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Published in:
Applied and Environmental Microbiology

Link to article, DOI:
10.1128/aem.01439-23

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

Citation (APA):
Roager, L., Athena-Vasileiadi, D., Gram, L., & Sonnenschein, E. C. (2024). Antagonistic activity of *Phaeobacter piscinae* against the emerging fish pathogen *Vibrio crassostreae* in aquaculture feed algae. *Applied and Environmental Microbiology*, *90*(3), Article e0143923. https://doi.org/10.1128/aem.01439-23

Applied and Environmental Microbiology

| Biotechnology | Full-Length Text

Antagonistic activity of *Phaeobacter piscinae* against the emerging fish pathogen *Vibrio crassostreae* in aquaculture feed algae

Line Roager,[1] Despoina Athena-Vasileiadi,[1] Lone Gram,[1] Eva C. Sonnenschein[1,2]

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ABSTRACT Aquaculture provides a rich resource of high-quality protein; however, the production is challenged by emerging pathogens such as *Vibrio crassostreae*. While probiotic bacteria have been proposed as a sustainable solution to reduce pathogen load in aquaculture, their application requires a comprehensive assessment across the aquaculture food chain. The purpose of this study was to determine the antagonistic effect of the potential probiotic bacterium *Phaeobacter piscinae* against the emerging fish pathogen *V. crassostreae* in aquaculture feed algae that can be an entry point for pathogens in fish and shellfish aquaculture. *P. piscinae* strain S26 produces the antibacterial compound tropodithietic acid (TDA). In a plate-based assay, *P. piscinae* S26 was equally to more effective than the well-studied *Phaeobacter inhibens* DSM17395 in its inhibition of the fish pathogens *Vibrio anguillarum* 90-11-286 and *V. crassostreae* DMC-1. When co-cultured with the microalgae *Tetraselmis suecica* and *Isochrysis galbana*, *P. piscinae* S26 reduced the maximum cell density of *V. crassostreae* DMC-1 by 2 log and 3–4 log fold, respectively. A TDA-deficient mutant of *P. piscinae* S26 inhibited *V. crassostreae* DMC-1 to a lesser extent than the wild type, suggesting that the antagonistic effect involves TDA and other factors. TDA is the prime antagonistic agent of the inhibition of *V. anguillarum* 90-11-286. Comparative genomics of *V. anguillarum* 90-11-286 and *V. crassostreae* DMC-1 revealed that *V. crassostreae* DMC-1 carries a greater arsenal of antibiotic resistance genes potentially contributing to the reduced effect of TDA. In conclusion, *P. piscinae* S26 is a promising new candidate for inhibition of emerging pathogens such as *V. crassostreae* DMC-1 in algal feed systems and could contribute to a more sustainable aquaculture industry.

IMPORTANCE The globally important production of fish and shellfish in aquaculture is challenged by disease outbreaks caused by pathogens such as *Vibrio crassostreae*. These outbreaks not only lead to substantial economic loss and environmental damage, but treatment with antibiotics can also lead to antibiotic resistance affecting human health. Here, we evaluated the potential of probiotic bacteria, specifically the newly identified strain *Phaeobacter piscinae* S26, to counteract these threats in a sustainable manner. Through a systematic assessment of the antagonistic effect of *P. piscinae* S26 against *V. crassostreae* DMC-1, particularly within the context of algal feed systems, the study demonstrates the effectiveness of *P. piscinae* S26 as probiotic and thereby provides a strategic pathway for addressing disease outbreaks in aquaculture. This finding has the potential of significantly contributing to the long-term stability of the industry, highlighting the potential of probiotics as an efficient and environmentally conscious approach to safeguarding aquaculture productivity against the adverse impact of pathogens.

KEYWORDS aquaculture, probiotics, fish pathogens, microalgae

Editor Jennifer F. Biddle, University of Delaware, Newark, Delaware, USA

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The authors declare no conflict of interest.

See the funding table on p. 14.

Received 21 August 2023
Accepted 21 December 2023
Published 13 February 2024

Aquaculture has for decades been a growing industry with 87.5 million tons of high-quality fish and shellfish protein being produced in 2020 (1). However, sustainable production is challenged by the spread of disease caused by fish pathogenic bacteria (2–4). Particularly, species of the Gram-negative gammaproteobacterial Vibrionaceae family are potent pathogens including *Vibrio anguillarum*, *Vibrio harveyi*, and *Vibrio parahaemolyticus* (5, 6). Also, *Vibrio crassostreae* belonging to the *Vibrio splendidus* group has been identified as an emerging pathogen having caused disease outbreaks and mortalities of several marine aquaculture organisms (7), such as European seabass (8), sea cucumbers (9), Pacific oysters (10–12), and Yesso scallops (13). In a challenge trial with blue mussel larvae, *V. crassostreae* killed 73% of challenged mussel larvae after 5 days (14). Pathogenic *V. crassostreae* strains have been isolated from diseased farmed turbot and European seabass in Norway (15), and from turbot larvae rearing units with high mortality in Norway and Spain (2, 16, 17). One of the well-studied strains, *V. crassostreae* DMC-1 (previously *V. splendidus*), was isolated at commercial hatcheries in Galicia, Spain, from the gut of moribund turbot larvae (2, 17, 18). Fish larvae are a particularly vulnerable stage in the trophic levels of aquaculture because their immature immune system does not render vaccination an effective disease control strategy and they are exposed to fish pathogens via their live feed (19–21). Current treatments involve the usage of detergents and antibiotics, causing potential environmental harm and development and spread of antibiotic resistance; probiotic bacteria such as lactic acid bacteria, bacilli, and roseobacters have been proposed as an efficient, sustainable alternative (22–25).

Members of the marine Gram-negative alphaproteobacterial *Roseobacter* group including *Phaeobacter* and *Tritonibacter* species have been investigated as potential fish probiotics. They are promising candidates for the reduction of fish pathogens in aquaculture (24, 26–28). They have repeatedly been isolated from aquaculture systems and thus occur naturally in this environment (29, 30). They efficiently antagonize fish pathogenic vibrios in direct challenge tests and also in the presence of aquaculture-relevant biological background such as algae, rotifers, crustaceans, fish eggs, and larvae (26–28, 31, 32). They have neutral or a positive effect on these eukaryotic hosts and a minor effect on the microbiome of the hosts (26, 32, 33). Several *Phaeobacter* and *Tritonibacter* species produce the potent antibacterial agent tropodithietic acid (TDA) (33), which has been linked to the antagonistic activity of *Phaeobacter* against *Vibrio* by comparing antibacterial activity to TDA-deficient mutants (34).

