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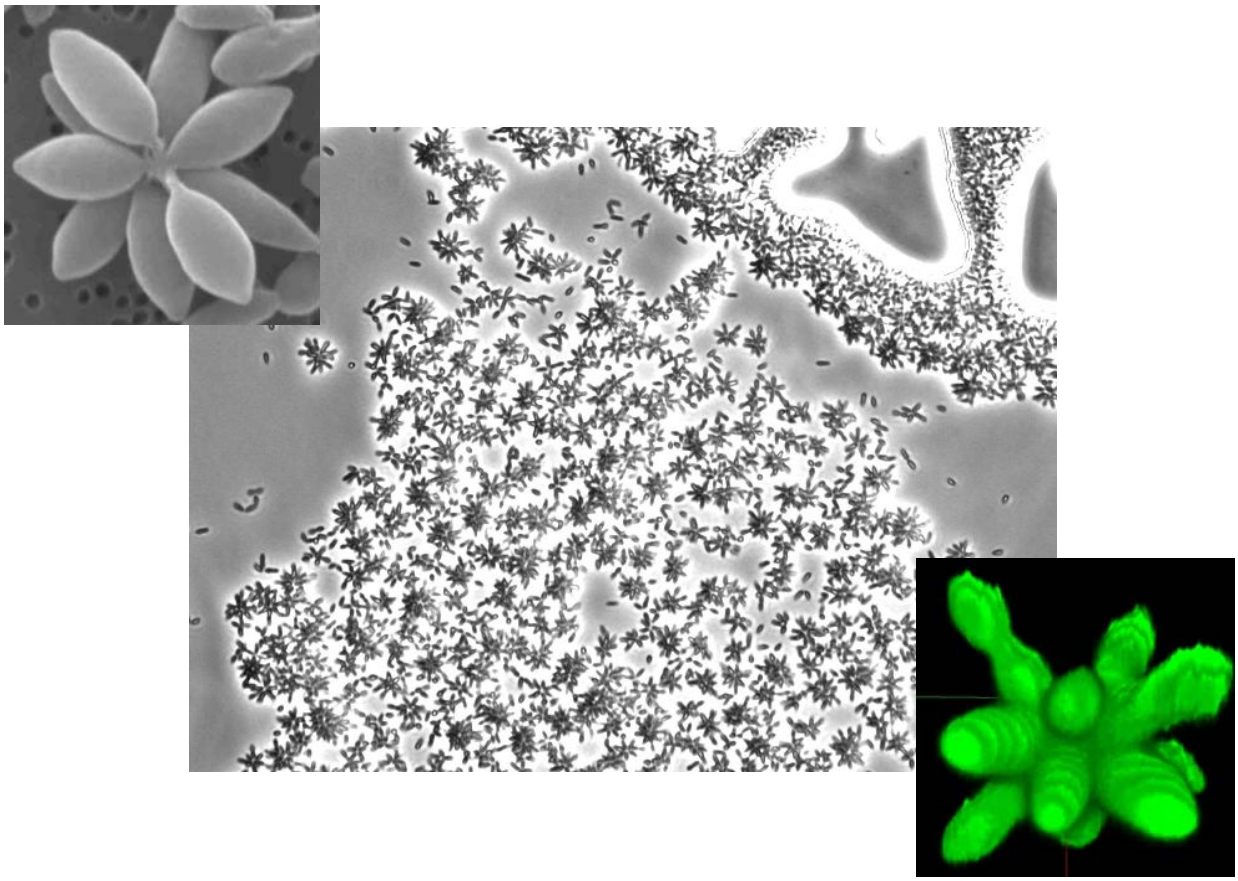
Ecology, antibacterial activity and biofilm formation of marine *Roseobacter* strains



Jesper Bartholin Bruhn
Department of Fisheries Research
Danish Institute for Fisheries Research, 2006

Cover Illustrations

Upper left: SEM picture of *Roseobacter* 27-4 grown under static condition. Picture taken by Michael Hansen and José Bresciani.

Lower right: CLSM picture of *Roseobacter* 27-4 attached to glass. Picture taken by Janus A. J. Haagensen.

Centre: phase contrast picture of *Roseobacter* strain 27-4 grown under static. Picture taken by Jesper Bartholin Bruhn.

Preface

The work presented in this thesis was carried out at the Dansih Institute for Fisheries Research (DIFRES), Department of Seafood Research in Kgs. Lyngby, Denmark under supervision of Professor Lone Gram (DIFRES). I was enrolled at The Royal Veterinary and Agricultural University.

This work was conducted in connection with the research network SCOFDA (Sustainable Control of Fish Diseases in Aquaculture) supported by the Danish Agricultural and Veterinary Research Council and the Danish Ministry of Food, Agriculture and Fisheries.

The work has been described in 4 papers, which are included in this thesis.

Jesper Bartholin Bruhn
December 2006

Abstract

The dramatic growth in the aquaculture sector has emphasized the importance of fish disease control. Due to the risk of development and transfer of antibiotic resistance alternative disease control measures to use of antibiotics must be implemented. The use of probiotics, which are live microorganisms that confer a health benefit to the host, may be an alternative to antibiotics in some situations. Mostly, potential probiotic bacteria have been selected due to their ability to antagonise pathogenic microorganisms, however, the mechanism by which they confer a health benefit are not known. Most studies rely on in vitro data and in vivo data, such as challenge trials, are scarce.

Several marine bacteria are capable of inhibiting other bacteria and some marine bacteria appear promising as probiotics in aquaculture. *Roseobacter* strain 27-4 a member of the *Roseobacter* clade, is a probiotic candidate. *Roseobacter* 27-4 was selected due to its strong anti *Vibrio* activity and it is capable of reducing mortality in turbot larvae infected with *Vibrio anguillarum*. It is hypothesized that the *Roseobacter* antibacterial activity, is an important mechanism underlying its probiotic effect.

The purpose of this thesis has been to investigate factors influencing *Roseobacter* 27-4 production of antibacterial compounds and to elucidate the structure, regulation and biosynthesis of the active compound.

The antibacterial activity of *Roseobacter* 27-4 was due to production of a sulphur containing compound, tropodithietic acid (TDA). TDA was also found in supernatants from *Silicibacter* TM1040, also a member of the *Roseobacter* clade. *V. anguillarum* did not become resistant to TDA even when continuously exposed to sublethal doses. Hence, production of TDA may have a persistent effect against this fish pathogen.

Static growth conditions enhanced TDA production and favoured a thick biofilm of rosette shaped aggregated for both *Roseobacter* 27-4 and *Silicibacter* TM1040. It is suggested that rosette/biofilm formation and production of TDA are connected phenotypes in both bacteria. The mechanisms or reason behind *Roseobacter* rosette formation is not known, but it is suggested that the rosette formation, may be a way for the bacteria to avoid grazing by protozoan. This defence can be enhanced by biofilm formation and production of TDA, as TDA also act as an antiprotozoan compound.

In this thesis it is hypothesised that colonization of surfaces can enhance the antibacterial effect of *Roseobacter* 27-4 and hence, create a stable long-term beneficial microbiota in

aquaculture environments. A novel way of quantifying both the probiotic and pathogenic bacteria during attachment is presented by using Real time PCR. Such a method will be useful in evaluating the effect of probiotic bacteria during colonization of surfaces.

All bacterial strains tested were sensitive to TDA, but non-*Roseobacter* strains were in general more sensitive to TDA as compared to members of the *Roseobacter* clade. Hence, production of TDA may give *Roseobacter* a selective advantage during interaction with other bacteria. Random transposon genomic mutagenesis was use to suggest a biosynthesis pathway for TDA. Several genes believed to be involved in the final formation of TDA are located on a plasmid, which may explain the loss of TDA production in spontaneously TDA negative mutants, as loss of the plasmid. TDA is a sulphur containing compound and some roseobacters metabolise the algal sulphur compound dimethylsulfoniopropionate (DMSP) and this metabolism has been linked to the global climate. The thesis hypothesised that TDA could be produced with DMSP as sulphur substrate but could not confirm this. Cysteine and sulphate was suitable substrates for TDA synthesis, whereas methonine could not be metabolised to TDA.

Several *Roseobacter* strains isolated from algae produced antibacterial compounds and some strains also formed rosette shaped aggregates, which improved their attachment capability. In the present thesis it is suggested that members of the *Roseobacter* clade may become dominant among algal associated bacterial, due to their production of antibacterial compound and high attachment capability.

The thesis concludes that the production of TDA by members of the *Roseobacter* clade is highly influenced by growth conditions. Enhanced production of TDA might improve the health of the aquatic organisms in aquaculture as these are unaffected by TDA whereas fish pathogenic bacteria are sensitive. Hence to facilitate and optimize the use of *Roseobacter* strains as probiotic bacteria, new ways of adding the bacteria which results in an enhanced TDA production will be an advantage. Further studies on the marine ecology of *Roseobacter* in relation to production of antibacterial compounds, will be of great interest due to their abundance in the oceanic environment.

Resume

Øget fokus på bekæmpelse af fiskesygdomme vil være nødvendigt, hvis den dramatiske stigning i akvakultur skal forsætte. Bakterielle sygdomme i akvakultur kan behandles med antibiotika, men grundet angst for udvikling og overførsel af resistens vil alternative metoder med fordel kunne implementeres. Brug af levende mikroorganismer, kaldet probiotika, som forbedre helbredet hos akvatiske organismer kan være et alternativ til antibiotika i nogle situationer. De fleste potentielle probiotiske bakterier er udvalgt baseret på deres evner til at hæmme fiskepatogene bakterier men det er uvist hvorfor stammerne har en helbredsforbedrende virkning på akvatiske organismer. De fleste undersøgelser er baseret på in vitro data og in vivo data er ofte ikke rapporteret.

Adskillige marine bakterier har evne til at hæmme andre bakterier og nogle af disse er ligeledes lovende probiotiske stammer til akvakultur. *Roseobacter* 27-4 som tilhører *Roseobacter* gruppen, er en af de lovende probiotiske kandidater. *Roseobacter* 27-4 blev udvalgt som probiotisk kandidat grundet dens evne til at hæmme fiskepatogene bakterier, samt at det er vist at den kan reducere dødeligheden hos pighvar larver inficeret med *Vibrio anguillarum*. Det er foreslået at den probiotiske effekt af *Roseobacter* 27-4s er forbundet med dens evne til at hæmme andre bakterier.

Formålet med nærværende opgave er at undersøge faktorer der influerer *Roseobacter* 27-4s produktion af antibakterielle stoffer og beskrive strukturen, reguleringen samt biosynthesen af de aktive antibakterielle stoffer.

Den antibakterielle aktivitet skyldes produktionen af et antibakterielt stof, der er identificeret som tropodithietic cid (TDA). TDA er ligeledes fundet i supernatanter fra *Silicibacter* TM1040, som også tilhører *Roseobacter* gruppen. Langvarig vækst af *V. anguillarum* i ikke dræbende koncentrationen af TDA resulterede ikke i resistens udvikling. Derfor kan TDA måske have en vedvarende effekt mod fiskepatogene bakterier.

I kulturer af *Roseobacter* 27-4 og *Silicibacter* TM1040 øges produktionen af TDA under statiske vækstbetingelser. Under disse vækstforhold dannes også en tynd biofilm af rosetteformede aggregater. Der synes at være en forbindelse mellem rosette/biofilm dannelsen og produktionen af TDA i begge bakterier. Mekanismerne bag og grunden til dannelsen af disse rosetter er ukendt men rosetterne er foreslået at medvirke som en forsvarsmekanisme mod græsning af protozoer. Denne forsvarsmekanisme kan blive forstærket af biofilmdannelse og produktion af TDA, da TDA også har en hæmmende virkning på protozoer.

Overfladekolonisering med probiotiske bakterier som *Roseobacter* 27-4 kan måske skabe en fordelagtig og stabil mikroflora i akvakulturmiljøet og dermed mindske udbrud af bakterielle sygdomme. Til evaluering af en sådan kolonisering er en Real time PCR metode udviklet, således det er muligt at kvantificere både probiotiske og fiskepatogene bakterier.

Bakterier der ikke tilhører *Roseobacter* gruppen er generelt mere følsomme overfor TDA sammenlignet med bakterier som tilhører *Roseobacter* gruppen. Produktion af TDA kan derfor give *Roseobacter* en selektiv fordel under interaktion med andre bakterier. Dannelse af TDA er undersøgt i nærværende opgave vha. transposon mutagenese. Denne analyse viser, at centrale gener i dannelsen af TDA er placeret på et plasmid, og at tab af dette plasmid derfor kan forklare hvorfor spontane TDA negative mutanter blev fundet. Strukturen af TDA indeholder to svovlatomer hvilket er interessant da nogle *Roseobacter* nedbryder det svovlholdige stof dimethylsulfoniopropionate (DMSP). Denne nedbrydning kan påvirke det globale klima og det er blevet foreslået at TDA kan produceres af *Roseobacter* med DMSP som svovlsubstrat. Denne hypotese blev dog ikke bevis i nærværende opgave. TDA kan produceres med cysteine og sulfate som svovlkilde, men ikke med methonine.

Adskillige *Roseobacter* stammer isoleret fra alger producerer antibakterielle stoffer og nogle af disse stammer viste sig ligeledes at have gode fasthæftelsesevner. Disse fænotyper kan være vigtige for *Roseobacter* bakterier i at opnå dominans blandt algeassocierede bakterier.

Det kan konkluderes at produktionen af TDA produceret af medlemmer af *Roseobacter* gruppen er meget påvirket at de fysiske vækstforhold. Forøget produktion af TDA kan måske forbedre overlevelsen af akvatiske organismer i akvakultur, da disse ikke er følsomme overfor TDA hvorimod fiskepatogene bakterier er meget følsomme. Skal brugen af *Roseobacter* stammer optimeres, vil det være en fordel at tilføre disse på en måde så TDA produktionen øges. Yderligere undersøgelser af *Roseobacter* gruppen i forhold til produktion af antibakterielle stoffer vil være af stor interesse, da *Roseobacter* er dominerende i mange forskellige akvatiske miljøer.

Table of Contents

Papers

Paper 1 **Jesper B. Bruhn**, Kristian F. Nielsen, Mette Hjelm, Michael Hansen, José Bresciani, Stephan Schultz and Lone Gram 2005. Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* Clade. Applied and Environmental Microbiology. **71:**7263-7270.

Paper 2 **Jesper B. Bruhn**, Janus A. J. Haagensen, Dorte Bagge-Ravn and Lone Gram 2006. Culture conditions of *Roseobacter* strain 27-4 affect its attachment and biofilm formation quantified by Real-time PCR. Applied and Environmental Microbiology. **72:**3011-3015.

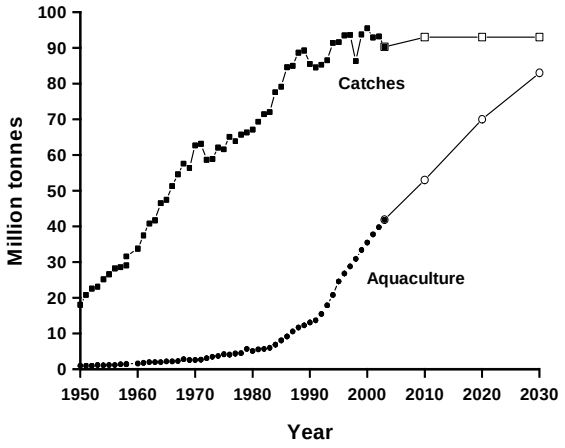
Paper 3 **Jesper B. Bruhn**, Lone Gram and Robert Belas 2007. Production of antibacterial compound and biofilm formation in *Roseobacter* species are influenced by culture conditions. Accepted in Applied and Environmental Microbiology.

Paper 4 Haifeng Geng, **Jesper B. Bruhn**, Kristian F. Nielsen, Lone Gram and Robert Belas 2007. Gene analysis of the regulation and biosynthesis of a sulphur-containing antibiotic by marine roseobacters (In prep).

1 Introduction

Aquaculture is farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants, and is one of the fastest growing food-producing sectors in the world, with an annual growth rate of 8.9% from 1970 to 2000 (FAO 2003) (Figure 1). The annual increase has been 6.1% from 2000 to 2002 and the total world aquaculture production was in 2002 51.4 million tons by quantity and US$60.0 billon by value (FAO 2004). It is estimated that this increase will continue during the next decade (Figure 1), if the aquaculture sector overcomes several challenges (FAO 2004). The production of aquatic organisms is intensive and microbial diseases may spread rapidly within the production sites. Infectious diseases are currently the most devastating problem in shrimp culture and present ongoing threats to other aquaculture sectors.

Figure 1: Production (in million tones of aquatic organisms) of captured or produced in aquaculture from 1950 until today. Estimates for the following decades are included (FAO 2004) (Figure made by Lone Gram).



Fish diseases are caused by different agents e.g. virus or bacteria, and the focus of this thesis will be on bacterial agents. As for terrestrial animals, antibiotics have been and are currently being used for the treatment of bacterial diseases. This use of antibiotics is giving rise to concern, as use of antibiotic in animal production increase the selective pressure exerted on the microbial environment and encourages the natural emergence of bacterial resistance. Resistant fish pathogenic bacteria are a problem in the aquaculture sector (Karunasagar et al., 1994), and possible transfers of resistance genes to human pathogens give rise to additional concern. Several alternative strategies for the use of antibiotics have been proposed and in particular vaccines have already been applied very successfully in aquaculture. Norway increased fish production from approximately 50,000 metric tons to 400,000 metric tons between 1987 and 1997 and decreased the use of antibiotic from 50 metric tons to about 750 kg (Verschuere et al., 2000b). However vaccination cannot be used in all cases. During hatching of fish and in the larvae stage the

size of the fish and slow development of the immune system makes it difficult to use vaccines. Crustaceans and molluscs lack acquired immunity and conventional vaccination cannot be used. For this reason new alternative strategies must be implemented and the use of probiotic organisms in aquaculture is promising. Probiotics are live microorganisms, which confer a health benefit for the host when administrated in adequate amounts (FAO/WHO 2001).

Many microbial strains have been tested as probiotic organisms and several bacterial strains have a disease reducing effect. This thesis will give an overview of probiotic bacteria applied within aquaculture. The focus will be on *Roseobacter* strain 27-4[1], which is one of the promising probiotic candidates. *Roseobacter* 27-4 was originally isolated from a turbot rearing facility due to its strong anti-*Vibrio* activity (Hjelm et al., 2004a). This strain and related strains appeared to constitute a relatively stable community within the rearing facility (Hjelm et al., 2004b) and were especially associated with the walls of the rearing tanks. To facilitate and optimize the use of probiotic bacteria such as *Roseobacter* 27-4 within aquaculture productions, it is necessary to understand their ecology. Since production of antibacterial compounds is often believed to be the mode of action of probiotic bacteria, it is important to understand how environmental factors affect the production of these.

Probiotics are generally administrated as live microbial feed supplements. Since *Roseobacter* 27-4 is associated with the tank wall of the rearing tanks, we suggest that this probiotic bacterium can be administrated by colonizing the surfaces of the rearing tanks. A well-established biofilm of probiotic bacteria might prevent the proliferation of fish pathogenic bacteria. However, if such a practice is to be applied, methods must be developed to quantify both the probiotic and pathogenic bacteria during biofilm formation to study the interaction. Hence, development of new ways to specifically quantify bacteria attached to surfaces will be investigated in this thesis.

It is unlikely that the bacteria produce antibacterial compound for protection of fish larvae. This behaviour must be important for the bacterium in its natural habitat. Members of the so-called *Roseobacters* clade are very common in marine environments and represent 20 – 30 % of the bacterial communities in the upper mixed layer of the ocean. This group is often most abundant in bacterial communities associated with marine algae and in coastal biofilms (Buchan et al., 2005). Members of the *Roseobacter* clade can affect the growth of

[1] *Roseobacter* 27-4 is based on 16S rDNA closely related to *Roseobacter gallaeciensis,* which recently was reclassified as *Phaeobacter gallaeciensis* (Martens et al., 2006b). Since two published papers uses *Roseobacter* 27-4 I have chosen to retain this in this thesis

the algae (Alavi et al., 2001) and play an important role in the global marine carbon and sulphur cycle (Wagner-Döbler and Biebl 2006). The fact that this group of bacteria is most abundant in algae communities, gives them a central role in the marine environment.

