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**Tropodithietic acid induces oxidative stress response, cell envelope
biogenesis and iron uptake in *Vibrio vulnificus***

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Originality-Significance Statement

The natural role of the antibiotic tropodithietic acid (TDA) and its impact on non-producing marine bacteria at *in situ* concentrations is currently unknown. Here we determine how a sub-lethal concentration of TDA affects the opportunistic fish and human pathogen *Vibrio vulnificus*, providing insight into the mechanism of action of TDA and its potential effects on both pathogenic and commensal bacteria.

Abstract

The *Roseobacter* group is a widespread marine bacterial group, of which some species produce the broad-spectrum antibiotic tropodithietic acid (TDA). A mode of action for TDA has previously been proposed in *Escherichia coli*, but little is known about its effect on non-producing marine bacteria at *in situ* concentrations. The purpose of this study was to investigate how a sub-lethal level of TDA affects *Vibrio vulnificus* at different time points (30 min and 60 min) using a transcriptomic approach. Exposure to TDA for as little as 30 min resulted in the differential expression of genes associated with cell regeneration, including the up-regulation of those involved in biogenesis of the cell envelope. Defense mechanisms including oxidative stress defense proteins and iron uptake systems were also up-regulated in response to TDA, while motility-related genes were down-regulated. Gene expression data and scanning electron microscopy imaging revealed a switch to a biofilm phenotype in the

presence of TDA. Our study shows that a low concentration of this antibiotic triggers a defense response to reactive oxygen species and iron depletion in *V. vulnificus*, which indicates that the mode of action of TDA is likely more complex in this bacterium than what is known for *E. coli*.

Introduction

The *Roseobacter* group is one of the most widespread marine bacterial groups and is often associated with eukaryotes such as algae (González *et al.*, 2000; Buchan *et al.*, 2005; Sonnenschein *et al.*, 2017; Dittmann *et al.*, 2018) and molluscs (Ruiz-Ponte *et al.*, 1998; Prado *et al.*, 2009; Wegner *et al.*, 2013). Members of this group have also been repeatedly detected in aquaculture systems (Hjelm, Riaza, *et al.*, 2004; Porsby *et al.*, 2008). Some species, including *Phaeobacter inhibens* and *Ruegeria mobilis*, produce the potent antibacterial compound tropodithietic acid (TDA) (Bruhn *et al.*, 2005; Porsby *et al.*, 2008), which is active against a range of Gram-positive and Gram-negative bacteria (Kintaka *et al.*, 1984; Porsby *et al.*, 2011; Rabe *et al.*, 2014). TDA has low toxicity to the roundworm *Caenorhabditis elegans* (Neu *et al.*, 2014), which is often used as a model organism for testing cytotoxicity (Sese *et al.*, 2009; Sprando *et al.*, 2009) resembling the effects induced in mammalian model organisms (Sprando *et al.*, 2009). However, TDA has also shown anticancer activity (Wilson *et al.*, 2016) and the compound can be cytotoxic to mammalian neuronal cells (Wichmann *et al.*, 2015), which indicates that the effect of TDA on eukaryotic cells and eukaryotes is dependent on the cell type and target organism. The activity towards neuronal cells is believed to be driven by disruption of the mitochondrial membrane potential and activation of oxidative stress response (Wichmann *et al.*, 2015). Altogether, the broad target range and toxicity towards mammalian cells should be taken into consideration if TDA and TDA-producers are to be used as treatment in humans as well as in rearing of fish and shellfish.

TDA-producing bacteria have potential as probiotics in aquaculture systems as they can prevent fish larvae mortality caused by pathogenic *Vibrio* spp. (Planas *et al.*, 2006a; D'Alvise *et al.*, 2013) without causing adverse effects on either the larvae (Hjelm, Bergh, *et al.*, 2004; Planas *et al.*, 2006b; D'Alvise *et al.*, 2010, 2012) or the live-feed organisms such as *Artemia* (Neu *et al.*, 2014; Grotkjær *et al.*, 2016), copepods (Rasmussen *et al.*, 2018), rotifers, and microalgae (D'Alvise *et al.*, 2012). The prospect of using antibiotic-producing bacteria in aquaculture raises the concern of resistance development in target organisms. However, previous studies have shown that human and fish pathogens are genetically and phenotypically unaffected by long-term exposure to TDA (Porsby *et al.*, 2011; Rasmussen *et al.*, 2016). Although TDA-tolerant bacteria have been co-isolated with TDA-producing *Pseudovibrio* spp. from marine sponges (Harrington *et al.*, 2014), their tolerance/susceptibility mechanisms and long-term stability remain unclear. Understanding such processes is crucial for a broader implementation of TDA-producing strains in the aquaculture industry.

