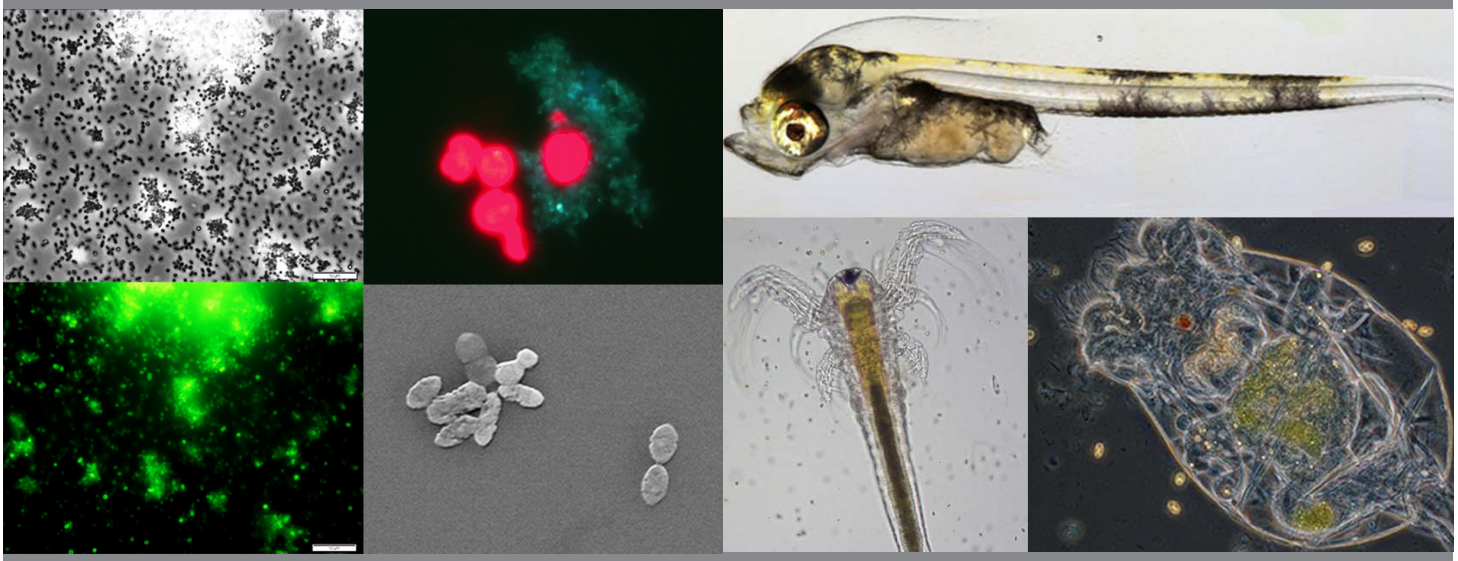
Aquaculture application and ecophysiology of marine bacteria from the *Roseobacter* clade



Paul D'Alvise
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National Food Institute

Aquaculture application and ecophysiology of marine bacteria from the *Roseobacter* clade

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Technical University of Denmark

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Division of Industrial Food Research

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Cover illustrations (from top left to bottom right):

Phase contrast micrograph of a static MB culture of *P. gallaeciensis* p*tda*C::*gfp*

Fluorescence micrograph of a co-culture of *T. suecica* and *P. gallaeciensis* p*tda*C::*gfp*

Cod larva 13 days after hatching, courtesy of J. Skadal, U. Bergen

Fluorescence micrograph of a static MB culture of *P. gallaeciensis* p*tda*C::*gfp* (same as top left)

Scanning electron micrograph of *Ruegeria mobilis* F1926 grown in static ½YTSS culture

Artemia nauplius 2 days after hatching, feeding on *T. suecica*

B. plicatilis, enriched with *T. suecica*

Preface

This PhD study has been carried out at the National Food Institute, Technical University of Denmark from October 2009 until January 2013 under the supervision of Professor Lone Gram.

The study was funded by the Danish Research Council for Technology and Production, project 09-066524 (Bioactive bacterial biofilm surfaces in aquaculture – disease prevention without antibiotics).

A part of the study was carried out in collaboration with Siril Lillebø at the University of Bergen, Norway, under the supervision of Dr. Heidrun Wergeland (University of Bergen) and Dr. Øivind Bergh (Institute for Marine Research, Bergen).

The results of this study are described in four manuscripts that are included in this thesis.

Paul D'Alvise

January 2013

Summary

Controlling bacterial disease is one of the major challenges for the global aquaculture industry. While juvenile and adult fish have a mature immune system and can be vaccinated, the early life stages, especially the larvae of marine fish and invertebrates, are prone to bacterial infections. Probiotic bacteria are a possible alternative to the use of antibiotics for preventing bacterial diseases. Marine bacteria from the *Roseobacter* clade (*α-Proteobacteria*) have shown potential as probiotic bacteria for marine larvae, and we have hypothesized that they could be used to antagonize pathogenic bacteria in the environment of the larvae.

The purpose of the present PhD study was to evaluate isolates from the genus *Phaeobacter* as probiotic bacteria for marine aquaculture, and to investigate the correlation of bacterial biofilm formation and antibacterial activity. Efficiency as probiotics was studied in cultures of microalgae, live feed organisms and fish larvae, placing an emphasis on a preventive application of the bacteria to the water. The influence of culture conditions and substrate components on biofilm formation and production of the antibacterial compound were investigated in pure cultures. Genetic and bioinformatic tools were used to construct mutants, to elucidate *in situ* gene expression, and to detect and manipulate a molecular mechanism regulating biofilm formation.

This study demonstrated that *Phaeobacter gallaeciensis* can be used to reduce numbers of the pathogen *Vibrio anguillarum* by three logarithmic units in gnotobiotic cultures of the microalgae *Tetraselmis suecica* and *Nannochloropsis oculata*. The same effect was achieved in gnotobiotic cultures of the rotifer *Brachionus plicatilis*. In challenge trials with cod (*Gadus morhua*) larvae, the probiotic bacterium was able to reduce the mortality of the larvae to the level of the untreated control. A mutant defective in production of the antibacterial compound tropodithietic acid (TDA) was included to elucidate the *in vivo* mechanism of action. The mutant was significantly less efficient at antagonizing the pathogen and protecting the larvae, indicating that TDA production makes a major contribution to the probiotic action.

The probiotic effect of different *Phaeobacter* strains and three introduction time points were compared in cod larvae challenge trials with *V. anguillarum*. Prophylactic application of the bacteria was found to be most efficient for preventing disease. The efficacy of the individual *Phaeobacter* strains correlated with their *in vitro* TDA production, as measured in a diffusion-inhibition assay, providing additional evidence for TDA as the *in vivo* mechanism of action.

TDA production is known to be most pronounced in static broth cultures, where many of the bacterial cells grow in biofilms. Most *Phaeobacter* strains produce some TDA also in shaken cultures, where they grow as single cells and multicellular aggregates, while isolates of the closely related species *Ruegeria mobilis* grow exclusively as single cells in shaken cultures and produce TDA only in static cultures. We hypothesized that attached growth in biofilms and aggregates as well as TDA production would be controlled by a central regulation mechanism. Presence of multiple genes involved in conversion of cyclic dimeric guanosinmonophosphate (c-di-GMP) indicated that motility and biofilm formation in *R. mobilis* and other *Roseobacter* clade species could be regulated via the intracellular concentration of c-di-GMP, as known from other bacteria. Indeed, chemical analyses of cellular c-di-GMP contents showed higher concentrations in static than in shaken broth cultures, and manipulation of c-di-GMP levels by introducing genes encoding enzymes for degradation or synthesis of c-di-GMP resulted in changed motility, biofilm formation, cell morphology and TDA production.

The influence of medium components on TDA production was investigated, and iron was found to be essential for antibacterial activity and production of the brown pigment. In absence of iron a non-inhibitory compound was formed, which could be converted to TDA by acid addition. Although neither this compound nor the brown pigment was structurally elucidated, there is indication that the compound is a precursor of TDA, and that iron enables or facilitates its conversion to TDA. The brown pigment may be a product of this conversion and might have a yet undiscovered ecological function.

In summary, this study demonstrates how *Phaeobacter* isolates can be used to prevent bacterial disease in marine larviculture, and elucidates some of the physiological mechanisms underlying the probiotic effect and ecological relations of these and other *Roseobacter* clade species.

Resumé

Kontrol af bakterielle sygdomme er en af de største udfordringer for den globale akvakulturindustri. Hvor fiskeyngel og voksne fisk har et fuldt immunforsvar og kan vaccineres, er især de tidlige stadier i fisks livscyklus udsatte for bakterielle infektioner. Probiotiske bakterier er et muligt alternativ til brugen af antibiotika for at forhindre bakterielle sygdomme. Marine bakterier fra *Roseobacter* cladden (*α-Proteobacteria*) har vist potentiale som probiotiske bakterier for marine larver, hvilket førte til hypotesen at de kunne anvendes til at hæmme patogene bakterier i larvernes naturlige miljø.

Formålet med dette PhD studie var at evaluere isolater af slægten *Phaeobacter* som probiotiske bakterier i marin akvakultur, og undersøge en korrelation mellem bakteriel biofilmdannelse og antibakteriel aktivitet. Den probiotiske effektivitet blev undersøgt i mikroalgekulturer, levende foderorganismer og fiskelarver med fokus på præventiv anvendelse af bakterierne i miljøet. Betydningen af dyrkningsbetingelser og enkeltkomponenter i dyrkningssubstratet for biofilmdannelse og produktionen af det antibakterielle stof blev undersøgt i renkulturer. Molekylærbiologiske metoder og bioinformatisk analyse blev anvendt til at konstruere mutanter, belyse *in situ* genekspression og førte til opdagelsen af en molekylær mekanisme involveret i reguleringen af biofilmdannelse.

Dette studie demonstrerede at *Phaeobacter gallaeciensis* kan reducere forekomsten af den patogene *Vibrio anguillarum* med tre logenheder i gnotobiotiske kulturer af mikoalgerne *Tetraselmis suecica* og *Nannochloropsis oculata*. Den samme effekt sås i gnotobiotiske kulturer af hjuldyret *Brachionus plicatilis.* I smitteforsøg med torskelarver (*Gadus morhua*) reducerede den probiotiske bakterie dødeligheden til samme niveau som den ubehandlede kontrol. En mutant uden evnen til at producere det antibakterielle stof tropodithietic acid (TDA) blev også undersøgt for at afdække *in vivo* mekanismen bag den probiotiske effekt. Mutanten var signifikant ringere til at hæmme den patogene bakterie og beskytte larverne, hvilket indikerede at produktion af TDA er en nøglefaktor i den probiotiske aktivitet.

Den probiotiske effekt af forskellige *Phaeobacter* stammer og tre introduktionstidspunkter blev sammenlignet i torskelarve smitteforsøg med *V. anguillarum*. Profylaktisk anvendelse af bakterierne viste sig at forhindre sygdom mest effektivt. Virkningsgraden af de enkelte

Phaeobacter stammer udviste korrelation med deres *in vitro* produktion af TDA målt i et diffusions-hæmningsassay, hvilket understøtter TDA produktion som *in vivo* mekanismen bag den probiotiske aktivitet.

TDA produktionen er størst i stillestående boullionkulturer, hvor mange af bakteriecellerne danner en biofilm. De fleste *Phaeobacter* stammer producerer også en vis mængde TDA i omrystede boullionkulturer, hvor de vokser både som enkeltceller og i multicellulære aggregater, mens stammer af den nært beslægtede art *Ruegeria mobilis* udelukkende vokser som enkeltceller i omrystede boullionkulturer og kun producerer TDA i stillestående boullionkulturer. Dette førte til vores hypotese at vækst i biofilm og celleaggregater samt produktion af TDA er kontrolleret af en central reguleringsmekanisme. Opdagelsen af adskillige gener involveret i behandlingen af cyclic dimeric guanosinmonophosphate (c-di-GMP) indikerede at motilitet og biofilmdannelse i *R. mobilis* og andre bakterier i *Roseobacter* claden kunne være reguleret af den intracellulære c-di-GMP koncentration som det kendes fra andre bakteriearter. Kemisk analyse af c-di-GMP niveauet i celler dyrket stillestående og omrystet viste at koncentrationen var højere ved stilestående dyrkning, ligesom manipulation af c-di-GMP niveauet opnået ved introduktion af gener, der koder for enzymer som henholdsvis nedbryder og syntetiserer c-di-GMP, resulterede i ændret bevægelighed, biofilmdannelse, cellemorfologi og TDA produktion.

Indflydelsen af substratkomponenter på TDA produktionen blev undersøgt og jern viste sig at være essentielt for dannelsen af antibakteriel aktivitet og et brunt pigment. Uden jern blev der dannet et ikke-hæmmende stof, som kunne omdannes til TDA ved at tilsætte syre. På trods af at hverken dette ikke-hæmmende stof eller det brune pigment blev strukturopklarede, er der indikationer på at stoffet er et forstadie til TDA, og at jern gør omdannelsen af stoffet til TDA mulig. Det brune pigment kan være et produkt af denne omdannelse, og har muligvis en endnu ukendt funktion i bakteriens økologi.

Dette studie demonstrerer hvorledes *Phaeobacter* stammer kan anvendes til at forhindre bakterielle sygdomme i marine larvekulturer, og afdækker nogle af de fysiologiske mekanismer bag den probiotiske effekt og økologiske rolle af disse og andre bakterier i *Roseobacter* claden.

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List of articles

1. **D'Alvise PW, Lillebø S, Prol-Garcia MJ, Wergeland HI, Nielsen KF, Bergh Ø, Gram L (2012) *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. PLoS ONE 7(8): e43996. doi:10.1371/journal.pone.0043996**

2. **D'Alvise PW, Lillebø S, Wergeland HI, Gram L, Bergh Ø (2013) Protection of cod larvae from vibriosis by *Phaeobacter* spp.: a comparison of strains and introduction times. Aquaculture, in press. doi: 10.1016/j.aquaculture.2012.12.013**

3. **D'Alvise PW, Magdenoska O, Melchiorsen J, Nielsen KF, Gram L (2013) Motility, biofilm formation and inhibitory activity in *Ruegeria mobilis* are influenced by cyclic di-GMP levels. Submitted manuscript**

4. **D'Alvise PW, Phipps R, Nielsen KF, Gram L (2013) Iron is essential for bioactivity and pigmentation of *Phaeobacter gallaeciensis* cultures. Manuscript in preparation**

1. Background, objectives and hypothesis of the PhD study

One of the great challenges of our time is providing food for a growing world population. Not only is the total demand for food increasing along with the global population, also quality demands rise as more people can afford protein-rich diets. Fish and other aquatic animals provide a substantial contribution to the world population's protein supply, and the demand for fish and shellfish is steadily increasing. The world's capture fishery production has not increased accordingly since 25 years, as more than 70% of all fisheries are maximally exploited or overexploited, fish stocks are being depleted or in the slow process of recovery from depletion, and even some formerly widespread and abundant fish species are threatened with extinction (Hutchings 2000; Musick *et al*. 2000; FAO Fisheries and Aquaculture Department 2012). Fishery alone cannot meet the demand for fish and shellfish, thus prices rise and aquaculture production becomes worthwhile. In plain terms, aquaculture is filling the gap between the overall constant output of capture fisheries and the steadily increasing demands for seafood (Figure 1).

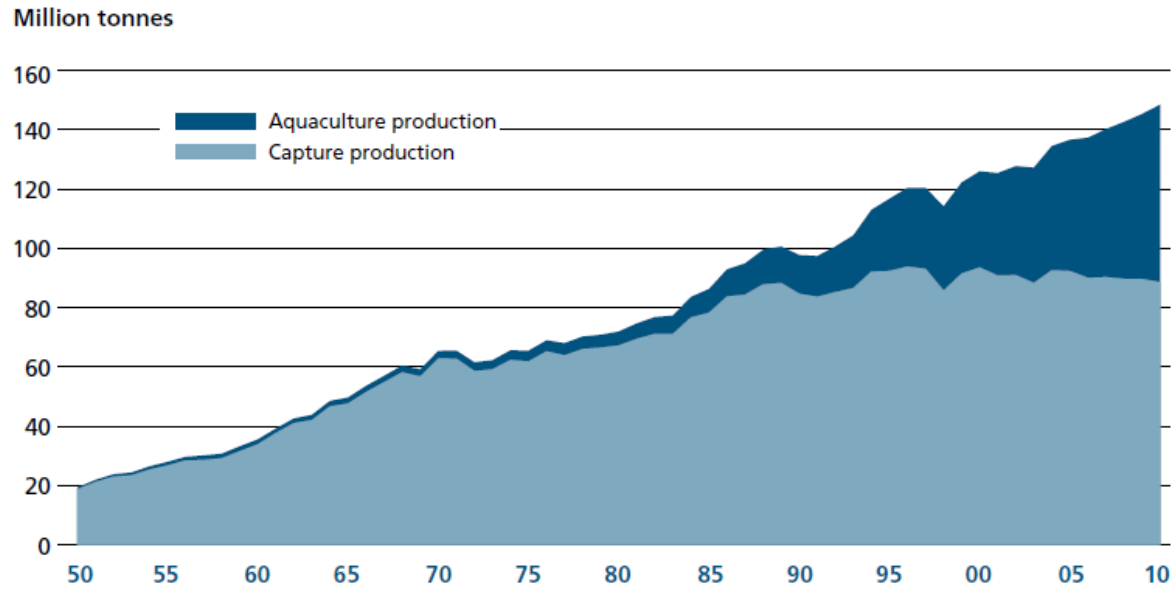

Figure 1: World capture fishery and aquaculture production. (FAO Fisheries and Aquaculture Department 2012)

While production volumes of capture fisheries remain constant, aquaculture production has increased steadily since 1970, and since the last decade, the yearly world aquaculture

production volumes (60 million tonnes) are in the same dimensions as the production volume of global fisheries dedicated to human consumption (70 mt) (FAO Fisheries and Aquaculture Department 2012). A large part of this is due to carp culture in Southeast Asia, while in the Western World salmon and trout culture take the top positions. A common feature of the traditional aquaculture species that account for the largest production volumes is that they are robust and easy to bring up, since they have relatively large eggs that develop in fresh water. However, during the last decades many new marine species were introduced to aquaculture. For example turbot (*Scophtalmus maximus*), halibut (*Hippoglossus hippoglossus*), cod (*Gadus morhua*), sea bass (*Dicentrarchus labrax*), red seabream (*Pagrus major*), Japanese amberjack (*Seriola quinqueradiata*), barramundi (*Lates calcarifer*), as well as a range of crustaceans such as the penaeid shrimps (*Penaeus* spp.), and mollusks such as abalone (*Haliotis* spp.) are now established aquaculture species. Many more are currently being evaluated for mass culture, among them truly challenging candidates such as cephalopods (e.g. *Octopus vulgaris*) or Pacific bluefin tuna (*Thunnus maccoyi*) (Garibaldi L. 1996; Fotedar R *et al*. 2011).

One of the most burning issues of marine aquaculture is poor survival of the larvae, which makes offspring production difficult, unreliable and expensive. Most marine species spawn very small eggs of around 1 mm diameter that hatch into tiny larvae, which in turn have to undergo extended periods of planktonic development. The immune system of the larvae is immature and infections with pathogenic bacteria are the major cause of larval mortality. Since the larvae require life feed such as microalgae, rotifers and *Artemia* that need to be present in high densities, levels of dissolved and particulate nutrients are high. Due to this, saprophytic and opportunistic pathogenic bacteria thrive in cultures of marine larvae and cause infections. Unlike in the oceans, infections are spread easily in larvae cultures, as an effect of the high host density. This can cause mass mortalities, where whole stocks of larvae are killed, or slow but continuous mortalities that limit production success (Figure 2)(Bolinches and Egidius 1987; Vandenberghe *et al*. 1998; Bergh *et al*. 2001; Reid *et al*. 2009).

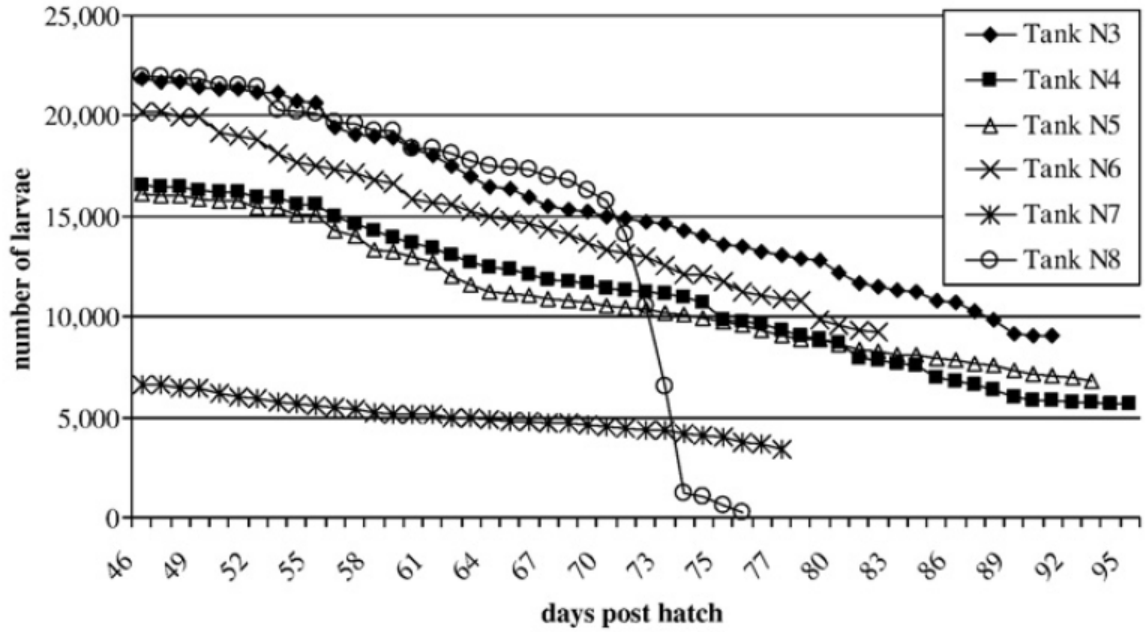

Figure 2: Mortality of cod (*Gadus morhua*) larvae in a Scottish hatchery from 46 to 95 days post hatch. All tanks initially contained 50,000 larvae, and total survival was 0 - 16 %. The mortalities in all tanks were caused by infections with *Vibrio* spp., and the mass mortality in tank N8 was due to *Vibrio anguillarum*. (Reid *et al.* 2009)

There are different approaches to mitigating the problem of bacterial infections in aquaculture. The practice of adding antibiotics to water or feed is effective in short-term perspective, but has to be avoided. Antibiotic-contaminated runoff and sludge from aquaculture has a devastating effect on ecosystems, and antibiotic use selects for resistant pathogens that make the treatment inefficient, besides posing a human health risk through spread of bacterial antibiotic resistance genes (Cabello 2006; Sapkota *et al*. 2008; Defoirdt *et al*. 2011a). A more cumbersome but sustainable approach to preventing diseases is the development of vaccines against specific pathogens, which can immensely reduce the demand for chemotherapeutics. As an example, the use of antibiotics in salmon aquaculture in Norway has decreased from 50 tonnes per year in 1987 to less than one tonne in 1997 due to development and introduction of efficient vaccines, while the production volume has increased nearly tenfold in the same period (Verschuere *et al*. 2000b). Vaccination of juvenile fish is now general and approved practice in aquaculture, although many more vaccines still need to be developed to protect the increasing number of farmed fish species from their most deleterious pathogens (Sommerset *et al*. 2005). However, vaccination can only be effective in animals that have an adaptive immune response. Fish larvae and

invertebrates lack an adaptive immune response, and rely only on their innate immune system (Chantanachookhin *et al*. 1991; Schrøder *et al*. 1998; Magnadottir 2006).

An alternative approach to reduce incidences of bacterial infections are probiotic bacteria that are live microorganisms, "which when administered in adequate amounts confer a health benefit to the host" (FAO and WHO 2001). This strategy was initially adapted from humans and other land animals, where mostly lactic acid bacteria are added to the feed to promote health and growth (Naidu *et al*. 1999; Simon *et al*. 2001; Heczko *et al*. 2006; Sans 2009). Probiotic bacteria for terrestrial animals are typically applied within the animals, as colonizers of the intestine, and this concept was transferred to the aquatic sector. Therefore, most research on probiotic bacteria in aquaculture has so far focused on intestinal bacteria (Verschuere et al. 2000b; Vine *et al*. 2006; Wang *et al*. 2008). Adding probiotic bacteria to feed or water has in a number of studies resulted in enhanced growth and increased resistance against infections with pathogenic bacteria, however the mechanism of action of the probiotics was hardly ever elucidated (Tinh *et al*. 2008a). Possible mechanism of action, as reviewed by Verschuere *et al.* (2000b) include competition for energy or nutrients, competition for space, enhancement of the host immune response, improvement of the water quality, and production of antagonistic compounds.

Aquatic animals are surrounded by water that, especially in intensive aquaculture operations, contains high concentrations of bacteria. Therefore is not only the intestinal microbiota, but also the external bacterial community highly relevant for the health of the animal (Olafsen 2001). Opportunistic pathogenic bacteria need to reach certain densities in the environment before being able to cause infections. The application of probiotic bacteria that do not colonize the intestine, but counteract pathogenic bacteria in the environment and constitute a beneficial exterior microbiota is a concept that has so far been neglected in aquaculture probiotics research.

A particularly promising group of probiotic bacteria for aquaculture are strains from the genus *Phaeobacter* of the *Roseobacter* clade (α-*Proteobacteria*), which have been isolated from different aquaculture operations in a number of studies due to their antagonistic effect against pathogenic bacteria (Ruiz-Ponte *et al*. 1998; Hjelm *et al*. 2004; Porsby *et al*. 2008). Their natural abundance in aquaculture systems in different climate zones, their excellent capacity to invade and dominate biofilms, their ability to engage in symbioses with

phytoplankton, and their pronounced antibacterial activity against *Vibrio* spp. and other aquaculture pathogens qualify them well for application as probiotics for marine larvae. Disease-preventing effects of a *Phaeobacter* sp. that were indicated in a challenge trial with turbot larvae (*Scophtalmus maximus*) confirmed this notion and encouraged further experimentation with these bacteria (Planas *et al*. 2006).

Concluding from the results of a previous study, we had hypothesized that *Phaeobacter* strains could be applied in the environment of fish larvae and their live feed cultures in order to prevent the formation of high concentrations of pathogenic bacteria (D'Alvise *et al*. 2010). The second hypothesis, that production of the antibacterial compound tropodithietic acid (TDA) may be restricted to cells growing in biofilms, was derived from the findings of an earlier study (Bruhn *et al*. 2007). The purpose of the present PhD study was (i) to optimize the application of *Phaeobacter* strains as probiotic bacteria for marine fish larvae, and (ii) to investigate how growth as biofilms influences TDA production, both with the perspective of enhancing the probiotic effect. The overall goal of this research is to provide a sustainable method for the global aquaculture industry that will reduce the prevalence of bacterial infections in larvae cultures, decrease the demand for antibiotics, and help to improve efficiency and sustainability.

This thesis contains a summary of the background and major results of the study, two published articles and two manuscripts in preparation. The published articles focus on the application of *Phaeobacter* strains as probiotics in marine larviculture, while the two unpublished manuscripts will contribute to the knowledge about the environmental physiology of biofilm formation and TDA production of *Roseobacter*-clade species.

2. Marine aquaculture

2.1 State of the industry, challenges and outlook

Aquaculture, the production of aquatic plants and animals, is the fastest-growing food-producing industry (FAO Fisheries and Aquaculture Department 2012). During the last 30 years aquaculture has expanded 12-fold with an annual growth rate of 8.8%, and has in 2010 reached a total volume of 60 million tonnes (mt - all numbers are subject to rounding) per year (FAO FIGIS 2012). A major share of global aquaculture production is covered by freshwater fish (56.4%), most notably by carp culture in China (16 mt). Marine aquaculture accounts for 38 % of the total aquaculture production, i.e. about 23 mt per year. The major share of this, 14 mt are mollusks, 4 mt are crustaceans, 4 mt are diadromous fish that reproduce in freshwater, and about 2 mt are truly marine fish. Not included in these numbers are the 19 mt of farmed marine algae that were harvested in 2010. Again, China has the world's biggest marine aquaculture production (13 mt/yr). All of Europe's marine aquaculture accounts for 2 mt/yr, of which about half dates from Norwegian salmon (*Salmo salar*) production. 185,000 tonnes of truly marine fish were produced in European aquaculture in 2010.

Marine aquaculture comprises a multitude of culture systems. The simplest form of marine aquaculture is ground culture of bivalves. Alternatively, the bivalves can be suspended in nets, on lines, or on poles into the water at a favorable location (Quayle and Newkirk 2012). Macroalgae are farmed similarly on lines or wide-mesh nets. As for fish culture, presently the most common form are floating net cages, which can contain large amounts of fish at minimal costs. Onshore tank and pond cultures are more expensive, however they are easier to access and are thus the best choice for labor-intensive cultures, such as larvae, juveniles and broodstock. Recirculating aquaculture systems are a further development of pond or tank cultures, where the water is cycled through the system and treated by mechanical and biological filters and conditioning systems. Recirculating aquaculture is a relatively new culture technique that presupposed the availability of durable technical equipment as well as biological and technical knowledge originating from wastewater treatment research (Bohl 1977).

2.1.1 Space requirements and pollution

Net cages for fish cultures are, as mentioned above, cost-efficient, but have the disadvantage that all waste products are released directly into the environment. Tanks and ponds offer the possibility of treating effluents before they are discarded into the environment, however this is seldomly practiced. Open-circuit marine pond or tank cultures are, for their demand of large quantities of water, confined to coastal sites and thus compete for the limited space in the densely inhabited, industrially utilized, or environmentally protected coastal zones. A deterrent example of the area requirements of coastal aquaculture is the destruction of vast areas of mangrove forest in Southeast Asia to make room for shrimp culture ponds. In Europe it was estimated that between 1960 and 1995 about one kilometer of natural coastline per day was developed, i.e. brought from a more or less natural state into human utilization (Airoldi and Beck 2007). This is only to the smallest extent due to aquacultural activities, however it highlights that coastal building ground is not an unlimited resource. Similarly, open cage cultures can only be sustained on a long-term basis in marine areas with strong currents or have to be frequently relocated, due to their unchecked waste release. The availability of suitable sites limits the growth of offshore aquacultures e.g. in the Norwegian salmon industry (FAO and Jones M 2004).

Recirculating aquaculture offers one possibility of moving marine aquaculture away from coastal zones. State-of-the-art marine recirculating systems need only little daily water exchange and can even be operated economically with artificial seawater if no saltwater source is available (Zohar *et al*. 2005). This enables seafood to be produced where it is consumed, allowing for short transit routes and exclusive pricing.

Dislocating cage cultures into the deep waters of the open oceans, where space is in abundant supply and waste products would be diluted to extinction, may present another possible solution to this problem. This is not an easy task, since the open ocean is a high energy environment with strong winds and steep wave patterns. Due to the long distance to the shore maintenance of open ocean systems has to be kept to a minimum for economic reasons. Nevertheless, functional model systems and commercial solutions have been developed (Figure 3), and the technology for automated feeding and remote stock control is progressing (Upton and Buck 2010).

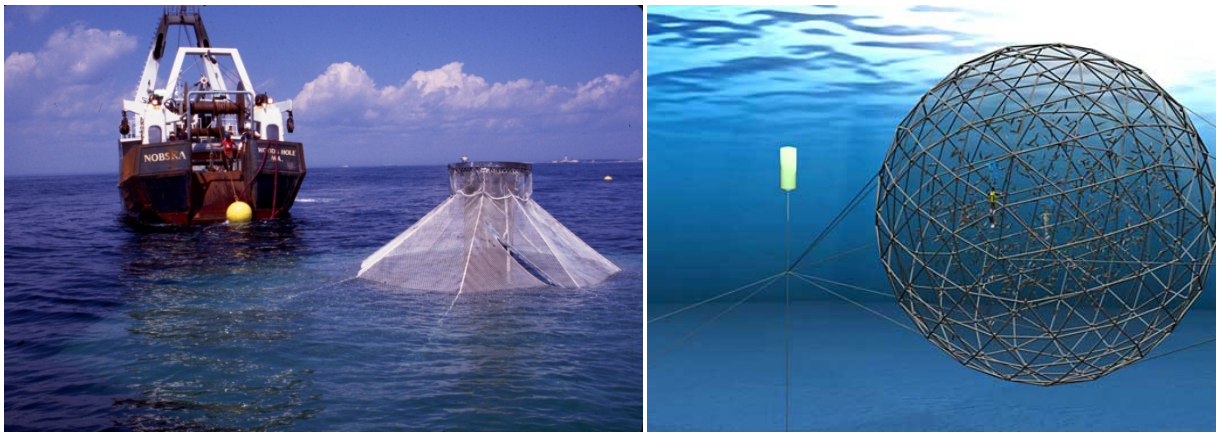

Figure 3: Experimental (left) and commercial (right) cage systems for open ocean aquaculture. (Ocean Engineering Laboratory, U. New Hampshire; Aquapod®, Ocean Farm Technologies)

Apart from structural issues, environmental pollution from aquaculture is a major problem. In some countries, local governments fail to establish or enforce environmental standards for the aquaculture industry. Uncontrolled growth of coastal aquaculture without wastewater management and unrestricted use of chemotherapeutics, such as antibiotics and copper compounds, lead to deterioration of coastal ecosystems through hypereutrophication and presents a human health hazard due to spread of resistant pathogenic bacteria and genetic resistance determinants, as exemplified by shrimp pond culture in Southeast Asia and by salmon cage culture in Chile (Gräslund and Bengtsson 2001; Martinez 2009; Cole *et al*. 2009; Buschmann *et al*. 2012). Wastewater treatment for mid- and large scale onshore aquaculture operations has to be implemented, and offshore aquacultures need to be operated within the environmental carrying capacity, in order to make coastal aquaculture sustainable (Wu 1995; Crab *et al*. 2007).

Also in respect to emission of nutrients, recirculating aquaculture systems offer a possibility for sustainable growth. The water use of recirculating systems is significantly lower than that of traditional aquaculture. Water treatment in recirculating aquaculture systems involves removal of particulate and colloidal waste, biofiltration to convert toxic ammonia to nitrate, denitrification, oxygenation, and water sterilization by ozonation and UV treatment (Timmons and Ebeling 2007). However, recirculating aquaculture systems do not reuse 100% of the water. A fraction of the water is discharged together with the concentrated solid waste as sludge, which can either be further treated, or used for energy production in biogas plants (Mirzoyan *et al*. 2010).

Another approach to reducing waste discharges is represented by integrated multi-trophic aquaculture, the culture of different species arranged in series or in close compartments, which enables one species to profit from the other species waste products and thus remove excess nutrients (Neori *et al*. 2004; Troell *et al*. 2009). As example, bivalves can be cultured in close proximity of fish cage cultures and thrive on excess feed and increased phytoplankton densities that develop in response to high nutrient levels. Also macroalgae cultures can profit from the elevated concentrations of dissolved nutrients. This concept can be realized by culturing bivalves and macroalgae next to or under floating net cages (Figure 4), or by installing algae or bivalve cultures downstream of onshore aquaculture tanks and ponds. Bivalve or algae cultures can also be integrated into recirculating systems. Advantages of integrated aquacultures are the reduced nutrient emissions and the economic value of the co-cultured crops, however often these systems are difficult to operate and diseases and parasites may be harder to control.

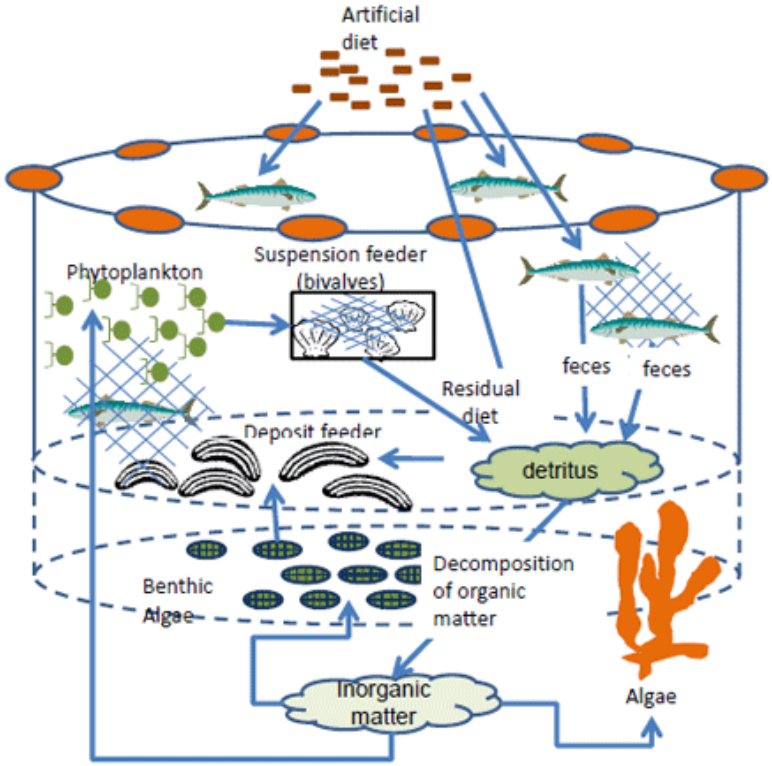

Figure 4: Schematic draft of an example for multi-trophic aquaculture, integrating the culture of bivalves, macroalgae and deposit-feeding sea cucumbers into fish cage cultures. (Japan International Research Center for Agricultural Sciences)

In order to reduce the nutrient emission of aquaculture operations, governments can set financial incentives by taxing nitrogen discharge into the environment, as practiced in Denmark.

2.1.2 Dependency of feed production on capture fisheries

One of the biggest issues that limit the sustainability of aquaculture is associated with the feed. Nearly all of the farmed high-value species are carnivorous predators that in nature feed on smaller fish and crustaceans. In captivity they have to be fed diets with high protein contents and specific amino acid profiles that resemble their natural prey in composition. Similarly, the lipid composition of the feed has to resemble that of the natural prey. High contents of highly unsaturated fatty acids (HUFA), such as docosahexaoenic acid (DHA) are required. For that reason, the main ingredients of fish feed are fish meal and fish oil, which are mainly produced from the Peruvian anchoveta (*Engraulis ringens*). Unfortunately, although this species is one of the most abundant organisms in the world, the Peruvian anchovy stocks are already maximally exploited and the catches have to be constrained since 1995 to prevent the populations from collapse (Thiel *et al*. 2012). The state of the Peruvian anchovy fishery also depends on climate conditions, since in El Niño years the cold, nutrient-rich water of the Humboldt current off the Peruvian coast is overlaid by warmer waters. This reduces the phytoplankton production in near-shore waters and thus the fish swarms disperse in response to the lower feed availability. Above that, an increasing share of the Peruvian anchovy catches is marketed as canned sardines in the recent years.

These circumstances force up the prices for fish meal and fish oil (Figure 5) and compromise the economic viability of aquaculture operations. In conclusion, aquaculture will either be limited in its further growth by the price and availability of fish meal and oil derived from dedicated fisheries, or it will have to decrease this dependency by finding ways of reducing and replacing fish meal and oil as main feed ingredients. There are different approaches to this, such as utilizing by-catch of other fisheries as source for fish meal, the use of oils from cultured microalgae and other microbial-derived feed ingredients, increasing the proportion of vegetarian proteins and lipids in feeds using agricultural by-products, the culture of fish species that can be fed inexpensive vegetarian diets, and the reduction of feed-conversion ratios by elaborate feed formulations and selective breeding (Benemann 1992; Carter and Hauler 2000; Hemre *et al*. 2002; Quinton *et al*. 2007; Turchini *et al*. 2009). However, the

dependency on fish meal and fish oil is strong due to its low price, thus most replacement strategies are, although technically feasible, not yet economically viable.

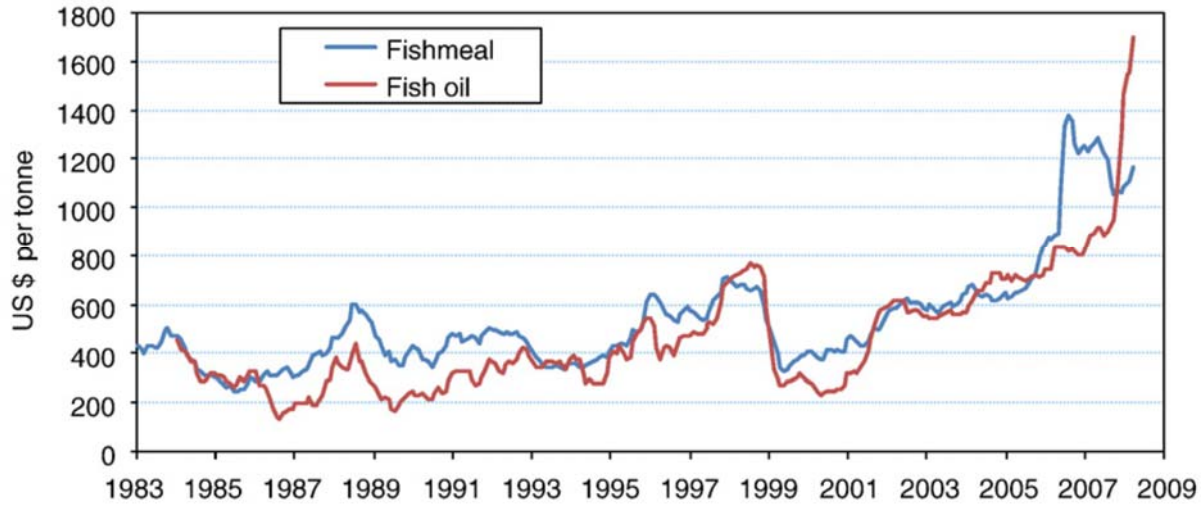

Figure 5: International market prices of fish meal and fish oil (Tacon and Metian 2008).

