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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

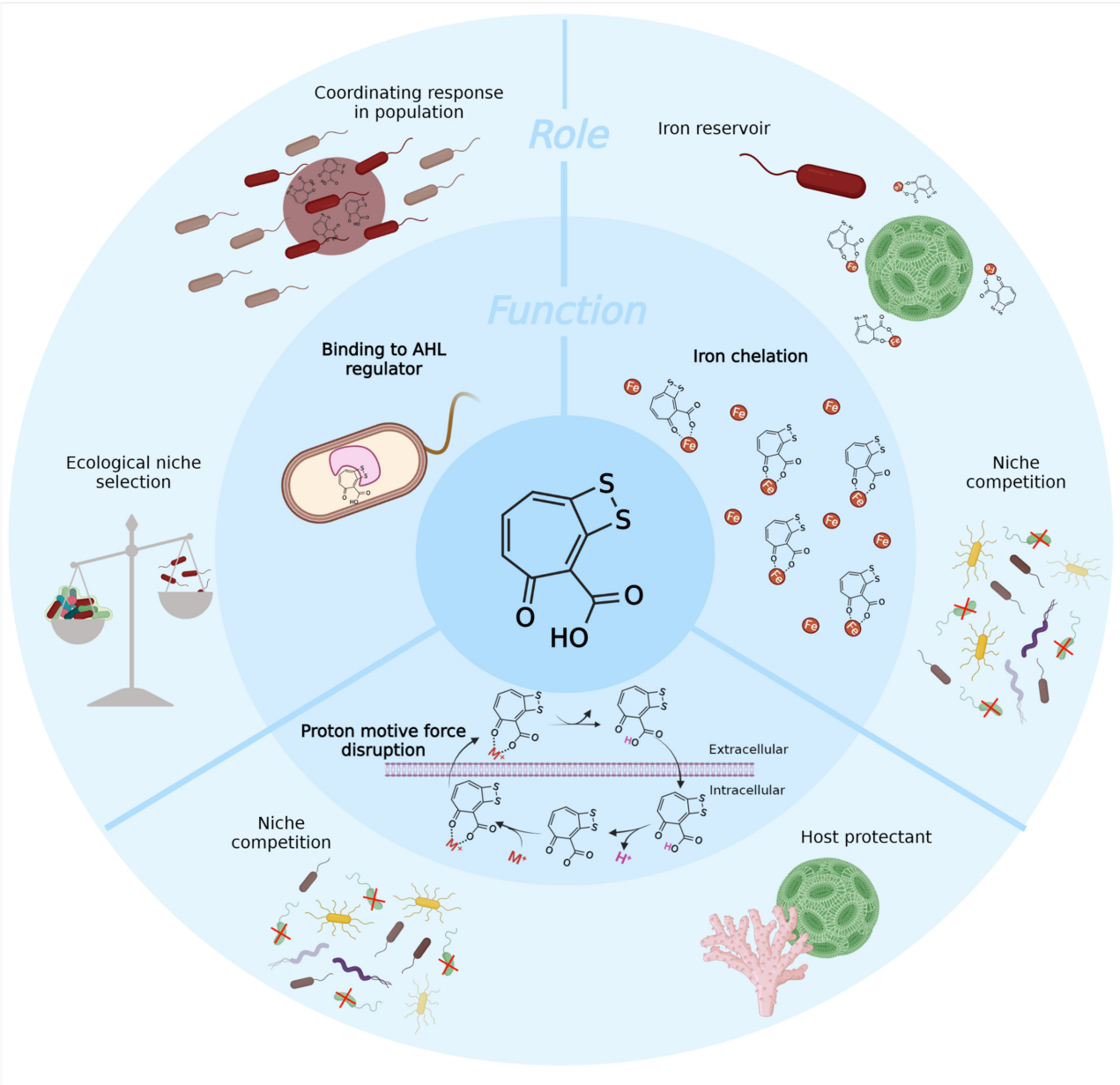

Figure 1. Proposal for three functions of TDA and potential ecological roles. Bold text indicate functions. Nonbold text indicate potential ecological roles. Created with Biorender.com.

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).

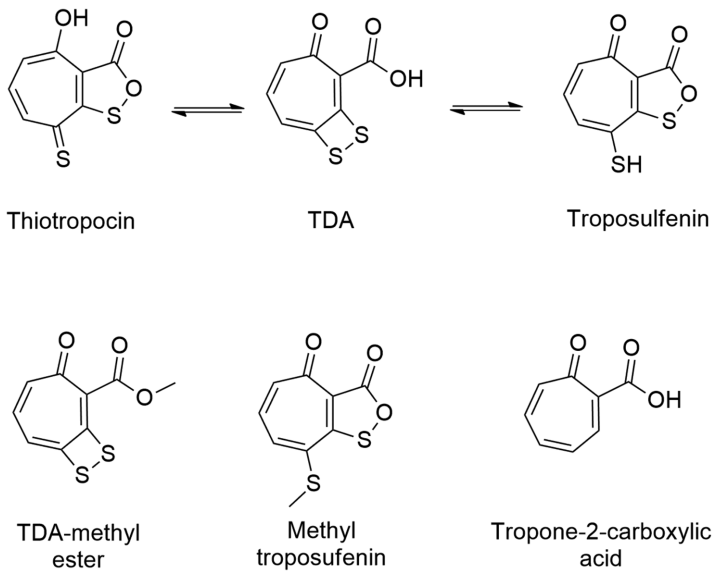

Figure 2. TDA and its tautomers (upper panel) and analogues (lower panel). Created with ChemDraw Professional (PerkinElmer Informatics).

