafA*	*gafA* from S26 under control of an IPTG-inducible *tac* promoter.	This study

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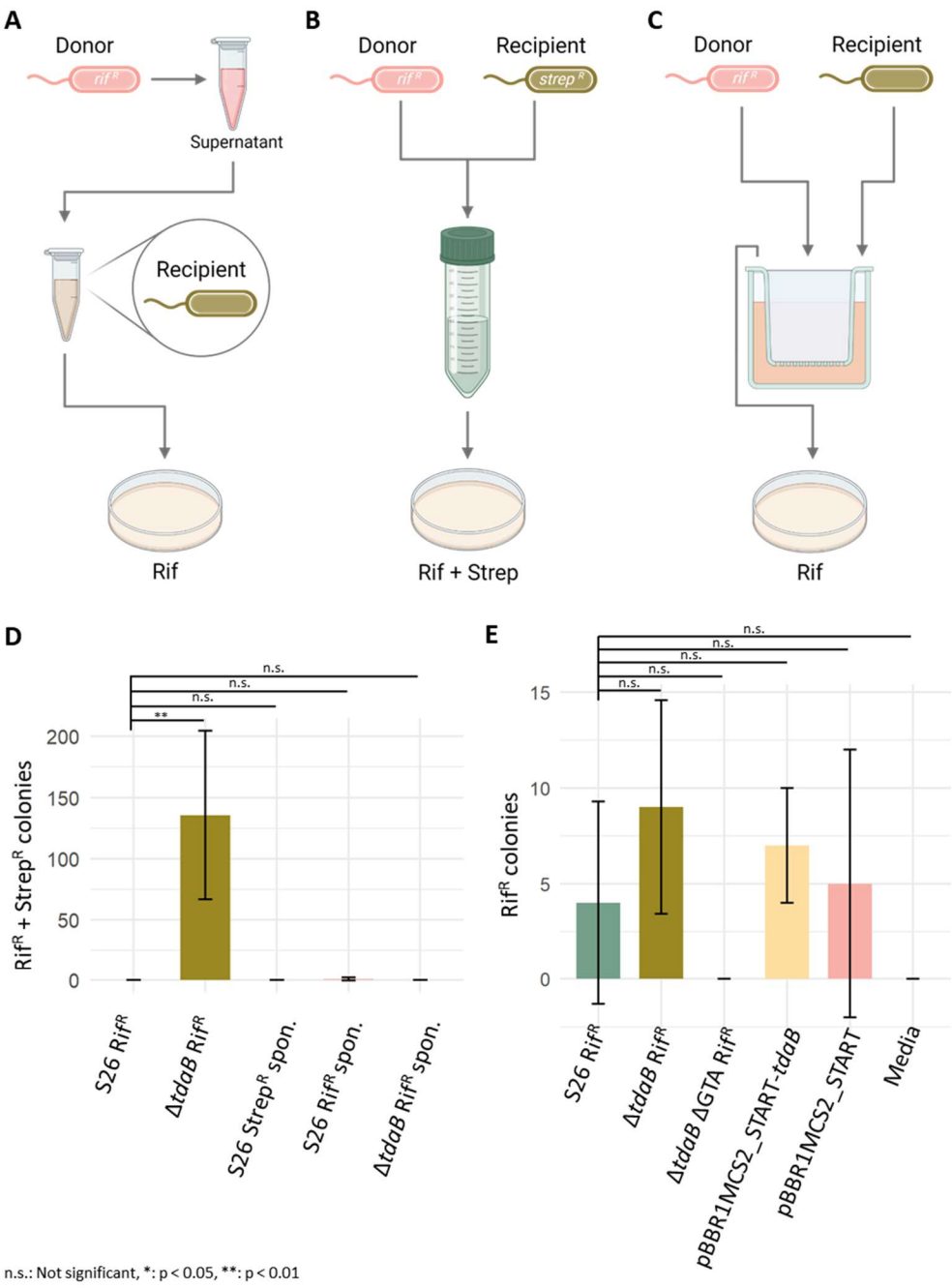

Figure 1. **A**. Schematic representation of the supernatant HGT assay. **B.** Schematic representation of the coculture HGT assay. **C.** Schematic representation of the membrane-separated coculture HGT assay. **D.** Preliminary data from a coculture HGT assay with S26 Strep^R as recipient and different donor strains (indicated on x-axis). **E.** Preliminary data from a membrane-separated co-culture setup. Δ*tdaB* was used as a recipient in co-culture with different donor strains (indicated on x-axis).