The most widely researched roseobacter probiotic candidate is the strain *Phaeobacter inhibens* DSM17395 (25); however, a novel promising probiotic candidate, *Phaeobacter piscinae* S26, was isolated from a Greek sea bass larval rearing unit and characterized as belonging to the new *Phaeobacter* species, *P. piscinae* (29, 35, 36). *P. piscinae* S26 produced the highest concentration of TDA among the tested *Phaeobacter* strains, including *P. inhibens* DSM17395, and caused the highest survival of *Artemia* in *Vibrio* pathogen trials (27). The majority of fish probiotic studies have used the pathogen *V. anguillarum* as target organism; however, as outlined above, a range of other vibrios, especially *V. crassostreae*, are emerging as pathogens in marine larviculture. Using a plate-based assay, Hjelm et al. (18) screened for antagonistic bacteria against *V. crassostreae* DMC-1 and isolated the strain *P. piscinae* 27-4. During co-cultivation, *P. piscinae* 27-4 inhibited *V. crassostreae* DMC-1 by 3 log units, while in comparison, inhibition of *V. anguillarum* 90-11-287 was 6–7 log fold. Therefore, the purpose of this study was to assess the effect of the new probiotic candidate, *P. piscinae* S26, against the fish pathogenic strain, *V. crassostreae* DMC-1, as a future sustainable biocontrol alternative in aquaculture. We investigated this antagonism in the microalgal systems of *Tetraselmis suecica* and *Isochrysis galbana*, as possible targets for probiotic application as these algae are commonly used as live feed in aquaculture. Furthermore, the genome of *V. crassostreae* DMC-1 was analyzed to suggest possible genotypes for the observed inhibition by *P. piscinae* S26.

RESULTS

Antagonistic activity of probiotic *Phaeobacter* against fish pathogenic vibrios

To analyze the antagonistic properties of the new probiotic candidate strain *P. piscinae* S26 wild type (WT) against the fish pathogens *V. crassostreae* DMC-1 and *V. anguillarum* 90-11-286, its activity in plate-based assays was compared to its TDA-deficient mutant S26 Δ*tdaB*, and the probiotic candidate *P. inhibens* DSM17395 WT and its TDA-deficient mutant DSM17395 Δ*tdaB::GmR* (Table 1). Both cell-free supernatants and cell suspensions of *P. piscinae* S26 inhibited *V. crassostreae* DMC-1 (Fig. 1A) and *V. anguillarum* 90-11-286 (Fig. 1B) in the plate-based assay as shown by halos in the bacterial lawn around the well or inoculum. Both cell-free supernatant and cell suspension of *P. piscinae* S26 produced inhibition zones of 17 and 19 mm in diameter, respectively, in *V. crassostreae* DMC-1 lawn. In contrast, *P. inhibens* DSM17395 produced smaller (8 mm for cell suspension) and no inhibition (cell-free supernatant) on *V. crassostreae* DMC-1 lawn. The inhibition of *V. anguillarum* 90-11-286 by *P. piscinae* S26 and *P. inhibens* DSM17395 was similarly strong with the inhibition zones of cell-free supernatant and cell suspension of 23 and 21 mm for *P. piscinae* S26, and 21 and 20 mm for *P. inhibens* DSM17395, respectively. No inhibition zones, thus, no antibacterial effect was observed for the TDA-deficient mutants of the *Phaeobacter* strains or the media control.

Antagonistic activity of *P. piscinae* S26 against the fish pathogenic *V. crassostreae* DMC-1 in algal systems

Without addition of *P. piscinae* S26, *V. crassostreae* DMC-1 grew within 2 days from 5.2 ± 0.7 to 6.2 log CFU/mL ± 0.1 in the *I. galbana* culture and remained at this cell concentration until day 7 (Fig. 2A). Addition of both *P. piscinae* S26 WT and Δ*tdaB* inhibited the growth of *V. crassostreae* DMC-1 throughout the experiment, and the cell concentration remained around the inoculum concentration of 4.5 log CFU/mL ($P < 0.0005$ after day 0).

A similar effect of *P. piscinae* S26 against *V. crassostreae* DMC-1 was observed in the *T. suecica* culture. Without addition of *P. piscinae* S26, *V. crassostreae* DMC-1 grew within 2 days from 4.4 ± 0.04 to 6.4 ± 0.03 log CFU/mL in the *T. suecica* culture and decreased to 5.1 ± 0.5 log CFU/mL on day 8 (Fig. 2B). Both *P. piscinae* S26 WT and Δ*tdaB* inhibited the growth of *V. crassostreae* DMC-1 throughout the experiment ($P < 0.05$ after day 0, except *V. crassostreae* DMC-1 monoculture vs *V. crassostreae* DMC-1/WT co-culture on day 5 [P = 0.07]); however, *V. crassostreae* DMC-1 was still able to grow from 4.4 ± 0.03 and 4.4 ± 0.1 to 5.4 ± 0.1 and 5.5 ± 0.03 log CFU/mL in the first 2 days followed by a decline to 1.3 ± 0.3 and 2.4 ± 0.4 log CFU/mL on day 8 for *P. piscinae* S26 WT and Δ*tdaB*, respectively (Fig. 2B). The inhibition by *P. piscinae* S26 Δ*tdaB* was slightly lower in comparison to the WT (P = 0.04 on day 8).

P. piscinae S26 WT and Δ*tdaB* grew in the presence of *V. crassostreae* DMC-1 in the *I. galbana* culture from 6.5 ± 0.1 log CFU/mL to 7.5 ± 0.3 log CFU/mL in 7 days (Fig. 3A). The growth of Δ*tdaB* was delayed as indicated by significantly lower cell concentration of Δ*tdaB* in comparison to the WT on day 4 (P = 0.003).

In the *T. suecica* culture, both *P. piscinae* S26 WT and Δ*tdaB* grew in the presence of *V. crassostreae* DMC-1 from 6.1 ± 0.1 and 6.3 ± 0.2 log CFU/mL to 7.2 ± 0.1 and 7.2 ± 0.1 log CFU/mL within 1 day followed by a decline to 6.7 ± 0.1 and 6.5 ± 0.04 log CFU/mL, respectively, on day 8 (Fig. 3B).

TABLE 1 Bacterial strains used in this study

Species	Strain	Genotype	Reference
P. piscinae	S26	Wild type (WT)	(29)
P. piscinae	S26	Δ*tdaB*	(36)
P. inhibens	DSM17395	Wild type (WT)	(37, 38)
P. inhibens	DSM17395	Δ*tdaB::GmR*	(39)
V. crassostreae (formerly *V. splendidus*)	DMC-1	Wild type	(2)
V. anguillarum	90–11-286	Wild type	(40)