The purpose of this thesis has been to investigate factors influencing *Roseobacter* 27-4 production of antibacterial compounds and to elucidate the structure, regulation and biosynthesis of the active compound.

We hypothesize that production of antibacterial compounds is an important phenotype for probiotic bacteria e.g. *Roseobacter* 27-4 and that this phenotype is correlated to the growth conditions of the bacteria. We also believe that if production of antibacterial compounds is a general phenotype among bacteria belonging to the *Roseobacter* clade it will influence their dominance within the marine environment. Hence, this thesis addresses the occurrence of antibacterial activity among marine *Roseobacter* strains.

2 Probiotic in aquaculture

2.1 Definition of probiotic microorganisms

The definition of the term probiotics has changed over time. The expression probiotic stems from combining the Latin word pro (for) with the Greek word bios (for life) (Zivkovic 1999). The term probiotics denotes bacteria that promote the health of other organisms. Lilly and Stillwell (1965) defined probiotic as “Substances produced by microorganisms which promote the growth of other microorganisms” but this has changed over the years as new understanding has emerged. In general, the interest has centred on terrestrial organisms, and the term probiotic inevitably referred to Gram-positive bacteria associated with the genus *Lactobacillus*. The use of Gram-positive bacteria in human feed gave rise to a definition by Fuller (1989), “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”. However, this definition is not applicable in aquaculture. The fact that the probiotic has been supplied as feed makes sense in human or other terrestrial animals, because the importance of the gastrointestinal tract is clearly recognized as onset of pathogen proliferation and invasion site. However, this is not the case for fish. Host and microorganisms are submerged in water and therefore areas such as skin and gills are important as site of proliferation and invasion site for fish pathogenic bacteria. Furthermore a review by Cahill (1990) reported that the bacterial present in the aquatic environment influence the composition of the gut microbiota and vice versa. Therefore, Verschurer et al., (2000b) claimed that in aquaculture systems the immediate ambient environment has a much larger influence on the health status than in terrestrial animals or humans. Probiotic in aquaculture may be more parallel to biocontrol, which is used for microbial cultures that prevent plant diseases. Based on the above the term probiotic will in this thesis be defined as “Probiotics are live microorganisms, which when administrated in adequate amounts, confer a health benefit for the host” (FAO/WHO 2001). This definition covers the supplement of a probiotic organism to the host environment or the feed.

The question is how to define beneficial effect, or how to determinate if a microorganism is successful as being a probiotic microorganism. Live microbial cultures may be administrated in aquaculture with different purposes e.g. disease prevention / minimization, as feed or as water quality improvement. All of these will directly or indirectly benefit the health and/or survival of the aquatic organism. For this reason, Gram and Ringø (2005) proposed that the effect of a probiotic could be measured by its ability to decrease frequency of disease and/or increase survival from lethal diseases.

Even though more than two thousand publications deal with the concept of using probiotic to benefit health of human and animals, the concept is still questioned (Berg 1998; Atlas 1999; Gomez-Gil et al., 2000; Gram and Ringø 2005).

Gomez-Gil et al., (2000) reviewed the use of probiotic bacteria in fish larvae cultures. They concluded that the available information is inconclusive, since few experiments with sufficient robust design have been conducted to permit critical evaluation. Gram and Ringø (2005) concluded that there is a general lack of in vivo studies. There is also a need for specific cause and effect relationships that can be substantiated scientifically (Atlas 1999). To understand the areas in which the probiotic concept is viable, we must understand the mechanisms by which such treatment works. This will require detailed knowledge of (Gram and Ringø 2005);

- The pathogen, its virulence, its proliferation and invasion sites
- The host, its immune defence, and its natural microflora
- The surrounding environment, including nutrients, microorganisms etc.
- The probiont, its functional features, its mechanisms of action, and its effect on the general microflora etc.

Today no studies dealing with probiotic in aquaculture have a complete understanding of the mechanisms by which the treatment works, however several studies have demonstrated a disease reducing effect in challenge or field trials.

2.2 Use of microorganisms for prevention of disease in aquaculture

A range of bacteria has either been suggested and/or evaluated as biological control agents in aquaculture. Many of the proposed strains belong to the lactic acid bacteria (LAB) to the genus *Vibrio*, to the genus *Bacillus* or the genus *Pseudomonas*, although many other genera or species have also been tested. This section gives a brief overview of some of the in vivo studies conducted with probiotic bacteria. For further information of the use of probiotic in aquaculture see the following reviews (Gatesoupe 1999; Verschuere et al., 2000b; Irianto and Austin 2002; Gram and Ringø 2005; Balcazar et al., 2006; Vine et al., 2006).

Several studies have evaluated the use of probiotic bacteria on grown fish, however, effective commercial vaccines are available for most fish pathogenic bacteria (Sommerset et al., 2005). Therefore the future focus of probiotic research should be aimed at problems, which cannot be solved with vaccines e.g. infectious diseases in production of fish larvae,

crustaceans and molluscs or diseases caused by bacteria where no vaccines are available (e.g. *Flavobacterium psychrophilum*). Investigations regarding cost benefit in the long term needs to be done, which will assure that it is an economically attractive way of decreasing infectious diseases. Since probiotic bacteria may not be effective in aquaculture in general, but may be effective in a specific production.

2.2.1 Finfish

Many different bacterial strains have been tested for their ability to reduce infectious diseases in finfish. All though positive results have been found, no specific strain(s) are broadly applied within finfish aquaculture.

Finfish larvae

The mortality of non-feeding turbot larvae (*Scophthalmus maximus*) was significantly reduced by eight out of 12 *Roseobacter* strains tested of which all showed antagonistic activity against *Vibrio* spp (Hjelm et al., 2004a). Also Makridis et al., (2005) found a positive effect of a *Roseobacter* strain on survival rate of gilthead sea bream larvae (*Sparus aurata*), but no significant difference was found between treatment with or without *Roseobacter*. One *Roseobacter* strain was tested in a challenge test against *V. anguillarum*. In turbot larvae receiving rotifers with both *Roseobacter* 27-4 and *V. anguillarum* the accumulated survival was significantly reduced compared to treatment with *V. anguillarum* alone (Figure 2) (Planas et al., 2006). Positive effects on the accumulated survival of turbot larvae have also been observed by addition of *Vibrio mediterranei* (Huys et al., 2001), *Vibrio pelagius* (Ringo and Vadstein 1998) and Lactic acid bacteria (LAB) (Gatesoupe 1994).

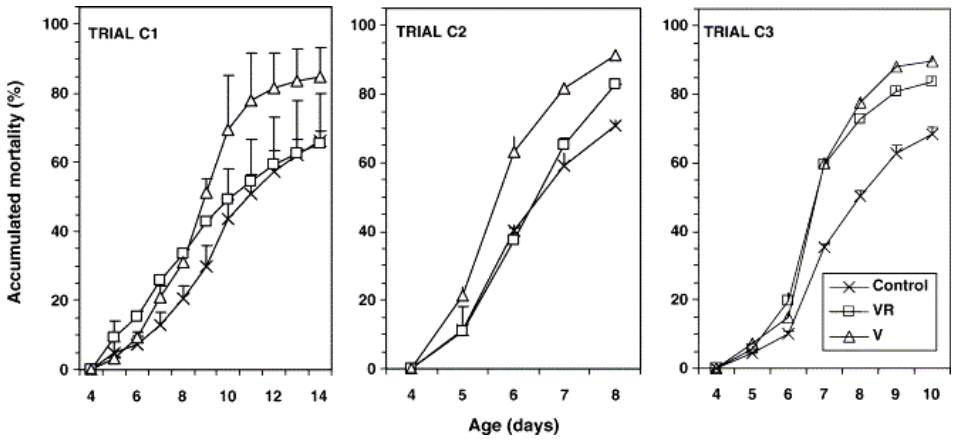


Figure 2: Accumulated mortalities in turbot larvae. VR: larvae were fed with rotifers loaded with *Roseobacter* 27-4 and *V. anguillarum*. V: larvae fed on rotifers loaded with *V. anguillarum*. Control: larvae fed with non-enriched rotifers (Planas et al., 2006).

Grown finfish

By adding a probiotic *Vibrio alginolyticus* strain, the survival increased in Atlantic salmon (*Salmo salar* L.) when challenged with *Aeromonas salmonicida* (from 0 to 82%), *V. anguillarum* (from 10 to 26%) and *Vibrio ordalii* (from 0% to 26%), however, no beneficial effect was found when challenged with *Yersinia ruckeri* (Austin et al., 1995). Using *Carnobacterium* spp. as a probiotic strain for Atlantic salmon, an increase in the survival was seen when challenged with *A. salmonicida* (from 0 to 20%), *V. ordalii* (from 23% to 74%) and *Y.ruckeri* (from 42 to 71%), but here no effect was seen when challenged with *V. anguillarum* (Robertson et al., 2000).

Several pseudomonas strains also have a disease reducing effect (Smith and Davey 1993; Spanggaard et al., 2001). The inhibition of *V. angiullarum* by *Pseudomonas fluorescens* AH2 shown in vitro was successfully repeated in vivo experiments with rainbow trout challenged with *V. anguillarum.* Here a significant reduction in mortality were seen for fish treated with AH2 (Figure 3A) (Gram et al., 1999). In vitro antagonism of *P. fluorescens* AH2 against *Aeromonas salmonicida* did however not confer Atlantic salmon protection against furunculosis as no differences were seen between treatment with and without AH2 (Figure 3B) (Gram et al., 2001). These results show that a promising probiotic candidate in one system may not automatically be applied in a new system.

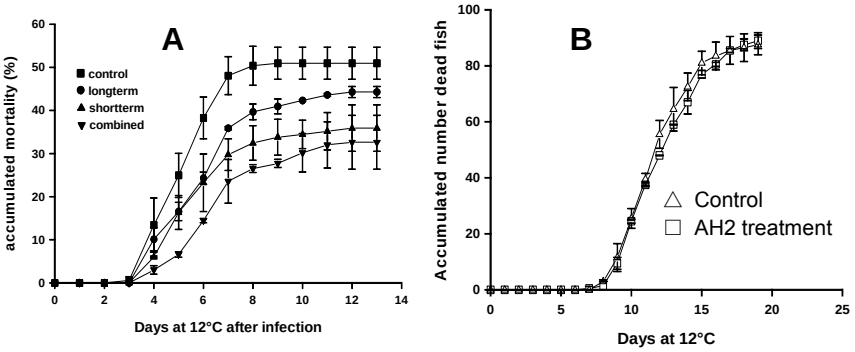


Figure 3: Accumulated mortality of A: rainbow trout in infected with *V. anguillarum* and B: atlantic salmon infected with *A. salmonicida.* Both with and without probiotic treatment with *P. fluorescens* AH2. Control: infected with the pathogenic bacteria (Gram et al., 1999; Gram et al., 2001).

2.2.2 Crustaceans

Marine shrimps are the dominant production in crustacean aquaculture. Today there is already a broad application of probiotic bacteria in commercial shrimp hatcheries. In general, most studies of probiotic bacteria for application in shrimp productions have focused on *Bacillus* strains. Commercial products such as BaoZyme-Aqua, Liqualife® and Promarine®, available for shrimp aquaculture, all contain one or several strain of *Bacillus* (Hong et al., 2005). *Vibrio* spp. is the most common cause of infections in shrimps industry. Moriarty (1998) compared survival of shrimps with and without addition of

Bacillus in the presence of luminous *Vibrio* strains. Mass mortality within 80 days was seen in cultures without *Bacillus* strains, whereas in the abundance of *Bacillus* strains the shrimps could be cultured for more than 160 days without problems. Likewise a positive effect on cultures of *Penaeus monodon* by exposing them to *Bacillus subtilis* BT23 or *Bacillus* S11 has been found. The addition of the *Bacillus* strains to shrimp cultures resulted in a reduced mortality when challenged with *Vibrio harveyi* (Rengpipat et al., 2003; Vaseeharan and Ramasamy 2003). The disease reducing effect of the *Bacillus* strains may be a result of improvement of the water quality e.g. by removal of ammonia (Chen and Chen 2001; Farzanfar 2006). Commercial products containing *Bacillus* spp. do indeed guarantee a water quality improvement. If this is the only mode of action in decreasing shrimp mortality, the *Bacillus* strains will not be considered as probiotic bacteria based on the definition used in this thesis.

2.2.3 Molluscs

Several different strains have been tested for their ability to reduce the heavy losses, which is experienced within oyster production due to disease outbreaks. Gibson et al., (1998) investigated the use of *Aeromonas media* A199*,* capable of producing an antibacterial compound, on the pacific cupped oyster (*Crassostrea gigas*). A199 was able to control infections with *Vibrio tubiashii,* as all larvae died in the presence of *V. tubiashii* alone whereas survival was approx. 95% when adding a combination of *V. tubiashii* and A199.

The marine *Roseobacter* strain BS107 had antagonistic activity *in vitro* against several fish and molluscs pathogenic bacteria. It was suggested that the inhibitory effect was displayed only in the presence of another bacterium as only supernatants from co-cultures gave inhibition zones in a well diffusion assay. Cell extracts of the *Roseobacter* strain lowered mortality rates of scallop (*Pectin maximus*) larvae in a challenge test with *Aeromonas salmonicida*. However, the presence of whole cells of the *Roseobacter* strain did not enhance survival of the larvae (Ruiz-Ponte et al., 1999).

In a recent study by Macey and Coyne (2005) a probiotic supplement containing both yeast and bacteria strains improved the growth rate of *Haliotis midae*. When challenging the abalone with *V. anguillarum,* 62% of the probiotic fed animals survival compared to 25% for non-treated animals, however, no statistic analysis was accomplished.

2.3 Isolation of probiotic bacteria

Probiotic microorganisms used in aquaculture are found in several microbial groups of bacteria (Verschuere et al., 2000b; Gram and Ringø 2005), bacteriophages (Nakai and Park 2002), yeasts (Tovar et al., 2002) and microalgae (Austin et al., 1992). The focus of this thesis is on the use of bacteria as probiotic organism.

For selection of effective probiotic bacteria, a collection of putative candidates is required. Such candidates can be isolated from the host or the environment in which the bacteria are supposed to exert their probiotic effect. However, there is no unequivocal identification that putative probiotics strains isolated from completely different environment to the cultured species or originating from very different habitats have a smaller success of being a probiotic bacteria (Verschuere et al., 2000b). Any collection of putative probiotic bacteria needs to be evaluated to find the optimal strain(s), however, no proven scientific rationale exists for the selection of the best probiotic species or strain (Gram and Ringø 2005; Vine et al., 2006). Conway (2006) listed several abilities, which are recommended to be included for the selection of functional probiotic strains. The list can be divided in in vivo effect e.g. that the probiotic candidates are non-toxic to the host and that it needs to have a demonstrable health effect. However these are not abilities, which are easily applied in screening methods. The in vitro abilities include antagonistic activity against pathogenic bacteria, survival and growth in situ and colonization potential. These are abilities, which have been used in screening protocols, even though there is no direct link between these abilities and the observed in vivo effects.

The most common way to select probiotics is selecting organisms, which show in vitro antagonism against the pathogen, which is causing the problem (Westerdahl et al., 1991; Riquelme et al., 1997; Spanggaard et al., 2001; Hjelm et al., 2004b). When detecting antagonism in a well diffusion assay one cannot conclude that this will be produced in situ, since the growth media can influence the degree of in vitro inhibition (Olsson et al., 1992; Hjelm et al., 2004a) and growth substrates are typically very different from the in situ environment. Even though a bacteria has the ability to inhibit pathogens in vitro, adding the bacteria will not necessarily improve health of the aquatic organisms in an in vivo system (Gram et al., 2001; Spanggaard et al., 2001; Suomalainen et al., 2005).

Testing pure cultures from fish environment, typically results in 4 - 28% having antagonistic activity against fish pathogenic bacteria (Westerdahl et al., 1991; Spanggaard et al., 2001). This selection procedure is very time consuming and screening directly for inhibiting strains, assuming that this is an important phenotype, will increase chances of

finding probiotic candidates. Replica plating is a rapid and easy way to screen multiple strains from environment by copying colonies from primary sample plates onto agar, which is cast with the pathogenic bacteria to be inhibited (Figure 4). By using replica plating 27,500 colonies isolated from a turbot rearing facility were screened for antagonistic activity. 537 colonies were pre-selected as potential antagonists strains of which all were Gram-negative. 205 of these had stable antagonistic activity and of these 147 belonged to the *Roseobacter* while 48 belonged to the *Vibrio* genus (Hjelm et al., 2004a; Hjelm et al., 2004b). Antagonistic *Roseobacter* strains have also been isolated from cultures of the scallop *Pecten maximus* (Ruiz-Ponte et al., 1998; Ruiz-Ponte et al., 1999; Ricardo et al., 2004).

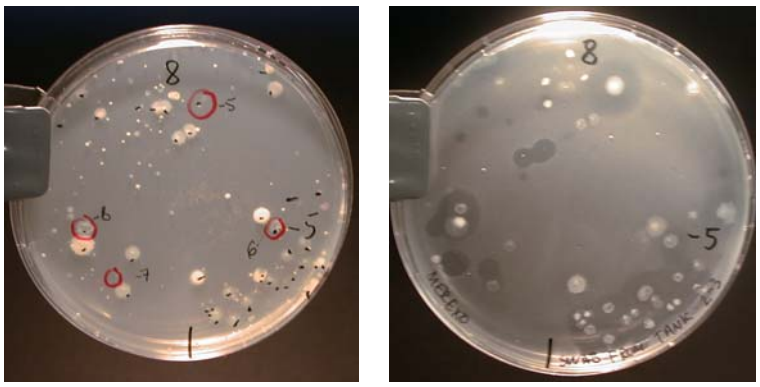


Figure 4: Primary plate with bacteria from water (left) and replica plate where inhibitory colonies cause a clearing in the turbid growth of the fish pathogen, *Vibrio anguillarum* (Hjelm et al., 2004a).

Colonization and growth within the gastrointestinal tract have also been applied as screening methods for probiotic bacteria, and a disease reducing effect has been observed for bacteria isolated by such an approach (Chabrillón et al., 2005; Chabrillón et al., 2006). Screening for colonization ability has been combined with screenings for antibacterial compounds (Olsson et al., 1992; Jöborn et al., 1997; Vine et al., 2004).

2.4 Possible mode of action of probiotic bacteria

Although some bacteria have probiotic effects in aquaculture productions, the mechanisms by which such treatment works is not known. No reports have completely elucidated the exact mode of action in vivo for the probiotic bacteria used in aquaculture. To elucidate the mode of action(s) knock out mutants in specific genes within the probiotic bacteria can be used. The following section describes the suggested mode of actions among fish probiotic bacteria. There is a high likelihood that a diseases reducing effect of a probiotic bacteria will be caused by a combination of these different suggested mode of actions.

2.4.1 Production of antibacterial compounds

Antagonistic compounds are defined as chemical substances produced by microorganisms (in this case bacteria) that are toxic (bactericidal) or inhibitory (bacteriostatic) towards other microorganisms. The presence of bacteria producing antibacterial compounds in the intestine of the host, on its surface, or in its culture water is thought to prevent proliferation of pathogenic bacteria and even eliminate these. The antibacterial compounds can be divided into compound with a direct (e.g. antibiotic, bacteriocins) or indirect (e.g. siderophore) effect on the pathogen. Siderophore are dealt with in the next section. Lactic acid bacteria often produce bacteriocins, however, these are often only active against closely related species (Klaenhammer 1993) and most pathogens involved in aquaculture are Gram-negative and bacteriocins from LAB may therefore not inhibit fish pathogenic bacteria.

Probiotic bacteria suggested as probiotic treatment in aquaculture can produce both proteinaceous and non-proteinaceous substrates (Table 1). The structure of the antibacterial compound is often not elucidated and their mode of action has not been reported. Furthermore none of these reports demonstrate that the antibacterial compound is produced in vivo. This will be of significant importance if production of these compounds is the mode of action. If the production of antibacterial compound is the only mode of action, it is possible that the pathogen eventually will develop resistance towards the compound. This will result in an ineffective treatment. The risk of the pathogen to develop resistance against the active compound has to be evaluated, to assure a stable effect of the probiotic bacterium.