2.1.3 Closing of life cycles and breeding

Although reproduction in captivity of the traditional aquaculture species such as carp, trout and salmon is trivial, most of the other cultured species are more difficult to propagate and for many species the juveniles still have to be collected from the natural environment. Besides of the impacts this practice has on wild stocks, it is not an acceptable state for a growing industry, which requires reliable and season-independent offspring production practices. For another reason, selective breeding has a tremendous potential of increasing the efficiency of aquaculture. In contrast to land animal husbandry, aquaculture is still widely relying on wild type animals, and an estimated 12% of growth rate increase per generation could be yielded and disease resistance could be improved by selective breeding, as achieved in Atlantic salmon (Table 1) (Gjedrem *et al*. 2012; Moss *et al*. 2012).

Table 1: Comparison of growth rates and feed utilization in wild type and selectively bred salmon (*Salmo salar*) (Thodesen *et al*. 1999).

Performance of the selected and wild lines of Atlantic salmon, mean ± s.e.m (weight: $n = 74$; thermal growth coefficient (TGC) and feed efficiency ratio (FER): $n = 3$)

	Selected	Wild	Difference
Weight, g			
0 d	814 ± 14	533 ± 12	
84 d	1455 ± 32	743 ± 20	
TGC, × 1000			
Period I	3.41[a]	1.44	1.97 ± 0.27*
Period II	3.23[a]	1.15	2.08 ± 0.26*
Period III	2.23[b]	1.73	0.50 ± 0.19*
Total	2.96	1.39	1.57 ± 0.11*
FER, g BW increase g^{-1} feed consumed			
Period I	1.29[a]	0.94	0.36 ± 0.09*
Period II	1.18[b]	0.83	0.35 ± 0.12*
Period III	1.01[c]	0.98	0.03 ± 0.06
Total	1.16	0.93	0.23 ± 0.03*

[abc] Different superscripts indicate significant difference among experimental periods ($P < 0.05$).
* Significant difference between the lines ($P < 0.05$).

The prerequisites for selective breeding are control over pairing, the ability to maintain livestock over many years, and prolific reproduction in order to have many different individuals to select from. Control over pairing is not a problem in most fish species, since the animals do not mate naturally in captivity and gametes are attained selectively by stripping. However, for many marine species it is still challenging to reproducibly raise large quantities of offspring, since survival rates from egg to juvenile are often rather low, and even the adult broodstock is regularly affected or wiped out by outbreaks of bacterial or viral diseases (Shields 2001; Stentiford *et al*. 2012).

2.1.4 Diseases in aquaculture

Infectious microbial diseases and parasites are not only a major obstacle to closing live cycles and breeding, but also inflict tremendous economic losses on the aquaculture industry. As an example, it is estimated that more than 3 billion US$ per year are lost as an effect of infectious diseases in shrimp culture alone (Stentiford *et al.* 2012). The most prevalent diseases in aquaculture are caused by bacteria (54.9%), followed by viruses (22.6%), parasites (19.4%) and fungi (3.1%) (Kibenge *et al*. 2012).

Examples of viral diseases that can cause tremendous economic losses in marine aquaculture are the viral white spot disease in penaeid shrimp, viral hemorrhagic septicemia (VHS), red sea bream iridoviral disease, or infectious salmon anemia (ISA) (Kibenge *et al.* 2012). The main weapons of the aquaculture industry against viral diseases are vaccines, however many vaccines need yet to be developed and/or consequently applied. Prebiotic feeds, which contain compounds that stimulate the immune defense, have also shown positive results in preventing viral disease outbreaks (Ringø *et al*. 2010; Kibenge *et al.* 2012).

Also parasites are a pressing problem, especially in offshore aquaculture, as exemplified by the high incidence of sea lice in salmon aquaculture that is a problem for the production and also impacts wild fish populations. Many salmon farms are located close to estuaries of rivers with natural salmon populations, and parasites from farmed salmon, such as sea lice (*Lepeophtheirus* spp., *Caligus* spp.), can have disastrous impacts on wild salmon populations (Figure 5) (Krkosek *et al*. 2007; Johansen *et al*. 2011). However, problems with parasites can be reduced, for example ballan wrasse (*Labrus bergylta*) is now being co-cultured with Atlantic salmon, as cleaner fish to remove sea lice from the fish bodies, and even a vaccine against sea lice is being developed (Raynard *et al*. 2002; Treasurer 2002). In recirculating aquaculture many parasite problems can be solved by mechanical ultra-filtration of the rearing water, which will remove eggs and larvae of the parasites. Biosafety measures can prevent introduction of parasites, and can also reduce introduction of pathogenic viruses and bacteria in closed systems.

Figure 6: Wild pink salmon (*Oncorhynchus gorbuscha*) smolt with parasitic sea lice (*Caligus* sp.). (S. Leahy)

2.1.4.1 Bacterial diseases in aquaculture

As stated above, the largest share of infectious diseases in aquaculture is due to bacterial pathogens. Hygiene and maintaining good water quality are the most important prerequisites for preventing bacterial infections. Unfortunately but necessarily, the majority aquaculture operations rely on intensive cultures, where high nutrient levels are an intrinsic property of the systems. The rise in nutrient concentrations of the water allows for rapid bacterial growth, favoring fast-growing, opportunistic bacteria. This opportunistic microbiota can to a large fraction consist of pathogenic bacteria that will cause infections when their concentrations are high enough.

The most prevalent causative agents of bacterial infections in marine aquaculture belong to the family *Vibrionaceae* of the *γ-Proteobacteria* (Gatesoupe 1999; Toranzo *et al*. 2005; Austin 2010). The worldwide most prominent and deleterious pathogen of marine fish, and also of mollusks and crustaceans, is *Vibrio (Listonella) anguillarum*. It has the ability to persist in nutrient-free seawater for more than one year and can increase 1000-fold in coastal seawater as an effect of carbohydrate-rich wastewater discharge, which may be a reason for its global abundance (Larsen 1985; Hoff 1989). *Vibrio harveyi* has the largest impact on crustacean culture, and multi-drug resistant *V. harveyi* strains are a major problem in Asian shrimp aquacultures. Other *Vibrio* species such as *V. alginolyticus* or *V. pectenicida* are the prevalent pathogens of bivalve larvae. Outbreaks of *Photobacterium damselae* ssp. *piscicida*, another member of the *Vibrionaceae* family, were also frequently reported from Mediterranean aquacultures (Shields 2001). *V. vulnificus* and *V. parahaemolyticus* are of special concern, since they do not only commonly account for mortalities in aquaculture, but some strains can also cause serious disease in humans. Since it is the most important fish pathogen, most efforts of developing vaccines have focused on *V. anguillarum*, and efficient vaccines have been developed (Kumar *et al*. 2007; Mikkelsen *et al*. 2011). Luckily, the members of the *Vibrionaceae* family have common antigens on their outer membrane that can be used to devise vaccines efficient against a broad range of *Vibrio* spp., and also cross-protection for other bacterial pathogens such as *Aeromonas hydrophila, Edwardsiella tarda*, *Streptococcus iniae* was achieved (Li *et al*. 2010; Zhou *et al*. 2010; Sun *et al*. 2011; Sun *et al*. 2012). An overview of the significant bacterial pathogens in marine aquaculture is provided in Table 2.

Table 2: The most important bacterial pathogens in marine aquaculture

Pathogen	Host	Disease	References
Vibrio spp.	Marine fish and invertebrates	Vibriosis; skin ulcers, topical and intestinal hemorrhages, muscle lesions, exophthalmia, septicemia	(Gatesoupe 1999; Toranzo *et al.* 2005; Austin 2010)
Aeromonas spp. e.g. *A. salmonicida, A. hydrophila*	Freshwater and marine fish, especially salmonids	Furunculosis, atypical furunculosis; similar to vibriosis	(Cornick *et al*. 1984; Austin *et al*. 1998; Magnadottir *et al*. 2002; Treasurer *et al*. 2007)
Francisella spp. e.g. *F. piscicida, F. noatunensis*	Freshwater and marine fish, such as cod (*Gadus morhua*), salmon (*Salmo salar*), tilapia (*Oreochromis* sp.) and mollusks, e.g. abalone (*Haliotis* sp.)	Granulomatous inflammation, septicemia	(Ottem *et al*. 2007; Birkbeck *et al*. 2011)
Edwardsiella tarda	Freshwater and marine fish Carrier animals: invertebrates, mammals	Edwardsiellosis; abnormal swimming, exophthalmia, organomegaly, hemorrhages, etc.	(Kim *et al*. 2004; Park *et al*. 2012)
Tenacibaculum spp. (formerly *Flexibacter* spp.) e.g. *T. maritimum, T. ovolyticum*	Marine fish, e.g. red seabream (*Pagrus major*), *T. ovolyticum* is a pathogen of eggs and larvae of marine cold-water fish	Tenacibaculosis; eroded mouth syndrome, black patch necrosis	(Wakabayashi *et al*. 1986; Bergh *et al*. 1992; Hansen *et al*. 1992; Suzuki *et al*. 2001; Avendano-Herrera *et al*. 2006)
Mycobacterium spp. e.g. *M. marinum*	Many species of marine fish	Tissue damage through production of mycolactones	(Kaattari *et al*. 2006)
Streptococcus iniae	Freshwater and marine fish	Streptococcosis; exophthalmia, skin lesions, septicemia, meningoencephalitis, hemorrhages, etc.	(Pier and Madin 1976; Romalde and Toranzo 1999; Shoemaker *et al*. 2001; Agnew and Barnes 2007)
Lactococcus garvieae	Freshwater and marine fish, crustaceans	Hyperacute, hemorrhagic septicemia	(Cheng *et al*. 2003; Vendrell *et al*. 2006)

All of these most prominent pathogenic bacteria cause serious disease and high mortalities. The majority is capable of fast propagation outside their host when enough nutrients are available and can thus be ecologically classified as saprophytic opportunistic pathogens.

Before introducing to methods for prevention of bacterial disease in marine aquaculture, especially in larvae cultures, it may be worthwhile to take a look at the way marine larvae are raised, and to consider the ecological interactions within marine larviculture systems.

2.2 Marine larviculture

Marine larviculture is the production of fish, crustacean, and mollusk juveniles in seawater systems. Most marine animals have one or more early planktonic life stage after hatching, which does not yet have the physiological and morphological features of adults. The larval stage of fish (Figure 7) is basically an extension of their embryonic development that ends when all organs but the gonads are fully developed. In contrast to fish, which do not have very specialized larval stages, most invertebrate larvae, such as of mollusks and crustaceans, may be morphologically completely different from the adult animals and may undergo different distinct larval stages before their final metamorphosis into the adult form (Figure 8).

Figure 7: Cod (*Gadus morhua*) larvae, 0 and 13 days post hatch. (FAO; J. Skadal, U. Bergen)

Marine larvae require live feed such as microalgae, protists and small invertebrates to mimic their natural feed, which is phytoplankton and zooplankton. Although the practical knowledge and the technological possibilities have increased during the last decades, hatcheries are still quite defenseless against viral and bacterial infections that reduce the production efficiency and can cause mass mortalities. This can lead to shortage of juveniles

and constitutes a bottleneck for the aquaculture industry in economic terms and also regarding the closing of life cycles of new species.

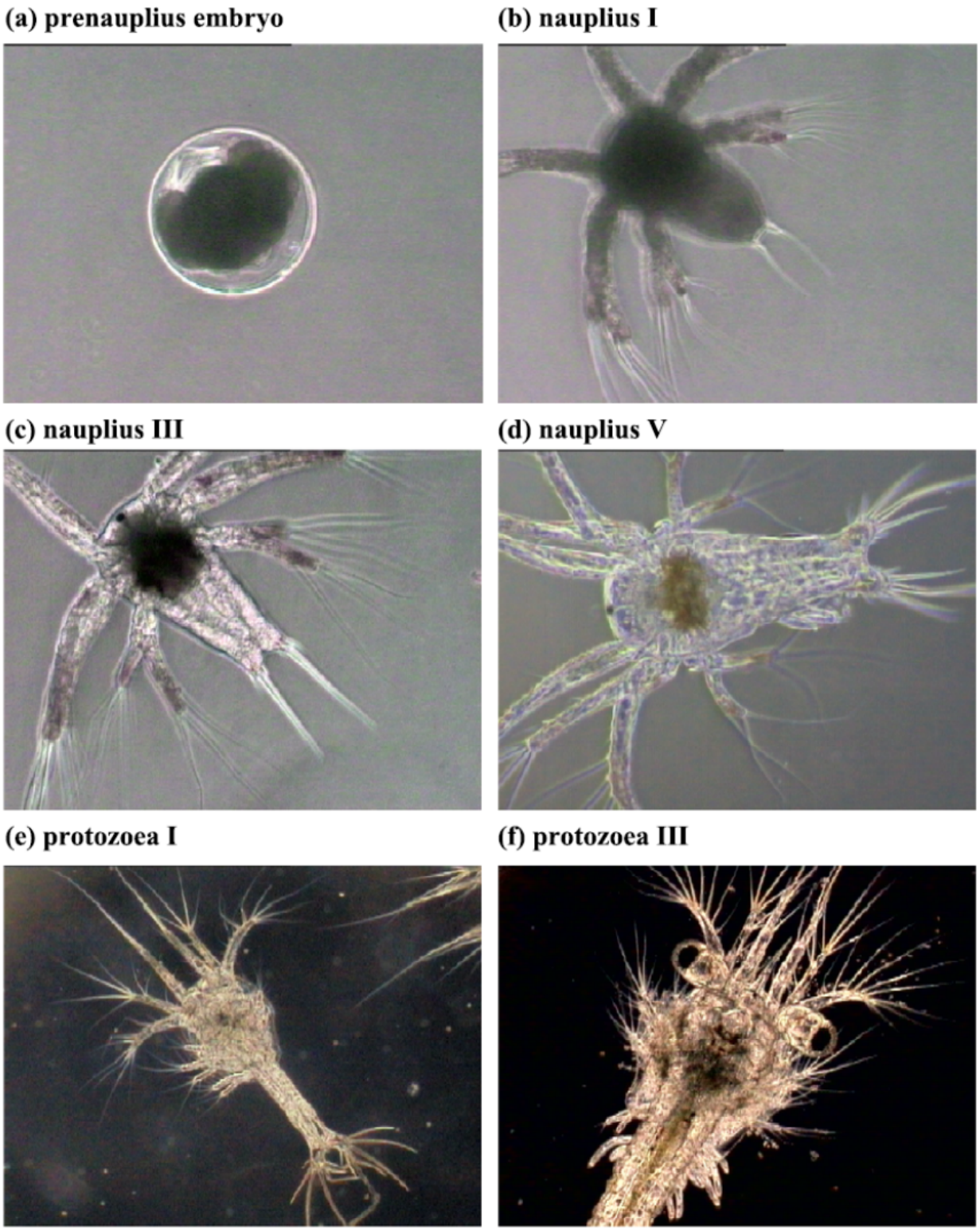

Figure 8: Different larval stages of the Pacific white shrimp *Litopenaeus vannamei*. Pictures a-d were recorded with 10-fold magnification and pictures e-f with 4-fold magnification. (Dong *et al*. 2004)

Newly hatched marine fish larvae are very small and often immature. During the first days after hatching, a yolk sac provides the larvae with nutrient and energy, and often the mouth is not yet opened during the early yolk sac phase (Evans and Claiborne 2006). After the mouth has opened, larvae start to drink and will soon begin to feed, if prey items of the right size are available (Mangor-Jensen and Adoff 1987). The period between the beginning of exogenous feeding and the end of the yolk reserves can be quite short in the larvae of many species, and critical shortage of nutrients can occur after the yolk sac has been consumed. During the last decades, major efforts were made to elucidate larval nutrition requirements (Shields 2001; Tocher *et al*. 2008). Since optimal nutrient composition and natural appearance of the diet is crucial for survival and growth of the larvae, living feed organisms are used. Most fish and crustacean larvae are carnivorous and feed on zooplankton. By far the most widely used live food for the early life stages of fish are the rotifer *Brachionus plicatilis* for small larvae, and the brine shrimp *Artemia* (e.g. *A. franciscana*) for larger larvae and early juveniles. Some hatcheries use natural zooplankton or cultured copepods as feed (Støttrup 2000), and nematodes (e.g. *Panagrellus redivivus*) are evaluated as inexpensive alternative to other live feed (Brüggemann 2012).

The rotifer *B. plicatilis* is ideally suited as prey item for first feeding for a number of reasons. Most remarkably, *B. plicatilis* has an extremely high rate of reproduction and grows well even in dense cultures. It can be fed cheap diets, such as baker's yeast (*Saccharomyces cerevisiae*) or formulated feeds. *B. plicatilis* moves slowly but steadily, so it triggers the prey capture reflexes of the larvae and yet it is easy to catch. Before being fed to the larvae, rotifers are enriched by feeding them microalgae that are rich in proteins and lipids. Also formulated preparations (e.g. SELCO – self-emulsifying concentrate, Inve Aquaculture, Belgium) are used for enrichment. Although the rotifer has a quite low nutrient content by itself, it can ingest large amounts of microalgae and thus serve as a vector to convey microalgae proteins and lipids into the larvae. Breeds of *B. plicatilis* in different sizes are available, ranging from 60 to 300 µm in length.

The brine shrimp *Artemia* is native to hypersaline waters and produces resting eggs (cysts) that are harvested in large quantities in many salt lakes all over the world. The cysts have a long shelf life and can easily be hatched by incubating them over night in aerated saltwater. The *Artemia* nauplii that hatch from the cysts can either be fed directly to the larvae, or they

can be enriched, similar to rotifers. Newly hatched *Artemia* nauplii (Instar I) are, depending on the strain, about 450 µm in length, and enriched *Artemia* (Instar II-III) are 600 – 1000 µm long, depending on the enrichment time. If larger prey organisms are needed, *Artemia* can be cultured for longer periods and reach about 1 cm in length (FAO 1996; Shields 2001).

The majority of marine larvae in aquaculture is cultured with addition of microalgae (Shields 2001; Conceicao *et al*. 2010). Microalgae are not only the essential feed for bivalves, but also carnivorous larvae, as of fish and crustaceans, thrive better with algae addition. For example, in halibut (*Hippoglossus hippoglossus*) and Californian yellowtail (*Seriola lalandi*) larvae growth and survival was enhanced in presence of microalgae (Naas *et al*. 1992; Stuart and Drawbridge 2011). This phenomenon is known as the green water effect and can be explained by different beneficial properties of the algae. A part of the effect may be due to improved nutrition of the larvae, since they start drinking shortly after hatching, thus algae are ingested. As an important factor, the nutritional value of the live feed is improved in steady presence of microalgae (Reitan *et al*. 1993; van der Meeren *et al*. 2007). Another advantage of the phytoplankton is its nutrient uptake that increases water quality, and the attenuation of light, which promotes natural feeding behavior (Naas *et al.* 1992; Øie *et al*. 1997). The main species of microalgae that are used in marine larviculture are *Nannochloropsis oculata*, *Isochrysis galbana, Tetraselmis* spp. and *Chlorella* spp., as well as a number of diatoms (e.g. *Skeletonema* spp.) for bivalves and crustaceans. However beneficial, live feed is also the main source of pathogenic bacteria that infect the larvae (Bergh *et al*. 1994; Skjermo and Vadstein 1999; Ringø and Birkbeck 1999).

As stated above, the pathogenic bacteria that cause the biggest problems in aquaculture are saprophytic opportunists, which propagate rapidly when enough nutrients are available. After hatching, larvae are quickly colonized by opportunistic bacteria, which can happen to be pathogens (Nicolas *et al*. 1989; Thomson *et al*. 2005; Reid *et al.* 2009). While stable supply of nutrients selects for a slow-growing and mainly non-pathogenic bacterial community, the rapid increase in nutrient concentrations in growing live feed cultures, and also in cultures of the larvae themselves, favor an opportunistic microflora, which can to a significant part consist of pathogenic bacteria (Munro *et al*. 1995; Skjermo *et al*. 1997; Skjermo and Vadstein 1999; Eddy and Jones 2002; Reid *et al.* 2009). The life feed organisms are relatively

insensitive towards most pathogenic bacteria, so the problem does not become visible until the contaminated feed has been fed to the larvae and mortalities occur.

2.2.1 Bacterial diseases of marine larvae

There is only a limited amount of scientific data available on bacterial diseases of marine larvae,, however intestinal infections with *Vibrio* spp. are accepted to be the most dominant problem (Bergh *et al.* 1992; Muroga 1995; Nogami *et al*. 1997; Hansen and Olafsen 1999; Olafsen 2001; Reid *et al.* 2009; Beaz-Hidalgo *et al*. 2010; Flegel 2012). The route of infection for *V. anguillarum* in turbot (*Scophtalmus maximus*) larvae was determined to be endocytosis in the intestinal epithelium of the larvae, and experiments in cod (*Gadus morhua*) larvae have confirmed this finding (Figure 9) (Grisez *et al*. 1996; Engelsen *et al*. 2008).

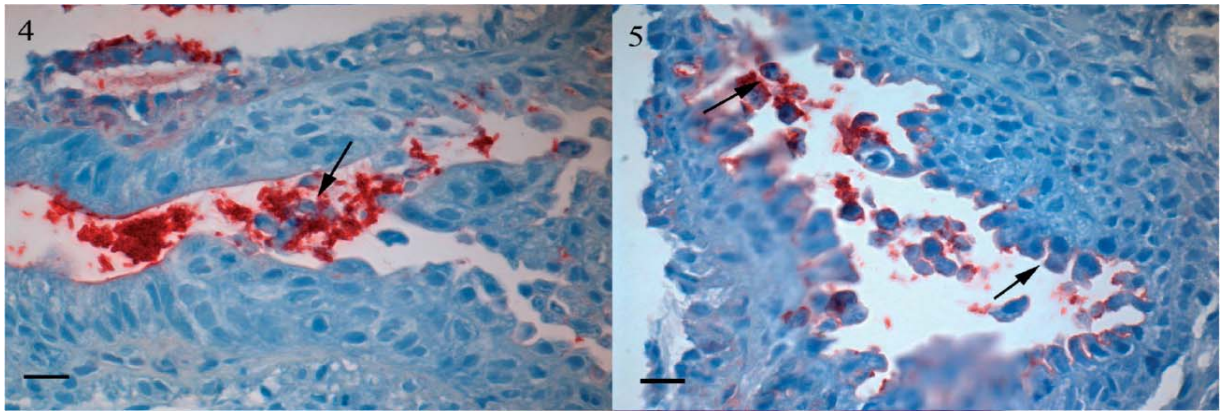

Figure 9: Immunohistochemical sections of cod (*Gadus morhua*) larvae intestines 4 days post hatch. The larvae were infected with *Vibrio anguillarum* HI-610, and the red color indicates *V. anguillarum* cells stained with specific rabbit antibodies, and the sections were counterstained with Shandon haematoxilin (blue). The pathogen is present in large numbers on the intestinal epithelium, associated with necrotic epithelial cells (arrows) and is about to penetrate the epithelium. (Engelsen *et al.* 2008)

Besides *Vibrio* spp., *Aeromonas salmonicida*, *Edwardsiella tarda,* and the egg pathogen *Tenacibaculum ovolyticum* (formerly: *Flexibacter ovolyticus*) were shown to infect fish larvae (Hansen *et al.* 1992; Bergh *et al*. 1997; Hansen and Olafsen 1999; Kim *et al.* 2004). A great diversity of bacteria, e.g. *Vibrio* spp., *Pseudomonas* spp., *Alteromonas* spp., *Flavobacterium* spp., *Cytophaga* spp., *Lactococcus* spp., *Bacillus* spp., have been isolated from larval fish of various species, however for most of them there is no indication of pathogenic properties (Hansen and Olafsen 1999). In order to actually identify bacteria as larval pathogens, studies should, in theory, prove Koch's postulates (the pathogen has to be isolated from diseased

organisms, cause the same disease in healthy organisms, and has to be re-isolated or detected in the host), however this is tedious and was only rarely accomplished.

Although not restricted to larvae, it should be mentioned that relatives of the bacteria from the *Roseobacter* clade (see chapter 3) that were used in this study to control fish pathogens were reported to cause disease in juvenile Pacific oyster (*Crassostrea virginiae*), which could be prevented by colonization of the oysters by another *Roseobacter* clade bacterium indentified as *Stappia stellulata* (Boettcher *et al*. 2000). A similar bacterium from the *Roseobacter* clade was associated with black band disease in corals, however no etiological experiments were conducted (Cooney *et al*. 2002; Pantos *et al*. 2003).

Although there is a growing body of scientific knowledge about bacterial pathogens in fish larvae, the global extent of the problem is hard to estimate. Infections are often difficult to spot, as infected larvae do not develop pronounced visible symptoms before dying. Bacterial infections do not necessarily result in mass mortalities, but can also lead to slow but continuous mortalities that are overlooked or accepted as normal (Reid *et al.* 2009). The reason for larval mortalities in commercial hatcheries is commonly not identified. Even if a bacterial infection is diagnosed, this may be interpreted as the result of increased susceptibility of the larvae that may have been due to other factors, such as bad water quality or malnutrition. The susceptibility to diseases of the larvae is indeed correlated to their well-being, nevertheless do high concentrations of pathogenic bacteria in the environment of the larvae inevitably cause infections.

2.3 Control of bacterial diseases in aquaculture

The first action that is normally taken by veterinarians facing problems with bacterial disease in fish is treatment by antibiotics. This makes sense as long as it is a rare event and the system is normally operated without antibiotics. However, in some types of aquaculture operations, bacterial diseases are such a prevalent problem that antibiotics are applied permanently or as prophylactic treatment, which leads to emergence of resistant pathogens and antibiotic residues in the environment (Cabello 2006; Sapkota *et al.* 2008).

Defoirdt *et al.* (2011a) have reviewed some of the possible alternative actions to control bacterial diseases in aquaculture. This includes manipulation of the host intestinal microbiota through feed additives i.e. prebiotics, the use of vibriostatic drugs, the use of pathogen-specific phages (see section 2.3.1), and interference with expression of virulence factors by inhibition of bacterial quorum-sensing (Figure 10).

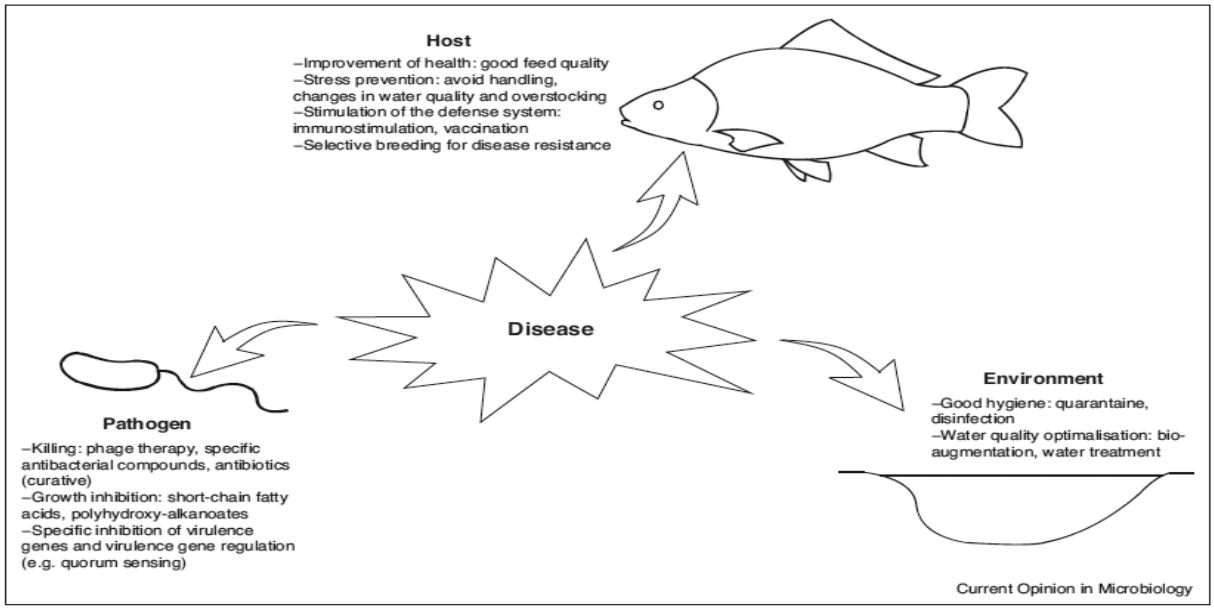

Figure 10: Possible approaches to reducing bacterial diseases in aquaculture. (Defoirdt *et al.* 2011a)

Many bacterial pathogens express their virulence genes only when present in high populations, which they detect by sensing the concentration of self-produced compounds, such as N-acyl homoserine lactones (AHLs) or the furanosyl borate ester (AI2), with specific receptor proteins (Cao and Meighen 1989; Natrah *et al*. 2011). Quorum-sensing inhibition can be achieved either by blocking or inactivating these receptors using structural analogs of their ligands (Rasmussen and Givskov 2006), or by degradation of the signal compounds (Tinh *et al*. 2007). It is questionable whether the preventive use of chemical quorum-sensing inhibitors in aquaculture will be an option, and there is evidence that two of the most important bacterial pathogens, *Vibrio anguillarum* and *Aeromonas salmonicida* do not require quorum-sensing to be virulent (Milton *et al*. 1996; Milton *et al*. 2001; Croxatto *et al*. 2002; Defoirdt *et al*. 2005; Rasch *et al*. 2007). However, recent studies using bacteria that degrade quorum-sensing signals to reduce disease in aquaculture organisms have shown very promising results for interfering with *V. harveyi* virulence (see section 2.2.1.2 Probiotic bacteria).

Whatever elaborate measures of disease-prevention may be appropriate in particular cases, it seems that the best general health-conserving measures for adult fish are animal welfare, hygiene and vaccination. Maintaining an optimal health status through hygienic conditions, optimal feeding, moderate stock densities and good animal welfare practices is the prerequisite for successful animal husbandry. However, this is often disregarded in aquaculture operations. Many disease outbreaks could be prevented if overstocking, overfeeding, rough handling of the animals and inacceptable water conditions would be avoided. Vaccines against many diseases are already available, and the progress in developing multivalent and combinatory vaccines, as mentioned above, will probably soon result in the possibility to protect juvenile and adult fish from the most prevalent diseases with a single injection.

While adult fish can be vaccinated against bacterial diseases, invertebrates and fish larvae are still vulnerable to bacterial infections since they lack an acquired immune response. Probiotic bacteria are interesting as feed additives that improve feed conversion or help to prevent intestinal infections in adult fish or invertebrates. Moreover, probiotics will probably find an important application in larvae cultures, where other measures of disease prevention do not apply, and probiotic microorganisms, especially those which antagonize pathogenic bacteria in the environment of the larvae, may play an important role in reducing bacterial diseases.

2.3.1 Control of bacterial diseases in marine larvae

Dismissing routine administration of antibiotics, which is now widely discredited in larviculture, a number of alternative strategies to prevent bacterial infections have been suggested, such as improving hygiene conditions and water quality, and improving the health of the cultured animals by better feed composition (Defoirdt *et al.* 2011a). However, all of the considerable advances in husbandry techniques and nutrition of fish larvae that were made during the two last decades have not lead to amelioration of bacterial diseases. Since live feed is recognized as the main source of pathogenic bacteria for the larvae, it may be indicated to tackle the problem where it originates, i.e. in the microbiota of live feed cultures. Disinfection of live feed, e.g. by UV exposure, has been tested as a method to reduce pathogen loads (Theisen *et al*. 1998; Munro *et al*. 1999). Although significant improvement in larval survival was reported, physical disinfection does not change the

qualitative composition of the bacterial community. Opportunistic pathogenic bacteria may be able to grow back to their initial density within a few hours and remain the prevalent bacteria in the cultures.

Reasoning that a biological problem requires a biological solution, probiotic microorganisms are recognized as a way of stabilizing the microbial community in larvae cultures. While most probiotic bacteria were selected for their ability to colonize intestines, prevention of bacterial diseases in marine larviculture can also be achieved with bacteria that efficiently colonize the environment of the larvae and their live feed cultures, and thus compete with the opportunistic pathogens for nutrients and space. Antagonism against the pathogens by production of antibacterial compounds is an additional advantage that helps to shift the microbial balance towards the non-pathogenic, beneficial bacteria. Understanding that proficient colonizers of larvae cultures are most probably already present in larvae cultures, the ambient microbiota of marine larviculture systems was screened for antagonistic bacteria in a number of studies. In many of these studies bacteria from the *Roseobacter* clade were isolated, which in some cases indeed proved to be promising probiotics. Chapter 3 gives an introduction to the bacteria of the *Roseobacter* clade and their intricate ecologic abilities that qualify them to prevent bacterial diseases in aquaculture.

2.3.2 Probiotic bacteria and phages in aquaculture

When probiotics are mentioned, people tend to think about lactic acid bacteria. This is quite appropriate, since the majority of probiotics used for land animals and in aquaculture are lactobacilli or Bacilli. However, many more microorganisms can be used as probiotics. This chapter gives an introduction to probiotic bacteria and bacteriophages as measures of preventing bacterial diseases in marine aquaculture.

2.3.2.1 Phage therapy

Bacteriophages are viruses that infect and kill bacteria. Phages are also one of the strongest driving forces of bacterial evolution and allow for horizontal gene transfer by transduction (Lang and Beatty 2007). From an ecologic point of view, there are two types of bacteriophages, lytic and lysogenic phages. Lytic phages inject their genome into the

bacterial cell, express their genes and replicate their genome using resources of the bacterial cell, and finally lyse the cell to liberate the new phages. Lysogenic phages have basically the same scheme of reproduction, however between infection of the cell and their own reproduction the genome of the phage is integrated into the host genome, as a so-called prophage. By this strategy the phage DNA can be replicated along with the host genome and will be contained in all of the daughter cells. Entering the lytic cycle is a rare event that is triggered by stress signals in the bacterium. Consequently, only lytic phages are of interest for phage therapy, since lysogenic phages do not efficiently reduce the bacterial population.

Shortly after the discovery of bacteriophages in the early 20th century, the possibility of treating bacterial infections with bacteriophages was realized. Many phage therapy experiments were carried out in the early years after this discovery, but since basic knowledge about phage biology was missing, many of these were not successful (Nakai and Park 2002; Oliveira *et al*. 2012) . Since antibiotics were discovered shortly thereafter and proved to be a nearly universal therapy for bacterial infections, phage therapy has remained a theoretical concept for a long time. The first serious application studies were not conducted until in the 1980s, however these showed promising results for treatment of open wound infections with known antibiotic-resistant bacteria (Slopek *et al*. 1987; Alisky *et al*. 1998). At the same time, Smith and colleagues (1982) demonstrated that phages could be used as treatments and prophylaxis against *E. coli* infections in mice. Many more successful animal trials, in which phage therapy was used to treat bacterial infections, have since been conducted (Soothill 1992; Barrow *et al*. 1998; Kutter *et al*. 2010; Pirnay *et al*. 2012).

Bacteriophages are highly abundant in natural seawater, indicating their important ecological role in the marine ecosystem (Bergh *et al*. 1989). However, the application of phages to treat or prevent diseases in aquaculture has only been studied for a short time, and, to the author's knowledge, there were so far no studies published on the use of phages in fish larvae. However, the insights gained from phage treatment of grown fish can probably be inferred to larvae.

The first successful study in the new field of probiotic phages for aquaculture successfully treated experimentally-induced *Lactococcus garvieae* infections in yellowtail (*Seriola quinqueradiata* – Figure 11), and attempts to treat *Pseudomonas plecoglossicida* infections

in ayu (*Plecoglossus altivelis*) with phages have been undertaken (Nakai *et al*. 1999; Park *et al*. 2000). Phages of *Flavobacterium psychrophilum* were successfully used to treat cold water disease in salmon (*Salmo salar*), and may also be used in treating infections in ayu (Kim *et al*. 2010; Castillo *et al*. 2012).

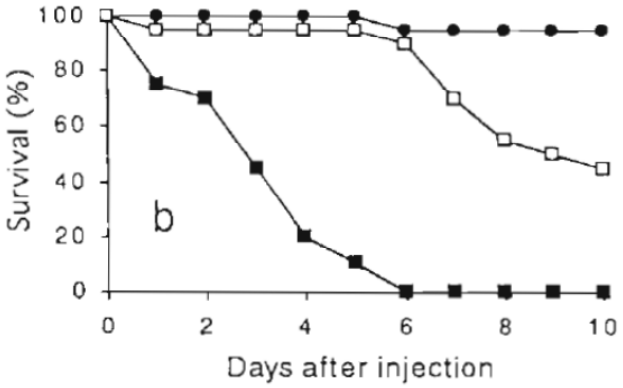

Figure 11: Effect of phage treatment in juvenile yellowtail (*Seriola quinqueradiata*) challenged with *Lactococcus garvieae*. Infected fish (n=20) treated with phage 1 hour after infection (●), infected fish treated with phage 24 hours after infection (□), and untreated infected fish (■). (Nakai *et al.* 1999)

A clear advantage of phage therapy over antibiotic therapy is that phages have a narrow host spectrum and thus only kill members of the targeted species. Another advantage is that phages are not used up by a high abundance of target bacteria, since their number is increased with every lysed bacterium. However, the narrow host spectrum can also be a problem, since sometimes even closely related strains of a target species cannot be infected by the same phages. Thus for treating or preventing real-life infections that are not due to a known bacterial isolate, a selection of phages for every possible pathogen species should ideally be used. Another disadvantage of phages is that they rely on specific sites on the bacterial surface for attachment, thus they impose a strong evolutionary pressure on their target bacteria, and often simple mutations can render the bacteria resistant towards the phages. Rapid appearance of phage-resistant strains of the pathogen is a problem for phage therapy (Nakai and Park 2002). Also here, the use of a mixture of phages that target different sites on the cell envelope could reduce the risk for resistance formation.

Due to these limitations it is questionable whether phage therapy alone has the potential to reduce the problem of bacterial infections in marine aquaculture, however it may find its

application for targeted control of persistent pathogens. This could be the case in marine larviculture, where phage therapy may prove to be a valuable weapon against the most prevalent pathogens, especially if applied in combinatory approaches together with other measures of disease prevention such as probiotic bacteria.

2.3.2.2 Probiotic bacteria in aquaculture

The practice of using beneficial bacteria to enhance survival and growth of cultured animals in aquaculture has been adopted from land animal husbandry. *Lactobacillus* spp. were known to enhance health and growth of fowl, swine and cattle when administered with the feed, and the concept of isolation, selection and application of beneficial bacteria was transferred to the aquatic environment (Verschuere et al. 2000b). Thus probiotic bacteria were initially defined as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller 1988).

Unlike terrestrial animals, aquatic organisms are always intimately exposed to the bacterial community in their environment. If fish are exposed to high enough concentrations of pathogens in the water, this will inevitably lead to infections (Olafsen 2001). In a pioneering study, Skjermo *et al.* (1997; 1999) found that the exterior microbiota of fish larvae cultures is one of the most important factors for the success of the cultures, and demonstrated that maturation of seawater, which promotes a non-opportunistic, oligotrophic microflora, increased survival and reproducibility between replicates. This study has emphasized the importance of controlling the ambient bacterial microbiota for the success of fish larvae cultures. Taking this into account, the definition of probiotics was extended by the FAO to include microbes that are added to the rearing environment; "Live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" (FAO and WHO 2001). This definition is very broad and includes bacteria, phages, microalgae, and even nematodes and rotifers, although this was probably not intended by the authors, and parallels the animal probiotics to protective biocontrol cultures used in horticulture.

Possible mechanisms of action of probiotic bacteria comprise improved digestion of feed and the nutritional value of the probiont itself, enhancement of the host immune response, competition with detrimental bacteria for nutrients or space, improvement of the water

quality by nutrient uptake and remineralization, interference with bacterial quorum-sensing, and production of antagonistic compounds (Verschuere *et al.* 2000b; Tinh *et al.* 2008a). Production of antibacterial substances that inhibit pathogenic bacteria is so far believed to be the most prevalent mechanism of action identified in efficient disease-preventing probiotic bacteria. It should be mentioned, as discussed below, that the vast majority of studies on aquaculture probiotics have not elucidated the actual mechanism of action *in vivo*. The focus on inhibitory compounds is almost exclusively based on *in vitro* pure culture studies. Inhibitory interactions among marine bacteria through production of antagonistic compounds are common, especially among particle-associated bacteria (Long and Azam 2001). Thus, marine bacteria have been recognized as a source of novel antimicrobial compounds and many pharmaceuticals are derived from substances discovered in marine bacteria (Jensen and Fenical 1996). The following section gives an overview of scientific studies evaluating the disease-preventing effect of probiotic bacteria, with a special focus on fish larvae and invertebrates.

2.2.1.2.1 Aquaculture application studies of probiotic bacteria

The use of *Lactobacillus* spp. In fish feed can indeed increase weight gain and reduce susceptibility to diseases in fish and crustaceans (Gatesoupe 1991; Gatesoupe 1994; Gildberg *et al*. 1995; Gildberg *et al*. 1997; Gildberg and Mikkelsen 1998; Ringo and Gatesoupe 1998; Gatesoupe 1999; Robertson *et al*. 2000; Verschuere *et al.* 2000b; Planas *et al*. 2004; Burr *et al*. 2005). Lactobacilli as feed additives have been well investigated, and many promising results in the relevant studies have encouraged the development of formulated feed with added probiotic *Lactobacillus* spp., which is now offered by many companies and increasingly being used. However, studies reporting reduction of mortalities by lactobacilli in challenge trials showed different success rates. For example, Gatesoupe *et al.* (1994) reported an increase in turbot (*Scophtalmus* maximus) larvae survival of 45% (53% vs. 8% in the control, 72 h after infection) by using *Lactobacillus*-enriched rotifers in a challenge trial with a *Vibrio* isolate. In contrast, Gildberg and Mikkelsen (Gildberg and Mikkelsen 1998) reported a merely temporal disease-reducing effect of using lactobacilli from two commercial probiotic feeds in postlarvae of cod (G*adus morhua*) challenged with *V. anguillarum* (Figure 12), although the two *Lactobacillus* isolates had shown pronounced *in vitro* antagonism.