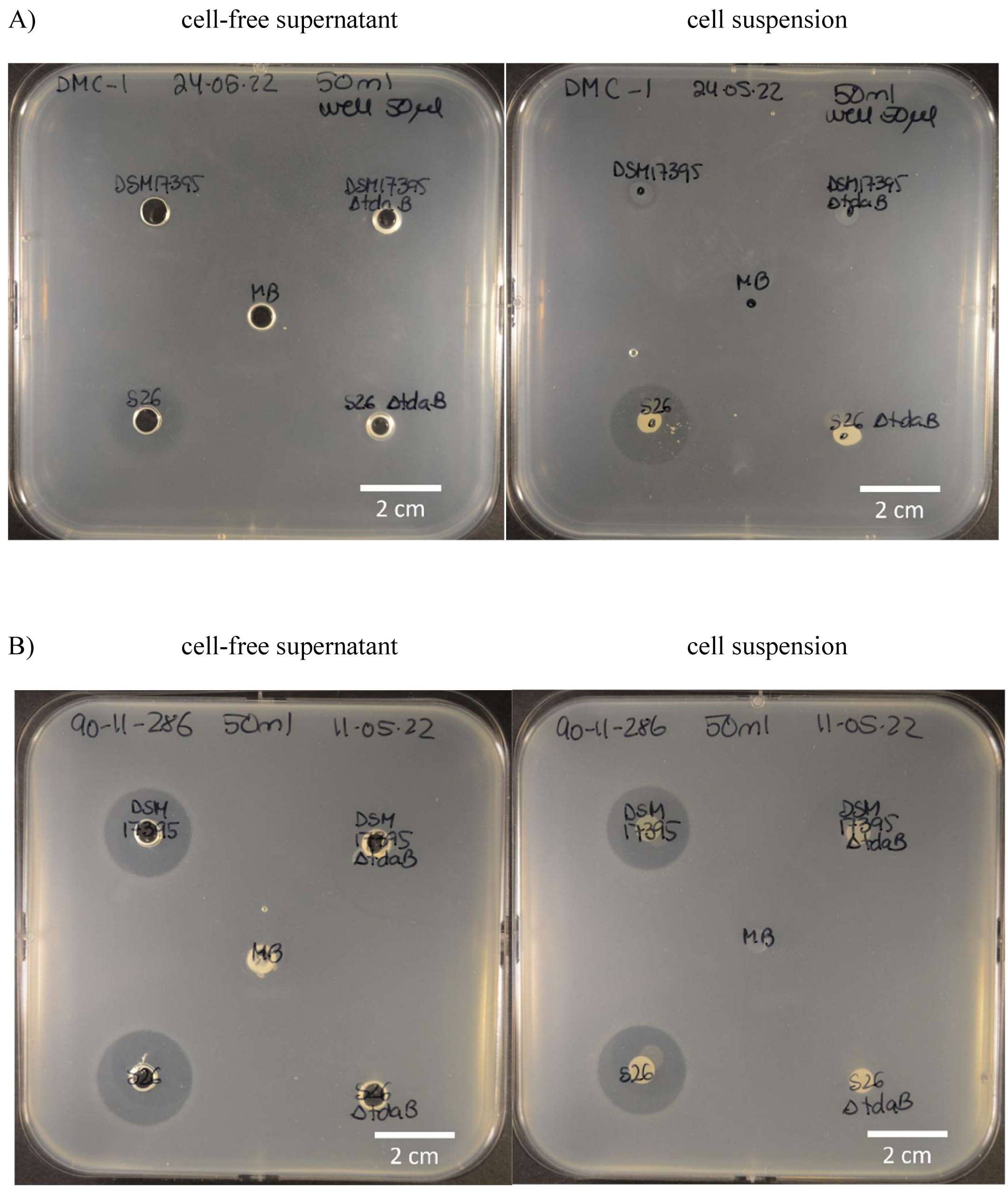

FIG 1 Plate-based antagonistic assay of cell-free supernatants and cell suspensions of probiotic *P. piscinae* S26 and *P. inhibens* DSM17395 and their TDA-deficient mutants Δ*tdaB* against the pathogenic vibrios (A) *V. crassostreae* DMC-1 and (B) *V. anguillarum* 90–11-286. Sterile MB was used as negative control.

The growth of the microalgae was generally not affected by the presence of the bacteria. *I. galbana* and *T. suecica* grew from 5.1 to 6.8 log cells/mL over 7 days and from 4.5 to 6.0 log cells/mL over 8 days ($P > 0.05$, except *I. galbana* axenic control vs *P. piscinae* S26 WT + *V. crassostreae* DMC-1 co-culture on day 2, $P = 0.02$) (Fig. 4).

Genomic analysis of *V. crassostreae* DMC-1

Although the growth of *V. crassostreae* DMC-1 was reduced by 2 log fold in the *I. galbana* and 1 log fold in the *T. suecica* system by *P. piscinae* S26, the inhibitory effect was less pronounced as previously observed for inhibition of *V. anguillarum* by *P. inhibens* DSM 17395 in algal system (25–27, 31, 32, 41). To investigate if *V. crassostreae* DMC-1 has the genetic potential to evade inhibition by *Phaeobacter* and/or TDA, we sequenced the

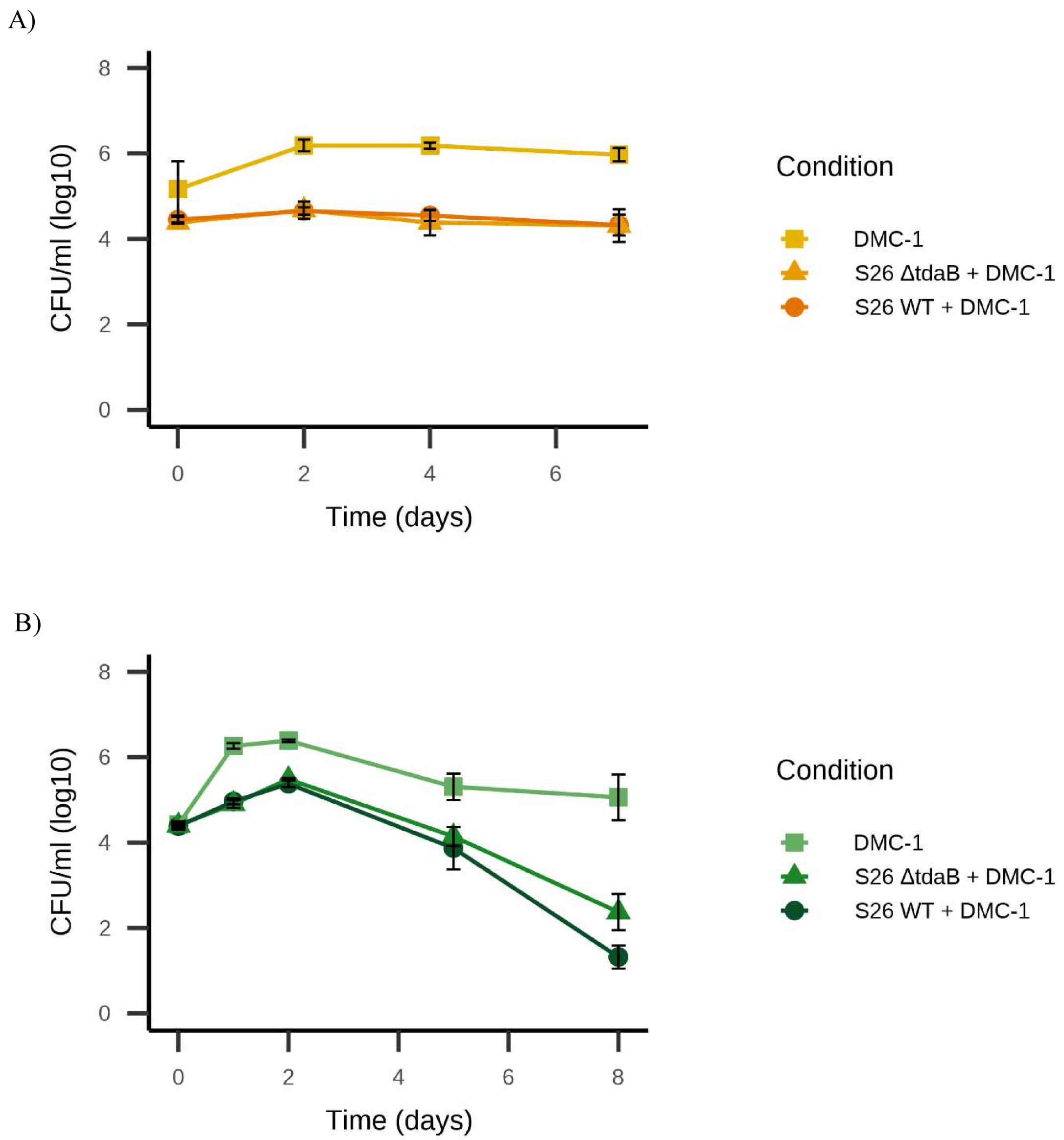

FIG 2 Growth of *V. crassostreae* DMC-1 measured as colony-forming units per milliliter over time in days in co-culture with *P. piscinae* S26 WT and Δ*tdaB* in (A) *I. galbana* and (B) *T. suecica* cultures. Condition: ● *P. piscinae* S26 WT + *V. crassostreae* DMC-1, ▲ *P. piscinae* S26 Δ*tdaB* + *V. crassostreae* DMC-1, ■ *V. crassostreae* DMC-1. *N* = 4 for *I. galbana*, *N* = 3 for *T. suecica*.