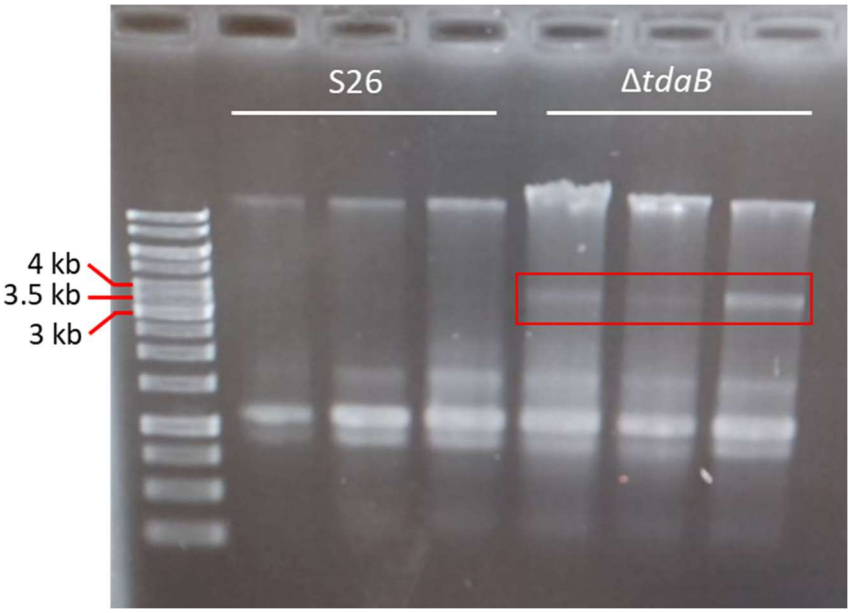

Figure 2. RNA extractions prior to DNase treatment. An approx. 4 kB band was present for Δ*tdaB* but not S26 WT samples.

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561 Figure 3. Log_2(fold change) of gene/protein abundance in Δ*tdaB* relative to WT of gene
562 homologues associated with GTA maturation, release, regulation, and recipient capability in
563 *R. capsulatus*. Samples were included in the heatmap if there was significant differential
564 abundance of transcripts/proteins in one of the three comparisons (transcriptome (T), cell
565 proteome (PC), or supernatant proteome (PS)).

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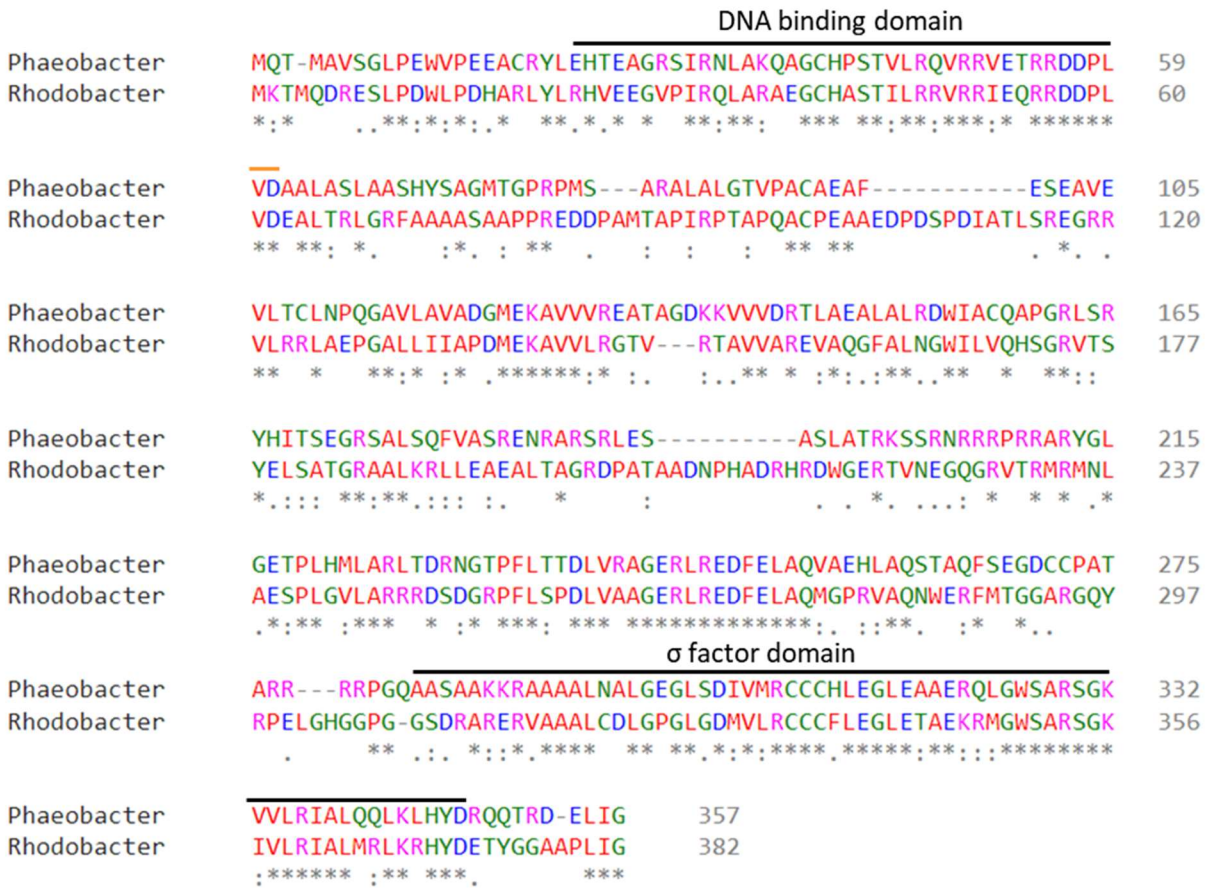

Figure 4. Clustalω alignment of *gafA* from *R. capsulatus* SB1003 and OL67_001522 from *P. piscinae* S26. A predicted DNA-binding domain and sigma factor domain are marked on the figure.

