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INHIBITION OF PRIMARY COLONIZERS BY
MARINE SURFACE-ASSOCIATED
BACTERIA

By

Vipra Nandani KUMAR

A Thesis Submitted in Partial Fulfilment of the
Requirements for the Degree of
Master in Science in Biology

School of Biological, Chemical and Environmental Sciences
Faculty of Science and Technology
The University of the South Pacific

2009
Declaration of Originality

I, Vipra Nandani Kumar, hereby declare that the work presented in this thesis, is to the best of my knowledge and belief original, except as acknowledged in the text, and that the material has not been submitted previously, either in whole or in part, for a degree at this or any other institution.

Vipra Nandani Kumar

Date

The research in this thesis was performed under my supervision and to my knowledge is the sole work of Miss Vipra Nandani Kumar.

Dhana Rao

Date

(Principal Supervisor)
Abstract

Surfaces immersed in seawater rapidly accumulate a complex biofouling community, of which bacteria and diatoms are among the first colonisers. However marine organisms have evolved several defence mechanisms and it has been suggested that green algae of the genus *Ulva* rely on microbial defence. The antibacterial properties of epiphytic bacteria are well established, but relatively little is known about their anti-diatom properties. In this study the hypothesis that surface-associated bacteria from tropical *Ulva* species have anti-fouling characteristics that may have a role in preventing surface fouling on the algae was investigated. Bacterial isolates from the surface of *Ulva* growing in tropical waters were obtained and tested for antibacterial and anti-diatom properties. It was found that 60% of the isolates expressed some inhibitory action against the remaining bacteria isolated in the study and 80% inhibited growth of the diatom *Cylindrotheca fusiformis*. Most effective bacteria were members of the *Pseudoalteromonas* genus. Also showing inhibitory properties were members of the genus *Bacillus*, *Vibrio* and *Shewanella*. Since *Pseudoalteromonas* spp. and the *Roseobacter* clade are model surface-associated bacteria, both groups were screened for anti-diatom property. Results showed that anti-diatom activity was present in 100% and 44% of tested *Pseudoalteromonas* and *Roseobacter* strains respectively. In order to better comprehend the anti-diatom property of marine surface-associated bacteria, a transposon mutant library of *Pseudoalteromonas tunicata* was generated and screened for mutants lacking in anti-diatom activity. Genetic analysis of transposon insertion sites into the *P. tunicata* genome was then used to identify loci linked with anti-diatom activity. Genes identified in this way include a cation/multidrug efflux pump, a beta-hexosaminidase protein, a RTX toxin-like gene and a member of the HemeO protein family. A hypothetical model for the regulation of anti-diatom activity in *P. tunicata* was suggested and this will form the basis of future studies that aim to identify the mechanism of anti-diatom activity in bacteria, especially in *P. tunicata*. Additionally, the presence of epiphytic bacteria engaged in antifouling activities on the surface of tropical *Ulva* sp. emphasizes the prevalence of microbial-mediated defence systems which can be manipulated to find solutions to current biofouling-associated problems.
Acknowledgement

I extend my appreciation to the Faculty of Science and Technology of the University of the South Pacific for awarding me with a Graduate Assistantship and funding this research. Sincere thanks are also conveyed to the Centre for Marine Bio-Innovation, University of New South Wales for collaborating in this research. To my supervisors, Dr. Dhana Rao of University of the South Pacific, Dr. Suheleen Egan and Prof. Staffan Kjelleberg of University of New South Wales, I am greatly honoured to have worked under you. Your constant guidance and companionship has brought out the best in this project. Please accept my utmost gratitude.

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I offer my highest salute to my devoted and loving parents for ensuring the best of everything for me. Thank you for your constant encouragement and endless support. And to my most treasured brother and sister-in-law, I do not know how to thank you enough. Thank you for looking after me and ensuring warm meals and a cosy bed. I am deeply indebted to your immense love and understanding.

Finally, thank you to my most beloved Swami for being my inspiration and guide. I humbly dedicate this thesis to you, dear Lord.

---

You are the all-pervading reality,
When the entire universe is filled with you,
How can I build a temple to you?
When you are effulgent like millions upon millions of suns,
How can I offer you my small candle light?
When you are the indwelling reality of all beings,
How can I call you with a particular name?
When the entire universe is in your stomach,

How can I offer you a little food in worship?
All I can offer you is my love,
All I can hope to do is to empty myself in you,
Who is the ocean of divine love.
I am nothing but an instrument through which you act,
Please direct me!
I will do as you command!
### List of Acronyms

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<th>Full Form</th>
<th>Unit</th>
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Chapter 1: Introduction

1.1 Biofouling in the marine environment

1.1.1 The process and its importance

Biofouling is the undesirable accumulation of microorganisms, plants and animals on surfaces immersed in water. It is a dynamic process involving a sequence of colonization events that lead to the formation of a mature fouling community. Dobretsov et al., (2006) viewed the colonization of a substratum in aquatic systems as a three-step process (Figure 1.1). The process involves (i) adsorption of dissolved organic molecules to a newly submerged or otherwise uncolonised surface, (ii) colonization of the surface by bacteria and microscopic eukaryotes (e.g., diatoms, fungi, and other heterotrophic eukaryotes) and (iii) settlement and subsequent growth of invertebrate larvae and algal spores.

Biofouling impacts on humans in a number of ways, perhaps most important are the potential economic effects. Any industry that is reliant upon or linked to aquatic environments must deal with the effects of biofouling (e.g., offshore oil and gas sectors, fishing and aquaculture industries and the transport industry). Perhaps the most common biofouling sites are ships hulls. A heavily fouled vessel suffers increased drag and decreased manoeuvrability due to the roughness of the hull. This has major economic implications, potentially resulting in a significant increase in fuel costs. Biofouling may also lead eventually to corrosion of the hull that may reduce the lifespan for the vessel. Other surfaces, in particular those exposed directly to water (e.g., heat exchangers, ballast tanks, and propellers), may also be subject to biofouling (Brizzolara, 2002). In addition, equipment used in fishing and fish farming (e.g., mesh cages and trawls) are also likely to harbour fouling organisms.

Living surfaces in the marine environment are also prone to biofouling. Macroalgae are particularly susceptible since they are sessile and often restricted to the photic zone where conditions for fouling are optimal (de Nys et al., 1995).
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1.1.2 The role of biofilms

Each of the three stages in the formation of a biofouling community is an important pre-requisite for the establishment of subsequent layers. In particular the microfouling stage (the second stage in Figure 1.1), which involves the formation of a microbial biofilm, initiates colonization for higher organisms. Studies have found that the bacterial component of marine biofilms are important for induction of larval settlement in several groups including echinoderms (Johnson et al., 1991), cnidarians (Negri et al., 2001), polychaetes (Unabia and Hadfield, 1999), gastropods (Rodriguez et al., 1995) and crustaceans (Neal and Yule, 1994). Hence an understanding of biofilm formation is essential for developing an overall understanding of the...
biofouling process. Although biofilms are composed of both bacteria and diatoms, our current general understanding of biofilm formation has been gained from bacterial biofilms. Biofilms are multicellular associations, consisting of closely spaced cells embedded in an extracellular matrix. The process of bacterial biofilm formation involves cell attachment, microcolony formation, biofilm maturation and cell dispersal (Figure 1.2).