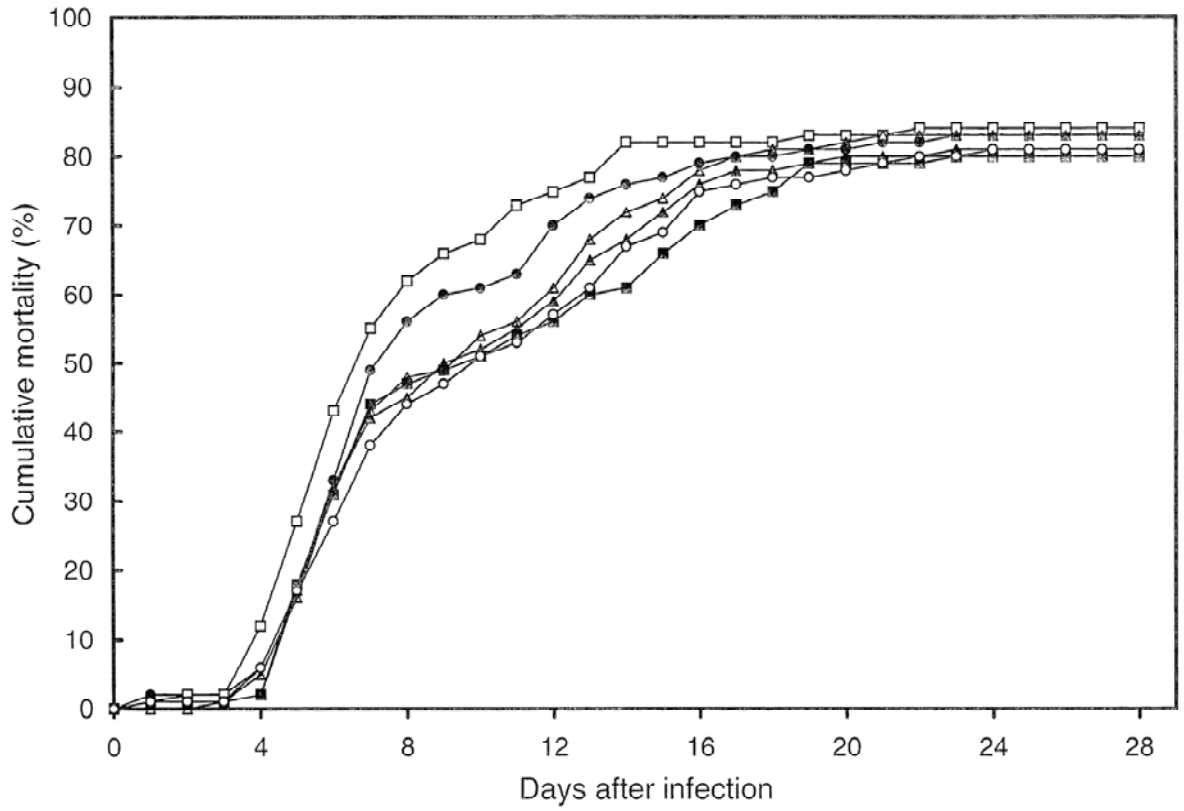

Figure 12: Cumulative mortalities of cod (*Gadus morhua*) postlarvae challenged with *Vibrio anguillarum*, fed on formulated diets with different additions: Control feed (●), *Lactobacillus* isolate from cod (□), *Lactobacillus* isolate from salmon (■), an immunostimulatory peptide from a fish protein hydrolysate (▲),*Lactobacillus* isolate from cod + peptide (○), *Lactobacillus* isolate from salmon + peptide (∆). (Gildberg and Mikkelsen 1998)

Correlating survival with the occurrence of bacterial taxonomic groups, Lauzon *et al.* (2010a) identified beneficial microbiota of cod larvae. *Lactobacillus, Enterococcus, Arthrobacter, Pseudoalteromonas* and *Lacinutrix* spp. were associated with well-performing cultures. A later study used a combination of three potentially beneficial isolates, an *Arthrobacter* sp., a *Carnobacterium* sp. and an *Enterococcus* sp. and found that the bacteria enhanced survival and growth of cod larvae (Lauzon *et al*. 2010b; Lauzon *et al*. 2010c). However, the mechanism of action of the probiotics was not investigated.

Focusing on antibacterial activity of the probiotic bacteria as their mechanism of action, one of the first studies of non-*Lactobacillus* fish probiotics used a non-pathogenic hatchery isolate of *V. alginolyticus*, which had shown *in vitro* antagonism against four bacterial pathogens, to reduce mortality of adult Atlantic salmon (*Salmo salar*) when used as exogenous preventive treatment before challenge with *Aeromonas salmonicida* (Austin *et al*.

1995). Taking this concept into fish larvae, Gatesoupe et al. (1997) reported significant improvement in survival of turbot larvae challenged with *V. splendidus* by using rotifers enriched with another non-pathogenic *Vibrio* sp. strain, which inhibited a pathogenic *Vibrio* isolate *in vitro* by production of siderophores. Production of siderophores as the mechanism of action was similarly reported in two studies with adult fish, which demonstrated mortality reduction in rainbow trout (*Oncorhinchus mykiss*) challenged with *V. anguillarum* (Gram *et al*. 1999; Spanggaard *et al*. 2001). Siderophores are iron-chelating, small-molecule compounds that are used by many bacteria, fungi and plants to facilitate uptake of iron that is bound in inorganic or organometallic complexes (Barton and Hemming 1993). Siderophores scavenge iron ions due to their strong affinity, and the diffusible siderophore-bound iron is subsequently assimilated through specific transporter proteins.

Antagonistic probiotics are used to prevent bacterial diseases also in crustaceans, as reviewed by Farzanfar (2006) and by Ninawe and Selwin (2009), and in bivalves, as reviewed by Kesarcodi-Watson (Kesarcodi-Watson *et al*. 2008) and by Prado *et al.* (2010). However, also bacterial strains which showed no *in vitro* antagonism, among them a *V. alginolyticus* and some unidentified isolates, were demonstrated by Verschuere *et al.* (2000a) to improve growth and survival of brine shrimp (*Artemia* sp.) challenged with *V. proteolyticus*.

A new approach to coping with the most prominent crustacean pathogen *V. harveyi* is the use of probiotic bacteria that interfere with its quorum-sensing. Tinh *et al*. (2007) isolated AHL-degrading bacteria from shrimp (*Litopenaeus vannamei*) intestines in enrichment cultures with AHLs as sole carbon source, and demonstrated that the bacteria degraded the homoserine lactone produced by *V. harveyi. V. harveyi* deploys three quorum-sensing signals, the homoserine lactone HAI-1, the furanosyl-borate-diester AI-2, and CAI-1, which was identified as (S)-3-hydroxytridecan-4-one (Waters and Bassler 2006; Higgins *et al*. 2007). The same bacterial isolates were also shown to neutralize the deleterious effect of AHL addition to turbot larvae cultures, which were probably due to virulence of unidentified bacterial pathogens in the larvae cultures (Tinh *et al*. 2008b). Later Dang *et al.* (2009b) isolated AHL-degrading bacteria from European sea bass (*Dicentrarchus labrax*) intestines and used the isolates to reduce the mortalities of *Artemia* challenged with *V. harveyi*, and demonstrated, similarly to the previous study, their ability to counteract the negative effect

of AHL addition in cultures of the giant river prawn *Macrobrachium rosenbergii* (Dang *et al*. 2009a). The isolates were later identified as *Bacillus* spp. (Defoirdt *et al*. 2011b).

In penaeid shrimp (e.g. *Penaeus* spp.) a *Bacillus* spp. was used to control infections with luminous *Vibrio* spp. and improved survival and total production volumes (Moriarty 1998). Similarly, another *Bacillus* sp. was found to increase growth, survival (Figure 13) and resistance to *V. harveyi* in the black tiger shrimp (*Penaeus monodon*) (Rengpipat *et al*. 1998).

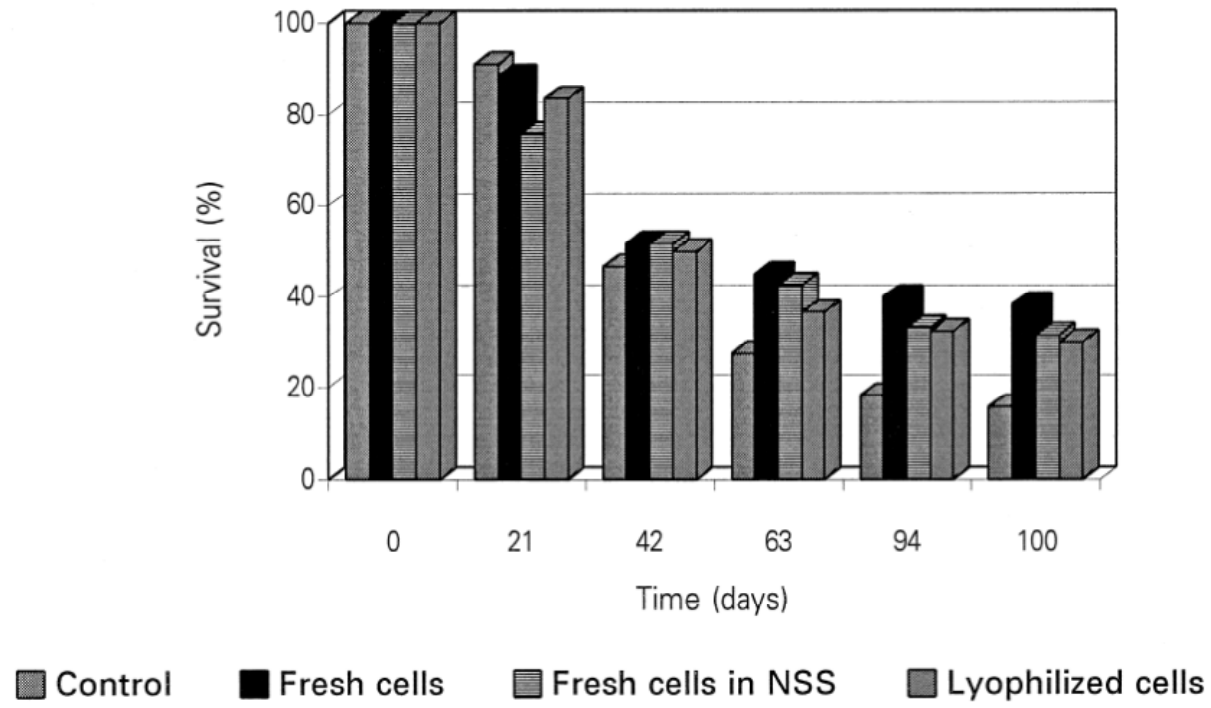

Figure 13; Increased survival of black tiger shrimp (*Penaeus monodon*) fed with formulated feed containing a probiotic *Bacillus* isolate, which also increased individual weight. (Rengpipat *et al.* 1998)

Although this pronounced effect could not be reproduced in a follow-up study, the mechanism of action was identified as stimulation of the immune response, and the concentration of *Vibrio* spp. in the water, shrimp intestines and faeces was found to be not reduced (Rengpipat *et al*. 2000). In another shrimp probiotics study, a *Bacillus subtilis* strain that was efficient in reducing infections with *V. harveyi* in black tiger shrimp had a clear antibacterial effect in *in vitro* and *in vivo* assays, indicating that probiotic bacteria from the large and diverse genus *Bacillus* can have different mechanisms of action as probiotics (Vaseeharan and Ramasamy 2003). Yet another study isolated a *Vibrio* sp. and a *Bacillus* sp. due to their *in vitro* antagonism, and found that *in vivo* the probiotic effect on shrimp (*P.*

vannamei) was due to both inhibition and immunostimulation in the *Bacillus* strain, while it was only due to inhibition of the pathogen in the *Vibrio* strain (Gullian *et al*. 2004).

Results like this, which indicate different mechanisms of action of one probiotic strain, can be misinterpreted to the effect that mechanisms of action are generalized and confused. For example, a study was conducted that isolated bacteria from shrimp (*P. monodon*) intestines due to production of siderophores and digestive exoenzymes, however the probiotic potential of these isolates was evaluated *in vivo* in immunoassays with the haemolymph of bacteria-exposed shrimp, and no positive effect was found (Alavandi *et al*. 2004). This illustrates the importance of understanding the mechanism of action in potentially probiotic isolates, and of evaluating these candidates in appropriate experimental systems. Tinh *et al*. (2008a) have, in a review of studies on probiotic bacteria in the larviculture food chain, emphasized the importance of evaluating the mechanism of action of *in vivo*, and suggested to investigate their behavior along the food chain in gnotobiotic systems, i.e. in absence of other bacteria than those studied.

In the present study a probiotic bacterium was evaluated in gnotobiotic cultures of live feed organisms for marine larvae and in experimental larvae cultures (D'Alvise *et al*. 2012). The mechanism of action was evaluated *in vivo* by using mutants deficient in production of an antibacterial compound, and protection of the larvae and feed cultures from *V. anguillarum* was achieved. The *in vivo* efficacy of three different strains of the bacterium was compared and preventive application to the environment was identified as most efficient way of application (D'Alvise *et al*. 2013a). A more detailed account of the results is given in section 3.3. This work was carried out in continuation of a previous study that demonstrated *in vitro* antagonism in a seawater system and indicated a possible application of the bacterium in the environment of the larvae (D'Alvise *et al.* 2010). However, the foundation of the present work are the three studies that have isolated the strains as potential probionts from aquaculture units due to their *in vitro* antagonism of *V. anguillarum* (Ruiz-Ponte *et al.* 1998; Hjelm *et al.* 2004; Porsby *et al.* 2008).

A study by Riquelme *et al*. (1997), aiming at isolating probiotic bacteria from and for Chilean scallop (*Argopectem pupuratus*), depicts how low the success rate of *in vitro* selection processes tends to be. 506 bacterial isolates were screened for antagonism of a *V.*

anguillarum-like pathogen, 11 of these were found to be inhibitory to the pathogen, and only 1 isolate, a *Vibrio* sp., proved to have a disease-preventing effect on the scallop larvae.

However cumbersome the selection and evaluation process may be, there are a few examples that show how antagonistic probiotic bacteria that were selected and evaluated in scientific studies stabilized the production and increased the economic success of aquaculture operations. For example, continuous full-scale hatchery application over many years has shown that infections with *Vibrio anguillarum* and the fungus *Haliphthoros* sp. in larvae cultures of the swimming crab *Portunus tricuberlatus* can be controlled by adding a probiotic bacterium to the pond water, which was identified as *Thalassobacter utilis* (Table 3) (Nogami and Maeda 1992; Nogami *et al.* 1997). The genus *Thalassobacter* belongs to the *Rhodobacteraceae* family and is related to the genus *Jannaschia* of the *Roseobacter* clade (Pujalte *et al*. 2005).

Table 3: Survival and production volumes of swimming crab (*Portunus trituberculatus*) larvae feeding on diatoms and rotifers in 200 m^3 tanks, as effect of addition of *Thalassobacter utilis* PM-4 (biocontrol). a) high mortality was due to cannibalism, not disease (Nogami and Maeda 1992)

Experiment	Treatment	Number of larvae (x 10^4)		Survival rate	Final production
		initial	final	[%]	[individuals/ m^3]
1	No addition	432	11	2.5	550
2	*T. utilis* 10^6cfu/ml	387	100	25.8	5,000
3	No addition	489	0	0	0
4	*T. utilis* 10^6cfu/ml	503	78	15.5	3,900
5	No addition	435	192	44.1	9,600
6	No addition	455	0	0	0
7	*T. utilis* 10^6cfu/ml	455	170	37.4	8,500
8	No addition	538	0	0	0
9	No addition	518	88	17.0	4,400
10	*T. utilis* 10^6cfu/ml	421	240	57.0	12,000
11	No addition	455	0	0	0
12	No addition	489	0	0	0
13	*T. utilis* 10^6cfu/ml	422	60	14.2	3,000
14	*T. utilis* 10^6cfu/ml	442	9	2.1[a)]	465
15	No addition	482	0	0	0
16	*T. utilis* 10^6cfu/ml	427	160	37.5	8,000
Total	*T. utilis* 10^6cfu/ml	3,057	831	**27.2**	
Total	No addition	4,263	292	**6.8**	

Among the probiotic bacteria for aquaculture, the members of the Roseobacter-clade are a particularly promising group. *Roseobacter*-clade species have shown potential as probiotics

in the present and in a number of other studies, and their application for marine larviculture will be the subject of chapter 3.3.

3. The *Roseobacter* clade

3.1 Introduction to the *Roseobacter* clade

Although there is a great bacterial phylogenetic diversity in the world's oceans, the majority of marine bacteria fall into nine distinct clades (Giovannoni and Rappé 2000). The members of most of these cannot be cultivated on traditional nutrient-rich microbiological media, however many members of one of the prevalent clades, the *Roseobacter* clade, can be cultivated on standard media and have thus received much attention during the last decade (Buchan *et al*. 2005). This may have to do with the fact that a large proportion of algae-associated bacteria could be cultivated, as found by Jensen *et al.* (1996), and many of the *Roseobacter* clade species indeed live in association with algae. Nevertheless, the cultivable part of the *Roseobacter* clade is no representative selection of the group, which harbors many oligotrophic specialists (Eilers *et al*. 2000; Newton *et al*. 2010).

3.1.1 Taxonomy and genomics

The first *Roseobacter*-clade species discovered were *Roseobacter litoralis* and *R. denitrificans*, two bacteriochlorophyll a-containing bacteria that were isolated from macroalgae (Shiba 1991). Gonzales and Moran (1997) found by comparison of 16S-sequences that a large share of the bacterial community in seawater, up to 28%, forms a distinct cluster within the *Rhodobacteraceae* family (*α-Proteobacteria*), which was named *Roseobacter* clade after the first described species within the group. The great majority of *Roseobacter* clade species are marine and some species were isolated from other saline or hypersaline environments; all *Roseobacter* clade species have an obligate requirement for sodium ions (Brinkhoff *et al*. 2008). The *Roseobacter* clade presently comprises 38 genera, many of which contain only one species. This is number is probably not final, since taxonomic classifications are being reconsidered as new species are discovered and more information on the described species becomes available (Brinkhoff *et al*. 2004; Martens *et al*.

2006; Yi *et al*. 2007; Newton *et al.* 2010). All species of the *Roseobacter* clade share at least 88% sequence homology of the 16S-rRNA sequence, and differences between genera that are often less than 4 %, and differences between species that may be less than 1% are hard to make out (Buchan *et al.* 2005; Martens *et al.* 2006; Brinkhoff *et al.* 2008). Within the *Roseobacter* clade, Newton *et al*. (2010) compared the sequences of 70 shared genes in 32 *Roseobacter* genomes and discovered that the clade can be subdivided into five distinct lineages (Figure 14).

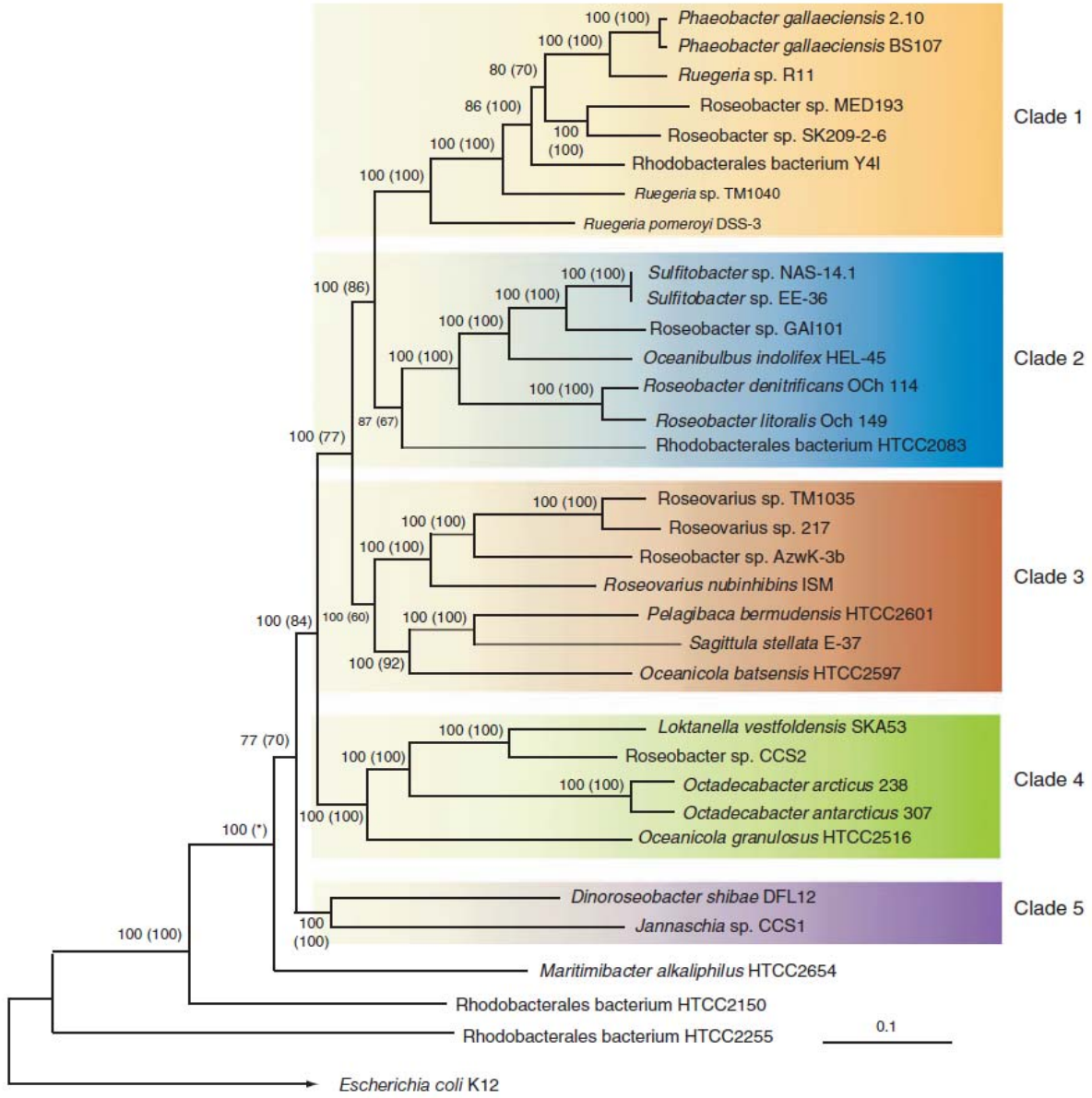

Figure 14: A consensus maximum likelihood tree of 32 sequenced Roseobacter genomes. The alignment for tree inference was created from a concatenation of 70 universal single-copy genes contained in each of the *Roseobacter* genomes and in *E. coli* K12, which was used as an outgroup. Bootstrap values of >50% for the maximum likelihood best-fit tree (200 iterations) and neighbor-joining tree (100 iterations) are listed at each node. The neighbor-joining bootstrap values are listed in parentheses. (*) demarcates nodes where the neighbor-joining tree did not agree with the maximum

likelihood tree. Designated Clades 1–5 are listed to the right of the tree. The scale bar represents 10% sequence divergence. (Newton *et al.* 2010).

Although most *Roseobacter* clade species inhabit the temperate areas of the oceans, there are also representatives of the clade found in Arctic and Antarctic waters and on polar sea ice, such as *Octadecabacter arcticus* and *O. antarcticus* (Gosink *et al*. 1997; Wietz *et al*. 2010). One of the larger coherent phylogenetic groups within the *Roseobacter* clade is the RCA cluster (*Roseobacter* Clade Affiliated), which is 98% identical on 16S level. The RCA clade is restricted to the polar and temperate ocean areas of the planet and can constitute up to 30% of the bacterial community in the Southern ocean (Selje *et al*. 2004; Buchan *et al.* 2005; Brinkhoff *et al.* 2008; West *et al*. 2008; Giebel *et al*. 2009a; Giebel *et al*. 2009b). Members of the RCA cluster are, together with the SAR11 clade (α-*Proteobacteria*) the most abundant bacteria in the oceans. The RCA cluster is closely related with *Octadecabacter* spp., and would thus in Figure 14 be contained in clade 4.

The *Roseobacter* clade offers a unique possibility of studying genomic characteristics and evolutionary processes in a group of closely related species, since many *Roseobacter* genomes have been or are currently sequenced (Brinkhoff *et al.* 2008; Thole *et al*. 2012; Moran and Sharma 2012; D'Alvise *et al*. 2013b). The genome of *P. gallaeciensis* BS107 contains two complete prophages and one GTA-like element (Thole et al. 2012). GTA (gene transfer agent) is a phage-like entity that packages genomic DNA of the bacterial cell in a capsid and transfers them to related bacteria, thus allowing for genetic exchange through modified transduction (Lang and Beatty 2007). GTA was discovered in *Rhodopseudomonas capsulata*, a *Roseobacter* clade member, and mediates gene exchange between different *Roseobacter* clade species (Marrs 1974). In contrast to lysogenic phages, GTAs do not contain genes that encode their own proteins and are described to have no adverse effect on the recipient cell. In the context of this indicator for frequent gene exchange, the striking genomic similarity between two *P. gallaeciensis* strains from opposite sides of the world, as found by Thole *et al.* (2012), suggest a relatively uniform global population of *P. gallaeciensis* that exchange genetic information.

3.1.2 Metabolism

In general, *Roseobacter* clade species have an aerobic metabolism, although some of the phototrophic species are facultative anaerobes (Allgaier *et al*. 2003; Buchan *et al.* 2005; Brinkhoff *et al.* 2008; Piekarski *et al*. 2009). There is a great metabolic diversity in the *Roseobacter* clade, comprising aerobic anoxygenic photosynthesis, lithotrophy on sulfur compounds, methylotrophy, and carbon monoxide oxidation (Kolber *et al*. 2001; Schaefer *et al*. 2002; Moran *et al*. 2003; Buchan *et al.* 2005; Wagner-Döbler and Biebl 2006; Martens *et al.* 2006). However, most members of the *Roseobacter* clade were characterized as metabolic generalists that are able to utilize a multitude of organic substrates (Moran *et al*. 2004; Martens *et al.* 2006; Newton *et al.* 2010). Among these nutritional generalists are the *Ruegeria* and *Phaeobacter* spp.. The metabolic versatility of the *Roseobacter* clade members plays a major role for the degradation of plant derived compounds, such as lignin, that are abundant in coastal ecosystems (Moran and Hodson 1994; Gonzalez *et al*. 1996; Buchan *et al*. 2000; Buchan *et al*. 2001).

Members of the *Roseobacter* clade produce a wide range of secondary metabolites such as signaling molecules, like acylated homoserine lactones (AHLs), and antibacterial compounds. A sponge symbiont from the *Phaeobacter*/*Ruegeria* cluster produces different cyclic dipeptides that are thought to play are role in the sponge-bacteria interaction (Mitova *et al*. 2004). Martens *et al.* (2007) screened the genomes of 22 *Roseobacter* clade species and found non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKS), indicating potential production of secondary metabolites. *Roseobacter* clade strains that live in symbiosis with toxic dinoflagellates (*Alexandrium* spp.) were shown to produce the sodium channel-blocking toxins responsible for paralytic shellfish poisoning (PSP) (Gallacher *et al*. 1997). However, none of the *Roseobacter* clade members and other bacteria that were associated with the toxic dinoflagellate *Gynosinium catenatum* showed autonomous PSP-toxin production in another study (Green *et al*. 2004). Although *Roseobacter* clade isolates are frequently reported to be antagonistic e.g. (Martens *et al*. 2007; Sharifah and Eguchi 2011), to the authors knowledge only two antibacterial inhibitory compounds were identified; tropodithietic acid (TDA) in *Phaeobacter* and *Ruegeria* spp., which will be the subject of section 3.2.3, and the antibiotic tryptantrin from *Oceanibulbus indolifex* (Wagner-Döbler *et al*. 2004).

The ability of many *Roseobacter* clade species to degrade dimethylsulfoniopropionate (DMSP), which is produced by many species of algae as osmolyte, has received increased attention during the last years and indicates the close association of many *Roseobacter* clade species with algae (Moran *et al.* 2003; Geng and Belas 2010b). There are two basic pathways for DMSP degradation (Figure 15); the demethylation pathway, where DMSP is degraded and fully utilized via 3-methyl-mercaptopropionate, and the cleavage pathway, where DMSP is cleaved into acrylate, which can be utilized by both bacteria and algae, and the volatile dimethylsulfide (DMS) (Wagner-Döbler and Biebl 2006). DMS molecules are thought to act as cloud condensation nuclei, and therefore contribute to cloud formation (Andreae and Crutzen 1997).

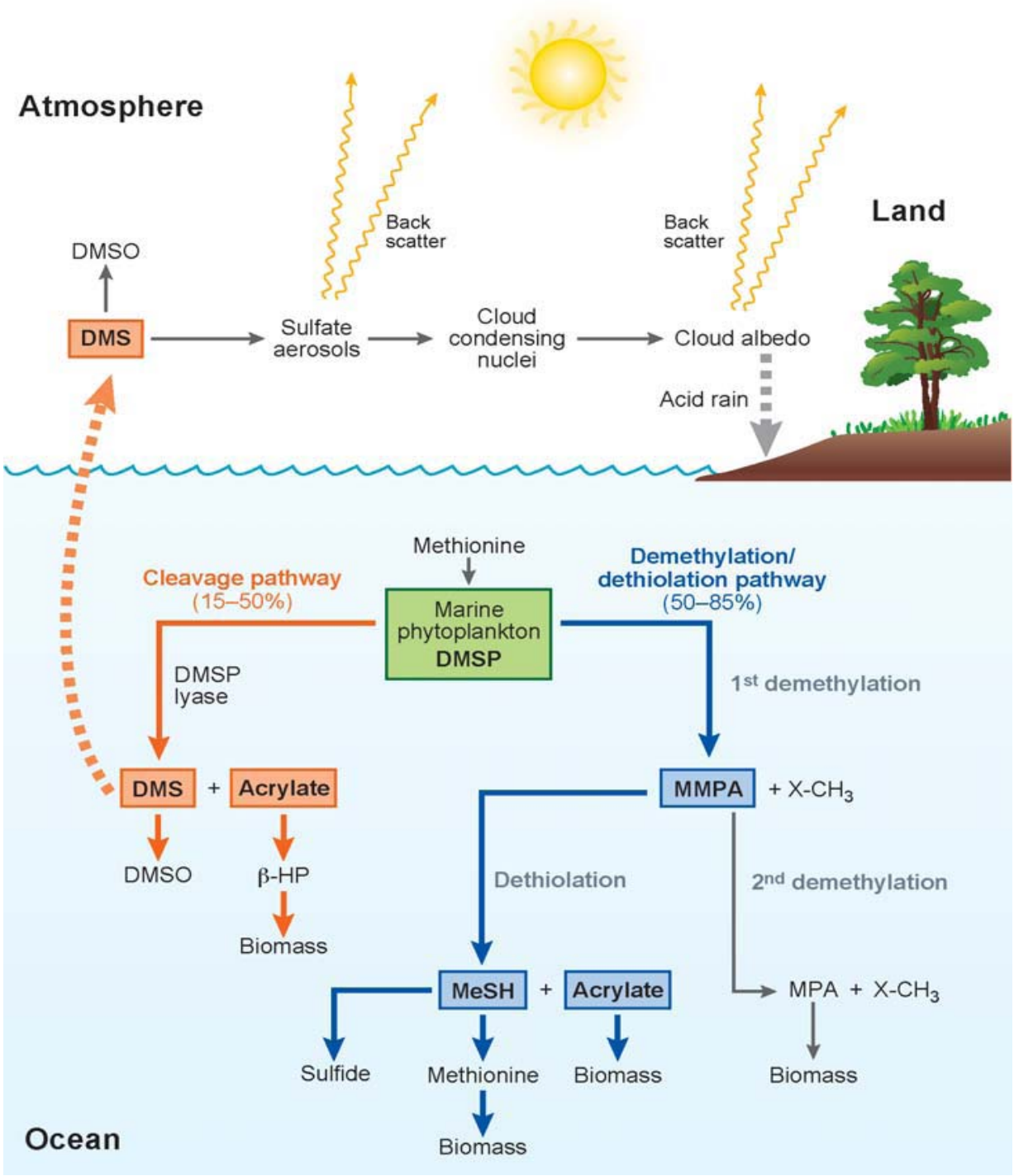

Figure 15: Role of Roseobacter clade bacteria in the global sulfur cycle. DMS, dimethylsulfide; DMSP, dimethylsulfoniopropionate; MMPA, 3-methyl-propionate; MeSH, methanethiol; X-CH3, unidentified molecule with a terminal methyl group; MPA, 3-mercaptopropionate; _-HP, _-hydrocypropionate; DMSO, dimethyl sulfoxide. The two pathways present in Roseobacter organisms are blue (demethylation/dethiolation pathway) and orange (cleavage pathway). (Wagner-Döbler and Biebl 2006)

Due to the possible increase of cloud-formation as result of bacterial DMSP degradation via the cleavage pathway, it has been hypothesized that natural phytoplankton communities

could possibly regulate the local climate through DMS production. This has become known as CLAW-hypothesis (an acronym from the surnames of the authors Charlson, Lovelock, Andreae and Warren) (Charlson *et al*. 1987). However, it has been argued that cloud formation is more complex than implied by the CLAW hypothesis and that a direct connection between DMS production and cloud formation is hard to establish (Quinn and Bates 2011). *Ruegeria mobilis* strain F1926, which was used in paper 3 of this study (D'Alvise *et al.* 2013b), has a gene encoding an analog of the DMSP-lyase *ddd*P (P. D'Alvise, unpublished result), indicating symbiotic association with algae (Todd *et al*. 2009; Todd *et al*. 2012).

Regardless of their potential impact on global or local climate, symbioses between marine phytoplankton and members of the *Roseobacter* clade are very important for the marine ecosystem, since they play a considerable role for plankton dynamics and primary production in the oceans.

3. 2 Ecophysiology of *Phaeobacter* and *Ruegeria* spp.

As mentioned above, a special trait of many members of the *Roseobacter* clade is their ability to engage in symbioses with phytoplankton. *Roseobacter* clade species were repeatedly isolated from cultures of microalgae, and *Roseobacter* abundance is correlated with natural or artificial algae blooms (Charlson *et al.* 1987; Gonzalez *et al*. 2000; Alavi *et al*. 2001; Porsby *et al.* 2008).

The representatives of the *Roseobacter* clade that were predominantly used in studies of algal-bacterial symbioses, as influenced by bacterial motility and biofilm formation, belong to the genera *Ruegeria* and *Phaeobacter*, which are the subject of the present study. *Ruegeria* species are a significant part of the culturable microbial community of all oceans, except from the polar zones (Gram *et al*. 2010), and *Ruegeria* (*Silicibacter*) sp. TM1040, a symbiont of the toxic dinoflagellate *Pfisteria piscicida,* was used as a model system to study symbiotic interactions (Alavi *et al.* 2001; Miller *et al*. 2004). Strains of the closely related genus *Phaeobacter* are of interest because of their outstanding ability to form and invade biofilms, their antifouling capacity, their antibacterial effect, and their potential application as probiotics in aquaculture (Brinkhoff et al. 2004; Hjelm et al. 2004; Rao *et al*. 2005; Rao *et*

al. 2006; Rao *et al*. 2007). One of the key characteristics of *Ruegeria mobilis* and *Phaobacter gallaeciensis* is their ability to produce the sulfur-containing tropone derivative tropodithietic acid (TDA), a potent antibacterial compound (Kintaka *et al*. 1984; Brinkhoff *et al.* 2004; Greer *et al*. 2008; Gram *et al.* 2010). As demonstrated in this thesis (see section 3.2.3) TDA is a major mechanism of the probiotic effect.

3.2.1 Symbiosis with algae

Ruegeria spp. are inhabitants of the open ocean waters and engage in symbiosis with microalgae (Alavi *et al.* 2001; Todd *et al.* 2012; Riclea *et al*. 2012; Magalhaes *et al*. 2012). Apart from that, *Ruegeria* sp. R11 was found on the surface of the marine macroalga *Delisea pulchra* (Case *et al*. 2011), and one strain, which was recently described as the new species *R. conchae*, was isolated from arc clam (*Scapharca broughtonii*) (Lee *et al*. 2011; Lee *et al*. 2012). Another *Ruegeria* strain was isolated from the sponge *Mycale laxissima* (Zan *et al*. 2011). From these findings it could be concluded that *Ruegeria* spp. generally tend to live in association with eukaryotes, of which most are algae. However, one could argue that filter-feeding animals like bivalves and sponges at least transiently harbor algae-associated microflora, and that the sponge and bivalve isolates may truly be associated with microalgae.

Although single *Phaeobacter* strains were isolated from the accessory nidamental glands (i.e. gonads) of the Hawaiian bobtail squid *Euprymna scolopes* (Collins and Nyholm 2011; Collins *et al*. 2012), and one strain was found on the cutaneous mucus of sea horses (*Hyppocampus guttulatus*) (Balcazar *et al*. 2010), most *Phaeobacter* strains have been isolated from coastal zones and seem to be symbionts of macroalgae and inhabitants of biofilms on solid surfaces (Rao et al. 2005; Rao et al. 2006; Thole et al. 2012). This is exemplified in a study performed by Porsby *et al*. (2008), where the microflora of a Danish turbot hatchery was sampled. *Phaeobacter* strains were predominantly isolated from the tank walls of fish larvae cultures, while *Ruegeria* strains were only isolated from microalgae cultures. In liquid laboratory cultures, *R. mobilis* shows faster growth and higher final densities than *Phaeobacter* strains grown in the same medium, while *Phaeobacter* strains generally produce more TDA, more EPS and have higher proportions of immotile, aggregate-forming cells in shaken cultures (unpublished results). From these findings, it could be hypothesized that *R. mobilis* strains

are adapted to the bloom-and-crash lifestyle of a microalgae symbiont in open waters, while *Phaeobacter* strains seem to be better adapted to a persistent life in biofilms.

P. gallaeciensis forms thick biofilms on the surface of *Fucus vesiculosus* (Thole *et al.* 2012), *Ulva australis* (Rao *et al.* 2006) and probably also on other macroalgae. New *P. gallaeciensis* strains have been isolated by Bernbom *et* al. (2011) from a Danish harbor, where they inhabit biofilms on solid surfaces that contain several species of diatoms (B.B. Rasmussen and P. D'Alvise, unpublished observation, Figure 16). All these findings indicate that *P. gallaeciensis* is well adapted to living in symbioses with algae and that this symbiosis may accelerate nutrient turnover in phytoplankton communities.

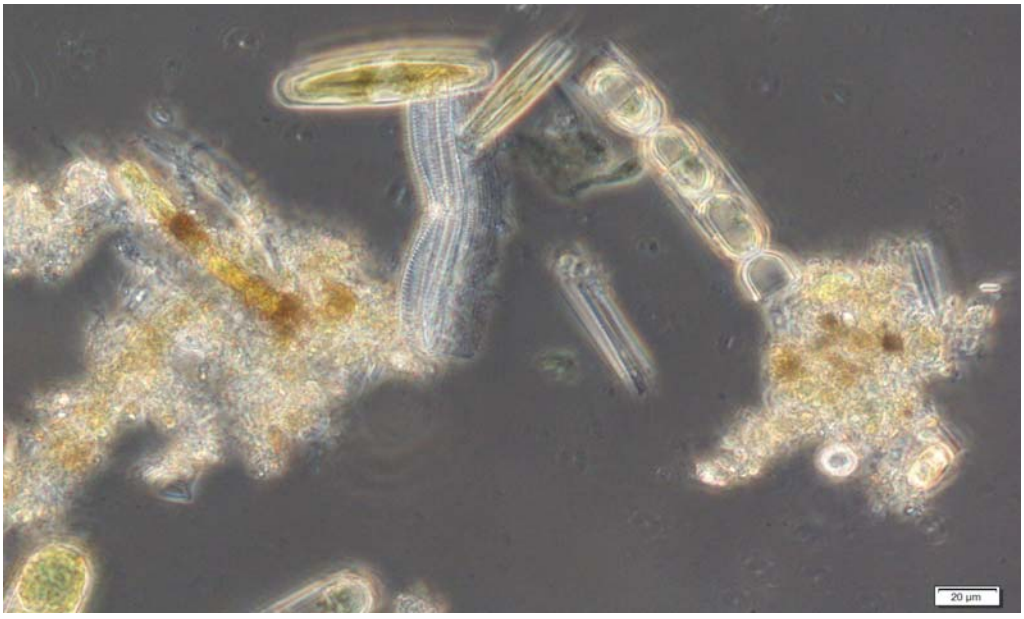

Figure 16: Micrograph of a biofilm sample from a solid surface in Jyllinge Harbor, Denmark, from which *Phaeobacter gallaeciensis* was isolated. Live and dead cells of several species of diatoms and biofilm-grazing flagellates were found in association with the bacterial biofilms.

As an additional hint towards its adaptation to association with algae, inoculation of axenic diatom (*Thalassiosira rotula*) cultures with natural seawater from the German Wadden Sea selected for algae-associated microflora containing *P. gallaeciensis* (Grossart *et al*. 2005). Growth of the diatom was slightly stimulated when the natural bacterial community including *P. gallaeciensis* was added to a culture in exponential growth phase, while adding the same to algae in stationary phase resulted in faster degradation of the algae. The growth-promoting effect may potentially be due to production of metabolites that enhance

algal growth, while the increased degradation of the senescent algae may have been due to production of algicides.