2.4.2 Nutrient competition

The bacteria inhabiting the aquaculture environment compete for available nutrients and this is suggested as a mode of action for probiotic bacteria. The most described competition for nutrients is the competition for iron by production of siderophores. Siderophores are low-molecular-weight ferric ion-specific chelating agents. Production of siderophores has been found in several *Pseudomonas* strains suggested to be probiotic strains (Smith and Davey 1993; Gram et al., 1999). A positive correlation was found between the production of siderophores and the protective action of *P. fluorescens* AH2 against *V. anguillarum* (Gram et al., 1999). Furthermore by using arbitrarily PCR-amplified gene transcripts it was concluded that production of siderophores was the cause of inhibition in *V. anguillarum* (Holmstrom and Gram 2003). These results suggest that competition for free iron is in fact the mode of action for *P. fluorescens* AH2 found in the in vivo experiments, however production of siderophores has not been demonstrated in vivo.

Table 1: Characteristic of the antibacterial compound produced by strains suggested as probiotic bacteria for use within aquaculture

Strain	Fish pathogens Inhibited	Compound	Parameters influencing the production	Mode of action	Reference
Roseobacter 27-4	*Vibrio* spp. (seen table 4)	Tropodithietic acid	Only produced in stationary phase under static growth conditions	Unknown	Bruhn et al., 2005b
Roseobacter CECT 5719 and. CECT 5718	*Vibrio anguillarum*	Five cyclic dipeptides	-	Unknown	Ricardo et al., 2004
Vibrio sp NM10	*Flavobacterium* spp. *Pseudomonas* spp. and *Vibrio* spp.	Heat labile proteinaceous > 5 kDa	Seawater in media, incubation time, temperature and pH	Unknown	Sugita et al., 1997
Micrococcus MCCB 104	*Vibrio* spp. and *Aeromonas* spp. do mot inhibit *Bacillus* spp., *Pseudomonas* spp. and *Vibrio splendidus*	Heat labile non-proteinaceous	Produced in the late exponential and stationary phase	Unknown	Jayaprakash et al., 2005
Pseudomonas PS-102	*Vibrio* spp. and *Aeromonas* spp.	Heat stable non-proteinaceous	Produced in the late exponential and stationary phase	Unknown[1]	Vijayan et al., 2006
Pseudoalteromonas strain X153	*Vibrio* spp. no effect on yeast and fungus	87 kDa Antimicrobial protein	-	Unknown	Longeon et al., 2004
Pseudomonas I-2	*Vibrio* spp.	Heat stable >10 kDa non-proteinaceous	Production was only seen in the stationery phase, and production was effected by salt conc. and pH.	Unknown	Chythanya et al., 2002
Vibrio C33	*Vibrio* spp. *Aeromonas hydrophila*	Several, one was identified as an aliphatic hydroxyl ether	-	Unknown	Jorquera et al., 2000
Aeromonas media A199	*Aeromonas* spp., *Vibrio* spp., *Yersinia ruckeri* and *P. damsella*	Bacteriocin-like	-	Unknown	Gibson et al., 1998
Roseobacter BS107	*Aeromonas* spp., *Vibrio* spp.	Heat stable proteinaceous	Inhibitory activity were only detected from co-cultures	Unknown	Ruiz-Ponte et al., 1999

[1] the mode of action was not elucidated however, siderophores were produced by the strain hence the inhibitory activity could be due to this production.

Production of siderophores has been recognized as an important factor in the disease suppression ability of fluorescent *Pseudomonas* spp. used as biocontrol cultures against plant pathogens (O´Sullivan and O´Gara 1992).

Since the bacterial community associated with aquaculture environment is dominated by heterotrophic bacteria, competition for organic substrates such as carbon and energy sources will take place. A bacterial strain SK-05 was selected based on its ability to grow in organic-poor environment, and the strain inhibited the growth establishment of *V. alginolyticus* in diatom culture. Since SK-05 had no inhibitory effect against *V. alginolyticus* in an in vitro system, it was concluded that SK-05 out-competed *V. alginolyticus* by maintaining a low organic concentration in the culture unsuitable for *V. alginolyticus* growth (Rico-Mora et al., 1998).

In a study by Verschuere et al., (2000a) *V. alginolyticus* were used as the probiotic bacterium. *V. alginolyticus* produced no extracellular antagonistic compounds against the Artemia spp. pathogen *V. proteolyticus*, even though the strain was able to have a negative effect on the colonization of *V. proteolyticus* in the culture water. The protective effect was only found when using living cells and therefore it was suggested that the mode of action were competition for substrates and available energy.

2.4.3 Competition for attachment sites

Successful colonization and invasion of the host by pathogenic bacteria rely on the ability to attach to the invasion sites and proliferate. If the pathogenic bacteria are prevented in this attachment by e.g. probiotic bacteria, chances for a successful invasion will be reduced. Several studies use attachment and growth, mostly to/in the mucus, as one of the criteria when selecting probiotic bacteria (Olsson et al., 1992; Chabrillón et al., 2005; Chabrillón et al., 2006).

Vibrio strain Pdp11 was selected as a probiotic candidate based on its ability to adhere in high numbers to the intestinal mucus. The strains showed no antagonistic activity against *V. anguillarum* in an in vitro assay. However a significant reduction in mortality was seen for Gilthead seabream challenged with *V. anguillarum* when adding Pdp11. It was suggested that the mode of action was colonization of the intestinal mucus and thereby preventing invasion of *V. anguillarum* (Chabrillón et al., 2006).

Members of the *Roseobacter* clade are the first to attach to surfaces in costal environment, and several members of the clade have been suggested as probiotic bacteria (Ruiz-Ponte et al., 1999; Ricardo et al., 2004; Luis et al., 2004; Hjelm et al., 2004a; Bruhn et al., 2005b;

Belas et al., 2006). *Roseobacter* 27-4 showed high attachment capability and formation of biofilm under growth conditions, which facilitates its production of an antibacterial compound (Bruhn et al., 2005b). This combination of phenotypes is suggested to be perfect for a probiotic bacterium. However, when administrating *Roseobacter* 27-4 through enrichment rotifers, the bacterium did not colonise the larval gut or intestine and was mostly detected in the culture water (Planas et al., 2006). Showing that high attachment to inert surfaces in general do not necessary give colonization abilities.

2.4.4 Quorum quenching

Blocking the virulence phenotypes in fish pathogenic bacteria may also be a mode of action of probiotic bacteria. Virulence is under the control of a so-called quorum sensing (QS) system in a number of human and plant pathogenic bacteria (Whitehead et al., 2001). However, QS systems also regulate suspected virulence phenotypes in some fish pathogenic bacteria (Swift et al., 1999; Freeman and Bassler 1999; Croxatto et al., 2002; Kim et al., 2003). QS systems rely on the ability of the bacteria to monitor their population cell density by release of small molecular mass signal molecules (mainly acylated homoserine lactones (AHLs) for Gram-negative bacteria) into the environment. The bacteria can sense their cell density by monitoring the concentration of signal molecules (by using AHL receptors), and thereby trigger specific phenotypes (for example virulence factors) at a particular cell density (Whitehead et al., 2001). AHLs compounds are produced by many fish pathogenic bacteria (Bruhn et al., 2005a). The QS systems can be disrupted by two different methods, interference with the AHL- receptors or by degrading the signal molecule.

Several molecules can react with the QS system e.g. cyclic dipeptides (Holden et al., 1999) long chain AHL molecules (McClean et al., 1997) and the so-called quorum sensing inhibitors (QSI). These molecules will block the AHL regulated systems, and thereby the expression of any virulence phenotype, which is regulated by QS, at concentrations that does not affect the growth of the bacteria. QSI compounds have successfully been applied to reduce mortality of fish and shrimp during challenge trials against pathogenic bacteria (Manefield et al., 2000; Rasch et al., 2004; Defoirdt et al., 2006). However, addition of QSI compounds is not always affective, as a known virulence factor in a high AHL producing strain of *Yersinia ruckeri* was unaffected by addition of QSI compounds (Kastbjerg et al., 2006).

Both cyclic dipeptides (Jørn Smedsgaard personal communication) and long chain AHL molecules (Bruhn et al., 2005b) are produced by *Roseobacter* 27-4 and other potential QS inhibitor compound have been detected within extracts of *Roseobacter* 27-4. Rasmussen

et al., (2005) developed a bioassay for testing extracts or compounds for QSI activity. If Quorum quenching is a mode of action for probiotic bacteria this bioassay is suitable for screening after bacteria which produce QSI compounds. A positive reaction is seen from a static grown supernatant of *Roseobacter* 27-4 (Figure 5), but also sterile media used at negative control gave a positive reaction. Indicating that no QSI compound which can be detected by this bioassay, is produced by *Roseobacter* 27-4 (Unpublished results by Bruhn and Rasmussen).

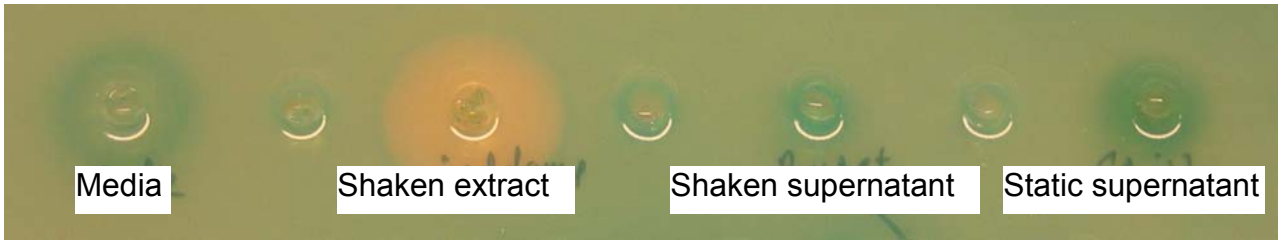


Figure 5: Supernatant from static and shaken grown cultures of *Roseobacter* 27-4 and extracts of a static culture tested in the QSI selector. Positive reaction (formation of blue colour) is seen for the static grown supernatant, however, also positive reaction is seen for the sterile media used at negative control.

Enzymatic degradation of AHL compounds are widespread among *Bacillus* spp., which produce specific AHL-lactonases that destroys the AHL molecule and thereby disrupt the QS system of other bacteria (Dong et al., 2000; Dong et al., 2001). Genes encoding AHL-lactonases have been found in several strains of *Bacillus thuringiensis,* which is used for biocontrol against plants pathogens (Lee et al., 2002). Furthermore, AHL-degrading *Bacillus* spp. can reduce virulence factors in the plant pathogens *Erwinia carotovora* and *Agrobacterium tumefaciens* (Molina et al., 2003). It can be suggested that degradation of AHL compounds may act as a mode of action for probiotic *Bacillus* used e.g. in shrimps farms.

2.5 Administration of probiotic bacteria

There are several ways in which probiotic cultures can be delivered to the aquaculture units. Vine et al., (2006) conclude that methods to deliver the probiotics to the larvae have not been optimized to achieve enhanced effects. Probiotic bacteria have been added by:

- Bathing the host in bacterial suspension
- Addition to diet e.g. by enrichment of live food (artemia/rotifers)
- Addition to culture water

There are only few studies in which the fish/animal has been bathed in a bacterial suspension of probiotic bacteria. Riquelme et al., (1997) found a disease reducing effect by bathing *Argopecten purpuratus* larvae in suspension of probiotic bacteria, however also ineffectual bathing has been reported (Suomalainen et al., 2005). Most studies have administrated the probiotic bacteria to aquaculture by adding them to the culture water or to the diet. Grisez et al., (1996) found that the important fish pathogen *V. anguillarum* was able to invade juvenile turbot through the intestine. In faecal extract from turbot juveniles the growth of *V. anguillarum* was inhibit by *Carnobacterium* cells. They concluded that the turbot intestinal tract and contents can serve as an enrichment site for *V. anguillarum*, and the use of intestinal bacteria with antagonistic activity against vibrios may be used to reduce the load of fish pathogenic *Vibrio* in turbot hatcheries (Olsson et al., 1998). Several studies have supplemented the diet with probiotic bacteria (Table 2).

Table 2: Minimum transience of probiotic candidates in the gut of fish and shellfish. j: juveniles, l: larvae (modified after Gatesoupe 1999).

Microbe	Host	Minimum persistence	Reference
Ae. Media	*Crassostrea gigas* (l)	2 days (20ºC)	Gibson et al., 1998
Aeromonas sp.	*Sc. Maximus* (l)	14 days (15-20ºC)	Munro et al., 1995
Ca. divergens	*G. morhua* (l)	9 days (8ºC)	Strom and Ringo 1993
Carnobacterium sp.	*Onchorhyncus mykiss* (j)	4 days (11ºC)	Jöborn et al., 1997
V. alginolyticus	*Sa. Salar* (j)	21 days (15ºC)	Austin et al., 1995
Vibrio Pelagius	*Sc. Maximus* (l)	14 days (17-20ºC)	Ringo et al., 1996
Roseobacter 27-4	*Sc. Maximus* (l)	3 days (18ºC)	Pérez-Lorenzo et al., 2006

In general, probiotic bacteria do not colonize and persist in the host and if a long-term exposure is required, they have to be supplied repeatedly. Probiotic bacteria such as *Roseobacter* 27-4 are detected in the culture water rather than in the intestine of the host, suggesting that adding the bacterium to the feed may not be the most effective way of adding this bacterium.

The skin has been suggested to be an important invasion site for fish pathogens such as *F. psychrophilum*, *V. anguillarum* and *A. sakmonicida* and especially skin damage enhances the infection by these pathogens (Svendsen and Bogwald 1997; Spanggaard et al., 2000; Madetoja et al., 2000). Adding the probiotic bacteria to the culture water could therefore be an effective way of adding probiotic bacteria. Smith and Davey (1993) concluded that *P. fluorescens* exerted its effect from the host´s exterior, since the strain did not significantly invade the fish following bath treatment. Also Spanggaard et al., (2000) suggested that the protective effect observed when using *Pseodumonas* AH2 against vibriosis is caused by bacterial antagonisms on the skin. *Roseobacter* 27-4 was found to have a protective effect on turbot larvae, however, the bacteria were almost only

detected in the culture water even though administrated through live food. This suggests that the antagonistic activity also for this bacterium is on the host's exterior.

As *Roseobacter* strains can be isolated from tank walls in costal fish farms (Hjelm et al., 2004b; Choi and Cho 2006), colonization of tank walls may be suggested as a novel way of administrating probiotic *Roseobacter,* as biofilm within rearing tanks can be a reservoir for potentially pathogenic bacteria (Bourne et al., 2006). In this thesis it is hypothesised that *Roseobacter* may exert its effect as a probiont when colonizing the inert surfaces of the aquaculture units.

2.6 Quantification of probiotic and pathogenic bacteria in aquaculture

Studying the interaction between probiotic bacteria and pathogens requires that each can be specifically quantified. If the probiotic bacteria are thought to exert its effect during colonization of tank walls, quantification of the bacterium during attachment is essential for evaluating the probiotic treatment.

Quantification of the probiotic bacterium and pathogenic bacteria both within the host and in the culture water is frequently done by growing the bacteria on selective or non-selective substrates (Gatesoupe 1994; Gildberg and Mikkelsen 1998; Riquelme et al., 2000; Riquelme et al., 2001; Rengpipat et al., 2003; Planas et al., 2006). The disadvantage of using this approach is that it is not strain specific e.g. it will not distinguish between pathogenic and non-pathogenic *Vibrio*. Furthermore, if plate counts are to be used for quantification of bacteria attached to an inert surface one must remove the bacteria before quantification (Dewhurst et al., 1986; Zips et al., 1990; Bourion and Cerf 1996). It is however impossible to know if all the bacteria have been removed or if some has been killed during the removal process.

By using Immunohistochemistry probiotic and pathogenic bacteria can be determined within the host (Gildberg and Mikkelsen 1998; Planas et al., 2006), which will give information regarding colonization capabilities and invasions sites. However, immunohistochemistry is time-consuming and not suitable for quantification.

Fluorescent in situ hybridization (FISH), can be used to investigate the bacterial diversity in aquatic animals (Hernandez-Zarate and Olmos-Soto 2006) and is suggested to be an important tool in future investigations of bacterial density in aquaculture. By using this method it is possible to investigate if non-cultureable bacterial strain is important in aquaculture production. Furthermore, fluorescence microscopy is used to quantify bacteria

attached to surfaces and in biofilms (Moller et al., 1995). In general, these methods have the disadvantage that a low number of attached bacteria cannot be quantified. Furthermore, quantification of a mixed species biofilm with several bacteria is difficult when using methods based on microscopy, even when different rRNA probes are available.

Real-time PCR have been used for quantification of humans pathogens in raw oysters (Lyon 2001; Campbell and Wright 2003; Blackstone et al., 2003) and Adams and Thompson et al., (2006) suggested that Real-time PCR offers an opportunity to quantify low levels of fish pathogenic bacteria. Real-time PCR also offers possibilities for quantification of bacteria adhered to the gastrointestinal mucosa (Huijsdens et al., 2002) and quantification of both pathogenic and probiotic bacteria during competition for attachment on intestinal epithelial cells (Candela et al., 2005), indicating that the method can be a strong tool for detecting interactions also within aquaculture. Real-Time PCR has also been used for quantification of bacteria in biofilm in e.g. dental plaque (Yoshida et al., 2003) or directly on an inert surface (Guilbaud et al., 2005).

2.6.1 Development of a Real-time PCR method for quantification of *Roseobacter* 27-4 and *Vibrio anguillarum*

As part of this work, a Real-time PCR procedure was developed for quantification of probiont and pathogen. Specific primers for Real-time PCR were designed within the virA gene for *V. anguillarum* (Jensen et al., 2005), primers designed in the 16S rRNA gene for *Roseobacter* 27-4 did not detect any fish pathogenic bacteria, however, five other bacteria from the *Roseobacter* clade gave positive, albeit delayed, detection (Bruhn et al., 2006). The primers designed for *Roseobacter* 27-4 can therefore only be used within a model system, as different strain of *Roseobacter* can be isolated for costal fish farms with high similarity within the 16S rRNA gene (Choi and Cho 2006).

The Real-time PCR quantification relies on a standard curve comparing either DNA concentration to amplification cycle (Ct value) or colony counts to amplification cycle (Figure 6). We concluded (Bruhn et al., 2006) that the cfu-Ct curve was required to reflect cell counts during growth of *Roseobacter* 27-4.

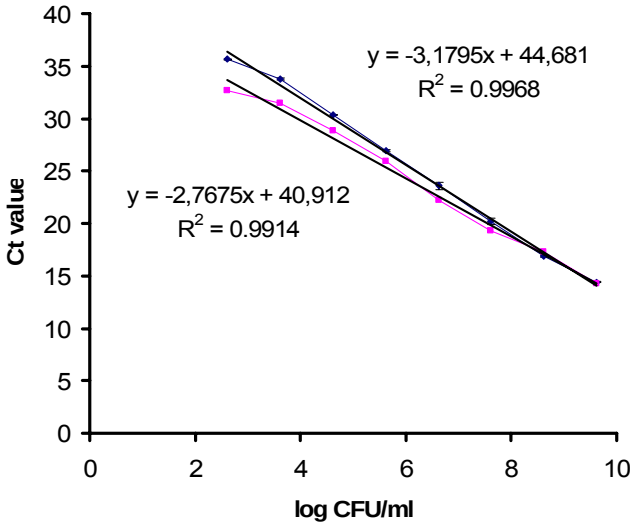


Figure 6: Standard curve representing the correlation between CFU/ml of *Roseobacter* 27-4 and Ct value detected with the Real-Time PCR method. ■ dilution of cells, ■ dilition of DNA (Bruhn et al., 2006).