The first stage in biofilm formation involves surface attachment. Upon encountering a suitable surface, planktonic cells adhere to the conditioning film and establish a weak interaction with the surface. This initial phase is referred to as “reversible attachment” (Busscher et al., 1992). Adhesion is influenced by many factors including the physico-chemical properties of the cell surface (e.g., cell surface hydrophobicity; Bruinsma, 2001), genetic determinants of the cell (e.g., the expression of cell surface components and matrix material; Caiazza and O’Toole, 2004) and hydrophobicity and charge of the substratum (Harkes et al., 1992; Mueller et al., 1992). Commitment, the next phase, which involves “irreversible attachment”, is a crucial step in biofilm formation since these initial colonizers form the foundation of the mature biofilm. After this transition, cells cannot be removed from the surface by simple washing procedures (Oliveira, 1992).

Following adhesion to a surface, bacterial cells aggregate, forming the basic structural unit of a biofilm, referred to as a microcolony (Davey and O’Toole, 2000). Microcolonies may form by one of three mechanisms - surface translocation, cell recruitment and clonal growth. The first two mechanisms involve recruitment of new cells to a microcolony. During surface translocation cells attached to the surface utilize swarming or twitching motility to join existing microcolonies. In contrast cell recruitment involves planktonic cells (Tolker-Nielsen et al., 2000) or cell flocs (Stoodley et al., 2001) attaching directly to cell aggregates from the bulk fluid. In clonal growth increase in microcolony size results from division of existing resident bacteria. The relative contribution of each mechanism varies depending on the organism involved, the surface being colonized and the environmental conditions (Stoodley et al., 2002).
Figure 1.2: Stages of biofilm development. (1) Surface attachment, (2) Microcolony formation, (3) Biofilm maturation and differentiation and (4) Cell dispersal.

During biofilm maturation the microcolonies reach their maximum dimensions. Cells within a given microcolony are non-motile and are usually segregated into a number of distinct cell clusters (Sauer et al., 2002). A microcolony is usually a simple conical structure or mushroom-shaped. Microcolony structure is dependant upon the presence of an extracellular cell-to-cell interconnecting matrix consisting mainly of exopolymers (e.g. polysaccharides, proteins and DNA). In addition, outer membrane proteins and cell appendages such as fimbriae, pili, and flagella may also form part of the biofilm matrix (Pamp et al., 2007). The interconnecting matrix is interspersed with highly permeable water-channels. These act as a “circulatory system,” delivering nutrients and removing metabolic waste from the microcolony (Lawrence et al., 1991; Costerton et al., 1994). Importantly biofilm growth form protects the biofilm-forming organisms against the negative effects of antimicrobial agents and predation by protozoans (Stewart, 2002; Matz and Kjelleberg, 2005). Additionally, biofilms facilitate horizontal gene transfer and intracellular communication (Hausner and Wuertz, 1999; Parsek and Greenberg, 2000) as well as promoting increased genetic diversity of the bacterial populations (Boles et al., 2004). Such characteristics improve the survival of bacterial communities in harsh environmental conditions.

For some bacteria, cell-cell communication is essential for the establishment of an ordered biofilm community. Bacteria achieve this using secreted signalling molecules called autoinducers in a process called “quorum sensing” (Nealson and Hastings, 1979). This enables the population to collectively regulate gene expression and, therefore, behave as a group. Quorum sensing is known to control bioluminescence,
secretion of virulence factors, sporulation, and conjugation. Thus, quorum sensing is a mechanism that allows bacteria to function much like a multi-cellular organism (Hammer and Bassler, 2007).

As a biofilm matures dispersal of cell aggregates from the main body occurs. The dispersal process may be either passive or active. Passive dispersal is a direct consequence of the immediate environmental conditions and usually occurs by erosion, sloughing, abrasion or predator grazing (Bryers, 1988). Cell aggregates are released as a result of physical disruption of the biofilm. On the other hand active dispersal mechanisms are used when environmental conditions become unfavourable. In this case release of cells or cell aggregates is initiated and regulated by the biofilm. For example, Pseudomonas spp., Escherichia coli, and Acinetobacter spp. biofilms will release cells in response to nutrient starvation (Delaquis et al., 1989; Sawyer and Hermanowicz, 2000; Jackson et al., 2002). Several studies have shown programmed cell death is responsible for active dispersal. In Pseudoalteromonas tunicata the antibacterial protein, AlpP, mediates cell death. This acts as a lysine oxidase resulting in the production of hydrogen peroxidase and cell death within the microcolony (Mai-Prochnow et al., 2004, 2006, 2008). Death of specific regions of the microcolony results in the release of isolated cell aggregates. Phaeobacter gallaeciensis biofilms, formerly Roseobacter gallaeciensis, also displays cell death within microcolonies (Martens et al., 2006).

Cells dispersing from biofilms often exhibit phenotypic and genotypic variation, a feature that is thought to enhance survival in the face of changing environmental conditions and competitive regimes (Boles et al., 2004; Webb et al., 2004; Ho, 2008). For example, dispersed cells of Ph. gallaeciensis express varying levels of antimicrobial activity against the competitive colonizer, P. tunicata. This is expected to lead to differential colonisation ability (Ho, 2008). In Pseudomonas aeruginosa biofilms, small colony variants exhibit enhanced attachment and accelerated biofilm development relative to the wild type strain (Webb et al., 2004).
1.1.3 Current biofouling control strategies and its impact on the marine ecosystem

To minimize the impact of biofouling on artificial structures in the marine environment (e.g. ship’s hulls) many are protected with antifouling coatings. These are paint-based and contain a biocide or toxin, often tributyl tin (TBT) or copper based compounds (Thomas, 2001; Yebra et al., 2004). The biocide is slowly released into the environment poisoning organisms that adhere to the surface.

However, since the biocides are non-specific they may also have harmful effects on non-fouling organisms (Evans, 1999; Yebra et al., 2004). In the case of TBT, organo-tin moieties are released as the coating degrades. These have a range of sublethal effects on non-target species. For example, low concentrations of TBT may cause defective shell growth in the oyster *Crassostrea gigas* and development of male characteristics in female dog whelk *Nucella lapillus* (Evans, 1995). Perhaps most alarming for the present study, studies indicate that the highest levels of TBT-contamination have been recorded in Fiji, the most contaminated site recording a TBT concentration of 360μgg⁻¹ (Maata and Koshy, 2001).

Due to the non-specificity of TBT and other tin containing biocides in antifouling paints, the International Maritime Organization and Marine Environmental Protection Committee (MEPC) have banned their usage (Champ, 1999). This has prompted a search for alternate antifoulants that are non-toxic and “environment-friendly.” An understanding of the natural defence mechanisms of marine organisms against biofouling is the first step towards “safer” antifoulants. It is hoped that these natural defence strategies and the associated bioactive compounds may be manipulated to develop novel antifouling technologies that are less harmful to the marine environment.

1.1.4 Natural defence mechanisms against biofouling

Marine algae are also prone to biofouling. Seaweeds employ a number of physical defence systems to prevent fouling. These include shedding of outer cell layers (Keats *et al.*, 1997), mucilaginous coverings on blades (Filion-Myklebust and Norton, 1981; Moss, 1982) and continuous erosion of the distal ends of blades (Mann, 1973; Ott,
Chapter 1: Introduction

1980). Water turbulence and abrasion may also limit fouling (Sieburth and Tootle, 1981).

