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Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures

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ABSTRACT

Fish-pathogenic *Vibrio* can cause large-scale crashes in marine larval rearing units and, since the use of antibiotics can result in bacterial antibiotic resistance, new strategies for disease prevention are needed. *Roseobacter*-clade bacteria from turbot larval rearing facilities can antagonize *Vibrio anguillarum* and reduce mortality in *V. anguillarum*-infected cod and turbot larvae. In this study, it was demonstrated that antagonistic *Roseobacter*-clade bacteria could be isolated from sea bass larval rearing units. In addition, it was shown that they not only antagonized *V. anguillarum* but also *V. harveyi*, which is the major bacterial pathogen in crustaceans and Mediterranean sea bass larvae cultures. Concomitantly, they significantly improved survival of *V. harveyi*-infected brine shrimp. 16S rRNA gene sequence homology identified the antagonists as *Phaeobacter* sp., and *in silico* DNA-DNA hybridization indicated that they could belong to a new species. The genomes contained genes involved in synthesis of the antibacterial compound tropodithietic acid (TDA), and its production was confirmed by UHPLC-TOFMS. The new *Phaeobacter* colonized live feed (*Artemia*) cultures and reduced *Vibrio* counts significantly, since they reached only 10^4 CFU mL^{-1}, as opposed to 10^8 CFU mL^{-1} in non-*Phaeobacter* treated controls. Survival of *V. anguillarum*-challenged *Artemia* nauplii was enhanced by the presence of wild type *Phaeobacter* compared to challenged control cultures ($89 \pm 1.0\%$ *vs* $8 \pm 3.2\%$). In conclusion, TDA-producing *Phaeobacter* isolated from Mediterranean marine larviculture are promising probiotic bacteria against pathogenic *Vibrio* in crustacean live-feed cultures for marine fish larvae.

Introduction

Aquaculture is the fastest growing agricultural industry, providing high-value protein-rich food for the growing world population. Currently, 50% of fish consumed are reared in aquaculture [26] and the culture of high value marine fish, crustacean and mollusk species is financially attractive. However, a major problem in aquaculture is represented by disease outbreaks caused by opportunistic pathogenic bacteria at the larval stage [6], where vaccination is not applicable. Pathogenic bacteria can be introduced into marine larviculture *via* live feed stock cultures, supply water, humans, or brood stock [24]. Fish larvae requiring live feed are mostly fed rotifers (*Brachionus plicatilis*), *Artemia* and often also live microalgae. Due to the high concentration of organic matter in *Artemia* cultures [52], bacteria can grow to high levels and may include opportunistic fish pathogens, such as the prominent fish and shellfish pathogens *Vibrio anguillarum* and *V. harveyi* [3,29,70]. High loads of pathogenic bacteria in live feed will eventually lead to infections that either spread rapidly and cause crashes of the larval population or induce a slow but steady mortality rate [63]. Such disease outbreaks constitute a major economic bottleneck in marine larviculture.

Bacterial diseases in fish larvae may be controlled by using antibiotics, either prophylactically or as acute treatment. However, since continuous use of antibiotics can result in development and spread of bacterial antibiotic resistance [13], new strategies for disease prevention are needed. Alternative treatment strategies

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include immunostimulants [7], use of bacterial quorum sensing inhibitors [20] or the use of probiotics, which are live microorganisms that exert a beneficial effect on the health of a host [25]. Many studies have addressed the potential use of probiotics in aquaculture [22,72], however, a reproducible reduction of mortalities has only occasionally been achieved [49]. Bacteria belonging to the marine *Roseobacter* clade have been isolated and/or detected in marine larviculture systems [38,42,55], and *Phaeobacter inhibens*, *P. gallaeciensis* and *Ruegeria mobilis* have been isolated due to their antagonism against pathogenic *V. anguillarum*. This antagonism is partially due to production of the antibacterial compound tropodithietic acid (TDA) [17]. Interestingly, while TDA has a broad inhibitory spectrum, resistance or tolerance in target pathogenic bacteria have not yet been detected and cannot be easily provoked, as would be the case for most other antibacterial agents [56]. Addition of *P. inhibens* to turbot or cod larvae infected with *V. anguillarum* has been shown to reduce mortality significantly, compared to infected larvae without *P. inhibens* addition [18,17,38].

Since introducing probiotic bacteria directly into larvae basins is challenging because of the large water volumes, and the fact that high pathogen concentrations are potentially already developing in the live feed cultures, we have suggested previously that the probiotics should be introduced already at the algae and rotifer culture stage where they are used as live feed [17]. Therefore, high pathogen concentrations in the live feed could be prevented and the probiotics would be present in all trophic stages of the hatchery. One purpose of the present study was to determine if pathogen reduction by *Phaeobacter* spp. would be effective in cultures of brine shrimp (*Artemia*), which are widely used as live feed for fish larvae and where high levels of organic material allow rapid growth of potentially pathogenic *Vibrio* spp., especially *V. harveyi.*

Studies on *Phaeobacter* as a fish larval probiont have been carried out in turbot and cod cultures infected with *V. anguillarum*. A range of other marine fish species, such as sea bream and sea bass, are reared under similar conditions using live feed, however, it is not known if roseobacters are part of the microbiota in this type of larval rearing. In sea bass and sea bream cultures, the temperature is often higher than in turbot and cod cultures, and *V. harveyi* is often the disease-causing pathogen [1]. Although the fish species are reared under different temperatures and water chemistry conditions, the live feed cultures (*Artemia*, algae, rotifers) are grown under similar conditions, which would allow cross-species development of feed probiotics. Therefore, a second purpose of the present study was to isolate potential probiotic bacteria from sea bass larval rearing units and evaluate if they would be effective against *V. harveyi*, which is the dominant pathogen of crustaceans, as well as sea bass and sea bream larvae.