genome of *V. crassostreae* DMC-1 and compared it against the genome of *V. anguillarum* 90-11-286. Phylogenetically, the strains are not closely related within the *Vibrio* genus sharing an average nucleotide identity (ANI) of 73%. Genes that contribute to virulence and possible resistance include those encoding biosynthetic gene clusters, virulence factors, or resistance genes: using antiSMASH analysis, the genome of *V. crassostreae* DMC-1 encodes four predicted biosynthetic gene clusters (BGCs) (classified as heterocyst glycolipid synthase-like PKS [with 26% similarity to eicoseicosapentaenoic acid], arylpolyene [with 85% similarity to APE_{Vf}], betalactone, and a siderophore [with 54% similarity to vibrioferrin]), while the genome of *V. anguillarum* 90-11-286 encodes six predicted BGCs (betalactone, homoserine lactone, ectoine [with 83% similarity], NRPS-PKS [with 100% similarity to piscibactin], arylpolyene [with 95% similarity to APE_{Vf}], and an NRPS [with 100% similarity to vanchrobactin]) (Table 2). The tool ARTS detected similar genes associated with resistance in both genomes, including those encoding ABC transporter efflux pumps, MexH, MexW-MexI, glyceraldehyde

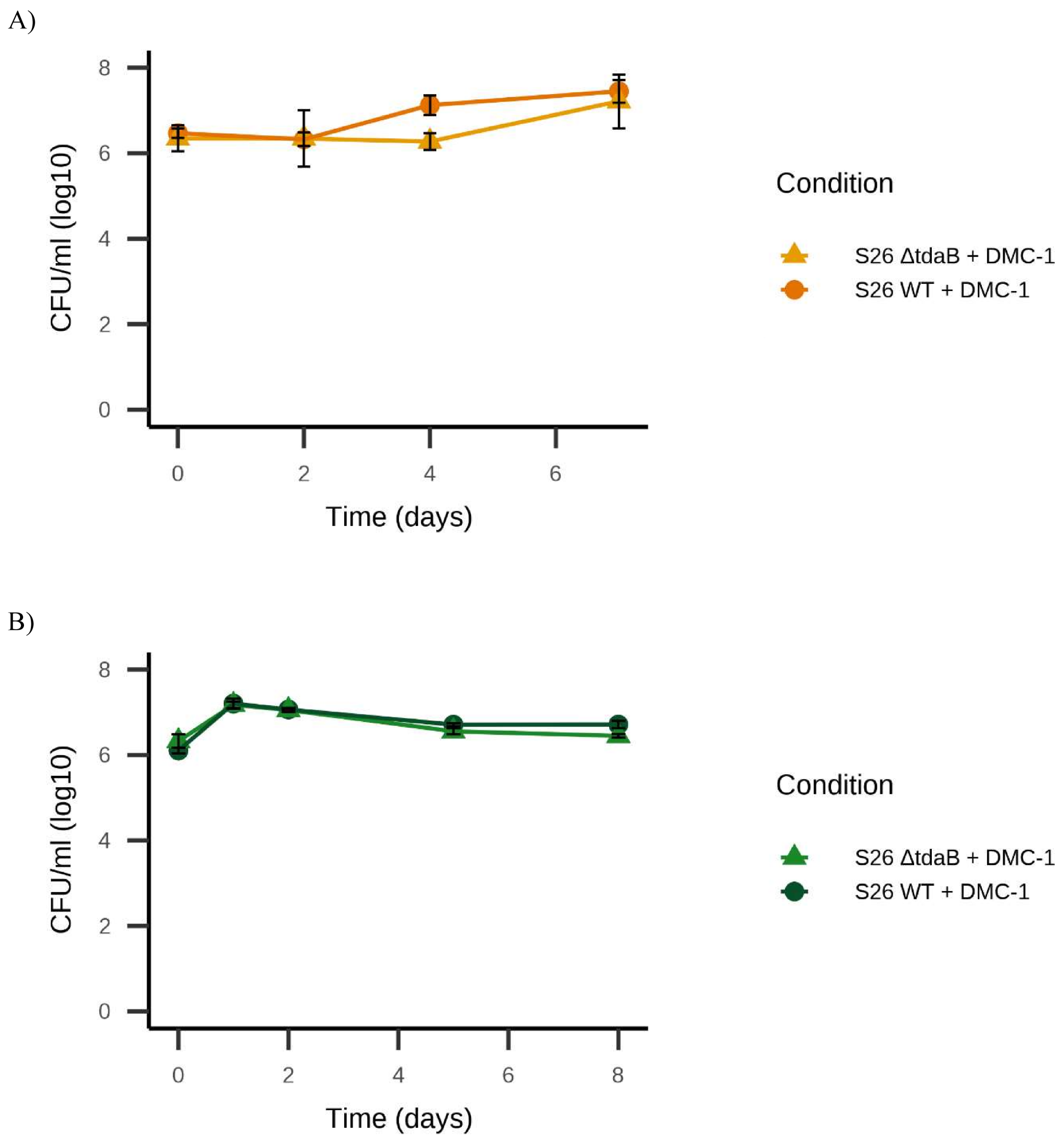

FIG 3 Growth of *P. piscinae* S26 WT and Δ*tdaB* measured as colony-forming units per milliliter over time in days in co-culture with *V. crassostreae* DMC-1 in (A) *I. galbana* and (B) *T. suecica* cultures. Condition: ● *P. piscinae* S26 WT + *V. crassostreae* DMC-1, ▲ *P. piscinae* S26 Δ*tdaB* + *V. crassostreae* DMC-1. *N* = 4 for *I. galbana*, *N* = 3 for *T. suecica*.