The antagonistic effect of TDA against a wide range of prokaryotes, in conjunction with the fact that resistance to this compound is rarely observed, suggest that TDA has multiple targets in the cell, and that at least one of the targets has a vital function, which is conserved and sensitive to mutations. Wilson *et al.* (2016) proposed that TDA in *Escherichia coli* acts as an electroneutral proton-antiporter creating an acidic cytosol by the import of H⁺ ions while exporting metal ions, which would be chelated in the extracellular space. As a result, the proton motive force (PMF) is disrupted and the cells are killed. The natural role, mode of action, and effect of TDA on bacteria in marine environments remain to

be understood. We have previously shown that the impact of TDA-producers on marine microbiomes is dependent on the complexity and composition of the established microbial community, with stronger influence on specific community members (Dittmann *et al.*, 2018). Particularly, genera known to include fast-growing opportunistic fish pathogens, such as *Vibrio* spp. and *Pseudoalteromonas* spp., decrease in abundance in the presence of TDA (Dittmann *et al.*, 2018). Within TDA-producers, TDA can also act as a quorum sensing molecule regulating motility, biofilm formation, and antibiotic production (Beyersmann *et al.*, 2017). Henceforth, TDA may have multiple functions and induce different responses depending on the sensing organism.

Altogether, TDA and TDA-producing bacteria are promising candidates for controlling pathogenic bacteria in aquaculture. However, it is crucial to understand the impact of TDA and the potential consequences of bacterial exposure to the compound – particularly how TDA-susceptible pathogens compensate metabolically to avoid mortality and if TDA exposure triggers expression of genes related to undesirable phenotypes such as virulence. Thus, the purpose of this study was to determine how a sub-lethal concentration of TDA affects the transcriptome of the human and fish pathogenic bacterium *Vibrio vulnificus* upon 30 min and 60 min exposure to the compound. This species was chosen as a model organism for vibrios, which are some of the most common causes of bacterial diseases in aquaculture. *V. vulnificus* is one of a few species causing major economic losses in rearing of several fish species (Thompson *et al.*, 2004; Toranzo *et al.*, 2005). Furthermore, vibrios are known to be particularly susceptible to TDA (Porsby *et al.*, 2011).

Results and Discussion

The effect of TDA on the transcriptional profiles of *V. vulnificus* CMCP6 was assessed by mRNA sequencing. A sub-lethal concentration of TDA (0.6 μ M; 260 times lower than the determined MIC value of 15.6 μ M) was chosen based on repeated growth experiments (data not included). This concentration had only a very marginal effect on growth (Supplementary File 2, Figure S1) and, hence, is likely to produce a metabolic effect. Cells exposed to TDA for 30 min and 60 min showed distinct gene expression profiles compared to control groups, i.e. cells exposed to DMSO (TDA solvent) for 30 and 60 min (Figure 1). Such a difference in gene expression between TDA-treated and control groups became more pronounced with increasing exposure time (TDA vs. control at 60 min compared to TDA vs. control at 30 min). Differential gene expression analysis comparing the transcriptome of the TDA-treated cells to the controls (FDR < 0.05, absolute $\log_2$ FC > 1) revealed 164 genes which were differentially expressed (DE) at 30 min of exposure (139 up-regulated, 25 down-regulated) and 687 DE genes at 60 min of exposure (417 up-regulated, 270 down-regulated). A total of 140 genes were DE at both time points; of these, 122 were up-regulated and 18 were down-regulated by TDA exposure. A full list of the DE genes with annotations can be found in Supplementary File 2 (Supplementary Table S1).