In addition, marine algae may produce inhibitory chemicals to prevent fouling and grazing (Dworjanyn, et al., 1999). A well studied example is the red alga *Delisea pulchra*, which produces an array of structurally related secondary metabolites known as halogenated furanones (Kazlausks et al., 1977; de Nys et al., 1993). These compounds interfere with bacterial colonization and prevent settling of invertebrate larvae and the spores of common fouling algae (Kjelleberg et al., 1997; Maximilien et al., 1998). Scanning electron microscopy of the alga reveals a significantly higher abundance of epibacteria near the holdfast than closer to the blade apices (Steinberg et al., 1997). This corresponds to a gradient in the concentration of halogenated furanones - which are highest close to apices. Studies indicate that furanones may control bacterial colonization by specifically interfering with acylated homoserine lactone (AHL)-mediated gene expression at the level of the LuxR protein (Manefield et al., 1999).

Although toxin mediated mechanisms are effective, they are energy expensive. Generally, defence costs are the sum of (1) the energy and nutrients consumed for defence production (and, therefore, lost to growth), (2) the energy necessary for sequestering the toxins away from active cell processes, (3) the interference of the defence with photosynthesis and (4) the loss of productivity from the tissue given it would have photosynthesized if it were not co-opted for defence (Coley, 1986). Hence, large amounts of energy are invested in toxin-mediated defence systems, and this is costly for smaller and simpler algal forms.

Organisms that lack chemical or physical defences are thought to rely on secondary metabolites produced by bacterial symbionts to provide defence against surface-colonizing organisms (Armstrong et al., 2001; Berland et al., 1972; Thomas and Allsopp, 1983). For example, symbiotic interactions have been found in the marine crustaceans *Palaemon macrodactylus* and *Homarus americanus* where symbiotic bacteria defend embryos from fungal infection (Gil-Turness and Fenical, 1992). More generally, Holmstrom et al., (1996) investigated the frequency with which bacterial strains isolated from living and inanimate surfaces displayed inhibitory activity
Inhibition of primary colonizers by marine surface-associated bacteria against fouling organisms. Results showed that 10% of isolates from rock surfaces inhibited the settlement of invertebrate larvae compared with 30% of isolates from marine animals and 74% from algal surfaces. These data suggest that many of the bacteria that form epiphytic communities on living surfaces are able to regulate fouling by other organisms (Egan et al., 2000).

Additionally, results from behavioral assays demonstrate that secondary metabolites may be produced to ensure colonization by preferred epibionts (Wahl et al., 1994; Bryan 1996; Engel et al., 2002). It is interesting to note that secondary metabolites may control the density of surface associated microbes, allowing growth of a community of preferred microbes rather than maintaining an axenic surface (Engel et al., 2002). For example, in D. pulchra halogenated furanones affect bacterial colonization differently. Attachment is inhibited in strains associated with surface-fouling while growth and swarming is inhibited in the preferred strains (Maximilien et al., 1998). The results explain why fouling strains are absent from the alga’s surface and preferred bacterial strains have limited surface distribution.

The ecology of marine algal surfaces is known to be highly complex. As space and nutrients are limited, colonization by bacteria often requires them to compete with one another (Egan et al., 2008). Bacterial strains known to be associated with algal surfaces include members of the Flavobacterium group of Bacteroidetes, members of the Roseobacter clade (Rao et al., 2005) as well as various Pseudoalteromonas and Alteromonas spp. (Holmstrom and Kjelleberg. 1999). Using a culture-independent method, Longford et al. (2007) compared bacterial diversity on the red macroalga D. pulchra with that of U. australis. Approximately 79 species from 7 phyla were isolated from D. pulchra while an estimated 36 species from only 4 phyla were isolated from Ulva. Alpha-, Delta- and Gammaproteobacteria were all well represented with Planctomycetes and Bacteroidetes common on both algae. However, there were very few species common to both algae (Longford et al., 2007).

One of the major arguments for preserving biodiversity is the potential for discovery of new bioactive compounds. For this reason, the genus Pseudoalteromonas has received a lot of attention in the last two decades. The focus reflects the bacterium’s frequent association with eukaryotic hosts in the marine environment. Studies of such
associations are useful for understanding the mechanisms underlying microbe-host interactions. Also, many pigmented species of \textit{Pseudoalteromonas} produce biologically active metabolites (Egan \textit{et al.}, 2002b). Species of \textit{Pseudoalteromonas} display antibacterial, bacteriolytic, agarolytic and algicidal properties, as well as various other pharmaceutically-relevant activities. Several \textit{Pseudoalteromonas} strains prevent the settlement and colonisation of marine surfaces by common fouling organisms (Holmstrom and Kjelleberg, 1999; Bowman, 2007).

Within the genus, \textit{Pseudoalteromonas tunicata} is thought to exhibit the broadest range of inhibitory activities (Holmstrom \textit{et al.}, 2002). This species produces a diverse range of biologically active compounds, many of which target marine fouling organisms (Holmstrom \textit{et al.}, 1998). To date a range of antifouling compounds have been isolated from \textit{P. tunicata}. The antifungal compound is a yellow, tambjamine-like alkaloid (YP1), the biosynthetic pathway of which is encoded by a cluster of 19 genes (\textit{tamA} to \textit{tamS}; Franks \textit{et al.}, 2005; Burke \textit{et al.}, 2007). Moreover, the autolytic antibacterial protein (AlpP) produces hydrogen peroxide which causes cell death, mediates differentiation, dispersal and phenotypic variation during the dispersal event (James \textit{et al.}, 1996; Mai-Prochnow \textit{et al.}, 2004, 2008). Other bioactive compounds include a polar, heat-stable anti-larval molecule (Holmstrom \textit{et al.}, 1992), a heat-sensitive anti-algal peptide (Egan \textit{et al.}, 2001) and an uncharacterized anti-diatom compound. With such a wide range of antifouling characteristics present in \textit{P. tunicata}, the antifouling potential of the remainder of the genus is worth investigating. More knowledge of the biologically active chemicals produced by \textit{Pseudoalteromonas} would also be potentially pharmacologically beneficial (Bowman, 2007).

The \textit{Roseobacter} clade is another group of marine bacteria that due to its worldwide distribution, abundance and physiological diversity is well studied (Brinkhoff \textit{et al.}, 2008). The group has been isolated from both coastal and open waters, a variety of micro- and macro-algae, microbial mats, sediments, polar sea ice, and marine invertebrates (Buchan \textit{et al.}, 2005; Wagner-Dobler and Biebl, 2006). Members of the group often form symbioses with higher organisms (Bruhn \textit{et al.}, 2007). For example, the symbiotic association between \textit{Silicibacter} sp. strain TM1040 (a member of the \textit{Roseobacter} clade) and the dinoflagellate \textit{Pfiesteria piscicida} involves bacterial
chemotaxis to dinoflagellate-produced dimethylsulfiniopropionate (DMSP), DMSP demethylation, and ultimately a biofilm on the surface of the dinoflagellate host (Alavi et al., 2001; Miller and Belas, 2004; Miller et al., 2004). Biofilm formation coincides with the production of an antibiotic, a sulfur-containing compound, tropodithietic acid (TDA). Since the genes critical for TDA biosynthesis are located on plasmids in both Silicibacter sp. strain TM1040 and Phaeobacter sp. strain 27-4, it is suggested that both members of the Roseobacter clade may use a common pathway for TDA biosynthesis that involves plasmid-encoded proteins (Geng et al., 2008). This suggests that investigating other members in the Roseobacter clade for bioactive properties is important.

*Phaeobacter gallaeciensis* (a member of the Roseobacter clade) is a commonly studied temperate, biofilm-forming strain. Together with *Pseudoalteromonas tunicata*, *Ph. gallaeciensis* benefits its algal host by producing compounds that inhibit common fouling organisms. Studies have shown *Ph. gallaeciensis* to be more competitive than *P. tunicata* during biofilm formation and having the capacity to invade and disperse pre-established biofilms (Rao et al., 2006). Hence, given strong competitive characteristics, participation in symbiotic interactions with eukaryotic hosts and wide distribution, members of the Roseobacter clade are also strong candidates for future antifouling solutions.