Materials and methods

Isolation of culturable marine bacteria and testing for their antimicrobial activity

Samples for microbial analyses were taken from two Greek sea bass aquaculture units in July 2013 (38°33′48″ N 23°36′16.5″ E). Swab samples were collected from tank walls and outlets, and water samples were taken from the outlet of the fish tanks and *Artemia* basins, as well as phytoplankton and rotifer cultures. Sterile gloves, swabs and tubes were used for sampling. All samples were 10-fold serially diluted in sterile artificial sea water and plated onto marine agar (MA, Difco 2216). Plates were incubated for 5 days at 25 °C and sent by courier to Denmark. As previously described [38], the MA plates were replica plated onto Instant Ocean® agar plates (30 g L^{-1} Instant Ocean® sea salts (Aquarium Systems Inc., Sarrebourg, France), 3.33 g L^{-1} casamino acids (Bacto, France), 4 g L^{-1} glucose, and 10 g L^{-1} agar) in which *V. anguillarum* serotype O1 strain 90-11-287 [67] was embedded. A total of 120 μL of a *V. anguillarum* overnight culture, grown in marine broth (MB, Difco 2216) at 25 °C, was mixed with 120 mL of molten, cooled (41.5 °C) agar before pouring the plates. The replica plates were incubated for 24 h at 25 °C. Colonies causing clearing zones in the *V. anguillarum*-seeded agar were isolated from the original MA plate. All isolated strains were stored at −80 °C in a freeze medium [33]. The strains were re-tested for antibacterial activity, using *Phaeobacter inhibens* strain DSM 17395 as a positive control. The potential production of antibacterial compounds in liquid cultures was tested by a diffusion-inhibition assay: cultures grown for 2 days in MB were sterile-filtered (0.2 μm) and 50 μL were added to 5 mm wells punched in *V. anguillarum*-seeded agar plates that were prepared as for replica plating [38]. Plates were inspected for clearing zones after 1 and 2 days incubation at 25 °C.

Characterization of the antagonistic isolates

Biochemical tests were used to tentatively identify the isolates that had antimicrobial activity. The Gram reaction was tested using 3% KOH, catalase by exposure to 3% H_2O_2, and cytochrome oxidase using BBL™ Dryslide™ Oxidase (Becton Dickson) on cultures grown for 1 day on MA at 25 °C. The ability to ferment or oxidize glucose was tested in OF basal medium (Merck 1.10282.0500) [39] supplemented with 2% Instant Ocean® sea salts. Shape, motility, and the *Phaeobacter* indicative ability to form rosettes [10] were examined by phase-contrast microscopy (1000-fold magnification using an Olympus BH2) of cultures grown in MB for 3 days at 25 °C under static conditions. 16S rRNA gene amplicons from a subset of 13 putative *Roseobacter*-clade isolates were sequenced at GATC Biotech GA (Köln, Germany) and their phylogenetic affiliations were determined by a BLASTN search in the NCBI nucleotide collection and through analyses of the V4-V5 region, as described by Buddruhs et al. [11]. The 16S rRNA gene sequences were deposited at the NCBI under accession numbers KT884052–KT884064.

Based on survival of *Artemia* challenged with *V. harveyi* and treated with the potential probionts, two isolates were chosen for further studies (*Phaeobacter* sp. S26 and S60; see below). To investigate antagonism against *V. anguillarum* and *V. harveyi* at different temperatures, the two strains were grown without shaking in MB at 5 °C for 168 h, 10 °C for 120 h, 15 °C for 96 h, 20 °C for 72 h, 25 °C for 72 h and 30 °C for 72 h together with seven reference strains (*Phaeobacter inhibens* DSM 16374^T, *Phaeobacter inhibens* DSM 17395, *Phaeobacter gallaeciensis* DSM 26640^T, *Phaeobacter* sp. 27-4, *Ruegeria mobilis* F1926, *Pseudovibrio* sp. FO-BEG1 and *Pseudophaeobacter arcticus* DSM 23566^T). Supernatants of these cultures were obtained by centrifugation (10,000 × *g*, 5 min) and 40 μL were tested in well-diffusion assays against the target organisms embedded in Instant Ocean® agar plates. All assays were performed in biological duplicates.

Detection of tropodithietic acid (TDA)

Marine broth culture samples of the 13 *Phaeobacter* sp. strains isolated from the Greek aquaculture unit and seven reference strains (*Phaeobacter inhibens* DSM 16374^T, *Phaeobacter inhibens* DSM 17395, *Phaeobacter gallaeciensis* DSM 26640^T, *Phaeobacter* sp. 27-4, *Ruegeria mobilis* F1926, *Pseudovibrio* sp. FO-BEG1 and *Pseudophaeobacter arcticus* DSM 23566^T) were extracted with ethyl acetate (HPLC grade) containing 1% formic acid (HPLC grade). The organic phase was evaporated to dryness at 35 °C with nitrogen flow. The samples were re-dissolved in 85% acetonitrile, and liquid chromatographic-high resolution (UHPLC-TOFMS) analysis was conducted in an Agilent 1290 UHPLC coupled to an Agilent 6550