3-phosphate dehydrogenase, HSP90, aspartate/ornithine carbamoyltransferase, DNA gyrase B, proteasome, biotin/lipoyl attachment domain, DNA topoisomerase IV, carboxyl transferase, RpoB, and DnaN (Table 2). Additionally, the genome of *V. crassostreae* DMC-1 carried genes associated with carbenicillin-hydrolyzing betalactamase, chloramphenicol acetyltransferase, MexE, the major facilitator superfamily efflux pump, quinolone resistance, and the resistance-nodulation-division superfamily efflux pump. Analyses with ResFinder and RGI identified resistance mechanisms against tetracycline, sulfonamide, and quinolone as well as two multidrug efflux complexes (AdeFGH and MdtEF) in the genome of *V. crassostreae* DMC-1, while no dedicated antibiotic resistance gene, but one multidrug efflux complex (MdtEF), was found in *V. anguillarum* 90-11-286. Three genes, *tdaR1-3*, have been linked to TDA resistance in the producer *P. inhibens* (42); however, these genes do not have any homologues in the genomes of *V. crassostreae* DMC-1 and *V. anguillarum* 90-11-286. However, *tdaR3* encodes for gamma-glutamylcy-

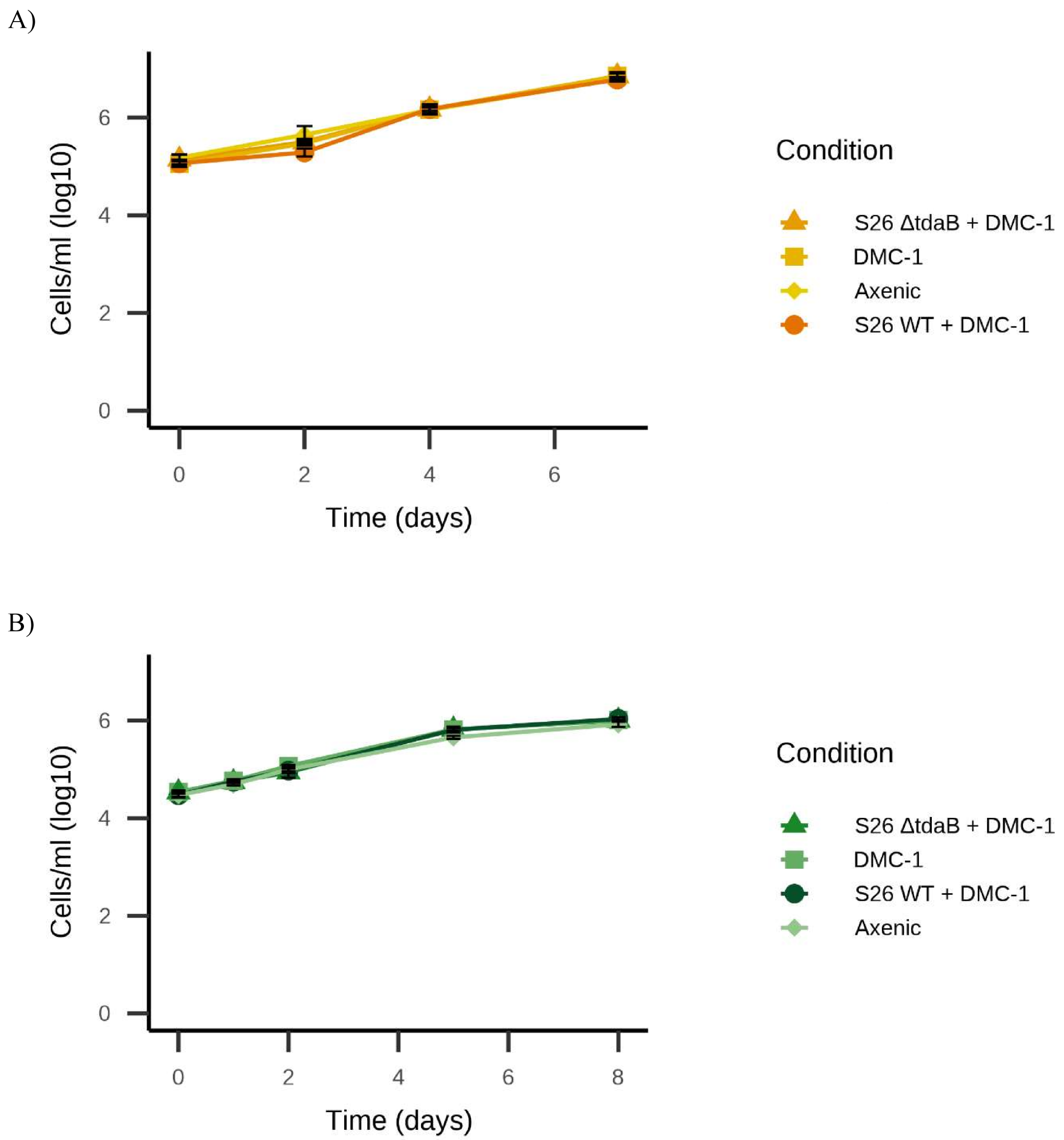

FIG 4 Average and standard deviation of concentration of algal cells per milliliter in *Phaeobacter-Vibrio* antagonistic assay for (A) *I. galbana* and (B) *T. suecica*. Condition: ● *P. piscinae* S26 WT + *V. crassostreae* DMC-1, ▲ *P. piscinae* S26 Δ*tdaB* + *V. crassostreae* DMC-1, ■ *V. crassostreae* DMC-1, ♦ axenic. *N* = 4 for *I. galbana*, *N* = 3 for *T. suecica*.

clotransferase activity, which is also predicted to be produced by YtfP encoded in the genomes of *V. crassostreae* DMC-1 and *V. anguillarum* 90-11-286.

Finally, as TDA does not appear to be the main driver of *V. crassostreae* DMC-1 inhibition in algal systems in contrast to the agar-based assay, potential metabolic competition between *Vibrio* and *Phaeobacter* was analyzed. *V. crassostreae* DMC-1 and *P. piscinae* S26 have unique genomic profiles for the degradation, utilization, and assimilation of—among others—amino acids, aromatic compounds, carbohydrates, and inorganic nutrients (Table 3). *P. piscinae* S26 has overall 19 unique full metabolic pathways for degradation, while *V. crassostreae* DMC-1 has only 10. This includes three unique pathways for the degradation of the aromatic compounds anthranilate, methyl salicylate, and salicylate in the *P. piscinae* S26's genome. Also, *P. piscinae* S26 has the unique genetic potential to degrade the sulfur-containing organic compounds dimethylsulfoniopropionate, methanesulfonate, and methyl thiopropionate. Furthermore, a major nutrient source for heterotrophic bacteria in algal systems are

TABLE 2 Secondary metabolite gene clusters and antibiotic resistance markers in the genomes of *V. crassostreae* DMC-1 and *V. anguillarum* 90–11-286 predicted by antiSMASH (43), ARTS (44), ResFinder (45), RGI, and CARD (46). Presence of antibiotic resistance hits indicated with a '+', absence with a '-'.