While it is acknowledged that the technology available to assess and exploit microbial diversity is limited, there is a need for studies that will enhance current understanding of microbial associations (Egan et al., 2008). It is recognized that many of the marine invertebrates (e.g. sponges, bryozoans and tunicates) that are sources of secondary metabolites also contain endo- and epibiotic microorganisms. Indeed, some invertebrate-derived natural products are structurally related to the bacterial metabolites (Sudek et al., 2007). With the complexity of associations in marine organisms, it is difficult to determine the biosynthetic source of many marine natural products (Konig et al., 2006). However, it is now recognised that many of these metabolites may well be of microbial origin (Sudek et al., 2007). The marine bryozoan *Bugula neritina* synthesizes bryostatins, complex polyketides that render the *B. neritina* larvae unpalatable to predators (Sharp et al., 2007). A recent study has shown that bryostatin, isolated from *B. neritina*, is actually produced by the
uncultured symbiotic bacterium “*Candidatus Endobugula sertula*” (Sudek *et al*., 2007). The finding highlights the important role of biotechnological advancement in the discovery and exploitation of microbial defence mechanisms. The need was recently re-emphasized when for the first time a strain of the *Roseobacter* clade-affiliated (RCA) cluster was successfully isolated and propagated (Mayali *et al*., 2008). This was accomplished through the application of novel techniques with algal cultures. Previous efforts to culture the RCA cluster as well as many other bacteria abundant in the marine environment, using traditional culture methods have not been successful. This recent finding stresses the need for developing novel molecular approaches to study uncultivated microbial diversity which could potentially lead to the discovery of new compounds (Egan *et al*., 2008) and improved biofouling-control techniques.

1.2 Diatoms as marine surface colonisers

Like bacteria, diatoms are also an important constituent of microfouling communities on marine surfaces. However, most studies have focused on the bacterial component. In the current search for improved-control strategies, it is essential to also develop an understanding of the role that diatoms play. This will be critical for finding more effective means of tackling the issue of biofouling.

1.2.1 Diatom biology

Diatoms are among the earliest eukaryotic colonizers of submerged surfaces and are among the most conspicuous components of natural biofilms (Evans 1988). Diatoms belong to the Bacillariophyceae with over 250 genera and perhaps as many as 100,000 species (Norton *et al*., 1996; Van Den Hoek *et al*., 1997). Characteristically, the cell walls are highly patterned with pores and ridges. They are unique among the algae due to the presence of silica-based cell walls. Most diatoms contain silicon transporters (SITs) for transferring Si(OH)$_4$ across lipid bilayer membranes (Hildebrand *et al*., 1997). Diatoms take up silicon predominantly as silicic acid that is then polymerized and deposited into the cell wall as silica (Del Amo and Brzezinski, 1999). Silicic acid is co-transported with sodium in marine diatoms with zinc also suggested to play a role in silicic acid uptake (Sullivan, 1977; Rueter and Morel,
1981). The diatom of interest in this research is *Cylindrotheca fusiformis*. This autotrophic, marine, pennate diatom is being used as a model organism for studying transport, deposition and patterning of silica in diatom cell walls. Biosilica from all diatom species investigated so far has shown to be a composite material containing proteins (mainly the silaffins) and long-chain polyamines as organic components. These organic constituents have been recognised as important players in silica biomineralisation. Several recent reviews have described the structure and properties of these organic molecules (mainly from *C. fusiformis*) as well as possible function in silica formation and patterning (Pohnert, 2002; Foo *et al*., 2004; Sumper *et al*., 2004; Sumper and Brunner, 2006).

The wall is constructed of two sections or thecae, with the smaller hypotheca fitting within a larger epitheca much like a Petri dish (Figure 1.3). In diatoms one of the most important cell wall proteins is pleuralin, which is involved in the cell cycle-dependent frustule development. To maintain the integrity of the frustule, coupling between biogenesis of new frustule components and cell cycle is required. The molecular mechanism by which this coupling occurs is unknown. Interestingly, although the thecae are morphologically similar, immunolocalisation with anti-pleuralin antibodies demonstrates that their protein composition is clearly different (Kroger and Wetherbee, 2000). It is hypothesized that pleuralins are involved in hypotheca-epitheca differentiation, a crucial process that ensures proper frustule development.

**Figure 1.3:** Schematic diagram of diatom structure and frustule terminology.

(Redrawn from Hasle and Syvertsen, 1997)
Generally, two types of diatoms are recognised, pennate or bilaterally symmetrical diatoms and centric or radially symmetrical diatoms. The former are mostly planktonic and the latter predominately benthic, associated with sediments or attached to rocks or macroalgae (Falciatore and Bowler, 2002; Leblanc et al., 1999). Diatoms can be unicellular or colonial and either autotrophic or heterotrophic (Gilabert, 2007). The brown colour of diatoms is due to the presence of the carotenoid pigment fucoxanthin, which is located together with chlorophyll $a$ and $c$ in their plastids (Round and Crawford, 1990). Reproduction in diatoms is mainly asexual, the daughter cells each receiving one half of the parental cell wall and constructing a new frustule half within it (Raven et al., 1999). Benthic diatoms are able to glide along surfaces; mucilage is secreted into furrow (known as a raphe) which allows movement (Falciatore and Bowler, 2002). In contrast, most planktonic diatoms are non-motile and rely on mixing of the water column to remain suspended (Stoermer et al., 2004).

### 1.2.2 Diatom adhesion, motility and extracellular polymeric substances

Diatoms are abundant in benthic habitats where they adhere to surfaces using copious quantities of mucilage. Depending on the nature of the surface, initial contact may or may not result in bonding by the diatom (Wetherbee et al., 1998). Adhesion in diatoms is $\text{Ca}^{2+}$ dependent and the process requires metabolic energy, protein and glycoprotein synthesis (Cooksey and Wigglesworth-Cooksey, 1995). Thus diatom bonding requires an active commitment involving the activation of specific adhesion mechanisms. In most benthic diatoms, cell-substratum adhesion occurs at the raphe, resulting in cell reorientation and a unique form of cell motility called “gliding” (Edgar and Pickett-Heaps, 1984). The mucilage secreted into the raphe links the cell cytoplasm to the substratum. This provides for ‘gliding’ motility via an actin-myosin system located adjacent to each raphe (Edgar and Pickett-Heaps, 1984; Poulsen et al., 1999). Cell-substratum adhesion at the raphe is a requirement for diatom gliding (Edgar and Pickett-Heaps, 1984; Wetherbee et al., 1998). As diatoms glide, the secreted mucilaginous strands are detached and left behind as diatom “trails” that eventually accumulate as a component of biofilms (Edgar and Pickett-Heaps, 1984; Higgins et al., 2000; Wetherbee et al., 1998). In addition, the trails remain adhesive and may aid in the accumulation of other biofouling agents (Lind et al., 1997).
The mucilage or extracellular polymeric substance (EPS) is produced by benthic diatoms both as part of the motility system and as a response to environmental conditions. The EPS is composed of polysaccharides, proteins, and glycoproteins (Chiovitti et al., 2003). Typically carbohydrates are the dominant component of EPS, but the constituent sugars are often complex and highly diverse (Hoagland et al., 1993). Characterization of EPS structure, serology, and lectin interactions, provides for a broad classification of EPS materials. Several subtypes are recognized including frustule EPS, outer capsular EPS, motility EPS, and matrix EPS (Wigglesworth-Cooksey and Cooksey, 2005). Combined, diatoms and their insoluble EPS are common features of biofouling communities.