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

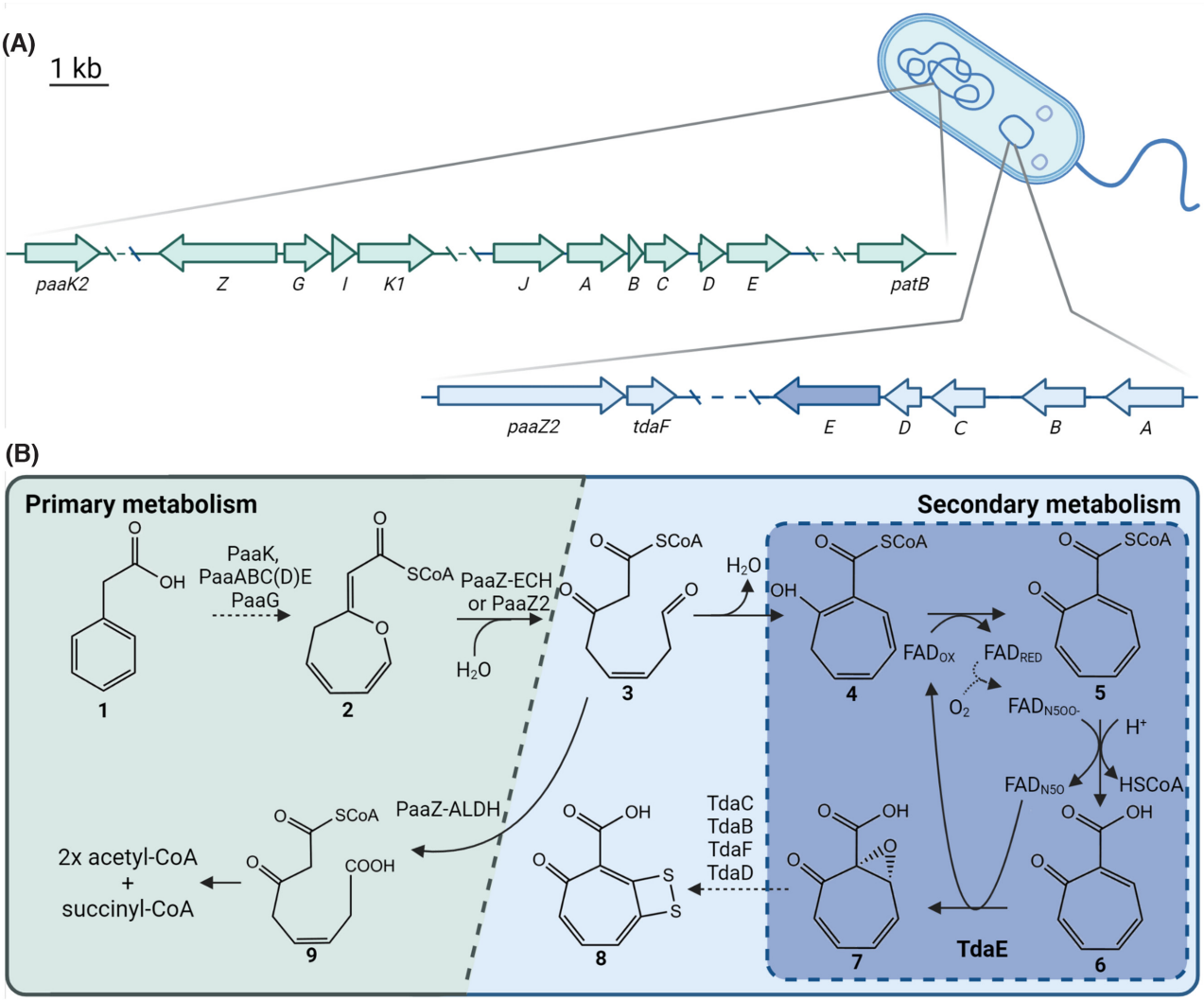

Figure 3. TDA biosynthesis and genes responsible for TDA production. **(A)** Biosynthetic genes involved in TDA biosynthesis in *P. inhibens* DSM17395. *paaABCDEIJK1K2* and *patB* (in green) are located on the chromosome, whilst *tdaABCDEF* and *paaZ2* (in blue) are located on a 262-kb megaplasmid. **(B)** 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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