The silica membrane used for DNA purification might be overloaded when high cell density samples are extracted. This will result in a lower efficiency at high cell densities compared to extracting from lower cell density samples. The result of this difference is seen in Figure 6. Guilbaud et al., (2005) prepared a standard curve by comparing C_t-values of DNA extractions from adherent cells with plate count from cells removed by sonication. A Ct-CFU/ml standard curve based on dilutions of *Listeria monocytogenes* DNA was different from the standard curve based on removal and quantification of the adherent cells in a similar manner (Bruhn et al., 2006). Hence, standard curve based on dilution of cells should be used when quantifying bacteria. By using a standard curve (R^2 = 0.98) for *V. anguillarum* it was possible to quantify *V. anguillarum* during growth under static and shaken conditions (Jensen et al., 2005) in a similar manner as for *Roseobacter* 27-4 (Figure 7).

Cell counts based on Ct Real-time PCR measurements were equal to DAPI stains of stainless steel surfaces (Figure 7; Bruhn et al., 2006). The Real-time PCR method detected as low as 35 CFU/cm^2, which is much lower then other reports where approx. 10^4 cells per cm^2 are the detection limit (Guilbaud et al., 2005; Jensen et al., 2005). The difference in sensitivity may partly be explained by the copy number of the detected gene as up to 4 16S rRNA genes have been reported in bacteria related to *Roseobacter* 27-4 (Fogel et al., 1999; Klappenbach et al., 2000) and only one gene copy is expected for the detected genes in *V. anguillarum* and *L. monocytogenes*.

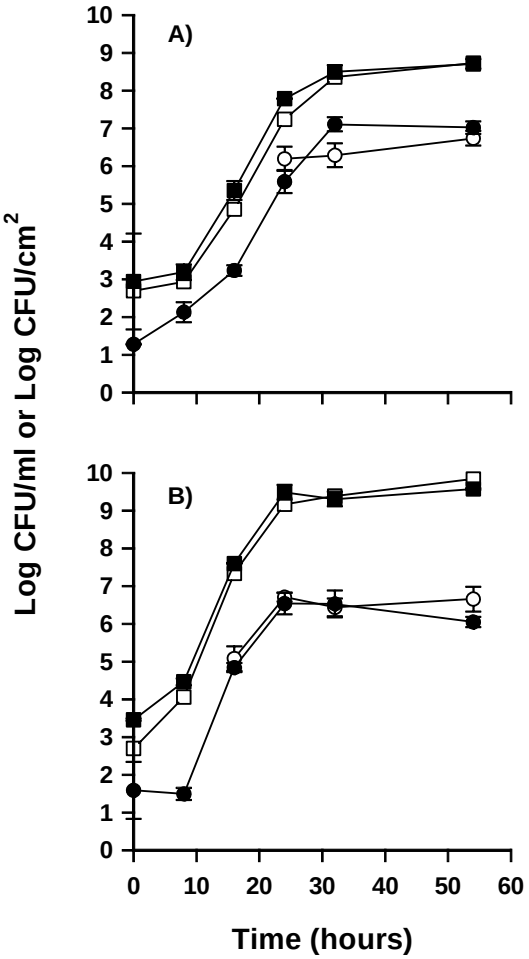


Figure 7: Quantification of *Roseobacter* 27-4 in liquid culture (Marine Broth) and attached to stainless steel coupons, A) under static conditions and B) under shaken conditions. □ CFU/ml determined by plate counts, ■ CFU/ml determined by Real-Time PCR (in duplicates), ○ CFU/cm^2 attached bacteria determined by DAPI staining, ● CFU/cm^2 attached bacteria determined by Real-Time PCR (in duplicates) (Bruhn et al., 2006).

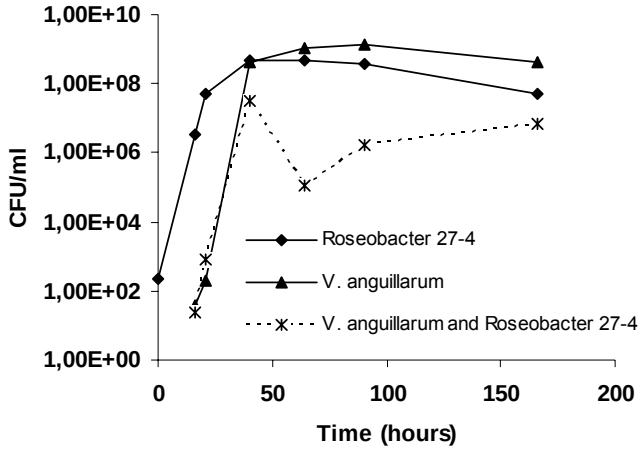
Interaction has been observed in liquid cultures when growing *Roseobacter* 27-4 together with *V. anguillarum,* as cell density of *V. anguillarum* decreased when *Roseobacter* 27-4 reached high cell counts (Figure 8). Interaction between bacteria is also seen during attachment and in biofilms (Bourion and Cerf 1996; Christensen et al., 2002; Hassan et al., 2004) and antagonistic interaction during attachment has been observed with bacteria belonging to the *Roseobacter* clade (Rao et al., 2005; Rao et al., 2006).

If the Real-time PCR procedure is to be applied for interaction experiments both in culture and on inert surfaces, the challenge is to remove or inactivate DNA from dead cells as dead bacteria may still be detected by Real-Time PCR. *V. anguillarum* density decrease during interaction with *Roseobacter* 27-4 (Figure 8 and Bruhn et al., 2005b) and removal of DNA from dead cell will be necessary if interaction is to be measured. By using Ethidium monoazide (EMA) it is possible to distinguish between viable and dead cells when quantifying bacteria with Real-time PCR (Nogva et al., 2003; Rudi et al., 2005; Wang and Levin 2006). The principle is that when EMA is added to samples containing both living and dead cells it will bind to DNA within dead cells (cells with damaged membranes). By

exposing the samples to light leads to covalent bindings between EMA and DNA, which inactivate the DNA for PCR amplification and eliminate free EMA.

DNase treatment of samples prior to DNA extraction has also been successfully applied to remove DNA from dead cells. A one-log reduction in estimated cell counts of *Vibrio choleras* was found when comparing DNase treated with non treated sampled (Lyon 2001).

Figure 8: Individually growth of *V. anguillarum* and in combination with *Roseobacter* 27-4 in Marine broth (unpublished results by Bruhn, Melchiorsen and Gram).



Attempts to remove or inactivate free DNA were done on heat treated *V. anguillarum* cells with both DNAses and Ethidium monoazide, however the Ct value remained at the same levels before and after heat treatment even thought the CFU/ml decreased (Jensen et al., 2005). Therefore further optimization of the method is needed before interaction experiments between *Roseobacter* 27-4 and *V. anguillarum* can be performed.

2.7 Conclusions from chapter 2

The dramatic growth in the aquaculture sector has emphasized the importance of fish disease control. Bacterial diseases are important constraints and may be treated with antibiotics. However, due to the risk of development and transfer of antibiotic resistance, alternative disease control measures must be implemented. Several studies have demonstrated a disease reducing effect of probiotic bacteria in fish rearing and one of several promising candidates is *Roseobacter* 27-4 a marine bacterium belonging to the *Roseobacter* clade.

A disease reducing effect is often only found in the presence of high numbers of probiotic bacteria. Furthermore, the probiotic bacteria do in most cases not colonize the host or

culture water, hence they have to be supplied repeatedly. This may be a limitation in the widespread use of probiotic bacteria. Creating a stable long-term beneficial microbiota in aquaculture environments would be an advantage. It is suggested in this thesis that *Roseobacter* 27-4 exerts its anti-pathogenic effect during biofilm formation at the tank walls, thus colonization of surfaces may be a novel way of administrating probiotic bacteria such as *Roseobacter* 27-4. A Real-time PCR procedure is presented as a method for evaluating the effect of surface colonized probiotic bacteria.

3 Tropodithetic acid produced by members of the *Roseobacter* clade

Several *Roseobacter* strains have antibacterial activity and the production of antibacterial compounds are in this thesis suggested as the mechanism for the disease reducing effect of *Roseobacter* 27-4, when added to *Vibrio* infected turbot larvae. Hence, the nature of the antibacterial compound, its synthesis and how it is regulated needs to be elucidated as this will be significant in understanding how *Roseobacter* 27-4 acts as a probiotic organism. The effect of the antibacterial compound on eukaryotic and prokaryotic cells will be discussed, as this is important knowledge if the production of antibacterial compound is to be enhanced. Also, the interaction with eukaryotic cells and animals is an important element of a risk assessment.

3.1 Antibacterial compound produced by Roseobacter

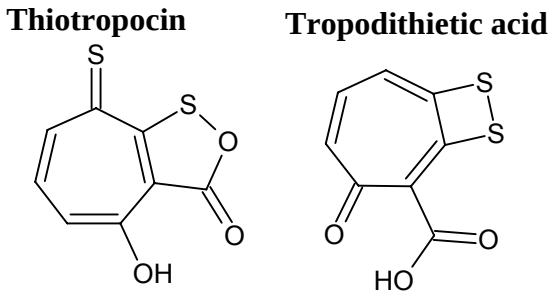
Several members of the *Roseobacter* clade produce antibacterial compounds (Ruiz-Ponte et al., 1999; Wagner-Döbler et al., 2004; Brinkhoff et al., 2004; Ricardo et al., 2004; Luis et al., 2004; Hjelm et al., 2004a; Rao et al., 2005; Martens et al., 2006a; Bruhn et al., 2007), however, this is not a general phenotype as about 50% of screened strains have no antagonistic activity (Martens et al., 2006a; Bruhn et al., 2007). Buchan et al., (2005) divided the *Roseobacter* clade into 41 lineages and production of antibacterial compounds is not linked to one specific lineage, but several positive strains belong to the so-called RGALL lineage; the *Roseobacter gallaciensis* lineage.

Several different compounds produced by members of the *Roseobacter* clade have antibacterial activity. *Roseobacter* strain BS107 produce an antibacterial proteinaceous molecule active against *Vibrio* spp. *Aeromonas* spp. and *Acinetibacter* spp. (Ruiz-Ponte et al., 1999). Five cyclic dipeptides produced by two *Roseobacter* strains inhibited *V. anguillarum* (Ricardo et al., 2004) and also Wagner-Dobler et al., (2004) detected antagonistic cyclic dipeptides and tryptanthrin in a member of the *Roseobacter* clade, *Oceanibulbus indolifex*.

Antagonistic activity is found in bacteria closely related to *Phaeobacter gallaeciensis* (Brinkhoff et al., 2004; Luis et al., 2004; Hjelm et al., 2004a; Hjelm et al., 2004b; Rao et al., 2005; Bruhn et al., 2005b), recently reclassified from *Roseobacter gallaeciensis* (Martens et al., 2006b). The antibacterial compound produced by *Rhaeobacter inhibens* T5, a member of the *Roseobacter* clade, is tropodithietic acid (TDA) (Figure 9) (Liang 2003; Brinkhoff et al., 2004). The structure of the antibacterial compound found in *Roseobacter*

27-4 was elucidated (Bruhn et al., 2005b), but it was not possible to distinguish between thiotropocin and tropodithietic acid (Figure 9) based on UV spectra and LC-MS-data. Both of these compounds may have antibacterial activity.

Figure 9: Structure of the antibacterial compounds Thiotropocin and Tropodithietic acid.



Thiotropocin is produced by a *Pseudomonas* strain (Kintaka et al., 1984; Tsubotani et al., 1984) and a *Caulobacter* strain (Kawano et al., 1997; Kawano et al., 1998). The structure of thiotropcin was not determined on the natural product but on a derivative thereof and when crystallizing the natural product directly, only tropodithietic acid was detected (Professor Dr. A. Zeeck personal communication; Liang 2003; Laatsch 2006). The antibacterial compound produced by both *Roseobacter* 27-4 and *Silicibacter* TM1040 has identical UV spectra (Figure 10) and the mass corresponds to the TDA found in *R. inhibens* T5 (Liang 2003; Bruhn et al., 2005b; Geng et al., 2007). This likely demonstrates that TDA is produced by these strains. Also a *Phaeobacter gallaeciensis* strain isolated from the surface of the marine alga *Ulva lactuca*, probably produces TDA (Rao et al., 2005).

It is not unlikely that the TDA producing *Caulobacter* strain PK654 (Kawano et al., 1997; Kawano et al., 1998) is actually a *Roseobacter* strain, as all phenotypic tests corresponded with *Roseobacter* strains and the strain produce a brown pigment, which is also found for TDA producing *Roseobacter* strains (Brinkhoff et al., 2004; Bruhn et al., 2005b; Bruhn et al., 2007). In addition the GC% of the strain does not match other *Caulobacter* strains (Kawano et al., 1997), but is similar to several *Roseobacter* strains.

TDA is not a stable compound and its activity declines with increasing temperature. The antibacterial activity in sterile culture supernatant containing TDA decreases over time at temperatures from 5°C to 37°C and no activity was measured after 8 days at 25°C. However, TDA is not inactivated by exposure to different pH values (Bruhn et al., 2005b).

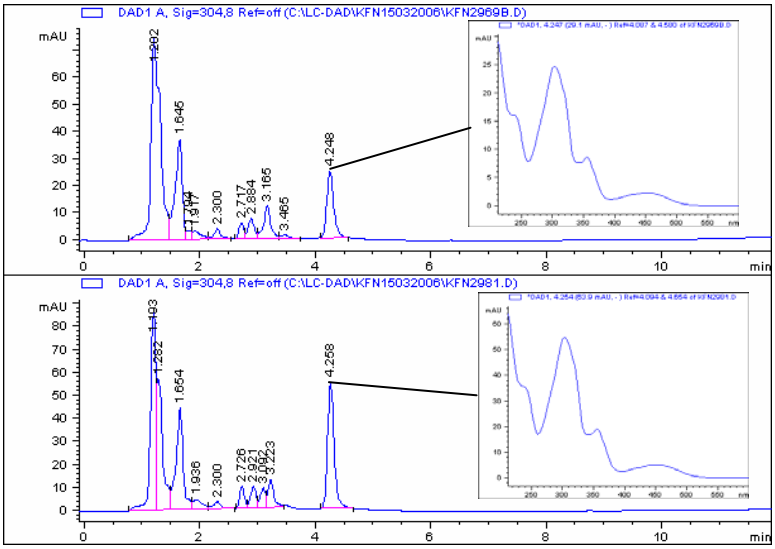


Figure 10: HPLC-DAD analysis showing the chromatogram of 0.20 µm filtered supernatant of *Silicibacter* TM1040 (upper) and *Roseobacter* 27-4 (lower). The UV spectra of the compound eluting at 4.2 min. have identical UV spectra with tropodithietic acid from *Rhaeobacter inhibens* T5 (Geng et al., 2007).

3.2 Effect of TDA on eukaryotes and prokaryotes

Several reports have demonstrated that TDA affects both eukaryotes and prokaryotes (Kintaka et al., 1984; Tsubotani et al., 1984; Kawano et al., 1998; Liang 2003; Hougaard et al., 2006; Bruhn et al., 2007). However, slightly contradictory results have been published in terms of the sensitivity of different organisms. For instance, *Candida albicans* was the most sensitive organism tested by Liang (2003), while Kazuhiko et al., (1984) found *C. albicans* to be almost unaffected by TDA. The growth substrate affects the amounts of by-products that potentially can contaminate the product of interest (Heidorn 2002) and as TDA has been extracted from cultures of TDA producing strains, different culture substrates may explain some of the difference found. By-products are best avoided by growing the TDA producing strain in a defined media with the amino acids L-phenyalanine and L-histidine as single amino acids (Heidorn 2002). Such a substrate was not used for growing the TDA producing strains *Pseudomonas* (Kintaka et al., 1984) and *Caulobacter* (Kawano et al., 1998) before purification of TDA.

3.2.1 Interaction with eukaryotes

Several fungi, yeast and algae are sensitive to TDA, however, fungi and yeast seem less sensitive as compared to algae (Kintaka et al., 1984; Kawano et al., 1997; Liang 2003). No toxic effect was observed when colonic carcinoma Caco-2 cells were grown in the presence of supernatant containing TDA (Hougaard et al., 2006), but the carcinoma cells HM2, MSF7 and HEL G2 were inhibited and cell numbers reduced when they were grown in the presence a of high concentration (>10µg/ml approx. 50µM) of TDA (Liang 2003). Non-carcinoma human cells were more sensitive, as strong inhibition in proliferation was found in concentrations at 1-2 µM TDA (Kawano et al., 1998). Although TDA affects

mammalian cells, they seem less sensitive than bacteria cells, as supernatant containing TDA in most cases is sufficient to inhibit or kill bacterial cells (Bruhn et al., 2005b; Bruhn et al., 2007).

Culture supernatant containing TDA causes no adverse reactions in zebrafish embryos and high concentration of supernatant resulted in lower mortality as compared to addition of sterile media (Table 3). Similar result was found for turbot larvae (Planas et al., 2006). *Caenorhabditis elegans* was also unaffected by addition of sterile TDA containing supernatant (Hougaard et al., 2006). However, Liang (2003) found that *C. elegans* was indeed affected when exposed to TDA with an IC_{50} of 25 µg/ml. High concentration of TDA also affected both *Artemia salina* and mice. One mg/ml TDA caused 100 % mortality of *A. salina* (Liang 2003) and the acute toxicity (LD_{50}) of TDA in mice was 50-100 mg/kg by oral administration and 8 mg/kg by subcutaneous injection (Kintaka et al., 1984). These results indicate that it is unlikely that TDA, if produced in the marine environment, will have any negative effect on aquatic organisms. Members of the *Roseobacter* clade are in general non-toxic to higher organisms in the marine environment, however some members are suggested to cause juvenile oyster disease (JOD), all though no toxins have been identified (Boettcher et al., 1999; Boettcher et al., 2000; Boettcher et al., 2005).

Table 3: The number of surviving zebrafish embryos exposed to dilutions of TDA containing supernatant from *Silicibacter* TM1040 or *Roseobacter* 27-4 (Belas et al., 2006).

Time	Supernatant from	Living zebrafish embryos out of six				
		Dilution of supernatant				
		1/4	1/8	1/16	1/32	1/64
24 hours	*Silicibacter* TM1040	0	0	6	6	6
	Roseobacter 27-4	0	0	6	6	6
	Marine broth	0	0	0	0	6
48 hours	*Silicibacter* TM1040	0	0	6	5	6
	Roseobacter 27-4	0	0	6	6	6
	Marine broth	0	0	0	0	6

3.2.2 Interaction with prokaryotes

All bacteria tested so far are sensitive to TDA (Table 4). In general, a *Roseobacter* supernatant containing TDA is sufficient to inhibit other bacteria, but members of the *Roseobacter* clade and a few other strains are only inhibited when exposed to extracts with higher concentration of TDA. The sensitivity seems to be independent of the Gram-reaction of the target cell. pH affected the sensitivity of the bacteria, as increasing pH in the assay media decreased the sensitivity (Kintaka et al., 1984).

Table 4: Inhibition of Gram-positive and Gram-negative bacteria by raw sterile filtered supernatants from TDA producing bacteria or by extracts or purified TDA (Kintaka et al., 1984; Tsubotani et al., 1984; Liang 2003; Brinkhoff et al., 2004; Bruhn et al., 2005b; Hougaard et al., 2006; Bruhn et al., 2007).

Gram negative Species	no. of strains	Sensitivity[1]	Gram positive Species	no. of strains	Sensitivity
Acinetobacter calcoaceticus	1	++	Actinobacteria	2	++
Bacteroides fragilis	1	+	*Bacillus* spp	5	++
Citrobacter freundii	1	++	*Clostridium*	1	++
E. coli	3	++	*Listeria monocytogenes*	5	++
Flavobacteria	3	++	*Mycobacterium marinum*	1	+
Halomonas spp.	1	++	*Staphylococcus aureus*	8	++
Klebsiella pneumoniae	1	++			
Proteus spp.	3	++			
Pseudomonas spp.	9	++[2]			
Roseobacter clade	17	+			
Salmonella spp.	6	++[2]			
Serratia marcescens	1	++			
Shewanella spp.	6	++			
Vibrio spp.	18	++[2]			

[1] ++ inhibited by supernatant from TDA producing strains or low concentration of TDA, + only inhibited by extracts from TDA producing strains or high concentration of TDA
[2] One or a few strains were only inhibited by extracts

Cell counts of *V. anguillarum* decreased rapidly within 2 hours when it was exposed to TDA-containing *Roseobacter* supernantat in a buffer system and no *Vibrio* were detected after 24 hours (Figure 11A). TDA also inhibited the growth of *V. anguillarum* in marine broth (Figure 11B). Similarly, the growth of *P. mirabilis* was immediately inhibited after addition of TDA, but no decrease in optical density was observed indicating that the bacteria were not lysed (Kintaka et al., 1984). The addition of TDA to *P. mirabilis* and *E. coli* did not cause lysis, but changed the morphology of the bacteria as they appeared swollen (Kintaka et al., 1984). Also the morphology of *V. anguillarum* changed when exposed to TDA containing supernatant. However, *V. anguillarum* cells appear to shrink when exposed to TDA (Hougaard et al., 2006).