1.3 Aims of this study

The green alga *Ulva* spp. does not produce secondary metabolites with recognised roles in fouling prevention (Awad, 2000; Abd El-Baky et al., 2008). *Ulva* spp. is found in temperate and tropical waters, including the shores of Fiji. While antibacterial activities have been identified for epiphytic bacteria isolated from temperate *Ulva* spp., the occurrence of anti-diatom properties has been less intensively investigated. The latter is likely due to the inherent, technical challenges of performing anti-diatom bioassays.

The major hypothesis addressed in this study is that surface-associated bacteria from tropical *Ulva* spp. have characteristics that have a role in limiting surface fouling of the algae. First the study aimed to isolate epiphytic bacteria from Fijian collection of *Ulva* spp. growing in tropical Fiji waters and characterise their inhibition of bacteria and diatoms. The second part concentrated on the prevalence of anti-diatom activity across two ecologically significant bacterial groups, namely *Pseudoalteromonas* and the *Roseobacter* clade. A further focus was the anti-diatom strategy of the model epibiont, *P. tunicata*, with gene identity information used to propose a preliminary model describing the mechanism.
To address the hypothesis the specific aims of this study were to:

1. Isolate and assess inhibitory activity of the epibionts against bacteria and diatoms.
2. Identify the taxonomy of the epibionts with inhibitory properties and establish a correlation with previous studies.
3. Screen *Pseudoalteromonas* and *Roseobacter* strains for anti-diatom activity.
4. Identify potential genes involved in the expression of the anti-diatom compound produced by *P. tunicata* and suggest a hypothetical model to describe the mechanism.
Chapter 2: Inhibitory activity of epiphytic bacteria isolated from Ulva

2.1 Introduction

Marine macroalgae are prone to colonization by fouling organisms. To limit fouling, some macroalgae produce secondary metabolites that mediate intra- and interspecific interactions (Harborne, 2001; Rosenthal and Berenbaum, 1992). Although such chemical defences are effective, they are expensive in terms of the energy required for metabolite production (Thomas, 2001). Instead simpler algal forms are suggested to have alternative mechanisms to prevent surface fouling.

Some bacteria produce inhibitory compounds that prevent surface fouling. Algae that lack physical or chemical defence mechanisms are thought to form symbioses with these bacteria (Holmström and Kjelleberg, 1999; Rao et al., 2005; Longford et al., 2007). Various studies show that epiphytic bacteria on marine surfaces display inhibitory activity against fouling organisms (Armstrong et al., 2001; Berland et al., 1972; Thomas and Allsopp, 1983; Holmstrom et al., 1996). For example, Lemos et al. (1985) isolated epibionts from five species of green and brown algae and found that 38 of 224 isolates displayed antibacterial activity.

Bacteria of the Flavobacterium group, various Pseudoalteromonas and Alteromonas spp. (Holmstrom and Kjelleberg, 1999) and members of the Roseobacter clade (Rao et al., 2005) are commonly found on marine surfaces. Amongst these bacteria, Pseudoalteromonas is unique. Fairly recently, the genus was established to contain various species that produced biologically active molecules (Holmstrom and Kjelleberg, 1999). In particular P. tunicata has the ability to influence the behaviour of higher organisms (Holmstrom et al., 1998). Another ecologically important group is the Roseobacter clade. The group includes the species Phaeobacter gallaeciensis, frequently isolated from the surface of U. australis (Shiba, 1992), marine snow particles (Gram et al., 2002) and dinoflagellates (Alavi et al., 2001; Lafay et al., 1995; Miller and Belas, 2004). The bacterium demonstrates antibacterial activity (Brinkhoff et al., 2004; Rao et al., 2005; Ruiz-Ponte et al., 1998). Advancement of current
biofouling-control strategies depends on the study of such inhibitory epiphytic bacteria which may lead to the discovery of new bioactive compounds.

Much is already known about the antibacterial, anti-algal and anti-larval properties of surface colonizing bacteria. However, anti-diatom capacity remains largely unexplored. Diatoms are amongst the early colonizers of marine substrates and are important components of the biofouling community. The lack of study largely reflects lack of suitable culture techniques of diatoms in bioassay screens.

The green algae, *Ulva* spp. does not produce secondary metabolites with recognised roles in fouling prevention (Awad, 2000; Abd El-Baky *et al.*, 2008) but does play host to antifoulant producing bacteria (Holmstrom *et al.*, 1996; Lemos *et al.*, 1985). Hence the alga is a suitable model system for exploring the role of inhibitory bacteria. *Ulva* is a cosmopolitan chlorophyte and found in temperate and tropical waters, including the shores of Fiji. Compared to temperate climates, tropical conditions may support a greater microbial diversity, but this remains unexplored. The study aimed to investigate whether *Ulva* growing in Fiji waters had surface microflora similar to that of temperate waters, which it relied upon for the prevention of surface fouling. The approach was to test isolated bacteria for inhibitory activity against both bacteria and diatoms. The epibionts with inhibitory properties were taxonomically classified and correlated with previous studies.

### 2.2 Materials and methods

#### 2.2.1 Isolation of epiphytic bacteria from *Ulva*

Samples of the green algae *Ulva*, were randomly collected from the intertidal zone of Laucala Bay, Suva, Fiji (18°06'S, 175°30'E). Collections were made at low tide and samples stored in sterile polyethylene bags for transport to the laboratory. The algal samples were washed with sterile seawater to remove loosely attached bacteria. To isolate bacteria that were tightly bound to the algal surface, samples were placed in vials containing 10 ml of sterile seawater and vortexed for 5 min. Aliquots of the cell suspensions were used to inoculate marine agar (Difco marine broth solidified with 1.5% agar) which were then incubated at 23°C for 48 hr. Morphologically distinct
bacterial colonies were selected and stored at -80°C in 30% glycerol (v/v). Isolates were routinely grown and maintained on marine agar at 23°C.

2.2.2 Bioassay against growth of bacteria

Bacteria isolated from the surface of Ulva were tested for antibacterial activity alongside three common laboratory strains; *E. coli*, *Pseudomonas aeruginosa* PAO1 and *Bacillus* strain CC6 (culture collection, Centre for Marine Bio-Innovation, University of New South Wales, Australia). The antimicrobial assay was modified from Rao *et al.*, (2005). Broth cultures of algal isolates were grown for two days at room temperature (23°C) and the supernatant isolated by centrifugation at 13 000 × g for 5 min. Supernatant samples were assayed for inhibitory activity using the drop assay. Briefly, 100 μl of 48 hr old target marine isolate and 24 hr old target laboratory isolate were spread on marine agar plates, and the plates were dried at 30°C for 30-60 min. Drops containing 10 μl of the test isolate supernatant, as well as a control (uninoculated marine broth) were placed on the agar surface and incubated at room temperature (23°C) for two days to allow formation of inhibition zones. Assays were conducted in triplicate.

2.2.3 Bioassay against growth of diatoms

The effect of the bacterial isolates on diatom growth was assessed using the pennate diatom, *Cylindrotheca fusiformis*, (CSIRO microalgae culture collection, Hobart, Australia). *C. fusiformis* was chosen as the target diatom as it grows rapidly on agar plates (Chan *et al.*, 1980). Diatoms were subcultured routinely by aseptically transferring 5 ml of the inoculum to 100 ml of the diatom culture solution-f/2 (Guillard and Ryther, 1962; Appendix I) and incubating at 20°C. A photoperiod of 16 hr light: 8 hr dark was provided (as per supplier’s instructions).