TDA exposure induced the expression of genes related to amino acid, carbohydrate, and lipid metabolism along with genes involved in biogenesis of the cell envelope, wall, and membrane (Figure 2). The up-regulation of these genes occurred regardless of the exposure time, being observed at both time points. In contrast, genes related to energy generation and conversion were down-regulated. Of the 547 DE genes unique for time point 60 min, 3.8% were involved in cell motility (down-regulated) and 1.3% in defense

mechanisms (up-regulated). Between 41% and 46% of the annotated protein sequences across the different time points were functionally annotated as “unknown” or had no comparable hit in the eggNOG database. This may not be surprising as proteins and protein domains of unknown function encompass a large fraction of the entries in biological data repositories (Nadzirin and Firdaus-Raih, 2012). The need for accurate functional annotation tools becomes inevitable with increasing amounts of –omics data being generated, and, while some open-source candidates exist, e.g. eggNOG (Huerta-Cepas *et al.*, 2016), BlastKOALA (Kanehisa *et al.*, 2016), and PANNZER2 (Törönen *et al.*, 2018), experimental validation of these predictions are still required.

It has recently been demonstrated that TDA can act as an electroneutral proton-antiporter disrupting the PMF by the import of H⁺ ions and export of 1⁺ metal ions in *E. coli* (Wilson *et al.*, 2016). The transcriptomic assessment of *V. vulnificus* CMCP6 performed in the current study indicates that the final parts of the electron transport chain were affected by TDA exposure, as well as genes related to oxidative stress and iron starvation. Genes encoding the Cytochrome C oxidase complex IV (*ccoN*, *ccoO*, *ccoP*, *ccoQ*) were down-regulated by 2.8 to 3.0-fold at 30 min and by 4.6 to 5.3-fold at 60 min, while the cytochrome bd oxidoreductase complex encoding genes (*cydA*, *cydB*, *cydX*) were down-regulated by 2.3 to 2.6-fold at 60 min of TDA exposure (Figure 3; Supplementary File 2, Supplementary Table S1). In general, blocking the electron transport chain, and particularly the terminal oxidases, results in energy depletion and increased levels of intracellular superoxide radicals (Poole and Cook, 2000). Our findings show that a predicted oxidative stress defense protein (WP_011079481.1) as well as a superoxide dismutase (WP_011079237.1) were highly up-

regulated in the presence of TDA at both time points (FC = 13.0 to 22.6 and 2.8 to 3.2, respectively). Furthermore, a gene annotated as a tellurite resistance TerB family protein was also up-regulated in response to TDA (WP_011079743.1, FC = 5.3 at 30 min, 14.9 at 60 min) and proteins from this functional category are also known to alleviate oxidative stress (Chasteen *et al.*, 2009). Given that TDA is a bactericidal antibiotic, these observations are in line with the theory by Kohanski *et al.* (2007); for *E. coli*, they proposed that bactericidal antibiotics induce cell death by stimulating the Fenton-mediated production of reactive oxygen species (ROS) through hyperactivation of the electron transport chain. This mode of action has been confirmed for other species (Thomas *et al.*, 2013; Van Acker *et al.*, 2013), and it might explain why components of the ROS stress-response are triggered by TDA. Alternatively, the mode of action of TDA proposed by Wilson *et al.* (2016), involving the export of protons, could explain why the down-regulation of cytochrome oxidases would be counteracting the change in membrane potential. Hence, even sub-lethal concentrations of TDA with insignificant effect on bacterial growth (Supplementary File 2, Figure S1) can induce a metabolic stress response that negatively affects the PMF, though the exact molecular interactions remain uncertain.

In addition to disruption of the PMF, TDA is potentially able to chelate +1 charged metal ions in the extracellular space of *E. coli* (Wilson *et al.*, 2016). Exposure of *V. vulnificus* CMCP6 to sub-lethal levels of TDA resulted in the up-regulation of several genes that play a role in iron transport and utilization. Three out of five core genes - a peptide synthetase (WP_011081748.1), an amino acid adenylation domain-containing protein (WP_052298478.1), and an isochorismatase (WP_011081755.1) - involved in production of