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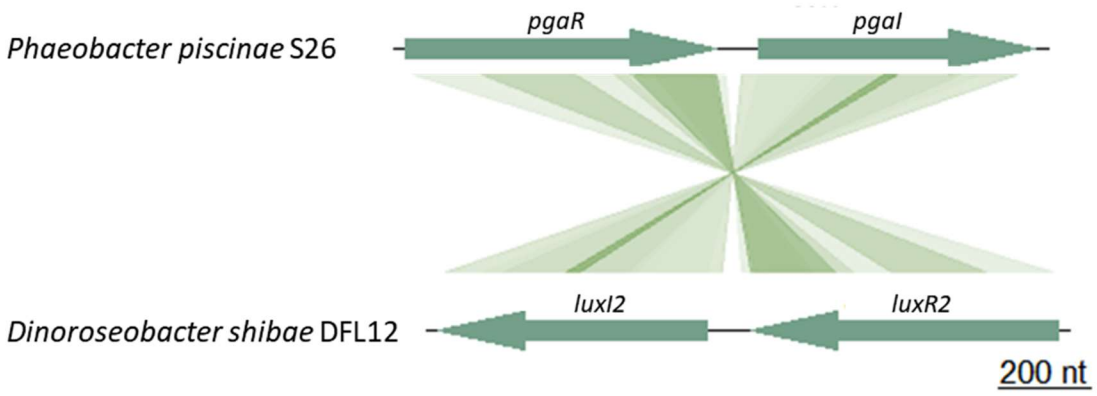

Figure 5. Genomic comparison (protein level) of the *pgaRI* system from *P. piscinae* S26 and the *luxIR2* system from *D. shibae* DFL12. Darker hues indicate higher degree of similarity.

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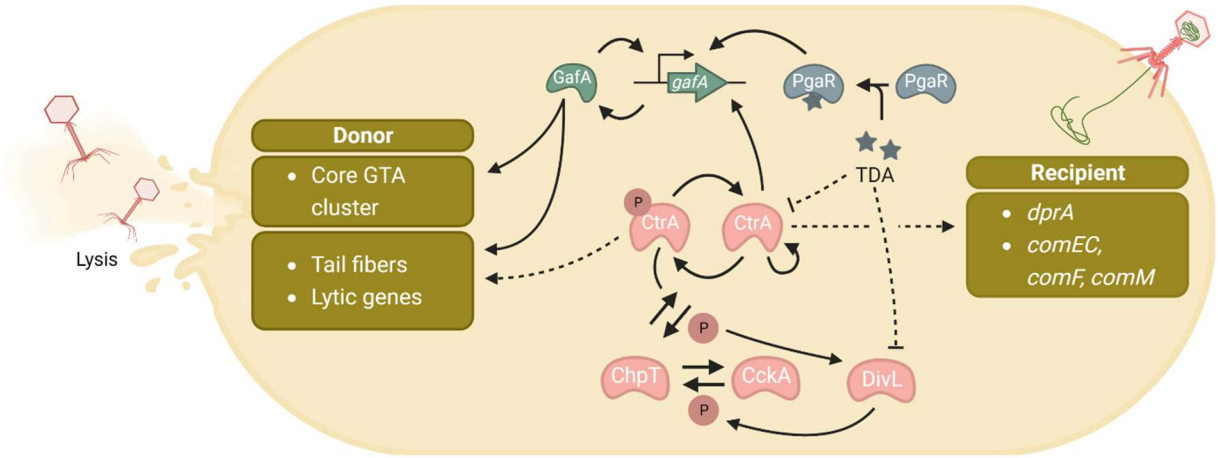

Figure 6. Proposed model of TDA-mediated regulation of GTA release. TDA interacts with the response regulator PgaR, which in turn represses gafA transcription. Since GafA is an activator of the expression of the core GTA cluster as well as the lytic gene operon, this ultimately results in a repression of GTA release. In addition, through an unknown mechanism TDA also represses expression of DivL and CtrA, which are part of a phosphorelay system controlling GTA maturation, cell lysis, and GTA recipience. Dashed lines indicate the regulatory route is unknown. Figure was made using Biorender.com.