3.2.1.1 Chemical interactions with symbiotic algae

P. gallaeciensis was shown to produce potent algicides, the roseobacticides, from p-coumaric acid, which is an algal cell-wall degradation product (Seyedsayamdost *et al*. 2011). Presence of specific genes in *P. gallaeciensis* DSM17395 (BS107) suggested the ability to produce acetoin and 2,3-butanediol (Thole *et al.* 2012). These compounds were described to be produced by bacteria in the rhizosphere of *Arabidopsis thaliana* and promoted growth or induced systemic resistance of the plants (Ryu *et al*. 2003; Rudrappa *et al*. 2010). Also production of phenylacetic acid, another plant growth stimulant was reported (Seyedsayamdost *et al.* 2011). *P. gallaeciensis* uses a petribactin-like siderophore to facilitate iron mobilization (Thole *et al.* 2012). The bacterium has two siderophore-specific transporter systems, one for its self-produced siderophore and another one, indicating that it is able to take up siderophores produced by other bacteria. It has been demonstrated that siderophore production by bacteria can facilitate algal iron uptake (Soria-Dengg *et al*. 2001; Amin *et al*. 2009).

Apart from these newly discovered abilities to interact with their algal symbiotic partners, it has been hypothesized that TDA and the brown pigment that is correlated with TDA production may play a role in the symbiotic interaction, other than its antibacterial effect.

3.2.1.2 Algal-bacterial symbioses in the present study

In the present study, *P. gallaeciensis* BS107 was co-cultured with two different species on microalgae (D'Alvise *et al*. 2012). The strain was inoculated into cultures of *Nannochloropsis oculata* and *Tetraselmis suecica* in their exponential growth phase. *P. gallaeciensis* readily colonized the algae cultures and neither increased nor reduced growth of the algae. No inhibition of algal growth was observed in any of the cultures, however no experiments with senescent algae were conducted. The bacterium was inoculated at high (10^7 cfu/ml) or low (10^2 cfu/ml) densities, but this did not affect the final bacterial concentrations (10^7 cfu/ml). *P. gallaeciensis* did not attach to the algae, probably due to their small size and their motility, but formed marine snow-like aggregates that contained debris from the algae, such as dead cells or the cell walls that *T. suecica* sheds during cell division (Figure 17). In many

instances the algae were observed to actively swim towards the bacterial aggregates and to attach to the particles, using their flagella. This may either be due to dissolved organic or inorganic nutrients leaching out from the particles, or may indicate production of beneficial compounds.

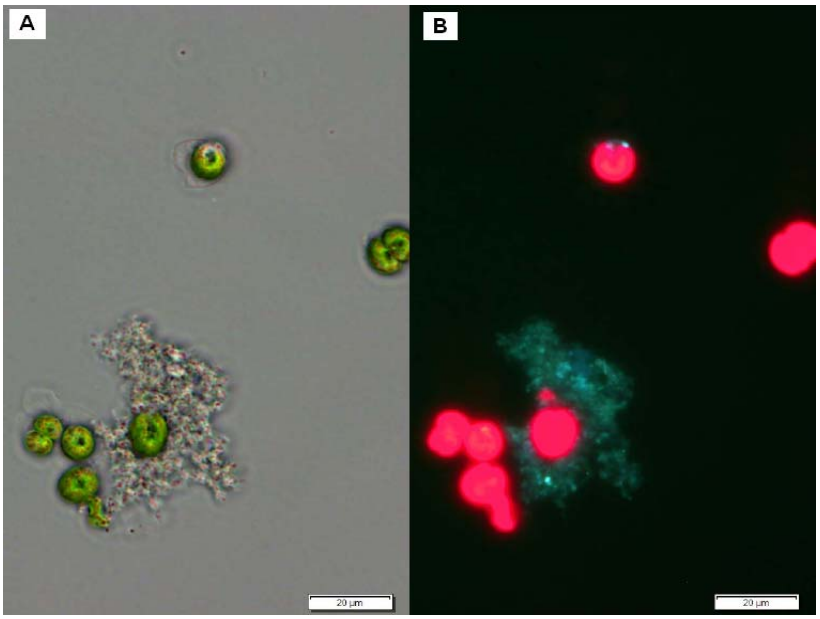

Figure 17: Phase contrast (A) and fluorescence (B) micrographs of *P. gallaeciensis* carrying the plasmid pPDA11 (*tdaCp::gfp*) in co-culture with *T. suecica*. The two panels show the same seven algal cells of which some are dividing, and a marine snow-like particle which is colonized by *P. gallaeciensis* carrying the promoter-fusion on a plasmid. The green fluorescence of *P. gallaeciensis* on the particle shows that the gfp gene is expressed from the tdaC promoter, indicating production of TDA. (D'Alvise et al. 2012)

Presence of *P. gallaeciensis* reduced concentrations of *V. anguillarum* by at least three logarithmic units, as compared to the control algae cultures with only *V. anguillarum*, where the pathogen reached concentrations of about 10^6 cfu/ml. The observation that *V. anguillarum* was reproducibly able to persist and propagate in the algae cultures is in contradiction to the findings of Kokou *et al.* (2012) who found that a selection of fish-pathogenic bacteria, such as *V. anguillarum*, were inhibited in axenic cultures of different microalgae, among them *Tetraselmis chui*, a close relative of *T. suecica*. In preliminary experiments to this study, *T. chui* was used and we have not observed inhibition of *V. anguillarum*. In cultures of *N. oculata* however, inhibition of *V. anguillarum* by the axenic algae alone was achieved, if the algae population was dense (D'Alvise *et al.* 2012).

3.2.1.3 Attachment to algae and particles

Many *Roseobacter* clade species have the ability to attach to particulate nutrient sources, such as microalgae that excrete DMSP and other organic compounds, or particulate detritus derived from phyto- or zooplankton (Slightom and Buchan 2009). Attached bacteria produce biofilm matrices consisting of extracellular polymeric substances (EPS) that make more detritus adhere to the particle and facilitate the conglomeration of particles, thereby forming larger aggregates known as marine snow (Simon *et al*. 2002). The increasing particle size of the marine detritus leads to enhanced sinking rates, thus the bacterial activity leads to export of organic matter into the ocean floor sediments (Alldredge and Silver 1988). This process is of great interest for modeling of global climate, since by incorporation of organic matter into the sediments large amounts of carbon are sequestered from the atmosphere. Attempts to increase sequestration of organic carbon from the atmosphere by means of iron fertilization of oceanic regions that are limited by lack of iron was successful in terms of increasing primary production, however the flux of organic matter into the sediments was not increased (Boyd *et al*. 2000). This demonstrates the importance of microbial processes that control distribution, utilization and remineralization of plankton biomass and detritus. Most of these processes are mediated by the microbial community associated with algae and particulate detritus, which consists to a significant part of *Roseobacter* clade bacteria.

The ability of members of the *Roseobacter* clade to adhere to algae is a central prerequisite for their symbiotic interaction. From a human perspective, nutrients in the marine environment seem to be evenly distributed, however on a microscopic scale there is a steep nutrient gradient between the nutrient-depleted water and particulate nutrient sources, and only attached bacteria have continuous access to these (Simon *et al.* 2002). In order to find rewarding places for attachment, a bacterium has to be able to sense and follow chemical cues that indicate a rewarding substrate, thus flagellar motility and chemotaxis are essential prerequisites for engaging in bacterial-algal symbioses (Miller and Belas 2006; Slightom and Buchan 2009; Geng and Belas 2010b).

3.2.2 Transition between motile and sessile lifestyle

Many *Roseobacter* species have alternating phases of planktonic life, where they are motile and chemotactic towards algal exudates, and phases of attached life, where they are

immobilized in a biofilm and utilize, or interact with, their substrate. This behavior was colloquially termed “the swim-or-stick lifestyle” (Belas *et al*. 2009). The molecular mechanisms underlying the alternation between the two distinctly different phases that each are attended with specific phenotypes have been investigated, however no convincing model of the decision-making between motile and sessile life had so far been proposed (Miller *et al.* 2004; Bruhn *et al.* 2007; Belas *et al.* 2009; Geng and Belas 2010a; Sule and Belas 2012). Nevertheless, a deepened knowledge of how *Roseobacter* species transition from planktonic life to biofilm formation is needed to understand carbon and nutrient cycling in the oceans (Slightom and Buchan 2009). The present study has contributed to this understanding by demonstrating that members of the *Roseobacter* clade deploy intracellular signaling based on cyclic dimeric guanosinmonophosphate (c-di-GMP), as the mechanism by which extracellular cues are integrated to reach a decision between motile and sessile life (Figure 18) (D'Alvise et al. 2013b).

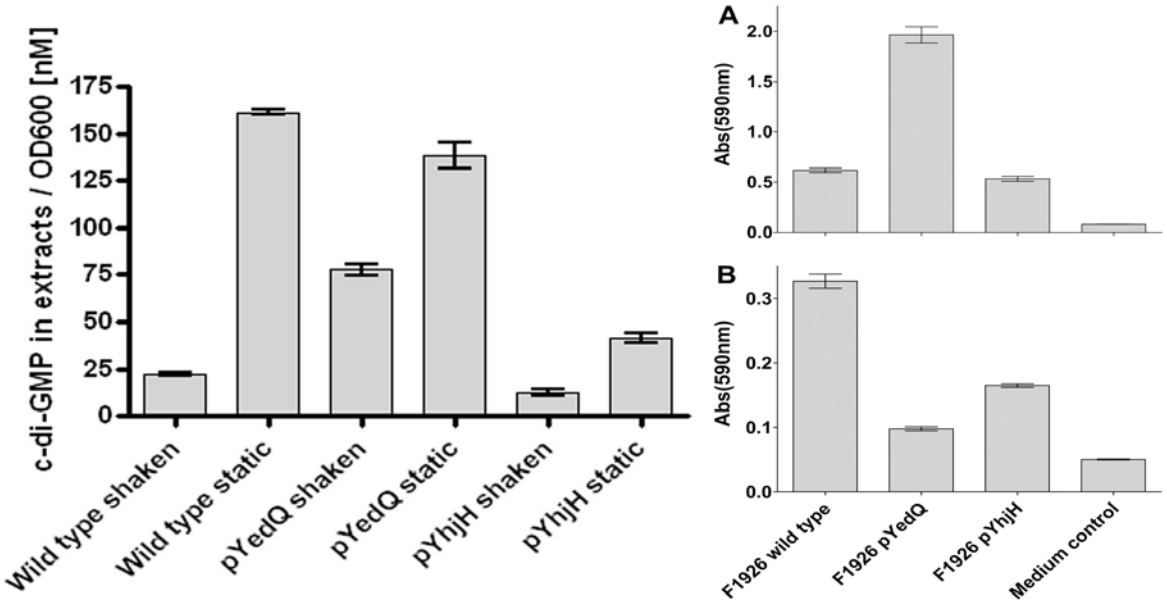

Figure 18: Left panel; intracellular concentrations of c-di-GMP in *Ruegeria mobilis* F1926 are influenced by culture conditions. C-di-GMP levels were manipulated using the plasmids pYedQ, which encodes a c-di-GMP-producing diguanyl cyclase, and pYhjH, which encodes a c-di-GMP-degrading diesterase. Right panel; Biofilm formation, as measured in a crystal violet assay (O'Toole *et al.* 2000), was increased by higher c-di-GMP levels (A), but the ability to attach to an inert surface was reduced both by increased and decreased c-di-GMP levels. (D'Alvise *et al.* 2013b)

C-di-GMP signaling as a way of switching between planktonic and attached life had been known from other bacteria, such as *Escherichia coli* and *Pseudomonas* spp., and increasing

evidence suggests that this mechanism is well-conserved in most bacterial species (Hengge 2009; McDougald *et al*. 2012). The basic mechanism of c-di-GMP signaling is that environmental signals are sensed by a multitude of specific receptors that individually modulate the activity of associated enzymes, which produce or degrade c-di-GMP. Instead of reacting to each single stimulus with phenotypic changes, which would result in uncoordinated expression of conflicting traits, bacterial cells process information from many relevant signals into a central indicator that concerts gene expression and activity of enzymes and organelles, thus giving rise to defined phenotypes. Among the signals that can potentially be integrated into the pool of c-di-GMP are concentrations of nutrients and other required substances, many different indicators of adverse conditions, light levels, dissolved gases and also cell to cell communication signals (Figure 19).

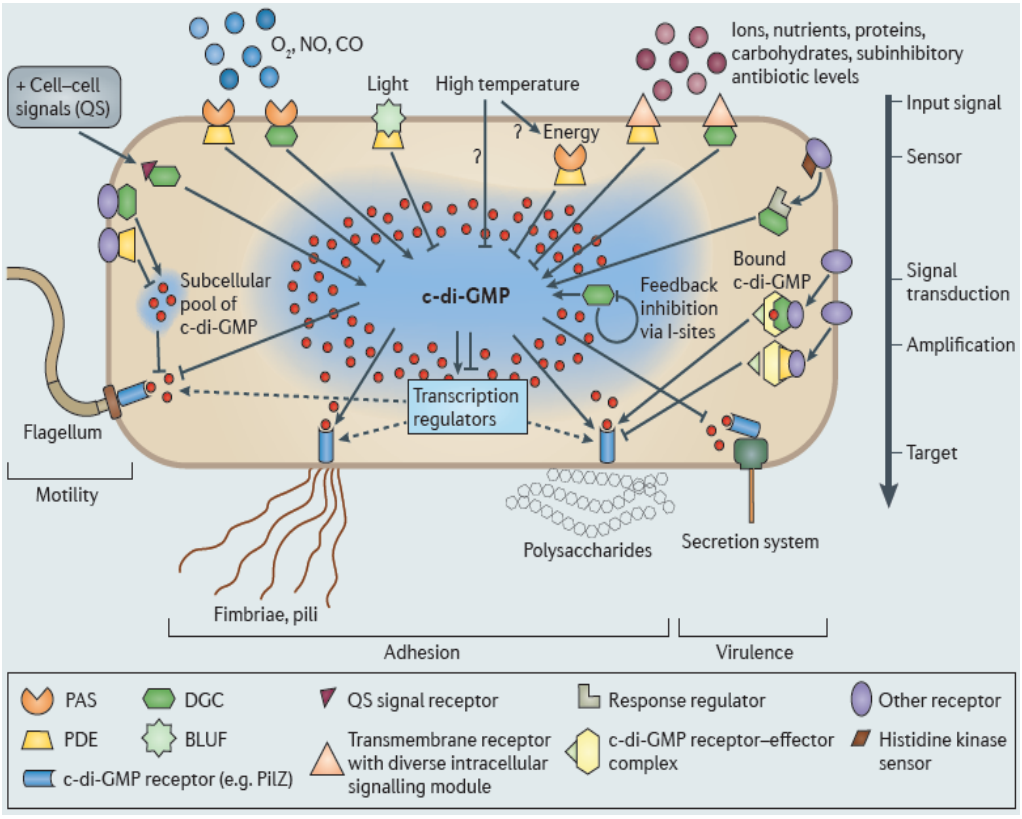

Figure 19: Schematic functionality of c-di-GMP signaling. Extracellular signals are sensed by different membrane-bound receptors (PAS = PER-ARNT-SIM-type gas receptors, BLUF = Blue Light Using Flavin receptor and other receptor types) that switch the activity of associated phosphodiesterases (PDE) or diguanylate cyclases (DGC). Depending on the stimulus, this increases or decreases c-di-GMP concentrations to a greater or lesser extent. Flagellar motility, production of extracellular polymeric substances (EPS) and other cellular activities are activated or deactivated by the altered c-di-GMP concentrations. C-di-GMP also binds to transcriptional regulators and acts as an inhibitor or activator for expression of specific genes. (McDougald *et al.* 2012)

In *R. mobilis* F1926 we found nine diguanyl cyclases and c-di-GMP-degrading phosphodiesterases, and the function of six of these alternates between degradation and synthesis of c-di-GMP, while the other three were predicted to have only diguanyl cyclase activity (D'Alvise *et al.* 2013b). We can only speculate what environmental cues activate or switch the function of these enzymes, however from the attachment experiment we can conclude that physical contact to a surface or other bacteria is among them. One of the enzymes contains an ammonium transporter-domain, indicating that either presence of ammonia in the environment may be a clue for attachment, or conversely, the cue may be ammonia production of the bacterium itself, which would only be egested in presence of a protein-containing substrate. Other cues that may influence the c-di-GMP turnover might be presence of other nutrients, e.g. algal exudates, oxygen or other gases, or quorum-sensing compounds. Since TDA seems to act as a quorum sensing compound, at least on its own production (Geng and Belas 2010a), it cannot be excluded that TDA, besides its production being regulated by c-di-GMP levels (see next section), may also be sensed and integrated via c-di-GMP signaling.

Irrespective of its physiological role, it could be hypothesized that some of the ecological success of *Phaeobacter* and *Ruegeria* spp. is due to TDA, which is, as far as known, within the *Roseobacter* clade a distinct attribute of the two genera.

3.2.3 Production of tropodithietic acid

Tropodithietic acid (TDA) is a tropone derivative with a carboxyl and a disulfur group. Tropothiocin is a synonym for TDA, the two compounds exist as a pair of rapidly interconverting tautomers (Figure 20) (Greer *et al*. 2008). Thiotropocin was first described to be produced by a *Pseudomonas* sp. from a soil sample, and had been patented as novel antibiotic (Kintaka *et al*. 1984; Tsubotani *et al*. 1984). Apart from this *Pseudomonas* isolate and the *Phaeobacter* and *Ruegeria* spp. in the *Roseobacter* clade, production of TDA was only reported in isolates of the closely related genus *Pseudovibrio* (*Rhodobacteraceae*), one isolate from the red macroalga *Delisea pulchra* and six from Atlantic sponges (Enticknap *et al*. 2006; Geng and Belas 2010a; Penesyan *et al*. 2011). Interestingly, all TDA-producing isolates live in close association with eukaryotes.

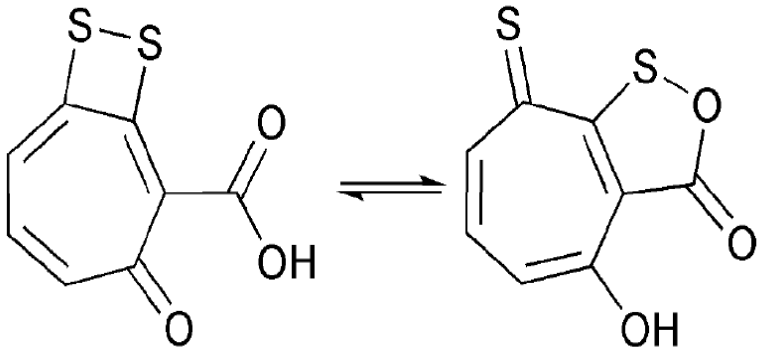

Figure 20: Tropodithietic acid (TDA, left) and thiotropocin (right) are interconverting tautomers. (Thiel *et al*. 2010)

Brinkhoff *et al.* (2004) identified the antibacterial compound of the seawater isolate *Roseobacter* sp. T5 as tropothiocin, and Bruhn et al. (2005) found that the antibacterial sulfur-containing compound of the probiotic isolate *Roseobacter* sp. 27-4 was identical. Both *Roseobacter* spp. were later reclassified as *Phaeobacter* spp. (Martens *et al.* 2006). The compound was also found to be produced by *Silicibacter* sp. TM1040, later reclassified as *Ruegeria* sp. TM1040 (Yi *et al.* 2007; Muramatsu *et al*. 2007; Brinkhoff *et al.* 2008; Newton *et al.* 2010), and twelve genes of its biosynthesis pathway were identified (Geng *et al*. 2008). Six of the genes in the lower, specific biosynthesis pathway *tda*A-F are located on a large plasmid, and this is also the case in *P. gallaeciensis* (Geng et al. 2008; Thole et al. 2012). In laboratory cultures the plasmid can be lost with a relatively high frequency, which results in white colonies on Marine Agar. However, nearly all natural *Phaeobacter* isolates harbor this plasmid, as indicated by their TDA production, indicating a strong selection pressure for TDA production. An NMR study of TDA biosynthesis in *Pseudomonas* sp. CB-104 revealed that TDA is derived from shikimic acid via phenylacetate, and it can be produced directly from phenylalanine (Cane). However, the lower steps in the TDA synthesis pathways have not yet been elucidated and the enzyme-encoding genes *tda*B-F have not been linked with biochemical reactions. One of the genes that were found to be involved in TDA biosynthesis is *cys*I, which encodes for a sulfite reductase that is normally involved in cysteine biosynthesis (Geng et al. 2008). In this study we have hypothesized that *cys*I would be involved in the last step of TDA production, possibly a modification of the two sulfur atoms. We speculated that *cys*I would not be functional in iron-depleted cultures, since an iron-sulfur cluster was described to constitute the active center of the enzyme (Ostrowski *et al*.

1989), and that this would lead to production of an inactive precursor of TDA, which we observed in cultures without iron addition. However, using a *cys*I-mutant constructed by Thole *et al*. (2012), the hypothesis could be falsified (D'Alvise *et al*. 2013c). It seems likely that *cys*I leads to deficiency in TDA production, because cysteine is needed as a precursor of TDA. Possibly the sulfur in the TDA molecule is incorporated via cysteine. It has been demonstrated that TDA can be produced from sulfate and this may take place via a cysteine intermediate (Heidorn 2002; Bruhn 2006; Geng et al. 2008).

TDA production in broth cultures depends on the cultivation conditions. High TDA production is only possible in static cultures, while shaking of the cultures prevents TDA formation in *Ruegeria* spp. and in *Phaeobacter* sp. 27-4 (Bruhn *et al*. 2006; Porsby et al. 2008). Shaking of the cultures also affects morphology, biofilm formation and motility. Static cultures are dominated by thick, EPS-containing biofilms that grow at the air-liquid interface and consist of star-shaped, rosette-like aggregates (Figure 21), in contrast to shaken cultures that contain predominantly single, motile cells.

Figure 21: Cell morphology of *Phaeobacter* sp. 27-4, grown as static liquid culture. (Bruhn *et al*. 2005)

The present study has demonstrated that these differences are due to unequal intracellular levels of c-di-GMP, and that manipulation of c-di-GMP levels affects TDA production (Table 4) (D'Alvise et al. 2013b). The perturbation in shaken cultures seems to prevent the single cells from making contact with a surface or each other, which is probably the critical

stimulus that would cause c-di-GMP levels to increase and thereby lead to expression of phenotypic traits that are associated with attached life, such as growth as rosettes, production of extracellular substances and TDA formation.

Table 4: Inhibition of *V. anguillarum* 90-11-287 by cell-free supernatants of *R. mobilis* F1926 wild type, F1926 pYedQ (overproduces c-di-GMP), and F1926 pYhjH (low c-di-GMP) cultures, grown in shaken or static MB for 72 h at 25°C (D'Alvise *et al.* 2013b)

	Inhibition zone diameter without well diameter [mm] first / second replicate	
Strain	Shaken cultures (200 rpm)	Static cultures
R. mobilis F1926 wild type	0 / 0	11 / 13.5
R. mobilis F1926 pYedQ	0 / 0	11 / 15
R. mobilis F1926 pYhjH	0 / 0	1 / 7

Production of TDA was also reported to be regulated in a density dependant manner. In *P. gallaeciensis*, AHL-based quorum-sensing influences TDA production (Berger *et al*. 2011), and Geng and Belas (2010a) showed that TDA production in *Ruegeria* sp. TM1040 is auto-inducible. However, during this PhD study we have tested the antagonistic abilities of *P. gallaeciensis* and two quorum-sensing deficient mutants (DSM17395 pgaR and DSM17395 pgaI (Berger *et al.* 2011)) in algae cultures, and the mutations did not compromise inhibition of *V. anguillarum* (Figure 22) (Prol-Garcia *et al*. 2012).

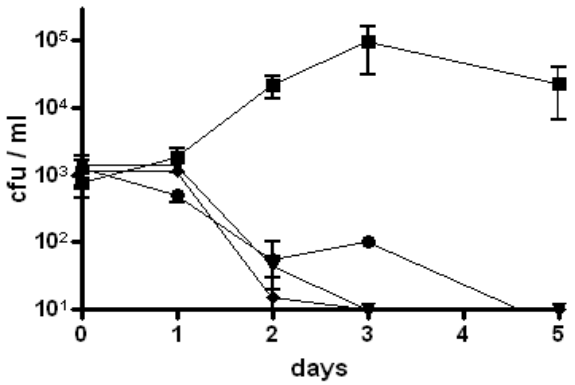

Figure 22: Concentrations of Vibrio *anguillarum* in cultures of the microalga *Tetraselmis suecica*, in the control (■), and in co-culture with *Phaeobacter gallaeciensis* quorum sensing mutants (pgaR ♦; pgaI ●) and wild type (▼).(Prol-Garcia *et al.* 2012)

Interestingly, the mutations in the quorum-sensing system did not affect TDA production in static liquid cultures, as measured in an agar-diffusion-inhibition assay, but only delayed the onset of TDA production in shaken cultures by one day (Figure 23). It could be speculated whether the effect of the mutations on TDA production may be mediated by the c-di-GMP signaling system, since interactions and connections between quorum sensing and c-di-GMP signaling are common, as reviewed by Srivastava and Waters (2012). The culture condition (shaken or static), which influences the amount of biofilm formed in the cultures, seems to interfere with the effect of quorum-sensing on TDA production, which might possibly indicate that the quorum-sensing signal could be integrated into the c-di-GMP signaling system.

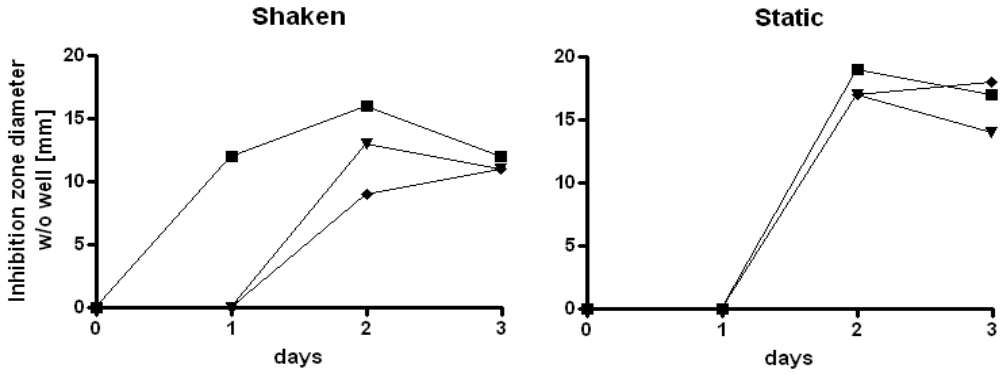

Figure 23: Production of tropodithietic acid (TDA), measured by inhibition of *Vibrio anguillarum*, in shaken and static Marine Broth cultures of *Phaeobacter gallaeciensis* DSM17395 wild type (■) and quorum sensing mutants (pgaR ▼; pgaI ♦). (Prol-Garcia *et al.* 2012)

TDA production in Marine Broth coincides with the appearance of a brown pigment, which is not produced by TDA-negative mutants (Brinkhoff et al. 2004; Geng et al. 2008). The brown pigment is not identical with TDA, and fractions of *Phaeobacter* sp. 27-4 supernatant that contained the pigment but not TDA were not bioactive, and the identity of the brown pigment has not yet been elucidated (Bruhn *et al*. 2005). The present study demonstrates that production of both TDA and the brown pigment depends on high iron concentrations (D'Alvise et al. 2013c). In media without iron addition a non-inhibitory TDA-like substance (pre-TDA) is produced that can be converted to TDA by acid addition. Although no final and conclusive chemical analysis of the pigment has been carried out yet, there is indication that the pigment may be a product of TDA and iron.

TDA does not have the vigorous iron-binding ability of a siderophore, yet it reacts with iron ions and forms a brown precipitate that is likely identical with the brown pigment in the iron-rich MB cultures. In a CAS-assay, which indicates mobilization of iron ions by e.g. siderophores, concentrated TDA (1mM) gives a positive reaction after prolonged incubation (Figure 24).

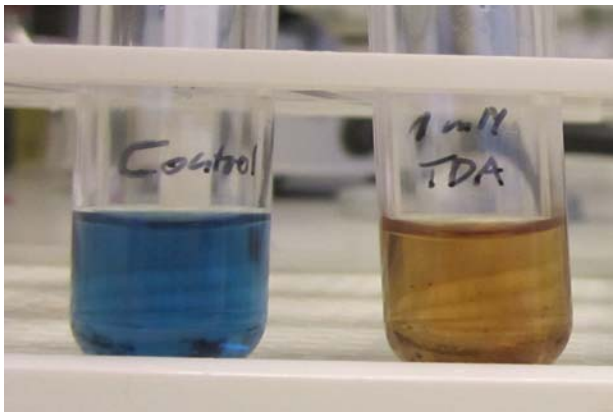

Figure 24: CAS-assay with water (control, left) and pure TDA (right) after 24h incubation.(D'Alvise *et al.* 2013b)

These results raise questions on the possible physiological and ecological functions of TDA and the pigment. How is TDA produced in natural settings, where iron concentrations are low? Does the putative TDA-iron complex facilitate or catalyze the conversion of pre-TDA? What role does the iron-affinity of TDA play for the symbiotic algae? For one thing, the iron-containing pigment precipitates out of seawater and is deposited in the biofilm matrix. This may constitute a pool of particle-bound iron that could be accessed using siderophores, thus increasing the availability of iron for the symbiotic algae and providing a selective advantage for both algae and the roseobacters.

Irrespective of the physiological details of its production, the TDA production of *Phaeobacter* spp. is crucial for their probiotic effect. The present study has demonstrated that the disease-preventing effect of a TDA-negative mutant of *P. gallaeciensis* was strongly reduced, and that the probiotic effect of *Phaeobacter* isolates can be predicted by assessing their TDA production in an agar-based diffusion assay (D'Alvise et al. 2012; D'Alvise *et al*. 2013a).

3.3 Application as probiotic bacteria

Phaeobacter spp. are prevalent in aquaculture operations in different climate zones, as *Phaeobacter* strains have been isolated from, or detected in, many aquaculture sites (Cytryn *et al*. 2005; Schulze *et al*. 2006; Brunvold *et al*. 2007; Prado *et al*. 2009; Michaud *et al*. 2009). The first indication of the probiotic potential of *Phaeobacter* spp. was observed when a bacterium with antibacterial activity, *Roseobacter gallaeciensis* BS107 (later renamed as *Phaeobacter* gallaeciensis BS107 - DSM17395 (Martens *et al.* 2006)), was isolated from scallop (*Pecten maximus*) larvae cultures (Ruiz-Ponte et al. 1998; Ruiz-Ponte *et al*. 1999). The isolate was tested in a scallop larvae challenge assay with a pathogenic *Vibrio* sp., but no disease preventing effect was achieved. However, the larvae in one of the controls that had received *P. gallaeciensis* showed a lower mortality than untreated larvae. Although this study was flawed by methodological difficulties, the prediction that a probiotic effect for marine larvae could be achieved with this strain proved to be right.

Later, an independent study with the objective of finding antagonistic probiotic bacteria for turbot larvae isolated *Phaeobacter* sp. 27-4 from tank walls of turbot larvae basins in Spain due to its antagonism against a *V. anguillarum* strain and demonstrated that the isolate was harmless for turbot larvae (Hjelm et al. 2004). Subsequently, *Phaeobacter* sp. 27-4 was tested in turbot larvae challenge trial with *V. anguillarum*, where it showed a significant disease-preventing effect in one out of three replicates (Planas et al. 2006). In that study *Phaeobacter* sp. 27-4 and the pathogen were used to enrich rotifers (*B. plicatilis*) before being fed to the larvae, thus both the pathogen and the probiont were delivered directly into the gastrointestinal system of the larvae. Moreover, the pathogen was administered three times during the first seven days of feeding (3-10 days post hatch) in very high doses (enrichment with 10^8 cfu/ml *V. anguillarum* for 3 hours), and either pathogen or probiont were delivered on one day, so that contact between *V. anguillarum* and *Phaeobacter* sp. was mostly restricted to the gastrointestinal tract. This was done because at that time most of the knowledge about probiotic bacteria was derived from experiments with lactobacilli, and it was assumed that all probiotic bacteria should colonize the gastrointestinal tract. However, the study did not find *Phaeobacter* attached to the intestinal mucus of the larvae in histological sections, which could possibly be explained by *Phaeobacter* spp. being unable to grow under anaerobic conditions. Given that we know today that the conditions for

probiotic activity of the strain were not ideal during that study, it is even more remarkable that *Phaeobacter* sp. 27-4 was able to reduce larval mortalities in that setting.

The present study has revisited the testing of *Phaeobacter* strains as probiotic bacteria for larviculture and questioned some of the experimental approaches in the initial experiments by Planas et al. (2006). One of these approaches was the mode of administration; the fact that *Phaeobacter* spp. are strictly aerobic and naturally occur in biofilms associated with abiotic substrates, algae, or particulate detritus argues against a role as intestinal probionts and indicates an application in the environment of the larvae. The second approach that we questioned was the challenge mode; a challenge with pathogenic bacteria delivered via rotifers presumes that presence of high amounts of pathogens in live feed is an unchangeable given fact of marine larviculture. We challenged this presumption and tested whether *P. gallaeciensis* BS107 could be used to control the microflora on all trophic levels. Therefore we used gnothobiotic algae (*Tetraselmis suecica*, *Nannochloropsis oculata*) and rotifer (*Brachionus plicatilis*) cultures to study the interaction between the probiont and a model pathogen (*V. anguillarum*) in these systems. We found that unchecked propagation of the pathogenic bacterium in the live feed cultures was prevented, and the pathogen concentrations were reduced by at least three logarithmic units in presence of P. gallaeciensis (Figure 25), while growth of the live feed organisms was not adversely affected (D'Alvise et al. 2012).

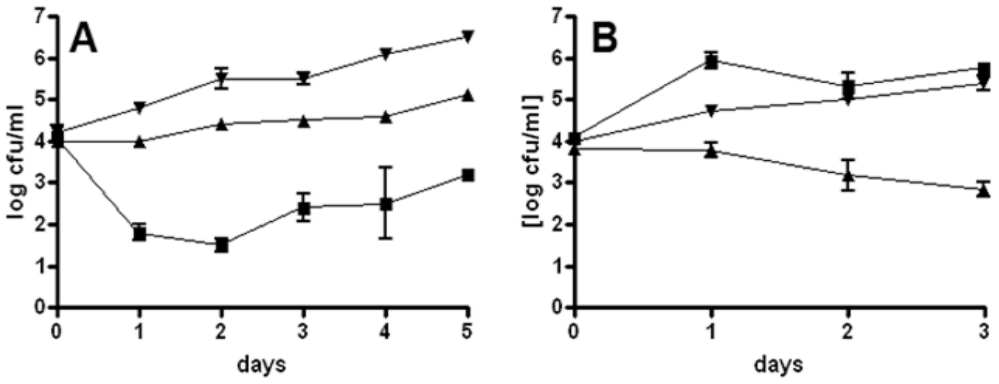

Figure 25: Concentrations of *Vibrio anguillarum* in cultures of the microalga *Tetraselmis suecica* (A) and the rotifer *Brachionus plicatilis* (B). The pathogen was introduced at 10^4 cfu/ml into axenic cultures (▼), and into cultures where *Phaeobacter gallaeciensis* wild type (■) or a TDA-deficient mutant (▲) was pre-established.

As an alternative challenge mode for experiments with fish larvae we chose administration of lower pathogen inocula to the environment of the larvae in a system that offers better control and higher reproducibility (Sandlund and Bergh 2008; Sandlund *et al*. 2010). In these single-larvae systems we were able to demonstrate that TDA-producing *Phaeobacter* strains, as opposed to TDA-deficient mutants, can completely prevent infections with *V. anguillarum*, if they are applied as prophylactic treatment when the cultures are set up (Figure 26) (D'Alvise et al. 2012; D'Alvise et al. 2013a).

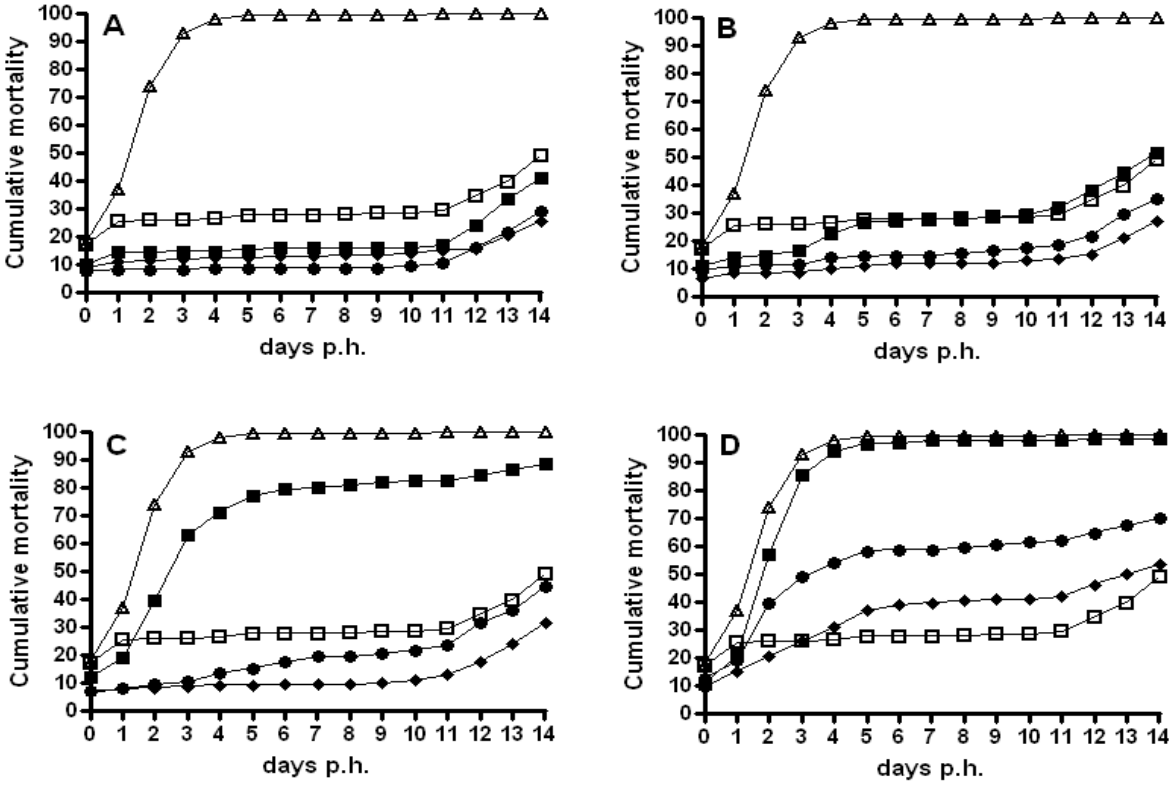

Figure 26: Mortality of cod larvae in challenge trials with V. anguillarum, mean values from three independent trials. Untreated larvae (□) and larvae inoculated with only V. anguillarum (Δ) are displayed as controls in all panels, and the effect of the three probiotic Phaeobacter strains 27-4 (■), M23-3.1 (♦), and BS107 (●) at different inoculation times is compared: A: Exposure to single strains. B: Challenge trial; Phaeobacter strains introduced 2 days before V. anguillarum. C: Challenge trial; Phaeobacter strains introduced simultaneous with V. anguillarum. D: Challenge trial; Phaeobacter strains introduced 48 hours after V. anguillarum. All bacteria were introduced before hatching (D'Alvise et al. 2013a).

Recent experiments showed that also in gnotobiotic *Artemia* cultures *P. gallaeciensis* BS107 prevented growth of *V. anguillarum* and reduced *Artemia* mortality in a challenge with *V. harveyi* to the level of the unchallenged controls (L.P. Lauridsen, N. Overby, P. D'Alvise, unpublished data, Figure 27). This adds *Artemia* to the list of live feed cultures in which *P.*

gallaeciensis can prevent the formation of infectious concentrations of *V. anguillarum* and completes the findings of the present study, since now it was demonstrated that the probiotic bacterium is able to antagonize pathogenic bacteria in all of the trophic levels in marine larviculture, i.e. in cultures of microalgae, rotifers, and fish larvae.