the siderophore vulnibactin were up-regulated by > 2-fold after 60 min of TDA exposure (Figure 3; Supplementary File 2, Supplementary Table S1). Additionally, several iron transporters and iron utilization systems were up-regulated by 2.3 to 4.6-fold (e.g. WP_043921119.1, WP_011081918.1, WP_011081754.1, WP_011082460.1). TDA is produced under iron-enriched conditions (D'Alvise *et al.*, 2016), and is therefore not considered to be a siderophore despite its iron-chelating ability. However, TDA production by roseobacters in marine broth is accompanied by the formation of a characteristic brown pigment (Prol García *et al.*, 2014), which is a TDA-iron complex (D'Alvise *et al.*, 2016). This complex is produced as a result of TDA's capacity to chelate ferric iron (D'Alvise *et al.*, 2016). Henceforth, the iron chelating effect of TDA could potentially trigger an iron-starvation response or induce iron scavenging as a defense mechanism in *V. vulnificus*.

In *E. coli*, disruption of the PMF by TDA exposure leads to several phenotypic changes, including decreased or eliminated motility (Wilson *et al.*, 2016). Our study shows that several flagella biogenesis-related genes are down-regulated by 2.0 to 2.5-fold in *V. vulnificus* due to TDA exposure. Motility assays confirmed decreased motility of *V. vulnificus* in the presence of a sub-lethal dose of TDA (Supplementary File 2, Figure S2). Our transcriptomic data revealed that the expression of genes involved in motility-to-biofilm phenotype (e.g. the outer membrane protein OmpU - WP_011079605.1 - and pilus assembly proteins - WP_011080200.1, WP_052298469.1, WP_011081080.1; Figure 3) was increased in response to TDA exposure (FC = 4.3 to 5.7 and 3.0 to 22.6). SEM analysis showed lack of flagella as well as pili-mediated cell-cell aggregation and cell-surface attachment following TDA exposure (Figure 4). Collectively, this indicates that TDA-exposed cells could have

switched to a biofilm phenotype. The biofilm matrix produced by vibrios consists mainly of extracellular polysaccharides (EPS) (Yildiz and Visick, 2009) and *V. vulnificus* also produces capsular polysaccharides when the cell density is high (Hayat *et al.*, 1993; Lee *et al.*, 2013). Multiple genes encoding capsular polysaccharide biosynthesis proteins (WP_011080173.1 and WP_011080171.1) or polysaccharide export protein (WP_043920971.1) were up-regulated by fold change above 5.7 at both tested time points, which further supports the shift of *V. vulnificus* to a biofilm phenotype in the presence of TDA (Figure 3; Supplementary File 2, Supplementary Table S1). Biofilm formation is one of many phenotypes that enable *V. vulnificus* to survive and proliferate in a variety of ecosystems, both during infection and when naturally occurring in the environment (Jones and Oliver, 2009; Yildiz and Visick, 2009). The phenotype is regulated by quorum sensing (McDougald *et al.*, 2006; Lee *et al.*, 2013), but its induction by antibiotics is, to the best of our knowledge, not described in this species. In TDA-producing species, TDA can act as a signaling molecule at low concentrations, regulating motility, biofilm formation, and antibiotic production (Beyersmann *et al.*, 2017). Therefore, it is possible that this molecule also induces biofilm formation in other marine species, and further studies should be performed to confirm this hypothesis.

In conclusion, a sub-lethal concentration of TDA has substantial effects on the transcriptome of *V. vulnificus*, particularly altering the expression of genes associated with a range of defense responses, such as oxidative stress response and biofilm formation. Our data support the previously reported model whereby TDA acts by disrupting the PMF in *V. vulnificus*, though some cellular reactions (e.g. lack of an acid response) do not fully concur with the

effect of TDA observed in *E. coli*. This suggests that the TDA mode of action is likely more complex than currently understood.

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The authors declare no conflict of interests.

References

- Van Acker, H., Sass, A., Bazzini, S., De Roy, K., Udine, C., Messiaen, T., et al. (2013) Biofilm-Grown *Burkholderia cepacia* Complex Cells Survive Antibiotic Treatment by Avoiding Production of Reactive Oxygen Species. *PLoS One* **8**: e58943.

Beyersmann, P.G., Tomasch, J., Son, K., Stocker, R., Göker, M., Wagner-Döbler, I., et al.