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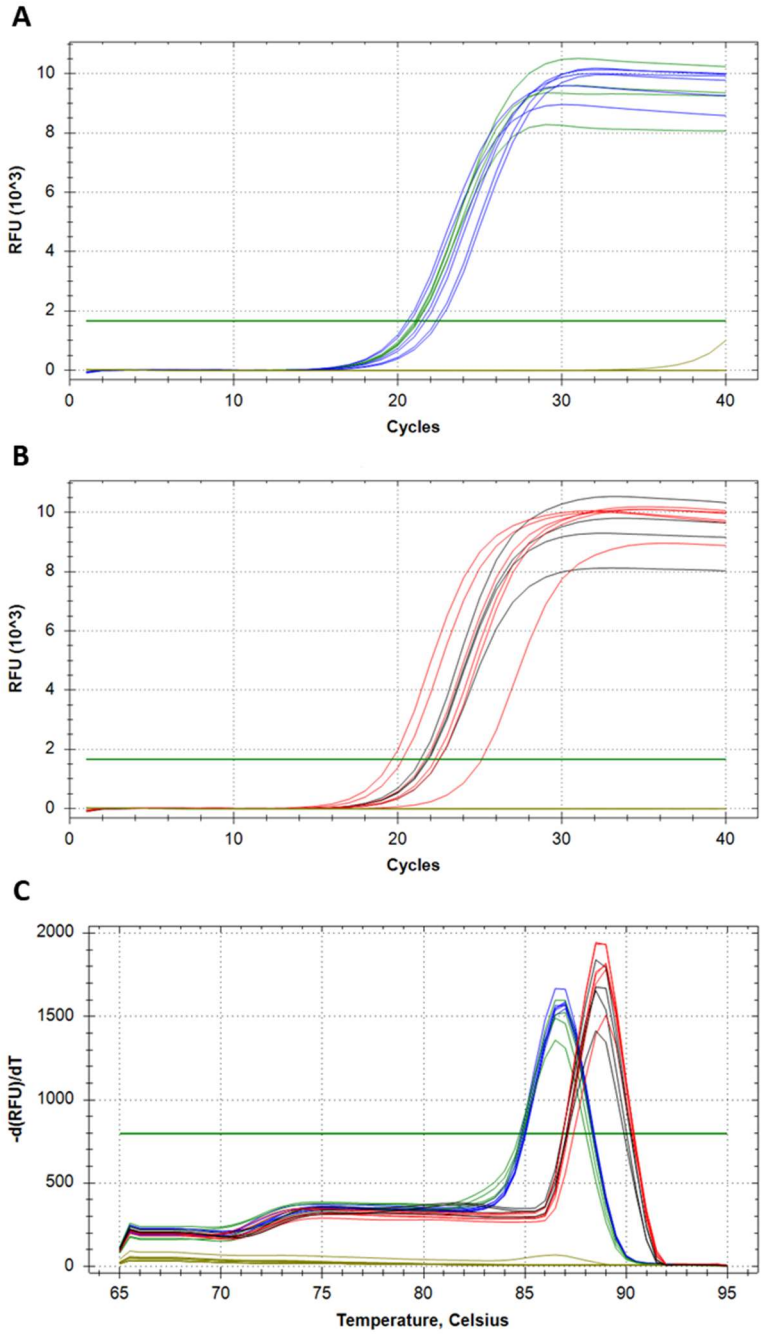
Supplementary figures and tables



Supplementary Figure 1. **A.** RT-qPCR plot for RpoB primers. WT: Green, Δ*tdaB*: Blue. Negative controls (RNA from samples) are olive colored in all plots. **B.** RT-qPCR plot for OL67_01823 primers. WT: Black, Δ*tdaB*: Red. **C.** Melting curve for RpoB primers (WT: green, Δ*tdaB*: blue) and OL67_01823 (WT: Black, Δ*tdaB*: Red) primers.

Manuscript IV

Monitoring production of tropodithietic acid using fluorescent gene reporter fusions

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In preparation

Monitoring production of tropodithietic acid using fluorescent gene reporter fusions

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Abstract

Activation of microbial secondary metabolism is tightly governed and regulation integrates both biotic and abiotic cues. Understanding how secondary metabolism is controlled is important for a number of reasons; first, the ability to activate secondary metabolism may lead to the discovery of compounds with industrially relevant bioactivities. Secondly, if we can identify *when* and *what* triggers the production of a compound, it may provide us with a clue as to the ecological role of this compound. However, chemical detection of secondary metabolites in natural systems can be difficult probably due to concentrations being low. An alternative approach is to use the expression of the biosynthetic genes as a proxy for secondary metabolite production, e.g. by using fluorescent gene-reporter systems. Here, we develop both a plasmid-borne transcriptional reporter, pBBR1MCS2_START-P*tdaC-gfp*, and a genome-integrated translational reporter, *tdaC-mCherry*, for tracking *tdaC*, an enzyme in the biosynthetic pathway of the secondary metabolite tropodithietic acid (TDA) produced by several members of the marine *Roseobacter* group. Fluorescence was observed on colony-level for both strains, but single-cell fluorescence could only be observed for the transcriptional reporter. These preliminary results provide the foundation for further development of gene-fusions indicative of TDA production..