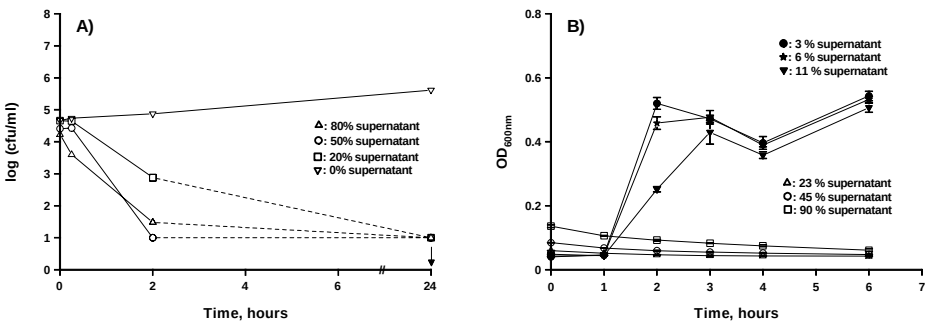


Figure 11: Survival in buffer (a) or growth in marine broth (b) of *V. anguillarum* when exposed to sterile filtered superantant from *Rosobacter* strain 27-4. (Bruhn et al., 2005b).

V. anguillarum did not become resistant to TDA even when continuously exposed to sublethal doses. Hence, production of TDA may have a persistent effect against this fish pathogen (Hougaard et al., 2006).

The effect of TDA on a target bacterial cell may depend on the ionic strength of the medium. If *V. anguillarum* is inoculated in a 1:1 mixture of *Roseobacter* supernatant and marine broth, growth occurs after two days. In contrast, *Vibrio* does not grow if *Roseobacter* supernatant is mixed with a M9 salts medium with 3% NaCl, glucose and casamino acids (M9GC). The stability of TDA in these two systems is equal as measured in a well diffusion assay, however the target cell appears protected by marine broth.

M9GC is high in phosphate buffer compared to marine broth and *V. anguillarum* is rapidly eliminated by TDA in a phosphate buffer, even thought the phosphate buffer by itself does not affect the *V. anguillarum* cells (Figure 11A). Phosphate buffers may inhibit enzymatic reactions and sequester divalent cations such as Ca^{2+} and Mg^{2+}, and as these ions line the exterior of the cell, this may result in the enhance effect of TDA found in M9GC.

3.2.3 Mode of action of TDA on prokaryotes

TDA is hydrophilic in the marine environment (pKa 2.0 calculated using ACD v9.04 pKa package Advanced Chemistry Development) and will not be diffusible through the bacterial membrane. An active transport in and out of the cell is therefore necessary. The compound is active against both Gram-negative and Gram-positive bacteria indicating that the target is not membrane specific as seen for e.g. bacteriocines (Klaenhammer 1993). Furthermore, the cells do not appear to lyse indicating that the cell membrane is intact. The bacterial cells shrank or swelled indicating that the compound may affect the osmotic pressure within the cell. Both Gram-positive and Gram-negative bacteria have sophisticated systems to survive osmotic stress, and these systems allow the bacteria to accumulate or release specific solutes (Poolman and Glaasker 1998). The cell volume will change, if these systems are disrupted e.g. by blocking of the transport proteins or by allowing inorganic ions to travel unrestricted through the cell wall (Poolman and Glaasker 1998). One can hypothesise that TDA react with ion channels or transporter proteins thereby destroying the ion gradient between the cytoplasm and the extracellular environment, eventually resulting in death of the bacteria. Genomic analysis of *Silicibacter pomeroyi,* a member of the *Roseobacter* clade, revealed an abundant number of transport proteins (Moran et al., 2004), which might give the roseobacters an advantage in resisting the effect of TDA as compared to non-*Roseobacters*. Identification of genes by DNA miroarray (Hansen et al., 2004) or arbitrarily PCR-amplified gene transcripts (Holmstrom

and Gram 2003), which are altered upon exposure to TDA, can be used to elucidate the mechanism of antibacterial action.

3.3 Substrates for TDA

3.3.1 *Roseobacter* in sulphur cycle

Tropodithietic acid contains two sulfur atoms, which is interesting as members of the *Roseobacter* clade play an important role in the sulphur cycle in marine environment and have more specifically been linked to the degradation of dimethylsulfoniopropionate (DMSP) (Moran et al., 2003; Wagner-Döbler and Biebl 2006). DMSP is an abundant organic sulphur compound produced by marine algae and coastal vascular plants as an organic osmolyte (Ledyard et al., 1993). There are two pathways for the degradation of DMSP; the cleavage pathway and the demethylation/demethiolation pathway (Figure 12). DMSP is degraded by several groups of marine bacterial, however only members of the *Roseobacter* clade have both pathways (Moran et al., 2003). During cleavage, DMSP is split into dimethyl sulphide (DMS) and acrylate. DMS is volatile and readily fluxes to the atmosphere, in which it plays a role in the formation of cloud condensation nuclei and backscatter of solar radiation (Bates et al., 1987) (Figure 12). The demethylation/ demethiolation pathway result in the formation of methanethiol (MeSH) or by double demethylation 3-mercaptopropionate (MPA) (Moran et al., 2003). If DMSP is degraded by the demethylation/demethiolation pathway, the sulphur is most likely to stay in the ocean in contrast to the cleavage pathway, and the degradation of DMSP therefore has a climatic role.

The TDA producer *Silicibacter* TM1040, can degrade DMSP and exhibit a positive chemotatic response towards DMSP, suggesting that this is an important compound for the bacterium (Miller et al., 2004; Miller and Belas 2004). Among four DMSP degrading *Roseobacter* strains, *Silicibacter* TM1040 was the only strain to produce detectable amount of antibacterial compound in supernatant (Miller and Belas 2004; Bruhn et al., 2007). This strain also differed in its degradation of DMSP as only 3-methylmercaptopropionate (MMPA) and no MeSH was detected. One can hypothesize that *Silicibacter* TM1040 is able to transfer sulphur from the DMSP degradation compounds to TDA and thereby use DMSP as sulphur source for TDA production.

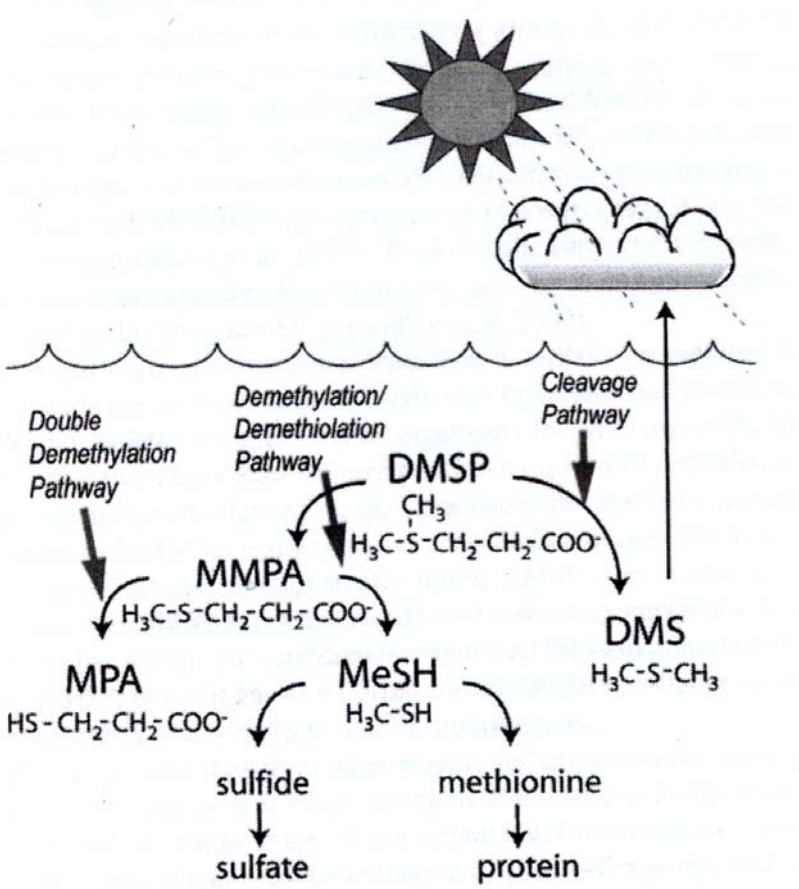


Figure 12: Pathways of bacterial DMSP degradation in seawater. *Roseobacter* isolated are known to carry out both cleavage (to DMS) and demethylation / demethiolation to MeSH (Figure from Moran et al., (2003)).

3.3.2 Sulphur source for TDA

To elucidate the origin of the sulphur in TDA, *Silicibacter* TM1040 and *Roseobacter* 27-4 were grown in Marine Basal Medium (MBM) (Baumann and Baumann 1981; Gonzalez et al., 1997), where sodium sulphate was replaced with sodium chloride. DMSP, MMPA, sulphate, amino acid, cysteine and methionine were added as sulphur source with glucose as carbon source. However, *Roseobacter* 27-4 was unable to produce TDA without addition of peptone and yeast extracts.

Formation of pigment (OD398) correlated linearly with sulphate concentration and inhibition zones were measured in the well diffusion assay when OD398 was above 0.3 (Table 5). This demonstrates that sulphate can be used as the sulphur source for TDA production. Sulphate is present in the marine environment at approx 28mM. Hence, this would be sufficient to act as TDA substrate (Geng et al., 2007).

Pigment and inhibition zones were also detected when mixed amino acids and cysteine (as single amino acid) acted as substrates (Table 5). *Silicibacter* TM1040 did not produce TDA and pigment when DMSP, MMPA or methionine were added as the only sulphur

source. Cell densities of *Silicibacter* TM1040 increased in the presence of DMSP indicating that the DMSP was degraded during the experiments. The data do not demonstrate that DMSP, MMPA or methionine as sulphur sources cannot be used in the marine environment, but TDA was not measured in MBM with either of these compounds as a single sulphur source.

Table 5: Log cell density, OD398 and inhibition zones from sterile supernatants in *Silicibacter* TM1040 cultures grown in MBM without sulphate, in the presence of varying sulphur sources (Geng et al., 2007).

Sulphur source	Log_{10} CFU/ml	OD398	Inhibition zone mm.
No sulphate added	6.8 ± 0.1	0.00 ± 0.00	0 ± 0
2 µM sulphate	6.6 ± 0.1	0.00 ± 0.00	0 ± 0
20 µM sulphate	5.2 ± 0.3	0.02 ± 0.00	0 ± 0
40 µM sulphate	5.7 ± 0.2	0.07 ± 0.03	0 ± 0
80 µM sulphate	7.1 ± 0.2	0.11 ± 0.01	0 ± 0
100 µM sulphate	7.9 ± 0.3	0.29 ± 0.03	17 ± 4
200 µM sulphate	8.3 ± 0.3	0.38 ± 0.02	19 ± 1
2 mM sulphate	8.4 ± 0.0	0.45 ± 0.06	21 ± 1
22 mM sulphate	8.4 ± 0.0	0.49 ± 0.02	21 ± 0
0,05% amino acid	7.4 ± 0.0	0.01 ± 0.00	0 ± 0
0,5% amino acid	8.4 ± 0.1	0.05 ± 0.01	0 ± 0
1% amino acid	8.1 ± 0.2	0.12 ± 0.01	12 ± 0
2% amino acid	8.3 ± 0.1	0.23 ± 0.02	14 ± 1
100 µM Methionine	5.2 ± 0.2	0.01 ± 0.00	0 ± 0
1 mM Methionine	5.8 ± 1.1	0.02 ± 0.00	0 ± 0
10 mM Methionine	7.6 ± 0.5	0.03 ± 0.00	0 ± 0
100 µM Cysteine	8.1 ± 0.3	0.14 ± 0.04	13 ± 1
1 mM Cysteine	8.5 ± 0.0	0.32 ± 0.04	19 ± 1
10 mM Cysteine	< 5.0	0.06 ± 0.01	0 ± 0
100 µM DMSP	6.4 ± 0.1	0.00 ± 0.00	0 ± 0
1 mM DMSP	7.4 ± 0.1	0.00 ± 0.00	0 ± 0
10 mM DMSP	7.3 ± 0.2	0.01 ± 0.00	0 ± 0
100 µM MMPA	6.4 ± 0.3	0.02 ± 0.00	0 ± 0
1 mM MMPA	6.4 ± 0.2	0.00 ± 0.00	0 ± 0
10 mM MMPA	< 5.0	0.00 ± 0.00	0 ± 0

Bacteria are only capable of incorporating sulphur from DMSP into protein if degrading DMSP to MeSH (Kiene et al., 1999). MeSH was not detected during degradation of DMSP by *Silicibacter* TM1040 (Miller and Belas 2004), however, the sulphur will mainly be incorporated into methionine if MeSH is produced under other environmental conditions (Kiene et al., 1999). *Silicibacter* TM1040 was not capable of utilizing methionine as a single sulphur source to form TDA, indicating that sulphur from DMSP cannot be incorporated into TDA.

Pigment and antibacterial compound were not detected if *Silicibacter* TM1040 was grown in MBM in the presence of 200µM sulphate without glucose. The low cell density at about 7.0 log CFU/ml may partly explain the absence of pigment and inhibition zones, however pigment was measured at the same cell density in the presence of glucose, suggesting that TDA was produced. Glucose may therefore acts as carbon source for the formation of TDA in the absence of amino acids, as inhibition zones and pigment were detected when *Silicibacter* TM1040 was grown with mixed amino acid in the absence of glucose. No pigment and inhibition zone were seen when varying concentrations of DMSP were added to samples containing 200µM sulphate, indicating that DMSP cannot be utilized as the carbon source for TDA.

3.4 Synthesis and regulation of TDA

Genes involved in synthesis of TDA were identified via Tn5 based transposon insertion library in both *Roseobacter* 27-4 and *Silicibacter* TM1040. Mutants devoid of antibacterial activity were selected for analysis, and all mutants devoid of antibacterial activity were non-pigmented, indicating a strong correlation between pigment and formation of antibacterial compounds (Brinkhoff et al., 2004; Bruhn et al., 2005b). The full genome of *Silicibacter* TM1040 has been sequenced, hence the analysis was conducted in this bacterium. The locations of the transposons in the TDA negative mutants in the *Silicibacter* TM1040 genome are shown in Figure 13. Some of the inserts were in genes located on a cryptic plasmid that has not been sequenced as part of the genome.

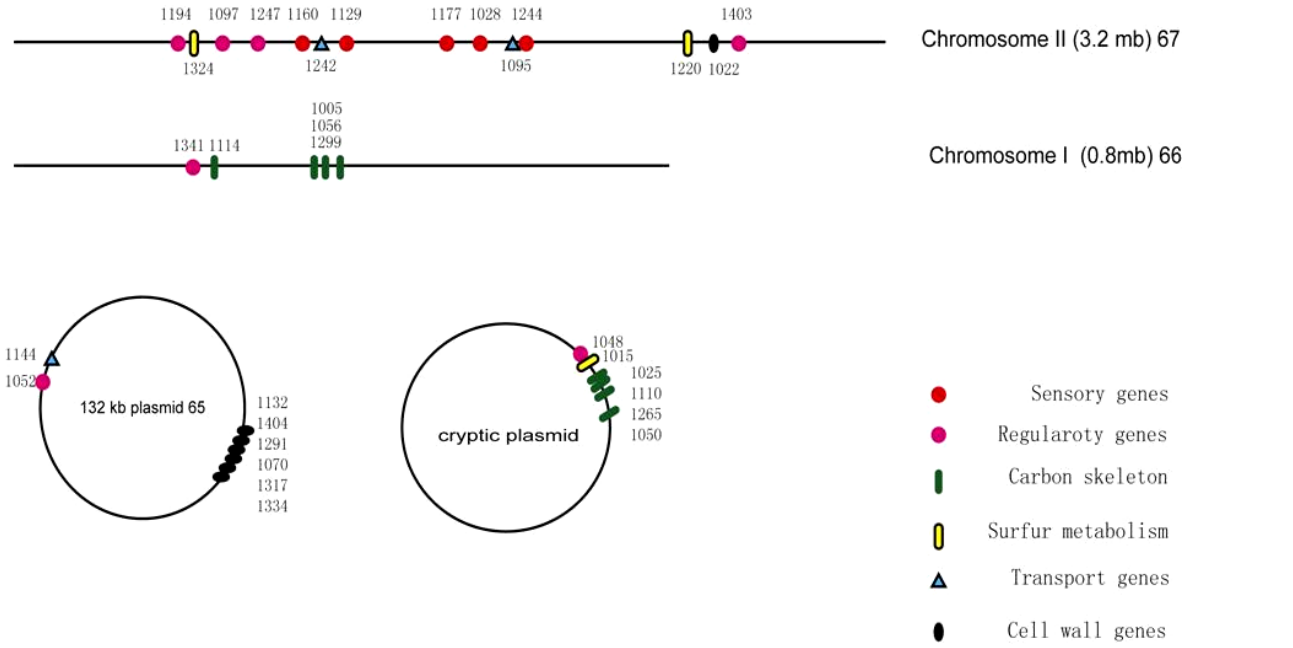


Figure 13: Locations of the transposons in the *Silicibacter* TM1040 genome. Genes are marked depending on function. Numbers refer to the mutant number (Geng et al., 2007).

A biosynthesis pathway and the regulation of TDA in *Silicibacter* TM1040 were suggested by combining information on the genes identified (Geng et al., 2007). All genes identified in *Roseobacter* 27-4 corresponded to genes found in *Silicibacter* TM1040 suggesting that the biosynthetic pathway and regulation functions are similar in this bacterium (Geng et al., 2007).

3.4.1 Biosynthesis of TDA

During biosynthesis of antibacterial compounds, organisms often use parts of pathways for primary metabolites (Martin and Demain 1980). This also seems to be true for TDA, as approx. 65% of the genes identified in non-TDA producing mutants of *Silicibacter* TM1040 were house keeping genes. Coenzyme A (CoA) and the shikimate pathway were essential in the biosynthesis and these two where combined via the phenylacetate-CoA ligase (PaaK) with phenylacetate as substrate from the shikimate pathway (Figure 14).