	V. crassostreae DMC-1		*V. anguillarum* 90–11-286	
Secondary metabolite hits				
Heterocyst glycolipid synthase-like PKS	1	26% similarity to eicoseicosapentaenoic acid	0	
Betalactone	1		1	
Homoserine lactone	0		1	
Ectoine	0		1	83% similarity to ectoine
NRPS-PKS	0		1	100% similarity to piscibactin
Arylpolyene	1	85% similarity to APE_{Vf}	1	95% similarity to APE_{Vf}
NRPS	1	54% similarity to vibrioferrin	1	100% similarity to vanchrobactin
Antibiotic resistance hits				
ABC transporter efflux pumps	+		+	
Efflux pump membrane transporter MexH	+		+	
Multidrug efflux RND transporter permease MexW-MexI	+		+	
Glyceraldehyde 3-phosphate dehydrogenase	+		+	
HSP90	+		+	
Aspartate/ornithine carbamoyltransferase	+		+	
Aspartate/ornithine carbamoyltransferase	+		+	
DNA gyrase B	+		+	
Proteasome	+		+	
Biotin/lipoyl attachment domain	+		+	
DNA topoisomerase IV	+		+	
Carboxyl transferase	+		+	
DNA-directed RNA polymerase subunit beta RpoB	+		+	
Beta sliding clamp DnaN	+		+	
Multidrug efflux complex, MdtEF	+		+	
Carbenicillin-hydrolysing betalactamase	+		–	
Chloramphenicol acetyltransferase	+		–	
MexE family multidrug efflux RND transporter periplasmic adaptor subunit	+		–	
Major facilitator superfamily efflux pump	+		–	
Quinolone resistance	+		–	
Resistance-nodulation-division superfamily efflux pump	+		–	
Tetracycline	+		–	
Sulfonamide	+		–	
Quinolone	+		–	
Multidrug efflux complex, AdeFGH	+		–	

carbohydrate exudates. Using a genomic analysis for carbohydrate-active enzymes (CAZymes) with dbCAN3, *P. piscinae* S26's genome harbors a total of 69 CAZymes including 21 glycoside hydrolases (GHs) and 42 glycosyl transferases (GTs). *V. crassostreae* DMC-1 has the potential to produce 79 CAZymes including 46 GHs and 24 GTs.

DISCUSSION

With fish pathogens such as vibrios causing significant economic loss to aquaculture systems and the need to prevent antibiotic usage, probiotic bacteria could represent a sustainable solution. For a safe application of such strains, we need to identify the specificity of their activity and test their efficiency in aquaculture-related systems. In this study, we found that the strain *P. piscinae* S26 is a promising candidate for probiotic application due to its antagonism against vibrios. This effect might even be more pronounced than for the previously tested strain *P. inhibens* DSM17395 as indicated

TABLE 3 Metabolic profiles for degradation, utilization, and assimilation encoded in the genomes of *P. piscinae* S26 and *V. crassostreae* DMC-1 analyzed with MicroScope (47)[a]

Super pathway	Pathway	*P. piscinae* S26	*V. crassostreae* DMC-1
Amino acid degradation	2-ketoglutarate dehydrogenase complex	1	1
	Alanine degradation I	0.5	1
	Alanine degradation II (to D-lactate)	0.33	1
	Alanine degradation IV	1	1
	Arginine degradation III (arginine decarboxylase/agmatinase pathway)	0.5	1
	Arginine degradation V (arginine deiminase pathway)	0.33	1
	Arginine degradation VII (arginase 3 pathway)	**1**	0
	Asparagine degradation I	1	1
	Aspartate degradation II	1	1
	Citrulline degradation	0.5	1
	D-serine degradation	0	**1**
	Glutamate degradation I	1	1
	Glutamate degradation II	0.5	1
	Glutamate degradation X	**1**	0
	Glutamine degradation I	1	1
	Glutamine degradation II	1	1
	Glycine cleavage complex	1	1
	Histidine degradation I	0.75	1
	Histidine degradation II	1	0.6
	L-cysteine degradation II	0	**1**
	L-cysteine degradation III	0.5	1
	L-serine degradation	1	1
	Methionine degradation II	**1**	0
	Ornithine degradation I (proline biosynthesis)	**1**	0
	Proline degradation	1	1
	Taurine degradation I	**1**	0
	Taurine degradation IV	0	**1**
	Threonine degradation I	0.25	1
	Threonine degradation II	1	1
	Threonine degradation IV	1	1
	Tryptophan degradation I (via anthranilate)	**1**	0
	Tryptophan degradation II (via pyruvate)	0	**1**
Aromatic compound degradation	**Anthranilate degradation II (aerobic**)	**1**	0
	Methyl salicylate degradation	**1**	0
	Phenylacetate degradation I (aerobic)	1	0.33
	Protocatechuate degradation II (ortho-cleavage pathway)	1	0.25
	Salicylate degradation I	**1**	0
C1 compound utilization and assimilation	CO_2 fixation into oxaloacetate (anapleurotic)	0.5	1
	Formaldehyde oxidation II (glutathione-dependent)	1	1
	Formaldehyde oxidation IV (thiol-independent)	**1**	0
	Formaldehyde oxidation V (tetrahydrofolate pathway)	1	1
	Formate oxidation to CO_2	1	1
Carbohydrate degradation	Acetoin degradation	0.5	1
	Chitin degradation II	0.4	1
	Chitobiose degradation	0	**1**
	D-mannose degradation	1	1
	Fructose degradation	0	**1**
	Lactose degradation III	1	1
	Melibiose degradation	1	1
	Ribose degradation	0.5	1

(*Continued on next page*)

TABLE 3 Metabolic profiles for degradation, utilization, and assimilation encoded in the genomes of *P. piscinae* S26 and *V. crassostreae* DMC-1 analyzed with MicroScope (47)[a] (*Continued*)

Super pathway	Pathway	*P. piscinae* S26	*V. crassostreae* DMC-1
	Xylose degradation I	**1**	0
Carboxylate degradation	2-methylcitrate cycle II	0.17	1
	Acetate conversion to acetyl-CoA	1	1
	Acetate formation from acetyl-CoA I	1	1
	Acetyl-CoA biosynthesis I (pyruvate dehydrogenase complex)	1	1
	D-gluconate degradation	0	**1**
	Glutaryl-CoA degradation	1	0.6
	Glycolate and glyoxylate degradation II	1	0.5
	Methylmalonyl pathway	**1**	0
Fatty acid and lipid degradation	Acetoacetate degradation (to acetyl CoA)	1	0.5
	Fatty acid beta-oxidation I	0.86	1
Inorganic nutrient metabolism	2-aminoethylphosphonate degradation I	0.33	1
	Dimethylsulfoniopropionate degradation I (cleavage)	**1**	0
	Methanesulfonate degradation	**1**	0
	Methyl thiopropionate degradation I (cleavage)	**1**	0
	Sulfate activation for sulfonation	0.5	1
	Sulfate reduction I (assimilatory)	0.75	1
	Sulfite oxidation I (sulfite oxidoreductase)	**1**	0
	Sulfoacetaldehyde degradation I	1	1
	Tetrathionate reduction I (to thiosulfate)	0	**1**
	Thiosulfate disproportionation III (rhodanese)	1	1
	Thiosulfate oxidation I (to tetrathionate)	0	**1**
	Two-component alkanesulfonate monooxygenase	1	0.5
Nucleoside and nucleotide degradation	**S-methyl-5-thioadenosine degradation II**	**1**	0
	Adenosine nucleotides degradation II	1	1
	Guanosine nucleotides degradation II	1	0.75
	Guanosine nucleotides degradation III	1	1
	Pseudouridine degradation	**1**	0
	Purine deoxyribonucleosides degradation	0.86	1
	Purine ribonucleosides degradation to ribose-1-phosphate	1	0.83
	Pyrimidine deoxyribonucleosides degradation	0.83	1
	Pyrimidine ribonucleosides degradation I	0.67	1
	Pyrimidine ribonucleosides degradation II	1	0.5
	Urate biosynthesis/inosine 5'-phosphate degradation	1	0.75
Secondary metabolite degradation	1,6-anhydro-N-acetylmuramic acid recycling	1	1
	N-acetylglucosamine degradation I	0.5	1
	N-acetylglucosamine degradation II	0.33	1
	D-galactonate degradation	1	0.67
	DIMBOA-glucoside degradation	1	1
	Mannitol degradation I	0	**1**
	Sorbitol degradation I	**1**	0

[a]Completeness of pathway indicated in the range of 0 to 1. Major differences between the strains and the corresponding pathways are highlighted in bold.

by larger inhibition zones in a plate-based inhibition assay. Indeed, Grotkjaer et al. (29) found *P. piscinae* S26 to produce higher concentrations of TDA than *P. inhibens* DSM17395, which could drive at least some of this anti-vibrio activity.