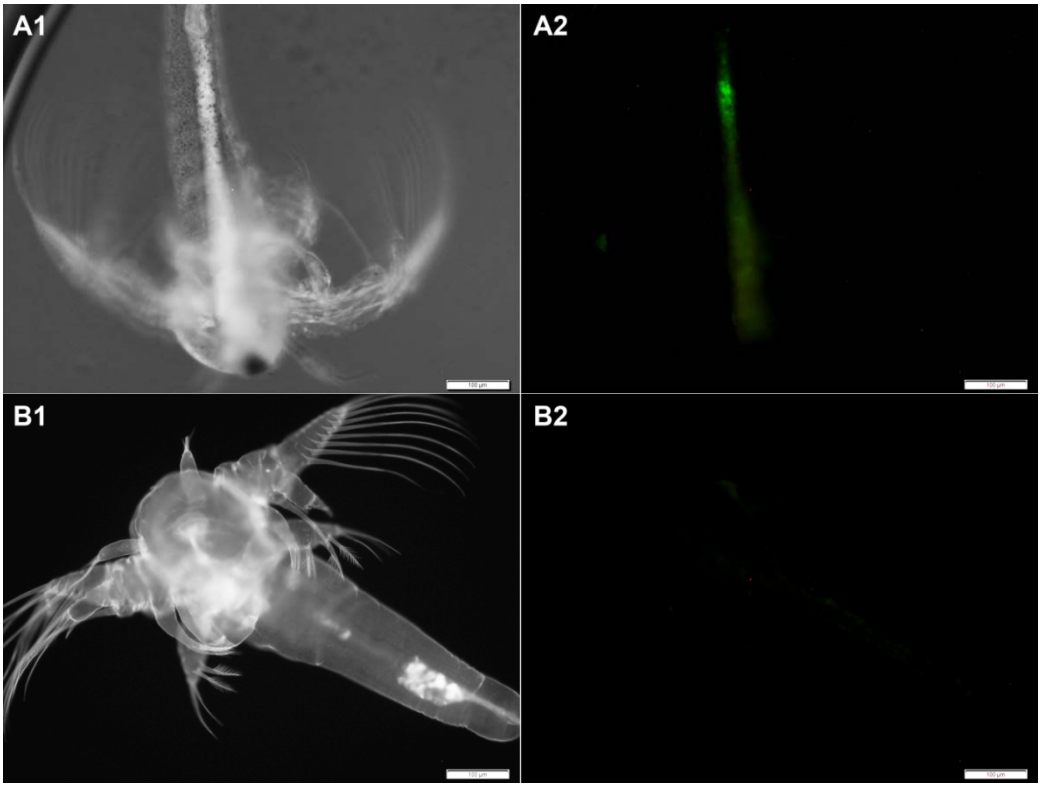

Figure 27: Reduction of gfp-tagged *Vibrio anguillarum* NB10 in *Artemia* guts by *Phaeobacter gallaeciensis* BS107. In gnotobiotic *Artemia* cultures with only *Vibrio anguillarum* the pathogen grew to levels of around 10^7 cfu/ml and colonized the intestinal system of the *Artemia* (A), while in presence of *P. gallaeciensis* BS107 its numbers were reduced to below 10^3 cfu/ml and the gut was not colonized. (Lauridsen, Overby, D'Alvise, unpublished results)

Although we used *V. anguillarum* as the model pathogen in most of our studies, the antibacterial effect of *Phaeobacter* spp. is not limited to this target strain. Inhibitory activity of *Phaeobacter* spp. against other pathogenic bacteria that cause problems in marine aquaculture have been demonstrated, among them a wide range of other *Vibrio* spp. like *V. harveyi, V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. pelagius, V. splendidus, V. tubiashii, V. neptunius, V. aesturianus, V. fluvialis, V. logei, V. proteolyticus, V. tapetis, Photobacterium damselae* ssp. *piscicida* (formerly: *V. damsela, Pasteurella piscicida*), *Moritella viscosa* (*V. viscosus*), *Aeromonas salmonicida* and other *Aeromonas* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Xanthomonas* spp., *Tenacibaculum maritimum,*

Lactococcus piscium, as well as the human pathogens *Salmonella enterica,* and *Staphylococcus aureus* (Ruiz-Ponte et al. 1999; Prado et al. 2009; D'Alvise et al. 2010).

A concern that is sometimes raised regarding the use of TDA-producing *Phaeobacter* spp. as probiotic bacteria is that this may also lead to formation of resistances, since TDA is after all still an antibiotic compound (Defoirdt *et al.* 2011a). It cannot be excluded that resistances to TDA might eventually arise, however Porsby et al. (2011) have tried to provoke TDA resistance formation by a number of thorough approaches using exposure to sub-inhibitory levels of TDA, however no resistant mutants could be isolated. Moreover, *Phaeobacter* spp. are natural inhabitants of coastal zones and aquaculture facilities. Using them as probiotics does not introduce a new element into the ecology of the system, but merely shifts the balance. *Phaeobacter* spp. have been competing with *Vibrio* spp. for energy and resources in coastal ecosystems for a very long time, and TDA has been a key metabolite in this interaction. It seems unlikely that the principles of this interaction will suddenly change.

4. Concluding remarks and future perspectives

During the last thirty years aquaculture has grown from a mostly negligible business into a full-sized industry and is now one of the largest food-producing sectors. As a result of its dramatic growth, the industry is facing a number of economic and ecological problems and challenges. New sustainable technologies have to be developed to replace some of the traditional practices that cannot be maintained at the scale of the current industry production. The use of antibiotics to treat and prevent bacterial diseases in aquaculture has to be limited to special cases and needs to be banned from the routine of aquaculture production. Juvenile and adult fish should be protected by multivalent vaccines, and in many cases husbandry conditions have to be improved to obviate frequent bacterial infections. For fish larvae and invertebrates, which cannot be vaccinated, probiotic bacteria have a great potential to prevent bacterial infections.

Although most of the probiotics used in aquaculture so far were bacteria that colonize the intestine, this study has been focusing on the external microflora of fish larvae and live feed cultures. Preventive application of *Phaeobacter* strains (*Roseobacter* clade, α-*Proteobacteria*) was shown to reduce concentrations of the pathogen *Vibrio anguillarum* in microalgae and rotifers and prevented vibriosis in challenged cod (*Gadus morhua*) larvae. Production of the antibacterial compound tropodithietic acid (TDA) was identified as the major mechanism of action by including mutants deficient in TDA synthesis. TDA as a pure compound was effective as well, however the antibacterial effect of a single TDA addition faded after a few hours. Application of TDA-producing bacteria is superior to using the pure compound, since concentration fluctuations are avoided and only a single, initial addition is required. The inhibitory effect is not only due to TDA production, as demonstrated by partial inhibition of pathogenic bacteria by the TDA-negative mutants, but also competition for nutrients and space may play a role, which argues for using the whole bacterium.

As suggested in previous studies, physical attachment of the cells was found to be a prerequisite for TDA production. The present finding that intracellular c-di-GMP concentrations are, as know from other bacteria, the central mechanism regulating transitions between sessile and motile life in *Roseobacter* clade species is of interest for the

aquaculture application of *Phaeobacter* strains, and beyond that it provides insight into how *Roseobacter* clade spp. interact with their algal symbionts. Understanding what cues and signals are integrated into c-di-GMP-based decision making of abundant environmental *Roseobacter* clade species would contribute to our knowledge of phytoplankton ecology. This could be achieved by studying the c-di-GMP-specific phosphodiesterases and diguanyl cyclases using bioinformatic approaches, molecular methods, and knock-out mutants in physiological experiments.

Also the finding that TDA interacts with iron, and might also in the oceanic environment require iron for its production, may be of ecological relevance. However, the insights that this study has gained into the complex topic of TDA production physiology are limited. Structure elucidation of the brown pigment, which may be a product of iron and TDA, and of the non-inhibitory precursor of TDA may elucidate the terminal step of TDA biosynthesis. Experiments addressing the utilization of iron and, possibly, the brown pigment in algae cultures with and without wild type and mutant *Phaeobacter* might be able to clarify the ecological role of the pigment.

As a future perspective from the present work, the application of *Phaeobacter* strains as probiotic bacteria in marine larviculture will have to be tested in real life. Pilot-scale or full-scale trials that will provide an unbiased assessment of the probiotic potential of the *Phaeobacte*r strains in marine hatcheries are planned within the framework of an upcoming project. The use of probiotic *Phaeobacter* strains will be part of a combinatory approach, which will include the use of pathogen-specific phages, to reduce bacterial infection in the larvae of different fish species.

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Paper 1

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***Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae.**

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Phaeobacter gallaeciensis Reduces *Vibrio anguillarum* in Cultures of Microalgae and Rotifers, and Prevents Vibriosis in Cod Larvae

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Abstract

Phaeobacter gallaeciensis can antagonize fish-pathogenic bacteria *in vitro*, and the purpose of this study was to evaluate the organism as a probiont for marine fish larvae and their feed cultures. An *in vivo* mechanism of action of the antagonistic probiotic bacterium is suggested using a non-antagonistic mutant. *P. gallaeciensis* was readily established in axenic cultures of the two microalgae *Tetraselmis suecica* and *Nannochloropsis oculata*, and of the rotifer *Brachionus plicatilis*. *P. gallaeciensis* reached densities of 10^7 cfu/ml and did not adversely affect growth of algae or rotifers. *Vibrio anguillarum* was significantly reduced by wild-type *P. gallaeciensis*, when introduced into these cultures. A *P. gallaeciensis* mutant that did not produce the antibacterial compound tropodithietic acid (TDA) did not reduce *V. anguillarum* numbers, suggesting that production of the antibacterial compound is important for the antagonistic properties of *P. gallaeciensis*. The ability of *P. gallaeciensis* to protect fish larvae from vibriosis was determined in a bath challenge experiment using a multidish system with 1 larva per well. Unchallenged larvae reached 40% accumulated mortality which increased to 100% when infected with *V. anguillarum*. *P. gallaeciensis* reduced the mortality of challenged cod larvae (*Gadus morhua*) to 10%, significantly below the levels of both the challenged and the unchallenged larvae. The TDA mutant reduced mortality of the cod larvae in some of the replicates, although to a much lesser extent than the wild type. It is concluded that *P. gallaeciensis* is a promising probiont in marine larviculture and that TDA production likely contributes to its probiotic effect.

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Introduction

One of the major challenges of marine aquaculture is the continuous and reliable production of juveniles. Severe losses in marine larviculture are caused by infection with opportunistic pathogenic bacteria, including several members of the *Vibrionaceae* family [1,2], that accounts for approximately 1.5% of the bacterial community in the oceans [3]. Only some *Vibrio* species are pathogenic to organisms reared in marine aquaculture and one of the most prominent fish and shellfish pathogens is *Vibrio (Listonella) anguillarum* that causes serious disease and mortalities [2]. The main source of pathogenic bacteria in marine aquaculture is supply water [4], but also brood stock, humans, or starter cultures are possible sources of pathogens [5]. The majority of marine fish larvae are reared intensively in presence of microalgae (green water), which improves feeding, growth, and survival of the larvae [6–9]. The larvae require live feed, and rotifers (*Brachionus plicatilis*) are typically used as first feed. The rotifers themselves are fed or enriched with live microalgae, such as *Tetraselmis suecica*, *Nannochloropsis oculata*, and *Isochrysis galbana*. Opportunistic pathogens can proliferate in larval feed cultures of phytoplankton and invertebrates due to high concentrations of organic matter. Algae, rotifer and *Artemia* cultures can therefore harbor high concentrations of pathogenic bacteria [1,5,10,11]. Prophylactic treatment of larvae or their feed cultures with antibiotics can reduce the pathogen load, but has to be avoided, since it leads to emergence of antibiotic-resistant pathogens, and since it impedes the establishment of a normal non-pathogenic microbiota [1,12,13].

The potential use of probiotic bacteria to limit outbreaks or effects of bacterial diseases in fish and invertebrate cultures has been investigated for more than two decades. Most studies have focused on the intestinal microbiota [14–19], although the use of probiotics is not confined to the intestinal tract of the cultured organisms [15,20]. Biotic and abiotic surfaces, algal and fecal particles, and the nutrient-rich water serve as habitat and reservoir of opportunistic pathogenic bacteria in cultures of fish larvae or their food organisms [1,5,10,11,20], and it is hypothesized that competition by non-pathogenic bacteria that are superior in colonizing and persisting in these habitats could reduce the incidence of pathogenic bacteria.

Phaeobacter gallaeciensis (formerly *Roseobacter gallaeciensis*) is a Gram-negative α-proteobacterium from the *Roseobacter*-clade [21]. The bacterium produces the antibacterial compound tropodithietic

acid (TDA) that is an efficient inhibitor of *V. anguillarum* and other fish-pathogenic bacteria [22–25]. *Phaeobacter* spp. are commonly isolated from larval cultures of marine fish and shellfish [26–28], and do not appear to adversely affect fish larvae [22,29]. *Ruegeria mobilis*, a close relative to *Phaeobacter* also producing TDA, is a cosmopolitan marine bacterium that can be isolated from most ocean waters, apart from polar waters [30].

In a previous study, it was demonstrated that *Phaeobacter* and *Ruegeria* isolated from a turbot hatchery [26] could eliminate *V. anguillarum* in a seawater-based combined liquid-surface system [22]. It was demonstrated, using a TDA-negative mutant, that TDA production was likely a key factor in the pathogen inhibition. The purpose of the present study is to determine if *P. gallaeciensis* BS107 (DSM 17395) could antagonize *V. anguillarum* in fish larvae and cultures of their feed organisms. To the authors' knowledge, no study on antagonistic probiotic bacteria has yet elucidated the mechanism of action *in vivo*. Therefore a non-antagonistic TDA-negative mutant of *P. gallaeciensis* BS107 (DSM 17395) was created to investigate the *in vivo* mechanism of action, as emphasized by Tinh *et al.* [15]. The type strain *P. gallaeciensis* BS107 (DSM17395) [28] was chosen, since its inhibition of *V. anguillarum* in *Tetraselmis* cultures was more pronounced than that of other *Phaeobacter* and *Ruegeria* strains, as assessed in a preliminary experiment (data not shown). Gnotobiotic algae and rotifers were used for studying probiotic and nutritional effects of the introduced organism, as recommended [15,31,32].

Materials and Methods

Bacterial strains and media

All strains and plasmids are listed in Table 1. *Phaeobacter (Roseobacter) gallaeciensis* BS107 (DSM17395) was isolated from seawater in scallop (*Pecten maximus*) cultures [28]. *Vibrio anguillarum* serotype O1 strain NB10 was used in algal and rotifer experiments. It was isolated from the Gulf of Bothnia and has caused disease in rainbow trout [33,34]. The strain has been tagged by insertion of plasmid pNQFlaC4-gfp27 (*cat*, *gfp*) into an intergenic region on the chromosome, and was kindly provided by D. Milton, University of Umeå [35]. *V. anguillarum* serotype O2α HI610 was used in challenge trials with cod larvae. The strain was isolated from diseased cod juveniles in the closed seawater basin Lake Parisvatn by the Institute of Marine Research (IMR), Norway, and has been used in challenge trials with cod [36–39]. It has been selected from a group of *V. anguillarum* strains of different serotypes, being the strain that caused the highest mortality in challenge trials with turbot, halibut and cod larvae [40].

Bacteria from frozen stock cultures (−80°C) were streaked on half-strength Marine Agar (½MA; 27.6 g Difco 212185 Marine Agar, 15 g Instant Ocean Sea Salts, 7.5 g Agar, 1 l deionized water). ½MA was also used for counting *P. gallaeciensis*. *V. anguillarum* was counted on Tryptone-Soy Agar (TSA; Oxoid CM0131) containing 6 mg/l chloramphenicol. The bacterial precultures for the algae and rotifer experiments were grown in 20 ml of ½YTSS (2 g Bacto Yeast extract, 1.25 g Bacto Tryptone, 20 g Sigma Sea Salts, 1 l deionized water) [41] at 25°C with aeration (200 rpm) until $OD_{600} = 1.0$. The cells were harvested at 5,000 x *g*, washed twice, and used as inoculum for algae and rotifer experiments. Bacteria were diluted and washed in artificial seawater (ASW; 2% Sigma Sea Salts). Axenicity of algae and rotifer cultures was controlled by plating 100 µl on ½MA and incubating for 7 days at 25°C.

For the challenge trials, *V. anguillarum* HI610 was grown in tryptone-soy broth with additional 0.5% NaCl at 20°C with shaking at 60 rpm to an OD_{600} of about 0.5. The *P. gallaeciensis* strains were grown in MB without shaking at 20°C until stationary phase was reached. All strains were harvested by centrifugation (1,825 x *g*), washed twice, and resuspended in aerated autoclaved 80% seawater. The bacterial concentrations in these suspensions were determined using a counting chamber for *V. anguillarum*, and for the *P. gallaeciensis* strains by measuring OD_{600} after centrifugation and dissolving in 0.1M NaOH.

Generation of a TDA-negative *Phaeobacter* mutant

A mutant library of *P. gallaeciensis BS107* was created by random transposon insertion mutagenesis using the EZ-Tn5 <R6Kγori/KAN>Tnp Transposome Kit (Epicentre, Madison, WI), following the procedure of Geng *et al.* [42]. Ten non-pigmented mutants were selected, and absence of TDA production was confirmed by UHPLC-TOFMS and in an agar-diffusion test against *V. anguillarum* [27]. Growth rates of selected mutants were compared to the wild type in aerated (200 rpm) ½YTSS cultures at 30°C, and one of the strains with a growth rate comparable to the wild type, BS107-Pda8, was chosen for further experiments. Using rescue cloning as described in the transposome kit manual, the mutated locus was identified as CDS104961, which encodes for a "periplasmic component of a TRAP-type C4-dicarboxylate transport system", as annotated on the BS107 genome on www.roseobase.org.

Fluorescence tagging of *Phaeobacter*

P. gallaeciensis BS107 was tagged chromosomally with a miniTn7(Gm)$P_{A1/04/03}$*DsRedExpress-a* cassette, using a mini-Tn7 tagging system [43,44]. The delivery and helper plasmids were electroporated into *P. gallaeciensis*, followed by selection on ½MA containing 75 µg/ml gentamicin. pPDA11, a transcriptional fusion of the *tdaC* promoter to a promoterless *gfp* gene ligated to the broad-host range plasmid pRK415, was constructed in an analogous manner to pHG1011 as described in Geng *et al.* 2010 [45].

Phaeobacter antagonism in algae

Tetraselmis suecica CCAP 66/4 (*Prasinophyceae*) was obtained as axenic culture from the Culture Collection of Algae and Protozoa (Oban, UK). It was cultured in B-medium [46], a mineral algae medium, based on ASW. The 250-ml culture bottles were closed with cotton plugs and slowly aerated through a 0.2 µm syringe filter and a silicone tube, to prevent settling of particles. Light intensity on the bottles was 13,000 lux (daylight spectrum). Algal concentrations were assessed by measuring absorption at 665 nm, and calibrating with counts of axenic reference cultures in a Neubauer-improved counting chamber, using formaldehyde as fixative (0.5% final concentration). For each *V. anguillarum* inoculum level tested, eight bottles of 150 ml of B-medium were inoculated with 6.6×10^4 cells/ml axenic *T. suecica*. Two bottles were inoculated with approximately 10^7 cfu/ml washed *P. gallaeciensis* BS107, two bottles with the same level of washed mutant *P. gallaeciensis* BS107-Pda8 cells, and four bottles were left axenic. The cultures were grown for 2 days and axenicity was checked. All cultures, except two axenic negative controls, were inoculated with *V. anguillarum* NB10 to concentrations of 10, 100, 1000, or 10^4 cfu/ml. Inoculum levels were verified by plate-counting. Concentrations of algae and both bacterial species were observed until day 5 after inoculation of the pathogen. Two independent experiments were performed for every initial concentration of *V. anguillarum*. ***Nannochloropsis oculata*** CCMP525 (*Eustigmatophyceae*) was obtained as axenic culture from the Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME). Since it did not grow in ASW-based B-medium, it

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype or relevant markers	Source or reference
Strains		
P. gallaeciensis BS107 (DSM17395)	Wild type	Ruiz-Ponte *et al.* 1998 [28]
P. gallaeciensis BS107-Pda8	CDS104961::EZ-Tn5, Kan^R	This study
P. gallaeciensis dsRed	MiniTn7(Gm^R)$P_{A1/04/03}$*DsRed*Express-a	This study
V. anguillarum NB10	Serotype O1, cm^R, PA1/04/03-RBSII-*gfp*mut3*-T1	Croxatto *et al.* 2007 [35]
V. anguillarum HI610	Serotype O2α	Samuelsen & Bergh 2004 [36]
Plasmids		
EZ-Tn5™ Transposome	EZ-Tn5<R6Kγori, Kan^R>Tnp	Epicentre Biotechnologies
pAKN132	miniTn7(Gm)$P_{A1/04/03}$DsRedExpress-a	Lambertsen *et al.* 2004 [43]
pUX-BF13	Helper plasmid: Tn7 transposase proteins	Bao *et al.* 1991 [44]
pPDA11	*tdaCp::gfp* ligated into broad host range vector pRK415	D'Alvise *et al.* (in preparation)

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was cultured in f/2-medium [47] based on Atlantic Seawater obtained from CCMP. *N. oculata* cultures were not aerated. Antagonism experiments were done as in *T. suecica*, but only one initial *V. anguillarum* concentration (10^4 cfu/ml) was tested. Two independent experiments with two different initial densities of algae (lower density: 4×10^6 cells/ml, higher density: 2×10^7 cells/ml) were carried out.

TDA analysis

Samples of *T. suecica* – *P. gallaeciensis* co-cultures (20 ml) were extracted in 50-ml falcon tubes with 30 ml ethyl acetate (HPLC grade) containing 1% formic acid (HPLC grade) on a shaking table for 1 h. The samples were centrifuged at 8,000 x *g*, and 26 ml of the upper phase was transferred to a new Falcon tube and evaporated to dryness at 35°C with nitrogen flow. The samples were resuspended in 300 µl 85% acetonitrile, vortexed for 5 sec, placed in an ultrasonication bath for 10 min, vortexed again for 5 sec and filtered through a standard 0.22 µm PFTE syringe filter into a HPLC vial. Subsamples of 2 µl were then analyzed by UHPLC-TOFMS on a maXis G3 quadrupole time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) ion source which was connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA). Separation was performed at 40°C on a 100 mm×2.1 mm ID, 2.6 µm Kinetex C_{18} column (Phenomenex, Torrance, CA) equipped with Kinetex pre-column using a water-acetonitrile gradient (both buffered with 20 mM formic acid) at a flow of 0.4 ml min^{-1} starting at 10% acetonitrile and increased to 100% in 10 min, keeping this for 3 min. MS was operated in ESI^+ with a data acquisition range of m/z 100–1000 at a resolution of 40,000 FMWH, the MS was calibrated using 20 mM sodium formate infused prior to each analytical run, providing a mass accuracy better than 1.5 ppm. TDA was detected and quantified from the extracted ion chromatograms of the $[M+H]^+$ ions (± m/z 0.001).

For quantification, *T. suecica* cultures were spiked to a final concentration of 4800, 2400, 1600, 800, 320, 160, and 0 (blank) nM TDA (BioViotica, Dransfeld, Germany) by adding a maximum of 80 µl TDA-acetonitrile solution, and treated as described above. Spiked samples were left at room temperature for at least 1 h prior to extraction. The method was validated on 3 different days using spiked samples as described, and no false positives or negatives were recorded. Relative standard deviation was 30% and the limit of detection was estimated to be <50 nM (signal/noise 5:1), based on the blank samples and lower calibration points. Sensitivity was greatly influenced by the age of the UHPLC column since TDA tailed (although a new pre-column was used) on columns which had been in use for only a few weeks. Samples from two individual biological experiments were analyzed independently.

Absence of TDA in static cultures of the TDA-negative mutant BS107-Pda8 was confirmed by UHPLC-TOFMS analysis.

Phaeobacter antagonism in rotifers, Brachionus plicatilis

A stock of the rotifer *B. plicatilis* (L-type) was obtained from Reed Mariculture (Campbell, CA). Axenic rotifers were attained by disinfecting approximately 50 amictic rotifer eggs in 1 ml of a strong antibiotic solution (150 µg/ml Tetracycline, 300 µg/ml Kanamycin, 60 µg/ml chloramphenicol, 1000 U/ml Penicillin in ASW) for 2 days. The hatched rotifers were filtered onto a sterile 50-µm polyamide mesh, rinsed with ASW and from then on fed with concentrated axenic *T. suecica*. No experiment with *N. oculata* as rotifer feed was conducted, since survival of *V. anguillarum* in presence of *N. oculata* was very variable (see Results section). Rotifer densities were determined by counting in a Sedgewick-Rafter counting chamber. Before counting, the cultures were thoroughly mixed, and 100 µl 5% formaldehyde added to a 1 ml sample. To set up co-culture experiments, axenic rotifer cultures were divided into eight 20-ml batches in 50-ml centrifuge tubes. The initial rotifer concentrations were 94 /ml (first replicate) and 30 /ml (second replicate). For each replicate, duplicate cultures were inoculated with washed wild type and mutant *P. gallaeciensis* at approximately 5×10^7 cfu/ml. On the next day, all cultures except the axenic controls were inoculated with 10^4 cfu/ml *V. anguillarum*. All rotifer cultures were fed daily with 10-fold concentrated *T. suecica* (1–2 ml depending on average rotifer density), so that the algae concentration was at approximately 10^6 cells/ml after feeding and did not drop below 2×10^5 cells/ml. The rotifers were counted daily, concentrations of *V. anguillarum* and *P. gallaeciensis* were determined, and axenicity of the negative controls was checked. The rotifer culture samples for enumeration of bacteria were homogenized by grinding and repeated pipetting through a 100-µl pipette tip. This was compared to homogenization with an Ultra-Turrax T25 (IKA, Germany) at 16,000 rpm and no significant differences in bacterial counts were found ($P = 0.74$).

Challenge trial

The protocol was adapted from [39,40]. Cod (*Gadus morhua*) embryos were obtained from the commercial hatchery Havlandet AS, in Florø, Western Norway. Transport of the embryos in polystyrene containers at around 8°C took 4 to 5 hours in total by boat and car. Two independent replicates of the challenge trial were conducted. The embryos used in the first trial were disinfected with Buffodine (Evans Vanodine, Preston, UK), the embryos for the second trial were left untreated. Upon arrival, the embryos were randomly picked and distributed to the wells of 24-well dishes (Nunc, Roskilde, Denmark) filled with 2 ml 80% autoclaved, aerated seawater, placing one embryo in each well. In each trial three dishes for each treatment (72 embryos) were prepared and inoculated immediately. The six treatment groups are listed in Table 2. All inocula were prepared in a volume of 100 µl, and the strains were not mixed before inoculation. Initial bacterial concentrations were 1×10^6 cfu/ml for *V. anguillarum* HI610 and about 10^7 cfu/ml for the *P. gallaeciensis* strains. The plates were incubated in the dark at 7°C. The day when 50% of the larvae had hatched was defined as day 0, which was 6 days after the start of the experiment. Dead larvae were registered daily for 14 days.

Statistics

Differences between concentrations of bacteria or algae were assessed using repeated measures ANOVA after log-transformation. Tukey's multiple comparison test was used for pairwise comparisons. To address the effects of *P. gallaeciensis* presence on concentrations of *V. anguillarum*, initial values (day 0) were omitted in the analysis and the experiments were analyzed separately. Rotifer numbers were not log-transformed before applying ANOVA, and initial values were omitted. Numbers of *P. gallaeciensis* and homogenization methods were compared using paired t-tests after log-transformation.

The cumulative mortalities in the challenge trials were compared at day 10, prior to the onset of starvation towards the end of the experiment. A chi-square test for 2^2 contingency tables was implemented, using the software R, version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Antagonism in algae cultures

Both wild type and mutant *P. gallaeciensis* colonized the cultures of *T. suecica* and *N. oculata*. In *T. suecica* cultures, *P. gallaeciensis* reached 10^7 cfu/ml (Figure 1). In the dense cultures of *N. oculata*, *P. gallaeciensis* concentrations were approximately 5×10^6 cfu/ml, and in the less dense cultures approximately 8×10^5 cfu/ml (Figure S1). The wild type *Phaeobacter* reached slightly higher numbers in

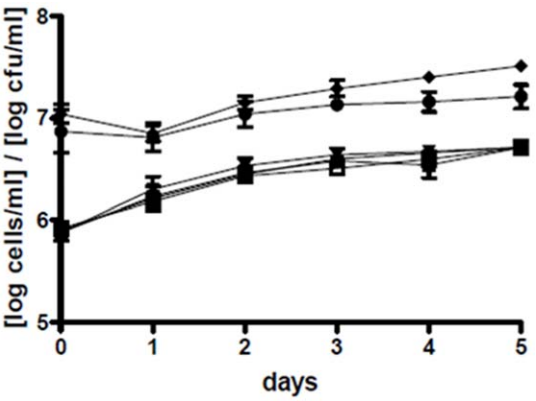

Figure 1. Concentrations of *Tetraselmis suecica* and *Phaeobacter gallaeciensis* in the co-cultures. Means and standard deviations of eight experiments: colony-forming units of *P. gallaeciensis* wild type (◆) and the TDA-negative mutant (●), and concentrations of *T. suecica* with *V. anguillarum* (▼), *T. suecica* with *P. gallaeciensis* wild type (■), *T. suecica* with *P. gallaeciensis* TDA-negative mutant (▲), and axenic *T. suecica* (□). The *P. gallaeciensis* strains were inoculated at 10^7 cfu/ml and remained as a steady population, while the algae went from late log into stationary phase.
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T. suecica than the TDA-negative mutant ($P = 0.0211$). This same slight difference was seen in one of the two *Nannochloropsis* experiments ($P = 0.0335$, $P = 0.9259$). *P. gallaeciensis* did not affect growth of the algae *T. suecica* ($P = 0.9977$) and *N. oculata* ($P = 0.9919$). Particles consisting of dead *T. suecica* and algal cell walls that were shed during cell division served as habitat for rosette forming *P. gallaeciensis* that formed dense biofilms on the particles (Figure 2 A–D).

V. anguillarum effectively colonized all *Tetraselmis* cultures that were not inoculated with *P. gallaeciensis* and numbers increased by up to 2.7 log units within the first day (Figure 3, Figure S2) and reached an average of 3×10^6 cfu/ml after 5 days. *V. anguillarum* did not colonize particles in the algae cultures, but remained in suspension (Figure 2 E). The numbers of *V. anguillarum* decreased markedly in presence of wild type *P. gallaeciensis* (Figure 3, Figure S2). *Vibrio* reductions were in the order of 3 log units, as compared to the monoxenic controls with only *V. anguillarum*, and complete elimination of the lowest *Vibrio* inoculum was achieved in 3 out of 4 replicates (Figure 3A). The effects of wild type *P. gallaeciensis* on *V. anguillarum* were highly significant in all *Tetraselmis* experiments, as compared to the controls ($P<0.001$) and to the mutant ($P<0.001$). Presence of the TDA-negative mutant did decrease concentrations of the pathogen by about one log unit, although this was only significant ($\alpha = 0.05$) for two of the initial *Vibrio* concentrations

Table 2. Group numbers and treatments in the challenge trial.

Group number	Treatment
T1	Negative control; no bacteria added
T2	Positive control; *V. anguillarum* O2α HI610 10^6 cfu/ml
T3	Wild type *P. gallaeciensis* BS107 (DSM17395) ~10^7 cfu/ml
T4	TDA-mutant *P. gallaeciensis* BS107-Pda8 ~10^7 cfu/ml
T5	*V. anguillarum* O2α HI610 10^6 cfu/ml and wild type *P. gallaeciensis* BS107 (DSM17395) ~10^7 cfu/ml
T6	*V. anguillarum* O2α HI610 10^6 cfu/ml and TDA-mutant *P. gallaeciensis* BS107-Pda8 ~10^7 cfu/ml

doi:10.1371/journal.pone.0043996.t002

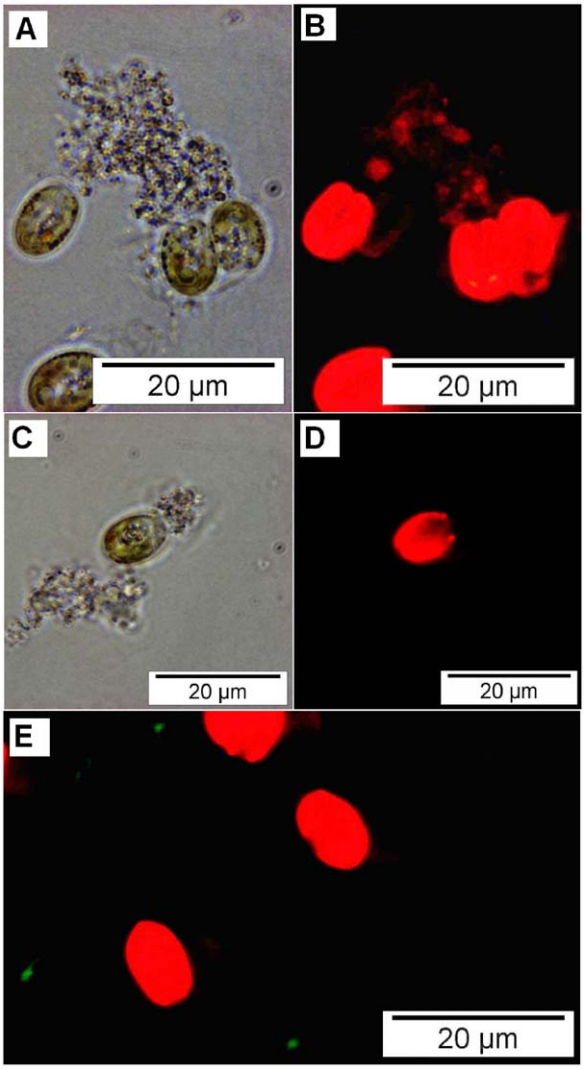

Figure 2. Localization of bacteria in cultures of *Tetraselmis suecica*. Phase-contrast (A,C) and fluorescence (B,D,E) micrographs. Co-culture of *Tetraselmis suecica* with *Phaeobacter gallaeciensis dsRed* (A,B), axenic *T. suecica* (C,D), co-culture of *T. suecica* with *V. anguillarum gfp* (E). Panel A and B show two single (left) and one dividing algal cell (right side), and a marine snow-like particle consisting of algae-debris which is colonized by red-fluorescent *P. gallaeciensis*. Red fluorescence of algae is due to chlorophyll. Panels C and D show an algal cell and particles from an axenic culture, recorded using the same settings as for the panels above. Panel E shows red-fluorescent algae cells and green-fluorescent *V. anguillarum*, which do not colonize particles, but remain in suspension as single, motile cells.
doi:10.1371/journal.pone.0043996.g002

(10^1: $P = 0.0518$, 10^2: $P = 0.0011$, 10^3: $P = 0.0517$, 10^4: $P = 0.0008$).

The marked difference in *V. anguillarum* inhibition by the wild type *P. gallaeciensis* and the TDA negative mutant suggested that TDA was a major effector molecule. However, TDA was not detected by chemical analysis of the *Phaeobacter–Tetraselmis* co-cultures, where triplicate cultures were each analyzed in triplicates with a detection limit <50 nM TDA. The experiment and analysis were repeated with the same result. To determine if the wild type did indeed produce TDA in the algal cultures, a *P. gallaeciensis* carrying pPDA11 (*tdaCp::gfp*) was co-cultured with *T.* suecica. The *tdaC* promoter, indicative of TDA production, was

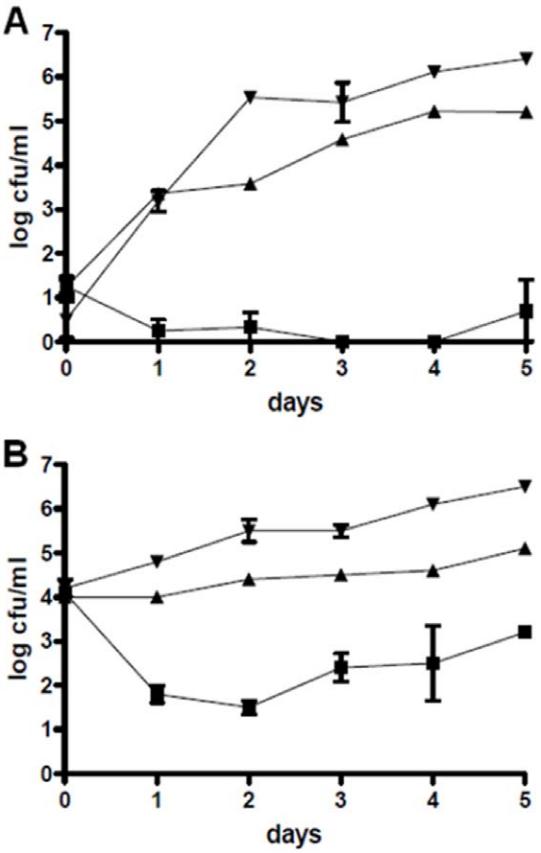

Figure 3. Reduction of *V. anguillarum* in cultures of *Tetraselmis suecica* by *Phaeobacter gallaeciensis*. Colony-forming units of *V. anguillarum* inoculated at 10^1 cfu/ml (A) and at 10^4 cfu/ml (B) in presence of *P. gallaeciensis* wild type (■), in presence of the *P. gallaeciensis* TDA-negative mutant (▲), and in the monoxenic control (▼).
doi:10.1371/journal.pone.0043996.g003

induced when growing on particles in a *T. suecica* culture, as indicated by Gfp fluorescence (Figure 4). Adding pure TDA to *Tetraselmis* cultures inoculated with *V. anguillarum* caused a complete killing of the *Vibrio* population, but also affected survival of algae (50 µM TDA). A 50-fold lower concentration (1 µM) had no effect on the algae, but temporarily reduced *V. anguillarum* below 10 cfu/ml. A concentration of 50 nM TDA, which was the detection limit of the chemical analysis, did not have any effect (data not shown).

V. anguillarum was completely eliminated in *Nannochloropsis oculata* cultures by wild type *P. gallaeciensis* within one or two days (Figure S3). However, *V. anguillarum* could only persist in dense cultures of *N. oculata*. In less dense *N. oculata* cultures *V. anguillarum* disappeared from the monoxenic control within 3 days. Consequently, the effect of the wild type *P. gallaeciensis* on *V. anguillarum*, as compared to the control, was significant in the experiment with high algae density ($P = 0.001$), but not in low density ($P = 0.2106$).

Antagonism in rotifer cultures

The concentrations of *P. gallaeciensis* and its mutant in the rotifer cultures were stable at about 10^6–10^7 cfu/ml, and no significant difference between the two strains was observed ($P = 0.3689$). The rotifers grew faster and reached higher densities in presence of *P. gallaeciensis* than in the axenic or monoxenic (*V. anguillarum*) controls ($P<0.05$) (Figure 5, Figure S4). Wild type *P. gallaeciensis* reduced *V. anguillarum* concentrations by 3 log units ($P<0.01$), in average from

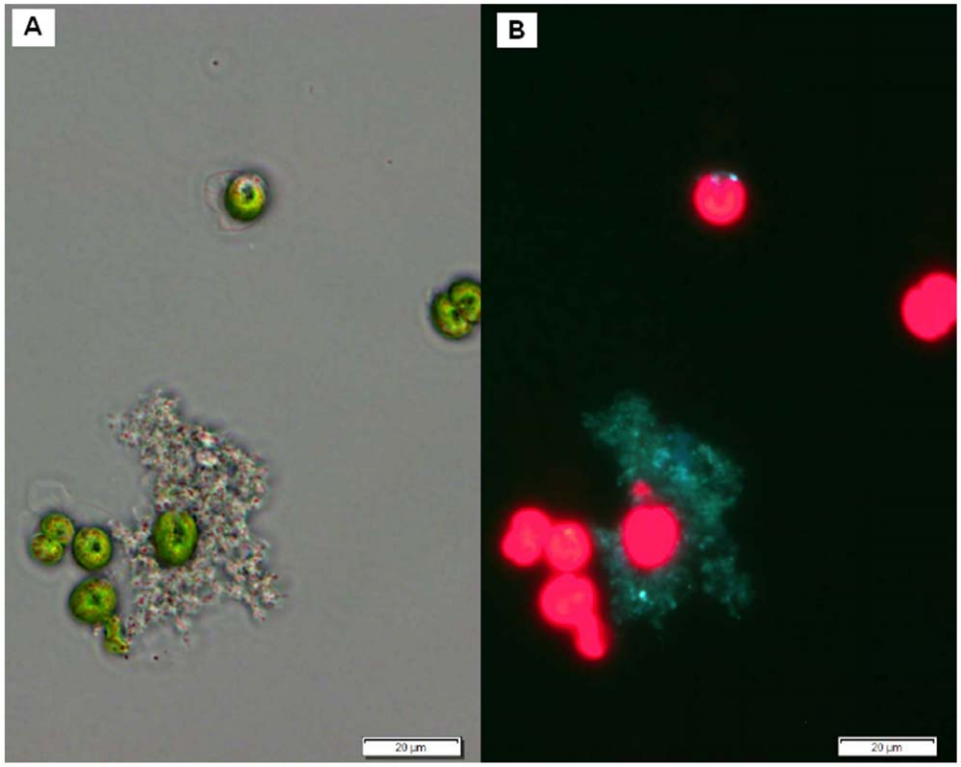

Figure 4. Expression of *tdaC* in co-culture with *Tetraselmis suecica*. Phase contrast (A) and fluorescence (B) micrographs of *P. gallaeciensis* pPDA11 (*tdaCp::gfp*) in co-culture with *T. suecica*. The two panels show the same seven algal cells of which some are dividing, and a marine snow-like particle which is colonized by *P. gallaeciensis* carrying the promoter-fusion on a plasmid. The green fluorescence of *P. gallaeciensis* on the particle shows that the gfp gene is expressed from the tdaC promoter, indicating production of TDA.
doi:10.1371/journal.pone.0043996.g004

6×10^5 to 9×10^2 cfu/ml (Figure 6). The effect of the TDA-negative mutant on the concentration of the pathogen was not significant ($P>0.05$).