To determine inhibitory activity, the diatom plating assay (Chan *et al.*, 1980) was used with some modifications. Briefly, 300 μl of exponential phase diatom culture (with optical density of higher than 0.06, at 600nm) was spread evenly over the surface of marine agar (Difco marine broth solidified with 1.5% agar) using a sterile
glass spreader and allowed to dry. The bacterial test culture was transferred to the centre of inoculated plates by spotting with an inoculating loop. Plates were incubated inverted at 20°C with a photoperiod of 16 hr light: 8 hr dark. Light was provided both from above and below the plates. Growth was monitored over 4 days. Anti-diatom activity was indicated by the presence of growth inhibition zones. Plates inoculated with diatom cultures only, served as a control for diatom growth. Assays were conducted in triplicate.

2.2.4 Identification of isolates with inhibitory properties

2.2.4.1 DNA extraction

DNA was extracted from bacterial isolates with inhibitory activity against bacteria and diatoms. Extractions were conducted using the XS-buffer method (Tillett and Neilan, 2000; Appendix I). A 2 ml aliquot of a 2 day old culture was pelleted by centrifugation, the supernatant discarded, and cells subsequently resuspended in 1 ml of XS-buffer. The suspension was incubated at 70°C for 60 min. After incubation, tubes were vortexed for 10 sec and placed on ice for 30 min. Tubes were then centrifuged at 21 000 × g for 10 min. The supernatant was transferred to a clean 2 ml microcentrifuge tube, 1 volume of isopropanol was added and the solutions mixed. The tubes were incubated at room temperature for 5 min and then centrifuged at 21000 × g for 10 min. The supernatant was decanted and the pellet washed with 70% (v/v) ethanol. The pellet was then air dried before being resuspended in 50-100 μl of sterile deionised water.

2.2.4.2 Agarose gel electrophoresis

The extracted DNA was examined by electrophoresis on 1% (w/v) agarose gel using λ-DNA digested with EcoRI/HindIII as a size marker and for concentration estimation. Gels were run in 1 × TBE buffer (Appendix I) at 80 volts for 20-30 min, stained with ethidium bromide, destained in TBE buffer, and then photographed using the Gel-Doc Imaging system (BioRad).
2.2.4.3 PCR amplification of 16S rRNA gene

The 16S rRNA gene was amplified using the polymerase chain reaction (PCR). Reaction volumes of 20 μl contained 1 × PCR Reaction Buffer (Invitrogen), 250 μM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each of F27 and R1492 primers (Appendix II), 2.5 mM MgCl₂ (Invitrogen), 0.05 unit Platinum Taq DNA Polymerase (Invitrogen) and 10 ng of extracted DNA template. Reaction mixture was thermocycled as follows: 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 2 min. A final extension step was performed at 72°C for 5 min and samples were held at 4°C.

PCR product concentration was estimated by agarose gel electrophoresis as described in section 2.2.4.2. For successful amplifications, the PCR product was purified using QIAquick PCR Purification Kit as per manufacturer’s instructions. Purified products were examined using gel electrophoresis as described above (section 2.2.4.2.).

2.2.4.4 DNA sequencing and sequence analysis

The purified PCR product were sequenced unidirectionally using 25 pmol of either of F27 or R1492 primers (Appendix II), 20 ng of DNA template, 5 × CSA sequencing buffer (Applied Biosystems), 1 unit of BigDye™ terminator cycle sequencing reaction mix v.3.1 (Applied Biosystems) and sterile deionised water in a final volume of 20 μl. Cycle sequencing was conducted using the following thermoprofile: 94°C for 10 sec, 50°C for 5 sec and extension at 60°C for 4 min in 99 cycles. Extension products were purified by ethanol precipitation. Specifically, 5 μl of 125 mM EDTA and 60 μl of 100% ethanol were added to each reaction tube and vortexed briefly. The extension products were left to precipitate for 30 min at room temperature. Tubes were then centrifuged at 21 000 × g for 20 min and the supernatant aspirated. The pellet was washed twice with 70% ethanol (v/v), the tubes briefly vortexed and centrifuged at 4°C, at 21 000 × g for 10 min. Samples were dried in a speedvac for 15 min. Sequencing was performed on an ABI 3730 DNA sequencing system at the Automated Sequencing Facility, UNSW. Sequences obtained were compared to sequences available in the NCBI BLAST 2.0 database (Altschul et al., 1990). Phylogenetic analysis was performed using the sequence data software, ARB (Ludwig...
et al., 2004) and the Greengenes database (DeSantis et al., 2006). Specifically, sequences were aligned to the Greengenes database in ARB using the integrated aligner. The sequence alignments were manually checked and redefined, if necessary. Placement of the sequences in the phylogenetic tree was determined by the maximum parsimony algorithm implemented in ARB. Taxonomic assignment was based on closely-related strains in the tree and the Hugenholtz taxonomy included in the Greengenes database. In addition, taxonomic classification was also undertaken by the Ribosomal Database Project II classifier based on a naïve Bayesian rRNA classifier (Wang et al., 2007).

2.3 Results

2.3.1 Epiphytic bacteria isolated from Ulva

Temporal replicates of culturing efforts from Fijian Ulva spp. yielded different bacterial morphotypes, of which 14 could be routinely sub-cultured. Using colony morphology and Gram staining, redundant isolates were eliminated. A total of 10 distinct bacterial isolates were chosen and used for further experiments.

2.3.2 Production of extracellular antibacterial compounds

Bacteria isolated from Ulva along with laboratory strains were used as target strains in assessing antibacterial activity of the Ulva epibionts. Table 2.1 summarizes the effect of epiphytic bacteria on the growth of 13 bacterial strains. Of the 10 isolates, 60% expressed some inhibitory action. The broadest ranges of antibacterial activity were displayed by U15 and U11, which were effective against 50% and 33% of the target strains, respectively. Isolates U8, U12, U13 and U14 failed to inhibit the growth of any of the bacterial strains tested.
2.3.3 Growth inhibition of diatoms

The effect of the 10 isolated epiphytic bacteria on the growth of *C. fusiformis* is summarized in Table 2.2. Eight of the ten isolates showed inhibitory activity against *C. fusiformis*. Isolates U7, U11 and U15 were the most effective whereas isolates U1 and U12 had no effect on *C. fusiformis*.

**Table 2.1:** Antibacterial activity expressed by bacteria isolated from *Ulva*

<table>
<thead>
<tr>
<th>Target Strain</th>
<th>Bacterial Isolate</th>
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<tbody>
<tr>
<td></td>
<td>U1 (mm)</td>
</tr>
<tr>
<td>U1</td>
<td>*</td>
</tr>
<tr>
<td>U3</td>
<td>0</td>
</tr>
<tr>
<td>U4</td>
<td>0</td>
</tr>
<tr>
<td>U7</td>
<td>0</td>
</tr>
<tr>
<td>U8</td>
<td>1</td>
</tr>
<tr>
<td>U11</td>
<td>0</td>
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<td>U12</td>
<td>0</td>
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<td>U13</td>
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</tr>
<tr>
<td>U14</td>
<td>0</td>
</tr>
<tr>
<td>U15</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0</td>
</tr>
<tr>
<td><em>PAO1</em></td>
<td>0</td>
</tr>
<tr>
<td><em>CC6</em></td>
<td>0</td>
</tr>
</tbody>
</table>

*Autoinhibitory activity was not tested.

^a^ The radius of growth inhibition measured in millimetres.