Vibrios, even within a species, may represent various levels of virulence to aquaculture organisms and carry a high genetic diversity (3, 48, 49). Similarly, vibrios have a varying level of sensitivity to the probiotic *Phaeobacter* and its bioactive compound TDA (25). This was also confirmed for *P. piscinae* S26 that inhibits both *V. anguillarum* 90-11-286 and *V. crassostreae* DMC-1; however, the latter to a lesser extent. For both targets, the activity can be attributed to the production of TDA, as no activity was observed for the TDA-deficient *P. piscinae* S26 mutants in a plate-based assay. Similarly, Hjelm et al. (18) demonstrated that the inhibitory effect of the TDA-producing strain *P. piscinae* 27-4 against *V. anguillarum* 90-11-287 was stronger over time than against *V. crassostreae* DMC-1 with a 6-log reduction in comparison to a 1-log-fold reduction after 6 days of co-cultivation.

When testing the efficacy of *P. piscinae* S26 to inhibit *V. crassostreae* DMC-1 in aquaculture-relevant algal cultures, *V. crassostreae* DMC-1 was reduced by 2 log and 3–4 log fold in *I. galbana* and *T. suecica* cultures, respectively. When Grotkjaer et al. (27) tested the activity of *P. inhibens* DSM17395 against *V. anguillarum* NB10 in xenic cultures of the algae *Dunaliella tertiolecta* and *T. suecica*, the reduction of vibrio was 3 log fold for both systems. Even more pronounced was the effect of *P. inhibens* DSM17395 against NB10 in a previous study in axenic cultures of *T. suecica* and *Nannochloropsis oculata*, which demonstrated a 3-log-fold reduction to complete elimination of the pathogen (26). The authors also observed that NB10 differed in its capability of inhabiting the two different algal systems. Although NB10 colonized *T. suecica* cells, it could only persist in dense cultures and disappeared from less dense cultures of *N. oculata*. We observed in our experiments that *V. crassostreae* DMC-1 would grow to a cell concentration of 6 log CFU/mL; however, although it could maintain this cell concentration in the *I. galbana* culture, the concentration reduced over time in the *T. suecica* culture.

Interestingly, both *P. piscinae* S26 and its TDA-deficient mutant inhibited the growth of *V. crassostreae* DMC-1 in both algal systems; however, less so for the mutant in the *T. suecica* culture. Although in direct challenge the inhibitory activity of *Phaeobacter* could be attributed to the production of TDA, previous work also demonstrated that in aquaculture-relevant systems, TDA is driving the antagonism, but does not fully explain the phenomenon (26). To obtain indications why *V. crassostreae* DMC-1 appears to be less affected by *P. piscinae* S26 than *V. anguillarum* 90-11-286 and why TDA is not the main driver of the antagonistic effect, we analyzed the *Vibrio* genomes. Although both strains are assigned to the genus *Vibrio*, they are not closely related and could accordingly have distinct differences in their metabolism, meaning that their overall fitness would be different in algal cultures. They carry a similar biosynthetic potential; however, our analyses demonstrate that *V. crassostreae* DMC-1 carries a larger arsenal of resistance genes in its genome, highlighting the need to further study this *Vibrio* species. Although the resistance mechanism to TDA has not been fully elucidated (42), gamma-glutamylcyclotransferase activity has been predicted to be involved in *Phaeobacter'*s native resistance. This activity is encoded within both genomes of *V. crassostreae* DMC-1 and *V. anguillarum* 90-11-286 and cannot therefore explain the reduced susceptibility of *V. crassostreae* DMC-1. However, *Phaeobacter* builds its native resistance by counteracting the TDA-induced proton influx with proton efflux, and our findings demonstrate that *V. crassostreae* DMC-1 has the greater ability to combat the effect of antibiotics, particularly due to any increased number of efflux pumps in comparison to *V. anguillarum* 90-11-286. Also, the complex metabolic interactions between the algae and the bacteria could lead to *P. piscinae* S26 outcompeting *V. crassostreae* DMC-1 for nutritional resources. The *P. piscinae* S26 genome carries a greater set of unique degradation, utilization, and assimilation pathways than *V. crassostreae* DMC-1, which would equip *P. piscinae* S26 with a broader adaptability to environmental nutrient sources, including those provided by microalgae. A high metabolic versatility is a generally accepted characteristic of bacteria of the *Roseobacter* group (50, 51). Specifically, *P. piscinae* S26 has the unique genetic potential to degrade the aromatic compounds anthranilate, methyl salicylate, and salicylate, which are involved in defense mechanisms and signaling in plants (52–54); however, less is known about the production and the role of these compounds by microalgae. Furthermore, *Phaeobacter* is well known for metabolizing dimethylsulfoniopropionate, a sulfur source produced by microalgae (55, 56), and an ability that was not found for *V. crassostreae* DMC-1. Additional genomic analysis identified that *P. piscinae* S26 and *V. crassostreae* DMC-1 harbor about a similar number of CAZymes; however, the *Phaeobacter* genome encodes twofold more GTs than GHs, while it is the other way around for *V. crassostreae* DMC-1. A diverse set of GTs could allow *Phaeobacter* to target a wide range of carbohydrates produced by the microalgae and could be important for its adaptation to this specific environment. Furthermore, the production of unknown antibacterial compounds by *Phaeobacter* could inhibit the growth of *V. crassostreae* DMC-1 (57–61). For instance, the algal compound dimethylsulfoniopropionate has some protective effect against TDA, which has previously been speculated to act as a protectant for eukaryotic hosts (62, 63). It is possible that similar effects are present in the systems studied here, but this remains a speculation and would need further investigation in future studies.

In conclusion, the potential of probiotic bacteria to address the economic losses caused by fish pathogens such as vibrios in aquaculture systems and the environmental burden of antibiotic and disinfectant usage holds significant promise for sustainable solutions. This study underscores the importance of specificity and efficacy testing for safe and effective application of probiotic strains. The strain *P. piscinae* S26 emerges as a strong contender for probiotic use due to its robust antagonistic activity against vibrios, potentially surpassing previously tested strains. Vibrios exhibit diverse levels of virulence and sensitivity to probiotics, which can be influenced by factors such as genetic diversity and metabolic interactions. The role of TDA as a primary driver of the antagonistic effects against vibrios is established, yet the interplay of other factors, such as resistance genes within *Vibrio* genomes and metabolic competition, demands further investigation. Future studies should investigate the intricate mechanisms underlying these interactions, shedding light on the effectiveness and limitations of probiotics in aquaculture settings, including the effect on algal products such as fatty acid composition. This will allow the development of tailored solutions capitalizing on the strengths of probiotics while navigating the complexities of aquatic ecosystems.