Challenge trial

Six days after the arrival of the embryos and inoculation, more than 50% of the larvae had hatched. Total cumulative hatching success was 79.2% (first trial 79.6% and second trial 78.7%). The initial mortality was lower in the first trial (16.6%) than in the second (24.8%). In the non-challenged and non-treated control, 34.7%±9.8% (average ± standard deviation) of the larvae had died by day 1, yet after the initial mortality only 2.8%±0% of the larvae died between day 2 and day 10, reaching an accumulated mortality of 37.5%±9.8% at day 10. The larvae challenged with *V. anguillarum* HI610 died rapidly and reached 100%±0%

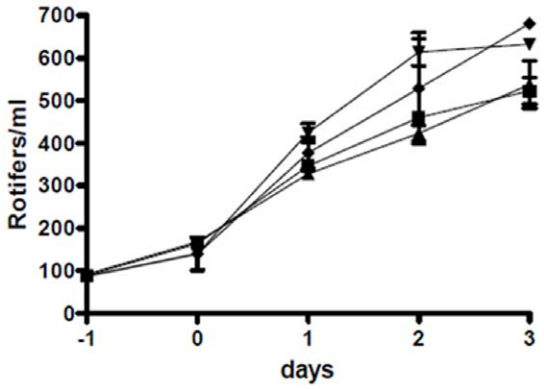

Figure 5. Influence of bacterial strains on rotifer growth. Rotifer numbers in co-culture with *P. gallaeciensis* wild type (▼), with the TDA-negative mutant of *P. gallaeciensis* (♦), with only *V. anguillarum* (▲), and axenic rotifers (■), first experiment. All bacteria were inoculated at day 0. Both *P. gallaeciensis* strains promoted rotifer growth, whereas *V. anguillarum* had no influence.
doi:10.1371/journal.pone.0043996.g005

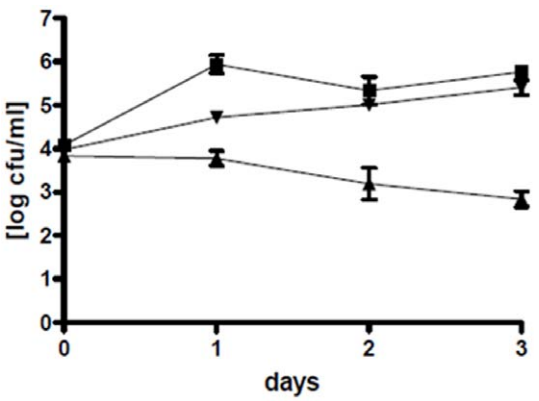

Figure 6. Reduction of *Vibrio anguillarum* by *Phaeobacter gallaeciensis* in rotifer cultures. Mean values of two duplicate experiments: colony-forming units of *V. anguillarum* in co-culture with *P. gallaeciensis* wild type (▲), with the TDA-negative mutant of *P. gallaeciensis* (▼), and in the monoxenic control (■).
doi:10.1371/journal.pone.0043996.g006

accumulated mortality. Treating *Vibrio*-challenged larvae with wild type *P. gallaeciensis* caused a significant reduction in accumulated mortality by day 10 to 12.5%±2.0% which was not only lower than in the challenged larvae but also lower than in the non-challenged (37.5%). 96.1%±1.1% of the hatched larvae that had received wild type *P. gallaeciensis* survived until day 10, when starvation set in (Figure 7, Figure S5). The larvae exposed to only *P. gallaeciensis* wild type or mutant had a cumulative mortality of 12.1%±3.1% at day 10. The TDA-negative mutant of *P. gallaeciensis* did reduce accumulated mortality of the challenged larvae to 68.8%±30.4% (Figure 7, Figure S5), but was not nearly as efficient as the TDA-producing wild type.

Discussion

The present study demonstrates that *Phaeobacter gallaeciensis* is harmless and beneficial for the early life stages of cod. Equally important, *P. gallaeciensis* is highly efficient at preventing infections with *V. anguillarum*, and this probiotic effect can be achieved at the low temperature (7°C) used for the cod embryos and yolk sac larvae. It has previously been demonstrated that a *Phaeobacter* sp. can protect turbot larvae against vibriosis at higher temperatures (18°C) [29]. Non-infected larvae showed some level of initial mortality, which may have been due to opportunistic bacteria introduced with the embryos. Both challenged and unchallenged cod larvae exposed to *P. gallaeciensis* had a significantly lower initial mortality, indicating that the inherently occurring microbiota of the chorion may be controlled by the probiont.

A key question in the use of probiotics in aquaculture is how and where the probiont should be introduced to the system. Several studies have emphasized the potential role of feed organisms as a vehicle for probiotic bacteria [29,48–51], or the potential of probiotic bacteria to control pathogenic bacteria in the feed cultures [51–53]. The majority of studies have focused on intestinal probiotic bacteria, and aimed at health-promoting effects within either the reared animal or the feed organism. In contrast, the present study takes a systems approach to preventing bacterial disease in aquaculture organisms, aiming at microbial control throughout the environment of the reared organism and the lower trophic levels of the production. Here it was found that

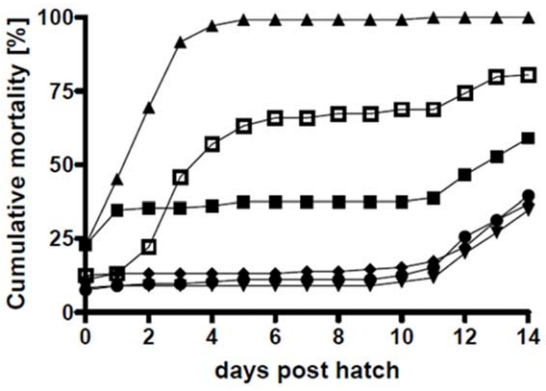

Figure 7. Mortality of cod larvae during the challenge trials. Mean values of two independent triplicate experiments. The single-larvae cultures were simultaneously inoculated with *P. gallaeciensis* wild type and *V. anguillarum* (T5, ●), or with the TDA-negative mutant of *P. gallaeciensis* and *V. anguillarum* (T6, □). Unexposed larvae and larvae exposed to single bacterial strains acted as controls: Negative Control (T1, ■), only *V. anguillarum* (T2, ▲), only *P. gallaeciensis* wild type (T3, ▼), and only *P. gallaeciensis* TDA-negative mutant (T4, ◆).
doi:10.1371/journal.pone.0043996.g007

cultures of two aquaculture-relevant algae and of the rotifer *B. plicatilis* can be colonized by *P. gallaeciensis* without compromising their growth, and that *P. gallaeciensis* in these cultures will strongly reduce, or eliminate fish-pathogenic *V. anguillarum*. Introducing *P. gallaeciensis* at this trophic level is very promising, since live feed is a common source of opportunistic pathogens [1,5,10,11]. These findings corroborate the hypothesis from a previous study, that algae and rotifers in aquaculture can be cultured together with probiotic *Roseobacters*, and thus prevent proliferation of pathogens [22]. A reduction of a pathogenic *Vibrio* sp. by 3 log units, as it was achieved in the present study, is very promising in terms of larval health promotion, as only a one log reduction of the bacterial load in rotifers through UV radiation resulted in higher survival of turbot larvae [54]. Using probiotic bacteria, as compared to UV treatment, offers the advantage that nutrients are consumed, niches are occupied, and rapid re-growth of pathogens is prevented. It should be noted that the present study was done using gnothobiotic systems to rule out the influence of the inherent microbiota of algae and rotifer cultures. Thus, it cannot be determined, if or to what extent *P. gallaeciensis* would affect the inherent microbial communities of algae and rotifer cultures.

The inhibition of *V. anguillarum* by a *Phaeobacter* sp. in a model aquaculture setting has been studied once before: Planas *et al.* [29] demonstrated that mortality in turbot larvae infected with *V. anguillarum* could be reduced by *Phaeobacter* sp. 27-4. A duplicate tank setup was used, and both the pathogen and the probiont were enclosed in rotifers and fed to the larvae. In spite of delivery with the feed, the probiont was only found in the lumen of the larval gut and did not colonize the intestinal epithelium. In contrast to this, the present study did not aim at a probiotic effect in the intestinal tract of the larvae, but assesses the potential of *Phaeobacter* to eliminate the pathogen in the environment of the larvae or embryos. It should be mentioned that, as larvae start to drink shortly after hatching [55], an intestinal presence of pathogens and probionts can occur. *Phaeobacter* sp. 27-4 is a TDA-producer, however, as opposed to *P. gallaeciensis* BS107, it produces TDA only in stagnant culture [26], suggesting that its TDA production may be more delimited and that BS107 could be more antagonistic *in vivo*.

In the challenge trial the TDA-negative mutant reduced the initial mortality as efficiently as the wild type, but could not prevent infection in the majority of the larvae. The probiotic effect of the mutant could be explained by competition for nutrients, space, or other resources or it could be attributed to a direct immunostimulatory effect on the larvae [56–59]. The mutated gene that renders *P. gallaeciensis* BS107-Pda8 unable to produce TDA belongs to an operon encoding the parts of a transport protein, which has not yet been reported to be involved in TDA production [42]. The role of this transmembrane protein in TDA production has not been investigated. It cannot be excluded that this mutation has pleiotropic phenotypic effects, and other functions than TDA production might be affected and could affect the antagonistic properties of *Phaeobacter gallaeciensis*. Nonetheless, the experiment using pure TDA indicated that this compound indeed has a major inhibitory effect against *V. anguillarum* in the algal system.

The difference in *Vibrio*-antagonism between the TDA-negative mutant and the wild type suggested that TDA production is the trait that enables *P. gallaeciensis* to antagonize *V. anguillarum*. However, TDA was not detectable by chemical analysis of the *Phaeobacter-Tetraselmis* co-cultures. Since a *tdaC*-promoter fusion (*tdaCp*::*gfp*), demonstrated that *tdaC* is expressed by *P. gallaeciensis* in particles in the algae cultures, the reason for the lack of chemical detection could be that the TDA concentration only reaches

inhibitory concentrations in *Phaeobacter*-colonized particles. TDA is likely concentrated within and around the particles, adhering to organic mass of the particle, or being kept within the EPS produced by *P. gallaeciensis*. From an ecological point of view, for a particle-associated marine bacterium the production of an antagonistic compound would be more efficient if the compound was not dispersed, but kept in the close vicinity to fend off possible competitors.

Although TDA-producing *Phaeobacter* and *Ruegeria* spp. are likely to be already present in larviculture systems, their antagonistic properties, which may depend on growth conditions, are probably different from *P. gallaeciensis* BS107 [22,26]. A preliminary experiment to this study showed that only a few of the tested *Phaeobacter* and *Ruegeria* strains were antagonistic in *T. suecica* cultures, whereas all of them did account for large inhibition zones in agar-based assays. Therefore, introduction of *P. gallaeciensis* BS107 in algae and rotifer cultures would likely enhance larval survival even though other *Roseobacters* are already present in the system. Its growth-promoting effect on rotifers may offer an unexpected additional advantage. Whether that is due to the nutritive value of the bacteria or to a potential role in the rotifer gut is not known. In the present study rotifer growth was not adversely affected by *V. anguillarum*. Nevertheless, a *V. anguillarum* strain was reported to cause pronounced growth inhibition of rotifers under suboptimal feeding schemes [60], which could possibly be remediated by *P. gallaeciensis*.

It cannot be predicted, if and how other pathogens in algae and rotifer cultures would be suppressed by *P. gallaeciensis*, however a range of fish pathogens are inhibited *in vitro* by *P. gallaeciensis* [22,25] indicating that it likely could protect against other pathogens than *V. anguillarum*. Porsby *et al.* [61] have addressed the concern that resistance to TDA could develop, and found, using several experimental approaches, that no resistant mutants or variants could be isolated, neither from short-term selection cultures containing different concentrations of TDA nor from long-term adaptation cultures (>300 generations) containing increasing concentrations of TDA.

A recent study demonstrated that *P. gallaeciensis*, when incubated with p-coumaric acid, produced potent algicides, the roseobactins, which were effective against different microalgae, among them *T. suecica* [62]. P-coumaric acid is a degradation product of lignin, which is contained not only in terrestrial plants, but also in algae. Production of the algicides was only possible in concentrations of p-coumaric acid above 0.4 mM. The authors hypothesized that *P. gallaeciensis* contributes to algal health and growth by secreting TDA and phenylacetic acid, but will produce algicides in presence of p-coumarate, which is an indicator for algal senescence, in order to utilize the algal biomass for its own growth [62]. However, in the present study no negative effect of *P. gallaeciensis* on algal growth was observed. Possibly the levels of p-coumaric acid in the cultures of microalgae were too low for roseobacticide production. The environmental ecological niche of *P. gallaeciensis* has not yet been described, although studies of Rao *et al.* [63–65] indicate its preference for the surface of macroalgae, which during their decay may account for higher local concentrations of p-coumaric acid than microalgae. In an aquaculture farm, *Phaeobacter* spp. have been found to "naturally" occur on solid surfaces, whereas only *Ruegeria* spp. were inherently associated with algae cultures [26].

Based on the present findings, it is hypothesized that *P. gallaeciensis* can be used in marine larviculture, as a means of controlling the ambient, potentially harmful microbiota in cultures of rotifers and microalgae, and as a prophylaxis against vibriosis in fish larvae.

Supporting Information

Figure S1 Concentrations of *Nannochloropsis oculata* and *Phaeobacter gallaeciensis* in the co-cultures. Colony-forming units of *P. gallaeciensis* wild type (•) and the TDA-negative mutant (□), and concentrations of *N. oculata* with *V. anguillarum* (▲), *N. oculata* with *P. gallaeciensis* wild type (▼), *N. oculata* with *P. gallaeciensis* TDA-negative mutant (♦), and axenic *N. oculata* (■) in the dense (A) and less dense (B) cultures.
(TIF)

Figure S2 Reduction of *Vibrio anguillarum* by *Phaeobacter gallaeciensis* in cultures of *Tetraselmis suecica*. Colony-forming units of *V. anguillarum* inoculated at 10^2 cfu/ml (A) and at 10^3 cfu/ml (B) in presence of *P. gallaeciensis* wild type (■), in presence of the *P. gallaeciensis* TDA-negative mutant (▲), and in the monoxenic control (▼).
(TIF)

Figure S3 Reduction of *Vibrio anguillarum* by *Phaeobacter gallaeciensis* in cultures of *Nannochloropsis oculata*. Colony-forming units of *V. anguillarum* in presence of *P. gallaeciensis* wild type (▲), in presence of the *P. gallaeciensis* TDA-negative mutant (▼), and in the monoxenic control (■), in dense (3×10^7 cells/ml; A) and less dense ($1–7\times10^6$ cells/ml; B) cultures of *N. oculata*.
(TIF)

Figure S4 Influence of bacterial strains on rotifer growth. Rotifer numbers in co-culture with *P. gallaeciensis* wild type (▼), with the TDA-negative mutant of *P. gallaeciensis* (♦), with only *V. anguillarum* (▲), and axenic rotifers (■), second experiment. All bacteria were inoculated at day 0. Both *P. gallaeciensis* strains promoted rotifer growth, whereas *V. anguillarum* had no influence.
(TIF)

Figure S5 Mortality of cod larvae during the challenge trials. Mean values of two independent triplicate experiments with error bars indicating standard deviations. The single-larvae cultures were simultaneously inoculated with *P. gallaeciensis* wild type and *V. anguillarum* (T5, •), or with the TDA-negative mutant of *P. gallaeciensis* and *V. anguillarum* (T6, □). Unexposed larvae and larvae exposed to single bacterial strains acted as controls: Negative Control (T1, ■), only *V. anguillarum* (T2, ▲), only *P. gallaeciensis* wild type (T3, ▼), and only *P. gallaeciensis* TDA-negative mutant (T4, ♦).
(TIF)

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Author Contributions

Conceived and designed the experiments: PD SL HIW KFN ØB LG. Performed the experiments: PD SL MJP KFN HIW ØB. Analyzed the data: PD SL KFN HIW ØB LG. Wrote the paper: PD LG.

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Paper 2

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Protection of cod larvae from vibriosis by *Phaeobacter* spp.: a comparison of strains and introduction times.

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Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparison of strains and introduction times

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ABSTRACT

Infections with *Vibrio anguillarum* and other pathogenic *Vibrio* spp. are a major problem for marine larviculture, and improved control of the microbiota in marine larvae cultures could ensure a more reliable and cost-effective production of juvenile fish. *Phaeobacter gallaeciensis* is capable of reducing *V. anguillarum* in live feed cultures and can, in challenge trials, protect fish larvae from vibriosis. The purpose of the present study was to estimate the probiotic potential of *Phaeobacter* isolates that produce different levels of the antagonistic compound tropodithietic acid (TDA). We compared the capability of three wild type *Phaeobacter* strains to reduce cod larvae mortalities in challenge trials with single cod (*Gadus morhua*) embryo/larvae cultures, and assessed the importance of the time point at which the probiotic bacteria were introduced relative to the pathogen. All three *Phaeobacter* strains reduced larvae mortalities, however to different degrees. The capability of the strains to prevent disease was correlated with their in vitro TDA production, as measured by in vitro inhibition of *V. anguillarum*. The most effective time to apply the probiotics was in advance of the pathogen, while simultaneous introduction was only effective for the two strains with the highest TDA production. This suggests that prophylactic use of *Phaeobacter* spp., where the probiotic bacterium is introduced early into the system, is most efficient in disease prevention.

1. Introduction

Most marine fish produce large quantities of small eggs, and their larvae require extended periods of planktonic development. In hatcheries, where fish larvae and their feed organisms are kept at artificially high densities, opportunistic bacterial pathogens can proliferate and infections can spread easily between the larvae. Thus, infection with opportunistic pathogenic bacteria still causes serious problems for marine hatcheries (Olafsen, 2001; Reid et al., 2009). High, variable, and unpredictable mortalities during the larval stages and first feeding period make juvenile production of many marine fish species unreliable (Reid et al., 2009; Shields, 2001; Skjermo and Vadstein, 1999; Vadstein et al., 2012). Enhanced control over the microbial community in larvae cultures and their feed organisms could not only make juvenile production more effective, but could also help to make new species with sensitive larvae available for aquaculture. Most bacterial diseases of marine fish larvae are caused by *Vibrio* spp., with *V. anguillarum* being the most prominent representative (Toranzo et al., 2005). This prevalence of *Vibrio* spp. among larval pathogens was confirmed in challenge experiments with larvae of cod and other species (Sandlund and Bergh, 2008; Sandlund et al., 2010). As an alternative to a prophylactic use of antibiotics, which should be avoided due to the risk of resistance and contamination of the environment, it has been suggested that probiotic bacteria could be applied to control bacterial communities in aquaculture and to reduce the amount of pathogenic bacteria (Tinh et al., 2008). A range of particularly promising probiotic candidates have been found in the genus *Phaeobacter*, which belongs to the *Roseobacter*-clade of the α-Proteobacteria.

Phaeobacter gallaeciensis produces the antibacterial compound tropodithietic acid (TDA) and is an efficient inhibitor of *V. anguillarum* and a range of other aquaculture-relevant pathogens in in vitro antagonism studies (Bruhn et al., 2005; D'Alvise et al., 2010; Prado et al., 2009). We have recently demonstrated that *P. gallaeciensis* BS107 (DSM17395) strongly reduces *V. anguillarum* in gnotobiotic cultures of two species of microalgae and in gnotobiotic rotifers. Moreover it reduced cod larvae mortality in challenge trials with *V. anguillarum* to a level beneath the uninfected controls (D'Alvise et al., 2012). The purpose of the present study was to compare, in the same experimental setup, the efficacy of *P. gallaeciensis* and two other *Phaeobacter* strains that produce different amounts of TDA, and to test how the probiotic effect is influenced by the time of introduction. In the previous study pathogen and probiont were

Abbreviation: tropodithietic acid, TDA.

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introduced at the same time, and in the present study we investigated whether the beneficial effect would be influenced by the time of introduction of the probiont, and more specifically, whether the probiotics would be as potent when applied preventively. We also assessed the antibacterial effect of the same *Phaeobacter* strains in vitro, to test the hypothesis that their capability of TDA production is the crucial feature for the probiotic effect.

P. gallaeciensis BS107 (DSM17395) was isolated from scallops at the Atlantic coast in Galicia, Spain. It is the type strain for *P. gallaeciensis* and was also used in the previous study. *Phaeobacter* sp. 27–4 was isolated from culture tanks of turbot larvae feeding on microalgae and rotifers in Galicia, Spain (Hjelm et al., 2004). In a challenge trial with turbot larvae, where the probiont as well as *V. anguillarum* were applied via rotifers, *Phaeobacter* sp. 27–4 could reduce larvae mortality in one out of three replicates (Planas et al., 2006). We chose strain 27–4, since it produces TDA in vitro only under static culture conditions, while *P. gallaeciensis* BS107 is able to produce TDA both in static and in shaken cultures. *Phaeobacter* sp. M23-3.1 was also isolated from turbot larvae basins, however from a Danish hatchery at the shore of Limfjorden (Porsby et al., 2008). Strain M23-3.1 was chosen since it produces more TDA than the type strain and since it was isolated from a low-temperature environment and thus might be more adapted to the rearing temperature of cod.

2. Material and methods

2.1. Bacterial strains and media

The inocula for the challenge trials were prepared as described earlier (D'Alvise et al., 2012): Briefly, *Phaeobacter gallaeciensis* BS107 (DSM17395), *Phaeobacter* sp. 27–4, and *Phaeobacter* sp. M23-3.1 were grown in 20 ml Marine Broth (Difco 2216 - MB) cultures without agitation at 20 °C until stationary phase was reached (approximately 48 h). *V. anguillarum* O2α HI610 was grown to an OD_{600} of about 0.5 in 20 ml tryptone-soy broth with addition of 0.5% NaCl and aeration at 60 rpm. All strains were harvested by centrifugation at 1,825× *g*, washed twice in aerated autoclaved 80% seawater, and resuspended in aerated autoclaved 80% seawater. The bacterial concentrations were determined with a counting chamber or by measuring OD_{600} after resuspending in 0.1 M NaOH to facilitate disintegration of the cohesive *P. gallaeciensis* pellets.

For measurements of tropodithietic acid (TDA) by *Vibrio* inhibition, the *Phaeobacter* strains were grown at 25 °C in 20 ml MB with and without aeration at 200 rpm for 72 h. Samples of 1 ml were taken from each culture, the cells were removed by centrifugation at 5,000× *g*, and the supernatant was filtered through 0.2-μm syringe filters (Sartorius, Germany).

2.2. Challenge trials

The challenge trials were conducted as described earlier (D'Alvise et al., 2012): Fertilized cod eggs (embryos) were obtained from the commercial hatchery Havlandet AS in Florø, Norway. A total of 3 challenge trials were conducted. The embryos used for challenge trial 1 were left untreated, whereas the embryos for trials 2 and 3 were disinfected with Buffodine (Evans Vanodine, UK). Single cod embryos were placed in the wells of 24-well dishes (Nunc, Denmark), filled with 2 ml aerated, autoclaved seawater. For each treatment and challenge trial three plates (72 embryos/larvae) were prepared, and three consecutive challenge trials with the same treatment groups were conducted, so all results are based on data from three independent biological replicates. The assays were incubated in the dark at 7 °C. Living and dead larvae were registered daily, and the day when more than half of the larvae had hatched was defined as day 0 post-hatch. Each of the *Phaeobacter* strains was tested alone and in challenges with *V. anguillarum* at three different time points of inoculation: *Phaeobacter* before: *Phaeobacter* was introduced at the start of the experiment and *V. anguillarum* was introduced 48 h later. Simultaneous: both *Phaeobacter* and *V. anguillarum* were introduced at the start of the experiment. *Phaeobacter* after: *V. anguillarum* was introduced at the start of the experiment and *Phaeobacter* was introduced 48 h later (Table 1).

2.3. Vibrio inhibition in vitro

Inhibition of *V. anguillarum* NB10 by was tested in a standard well-diffusion assay, as adapted from (Hjelm et al., 2004). *V. anguillarum* NB10 was grown in MB for 1 day at 25 °C with aeration at 200 rpm. 50 μl of the *V. anguillarum* preculture was added to 50 ml molten Instant Ocean-Agar (1.5 g Instant Ocean Sea Salts, 0.1 g Casamino Acids (Bacto, France), 0.2 g glucose, 0.5 g agar) at 41.5 °C and poured into a 14-cm petri dish. Wells of 6 mm diameter were punched into the solidified agar and filled with 50 μl of *Phaeobacter* culture supernatant. The assay was incubated for 1 day at 25 °C and diameters of inhibition zones were measured.

2.4. Statistics

The cumulative mortalities in the challenge trials were compared at day 10 post-hatch, prior to the onset of starvation towards the end of the experiment. A chi-square test for 2^2 contingency tables was implemented, using the software R, version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Challenge trials

Cumulative mortality kinetics of cod larvae in the three challenge trials are shown in Fig. 1. In challenge 1 and 2, more than 50% of the larvae had hatched after 7 days of incubation; in challenge trial 3 the majority of the larvae had hatched already after 3 days. Total cumulative hatching success was 84.7% (82.3, 78.6, and 93.17%). Average initial mortality in the negative controls was 17.1% (18.1, 27.8, and 5.6%), and average mortality on day 10, before the onset of starvation, was 28.7% (30.6, 44.4, and 11.1%; Table 1). None of the *Phaeobacter* strains had adverse effects on cod larvae; average mortalities of cod larvae that had received *Phaeobacter* as single strains were statistically either not different from, or lower than in the negative control.

All *Phaeobacter* strains significantly reduced mortalities of challenged larvae as compared to the positive infection control, with the exception of *Phaeobacter* sp. 27–4 when it was introduced 2 days after the pathogen. When introduced 2 days in advance of the pathogen, all *Phaeobacter* strains reduced the average mortality of challenged larvae to levels of the untreated control or below (13–30% cumulative mortality 10dph). When introduced at the same time as the pathogen, all *Phaeobacter* strains still reduced mortalities of challenged larvae; however, only *P. gallaeciensis* BS107 and *Phaeobacter* sp. M23-3.1 reduced mortalities to the level of the negative controls or below (22 and 11%). When introduced 2 days after the pathogen, only *Phaeobacter gallaeciensis* BS107 (62%) and *Phaeobacter* sp. M23-3.1 (41%) were effective, yet they did not reduce mortalities to the level of the negative control. Consequently, introducing *Phaeobacter* in advance of *V. anguillarum* was the most efficient way of reducing mortalities in challenged larvae.

Comparing the probiotic effect of the *Phaeobacter* strains, *Phaeobacter* sp. M23-3.1 was the most efficient probiont. *Phaeobacter gallaeciensis* BS107 was very efficient as well, but when it was introduced 2 days later than the pathogen, it failed to prevent mortality in the third challenge trial. *Phaeobacter* sp. 27–4 did only reduce mortality efficiently when introduced in advance of the pathogen, while

Table 1
Treatment groups and cumulative mortalities of cod larvae at 10 days post-hatch in the challenge trials.

Treatment	*Vibrio anguillarum* HI610 added	*Phaeobacter* sp. added	Cumulative mortalities 10 d.p.h.			Average	Standard deviation
			Challenge 1	Challenge 2	Challenge 3		
Negative control; No bacteria added	None	None	30.6%	44.4%	11.1%	28.7%	16.7%
Positive control; *V. anguillarum* alone	10^6 cells/ml at start of experiment	None	98.6%	100.0%	100.0%	99.5%	0.8%
27–4 alone	None	~10^7 cfu/ml *Phaeobacter* sp. 27–4 at start of experiment	12.5%	27.8%	8.3%	16.2%	10.2%
BS107 alone	None	~10^7 cfu/ml *P. gallaeciensis* BS107 at start of experiment	11.1%	9.7%	8.3%	9.7%	1.4%
M23 alone	None	~10^7 cfu/ml *Phaeobacter* sp. M23-3.1 at start of experiment	19.4%	11.1%	11.1%	13.9%	4.8%
27–4 before	10^6 cells/ml 48 h after start of exp.	~10^7 cfu/ml *Phaeobacter* sp. 27–4 at start of experiment	50.0%	23.6%	15.3%	29.6%	18.1%
BS107 before	10^6 cells/ml 48 h after start of exp.	~10^7 cfu/ml *P. gallaeciensis* BS107 at start of experiment	16.7%	13.9%	22.2%	17.6%	4.2%
M23 before	10^6 cells/ml 48 h after start of exp.	~10^7 cfu/ml *Phaeobacter* sp. M23-3.1 at start of experiment	13.9%	12.5%	12.5%	13.0%	0.8%
27–4 simultaneous	10^6 cells/ml at start of experiment	~10^7 cfu/ml *Phaeobacter* sp. 27–4 at start of experiment	63.9%	88.9%	94.4%	82.4%	16.3%
BS107 simultaneous	10^6 cells/ml at start of experiment	~10^7 cfu/ml *P. gallaeciensis* BS107 at start of experiment	13.9%	11.1%	40.3%	21.8%	16.1%
M23 simultaneous	10^6 cells/ml at start of experiment	~10^7 cfu/ml *Phaeobacter* sp. M23-3.1 at start of experiment	16.7%	9.7%	6.9%	11.1%	5.0%
27–4 after	10^6 cells/ml at start of experiment	~10^7 cfu/ml *Phaeobacter* sp. 27–4 48 h after start of experiment	97.2%	97.2%	100.0%	98.1%	1.6%
BS107 after	10^6 cells/ml at start of experiment	~10^7 cfu/ml *P. gallaeciensis* BS107 48 h after start of experiment	16.7%	69.4%	98.6%	61.6%	41.5%
M23 after	10^6 cells/ml at start of experiment	~10^7 cfu/ml *Phaeobacter* sp. M23-3.1 48 h after start of experiment	16.7%	19.4%	87.5%	41.2%	40.1%

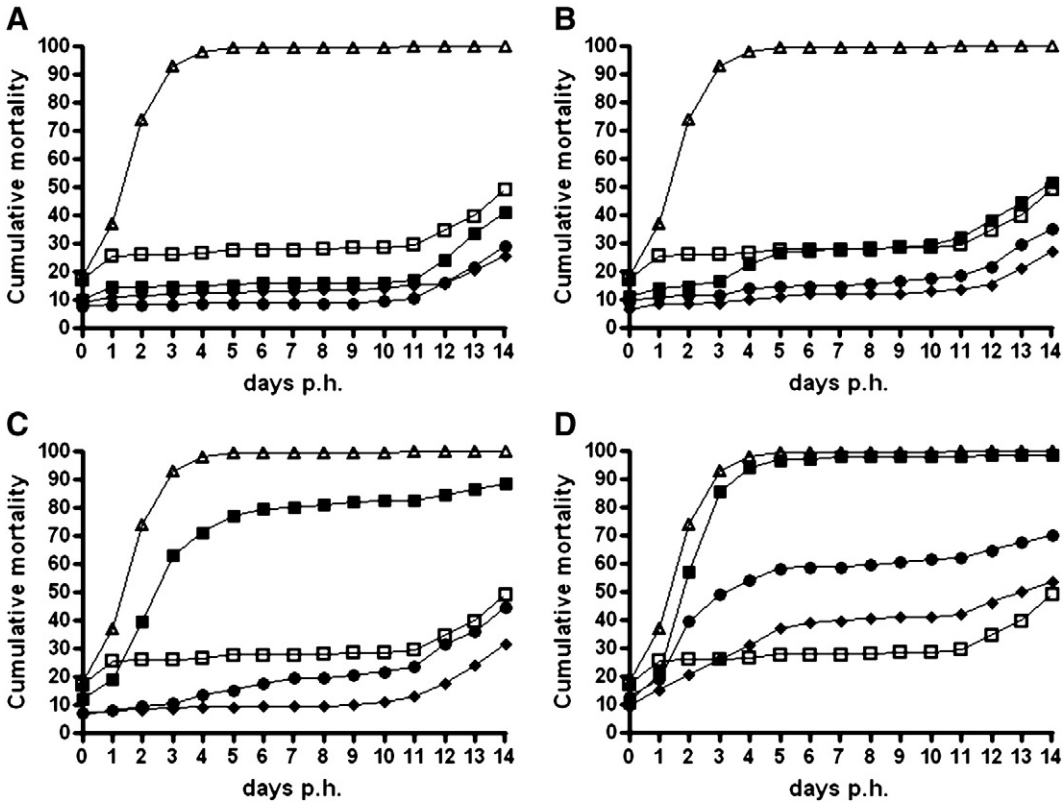

Fig. 1. Mortality of the cod larvae in the challenge trials. Mean values from three independent trials. Untreated larvae (□ Negative Control) and larvae inoculated with *V. anguillarum* HI610 only (Δ Positive Control) are displayed in all panels. A: Exposure to single strains. At the start of the experiment the single-larvae cultures were inoculated with single bacterial strains: *Phaeobacter* sp. 27–4 (■ 27–4 alone), *Phaeobacter* sp. M23-3.1 (♦ M23 alone), and *Phaeobacter gallaeciensis* BS107 (● BS107 alone). B: *Phaeobacter* strains were introduced 48 h before *V. anguillarum*. *Phaeobacter* sp. 27–4 and *V. anguillarum* HI610 (■ 27–4 before), *Phaeobacter* sp. M23-3.1 and *V. anguillarum* HI610 (♦ M23 before), *Phaeobacter gallaeciensis* BS107 and *V. anguillarum* HI610 (● BS107 before). C: *Phaeobacter* strains were introduced simultaneous with *V. anguillarum*. *Phaeobacter* sp. 27–4 and *V. anguillarum* HI610 (■ 27–4 simultaneous), *Phaeobacter* sp. M23-3.1 and *V. anguillarum* HI610 (♦ M23 simultaneous), *Phaeobacter gallaeciensis* BS107 and *V. anguillarum* HI610 (● BS107 simultaneous). D: *Phaeobacter* strains were introduced 48 h after *V. anguillarum*. *Phaeobacter* sp. 27–4 and *V. anguillarum* HI610 (■ 27–4 after), *Phaeobacter* sp. M23-3.1 and *V. anguillarum* HI610 (♦ M23 after), *Phaeobacter gallaeciensis* BS107 and *V. anguillarum* HI610 (● BS107 after).

Table 2
Inhibition of *V. anguillarum* by *Phaeobacter* spp. supernatants in a well-diffusion assay.

Culture condition	Diameter of inhibition zones without well diameter [mm]		
	Phaeobacter sp. 27–4	*P. gallaeciensis* BS107	*Phaeobacter* sp. M23-3.1
Shaken	0	10	13
Static	18	17	17

its effect was unreliable for simultaneous introduction and insignificant for later introduction. This ranking of the *Phaeobacter* strains' probiotic efficacy was not only found by comparison of averages, but as well when the three challenge trials are regarded as separate experiments (Table 1).

3.2. Vibrio inhibition in vitro

In vitro TDA production in shaken and static cultures of the three *Phaeobacter* strains, as assessed in a diffusion-inhibition assay, ranked in the same order as their efficacy in the challenge trials (Table 2). The supernatants from static cultures of all three strains caused inhibition zones of about the same size, indicating about the same level of TDA production. *Phaeobacter* sp. 27–4 supernatants from shaken cultures did not cause any measurable inhibition zones, while *P. gallaeciensis* BS107 and *Phaeobacter* sp. M23-3.1 did produce TDA in shaken cultures, with strain M23-3.1 causing somewhat larger inhibition zones than the type strain (13 and 10 mm).

4. Discussion

In a previous study we observed that *Phaeobacter gallaeciensis* strain BS107 was able to inhibit *Vibrio anguillarum* in gnotobiotic live feed cultures for marine fish larvae and found that mortality of challenged cod larvae could be reduced when *P. gallaeciensis* BS107 was added together with the pathogen (D'Alvise et al., 2012). In the present study, we demonstrate that the probiotic effect on cod larvae is not dependent on this specific strain and that other *Phaeobacter* strains reduce cod larval mortality as well. Furthermore we show that the extent of this effect is influenced by the time of introduction of the probiotic bacteria in respect to introduction of the pathogen.

We have hypothesized that TDA production is the most important in vivo mechanism of action for the probiotic effect of *P. gallaeciensis*, since a TDA negative mutant was less protective of the cod-larvae in challenge trials; however, we could not chemically detect TDA in concentrations that are thought to be effective (D'Alvise et al., 2012). In the present study we found that the probiotic impact of different *Phaeobacter* strains was correlated with their capability of TDA production, as measured by in vitro inhibition of *V. anguillarum*, supporting the hypothesis that TDA production is a key mechanism of probiotic action. Previously published measurements of TDA production in the strains are in accordance with the inhibition measurements in this study (Porsby et al., 2008). The correlation between in vitro and in vivo inhibition could also prove to be valuable information for selecting *Phaeobacter* strains from a larger collection of isolates for in vivo evaluation as probiotics. In vitro TDA production in shaken and static MB cultures, which can easily be assessed in a diffusion-inhibition assay, might serve as a predictor of in vivo antagonistic potential.

As for the time of application, introduction of the probiotic *Phaeobacter* strains before the pathogen proved to be most efficient. This implies that using *Phaeobacter* spp. as a prophylactic treatment against bacterial infections may be the most promising way of utilizing its antagonistic potential. In summary with the findings of the previous study, *Phaeobacter* spp. could be used as a probiotic additive for both larvae and live feed cultures that should be added when new cultures are set up. In contrast to precautionary treatment with antibiotics, this would probably not lead to emergence of resistant pathogens. In a study that deployed thorough experimental approaches to provoke development of resistance against TDA by continuous exposure to sub-lethal concentrations, no resistance or persistent tolerance was developed (Porsby et al., 2011). TDA-forming *Phaeobacter* and *Ruegeria* spp. occur naturally in larviculture sites in different climate zones (Hjelm et al., 2004; Porsby et al., 2008), thus by adding *Phaeobacter* spp. as probiotics a feature of the inherent ecological community would be utilized, rather than an artificial element introduced. This would resemble biological pest-control in organic agriculture, where beneficial microbes or invertebrates are used to control plant diseases and parasites.

Skjermo and Vadstein introduced the r/K-concept to marine larviculture, arguing that opportunistic pathogenic bacteria may become dominant when the organic load of the rearing water in relation to the standing stock of bacteria increases, while a constant organic load selects for non-pathogenic equilibrium species (Skjermo and Vadstein, 1999). The use of microbial matured water, a reduction in disinfecting procedures, recirculation of water, and the addition of probiotic bacteria were suggested as measures to control the microbial community (Attramadal et al., 2012; Skjermo and Vadstein, 1999). *Phaeobacter gallaeciensis* is an r-selected (equilibrium) species, as characterized by a relatively slow growth rate and by being a proficient, competitive, and persistent biofilm former (Rao et al., 2006). Above their capability of reducing pathogenic bacteria at the different trophic levels of marine larviculture, these ecophysiological traits emphasize the probiotic potential of *Phaeobacter* strains.

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Paper 3

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Motility, biofilm formation and inhibitory activity in *Ruegeria mobilis* are influenced by cyclic di-GMP levels.

Submitted manuscript

Motility, biofilm formation, and inhibitory activity in *Ruegeria mobilis* are influenced by cyclic di-GMP levels

Running title: c-di-GMP signaling in *Ruegeria mobilis*

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Abstract (250 words)

The ability to attach to phytoplankton and particles is an important physiological property of *Roseobacter* clade species, and periods of attached life are interspersed with planktonic phases. Many bacteria regulate transitions between motile and sessile life stages by intracellular concentrations of cyclic dimeric guanosinmonophosphate (c-di-GMP). The purpose of this study was to investigate whether shifts between motile and sessile life in the globally abundant *Roseobacter* clade species *Ruegeria mobilis* are associated with c-di-GMP signaling. Genes for GGDEF- and EAL-domain proteins were found in the genome of *Ruegeria mobilis* strain F1926. Ion-Pair chromatography-tandem mass spectrometry revealed sevenfold higher c-di-GMP concentrations per cell in biofilm-containing cultures than in planktonic cells. Plasmid-borne GGDEF- and EAL-domain genes were introduced into *R. mobilis*. The GGDEF-domain gene increased c-di-GMP contents, enhanced biofilm formation and reduced motility. The EAL-domain gene decreased c-di-GMP, increased motility and prevented formation of multicellular rosettes that are typical for biofilms. Production of the potent antibiotic tropodithietic acid (TDA) in *R. mobilis* coincides with biofilm formation. Introduction of the EAL-domain gene reduced the antibacterial activity of cell-free supernatants, indicating that TDA formation might be controlled by c-di-GMP. Expression of *tda*C, a key gene for TDA biosynthesis, was monitored using a promoter-*gfp* fusion. The *tda*C-promoter was only expressed in attached or biofilm-forming cells, and expression was induced by attachment to a surface. In conclusion, c-di-GMP signaling controls biofilm formation and expression of biofilm-associated traits in *R. mobilis* and, as suggested by presence of GGDEF and EAL domain genes, also in other *Roseobacter* clade species.

Keywords: c-di-GMP / tropodithietic acid / *Roseobacter* clade / *Ruegeria mobilis*

Introduction

The *Roseobacter* clade (α-Proteobacteria) accounts for a significant part of the microbiota in the world's oceans, especially in coastal zones and surface waters (Brinkhoff *et al.*, 2008; Wietz *et al.*, 2010; Gonzalez and Moran, 1997; Buchan *et al.*, 2005; Newton *et al.*, 2010). *Roseobacter* clade species are metabolically and ecologically diverse, comprising anoxygenic phototrophs, sulfur metabolizers, carbon monoxide oxidizers, and degraders of aromatic compounds (Shiba *et al.*, 1979; Moran *et al.*, 2003; Moran *et al.*, 2004; Allgaier *et al.*, 2003; Sorokin and Lysenko, 1993; Moran and Hodson, 1994; Buchan *et al.*, 2001). However, most species of the *Roseobacter* clade were classified as ecological generalists (Newton *et al.*, 2010; Moran *et al.*, 2004). Abundance and activity of many *Roseobacter* clade members is correlated with phytoplankton population densities, and one prominent ability of many *Roseobacter* clade members is the conversion of the phytoplankton osmolyte dimethylsulfoniopropionate (DMSP) to the volatile dimethyl sulfide (DMS), which impacts the local and global climate (Moran *et al.*, 2003; Gonzalez *et al.*, 2000; Charlson *et al.*, 1987; Gonzalez and Moran, 1997; Geng and Belas, 2010b).