Introduction

Bacteria produce a wide variety of small, bioactive compounds, collectively termed secondary or specialized metabolites. These metabolites are involved in a number of cellular processes, including nutrient acquisition, signaling, and antagonism against competitors (1–7). Secondary metabolism is tightly governed by different regulatory systems: From global cellular regulatory systems to localized regulation by cluster-specific regulators encoded alongside the biosynthetic machinery, secondary metabolism integrates a number of cues. Some of these cues are abiotic and include pH, nutrient availability, or temperature. Others arise from biotic cues from other organisms, e.g. sub-inhibitory concentrations of antibiotics (8–10) or quorum sensing signals (11–13). The cues that govern secondary metabolite production are anything but random; instead, they may provide valuable input toward understanding the roles these compounds play in natural systems. A typical case is siderophores, iron-chelating secondary metabolites that sequester iron from the environment and deliver it back to the cells through specific receptors (14). Siderophore production is initiated when iron availability is low, reflecting that siderophores likely evolved to aid nutrient acquisition (15).

A key obstacle in studying the regulation of secondary metabolism is the detection of the molecules. Chemical detection of the compounds is the most direct way of doing this, but the concentration of these molecules is often, at bulk extraction level, low and hence, escape detection. Recently, advances have been made in e.g. resin-based extraction (16) and mass spectrometry imaging (17, 18), however, these methods may not be widely applicable. An alternative to directly chemical detection is the monitoring of expression of the biosynthetic genes that can be used as a proxy for production, and that can be monitored e.g. through RT-qPCR or the use of reporter genes.

Tropodithietic acid (TDA) is a secondary metabolite produced by members of the marine *Roseobacter* group (19). This group of bacteria is often found in association with algal blooms and displays a 'swim-and-stick' lifecycle (20–22). TDA is a multifunctional compound, exhibiting both antibacterial, anticancer, iron-chelating and quorum-sensing properties (5, 23, 24). Its production is regulated by a number of factors; notably, stagnant growth, biofilm formation, and attachment to a surface promote expression of the TDA biosynthesis genes in *Tritonibacter mobilis* (25). However, this link is not universal, as stagnant growth and biofilm formation are not prerequisites for TDA production in most TDA-producing *Phaeobacter* spp. (26). This has been speculated to reflect niche-specific regulation of TDA (27).

In a previous study, we observed that TDA-production led to a number of physiological changes in the producing bacteria related to colonization and adaptation, and we hypothesized that TDA

coordinates these processes (28). We, therefore, set out to develop a fluorescent gene reporter-fusion system for tracking TDA production *in situ* during colonization to further explore this hypothesis. Furthermore, the system can be used for high-throughput screening conditions affecting TDA production.

Results (preliminary)

The *tda* gene cluster consists of *tdaA-F* and *paaZ2*. The *tdaC* gene has previously been used as a proxy for TDA-production (25), and we, therefore, chose a similar strategy for our reporter systems. We constructed a translational reporter-fusion by introducing an *mCherry* gene upstream of the *tdaC* gene (*tdaC-mCherry*, **Figure 1A**), as well as a plasmid-borne transcriptional reporter with a *gfp* gene fused to the *tdaC* promoter (P*tdaC-gfp*, **Figure 1B**). The reporter strains were inspected through fluorescence microscopy and colony-level fluorescence could be observed for both P*tdaC-gfp* and *tdaC-mCherry* (**Figure 2**). The wild-type S26 was included as a control for *tdaC-mCherry*, whilst an empty vector control was included for P*tdaC-gfp*. None of the controls exhibited significant background fluorescence.

Secondary metabolite expression patterns may differ even within adjacent cells of a population (29). Additionally, a previous study used a P*tdaC-gfp* reporter in *T. mobilis* to demonstrate that *tdaC* expression coincided with attachment to a surface (25). We, therefore, aimed at a gene reporter system which could be detected not only on colony level but also on single cell level to facilitate a detailed investigation of TDA production dynamics. We were only able to observe a fluorescent signal from P*tdaC-gfp* (**Figure 3**), whilst *tdaC-mCherry* did not emit a visible fluorescent signal at the single cell level (data not shown).

Preliminary discussion and future plans

Whilst bulk extractions can be a good starting point for detecting presence of microbial secondary metabolites, it does not inform about the spatial distribution of the compounds *in situ*. However, the rapid developments within chemical detection have, at least to some extent, conquered this issue by developing methods such as mass spectrometry imaging, where metabolites can be visualized *in situ* (17, 18). The drawback of this type of method is the cost and labor associated; genetic reporter systems serve as a cheaper and faster alternative, permitting high-throughput studies of, for instance, factors influencing secondary metabolite production.

Here, we set out to develop a reporter system to track *tdaC* expression as a proxy for production of the secondary metabolite TDA. Whilst the P*tdaC-gfp* fusion can be used for monoculture-studies of

tdaC expression, the plasmid-based system requires selective pressure for ensuring plasmid maintenance, and thus, the system is not suitable for tracking *tdaC* expression dynamics in microbial communities. Our integrated translational reporter fusion, *tdaC-mCherry*, overcomes this obstacle, but fluorescence was only weakly be detected on colony-level and not at all on single-cell level. To reliably track *tdaC* expression, a higher fluorescent signal would be necessary. A third approach that could potentially overcome the challenges of both of these systems is to integrate the transcriptional P*tdaC-gfp* system into an alternative integration site on the chromosome of S26. Still, the pBBR1MCS2_START-P*tdaC-gfp* system can be used for screening of conditions that induce *tdaC* expression.