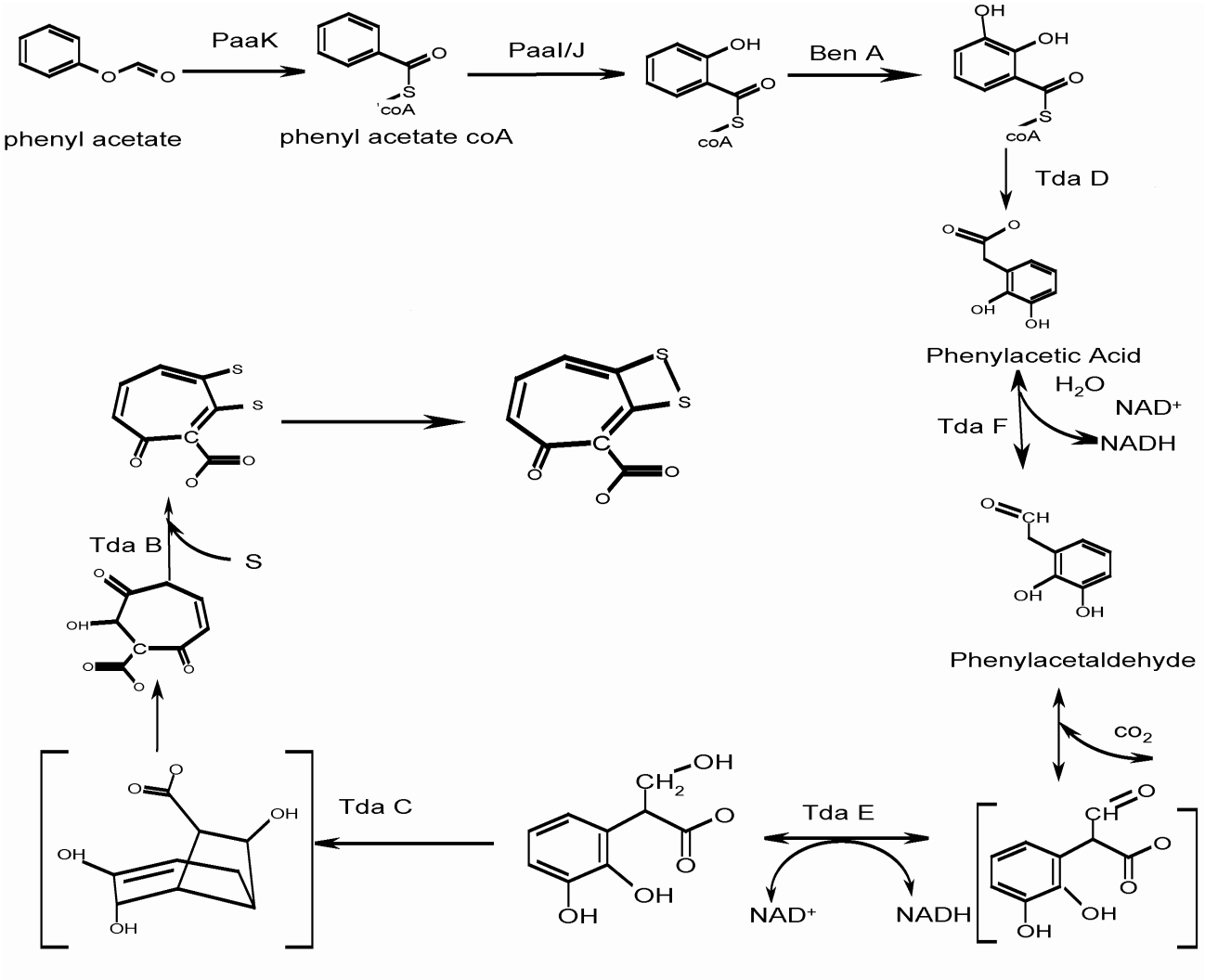


Figure 14: Suggested biosynthesis pathway of TDA in *Silicibacter* TM1040 (Geng et al., 2007).

The shikimate pathway is an essential metabolic route by which bacteria synthesize the aromatic amino acids phenylalanine, tyrosine and tryptophan as well as a number of other aromatic compounds (Floss 1997). Substrates to the shikimate pathway originate from the glycolysis, which agrees with that glucose may act at carbon source for TDA. By labelling glucose Cace et al., (1992) demonstrated that the shikimate pathway is part of the synthesis of TDA in *Pseudomonas* CB-104. The phenylacetate can also originate directly from the amino acid phenylalanine (Cane et al., 1992) and this may explain why growth substrates supplemented with L-phenyalanine resulted in a more pure TDA with less by-products (Heidorn 2002).

The final five steps in the synthesis are conducted by the genes *tdaB-tdaF.* These genes are regulated by *tdaA*, and *tdaA-tdaF* is located in an operon on a cryptic plasmid (Figure 13 and 14) (Geng et al., 2007). The location of these important genes on a plasmid indicate that the loss of antagonistic activity found in several stable spontaneous mutants (Bruhn et al., 2007), may be a result of the loss of this plasmid. Brinkhoff et al., (2004) found stable spontaneous mutants of *R. inhibens* T5 lacking the ability to produce TDA. Furthermore one could suggest that the transfer of such a plasmid could give other *Roseobacter* the ability to produce TDA.

Cace et al., (1992) did not provide experimental data demonstrating how the two sulphur atoms are added to TDA, and our genetic analysis did not reveal the source of sulphur. However, we suggested that cysteine or its intermediates may be the source (Geng et al., 2007). Cysteine acts as sulphur source for TDA. Also TDA is produced when sulphate is added, but this is probably because sulphate can be assimilated into cysteine by the reductive sulphur metabolism (Truper 1975; Leustek et al., 2000). The reductive sulphur metabolism was disrupted by a transposon in the *cysI* (Sulfite reductase) gene. This mutant was unable to produce TDA in medium containing sulphate or methionine as the only sulphur source, however, when adding exogenous cysteine the mutants were able to produce TDA. This demonstrates that cysteine is an important substrate for TDA and indicates that methionine cannot be converted to cysteine. However, this doesent demonstrate that methionine cannot act as substrate in the natural habitat.

3.4.2 Regulation of TDA production

The genetic analysis indicate that TDA loss of function mutations affected genes encoding regulatory proteins and strongly suggests that TDA synthesis is regulated at the level of transcription (Geng et al., 2007). The genetic analysis revealed that tranposons hit homologs of regulatory genes such as *rpoE*, *rsbU* and *hrcA*, these genes are part of a stress response within several bacteria (Raina et al., 1995; Chen et al., 2003; Susin et al.,

2004). Stress often induces antibiotic production in bacteria (Marahiel et al., 1993; Sarniguet et al., 1995; Haas and Keel 2003). The regulation of antibiotic in several *Bacillus* spp is an interaction of diverse factors (e.g. nutritional stress), which also control other stationary phase induced processes in *Bacillus* spp e.g. sporulation (Marahiel et al., 1993). High levels of antibiotics are usually only produced in the stationery phase when antibiotic producing bacteria are grown in nutrient rich media (Martin and Demain 1980). This is also the case for *Silicibacter* TM1040 (Bruhn et al., 2007).

If one believes that antibacterial compounds are a competitive phenotype produced to inhibit other organisms, it should be absent in the exponential growth phase where rapid growth is the best way to compete. In some experiments, growth rate is a major parameter controlling the long term bacterial population density (Grossart et al., 2003). The absence of antibacterial compound in the exponential phase may also be explained by the bacteria themselves being more sensitive in the exponential phase. Thus, they might be inhibited by their own production of antibacterial compounds (Martin and Demain 1980).

LysR-type family represents a common class of transcriptional regulatory proteins and members function as both activators and repressors of a wide variety of target genes e.g. virulence genes (Kovacikova and Skorupski 2002; Heroven and Dersch 2006), genes encoding antibacterial and antifungal compounds (Naas et al., 1995; Silby et al., 2005) and genes involved in sulphur utilization (Jovanovic et al., 2003). A LysR-type family transcriptional regulator homolog was also hit by a transposon resulting in loss of TDA production, indicating that this is part of the regulation of TDA in *Silicibacter* TM1040 (Geng et al., 2007).

Antifungal activity is regulated by a LysR-type regulator in *Pseudomonas aureofaciens* and a mutant in the LysR regulatory gene does not produce antifungal activity, but the mutant grew to higher numbers in the stationary phase as compared to the wild type (Silby et al., 2005). Similar findings were seen for both *Roseobacter* 27-4 and *Silicibacter* TM1040 as antibacterial activity negative mutants grow to about ½ log unit higher cell density in the stationary phase (unpublished results Bruhn). As production of an antibacterial compound may be a “burden” for the bacteria, it must provide the producer cell with an advantage, otherwise the negative mutants would take over and become dominant.

Production of antibacterial compound may give the bacteria other advantages than just inhibiting other bacteria, as these compounds may also act as signal compounds (Yan et al., 2003; Mai-Prochnow et al., 2006). The antibacterial compound in a *Bacillus* strain serves as a signal, which induce planktonically grown cells to behave as if they were in a biofilm (Yan et al., 2003). An antibacterial and autocidal protein produced by

Pseudoalteromonas tunicate was suggested to confer an ecological advantage to the bacteria dispersed from biofilms. These cells were metabolically highly active in phenotypes suggested to be important for colonization of new surfaces (Mai-Prochnow et al., 2006).

3.5 Conclusions from chapter 3

The antibacterial compound produced by *Roseobacter* 24-7 and some other members of the *Roseobacter* clade is tropodithietic acid (TDA). TDA affects both eukaryotic and prokaryotic cells, but eukaryotes seem less sensitive as compared to prokaryotes. TDA affected *Roseobacter* and a few other strains to a lesser degree than a range of other tested strains, hence production of TDA may give members of the *Roseobacter* clade a selective advantage during interaction.

It was suggested that DMSP might act as sulphur source for TDA, however it was not possible to demonstrate this. It was demonstrated that both sulphate and cysteine act as sulphur source for TDA. Analysis of mutants defective in production of TDA indicated that stress factors are important in the regulation of TDA in *Silicibacter* TM1040. Production of antibacterial compounds reduces the maximum cell density of the population, hence, bacterial strains may regulate their production of antibacterial compound under different growth condition to optimize their growth. One may speculate that production of antibacterial compound is only beneficial under certain growth conditions.

4 Factors affecting production of antibacterial compounds and biofilm in marine bacteria

It is hypothesized in this thesis that the *Roseobacter* antibacterial compound, TDA is an important mechanism underlying its probiotic effect. In order to facilitate and optimize the use of *Roseobacter* strains as probiotic bacteria it is therefore necessary to understand how growth conditions influence the production of TDA. It is furthermore speculated that the biofilm mode of growth could facilitate antagonism and that the probiont may be added as a colonizer of the aquaculture environment. If *Roseobacter* strains are to be administered to tank walls in rearing facilities, phenotypes important for attachment also have to be elucidated. *Roseobacters* are, however, not the only group of marine bacteria with antagonistic effects against other bacteria and the present chapter introduce several marine bacteria that produce antibacterial compounds and discuss how environmental factors can influence the production.

4.1 Antibacterial compounds produced by marine bacteria

Already in 1889, marine bacteria with inhibitory effects against other bacteria were described (Giaxa 1889), and subsequent Rosenfeld and Zobell (1947) described antibiotic producing marine microorganisms. Antibacterial compounds are produced by several marine bacterial groups including *Alteromonas*, *Pseudoalteromonas*, *Pseudomonas*, *Vibrio* all belonging to the γ-Proteobacteria, and also members of the *Roseobacter* clade *Bacillus* spp and *Choromobacterium* spp produce antibacterial compounds (Ivanova et al., 1998; Jensen and Fenical 2000; Imada et al., 2002; Isnansetyo and Kamei 2003; Bruhn et al., 2005b). *Pseudomonas* spp. and/or *Alteromonas* spp. are often most abundant among culturable marine bacteria with antibacterial activity (Lemos et al., 1985; Nair and Simidu 1987; Jayanth et al., 2002), however a range of other culturable bacteria may also produce antibacterial compounds (Grossart et al., 2004; Anand et al., 2006).

Many different antibacterial compounds have been isolated from marine bacteria (Table 6). Most of these compounds are low molecular weight, thermolabile compounds. They are not affected by proteolytic enzymes indicating that they are not proteins (Lovell 1966; Burkhold et al., 1966; Lemos et al., 1985; Jensen and Fenical 2000; Nakashima et al., 2005; Bruhn et al., 2005b; Saha et al., 2006), however, also antibacterial proteins are produced by marine bacteria (Barja et al., 1989; James et al., 1996). There is variation in the antibacterial spectrum of these antibacterial compounds, as some have a broad spectrum and others are more specific (Table 6) (Lemos et al., 1985; Nakashima et al., 2005; Anand et al., 2006; Bhattarai et al., 2006; Saha et al., 2006).

Table 6: Type and spectrum of antibacterial compound produced be marine bacteria.

Marine Bacteria	Antibacterial compound(s)	Active against	Reference
Actinobacterium	Non-proteinaceous (low molecular weight compound)	*E. coli*; *Salmonella enterica; S. aureus*; *Klebsiella pneumoniae*	Saha et al., 2006
Alteromonas spp.	Glycoprotein	Several Gram-positive and Gram-negative strains, did not inhibit *Enterobacter* spp; *Bacillus* spp and *C. albicans*	Barja et al., 1989
Bacillus cereus	Thiocillin (peptide)	Gram-positive bacteria	Nagai et al., 2003
Hahella spp.	Prodigiosin (low molecular weight compound)	Gram-positive bacteria	2005 Nakashima et al., 2005
Halomonas spp.	Aminophenoxaxinones (low molecular weight compounds)	*Escherichia coli; Bacillus subtilis; S. aureus; C. albicans*	Bitzer et al., 2006
Pseudoalteromonas spp	Korormicin (low molecular weight compound)	*Salinivibrio costicola* and *Pseudoalteromonas haloplanktis* did not inhibit *E. coli*	Yoshikawa et al., 2003
Pseudoalteromonas tunicata	a 190-kDa protein	*E. coli*; *Bacillus* spp; *Pseudomonas* spp; *Proteus mirabilis* did not inhibit *Serratia liquefaciens*	James et al., 1996
Pseudomonas bromoutilis	Pentabromopseudiline (low molecular weight compound)	*Staphylococcus* spp; *Streptococcus* spp; *Mycobacterium tuberculosis*; *C. albicans*	Lovell 1966; Burkhold et al., 1966
Roseobacter spp.	Thiodithietic acid (low molecular weight compound)	see Table 4	Brinkhoff et al., 2004; Bruhn et al., 2005b

TDA produced by *Roseobacter* strains has a broad antibacterial spectrum, as all tested bacteria are sensitive to the compound (Table 4). Bruhn et al., (2007) stated that members of the *Roseobacter* clade are less sensitive compared to non-*Roseobacter.* However the sensitivity to TDA was only tested against culturable members and it can therefore not be concluded that the members of the *Roseobacter* clade in general are less sensitivity to TDA, as many members are not culturable (Buchan et al., 2005).

4.1.1 Environmental conditions affecting production of antibacterial compounds

Environmental conditions affect production of antibacterial compounds in several marine

bacteria (Sugita et al., 1997; Ivanova et al., 1998; Marwick et al., 1999; Yan et al., 2002; Yan et al., 2003; Bruhn et al., 2005b; Bitzer et al., 2006). E.g. biofilm formation affect production in a *Bacillus licheniformis*, concentration of marine salt affects production in a *Vibrio* strain and presence of oxygen affects the production in a *Bacillus brevis*. The production of TDA by *Roseobacter* strains is influenced by several factors including temperature, marine salts and physical growth conditions.

Coastal bacteria that produce antibacterial compounds are often pigmented (Nair and Simidu 1987; Jayanth et al., 2002) and in some bacteria, the pigment is the antibacterial compound (Duran and Menck 2001; Nakamura et al., 2002). A strong correlation is found between a brown/black pigment and antibacterial activity for several TDA producing *Roseobacter* strains (Figure 15) (Brinkhoff et al., 2004; Bruhn et al., 2005b; Bruhn et al., 2007), which may indicate that the pigment has an antibacterial activity.

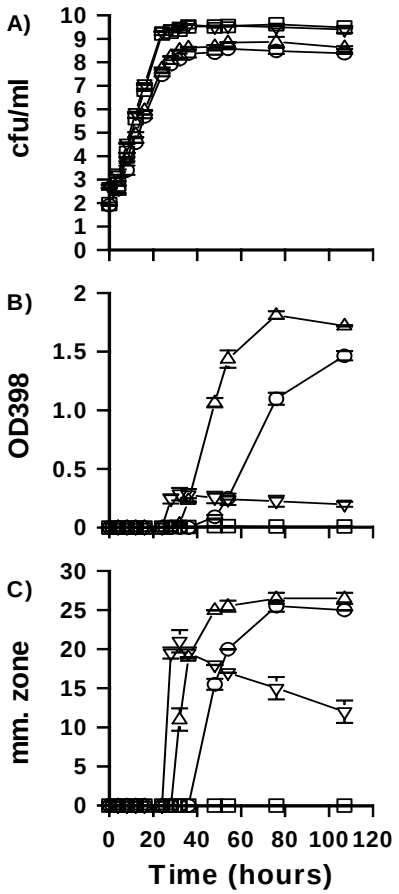


Figure 15: Influence of culture conditions on the growth, production of pigment, and antibacterial activity of *Roseobacter* 27-4 and *Silicibacter* TM1040 in MB at 25°C. A) Growth measured by plate counts: □ shaken culture of *Roseobacter* 27-4, ○ static culture of *Roseobacter* 27-4, ▽ shaken culture of *Silicibacter* TM1040, △ static culture of *Silicibacter* TM1040. B) Pigment formation, as measured by absorbance at OD_{398}. C) Antibacterial activity represented by the zone of inhibition (in mm), as determined using the well diffusion assay with *V. anguillarum* (Bruhn et al., 2007).

Pigmented compounds can indeed have active antibacterial activity e.g. violacein produced by *Chromobacterium violaceum* (Duran and Menck 2001; Nakamura et al., 2002). However the brown pigment produced by *Roseobacter* strains was not the

antibacterial compound (Bruhn et al., 2005b; Professor Dr. A. Zeeck personal communication), as the pigment was retained on the purification column, whereas the active compound was not.

The pigment production and TDA antibacterial activity were absent or reduced under shaken growth conditions in *Roseobacter* 27-4 and *Silicibacter* TM1040 (Figure 15) (Bruhn et al., 2005b; Bruhn et al., 2007). Also shaken growth conditions affected both production of antibacterial activity and pigment in a *B. licheniformis* as none of these were produced under shaken growth conditions (Yan et al., 2002; Yan et al., 2003).

The surface volume ratio affected the production of pigment and TDA in *Roseobacter* 27-4 (Bruhn et al., 2005b) and pigment formation always started at the surface media interface (Figure 16), which suggests that the atmosphere may affect the production.

Figure 16: Production of pigment by *Roseobacter* 27-4 depending on the surface volume-ratio of marine broth. Biofilm and pigment formation was visible at the air-media interface after a few days incubation at 25°C (Bruhn et al., 2005b).

Atmosphere can be important for production and stability of antibacterial compounds. The presence of oxygen inhibits the production and accelerated the breakdown of the antibacterial compound gramicidin in *B. brevis* (Friebel and Demain 1977), but had no effect on production of pigment and antibacterial compound in *Roseobacter* 27-4 (Table 7). Growth of *Roseobacter* 27-4 stopped when the atmosphere was changed using carbondioxide.

Table 7: Production of pigment by *Roseobacter* 27-4 when grown under different atmospheres (Bruhn, unpublished results).

% composition				Pigment and TDA production
Air, atmosphere	Oxygen	Nitrogen	Carbondioxide	
56	44			+[1]
40	60			+
15		85		+
10		90		+
4		96		+
80			20	-
50			50	-

[1]+: Similar levels of pigment and antibacterial activity as compared to normal atmosphere

Several members of the *Roseobacter* clade produce pigments to utilize light (Wagner-Döbler and Biebl 2006) and accumulation of the phototynthetic pigments in *Roseobacter denitrificans* occurred only in darkness (Takamiya et al., 1992). It is not known if these photosynthetic pigments have antibacterial activity, but we investigated if light affected the production of pigment in *Roseobacter* 27-4. Results were inconclusive as light appeared to affect pigmentation negatively when the organism was grown on agar-plates, but these data could not be reproduced in liquid medium (unpublished results by Bruhn).

Marine salt influences the production of antibacterial compounds in marine bacteria as a higher degree of antibacterial activity was observed in a streak test on agar plates when using 3.5% marine salts in the growth substrate compared to 1% (Strahl et al., 2002). If the influence of marine salt is due to enhanced production of antibacterial compound or higher sensitivity by the target bacteria is unknown. The concentration of marine salts affected *Vibrio* NM10 production of antibacterial compound, where the highest activity was seen when adding 50% seawater to the media (Sugita et al., 1997). Sterile filtration of marine broth decreased the production of TDA in *Roseobacter* 27-4, suggesting that insoluble salts are important for TDA production (Bruhn et al., 2005b). Several of the marine salts may be important for instance iron, which induced the production of antibacterial compound in *B. licheniformis* and *Pseudomonas syringae* (Palmer and Bender 1993; Yan et al., 2003). The NaCl concentration may also affect the antibacterial activity, as increased NaCl concentrations above 1% caused a decrease in antibacterial activity in *Pseudomonas* I-2 and *P. syringae* (Palmer and Bender 1993; Chythanya et al., 2002).