^b^ *Escherichia coli*

^c^ *Pseudomonas aeruginosa* PAO1

^d^ *Bacillus* strain CC6

^e^ Growth inhibition radii of a value greater than zero and less than one millimetre.
Table 2.2: Inhibition of *C. fusiformis* as expressed by bacteria isolated from *Ulva*

<table>
<thead>
<tr>
<th>Bacterial Isolate</th>
<th>Inhibition Zone Radii (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>U₁</td>
<td>0</td>
</tr>
<tr>
<td>U₃</td>
<td>0-1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U₄</td>
<td>0-1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U₇</td>
<td>0-2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U₈</td>
<td>0-1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U₁₁</td>
<td>0-2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U₁₂</td>
<td>0</td>
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<tr>
<td>U₁₃</td>
<td>0-1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U₁₄</td>
<td>0-1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U₁₅</td>
<td>0-2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*The radius of growth inhibition measured in millimetres.

<sup>a</sup> Growth inhibition radii of a value greater than zero and less than one millimetre.

<sup>b</sup> Growth inhibition radii of a value greater than zero and less than two millimetres.

2.3.4 Characterization of active bacterial strains

On the basis of screening results, 8 active strains were chosen for species identification. Table 2.3 summarises the identity of the isolates based on comparison to NCBI, Ribosomal Project and ARB Project databases. Isolates were found to have high identity (99-100% base identity) with those of previously sequenced marine bacteria. Phylogenetic relations of the isolates to their closest groups are given in Figures 8.1-8.6 (Appendix III).
### Table 2.3: 16S rRNA gene identification of bacteria isolated from Ulva

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sequence similarity with NCBI¹</th>
<th>Taxonomical Hierarchy (Ribosomal Project)²</th>
<th>Phylogeny (ARB Project)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>U₃</td>
<td><strong>Bacillus</strong> sp. (deposited under GenBank accession FJ235130)</td>
<td>» » domain Bacteria&lt;br&gt; » » » phylum Firmicutes&lt;br&gt; » » » » class Bacilli&lt;br&gt; » » » » order Bacillales&lt;br&gt; » » » » » family Bacillaceae&lt;br&gt; » » » » » genus Bacillus</td>
<td><em>Bacillus subtilis</em> str. CTA8-17-1&lt;br&gt;<em>Bacillus thurigiensis</em> str. Al Hakam&lt;br&gt;<em>Bacillus cereus</em> str. WXZ-8&lt;br&gt;<em>Bacillus cereus</em> str. WGPSB2</td>
</tr>
<tr>
<td>U₄</td>
<td><strong>Shewanella oneidensis</strong> (deposited under GenBank accession FJ235131)</td>
<td>» » domain Bacteria&lt;br&gt; » » » phylum Proteobacteria&lt;br&gt; » » » » class Gammaproteobacteria&lt;br&gt; » » » » order Alteromonadales&lt;br&gt; » » » » » family Shewanellaceae&lt;br&gt; » » » » » genus Shewanella</td>
<td><em>Shewanella</em> sp. str. BR2&lt;br&gt;<em>Shewanella</em> sp. str. B21&lt;br&gt;<em>Shewanella</em> sp. str. 8027&lt;br&gt;<em>Shewanella</em> sp. str. 8005</td>
</tr>
<tr>
<td>U₇</td>
<td><strong>Pseudoalteromonas</strong> sp. (deposited under GenBank accession FJ235132)</td>
<td>» » domain Bacteria&lt;br&gt; » » » phylum Proteobacteria&lt;br&gt; » » » » class Gammaproteobacteria&lt;br&gt; » » » » order Alteromonadales&lt;br&gt; » » » » » family Pseudoalteromonadaceae&lt;br&gt; » » » » » genus Pseudoalteromonas</td>
<td><em>Pseudoalteromonas</em> sp. str. ANT9388&lt;br&gt;<em>Pseudoalteromonas</em> sp. str. 8057&lt;br&gt;<em>Pseudoalteromonas</em> sp. str. E36&lt;br&gt;<em>Pseudoalteromonas</em> sp. 8</td>
</tr>
</tbody>
</table>
| U_8  (400 bp) | Shewanella sp.  (deposited under GenBank accession FJ235133) | »  »  domain Bacteria  
»  »  »  phylum Proteobacteria  
»  »  »  »  class Gammaproteobacteria  
»  »  »  »  »  order Alteromonadales  
»  »  »  »  »  »  family Shewanellaceae  
»  »  »  »  »  »  »  genus Shewanella | - Shewanella putrefaciens str. PO14  
- Shewanella sp. G5  
- Shewanella baltica S5-16  
- Shewanella sp. str. ES-5 |
| U_11 (400 bp) | Pseudoalteromonas sp.  (deposited under GenBank accession FJ235134) | »  »  domain Bacteria  
»  »  »  phylum Proteobacteria  
»  »  »  »  class Gammaproteobacteria  
»  »  »  »  »  order Alteromonadales  
»  »  »  »  »  »  family Pseudoalteromonadaceae  
»  »  »  »  »  »  »  genus Pseudoalteromonas | - Pseudoalteromonas sp. str. ANT9388  
- Pseudoalteromonas sp. str. 8057  
- Pseudoalteromonas sp. str. E36  
- Pseudoalteromonas sp. 8 |
| U_13 (500 bp) | Vibrio sp.  (deposited under GenBank accession FJ235135) | »  »  domain Bacteria  
»  »  »  phylum Proteobacteria  
»  »  »  »  class Gammaproteobacteria  
»  »  »  »  »  order Vibrionales  
»  »  »  »  »  »  family Vibrionaceae  
»  »  »  »  »  »  »  unclassified Vibrionaceae | - Vibrio rumoiensis sp. str. S-4  
- Vibrio rumoiensis sp. str. S-4  
- Vibrio rumoiensis sp. str. SYS6-01  
- Vibrio rumoiensis sp. ANG.218 |
### Chapter 2: Inhibitory activity of epiphytic bacteria isolated from *Ulva*

| U_14 (500 bp) | **Vibrio** sp. *(deposited under GenBank accession FJ235136)* | » » domain Bacteria  
» » » phylum Proteobacteria  
» » » » class Gammaproteobacteria  
» » » » » order Vibrionales  
» » » » » » family Vibrionaceae  
» » » » » » » genus *Vibrio* | - *Vibrio rumoiensis* sp. str. S-4  
- *Vibrio rumoiensis* sp. str. S-4  
- *Vibrio rumoiensis* sp. str. SYS6-01  
- *Vibrio rumoiensis* sp. ANG.218 |
| U_15 (500 bp) | **Pseudoalteromonas rubra** *(deposited under GenBank accession FJ235137)* | » » domain Bacteria  
» » » phylum Proteobacteria  
» » » » class Gammaproteobacteria  
» » » » » order Alteromonadales  
» » » » » » family Pseudoalteromonadaceae  
» » » » » » » genus *Pseudoalteromonas* | - *Pseudoalteromonas* sp. str. 8041  
- *Pseudoalteromonas rubra* str. ATCC29570T  
- *Pseudoalteromonas* sp. str. QD1-2  
- *Pseudoalteromonas luteoviolacea* str. S2 |

1 Percentage base identity of 99-100% for all isolates  
2 Confidence threshold of 95% for all isolates.  
3 With reference to four most closely related species in ARB Project database
Chapter 2: Inhibitory activity of epiphytic bacteria isolated from Ulva

2.4 Discussion

2.4.1 Antibacterial activity of bacterial isolates

Antibacterial activity is known to be present in many surface-associated bacteria (Holmström and Kjelleberg, 1999; Rao et al., 2005; Longford et al., 2007). Many isolates in this study also displayed antibacterial activity (Table 2.1). Additionally, U15 which inhibited the largest number of isolates was pigmented. This correlates with a previous study where pigmentation was linked to the production of antifouling compounds. The study by Egan et al., (2002b) observed that colour mutants of P. tunicata differed in antifouling characteristics. Moreover, loss of antifouling activities and pigmentation was the result of disruption to genes with sequence similarities to transcriptional regulators, ToxR from Vibrio cholerae and CadC from Escherichia coli (Egan et al., 2002a).