Methods and Materials

Strains and culture condtions. A list of strains used in this study can be found in **Table 1**. *Phaeobacter* strains were cultured on Marine Agar (MA, BD Biosciences), or in Marine Broth (MB, BD Biosciences). Cultures were grown at 25°C with shaking at 200 rpm, unless otherwise indicated. *Escherichia coli* strains were routinely cultured in LB broth (BD Biosciences) or LB agar (BD Biosciences) during conjugation. *E. coli* WM3064 used for electroporation was cultured in low salt LB broth (1 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) or agar (1% w/v bacteriology grade agar) supplemented with 0.3 mM diaminopimelic acid (DAP). *E. coli* strains were grown at 37°C with shaking at 200 rpm. Antibiotics were added to selective media in the following concentrations: 10 µg/mL (liquid) or 15 µg/mL (agar plates) chloramphenicol; 100 µg/mL (liquid) or 200 µg/mL (agar plates) ampicillin; 50 µg/mL (liquid and agar plates) kanamycin for *E. coli* strains and 200 µg/mL (liquid and agar plates) kanamycin for *P. piscinae* strains.

DNA manipulation. Plasmids and primers used in the study are listed in **Table 2 and 3**. Genomic DNA extractions were performed using the NucleoSpin® Tissue kit (Macherey-Nagel, Fisher Scientific). The Monarch® Plasmid Miniprep Kit (NEB, Bionordika) was used for plasmid extractions. Primers were synthesized by Integrated DNA Technologies (Belgium). Amplification of fragments for cloning were performed using Q5® High-Fidelity 2X Master Mix (NEB, Bionordika, Denmark), whilst diagnostic PCRs were performed with TEMPase Hot Start 2X Master Mix K (Ampliqon, VWR). PCR products were purified with the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Restriction enzymes were acquired from New England Biolabs (NEB, Bionordika).

Plasmids construction. Plasmids were designed using ApE - A plasmid Editor (v2.0) (30). Plasmids were assembled using direct RecET-mediated cloning, as described by Wang *et al.* 2016 (31). For cloning of pDM4-*tdaC-mCherry*, fragment inserts were amplified using the primer pairs 5' arm

Fw/Rv, mCherry Fw/Rv, and 3' arm Fw/Rv and joined to a single insert through two rounds of overlap PCR. The resulting intermediates were subcloned into pJET1.2 using the CloneJET PCR Cloning Kit (ThermoFisher Scientific) and transferred to *E. coli* TOP10 (Invitrogen). The resulting plasmids were used for subsequent rounds of amplification. The pDM4 backbone was amplified using the primer pair pDM4 Fw/Rv. To construct the pBBR1-MCS2-P*tdaC-gfp* plasmid, the promoter of the *tdaC* (P*tdaC*) *was* amplified using primer pairs PtdaB'C-FW and PtdaC-gfp-Rv. The promoter-less gfpmut3* gene was amplified using the primer pair GFPmut3-Fw and GFPmut3-Rv and the subsequent PCR products were fused to the P*tdaC* via overlap PCR with primers PtdaB'C-FW and GFPmut3-Rv. The fused P*tdaC-gfp* segments were cloned between the XhoI and XmaI sites of the pBBR1-MCS2 vector via restriction digestion cloning using the Quick Ligation Kit (New England Biolabs, M2200, BioNordika, Denmark), which resulted in plasmid pBBR1-MCS2-P*tdaC*-gfp. Plasmids were verified by colony PCR and sequencing using Primer set Check-Pgfp (up) and Check-Pgfp (down). The recombined plasmid and the empty vector the pBBR1-MCS2, were transferred into strain *E. coli* WM3064 and the transformants was subsequently used as donors to conjugate the plasmids into the wild type S26 strain as described by Lindqvist *et al.* 2023 (28). This resulted in the strains S26 P*tdaC-gfp* and S26 pBBR1MCS2_START.

Conjugation and homologous recombination. Conjugations were carried out as described by Lindqvist *et al.* 2023 (28). For integration of *mCherry* into the chromosome using pDM4-*tdaC-mCherry*, two rounds of selection were performed; a first selection using chloramphenicol, and a counter-selection using ½YTSS (3 g/L yeast extract, 3 g/L tryptone, 30 g/L sea salts (Sigma-Aldrich)) with 10% sucrose. The succesful homologous crossover was confirmed through diagnostic PCR using Check P1-4.

Microscopy. Fluorescence images of colonies were obtained using a motorized fluorescence stereo zoom microscope, Axio Zoom.V16 (Zeiss Microscopy, Oberkochen, Germany). The microscope was equipped with a PlanApoZ 0.5X/0.125NA FWD 114mm objective and a monochromatic Axiocam 503 camera. Zeiss software ZEN was used to capture photos of colonies on MA plater (Brightfield and GFP at ex. 470 nm, em. 525 nm). The fluorescent cells were imaged using a 60× oil objective on Nikon Inverted Fluorescence Microscope-EclipseTi2 (Nikon, Tokyo, Japan; WIB ex. 457–487 nm, em. 502–537 nm). The NIS-Element software was used to acquire representative fluorescence micrographs with an exposure time of 70 ms. Images were processed using Fiji (32) with adjustments applied equally across samples.