Growth substrates may also affect production of antibacterial compounds by marine bacteria as using a carbon sources which is slowly utilized, gives an improved antibiotic yield as compared to glucose (Martin and Demain 1980; Marwick et al., 1999). Specific carbon and nitrogen sources may also have an influence on production of antibacterial compounds. E.g. only with anthranilic acid, as both carbon and nitrogen source, did *Halomonas* spp produce five antibacterial aminophenoxazinones (Bitzer et al., 2006). Furthermore, when utilizing cysteine as a single amino acid source *Silicibacter* TM1040 produced TDA, however in the presence of methionine as single amino acid source no TDA was produced (Geng et al., 2007).

Both the general microbiota of sponge surface associated bacteria and bacteria with antibacterial activity change over the year, which may be a temperature effect (Thakur et al., 2004). Temperature can also influence production of antibacterial compounds in several marine bacteria. Production of TDA in *Roseobacter* 27-4 decreased at 30°C as compared to temperatures at 20 or 25°C (Bruhn et al., 2005b). Similarly, the antibacterial

activity in *Vibrio* strain NM10 and *P. syringae* was decreased at 30°C as compared to lower growth temperatures, however in *Pseudomonas* I-2 the highest activity was seen at 30 and 37°C (Palmer and Bender 1993; Sugita et al., 1997; Chythanya et al., 2002).

Antibacterial activity was only detected in late exponential and stationary phases for several marine probiotic bacteria (Figure 15) (Chythanya et al., 2002; Jayaprakash et al., 2005; Vijayan et al., 2006; Bruhn et al., 2007). This may be due to detection limits of the assay or indeed that the compound is only produced in the stationary phase. Brinkhoff et al., (2004) stated that TDA was produced by *R. inhibens* T5 in the complete growth phase, but based on the presented data they only documented that TDA was produced in the late exponential and stationary phase.

4.1.2 Quorum sensing control of antibacterial compounds

The detection of TDA in late exponential/stationary phase could lead one to speculate that the compound is quorum sensing regulated. The production of antibacterial compounds may be controlled in a quorum dependent manner (Bainton et al., 1992; Throp et al., 1995; Wood et al., 1997) e.g. the probiotic bacteria *P. aureofaciens,* which is used to protect wheat against filamentous fungi, regulate the expression of three antibiotics in situ on plant roots by using a quorum sensing system (Wood et al., 1997). In a collection of 13 members of the *Roseobacter* clade, AHLs were only detected in strains that produced antibacterial activity (Bruhn et al., 2007). But there was no direct link between AHL production and antibacterial activity as antibacterial activity was also detected in strains that did not produce AHLs e.g. *Silicibacter* TM1040. Quorum sensing signal compounds are produced by several members of the *Roseobacter* clade (Gram et al., 2002; Wagner-Döbler et al., 2004; Bruhn et al., 2005b). In *Roseobacter* 27-4 *N*-(3-hydroxydecanoyl)-L-homoserine lactone is produced, which is also detected in the TDA producing *R. inhibens* T5 (Wagner-Döbler et al., 2004; Bruhn et al., 2005b). Quorum sensing regulation of antibacterial compounds could be a way for the bacteria to optimize their competitive ability. At low cell densities there may be less need for competition, whereas at higher cell densities it may be beneficial to produce antibacterial compounds when the nutrient levels are decreasing. To date no studies have demonstrated quorum sensing controlled phenotypes in members of the *Roseobacter* clade.

4.2 Cell morphology and biofilm formation of Roseobacter

An intriguing characteristic of several *Roseobacters* is their growth as starshaped rosettes (Figure 17). Rosette forming bacteria are often found in the marine environment and

especially members of the *Roseobacter* clade form rosettes (Leifson et al., 1964; Ruger and Hofle 1992; Gonzalez et al., 1997; Uchino et al., 1998; Labrenz et al., 1998; Pukall et al., 1999; Gonzalez et al., 2003; Pladdies et al., 2004; Larimer et al., 2004; Amaro et al., 2005; Bruhn et al., 2005b; Jansen 2006; Bruhn et al., 2007), however, it is not a specific marine phenomena as also soil bacteria produce rosettes (Whang and Hattori 1988).

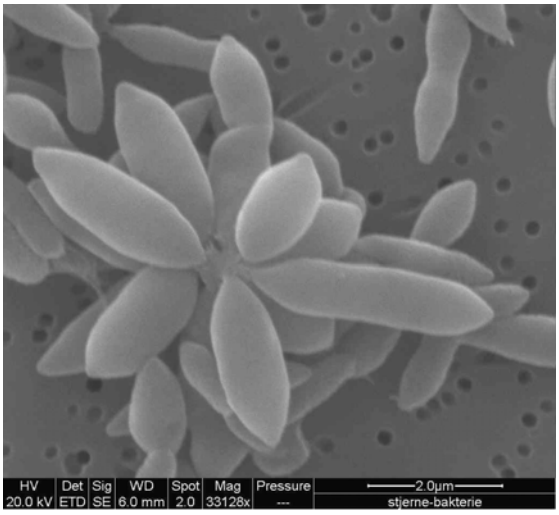


Figure 17: Scanning electron micrographs of *Roseobacter* strain 27-4 grown in Marine Broth under static conditions. Standard fixation and dehydration procedure was used (Bruhn et al., 2005b).

One can speculate on which advantages the bacteria obtain by forming this structure, and it can be suggested that the rosette formation reduce grazing of the bacteria. Size of bacteria is suggested as a defence mechanism for bacteria to avoid grazing (Lebaron et al., 1999). Optimal bacterial grazing for *Spumella guttula* occurs at bacterial sizes between 0.8 to 1.5 µm and grazing is rapidly decreased when the size of the bacteria exceeds 1.5 µm (Kinner et al., 1998). When forming the rosette structure the size of the aggregates is about 5 µm in diameter (Figure 17) (Bruhn et al., 2005b), which can make them unattractive for grazing. Members of the *Roseobacter* clade have been detected before and after grazing, while others were not detected after grazing indicating that this group has a defence mechanism, however it is unknown if these isolates formed rosettes (Lebaron et al., 1999).

The mechanism of rosette formation are only partially described, but may be linked to the flagella system of bacteria (Leifson et al., 1964). Several strains of motile marine bacteria are capable of forming rosettes (Leifson et al., 1964). Furthermore, Bruhn et al., (2007) found a correlation between rosette formation and motility as all rosette forming strains were motile and only one strain was motile without forming rosettes. The flagella were important for the rosette formation in strains belonging to *Caulobacter* as the cell attached to each other by using the flagella (Leifson et al., 1964). The rosette formation in *Agrobacterium luteum* was due to polar fibrils, which are spread around the rosette shaped

aggregate. These fibrils were up to 10.5 µm long (Ahrens et al., 1968). Also the cells of *Roseobacter* 27-4 attached to each other by a fibril (Figure 17), however, the cells seemed to divide into the rosette formation rather than single cells attaching to each other. No flagella are observed on cells in the rosette formation, but during planktonic growth both *Roseobacter* 27-4 and *Silicibacter* TM1040 are highly motile. It can be speculated that the flagella system in bacteria is part of the formation of the fibril by which the cells are attached. This may explain the correlation between rosette formation and motility.

Growth conditions affected the formation of rosettes in *Roseobacter* 27-4 as static grown cultures are dominated by rosette forming aggregates in contrast to shaken cultures, which mostly consist of motile planktonic cells (Figure 18). Also the TDA producing *R. inhibens* T5 formed similar rosette structure in static culture (Bruhn personal observation). *Silicibacter* TM1040 also forms TDA, pigment and rosettes when grown as a shaken culture, albeit at a much lower level than in stagnant cultures (Figure 15 and 18). However the rosette formation is not directly correlated to TDA production as the non-pigmented TDA negative spontaneously mutants in *Silicibacter* TM1040 still form rosettes (Bruhn et al., 2007).

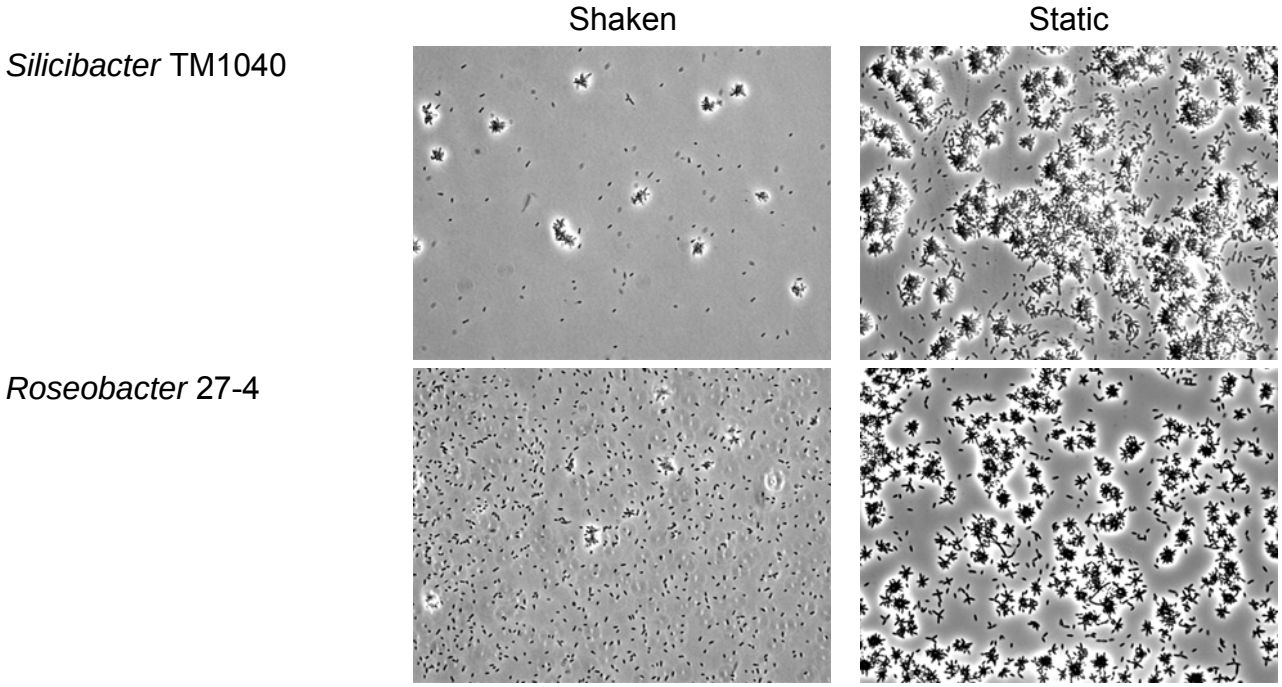


Figure 18: *Silicibacter* TM1040 and *Roseobacter* strain 27-4 grown in Marine Broth at 20ºC under static and aerated conditions. Pictures are from phase contrast microscope at x 1000 magnification (Bruhn et al., 2007).

Pladdies et al., (2004) investigated rosette forming bacteria in the neuston, which are bacteria which float on or drift immediately under the water surface, and found three different rosette morphotypes. They believed that these morphotypes enable the bacteria

to grow at the air-water interface, which is also where the highest concentration of rosettes are found in cultures of *Roseobacter* (Bruhn et al., 2005b; Bruhn et al., 2007). Furthermore, members of the *Roseobacter* clade are most often found in the upper mixed layer in the marine environment (Buchan et al., 2005).

Roseobacter are the most rapid colonizer of surfaces in the coastal environments (Dang and Lovell 2000; Dang and Lovell 2002). Bruhn et al., (2007) believed that this high attachment capability is caused by the ability to form rosettes, as *Roseobacter* strains with a pronounced rosette formation under static growth conditions were most affective in attachment to surfaces and producing a biofilm at the air liquid interface (Figure 19) (Bruhn et al., 2006; Bruhn et al., 2007).

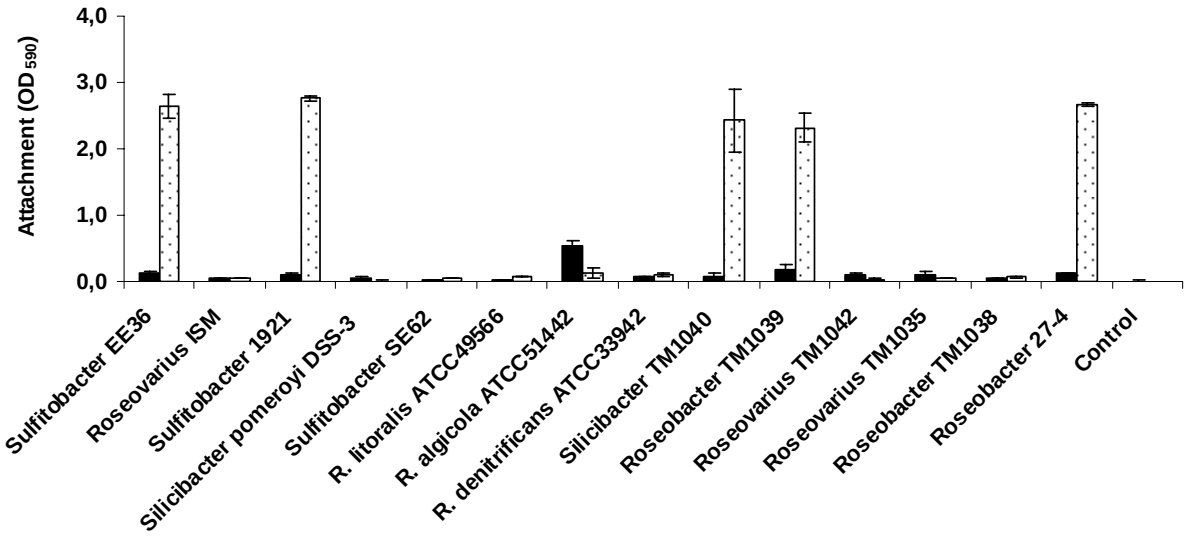
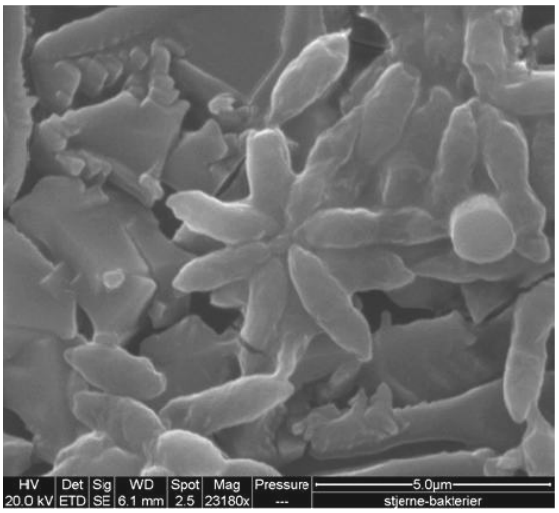


Figure 19: Attachment of *Roseobacter* clade bacteria from static (Dotted bars) or shaken (Filled bars) cultures to glass cover slides measured by crystal violet at OD_{590}. *Sulfitobacter* EE36, *Sulfitobacter* 1921, *Silicibacter* TM1040, *Roseobacter* TM1039 and *Roseobacter* 27-4 all had a pronounced rosette formation under static growth conditions (Bruhn et al., 2007).

The biofilm formed at the air liquid interface contained rosettes attached to each other (Bruhn et al., 2005b). The slime layer covering the rosette seen in Figure 20 may be one reason for rosette-rosette attachment. This slime may be exopolysaccharide (ESP), which is an important phenotype during attachment and formation of biofilm (Pratt and Kolter 1999; Jefferson 2004) and ESP is produced by members of the *Roseobacter* clade (Kwon et al., 2002). We have, however, not yet investigated the EPS production in *Roseobacter*.

Figure 20: Scanning electron micrographs of *Roseobacter* strain 27-4 grown in Marine Broth under stagnant conditions. Cells were only exposed to osmium vapour and subsequently coated with gold-palladium (Bruhn et al., 2005b).



The rosette structure is also correlated to the ability of *Roseobacter* 27-4 to form biofilm on inert surfaces, as inoculating static cultures into a flow chamber system resulted in a three-dimensionally biofilm (Figure 21) (Bruhn et al., 2006). In contrast, when inoculating of a shaken culture no three-dimensionally biofilm was observed, even though attached planktonic cells were seen.

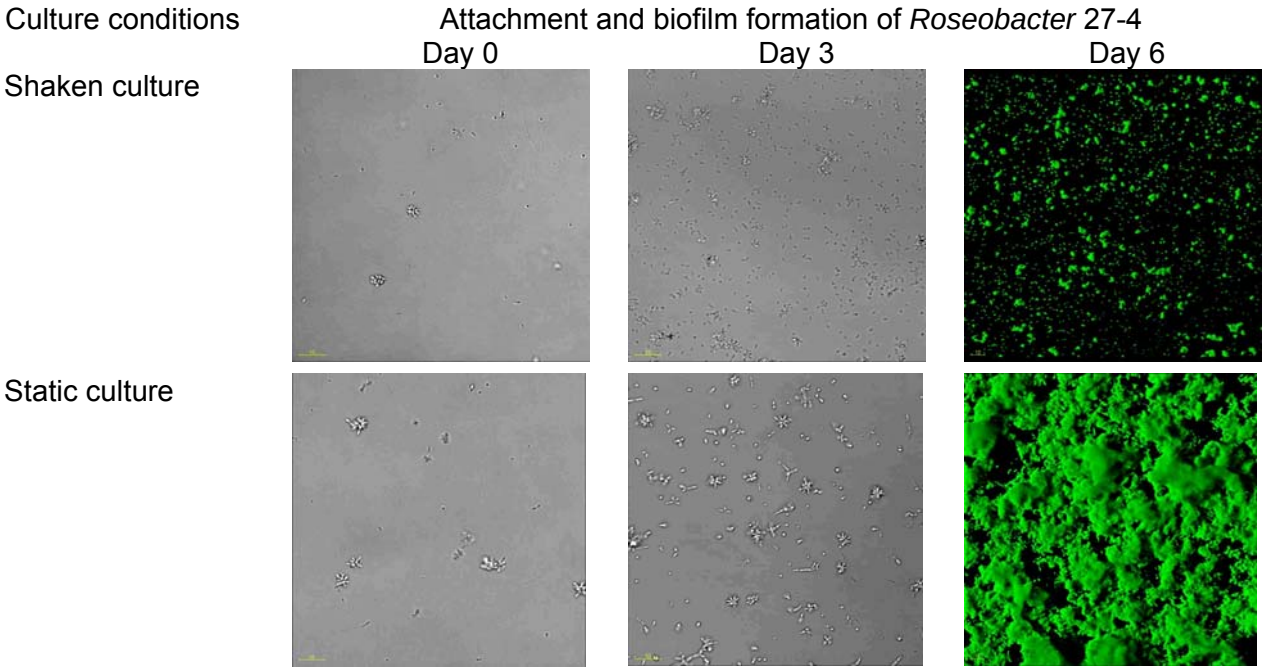


Figure 21: Comparison of biofilm formation of *Roseobacter* 27-4 inoculated from a shaken or static preculture. Pictures from day zero and day three are from phase contrast microscope at x 1000 magnification, pictures from day six are from confocal laser scanning microscopy (Bruhn et al., 2006)

The correlation between rosette and biofilm formation may be a further link to a grazing defence as formation of biofilms is a protective agent against grazing for e.g. *Vibrio cholerae* (Matz et al., 2005). During biofilm formation *V. cholerae* secrete an antiprotozoal

factor, which is part of the defence against grazing. TDA also has an antiprotozoal activity (Tsubotani et al., 1984) and the production of TDA is most pronounced under conditions that facilitate biofilm and rosette formation. The combination of forming both rosette and biofilm and producing TDA may be optimal in avording grazing.