Belas *et al.* (2009) noticed that many *Roseobacter* clade species have a "biphasic swim-or-stick lifestyle" that enables their symbiosis with phytoplankton, and suggested that a central regulation mechanism coordinated the shift between planktonic and attached phenotype. Accumulating evidence indicates that bis-(3'-5')-cyclic dimeric guanosin monophosphate (c-di-GMP) functions as a nearly universal second messenger in bacteria, regulating transitions between planktonic and sedentary phases by controlling phenotypic features like flagellar motility and EPS production (Hengge, 2009; McDougald *et al.*, 2012). Above that, c-di-GMP regulates important functions that are associated with either one of the lifestyles, such as virulence or antibiotic production (Cotter and Stibitz, 2007; Tamayo *et al.*, 2007; Schmidt *et al.*, 2005). The intracellular pool of c-di-GMP is balanced by diguanylate cyclases (GGDEF-

71 domain proteins) that synthesize the compound and by specific phosphodiesterases (EAL-
72 domain proteins) that degrade it (Gjermansen *et al.*, 2006; Ryjenkov *et al.*, 2005; Ausmees *et*
73 *al.*, 2001). The activity of these antagonistic enzymes is controlled by a range of sensory
74 proteins that allow a multitude of external or internal stimuli to act on the intracellular pool of
75 c-di-GMP and thus influence the decision between sessile and motile life. Here we
76 hypothesized that the transition between planktonic and attached lifestyle in *Roseobacter*
77 clade species is induced by intracellular c-di-GMP levels. To test this, we introduced the
78 plasmids pYedQ and pYhjH that had been used as tools to demonstrate that c-di-GMP
79 signaling regulated *Pseudomonas putida* biofilm formation and dispersal (Gjermansen *et al.*,
80 2006). The plasmids contain either one of the *E.coli* genes *yed*Q and *yhj*H, which encode a
81 diuguanylyl cyclase that synthesizes c-di-GMP and a c-di-GMP-degrading diesterase,
82 respectively. Intracellular levels of c-di-GMP in *Ruegeria mobilis* F1926 wild type and
83 plasmid-carrying mutants were assessed using ion-pair liquid chromatography-tandem mass
84 spectrometry.

85 The *Roseobacter* clade genera *Ruegeria* and *Phaeobacter* have been of particular interest due
86 to their ability to form the antibacterial compound tropodithietic acid (TDA). *Phaeobacter*
87 strains have been isolated from coastal zones, especially from biofilms in fish and
88 invertebrate larvae cultures, and have been studied for their antibacterial activity and as
89 probiotics for marine aquaculture (Hjelm *et al.*, 2004; Planas *et al.*, 2006; Ruiz-Ponte *et al.*,
90 1998; D'Alvise *et al.*, 2010; Porsby *et al.*, 2008; Brinkhoff *et al.*, 2004). *Ruegeria*
91 *(Silicibacter)* sp. TM1040 is a symbiont of the dinoflagellate *Pfisteria piscicida*, and was the
92 first TDA-producing bacterium in which parts of the TDA biosynthetic pathway were
93 elucidated (Alavi *et al.*, 2001; Bruhn *et al.*, 2007; Geng *et al.*, 2008; Geng and Belas, 2010a;
94 Yi *et al.*, 2007; Moran *et al.*, 2007; Miller and Belas, 2006). *Ruegeria mobilis* (*R. pelagia*)
95 occurs in all of the world's oceans, except Arctic and Antarctic waters, both in coastal zones

and open oceans (Gram *et al.*, 2010; Lai *et al.*, 2010). Several strains of *R. mobilis*, which all produce TDA, have been isolated from microalgae cultures, highlighting their preference for phytoplankton (Porsby *et al.*, 2008).

Studying *Phaeobacter* sp. 27-4, a strain isolated from an aquaculture site due to its inhibitory effect on the fish pathogen *V. anguillarum*, Bruhn *et al.* (2005) found that TDA production in Marine Broth occurred only under static growth conditions, but not in shaken broth cultures. TDA production coincided with formation of a thick layer of biofilm at the air-liquid interface consisting of multicellular, star-shaped aggregates. A later study demonstrated that attachment to an inert surface was affected by culture conditions as well (Bruhn *et al.*, 2006). However, although the tight association between biofilm formation and TDA production strongly indicates that TDA is only produced by attached or biofilm-forming cells, this was never verified. The present finding that c-di-GMP plays a major role in the transition from motile to sessile state in *R. mobilis* lead us to hypothesize that formation of TDA, which is associated with biofilms, depends on c-di-GMP. Consequently, investigating whether TDA production was affected by altered c-di-GMP levels became a second aim of this study. Therefore we measured the impact of changed c-di-GMP levels on antibacterial activity and pigmentation as indicators of TDA production and studied expression of the *tda*C gene in attached and planktonic cells.

Material and Methods

Strains, plasmids and media. An overview of strains and plasmids is provided in Table 1. *Ruegeria mobilis* F1926 was isolated from the Indian Ocean (-31.4061, 91.17758) during the Galathea III expedition (Gram *et al.*, 2010). The strain was revived from frozen stock cultures (-80°C) on half-strenght Marine Agar (½MA; 27.6 g Difco 212185 Marine Agar, 15 g Instant

120 Ocean Sea Salts, 7.5 g Agar, 1 l deionized water). Plasmids pYedQ and pYhjH were obtained
121 from Tim Tolker-Nielsen (University of Copenhagen) and electroporated into *R. mobilis*
122 F1926, as described below. ½YTSS broth and agar (Gonzalez *et al.*, 1996) containing 50
123 µg/ml tetracycline were used for selecting transconjugants after the electroporations and for
124 routine culturing of the plasmid-carrying mutants. Cultures for microscopy and for chemical
125 measurements of c-di-GMP were grown in full strength Marine Broth (MB) that contained 50
126 µg/ml tetracycline for the plasmid-carrying mutants. ½YTSS with and without tetracycline
127 was used for biofilm and attachment assays. All cultures were grown as 20 ml batches in 250-
128 ml glass bottles at 25°C, except as noted otherwise, and shaking velocity was 200 rpm.

129 **Detection of genes with GGDEF- and EAL-domains.** Genomic DNA was obtained from
130 strain F1926 by successive phenol-chloroform purification steps. Mate-pair library
131 preparation, Illumina Hi Seq 2000 sequencing and initial data processing were done by BGI
132 (Beijing, China). The genome draft was annotated by the Joint Genomic Institute (http://
133 http://www.jgi.doe.gov/), and genes encoding GGDEF- and EAL-domain proteins are listed
134 in Table 2. Submission of the annotated genome draft to EMBL/EBI is in progress, an
135 accession number will be provided.

136 **Electroporations.** The electroporation method was adapted from Miller & Belas (2006).
137 Recipient cells were grown in 50 ml ½YTSS (*R. mobilis*) or LB medium (*E. coli,* 37°C) until
138 OD600 was about 0.5, chilled on ice for 30 min, harvested by centrifugation at 2,380 x *g*,
139 washed twice in 10 ml autoclaved, ice-cold MilliQ-water, and resuspended in 0.5 ml ice-cold
140 10% glycerol. Aliquots of 70 µl were stored at -80°C until use. Electrocompetent cells were
141 mixed with 180 - 230 ng plasmid DNA, incubated 30 min on ice, transferred to a 0.2 cm
142 electroporation cuvette (165-2086 Biorad, Hercules, CA), and electroporated at 2.5 kV cm^{-1},
143 200 Ω, 25 µF using a Biorad Gene Pulser. The cells were immediately transferred to liquid
144 growth medium without antibiotics, recovered for 2 - 4 hours, and plated on selective agar.

C-di-GMP extraction. Shaken and static cultures of *R. mobilis* F1926 wild type and the pYedQ and pYhjH-carrying mutants were grown for 24 hours. The cultures were cooled on ice, and static cultures were shaken briefly to break up the biofilms. One ml was sampled and vortexed vigorously to further break up aggregates before measuring OD600. Cultures were harvested (5,000 × *g*) and the pellets were extracted with 10 ml 75% (v/v) boiling ethanol/water containing 10 µM HEPES. The pellets were resuspended in 75% ethanol by vortexing and the suspensions were left in a 80°C water bath for 5 minutes. Subsequently the suspensions were centrifuged (4250 × g) and the supernatants were evaporated to dryness under nitrogen. The samples were dissolved in 100 µl mobile phase and filtered through 0.2 µm PTFE hydrophilic filters before analysis.

Ion pair UHPLC-MS/MS analysis of C-di-GMP. The analysis was carried out on an Agilent 1290 binary UHPLC system coupled with an Agilent 6460 triple quadrupole (Torrance, CA, USA). The triple quadrupole was equipped with an Agilent jet stream ESI source and was operated in negative ion and multiple reactions monitoring mode. $[M-H]^-$ m/z 689.1 was used as parent ion, m/z 149.9 and 537.9 were used as quantifier and qualifier, respectively. The collision energy and fragmentor voltage for the quatifier ion were 35 V and 100 V respectively. An Agilent Poreshell 120 phenyl-hexyl 2.7 µm, 100 mm x 2.1 mm column was used. Mobile phase A was 10 mM tributylamine and 10 mM acetic acid (pH 5.5), and mobile phase B was 90% (v/v) methanol containing 10 mM tributylamine and 10 mM acetic acid. The gradient used was 0-5 min 0% B, 5-10 min 0-2% B, 10-11 min 2-9% B, 11-16 min 9% B, 16-24 min 9-50% B, 24-28.5 min 100% B, 28.5-30 100% B, 30-30.5 min 100-0% B, 30.5-36 min 0% B. The column temperature was kept at 40 °C and the injection volume was 10 µl. Two shaken cultures of the pYhjH-carrying mutant, which produced the lowest amounts of c-di GMP, were used for matrix-matched calibration. One mg/ml c-di-GMP in water was used to prepare the spiking solutions. The cultures were mixed and

divided into four equal portions. After centrifugation and removal of the supernatants, three of the portions were spiked with 100 µl of 25 ng/ml, 75 ng/ml, and 250 ng/ml c-di-GMP standard, and the forth portion was kept as a blank. The spiked cultures were extracted and prepared for analysis as described above. The amount of c-di-GMP detected in the blank was subtracted from the spiked calibrants, and the analysis was calibrated by linear regression (r^2=0.998). To obtain a relative estimate of c-di-GMP concentrations per cellular biomass, c-di-GMP concentrations were divided by the measured OD600 of the original cultures.

Biofilm and attachment assay. Biofilm formation in *R. mobilis* F1926 wild type, F1926 pYedQ and F1926 pYhjH was measured by a crystal violet method (O'Toole *et al.*, 2000). Briefly, shaken precultures were diluted with fresh medium to an OD600 of 0.1, pipetted into a 96-well microtiter plate, and incubated for 24 hours. Culture liquid was removed, and biofilms were washed and stained in 1% (w/v) crystal violet solution. After washing, the crystal violet was extracted from the stained biofilms with 96% ethanol and quantified by measuring absorption at 590 nm. Attachment to an inert surface was measured in a modified crystal violet assay. Static cultures were grown in 96-well plates as described. Shaken cultures were grown in glass bottles for 24 hours, OD600 was adjusted to 1.0, and 200 µl were pipetted into the wells of a micotiter plate. A lid with 96 polystyrene pegs (Innovotech, Edmonton, Canada) was placed on the plate and the cells were allowed to attach to the pegs for 1 minute in static cultures and 10 minutes in shaken cultures. Adherent biofilms on the pegs were washed twice by dipping into water, dried for 5 minutes, and stained in crystal violet solution. After triple washing in water, the crystal violet was extracted from the stained biofilms on the pegs in each 200 µl ethanol, and absorption was measured at 590 nm.

Statistics. Cellular c-di-GMP concentrations were compared between wild type and mutants, and between shaken and static cultures by t-tests. Differences in average crystal violet absorption values in the biofilm and attachment assays were examined by one-way ANOVA

195 with Tukey's multiple comparison test using the software Prism version 4.03 (GraphPad
196 Software, La Jolla, CA).

197 **Measurement of antibacterial activity.** Inhibition of *V. anguillarum* 90-11-287 in a
198 standard well-diffusion assay, as adapted from (Hjelm *et al.*, 2004), was used as an
199 approximation for TDA production. *V. anguillarum* 90-11-287 was grown in MB for 1 day at
200 25°C with aeration at 200rpm. 50 µl of the *V. anguillarum* preculture was added to 50 ml
201 molten Instant Ocean-Agar (1.5 g Instant Ocean Sea Salts, 0.1 g Casamino Acids (Bacto,
202 France), 0.2 g glucose, 0.5 g agar) at 41.5°C and poured into a 14-cm petri dish. Wells of 6
203 mm diameter were punched into the solidified agar and filled with 50 µl of *R. mobilis* F1926
204 culture supernatant. The assay was incubated for 1 day at 25°C and diameters of inhibition
205 zones were measured.

206 **Construction of a *tda*Cp::*gfp* reporter fusion.** A transcriptional fusion between the
207 promoter of *tda*C and a promoterless *gfp* gene was constructed similarly to pHG1011 (Geng
208 and Belas, 2010a). The promoter sequence of the *tda*C gene was amplified using the primers
209 ptdacF (5'-GTCCCAGAGACCAACGCAATGAGTAAAGGAGAAGAA-3') and ptdacR
210 (5'-TTCTTCTCCTTTACTCATTGCGTTGGTCTCTGGGAC-3'). The *gfp* open reading
211 frame in pAKN137 (Lambertsen *et al.*, 2004) was amplified using the primers gfpF (5'-
212 GTCCCAGAGACCAACGCAATGAGTAAAGGAGAAGAA-3') and gfpR (5'-
213 TGATAAGCTTTTATTTGTATAGTTCATCCATGCCATGT-3'). Primer ptdacF created a
214 PstI-restriction site and gfpR created a HindIII-site. Primer ptdacR and gfpF created identical
215 36-bp-sequences in the adjacent ends of the two amplicons, each containing the end of the
216 promoter and the start of the *gfp* open reading frame. This allowed seamless cloning of
217 promoter and *gfp* gene by overlap-extension PCR. The product was cloned into the broad-
218 host range vector pRK415 (Keen *et al.*, 1988), after both had been digested with PstI and
219 HindIII (New England Biolabs, Ipswich, MA) to yield plasmid pPDA11.

Microscopy. Shaken and static cultures of *R. mobilis* F1926 wild type and the mutants carrying pYedQ and pYhjH were grown in duplicates for 24 hours and were compared by phase contrast microscopy. Before specimen preparation, static cultures were agitated briefly to break the biofilms into smaller pieces for sampling. Images that were representative of the specimen were recorded. Screening the whole specimen, motility, rosette formation, and the proportion of single cells and cells in biofilms were registered. Absence of motility or rosettes was stated, if no motile cell or rosette was detected in either of the duplicate samples. Gfp-fluorescence of *R. mobilis* F1926 pPDA11 was detected by microscopy using a WIB cube (Olympus, ex.460-490, em.>515). Shaken and static cultures were grown for three days. Again, static cultures were briefly shaken before specimen preparation. Representative fluorescence micrographs were recorded with 1.5 s exposure, and a phase contrast image of the same area was recorded right thereafter. To record a time series showing the onset of gfp-expression in response to attachment, a three day old shaken culture was diluted 1:2 with fresh medium and grown for four hours at the same conditions. A specimen was prepared, and a time series was recorded, as described. For better display, contrast in the phase contrast pictures and brightness of the fluorescence micrographs was enhanced using Adobe Photoshop. The same adjustments were made on all images of the same type.

Accession number. (will be provided)

Results

Bioinformatic analysis. The *Ruegeria mobilis* F1926 genome was assembled in 266 contigs with a total length of 4.9 Mb. 18 contigs were larger than 100 kb and contained together 3.8 Mb. For comparison, *Ruegeria* sp. TM1040 contains 4.2 Mb of genomic DNA, a

megaplasmid of 0.8 Mb, and a plasmid of 0.1 Mb (Moran *et al.*, 2007). Parts of the largest contig (350 kb) in the F1926 genome draft aligned with parts of the megaplasmid of *Ruegeria* sp. TM1040 (results not shown). Nine genes encoding diguanylate cyclases and c-di-GMP-specific phosphodiesterases were identified in *R. mobilis* F1926, based on Pfam (Wellcome Trust Sanger Institute) classification (Table 2). Six of these encoded for proteins that contained both a GGDEF- and an EAL-domain, indicating that the activity of these enzymes can be switched from synthesizing to degrading c-di-GMP. Additional domains were identified in some of the genes, such as the MYHT-domain, a sensor domain in bacterial signaling proteins that is often found together with EAL or GGDEF domains, and the HAMP-domain, which is typically found in integral membrane proteins that are part of signal transduction pathways. One of the GGDEF/EAL genes was identified as an ammonium-transporter.

Ion-Pair UHPLC-MS/MS. Plasmids pYedQ and pYhjH were introduced into *R. mobilis* F1926 with the aim of manipulating intracellular c-di-GMP concentrations, which was subsequently verified by ion-pair Chromatography-MS/MS in extracts of 24 hour old MB cultures of F1926 wild type, F1926 pYedQ and F1926 pYhjH (Fig. 1). The compound was detected in all extracts (Fig. 1). Concentrations in the extracts ranged from 16 to 140 nM and were divided by OD600 of the original culture to obtain a relative measure of c-di-GMP per cell (Fig. 2). Static cultures of the wild type, where thick air liquid interface biofilms were observed, contained six times more c-di-GMP per cell than shaken cultures, which did not form biofilms. In the mutant carrying plasmid pYhjH c-di-GMP concentrations per cell were reduced to about half (shaken, $p=0.02$) and a quarter (static, $P<0.001$) of the concentrations measured in the wild type grown under the same conditions. Plasmid pYedQ increased c-di-GMP per cell to the threefold of the wild type under shaken conditions ($p=0.003$). Under static conditions c-di-GMP concentrations in F1926 pYedQ were not significantly different

from the wild type. However, this increased to 470 nM after three days, while c-di-GMP in the other strains decreased (data not shown). Regardless of the introduced plasmids, cultivation conditions influenced c-di-GMP levels. For all strains concentrations in static cultures were higher than in shaken cultures ($p<0.001$).

Phenotypic effects of altered c-di-GMP levels. Shaken cultures of *R. mobilis* F1926 wild type were dominated by single cells, and about half of these were motile (Fig. 3). Introduction of pYedQ, which increased c-di-GMP concentrations, caused complete disappearance of motile cells and increased formation of multicellular aggregates in shaken cultures. *R. mobilis* F1926 pYhjH, which contained less c-di-GMP, grew almost exclusively as motile cells in shaken cultures. Static cultures of the wild type were dominated by biofilms consisting of multicellular, star-shaped aggregates (rosettes), but also motile single cells were found. In static cultures of the pYedQ-carrying mutant even thicker rosette-containing biofilms were formed, however no motile cells were observed. Introduction of pYhjH increased the proportion of motile cells and prevented formation of rosettes in static cultures (Fig. 3F).

Biofilm formation and attachment were assessed in mutant strains and wild type (Fig. 4). Biofilm formation was significantly increased in the pYedQ-carrying strain ($p<0.001$) whereas pYhjH was comparable to wt ($p>0.05$). Attachment of cells from shaken and static cultures was measured as stained biomass of cells attaching to polystyrene pegs. In shaken cultures the wild type attached most efficiently, while attachment was reduced by a third in the pYhjH-carrying mutant and by two thirds in the mutant carrying pYedQ ($p<0.001$), indicating that both a decrease and an increase of intracellular c-di-GMP levels interfere with attachment. In static cultures, no significant differences in attachment were found between mutants and wild type (data not shown).

293 The antibacterial effect of the plasmid-carrying mutants and the wild type was compared in
294 an agar-diffusion-inhibition assay. In static cultures, the pYedQ-carrying mutant caused
295 inhibition zones of equal or larger size than the wild type, while the mutant carrying pYhjH
296 was less inhibitory (Table 3). This indicated that TDA production was indeed influenced by
297 intracellular c-di-GMP concentrations. However, this could only be confirmed under static
298 conditions, since shaking abolished TDA production in all cultures. Brown pigmentation of
299 the cultures, which is indicative of TDA production (Bruhn *et al.*, 2005), was correlated with
300 inhibition in the biotests (Fig. S1).

301 **Expression of *tda*C on single cell level.** The observation that inhibitory activity was
302 influenced by c-di-GMP prompted us to study the expression of a key gene involved in TDA
303 biosynthesis on single cell level. In shaken cultures, *R. mobilis* F1926 pPDA11 cells were
304 predominantly planktonic, and approximately half the cells were motile, as described above
305 for the wild type. The *tda*C promoter was not expressed, as indicated by lack of green
306 fluorescence (Fig. 5), correlating with a lack of inhibition (Table 3). A few small
307 multicellular aggregates where *tda*C was expressed were found. In contrast, major
308 proportions of the cells in static cultures were contained in biofilms or multicellular
309 aggregates and expressed the *tda*C gene. However, a part of the single cells in samples from
310 static cultures were not fluorescent, and a fraction of these was motile. Expression of the
311 *tda*C promoter was never observed in motile cells. This indicated that *tda*C, a gene encoding
312 a central enzyme in the TDA biosynthesis pathway, is expressed differently in cells within
313 the same cultures, and that its expression coincides with life in biofilms or aggregates where
314 high c-di-GMP levels are found.

315 **Induction of *tda*C expression in response to attachment.** When a sample of a shaken *R.*
316 *mobilis* F1926 pPDA11 culture was prepared for microscopy, some of the non-motile
317 bacterial cells attached immediately to the glass surfaces. A time series of fluorescence and

phase contrast micrographs showing newly attached cells on a glass cover slip was recorded (Fig. 6). Gfp expression was initiated in the newly attached cells, and green fluorescence could be observed already 10 minutes after preparation of the microscope slide. Maximal fluorescence of the initially attached cells was reached about 15 - 20 minutes after start of the experiment. Considering the time needed for *gfp* gene expression and maturation, expression must have been triggered immediately when the cells attached. This instant initiation of *tda*C expression suggests a regulatory connection between TDA production and initial attachment, which is likely induced via enhanced c-di-GMP levels.

Discussion

Attachment to surfaces and biofilm formation are characteristic features of many *Roseobacter* clade species, and a more comprehensive understanding of the transition between motile and sessile life stages in the *Roseobacter* clade is needed to understand carbon and nutrient cycling in the oceans (Slightom and Buchan, 2009). Also, production of the antibacterial substance TDA in *Phaeobacter* spp. has been associated with biofilms (Bruhn *et al.*, 2005), and since TDA is a key component for their probiotic effect on fish larvae (D'Alvise *et al.*, 2012), understanding the transition between motile and sessile stages may provide new perspectives on their practical application.

Many bacteria have two distinct lifestyles, a sessile or biofilm stage on a substrate or within a host, characterized by increased metabolic activity and proliferation, and a mobile stage where the cells are metabolically less active, disperse into the wider environment, and persist until a new substrate or host is found. The transition between these two distinct states is in many bacteria controlled by a pool of intracellular c-di-GMP that binds to transcriptional regulators, activating or repressing transcription of a multitude of genes, e.g. for motility,

342 EPS production, extracellular appendages, virulence, or antibiotics (Hengge, 2009;
343 McDougald *et al.*, 2012). The results of our study suggest that c-di-GMP signaling plays a
344 similar role in *R. mobilis*. Intracellular concentrations of c-di-GMP changed with cultivation
345 conditions that favored or prevented biofilm formation. Manipulation of c-di-GMP levels
346 altered the proportions of motile and sessile cells under different culture conditions and
347 affected expression of phenotypes that are associated with either planktonic or attached
348 lifestyle. Increased levels of c-di-GMP promoted biofilm formation and repressed motility,
349 while a decrease in c-di-GMP concentrations stimulated motility, prevented formation of star-
350 shaped aggregates, and reduced inhibitory activity. The finding that an introduced GGDEF-
351 domain protein increased biofilm formation and interfered with motility is consistent with
352 previous studies (Ausmees *et al.*, 2001; Gjermansen *et al.*, 2006; Hengge, 2009; McDougald
353 *et al.*, 2012; Wolfe and Visick, 2008) and indicate a functional c-di-GMP signaling system in
354 *R. mobilis*. This is confirmed by presence of genes encoding GGDEF- and EAL-domain
355 proteins in the genome of *R. mobilis* F1926. Presence of similar genes annotated as
356 diguanylyl cyclases and c-di-GMP-specific phosphodiesterases in the closely related strains
357 *Ruegeria* sp. TM1040 and *Phaeobacter gallaeciensis* BS107, as well as in more remote
358 *Roseobacter* species like *Roseobacter litoralis* Och149 and *Octadecabacter antarcticus* 238
359 (www.roseobase.org), suggests that c-di-GMP signaling is a universal feature of the
360 *Roseobacter* clade. We conclude that in the *Roseobacter* clade, in analogy with many other
361 bacteria, intra- and extracellular cues are integrated via a c-di-GMP second messenger system
362 and that expression of phenotypic traits specific for either planktonic or attached life are
363 regulated in response to c-di-GMP concentrations. Belas *et al.* (2009) have introduced the
364 term “swim-or-stick switch” for the molecular mechanism that regulates transitions between
365 motile and sessile stage in *Roseobacter* clade species. We think that the intracellular
366 concentration of c-di-GMP is the swim-or-stick switch.

Recently, Zan *et al*. (2012) revealed that in *Ruegeria* sp. KLH11, a sponge symbiont, motility and biofilm formation are controlled by N-acyl homoserine lactone-based quorum sensing (QS). Similarly, Sule and Belas (2012) found that in *Ruegeria* sp.TM1040 motility and biofilm formation are controlled by a QS-like system based on a diffusible signal compound with a molecular mass of about 226 Da. *Ruegeria mobilis* strain F1926 may utilize the same or a similar QS system as *Ruegeria* sp. TM 1040, however, since QS and c-di-GMP signaling control the same phenotypes, the two regulation systems are likely connected at some level. In a review of connections between QS and c-di-GMP signaling, Srivastava and Waters (2012) propose that QS signals are generally integrated by the epistatic c-di-GMP signaling system, allowing information about local cell density to be merged with other environmental cues for making a decision between attached and planktonic life. Thus, studying the connection between QS and c-di-GMP signaling may provide further insight into how motility and biofilm formation are controlled.

Most studies of c-di-GMP signaling have approached the role of the compound using bioinformatic and transcriptional tools or genetic manipulation. Only few studies actually measured concentrations of c-di-GMP to substantiate their findings, e.g. (Merritt *et al.*, 2010). Low c-di-GMP concentrations in a sample with high amounts of other nucleotides can only be analyzed by chromatographic separation followed by specific detection through tandem mass spectrometry (MS/MS) or high resolution mass spectrometry, while UV detection will not provide specific results. Due to the ionic nature of c-di-GMP it is difficult to obtain reproducible retention times, as well as sharp symmetrical peaks using conventional reversed phase HPLC/UHPLC (Waters *et al.*, 2008; Luo *et al.*, 2007). Thus, we applied ion-pair liquid chromatography-MS/MS for chemical analysis of c-di-GMP in this study, and this allowed us to quantify the compound under different growth conditions and after genetic manipulations.

391 A relative of the *Roseobacter* clade within the α-Proteobacteria, *Caulobacter crescentus*, has
392 a very sophisticated variant of c-di-GMP-mediated "swim-and-stick" life (Duerig *et al.*,
393 2009; Aldridge *et al.*, 2003; Paul *et al.*, 2004; Huitema *et al.*, 2006; Viollier *et al.*, 2002). The
394 cell cycle comprises a stage of flagellated swarmer cells, in which replication is inhibited by
395 low c-di-GMP levels, and a stage of sessile, stalked cells that form new swarmer cells at their
396 non-attached end. In the stalked cells c-di-GMP is unequally distributed, as an effect of
397 antipodal location of GGDEF- and EAL-domain proteins, restricting cell division to the non-
398 attached pole. In the present study, formation of star-shaped rosettes was observed to be
399 prevented in a mutant with lowered c-di-GMP levels, indicating an involvement of the
400 compound in producing that phenotype. Rosette formation in *Roseobacter* clade species
401 could be the result of a process involving polar differences in c-di-GMP contents, where cell
402 division is possibly restricted to one pole of the rosette-forming cell and its daughter cells.

403 Bruhn *et al.* (2007) demonstrated that cells from static cultures attached better to a glass
404 surface than cells from shaken cultures. The same pattern was observed in this study.
405 However, we also compared attachment between shaken cultures of wild type and mutants
406 and found that attachment was compromised both by increased and decreased c-di-GMP
407 levels. Miller and Belas (2006) demonstrated that in *Ruegeria* sp. TM1040 motility is crucial
408 for initiating the *Ruegeria*–dinoflagellate symbiosis. Consequently, the mutant with increased
409 c-di-GMP levels may have a reduced capability of attaching, because motility was repressed.
410 Interestingly, attachment of the pYhjH-carrying mutant with increased motility was reduced
411 as well, even if not to the same extent. It stands to reason that c-di-GMP plays a role in the
412 transition from motile to sessile state. Initial contact with the substrate is possibly sensed by a
413 surface receptor that switches the activity of a diguanylyl cyclase/phosphodiesterase from
414 degrading to synthesizing c-di-GMP. In effect, c-di-GMP levels would rise and initiate
415 attachment, e.g. by switching off flagellar motility, producing EPS, etc. This rise of c-di-

GMP in response to surface contact would be impaired in F1926 pYhjH by the c-di-GMP-degrading activity of the phosphodiesterase YhjH.

Antibacterial activity and production of the brown pigment, both indicative of TDA production, were reduced by decreased intracellular c-di-GMP level, suggesting that the association between the biofilm stage and TDA production would be c-di-GMP mediated. This led us to study expression of *tda*C as indicator of TDA production on single-cell level, and we found that *tda*C is expressed differently within the same cultures. *tda*C was expressed only in biofilms or aggregates, and its expression could be triggered by attachment. Thus, *tda*C was expressed where high levels of c-di-GMP would be found. Based on these results, we hypothesize that TDA production is triggered by elevated intracellular concentrations of c-di-GMP. Geng & Belas (2011) showed that TdaA, a LysR-type transcriptional regulator, binds to the *tda*C promoter and activates expression of *tda*C. Normally the ligands of LysR-type transcriptional regulators are small molecules (Schell, 1993), and one could speculate that c-di-GMP might be the ligand of TdaA.

Two possible regulation mechanisms for TDA production were identified on community level. Quorum sensing was found to activate production of TDA in *P. gallaeciensis*, and also TDA itself was observed to act as an autoinducer, causing increased expression of genes necessary for its own production (Geng and Belas, 2010a; Berger *et al.*, 2011). However, both mechanisms fail to explain how *tda*C can be expressed differently in adjacent cells. The hypothesis that TDA production is regulated by c-di-GMP provides an alternative explanation for how TDA production can be different in cells within the same culture, and for how it can, on single-cell level, be spontaneously induced by attachment, despite absence of TDA.

In conclusion, our study adds organisms from the Roseobacter clade to the growing number of bacteria that use c-di-GMP as a key secondary messenger. Notably, c-di-GMP may be the key molecule in the often described "stick-and-swim" lifestyle of several roseobacters.

Acknowledgements

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5106 words (incl. abstract, excl. references)

452 **Tables and figures**

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant markers	Source or reference
Strain		
Ruegeria mobilis F1926	Wild type	Gram *et al.* 2010
E. coli TransforMax EC100D *pir*$^{+}$		Epicentre
Plasmids		
pPDA11	tdaCp::gfp ligated into pRK415, Tet^{R}	This study, D'Alvise *et al* 2012
pRK415	Conjugative broad host range vector, Tet^{R}	Keen *et al.* 1988
pYedQ	*E. coli* gene *yed*Q (diguanylyl cyclase) ligated into pRK404A, Tet^{R}	Ausmees *et al.* 2001, Gjermansen *et al.* 2006
pYhjH	*E. coli* gene *yhj*H (c-di-GMP-specific phosphodiesterase) ligated into pBBR1-MCS3, Tet^{R}	Gjermansen *et al.* 2006

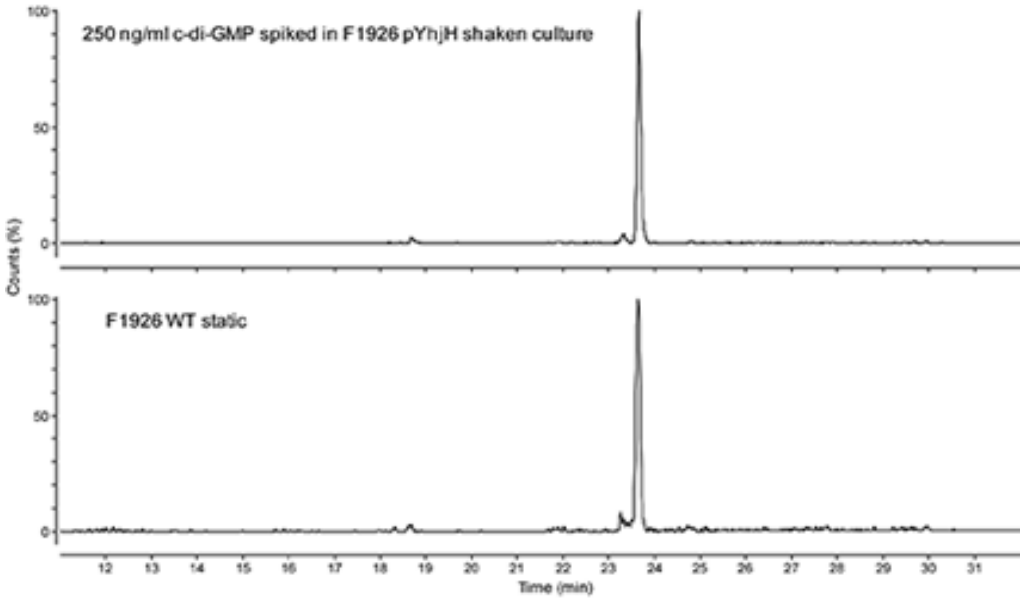
453

Table 2. GGDEF- and EAL-domain genes in Ruegeria mobilis F1926

Locus tag	Predicted function	Pfam domain (E-score)	Length [bp]
00678	diguanylate cyclase domain	GGDEF (3.8e-29)	942
01033	diguanylate cyclase/phosphodiesterase	GGDEF (4.2e-38) EAL (4.7e-56) PAS_7 (1.8e-7)	2382
01097	Response regulator receiver modulated diguanylate cyclase	GGDEF (7.4e-46) Response_Reg (2.8e-14)	1404
01102	diguanylate cyclase domain	GGDEF (3.0e-39)	1029
02701	diguanylate cyclase/phosphodiesterase	GGDEF (2.6e-34) EAL (5.1e-67)	2055
02713	diguanylate cyclase/phosphodiesterase	GGDEF (1.6e-45) EAL (1.1e-59) HAMP (4.5e-10)	2103
02898	diguanylate cyclase/phosphodiesterase	GGDEF (2.3e-35) EAL (5.6e-66) MHYT (6.1e-9) MHYT (1,2e-6) MHYT (1.1e-4)	2055
03555	diguanylate cyclase/phosphodiesterase/ ammonium transporter	GGDEF (5.2e-35) EAL (5.5e-59) Ammonium_transp (6.1e-96)	2715
03722	diguanylate cyclase/phosphodiesterase	GGDEF (7.6e-23) EAL (2.7e-57)	1545

Table 3. Inhibition of *V. anguillarum* 90-11-287 by cell-free supernatants of *R. mobilis* F1926 wild type, F1926 pYedQ, and F1926 pYhjH cultures, grown in shaken or static MB for 72 h at 25°C

	Inhibition zone diameter without well diameter [mm] first / second replicate	
Strain	Shaken cultures (200 rpm)	Static cultures
R. mobilis F1926 wild type	0 / 0	11 / 13.5
R. mobilis F1926 pYedQ	0 / 0	11 / 15
R. mobilis F1926 pYhjH	0 / 0	1 / 7



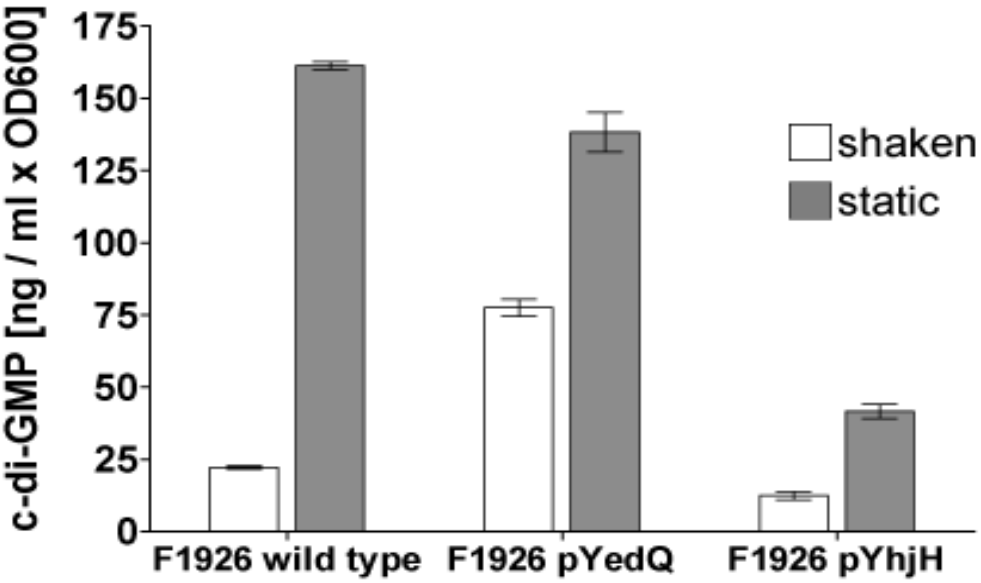
458

459 **Figure 1:** Cyclic di-GMP detection in extracts of *R. mobilis* F1926. Chromatograms of

460 F1926 pYhjH shaken culture spiked with c-di-GMP (top) and F1926 wild type static culture

461 (bottom). The MRM transition monitored is m/z 689.1→149.9.

462



463

464 **Figure 2:** Cyclic di-GMP concentrations in extracts of 24 hours old shaken and static cultures

465 of *R. mobilis* F1926, F1926 pYedQ, and F1926 pYhjH divided by OD600 of the cultures.

466

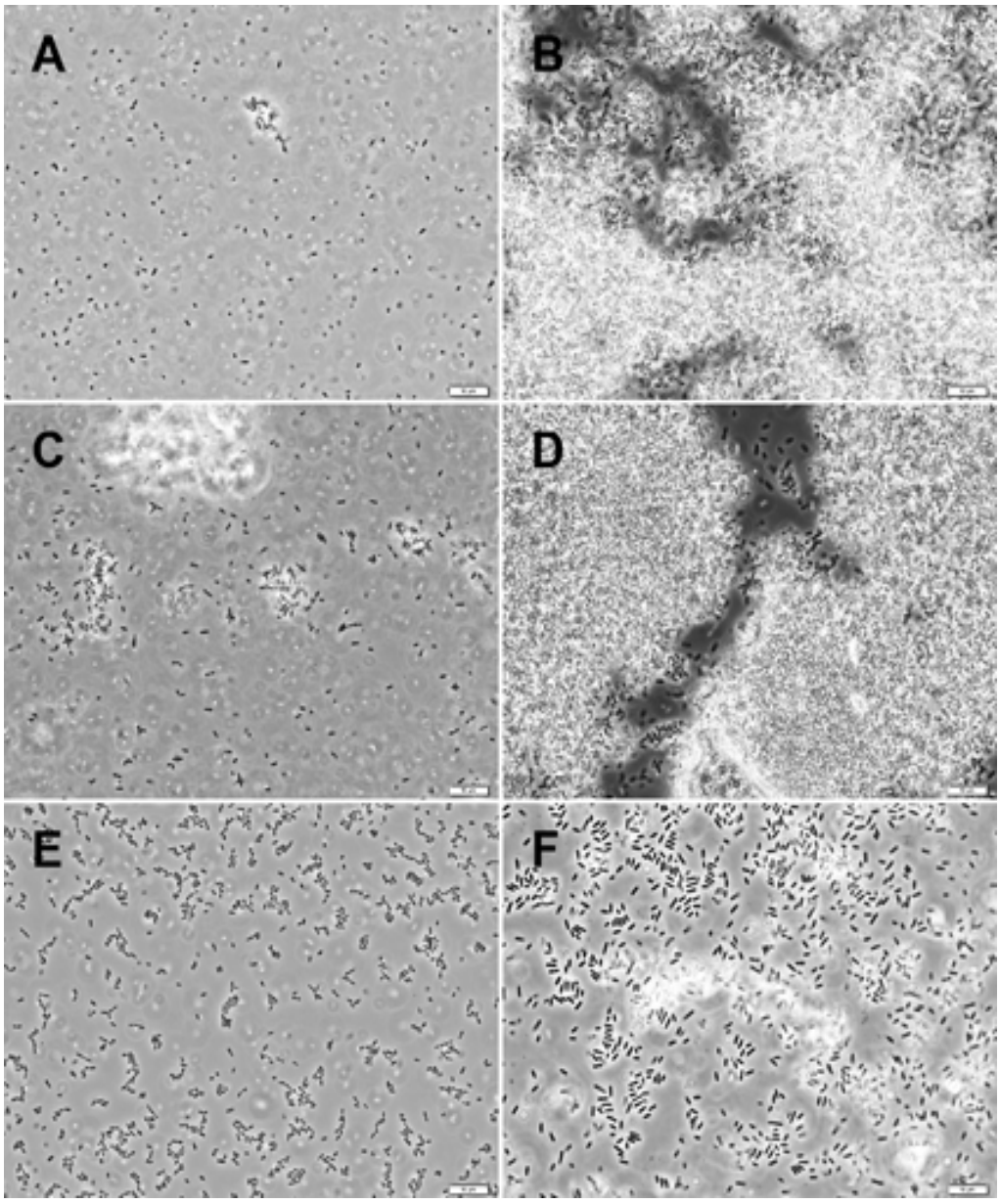


Figure 3: Phase-contrast micrographs of shaken (A, C, E) and static (B, D, F) Marine Broth cultures of *R. mobilis* F1926 wild type (A, B), F1926 pYedQ (C, D), and F1926 pYhjH (E, F).