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259 **Figures and tables**

260 **Table 1.** Strains used in this study.

Strain	Genotype	Reference
***Phaeobacter piscinae* S26**		Grotkjær *et al*. 2016 (33)
tdaC-mCherry	tdaC-mCherry	This study
P*tdaC-gfp*	Wild type S26 carrying plasmid pBBR1MCS2-P*tdaC-gfp. KanR*	This study
pBBR1MCS2	Wild type S26 carrying plasmid pBBR1MCS2*. KanR*	This study
Escherichia coli WM3064	*thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD)567 ΔdapA1341::[erm pir]*	Strain developed by William Metcalf at UIUC
Escherichia coli TOP10	F- *mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG*	ThermoFisher Scientific, C404010
Escherichia coli GBdir-pir116	F^- Δ*lac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMluI)::pir-116*	Wang *et al*. (2016) (31)

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263 **Table 2.** Plasmids used in this study.

Plasmid	Features	Reference
pDM4	*sacB*. *cat*. R6Kγ origin.	Milton *et al.* (1996) (34)
pDM4- tdaC-mCherry	pDM4 backbone carrying homology arms and *mCherry* for tagging *tdaC*.	This study
pBBR1MCS2_START	pBBR1 origin of replication, Kan^r, P_{lac}, *lacZα*	Obranić *et al.* (2013) (35)
pBBR1MCS2_START-P*tdaC-gfp*	Expression plasmid carrying *gfp* under control of the *tdaC* promoter.	This study

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266 **Table 3.** Primers used in this study.

Primer	Sequence (5'-3')	Notes
*Primers for construction of pBBR1MCS2_START-P*tdaC-gfp		
GFPmut3-Fw	ACGATGGAACAGGCCATGCGTAAAGGAGAAGAACTTTTC	Amplification of *gfp*
GFPmut3-Rv	TCCCGGGTTATTTGTATAGTTCATCCATGC	
PtdaB'C-Fw	AAAACTCGAGCTGGTTTGAGCGCTGTCT	Amplification of *tdaC* promoter
PtdaC-gfp-Rv	TTCTCCTTTACGCATGGCCTGTTCCATCGTCC	
Check-Pgfp (up)	ATGCGTAAAGGAGAAGAACTTTTC	For diagnostic PCR and sequencing.
Check-Pgfp (down)	TTATTTGTATAGTTCATCCATGC	
Primers for construction of S26 tdaC-mCherry		
5' arm Fw	GTTACCCGCATGCAAGATCCGACGCCGAGTATATTCC	Amplification of 5' homology arm
5' arm Rv	TCGCCCTTGCTAGCCATATTGTCGGGGCAGGATCAG	
mCherry Fw	TGATCCTGCCCCGACAATATGGCTAGCAAGGGCGAG	Amplification of *mCherry*
mCherry Rv	CCCTCCTGTTGGTGTAGTTACTTGTACAGCTCGTCCATGC	
3' arm Fw	ATGGACGAGCTGTACAAGTAACTACACCAACAGGAGGGAC	Amplification of 3' homology arm
3' arm Rv	CTTATCGATACCGTCGACCGATCATCCGCCACAAGC	
pDM4 Fw	CTTGTGGCGGATGATCGGTCGACGGTATCGATAAG	Amplification of pDM4
pDM4 Rv	AATATACTCGGCGTCGGATCTTGCATGCGGGTAAC	
Check P1 Fw	GAAGAGACGAGTGATGGATC	Primers for checking integration of *mCherry* into S26 genome
Check P2 Rv	GATGATGGCCATGTTATCCTC	
Check P3 Fw	TACACCATCGTGGAACAGTAC	
Check P4 Rv	GCTGGCATAGAGATCCAATG	
CmR Fw	GGCATTTCAGTCAGTTGCTC	
CmR Rv	CCATCACAAACGGCATGATG	