MATERIALS AND METHODS

Bacterial and algal strains and culturing conditions

The bacterial strains *P. piscinae* S26 wild type (WT) (29) and TDA-deficient, scarless mutant Δ*tdaB* (36), *P. inhibens* DSM17395 WT (37, 38) and TDA-deficient, insertion mutant Δ*tdaB::GmR* (39) were grown on Marine Agar (MA; Difco2216 BD) at 25°C or in Marine Broth (MB; Difco2216 BD) at 25°C and 200 rpm (Table 1). *V. crassostreae* (formerly *V. splendidus*) DMC-1 (2) and *V. anguillarum* 90-11-286 (40) were grown on MA or Tryptone Soy Agar (TSA; Sigma-Aldrich) at 25°C or in MB at 25°C and 200 rpm.

Axenic *I. galbana* CCMP 1323 and *T. suecica* CCMP 906 were obtained from the Bigelow National Center for Marine Algae and Microbiota (NCMA) and were cultivated in 3% instant ocean (IO; Instant Ocean sea salts; Aquarium Systems, Inc.) with f/2 without silicate (f/2 – Si; NCMA [64]) at 18°C and constant light at ~50 µE m^{-2} s^{-1}. Pre-cultures were plated on TSA and MA before each experiment to confirm their axenic status.

Antagonistic activity of probiotic *Phaeobacter* against fish pathogenic vibrios

The antibacterial activity of *P. piscinae* S26 WT and Δ*tdaB* and *P. inhibens* DSM17395 WT and Δ*tdaB::GmR* against *V. crassostreae* DMC-1 and *V. anguillarum* 90-11-286 was tested using an agar-based assay (65). For preparation of *V. crassostreae* DMC-1 embedded agar plates (0.1% final concentration of overnight culture), the Petri dishes were placed on ice when pouring the plates as *V. crassostreae* DMC-1 was very sensitive to the temperature of the agar. Bacterial strains were grown overnight in MB at 25°C and 200 rpm, and either 10 µL of probiotic strain was spotted on top of the *Vibrio* agar or 50 µL of sterile-filtered supernatant was suspended into wells punched into the *Vibrio* agar. Inhibition zones were measured after overnight incubation at 25°C.

Antagonistic activity of *P. piscinae* S26 against the fish pathogenic *V. crassostreae* DMC-1 in algal systems

To determine the probiotic effect of *P. piscinae* S26 and its TDA-deficient mutant Δ*tdaB* against *V. crassostreae* DMC-1 in algal cultures, four treatments were tested in the algal systems: (i) *P. piscinae* S26 WT + *V. crassostreae* DMC-1, (ii) *P. piscinae* S26 Δ*tdaB* + *V. crassostreae* DMC-1, (iii) *V. crassostreae* DMC-1, and (iv) axenic algae. Cultures were set up in biological triplicates with *T. suecica* or quadruplicates with *I. galbana*, resulting in 28 cultures in total with each culture having a volume of 50 mL prepared in a 250-mL Erlenmeyer flask. The estimated starting concentration of the algae was 10^5 algal cells/mL in 3% IO + f/2 – Si medium. *V. crassostreae* DMC-1 was added to the algal cultures at 0.1% of an overnight culture to an estimated starting concentration of 10^4 *Vibrio* cells/mL. Either 1% of *P. piscinae* S26 WT or Δ*tdaB* was added to an estimated starting concentration of 10^6 *Phaeobacter* cells/mL. All cultures were incubated at 18°C and constant light at ~50 µE m^{-2} s^{-1}, and algal and bacterial concentrations were determined on days 0, 1, 2, 5, and 8 (*T. suecica*) and 0, 2, 4, and 7 (*I. galbana*). Bacterial colony forming units were determined after 10-fold serial dilution in 3% IO and plating on TSA (*Vibrio* CFU) and MA (*Phaeobacter* CFU). Plates were incubated overnight or for 2–3 days at 25°C, respectively, before counting. For algal cell counts, samples were fixed with 1% formaldehyde and were stored at 4°C until flow cytometry on a Miltenyi MACSQuant VYB. Statistical analysis was performed with an unpaired, two-tailed Student's *t*-test.

Genomic analysis of *V. crassostreae* DMC-1

Genomic DNA was extracted from 1 mL of an overnight culture of *V. crassostreae* DMC-1 in MB using the NucleoSpin tissue kit (740952; Macherey-Nagel). DNA (114 ng/µL) was submitted to Novogene (UK) for 150 bp paired-end sequencing on a NovaSeq Illumina platform. Additionally, long reads were produced on a R9 flow cell using the MinION sequencer (Oxford Nanopore Technologies). Adapters of short reads were removed using AdapterRemoval, and ends were trimmed using fastp. The long reads were trimmed using porechop and were filtered using filtlong. Finally, the short and long reads were assembled using unicycler v0.4.7. The assembly was submitted to NCBI for annotation using the Prokaryotic Genome Annotation Pipeline (PGAP). The genome sequence has been deposited at NCBI under the accession number JAMHIT000000000. BLAST-based average nucleotide identity (ANIb) to *V. anguillarum* 90-11-286 (Genbank acc. no. GCF_001660505.2) was performed with JSpeciesWS (66). Functional traits encoded in the genomes of *V. crassostreae* DMC-1 and *V. anguillarum* 90-11-286 were identified using antiSMASH 7.0.0 (43), ARTS (44), and ResFinder 4.1 (database version 2022-05-10) (with default settings of 90% identity threshold and 60% minimum length) (45). Antibiotic resistance genes were predicted with RGI version 5.0.0 and CARD version 3.0.2 (46) on the Genoscope platform (47). Metabolic profiles of the *V. crassostreae* DMC-1 and *P. piscinae* S26 genomes for degradation, utilization, and assimilation were evaluated with the Metabolic Profile Tool using MicroCyc pathways (67) on the MicroScope platform (47), considering only pathways with a completion level of ≥1. Genomic profiles for carbohydrate degradation of *V. crassostreae* DMC-1 and *P. piscinae* S26 (Genbank acc. no. GCF_000826835.1) were analyzed using dbCAN3 (68) with HMMER- and DIAMOND-based searches. Hits were considered for comparison if recognized by both searches.

ACKNOWLEDGMENTS

The authors would like to thank Mohammad Seyedsadamdost (Princeton University) for providing the TDA-deficient mutant of *Phaeobacter inhibens* DSM17395. Thanks to Helena Falholt Elvebakken for support with Nanopore sequencing and Jette Melchiorsen for help with laboratory experiments.

This work was supported by the Danish National Research Foundation to the Center for Microbial Secondary Metabolites (DNRF137) and Novo Nordisk Foundation (grant numbers NNF20OC0064249 [EngAlMi] and NNF19OC0055625 [IMLiB]).

L.R., L.G., and E.C.S. designed the work. L.R. and D.A.V. executed the work. L.R., D.A.V., and E.C.S. analyzed the data. L.R., D.A., L.G., and E.C.S. wrote the manuscript.

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FUNDING

Funder	Grant(s)	Author(s)
Danmarks Grundforskningsfond (DNRF)	DNRF137	Lone Gram
Novo Nordisk Fonden (NNF)	NNF20OC0064249, NNF19OC0055625	Lone Gram

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DATA AVAILABILITY

The genome data of *V. crassostreae* DMC-1 is available at NCBI under the accession number JAMHIT000000000.

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