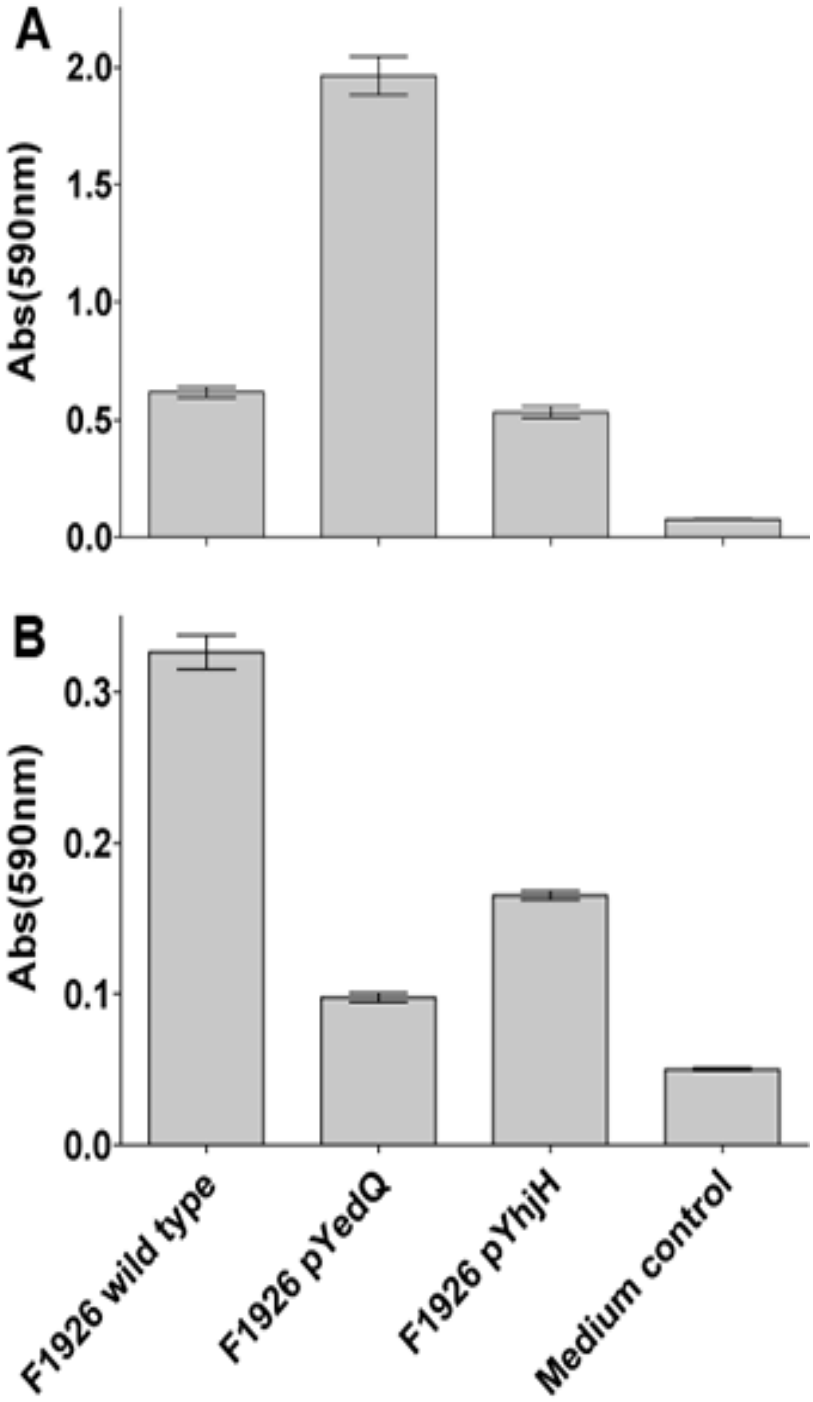


Figure 4: Biofilm formation (A) and attachment (B) of *R. mobilis* F1926, F1926 pYedQ, and F1926 pYhjH, measured in crystal violet assays. Cells from shaken cultures were used in the attachment assay. Sterile medium (½YTSS) was used as control.

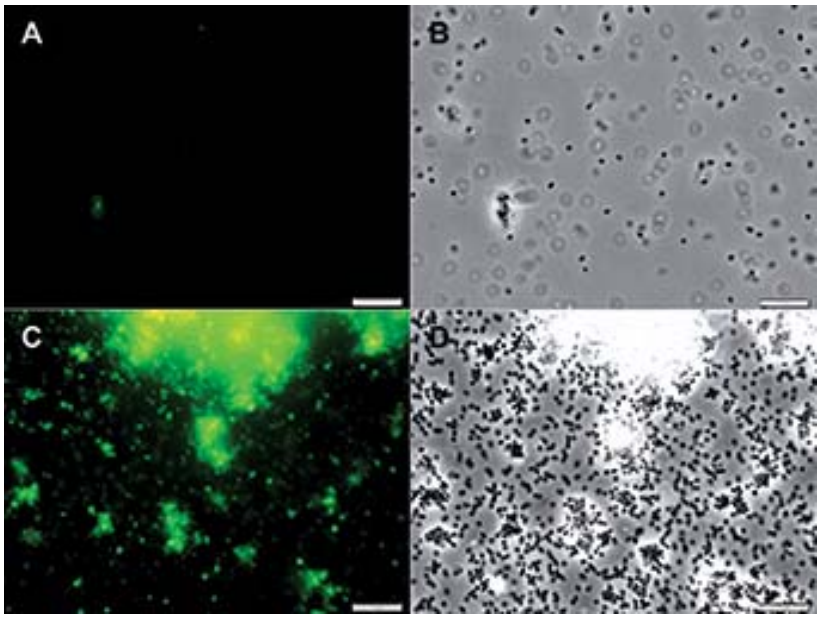


Figure 5 (legend: next page)

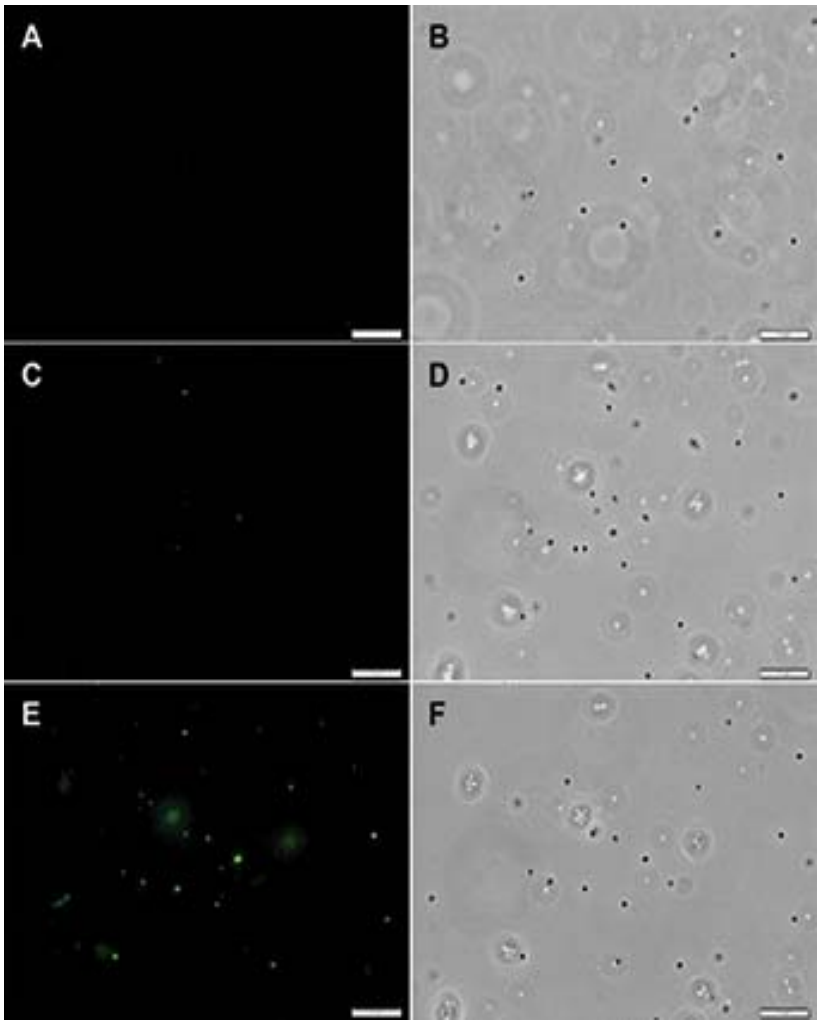


Figure 6 (legend next page)

Figure 5: Expression of *tda*C in *R. mobilis* F1926 grown in shaken (A, B) and static (C, D) MB cultures, monitored by a plasmid-bound reporter fusion of the *tda*C-promoter with a promoterless *gfp*-gene (pPDA11). Fluorescence (A, C) and phase contrast (B, D) micrographs were each recorded using the same settings.

Figure 6: Time series of *tda*C expression in newly attached *R. mobilis* F1926 pPDA11. The images were recorded 1 minute (A, B), 10 minutes (B, C) and 20 minutes (E, F) after preparing the specimen. Fluorescence (A, C, E) and phase contrast (B, D, F) micrographs of the same area were each recorded using the same settings. The focal plane was set right beneath the cover slip to record attached cells. White arrows indicate the position of the same cell in all images.

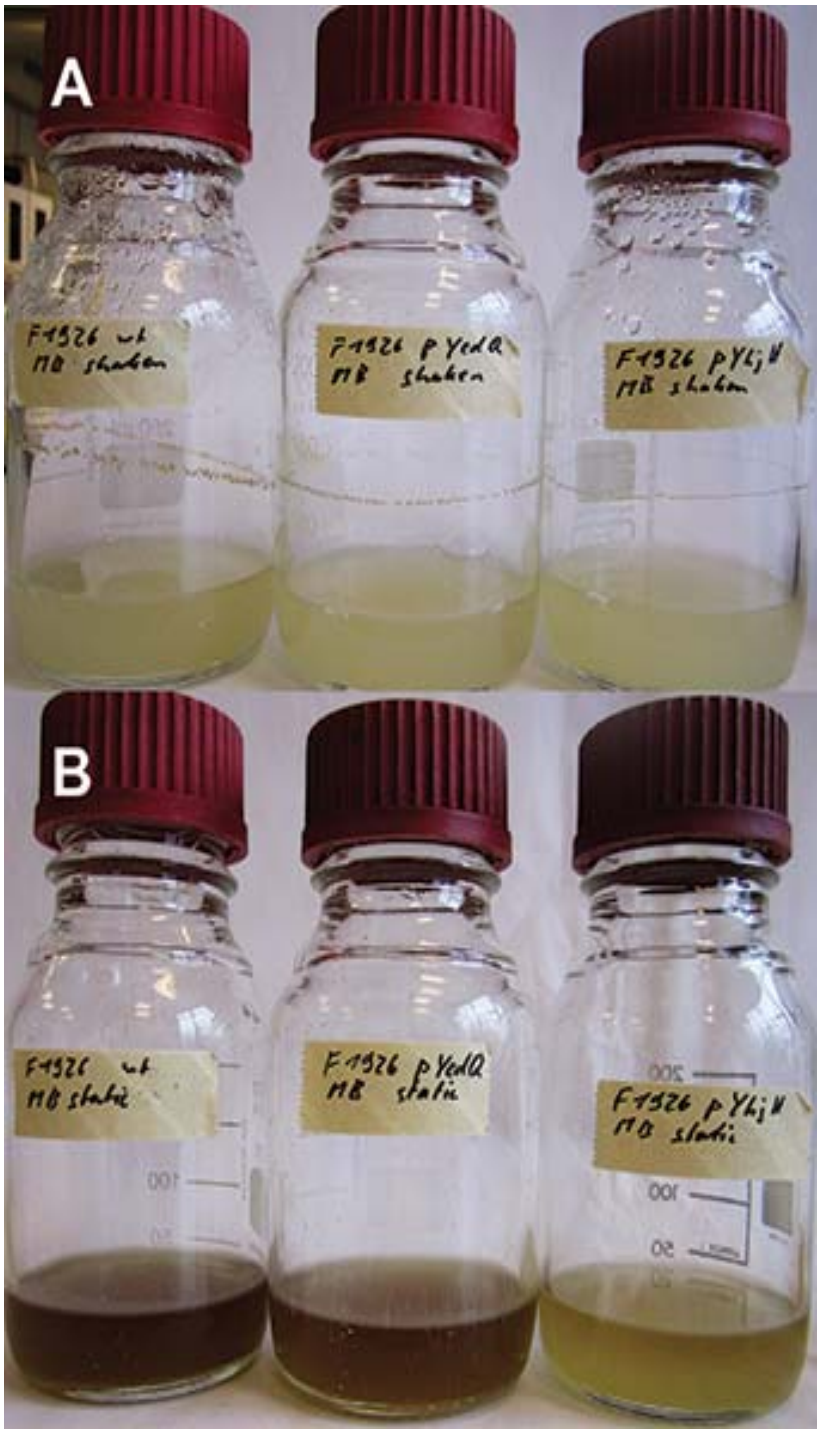


Figure S1: Shaken (A) and static (B) cultures of *R. mobilis* F1926 (left), F1926 pYedQ (middle), and F1926 pYhjH (right) grown in Marine Broth for 3 days at 25°C. Formation of the brown pigment is indicative of TDA production.

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Paper 4

D'Alvise PW, Phipps R, Nielsen KF, Gram L (2013)

Iron is essential for bioactivity and pigmentation of *Phaeobacter gallaeciensis* cultures.

Manuscript in preparation

Iron is essential for bioactivity and pigmentation of *Phaeobacter gallaeciensis* cultures

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Abstract

Tropodithietic acid (TDA) is the antibacterial compound produced by members of the genera *Phaeobacter* and *Ruegeria* of the *Roseobacter* clade. Aeration, biofilm formation and quorum sensing signals influences TDA production, however, little is known about how nutritional factors influence TDA production, and the purpose of this study was to determine how TDA production is influenced by substrate components. High concentrations of ferric citrate, as present in Marine Broth, or other iron-containing compounds were required for TDA formation, as assessed by inhibition of *Vibrio anguillarum* and by chemical detection of TDA. When *P. gallaeciensis* DSM17395 was grown in tryptone, yeast and sea salt-medium without iron addition, no inhibition was measured in the bioassay. However, if supernatants of these cultures were acidified, high concentrations of TDA were detected. We hypothesize that this is due to production a non-inhibitory, TDA-like compound (pre-TDA) that is produced at low iron concentrations and is converted to TDA at low pH. The production of TDA in presence of iron coincides with formation of a brown pigment, and a similar brown substance precipitated when pre-TDA was converted to TDA by acid addition. Elemental analysis of the brown precipitate showed elevated sulfur and iron contents. Pure TDA interacted with ferric iron, and a brown precipitate was produced. These findings suggest that the brown pigment may be a product of TDA and iron. Our results raise questions about how TDA is produced in natural marine settings where iron is typically limited, and whether the affinity of TDA to iron points to a different physiological or ecological function than as an antibacterial compound.

262 words

Introduction

Tropodithietic acid (TDA) is the antibacterial compound produced by most members of the genera *Ruegeria* and *Phaeobacter* of the *Roseobacter* clade (*α-Proteobacteria*) [1,2]. A number of studies have focused on TDA-producing bacteria, especially as colonizers and symbionts of micro- and macroalgae [3–7], and as potential probiotics for marine aquaculture [8–12]. Thiotropocin, a structural analog of tropodithietic acid, was discovered as the antibiotic produced by a *Pseudomonas* sp. isolate from a Japanese soil sample [13,14]. A computational study has revealed that thiotropocin and tropodithietic acid exist as a pair of interconverting tautomers [15]. To simplify matters, in the present study we refer to both compounds as TDA. The complete biosynthetic pathway of TDA is not known, however biosynthesis of TDA from glucose was investigated in a *Pseudomonas* sp. and shikimate and phenylpyruvate were identified as intermediate compounds [16]. Twelve genes that are necessary for TDA production were found by random transposon mutagenesis in a *Ruegeria* (*Silicibacter*) sp. [4], however the lower steps in the biosynthesis pathway of TDA and direct precursors of the compound remain unknown. Appearance of a brown, non-bioactive pigment coincides with TDA production in Marine Broth or on Marine Agar, and the pigment is never produced in mutants deficient in TDA production [17].

Since TDA is a key component of the antibacterial and probiotic effect of *P. gallaeciensis* [11], understanding how to facilitate TDA production would be of great applied interest. The influence of environmental factors on TDA production and genetic regulation of TDA production is only partly elucidated. Culture conditions, such as shaking, influence TDA production in many *Phaeobacter* and *Ruegeria* spp. and also quorum sensing signals increase TDA production [17–19]. In a recent study we showed that roseobacters utilize c-di-GMP signaling to decide between motile and sessile life, and that TDA production is associated

with the sessile life stage and probably requires high intracellular c-di-GMP concentrations [20].

We have observed that antibacterial activity of cell-free supernatants from *P. gallaeciensis* cultures strongly depended on the growth medium. Supernatants from cultures grown in Marine Broth (MB, Difco 2216) always caused pronounced inhibition of a target bacterium, while supernatants from less complex culture media lacked antibacterial activity and pigmentation. This led us to hypothesize that one or more components of MB were critical for TDA formation. Consequently, the initial purpose of this study was to investigate which of the non-essential MB components promoted TDA production. Measuring TDA production in different culture media both by chromatography-MS and, as a quick and easy approximation, in an agar diffusion-inhibition assay, we noticed that the results of chemical TDA quantification did not always agree with inhibitory activity and pigmentation. While investigating this effect, we found that *P. gallaeciensis* and other TDA-producing roseobacters are capable of producing a non-inhibitory compound, which can be converted to TDA by lowering pH. Thus, studying this precursor or structural analog of TDA became the second motive of this study.

Material and Methods

Strains and Media. *Phaeobacter gallaeciensis* DSM 17395 (BS107) from -80°C freeze stocks was cultured by streaking on Marine Agar (MA, Difco 2216), and subsequent inoculation into Marine Broth (MB, Difco 2216). For testing which components of MB are essential for TDA production, a range of media based on the two basic recipes ½YTSS (2 g/l yeast extract, 1.25 g/l tryptone and 20 g/l Sigma sea salts [21]) and IO+ (4 g/l D-glucose, 3.5 g/l casamino acids and 30 g/l sea salts [10]) was prepared. The additional components of MB

(0.1 g/l ferric citrate, 22 mg/l boric acid, 4 mg/l sodium silicate, 2.4 mg/l sodium fluoride, 1.6 mg/l ammonium nitrate, and 8 mg/l disodium phosphate) were added to different batches of the media, leaving out one of the components at a time. *P. gallaeciensis* was cultured in ½YTSS medium with and without addition of 0.5mM ferric chloride to assess the effects of iron on TDA formation. *Ruegeria mobilis* F1926 [22] and *Phaeobacter* so. 27-4 [8], as well as the TDA-deficient mutants *P. gallaeciensis* BS107-Pda8 [11], BS107-MJG-G6 (*tda*B::EZ-Tn5) [23], and *Phaeobacter* 27-4-JBB1001 [4] were cultured under the same conditions. Since we hypothesized (see result section) that the *cysI* gene was key in the production of a TDA-precursor, a *cys*I mutant of *P. gallaeciensis* DSM 17395 (BS107), strain WP45 [24], was obtained and cultured in ½YTSS to test for formation of pre-TDA. The reporter plasmid pHG1011 ([18], *tda*C::*lac*Z, courtesy of H. Geng) was electroporated into *P. gallaeciensis* DSM 17395, and expression of the *lac*Z gene was detected on ½YTSS containing 50 µg/ml XGal. *Vibrio anguillarum* NB10, which was used as target bacterium in an inhibition assay, was cultured in MB [25]. Culture volumes were 20 ml and all incubations were carried out at 25°C. *P. gallaeciensis* was incubated for 72 h before analysis and *V. anguillarum* was grown for one day (18-24h).

Preparation of supernatants and acidification. Cell-free supernatants were prepared from cultures of *P. gallaeciensis* by centrifugation (5,000 x *g*) and filtered through Minisart 0.2µm syringe filters (Sartorius, Göttingen, Germany). To convert pre-TDA contained in supernatants from ½YTSS and IO+ cultures, hydrochloric acid was added to a final concentration of 30 mN. For inhibition testing 100 µl supernatant were acidified by adding 10 µl of a 0.3 N HCl solution, and for larger assays 150 µl 2 N HCl was added to 10 ml supernatant. Controls in the inhibition assay included sterile medium acidified with the same amount of HCl.

Inhibition assay. Inhibition of *Vibrio anguillarum* NB10 by cell-free culture supernatants was tested in an agar-diffusion assay as described in [26].

Chemical TDA detection. For chemical measurements of TDA, *P. gallaeciensis* cultures were extracted with ethyl acetate containing 1% formic acid, the organic phase was evaporated to dryness and redissolved in 85% acetonitrile. The extracts were analyzed by UHPLC-DAD-TOFMS on a maXis G3 quadrupole time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) ion source which was connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA), as described in [11].

Attempts to extract pre-TDA. *P. gallaeciensis* was grown in 1 liter ½YTSS with a high surface/volume ratio for 3 days. Supernatants were prepared as described above, and extraction of pre-TDA was attempted with organic solvents, 2-butanol, ethyl acetate, and QuEChERS (acetonitrile and salts to make a 2-phase system). Further attempts were made with solid phase extraction on amino columns (HILIC mode, formate elution, elution at high pH using NH_4OH water), cyanopropyl, diol, HyperCarb graphite, Strata X, and Sephadex G10 columns. Normal phase SPE was not possible, since the compound was not soluble in dry organic solvents.

Elemental analysis. Precipitates from 160 ml (8 x 20 ml) acidified ½YTSS supernatants of *P. gallaeciensis* DSM 17395 wild type and mutant MJG-G6 were dried, dry weights were recorded, and the samples were analysed for carbon, hydrogen, nitrogen, sulphur, oxygen, and iron contents by DB Lab A/S (Odense, Denmark).

Iron-scavenging activity of TDA. Sterile filtered supernatants of DSM 17395 and F1926 grown in MB, ½YTSS and IO+ without iron addition, as well as pure TDA in concentrations of 1 mM, 100 µM and 10 µM were mixed 1:1 with the chrome-azurol-S-scanning solution

[27]. *Pseudomonas aeruginosa* PAO1 grown in IO+ with only 1% sea salts served as a positive control. A colour change from blue to orange indicated iron chelating activity.

Results

Influence of substrate components on TDA production. The non-essential components of MB were tested for their influence on TDA production. The two marine media IO+ and ½YTSS were used as basic recipes and the additional compounds of MB were included, leaving out one at a time. TDA production by *Phaeobacter gallaeciensis* DSM 17395 was tested in variants of these media, in each of which one of the additional MB components was missing. Brown pigmentation and inhibitory activity of the supernatant against *Vibrio anguillarum* served as indicators for TDA production. When ferric citrate was not added, the cultures lacked brown pigmentation (Fig. 1 and no antibacterial activity of the supernatants was detected (Fig. 2). Both pigment and inhibitory activity were formed when different iron-containing compounds were contained in the medium, such as ferric chloride, ferrous sulfate, Fe(III)-EDTA, and elemental iron powder, all of which likely form ferric hydroxide in seawater [28]. Unnaturally high concentrations of iron (>50µM) were needed to yield the same extent of antibacterial activity as in MB. 408 µM iron (0.1g/l ferric citrate) is contained in MB, while iron concentrations in natural seawater are in the range of pM to nM [28–30]. We tested whether the antibacterial activity of the supernatant from a low-iron culture could be restored in absence of cells by adding iron. Addition of ferric chloride solution did indeed restore the inhibitory activity of the supernatant. However, addition of other iron-salts did not restore inhibitory activity and we found later that the activity-restoring effect of ferric chloride was not due to iron, but due to the acidity of the solution.

Production of a non-inhibitory precursor of TDA. When supernatants from cultures without iron addition, which were neither pigmented nor inhibitory, were extracted with ethyl acetate containing 1% formic acid, substantial amounts of TDA were detected by UHPLC-DAD-TOFMS and UHPLC-DAD. Accordingly, inhibitory activity of the supernatants was restored by acid addition, e.g. hydrochloric acid (Fig. 2), while elemental iron powder, Fe(III)-EDTA, hydrogen peroxide, or proteinase K were not effective. This indicated a possible explanation for the high TDA values measured in the extracts of the non-inhibitory supernatants from low-iron cultures: a non-inhibitory compound that was produced in the low-iron cultures was converted to TDA in presence of the formic acid contained in the extracting agent.

The non-inhibitory analog or precursor of TDA was produced in all tested growth media without iron addition. For lack of structural information, we named the yellow, diffusible compound “pre-TDA”, which has the same absorption maximum of visible light as TDA (385nm). It is, apart from presence of iron, produced under the same conditions as TDA, i.e. its production is strongest in static liquid cultures, and in *Ruegeria mobilis* F1926 and *Phaeobacter* sp. 27-4 the compound is not produced in shaken cultures.

Mutants deficient in TDA production did not form pre-TDA. We hypothesized that the *cys*I gene would be responsible for the conversion of pre-TDA to TDA (see discussion), however also the *cys*I-mutant *P. gallaeciensis* DSM 17395 WP45 [24] did not produce pre-TDA.

Chemical characteristics of pre-TDA. Aiming to elucidate the structure of pre-TDA, we attempted to extract the compound. However this was problematic, since extracting agents and mobile phases for good retention of TDA need formic, phosphoric or trifluoroacetic acid, which would convert pre-TDA to TDA. Concentration and purification by extraction with organic solvents, 2-butanol, ethyl acetate, and acetonitrile was not successful, as well as Solid

Phase extraction on amino columns, cyanopropyl, diol, HyperCarb graphite, Strata X, and Sephadex G10 columns. The only partially successful step was to precipitate the active compound at neutral pH with isopropanol, however this did not work consistently.

Induction of the *tda*C promoter by pre-TDA. Despite its lack of inhibitory activity, pre-TDA was able to induce the *Ruegeria (Silicibacter)* sp. TM1040 *tda*C promoter (pHG1011 *tda*Cp::*lac*Z), which was shown to require TDA for induction [18]. *P. gallaeciensis* DSM 17395 carrying pHG1011 expressed *lac*Z from the *tda*C promoter during growth in ½YTSS without iron addition (Fig. 3). This suggested structural resemblance of pre-TDA to TDA.

Clues on the brown pigment coinciding with TDA production. When a pre-TDA-containing supernatant from a ½YTSS culture without iron addition was acidified, a brown precipitate was formed. The precipitate was removed by centrifugation (Fig. 4), redissolved in dilute NH_4OH solution, and tested for antibacterial activity at different pH levels. No antibacterial activity was found, as compared to NH_4OH water of the same pH (data not shown). In the supernatant of a TDA-negative mutant that was used as control, a smaller amount of a white precipitate were formed by acidification (Fig. 4). Elemental analysis of both precipitates (Tab.1) revealed significantly higher relative contents of iron (667ppm vs. 29ppm) and sulfur (2.7% vs. 0.4%) in the brown precipitates.

Adding $Fe(III)Cl_3$ in excess (10mM) to a solution of pure TDA (1mM in 12.5% DMSO) resulted in a color change from yellow to deep red (Fig. 5 A and B). Chemical analysis of the TDA solutions showed a decrease in TDA concentration in response to ferric chloride addition. When subsequently pH was raised by adding ammonia water, the red putative TDA-iron complex was resolved, the colour of the solution reverted to yellow, and a high TDA-specific peak reappeared in the chemical analysis (Fig. 6). After prolonged incubation the red iron-TDA mixture turned brown, and a brown precipitate was formed (Fig. 5 C), which

resembled the pigment that coincides with TDA production in iron-rich media. However inconclusive, these observations suggest a disposition of TDA to react with Fe(III) ions. An iron-TDA complex may be formed. As a methodological note, we observed that for TDA analysis on C_{18} and Phenyl columns (Phenomenex Kinetex Luna as well as Waters BEH columns) need to be new (<1-2 weeks), since extensive tailing with peak widths up to 1 minute was observed on older columns, even with fresh pre-columns and even though other acidic analytes (e.g. fumonisins, ochratoxins) gave perfectly sharp peaks. Again, iron (and other metal ions) deposited in the head of the columns could be the cause of this problem, owing to TDA- metal interactions.

CAS assay. Pure TDA showed iron-chelating activity, however this could not be detected after 10 minutes like in the positive control, but only after prolonged incubation (16-24h). A positive reaction was only observed for the highest TDA concentration tested (1mM).

Discussion

The present study demonstrates that *P. gallaeciensis* requires iron to produce antibacterial active TDA, while in absence of iron a non-antagonistic TDA-like compound is produced, which can be converted to TDA in presence of acid. As mentioned, iron concentrations in seawater are much lower than what is required for TDA production in pure cultures. This raises the question whether under natural settings TDA or pre-TDA is produced. Either the requirement for iron is relative to the local density of the bacteria, meaning that TDA can be produced at low iron concentrations by a sparse population of roseobacters, or the concentration requirements are absolute, which would mean that TDA is probably never produced in natural seawater in the way it is known from pure cultures grown in MB. However, pronounced antibacterial effects of *Phaeobacter* sp. were observed in artificial

seawater, indicating TDA production [10]. Pre-TDA may be converted to TDA in a natural process that is not yet understood, possibly while it exerts a yet undiscovered function.

Geng *et al*. [4] described random transposon insertion mutants of *Ruegeria* sp. TM1040 deficient in TDA production, as detected by lack of inhibition of *V. anguillarum*. One of the non-inhibitory mutants was a *cys*I-mutant, which in a later study was shown to express the *tda*C gene that is normally induced by TDA [18]. To address this contradiction, culture supernatant of the *cys*I-mutant was extracted using hydrochloric acid, and TDA could be detected by HPLC. This pattern of lacking inhibition combined with induction of the *tda*C promoter and TDA being detected after acid treatment resembled our present observation and could possibly be explained with pre-TDA being formed by the *cys*I-mutant strain. Thus, we pursued the hypothesis that the *cys*I gene may not only be involved in cysteine synthesis but may also account for the conversion of pre-TDA to TDA, and that a *cys*I-mutant would be deficient in the last step of TDA biosynthesis, presumably the conversion of pre-TDA to TDA. Encouraging this hypothesis, introduction and modification of the two sulfur atoms into the TDA molecule is a late event in TDA biosynthesis [16], and the *Cys*I enzyme is a sulfite reductase that contains a siroheme and an S_4Fe_4 cluster [31], which could possibly be inactivated by lack of iron. However, our experiments showed that the *cys*I-mutant *P. gallaeciensis* WP45 did not form pre-TDA and thus failed to support this hypothesis.

When pre-TDA is converted to TDA by adding acid to cell-free supernatants, a brown precipitate is formed. The color of brown pigment reminds of melanin compounds that, although having specific biosynthetic steps, are polymers of compounds with phenolic and indolic structures [32]. The tropolene ring of TDA and the acid group could possible facilitate a polymerization. It cannot be ascertained from the present results that the precipitate formed after acid addition is identical with the pigment in iron-rich cultures, however the observation

indicates that the brown pigment correlated with TDA production might be a by-product of the conversion from pre-TDA to TDA. Increased sulfur contents of this precipitate suggest that it might originate from TDA. Together with its elevated iron content this suggests that the brown precipitate might be derived from freshly formed TDA and dissolved iron, which is contained in the medium in low amounts. A similar brown precipitate was formed from pure TDA and ferric chloride after prolonged incubation, which also points to TDA-iron interactions as source of the brown pigment. What role does iron play in the conversion from pre-TDA to TDA? It could be speculated that under standard culture conditions pre-TDA would form an iron complex, from which TDA may be liberated as more pre-TDA is produced, thus iron would act as a catalyst facilitating pre-TDA conversion at neutral pH. Since pre-TDA does not have the same mass as TDA, a part of the molecule might be removed by the iron binding reaction, which might also be achieved by acid addition.

The affinity of TDA to iron may also indicate an ecological role that is not yet understood. The iron-mobilizing effect that was observed in the CAS assay indicates that TDA might act as a siderophore. Yet, the iron-binding reaction seemed to be quite slow, while siderophores typically have very high iron affinities. Beyond that, siderophores are normally produced under iron-starved conditions, while TDA is produced in presence of iron. However, if the brown pigment that is precipitated in the extracellular matrix of the bacteria contains iron, this could represent a pool of iron that can possibly be utilized by the bacteria or the symbiotic algae.

The results of this study indicate that TDA and its presumed precursor are closely connected to iron and the brown pigment, however no definite conclusions can yet be drawn and further chemical analysis of pre-TDA and the brown pigment will be needed to elucidate their identity.

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Table and figures

Table 1: Elemental analysis of precipitates from acidified supernatants

	Brown precipitate from DSM17395 wild type	White precipitate from DSM17395 tdaB::EZ-Tn5
C	27.1 %	22.5 %
H	3.42 %	4.43 %
N	6.85 %	6.01 %
O	23.6 %	25.6 %
S	2.68 %	0.41 %
Fe	667 ppm	29 ppm

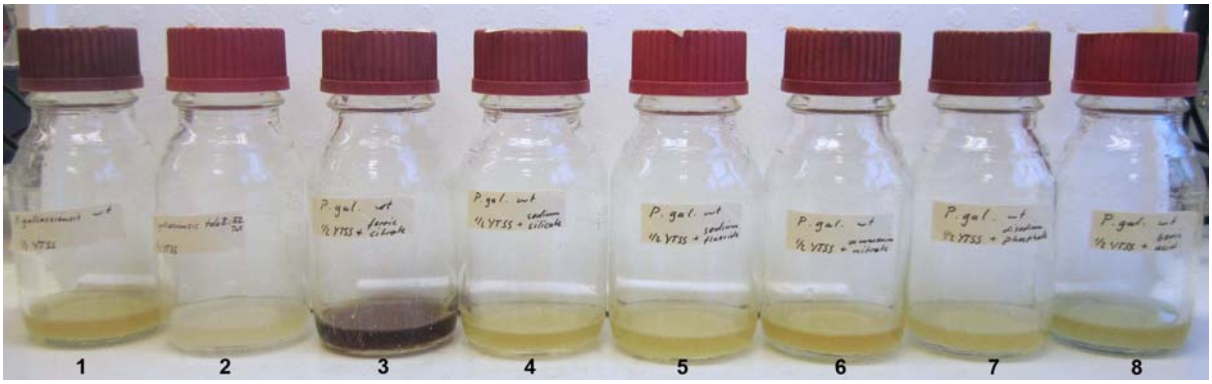

Figure 1: Pigmentation of static liquid *Phaeobacter gallaeciensis* DSM 17395 (BS107) cultures.
1: wild type in ½YTSS;
2: *tda*B mutant in ½YTSS;
3: wild type in ½YTSS with 0.1g/l ferric chloride;
4: wild type in ½YTSS with 4mg/l sodium silicate;
5: wild type in ½YTSS with 2.4 mg/l sodium fluoride;
6: wild type in ½YTSS with 1.6 mg/l ammonium nitrate;
7: wild type in ½YTSS with 8 mg/l disodium phosphate;
8: wild type in ½YTSS with 22 mg/l boric acid

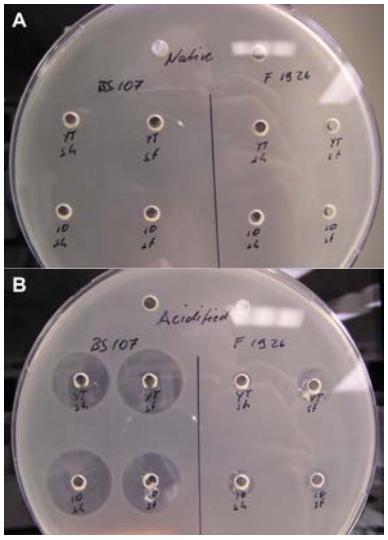

Figure 2: Supernatants from shaken (sh) and static (st) ½YTSS (YT) and IO-medium (IO) cultures of Phaeobacter gallaeciensis DSM 17395 (BS107) and Ruegeria mobilis F1926, tested as native supernatants (A) and with acid addition (B).

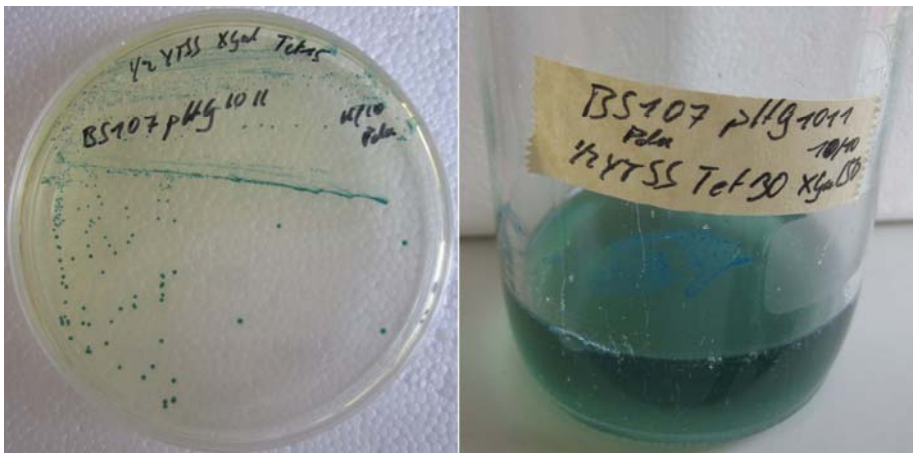

Figure 3: Induction of the *tda*C promoter of pHG1011 in *P. gallaeciensis* DSM 17395 (BS107) in relative absence of iron. The diffusible yellow substance around the colonies is pre-TDA.

Figure 4: Supernatants from *P. gallaeciensis* DSM 17395 (BS107) wild type (left) and the TDA-deficient mutant DSM 17395 MJG-G6 (right) were acidified and centrifuged. A brown precipitate was formed in the wild type supernatant, while the small precipitate formed in the mutant supernatant was white.

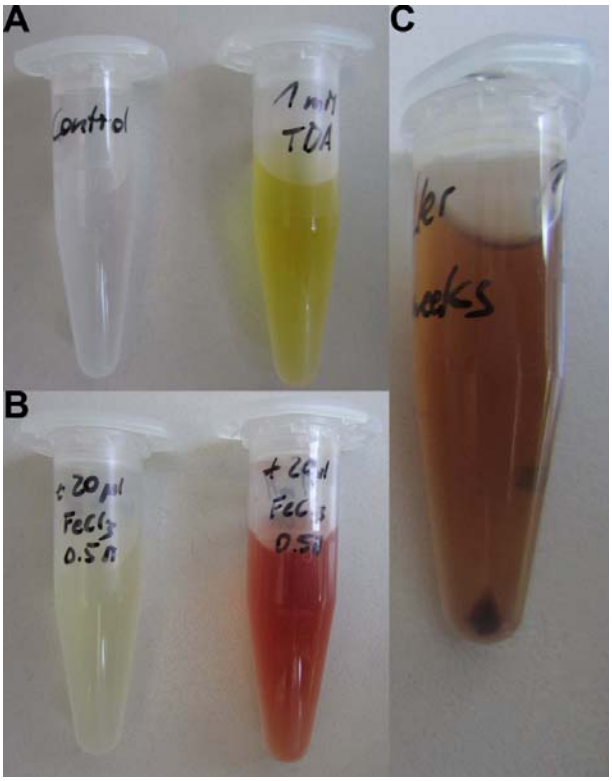

Figure 5: 1 mM TDA and a water control (A) were added 10mM ferric chloride (B). After 4 weeks the TDA and ferric chloride solution had turned brown and a brown precipitate could be removed by centrifugation (C).

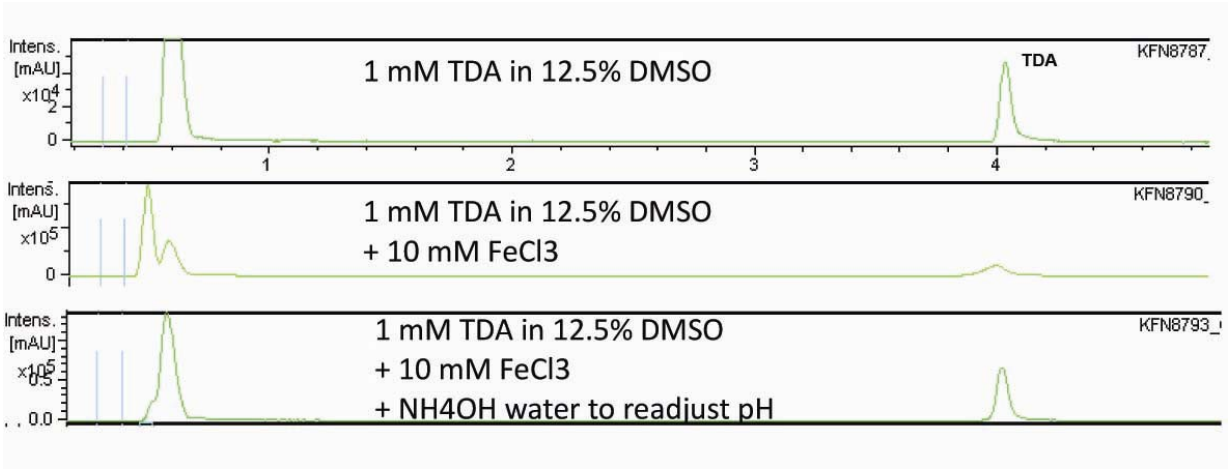

Figure 6: TDA-detection by UHPLC-MS in the TDA-containing solutions from Figure 4B, and in the red TDA-containing solution from figure 4B after the pH was readjusted to neutral by adding ammonia-water.

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