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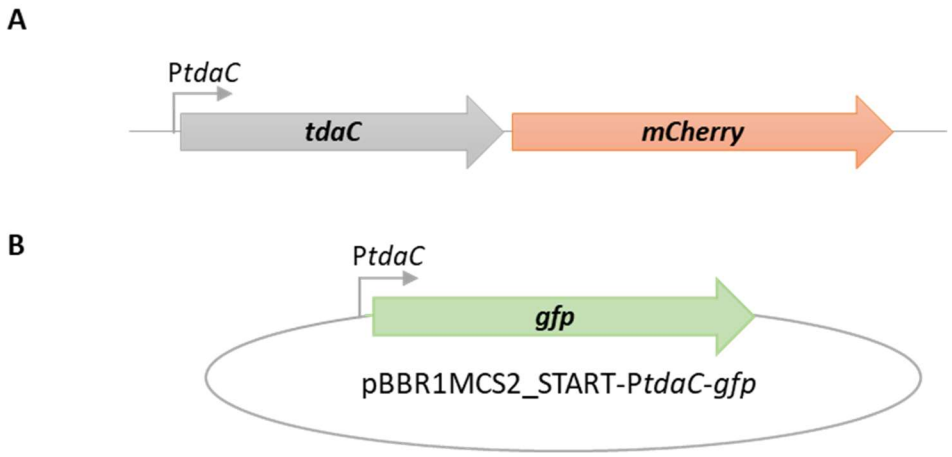
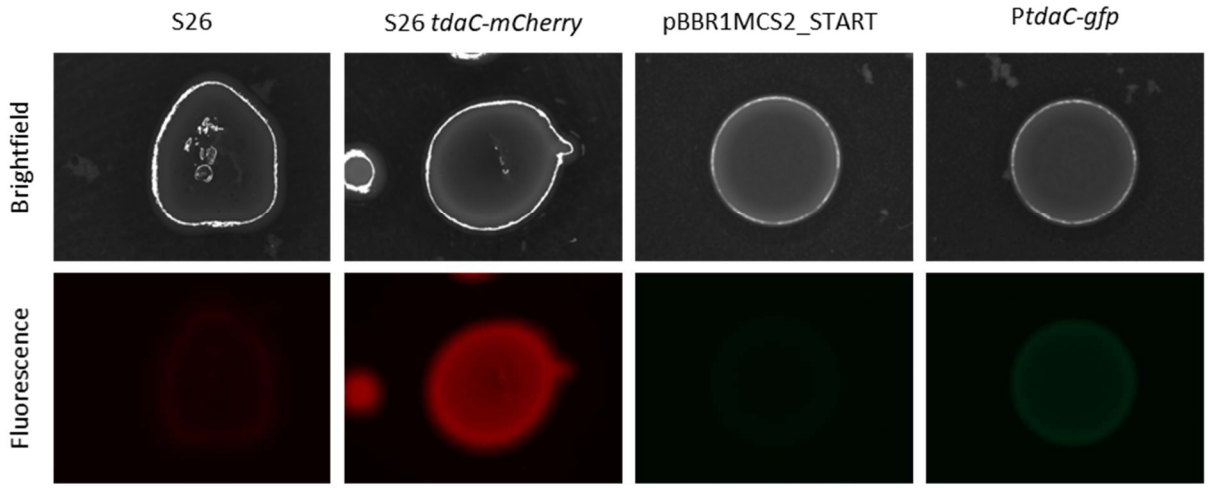


Figure 1. A. Schematic representation of the *tdaC-mCherry* translational reporter fusion. B. Shematic representation of the transcriptional P*tdaC-gfp* reporter fusion.



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275 **Figure 2**. Brightfield- and fluorescence imaging of colonies of reporter-fusions and corresponding negative
276 controls. S26 was included as a negative control for S26 *tdaC-mCherry*, whilst an empty vector control, S26
277 carrying pBBR1MCS2_START, was included as a negative control for P*tdaC-gfp*.

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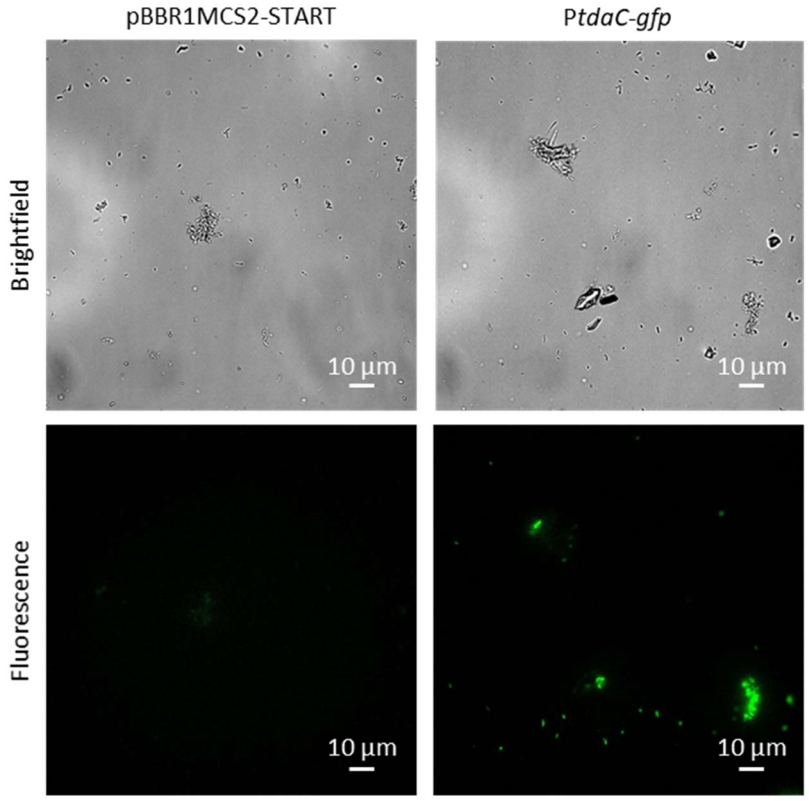

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280 **Figure 3**. Inverted brightfield and fluorescence microscopy of P*tdaC-gfp*. S26 carrying pBBR1MCS2_START was
281 included as an empty-vector control.

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