(2017) Dual function of tropodithietic acid as antibiotic and signaling molecule in global gene regulation of the probiotic bacterium *Phaeobacter inhibens*. *Sci Rep* **7**: 730.

Bruhn, J.B., Nielsen, K.F., Hjelm, M., Hansen, M., Bresciani, J., Schulz, S., and Gram, L.

(2005) Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* clade. *Appl Environ Microbiol* **71**: 7263–7270.

Buchan, A., Gonzalez, J.M., and Moran, M.A. (2005) Overview of the Marine *Roseobacter* Lineage. *Appl Environ Microbiol* **71**: 5665–5677.

Chasteen, T.G., Fuentes, D.E., Tantaleán, J.C., and Vásquez, C.C. (2009) Tellurite: history, oxidative stress, and molecular mechanisms of resistance. *FEMS Microbiol Rev* **33**: 820–832.

D'Alvise, P.W., Lillebø, S., Prol-Garcia, M.J., Wergeland, H.I., Nielsen, K.F., Bergh, Ø., and Gram, L. (2012) *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. *PLoS One* **7**: e43996.

D'Alvise, P.W., Lillebø, S., Wergeland, H.I., Gram, L., and Bergh, Ø. (2013) Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparison of strains and introduction times. *Aquaculture* **384–387**: 82–86.

D'Alvise, P.W., Melchiorson, J., Porsby, C.H., Nielsen, K.F., and Gram, L. (2010) Inactivation of *Vibrio anguillarum* by attached and planktonic *Roseobacter* cells. *Appl Environ Microbiol* **76**: 2366–70.

- D'Alvise, P.W., Phippen, C.B.W., Nielsen, K.F., and Gram, L. (2016) Influence of Iron on Production of the Antibacterial Compound Tropodithietic Acid and Its Noninhibitory Analog in *Phaeobacter inhibens*. *Appl Environ Microbiol* **82**: 502–9.
- Dittmann, K.K., Sonnenschein, E.C., Egan, S., Gram, L., and Bentzon-Tilia, M. (2018) Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities. *Environ Microbiol Rep* **00**..
- González, J.M., Simó, R., Massana, R., Covert, J.S., Casamayor, E.O., Pedrós-Alió, C., and Moran, M.A. (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. *Appl Environ Microbiol* **66**: 4237–46.
- Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P.W., Dourala, N., Nielsen, K.F., and Gram, L. (2016) Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures. *Syst Appl Microbiol* **39**: 180–188.
- Harrington, C., Reen, F., Mooij, M., Stewart, F., Chabot, J.-B., Guerra, A., et al. (2014) Characterisation of Non-Autoinducing Tropodithietic Acid (TDA) Production from Marine Sponge *Pseudovibrio* Species. *Mar Drugs* **12**: 5960–5978.
- Hayat, U., Reddy, G.P., Bush, C.A., Johnson, J.A., Wright, A.C., and Morris, J.G. (1993) Capsular Types of *Vibrio vulnificus*: An Analysis of Strains from Clinical and Environmental Sources. *J Infect Dis* **168**: 758–762.
- Hjelm, M., Bergh, O., Riaza, A., Nielsen, J., Melchiorson, J., Jensen, S., et al. (2004)

- Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Syst Appl Microbiol* **27**: 360–371.
- Hjelm, M., Riaza, A., Formoso, F., Melchiorson, J., and Gram, L. (2004) Seasonal incidence of autochthonous antagonistic *Roseobacter* spp. and *Vibrionaceae* strains in a turbot larva (*Scophthalmus maximus*) rearing system. *Appl Environ Microbiol* **70**: 7288–7294.
- Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M.C., et al. (2016) EGGNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res* **44**: D286–D293.
- Jones, M.K. and Oliver, J.D. (2009) *Vibrio vulnificus*: Disease and Pathogenesis. *Infect Immun* **77**: 1723–1733.
- Kanehisa, M., Sato, Y., and Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J Mol Biol* **428**: 726–731.
- Kintaka, K., Ono, H., Tsubotani, S., Harada, S., and Okazaki, H. (1984) Thiotropocin, a new sulfur-containing 7-membered-ring antibiotic produced by a *Pseudomonas* sp. *J Antibiot (Tokyo)* **37**: 1294–1300.
- Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A., and Collins, J.J. (2007) A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics. *Cell* **130**: 797–810.