4.3 Conclusions from chapter 4

Production of antibacterial compound is widespread among marine bacteria. Most of these compounds are low molecular weight non-proteinaceous substrates, however, also proteinaceous compounds are found. The ability to produce antibacterial compounds is often associated with the ability to produce pigments. The production of antibacterial compounds is in several marine bacteria affected by growth conditions such as concentration of marine salts, atmosphere, available nutrients and physical growth conditions. The marine environment is however not unique in containing bacteria with antibacterial activity as e.g. soil also contains antibacterial producing strains especially *Bacillus* spp. and *Pseudomonas* spp. The compounds in these bacteria are affected in a similar manner as for the marine bacteria (Duffy and Defago 1999; Raaijmakers et al., 2002; Haas and Keel 2003; Urdaci and Pinchuk 2004).

During growth conditions that enhanced production of antibacterial compounds a rosette shaped aggregate was dominating in *Roseobacter* cultures. This rosette shape structure is correlated to biofilm formation, which could indicate that a biofilm mode of growth facilitate antagonism of *Roseobacter.* Correlations between biofilm formation and antibacterial compound can also be found in other marine bacteria (Yan et al., 2002; Yan et al., 2003), hence one may suggest that these phenotypes could be important for colonisation of the marine environment. Rosette forming bacteria are found both in marine environment as well as in other environments. However, most reports of rosette forming bacteria deal with members of the *Roseobacter* clade. The bacteria may have different advantages by producing the rosette shape e.g. it may reduce grazing.

5 Habitats of *Roseobacter* in the marine environment

It is likely that phenotypes such as ability to attach, form biofilms and produce antibacterial compounds are important for members of the *Roseobacter* clade in their natural habitat. This chapter will describe the habitats of *Roseobacter* in the marine environment as the *Roseobacter* clade is exclusively marine or hypersaline (Buchan et al., 2005).

The marine environment contains approximately 10^6 cells/ml of surface seawater and the diversity of bacteria strains in the marine environment is enormous, however the majority (80%) of marine bacteria fall into only nine major phylogenetic groups (Figure 22). These groups include the SAR11 (*Pelagibacter ubique*), which is the most abundant bacteria in the marine environment and the *Roseobacter* clade, both members of the α-proteobacterial group (Giovannoni and Rappe 2000; Morris et al., 2002; Giovannoni and Stingl 2005).

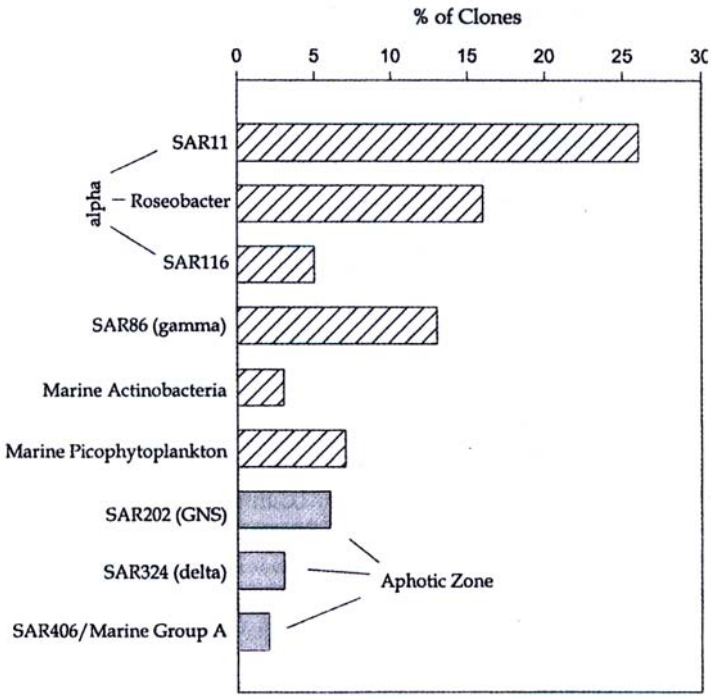


Figure 22: Frequency of the most common bacterioplankton SSU rRNA gene clusters. Frequencies were determined by dividing the number of clones from a particular gene cluster by the total number of clones in the data set. Clones from marine snow and sediments, and Archaea, were not included in the data set (Figure from Giovannoni and Rappe 2000).

It is debated if marine bacterioplankton are cosmopolitans in other words if all bacteria are everywhere and if it is just a question of detecting them or if real distribution are found in the marine environment (Pedros-Alio 2006). It seems that some marine bacterioplankton are cosmopolitans e.g. members of the SAR11 (*Pelagibacter ubique*), one member of the *Roseobacter* clade and a member of the SAR86 clade, where as other are restricted to

some regions (Pommier et al., 2005). Distribution is also found within the *Roseobacter* clade as a newly discovered cluster affiliated to the *Roseobacter* clade is only found in the arctic and southern oceans (Selje et al., 2004).

Our understanding of the marine ecology is to some degree based on culturable marine bacteria, however only 0.1% to 6% of marine bacteria are so far found to be culturable (Lanoil et al., 2000; Bernard et al., 2000; Simu et al., 2005). Until recently, only the *Roseobacter* clade and the marine Picophytoplankton had members that were culturable, among the nine most abundant bacterialplankton groups, giving these two groups a central role when investigating marine ecology, however the first culturable member *Pelagibacter ubique* of SAR11 is now found (Giovannoni et al., 2005). Most culturable bacteria in the marine environment belong to the γ-Proteobacteria (Giovannoni and Rappe 2000). *Alteromonas* spp and *Vibrio* spp were found to be the most abundant among culturable strains isolated from the Sargasso Sea (Lanoil et al., 2000), and members of the *Roseobacter* clade only accounted for a small proportion. However, members of the *Roseobacter* clade dominated among culturable bacteria in the Baltic and Skagerrak Sea (Simu et al., 2005). This may be explained by that members of the *Roseobacter* clade were the most active in the North Sea to incorporate glucose, which is typically the most abundant free neutral aldose in seawater (Alonso and Pernthaler 2006).

Members of the *Roseobacter* clade are mostly non-culturable and 68% of the members have presently no close relatives in cultures, however, this clade is still one of the most accessible of the major marine taxa. Insights gained form the culturable members of the *Roseobacter* clade will continue as the basis of testable hypotheses for illuminating the ecological roles of this group (Buchan et al., 2005).

The representation of *Roseobacter* in upper mixed layer of seawater ranges from not detected and up to 27%. The highest density is found in costal environment and the population decrease with depth (Buchan et al., 2005). Members of the *Roseobacter* clade are often the most abundant in bacterial communities associated with marine algae, including phytoplankton blooms and algal cultures (Table 8). Harmful algal booms are increasing in frequency along the coastal regions around the world. The algae are typically colonized by bacterial communities, which may play a role in the development of the algal bloom. Members of the *Roseobacter* clade are known to interact with algae, and enhanced growth of the algal is found in the present of some *Roseobacter* strains (Alavi et al., 2001; Alavi 2004). Other *Roseobacter* strains produce algal lytic compounds (Amaro et al., 2005) and also TDA can be toxic to algal (Kawano et al., 1997; Liang 2003).

Table 8: Bacterial groups dominating in association with phytoplankton, estimated by numbers of sequences or culturable bacteria.

Phytoplankton	Dominating among isolated sequences or culturable bacteria	Detection method(s)	Isolated from	Reference
Alexandrium spp.	46% *Roseobacter* clade, 31% Cytophaga-Flavobacteriym-Bacteroides (CFB)	PCR and DGGE analysis of the 16S rRNA gene	Gulf of maine (US)	Jasti et al., 2005
Alexandrium spp.	42% *Roseobacter* clade, 30% *Pseudoalteromonas*	PCR and DGGE analysis of the 16S rRNA gene	Orkney Isles (UK)	Wichels et al., 2004
Mixed Phytoplankton	67% *Roseobacter* clade, 33% CFB group	PCR and DGGE analysis of the 16S rRNA gene	Bay of Fundy (US)	Rooney-Varga et al., 2005
Pfiesteria piscicida	50% α-proteobacteria of which 33% were reported to belong to the *Roseobacter* clade	PCR of the 16S rRNA gene Restiction fragment length polymorphism analysis (RFLP)	Laboratory culture	Alavi et al., 2001
Thalassiosira rotula and *Skeletonema costatum*	42% *Roseobacter* clade, 31% CFB group	PCR and DGGE analysis of the 16S rRNA gene	Laboratory cultures	Grossart et al., 2005
Thalassiosira rotula	31% α-proteobacteria (of which >50% *Roseobacter* clade) dominated the free living bacteria in *T. rotula* cultures in contrast 54% at the attached bacteria belonged to the CFB group.	Fluorescent in situ hybridization (FISH)	Laboratory culture	Grossart et al., 2005
Thalassiosira spp.	38% α-proteobacteria (of which 33% *Roseobacter* clade), 25% CFB group	PCR and DGGE analysis of the 16S rRNA gene	San Diego (US)	Riemann et al., 2000
Eight different Phytoplankton	Isolated belonging to the *Rhodobacter* group of which more then 50% were members of the *Roseobacter* clade dominated in six out of eight algal cultures with an average of 36% of the total culturable density	16S rRNA analysis of culturable bacteria	Commercial hatcheries	Nicolas et al., 2004
Oceanic algal blooms	75% α-proteobacteria (of which 35% *Roseobacter* clade), 11% γ-*Proteobacteria*	Group-specific 16S rRNA-targeted oligonucleotides, 16S rDNA clone libraries and RFLP analysis	North Atlantic	Gonzalez et al., 2000
Cymnodinium catenatum	49% α-proteobacteria (of which >55% *Roseobacter* clade), 26% CFB group	16S rRNA analysis of culturable bacteria	Laboratory cultures	Green et al., 2004

It has been suggested that some bacteria are involved in the production of toxin or modification of these compounds (Gallacher et al., 1997; Gallacher and Smith 1999), and members of the *Roseobacter* clade may produce sodium channel-blocking (SCB) toxins (Vasquez et al., 2001). In contrast some *Roseobacter* strains degrade or transform algal toxins (Smith et al., 2001). Wagner-Dobler and Biebl (2006) suggested that *Roseobacter* strains might have an important role for controlling toxicity, development and duration of algal blooms, however the ecology behind their dominance is unknown.

Algae can reach high intracellular concentration of DMSP (Miller and Belas 2004) and members of the *Roseobacter* clade have been linked to the de degradation of DMSP especially in the costal regions where DMSP concentration is high (Moran et al., 2003; Howard et al., 2006). Furthermore, motility is an important phenotype for the interaction between algal and *Roseobacter* (Miller and Belas 2006) and a DMSP degrading member of the *Roseobacter* clade has chemotaxis towards DMSP (Miller et al., 2004). However, only about 50% of tested *Roseobacter* strains isolated from algal were motile, hence other phenotypes may be important in their dominance (Bruhn et al., 2007). Bruhn et al., (2005b) suggested that the ability to degrade DMSP could influence the production of TDA, as sulphur from DMSP may be used as sulphur source. Even though it has not been possible to demonstrate that sulphur from DMSP is used in the formation of TDA (Geng et al., 2007), it may still be true in the marine environment.

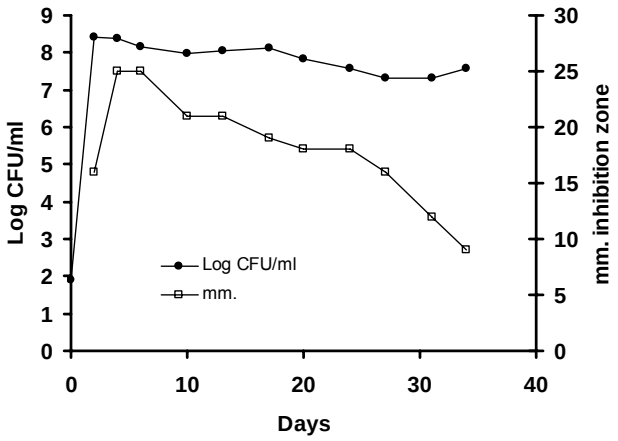
5.1 Antagonistic Interaction among marine bacteria

Bruhn et al., (2007) suggested that the dominance of the *Roseobacter* clade in the algal microbiota may partly be explained by antagonistic interaction between *Roseobacter* and other marine bacteria.

During attachment to particles in the sea interactions among bacteria affect the microbial dynamics (Simon et al., 2002). Particle associated isolates showed greater antagonistic activity compared to free-living bacteria (Nair and Simidu 1987; Long and Azam 2001), which may reflect that particle associated and freely suspended marine bacteria are in general different (Giovannoni and Rappe 2000). For members of the *Roseobacter* clade, attached bacteria are 13 times more likely to produce antimicrobial compounds compared to their free-living counterparts, in contrast to non-roseobacter marine species (Long and Azam 2001). Furthermore, high attachment and production of antibacterial compound were correlated for several members of the *Roseobacter* clade (Bruhn et al., 2005b; Bruhn et al., 2007), which may give these bacteria a selective advantage in colonization of algal and marine particles. TDA is however, very termolabile also at temperatures found in

marine environments, which might question the role of TDA. Following the growth and inhibitions zones found in a well diffusion assay for *Roseobacter* 27-4 and *Silicibacter* TM1040, TDA was detected for about 30 days (Figure 23), indicating a continues production within the stationary phase or that the cells somehow stabilise the compound. We therefore suggested that TDA have a central role for *Roseobacter* in antagonistic interactions in the marine environment.

Figure 23: CFU/ml of *Roseobacter* 27-4 in marine broth. Sterile supernatant tested in a well diffusion assay with *V. anguillarum* as target organism (unpublished results by Bruhn, Melchiorsen and Gram).



Competitive relationships between antibiotic producing marine bacteria and other non-producers have been studied in seawater. Producing strains showed a competitive advantage against non-producers, as the latter was strongly inhibited (Lemos et al., 1991). Production of antibacterial compounds has a role in the colonization of *Ulva australis* by *Pseudalteromonas tunicate* (Rao et al., 2005). Studies have shown that antagonistic attached marine bacteria directly inhibited *V. cholerae* colonization of particles (Long et al., 2005). A suggested TDA producing strain of *P. gallaeciensis* was able to prevent the growth of others non-roseobacter bacteria on surfaces, while growing in a biofilm (Rao et al., 2005). This strain was also an aggressive colonizer of the alga *Ulva australis,* since it was able to invade and disperse pre-established biofilms of other marine bacteria (Rao et al., 2006), even though enhanced resistance to antimicrobial compounds have been found by marine bacteria in biofilms (Burmolle et al., 2006). In contrast, Grossart et al., (2003) found that bacterial growth rate was the most important parameter controlling the long term bacterial population density on agar particles and found no effect of production of antibacterial compound.

Not only bacteria-bacteria interactions may be important in the competition in algal communities, as marine algal also can produce antibacterial compounds, which may play a part in the selection of their associated bacteria (Lustigman and Brown 1991).

Furthermore, competition against yeast and fungal may also be important for colonization in the marine environments and secondary metabolites produced by *P. tunicate* were affective in competition against fungal and yeast surface communities in the marine environment (Franks et al., 2006). TDA produced by *Roseobacter* stains may also inhibit both yeast and fungal (Kintaka et al., 1984; Kawano et al., 1997; Liang 2003).

5.2 Conclusions from chapter 5

Members of the *Roseobacter* clade are often found in the marine environment and are abundant in algal associated communities. Algal associated bacteria may have an important role in controlling toxicity, development and duration of algal blooms, hence understanding the ecology behind the dominance of *Roseobacter* may give us an understanding of algal blooms. Interactions within the marine environment may play an important role in marine ecology and it is suggested that the abilities of *Roseobacter* to attach and produce antibacterial compound gives them a selective advantage in colonization of algal, however, this remains to be demonstrated.

6 Conclusions and perspectives

The dramatic growth in the aquaculture sector has emphasized the importance of fish disease control. Bacterial diseases are important constraints and may be treated with antibiotics. However, due to the risk of development and transfer of antibiotic resistance, alternative disease control measures must be implemented. The use of probiotics, which are live microorganisms that confer a health benefit for the host, may be an alternative to antibiotics in some situations. *Roseobacter* strain 27-4, which has been isolated from a turbot larval rearing unit, is a promising probiotic candidate, as it is capable of reducing mortality in turbot larvae infected with *V. anguillarum*.

Roseobacter 27-4 was selected as a putative probiotic candidate due to its strong anti *Vibrio* activity. This inhibitory activity is caused by the antibacterial compound tropodithietic acid (TDA), which is produced by several *Roseobacter* stains. We hypothesize that TDA is an important mechanism underlying the probiotic effect of *Roseobacter* 27-4. Static growth conditions enhanced TDA production in several *Roseobacter* strains. Also, static growth conditions favoured the formation of a thick biofilm of rosette shaped aggregates formed at the air-liquid interface. The growth in rosettes and biofilm formation appeared to be linked, and these growth patterns also appeared connected to the formation of TDA.

Using *Roseobacter* 27-4 as a probiotic culture requires that it is established under conditions that promote TDA production e.g. in biofilm. It is hypothesised in this thesis that its colonization of surfaces can enhance its anti-pathogenic effect and hence, create a stable longterm beneficial microbiota in aquaculture environments. This may be an advantage as probiotic bacteria often have to be added repeatedly to have a disease reducing effect. This thesis presents a novel way of quantifying both the probiotic and pathogenic bacteria during attachment, by using Real time PCR. Such a method will be useful in evaluating the effect of probiotic bacteria during colonization of surfaces.

TDA affects both eukaryotic and prokaryotic cells, but eukaryotes seem less sensitive compared to prokaryotes. All bacterial strain tested were sensitive to TDA but non-*Roseobacter* strains were in general more sensitive to TDA, as compared to members of the *Roseobacter* clade. Hence production of TDA may give *Roseobacter* a selective advantage during interaction with other bacteria. The mode of action is unknown, but it is suggested that TDA react with ion channels and transport proteins. As part of a new project we will elucidate the molecular mechanisms by which TDA affects the fish pathogens.

Members of the *Roseobacter* clade participate in the sulphur cycle in the marine environment, especially the breakdown of the algal compound, dimethylsulfoniopropionate (DMSP). Since TDA contains two sulphur atoms one could speculate that DMSP could act as substrate for the TDA synthesis. The biosynthesis pathway suggested from transposon mutants did not reveal the origin of the sulphur in TDA, but a TDA producing *Roseobacter* strain (*Silicibacter* TM1040) degrades DMSP. It was, however, not possible to demonstrate that DMSP could be used as sulphur source for TDA production. Both cysteine and sulphate were suitable substrates for TDA synthesis whereas methionine could not be metabolised to TDA. Sulphate is present in the marine environment at concentrations sufficient for TDA production and TDA may therefore be produced in the marine environment if the required energy is available.

Stabile spontaneously TDA negative mutants are found in several TDA producing *Roseobacter* strains. The biosynthesis pathway showed that the last steps in the formation are encoded by genes located on a plasmid, which may explain the loss of TDA production as loss of the plasmid. A transfer of this plasmid to other *Roseobacter* could spread the occurrence of TDA producing strains.

Members of the *Roseobacter* clade are abundant in several marine environments and their dominance in algal blooms has been of special interest. It has been suggested that members of the *Roseobacter* clade play an important role in algal blooms, however the ecology behind their dominance is not known. Several *Roseobacter* strains isolated from algae produced antibacterial compounds, and some strains also formed rosette shaped aggregates, which improved their attachment capability. It is suggested in the present thesis that members of the *Roseobacter* clade may become dominant among algal associated bacteria, due to their production of antibacterial compound and high attachment capability.

Other marine bacteria than members of the *Roseobacter* clade produce antibacterial compounds. Seen from a biological or ecological point of view, a better understanding of these antibacterial compounds may also guide us to an understanding of the balance between different marine organisms. Hence an ongoing project will determine the mechanisms that govern the interaction between culturable marine bacteria. This will be conducted as a part of the Galathea 3 cruise where samples from all around the world will be analysed for the presence of bacteria capable of producing antibacterial compounds. Antibacterial compounds may be a key to the success of different bacteria in different aquatic environments. Also such compounds are likely candidates for development of novel antibiotics and other antimicrobial compounds. They have a potential broad use – in the clinical sector and animal production as well as in food and biotech processing.

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