Lee, K.-J., Kim, J.-A., Hwang, W., Park, S.-J., and Lee, K.-H. (2013) Role of capsular polysaccharide (CPS) in biofilm formation and regulation of CPS production by quorum-sensing in *Vibrio vulnificus*. *Mol Microbiol* **90**: 841–857.

McDougald, D., Lin, W.H., Rice, S.A., and Kjelleberg, S. (2006) The role of quorum sensing and the effect of environmental conditions on biofilm formation by strains of *Vibrio vulnificus*. *Biofouling* **22**: 161–172.

Nadzirin, N. and Firdaus-Raih, M. (2012) Proteins of unknown function in the Protein Data Bank (PDB): an inventory of true uncharacterized proteins and computational tools for their analysis. *Int J Mol Sci* **13**: 12761–72.

Neu, A.K., Månsson, M., Gram, L., and Prol-García, M.J. (2014) Toxicity of Bioactive and Probiotic Marine Bacteria and Their Secondary Metabolites in *Artemia* sp. and *Caenorhabditis elegans* as Eukaryotic Model Organisms. *Appl Environ Microbiol* **80**: 146–153.

Planas, M., Pérez-Lorenzo, M., Hjelm, M., Gram, L., Uglenes Fiksdal, I., Bergh, Ø., and Pintado, J. (2006a) Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture* **255**: 323–333.

Planas, M., Pérez-Lorenzo, M., Hjelm, M., Gram, L., Uglenes Fiksdal, I., Bergh, Ø., and Pintado, J. (2006b) Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture* **255**: 323–333.

Poole, R.K. and Cook, G.M. (2000) Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation., pp. 165–224.

Porsby, C., Nielsen, K., and Gram, L. (2008) *Phaeobacter* and *Ruegeria* species of the *Roseobacter* clade colonize separate niches in a Danish Turbot (*Scophthalmus maximus*)-rearing farm and antagonize *Vibrio anguillarum* under different growth conditions. *Appl Environ Microbiol* **74**: 7356–7364.

Porsby, C.H., Webber, M. a, Nielsen, K.F., Piddock, L.J. V, and Gram, L. (2011) Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select. *Antimicrob Agents Chemother* **55**: 1332–7.

Prado, S., Montes, J., Romalde, J.L., and Barja, J.L. (2009) Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria. *Int Microbiol* **12**: 107–114.

Prol García, M.J., D’Alvise, P.W., Rygaard, A.M., and Gram, L. (2014) Biofilm formation is not a prerequisite for production of the antibacterial compound tropodithietic acid in *Phaeobacter inhibens* DSM17395. *J Appl Microbiol* **117**: 1592–1600.

Rabe, P., Klapschinski, T.A., Brock, N.L., Citron, C.A., D’Alvise, P., Gram, L., and Dickschat, J.S. (2014) Synthesis and bioactivity of analogues of the marine antibiotic tropodithietic acid. *Beilstein J Org Chem* **10**: 1796–801.

Rasmussen, B.B., Erner, K.E., Bentzon-Tilia, M., and Gram, L. (2018) Effect of TDA-producing *Phaeobacter inhibens* on the fish pathogen *Vibrio anguillarum* in non-axenic algae and copepod systems. *Microb Biotechnol* **11**: 1070–1079.

Rasmussen, B.B., Grotkjær, T., D'Alvise, P.W., Yin, G., Zhang, F., Bunk, B., et al. (2016)

Vibrio anguillarum is genetically and phenotypically unaffected by long-term continuous exposure to the antibacterial compound tropodithietic acid. *Appl Environ Microbiol.*

Ruiz-Ponte, C., Cilia, V., Lambert, C., and Nicolas, J.L. (1998) *Roseobacter gallaeciensis* sp. nov., a new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*. *Int J Syst Bacteriol* **48**: 537–542.

Sese, B.T., Grant, A., and Reid, B.J. (2009) Toxicity of Polycyclic Aromatic Hydrocarbons to the Nematode *Caenorhabditis elegans*. *J Toxicol Environ Heal Part A* **72**: 1168–1180.

Sonnenschein, E.C., Nielsen, K.F., D'Alvise, P., Porsby, C.H., Melchiorson, J., Heilmann, J., et al. (2017) Global occurrence and heterogeneity of the *Roseobacter*-clade species *Ruegeria mobilis*. *ISME J* **11**: 569–583.

Sprando, R.L., Olejnik, N., Cinar, H.N., and Ferguson, M. (2009) A method to rank order water soluble compounds according to their toxicity using *Caenorhabditis elegans*, a Complex Object Parametric Analyzer and Sorter, and axenic liquid media. *Food Chem Toxicol* **47**: 722–728.

Thomas, V.C., Kinkead, L.C., Janssen, A., Schaeffer, C.R., Woods, K.M., Lindgren, J.K., et al. (2013) A Dysfunctional Tricarboxylic Acid Cycle Enhances Fitness of *Staphylococcus epidermidis* During β -Lactam Stress. *MBio* **4**: e00437-13.

Thompson, F.L., Iida, T., and Swings, J. (2004) Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**: 403–31, table of contents.

- Toranzo, A.E., Magariños, B., and Romalde, J.L. (2005) A review of the main bacterial fish diseases in mariculture systems. *Aquaculture* **246**: 37–61.
- Törönen, P., Medlar, A., and Holm, L. (2018) PANNZER2: a rapid functional annotation web server. *Nucleic Acids Res* **46**: W84–W88.
- Wegner, K., Volkenborn, N., Peter, H., and Eiler, A. (2013) Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiol* **13**: 252.
- Wichmann, H., Vocke, F., Brinkhoff, T., Simon, M., Richter-Landsberg, C., Wichmann, H., et al. (2015) Cytotoxic Effects of Tropodithietic Acid on Mammalian Clonal Cell Lines of Neuronal and Glial Origin. *Mar Drugs* **13**: 7113–7123.
- Wilson, M.Z., Wang, R., Gitai, Z., and Seyedsayamdost, M.R. (2016) Mode of action and resistance studies unveil new roles for tropodithietic acid as an anticancer agent and the γ -glutamyl cycle as a proton sink. *Proc Natl Acad Sci* **113**: 1630–1635.
- Yildiz, F.H. and Visick, K.L. (2009) *Vibrio* biofilms: so much the same yet so different. *Trends Microbiol* **17**: 109–118.

Figure legends

Figure 1. Gene expression profiles of *Vibrio vulnificus* CMCP6 upon exposure to 0.6 μ M tropodithietic acid (TDA). The multi-dimensional scaling plot depicts distances as leading log-fold-changes (logFC); the root-mean-square of the largest absolute log-fold-changes between each sample pair. Circles indicate samples exposed to TDA, triangles indicate controls exposed to DMSO, the solvent of TDA. Each triplicate culture was sampled upon 30 min (green) and 60 min (blue) exposure. The plot is based on the top 500 genes.

Figure 2. Functional categories of differentially expressed genes in *Vibrio vulnificus* CMCP6 upon exposure to 0.6 μ M tropodithietic acid (TDA). The bars represent the percentage of annotated, differentially expressed genes (FDR < 0.05, absolute $\log_2$ FC > 1) upon 30 min (green) and 60 min (blue) exposure to TDA. The up-regulated genes are represented by the lighter shade of color, the down-regulated genes are represented by the darker shade of color. The functional categories were assigned using the eggNOG 4.5.1 tool (Huerta-Cepas *et al.*, 2016) on protein sequences of the annotated genes. Genes with multiple functions have been excluded from the diagram.

Figure 3. Expression levels of a set of target genes in *Vibrio vulnificus* CMCP6 upon exposure to 0.6 μ M tropodithietic acid (TDA) or solvent (DMSO) acting as control at time point 30 min and 60 min. The heatmaps visualize Z-scores calculated from the normalised

counts per million (CPM). Each treatment-time point column is based on mRNA sequencing data from three biological replicates.

Figure 4. Scanning electron microscopy of *Vibrio vulnificus* CMCP6 upon exposure to 0.03 mM TDA tropodithietic acid (TDA). A-C) Controls exposed to solvent (DMSO); D-F) Cells exposed to TDA. The scale bars are 10 μm (A and D), 4 μm (B and E), and 2 μm (C and F).