During colonization, epiphytic bacteria compete for space and nutrients. Having antimicrobial properties confers selective advantages during colonization. It also provides protection to the host by reducing colonization of fouling organisms. Numerous studies show that bacteria produce active compounds against other microorganisms as well as against higher organisms (Egan et al., 2000; Kjelleberg et al., 1997). This characteristic may contribute to the overall microbial diversity on an algal surface since colonization and settlement of other organisms becomes highly regulated. Generally, a multitude of factors such as chemical-mediated interactions, communication, space and nutrient limitation and competition may shape the composition and properties of a surface community (Egan et al., 2008).

2.4.2 Anti-diatom activity of bacterial isolates

Anti-diatom activity was observed to occur widely amongst the bacterial isolates (Table 2.2). The presence of growth inhibition zones on assay plates may indicate the production of inhibitory substances by these isolates. A few studies have investigated anti-diatom activity in marine bacteria. Silva-Aciaries and Riquelme (2007) recently studied the effect of bacterial biofilms of Alteromonas sp. strain Nil-LEM on the settlement of 8 marine benthic diatoms. Comparison was made against Halomonas.
Inhibition of primary colonizers by marine surface-associated bacteria

*marina* (ATCC 25374) and *Pseudoalteromonas tunicata*, reference strains with proven antifouling properties. The highest antifouling activity was found for the *Alteromonas* strain. Similarly, a study of diatom settlement responses to crude extracts of several sponge species linked laboratory results with field evidence. In the laboratory, 6 out of 7 sponge extracts inhibited growth and caused mortality of the pennate diatom *Nitzschia paleacea* at tissue-level concentration. For field experiments, sponge metabolites immobilized in a gel matrix were exposed to natural microbial communities. After 7 days of exposure, 6 extracts suppressed the recruitment of diatoms (Dobretsov et al., 2005). These results indicate that anti-diatom activity is a common feature of marine surface-associated organisms. However, lack of suitable assay techniques has limited diatom-related studies.

The ecological significance of anti-diatom activity may be that bacteria are able to symbiotically exist with the host by preventing settlement and growth of diatoms in exchange for space and nutrients. Additionally, bacteria with the ability to inhibit diatom growth may be at an advantage during colonization with more effective isolates being more competitive than other surface colonizers.

### 2.4.3 Identification of active bacterial strains

Most of the isolates characterised in this study share sequence similarity to surface-associated marine bacteria previously isolated from temperate habitats. The majority of the isolates in the present study fall within the Proteobacteria (Table 2.3), a finding consistent with the global distribution of this group (Britschgi and Giovannoni, 1991; Schmidt et al., 1991; Field et al., 1997). As noted by Longford et al., (2007) comprehensive studies have led to the recognition of bacterial distribution in planktonic communities. In contrast, the study of living surface-associated biofilms is still in its infancy. Hence there is insufficient data to make similar large-scale comparisons of epibionts.

Several studies have looked at the diversity of bacteria on *Ulva* (Longford et al., 2007; Skovhus et al., 2007; Egan et al., 2000). Using a culture-independent method, Longford et al., (2007) found bacteria from an estimated 36 species and 4 phyla present on the surface of *U. australis*. These included Alpha-, Delta- and
Chapter 2: Inhibitory activity of epiphytic bacteria isolated from Ulva

Gammaproteobacteria along with Planctomycetes and Bacteroidetes. Moreover, bacteria of the genus Pseudoalteromonas has also been isolated from the surface of Ulva (Skovhus et al., 2007; Egan et al., 2000). In addition, a study showed that bacterial cells on the surface of U. australis consisted of approximately 70% Alphaproteobacteria and 13% Cytophaga-Flavobacteria (Tujula, 2006). In this study, except for one isolate, all were identified as Gammaproteobacteria.

It is speculated that expertise in culture-independent studies may be useful for demonstrating a greater microbial diversity on tropical Ulva spp. that has potential for more antifouling activities. Since marine bacteria have low culturability and are slow growing, cell recovery may be increased by increasing incubation period. In addition, using alternative media (e.g. a polysaccharide containing marine medium with a variety of substrates such as sea salts) could lead to the isolation of a wider range of bacteria.

Two inhibitory isolates (U_4 and U_8) had highest sequence similarity with Shewanella sp. (Table 2.3; Figures 8.2 and 8.4, Appendix III). Isolate U_4 showed 100% similarity to Shewanella oneidensis SCH0402, Genbank accession AY881235. This bacterium was originally isolated from temperate South Korean waters. The strain was found to be most active against a range of target bacteria and to have stronger repellent activity than tributyltin oxide (Bhattarai et al., 2006). Two antifouling compounds have been isolated from Shewanella oneidensis SCH0402, identified as 2-hydroxymyristic acid and cis-9-oleic acid (Bhattarai et al., 2007). These may or may not be the same compounds responsible for antifouling activity observed in this study. Isolate U_8 also showed high sequence similarity (99%) to Shewanella sp. However, based on phylogenetic analysis on the ARB database, U_8 appeared to be a different species to U_4 (Figures 8.2 and 8.4, Appendix III).

Also identified in this study were 3 isolates (U_7, U_11, and U_15) belonging to the genus Pseudoalteromonas (Table 2.3; Figures 8.3 and 8.6, Appendix III). Bacteria of the genus Pseudoalteromonas are often present on the surface of Ulva spp. (Egan et al., 2000; Skovhus et al., 2007). Pseudoalteromonas sp. influence biofilm formation in various marine niches; are involved in predator-like interactions within the microbial loop; influence settlement, germination and metamorphosis of various invertebrate
and algal species; and are adopted by marine flora and fauna as defence agents (Bowman, 2007).

Pigmented species of *Pseudoalteromonas* produce an array of both low and high molecular weight compounds with anti-fouling activities. The compounds formed include toxic proteins, polyanionic exopolymers, substituted phenolic and pyrrole-containing alkaloids, cyclic peptides and a range of bromine-substituted compounds (Bowman, 2007). In the current study, isolate U15, that had 100% sequence similarity with *Pseudoalteromonas rubra*, was also red-pigmented and had the strongest antibacterial and antidiatom activities (Tables 2.1 and 2.2). The bright red pigment of *P. rubra*, is a low molecular weight substance, identified as cycloprodigiosin HCl (Gerber and Gauthier, 1979; Kawauchi *et al.*, 1997). This compound is of pharmaceutical importance as an immuno-proliferation suppressesor (Magae *et al.*, 1996), for displaying anti-malarial activity (Kim *et al.*, 1999) and for inducing apoptosis in several cancer cell lines (Campàs *et al.*, 2003; Perez-Tomas *et al.*, 2003). Substituted phenylalkenoic acids, referred to as rubrenoic acids, purified from *P. rubra* show bronchodilatatoric activity. *P. rubra* also forms a high molecular weight substance, which is possibly a glycoprotein or polysaccharide known to expresses antibacterial activity. Growth inhibition by the antibiotic is due to the induction of oxidative stress in target cells through increased O2 uptake and an accumulation of hydrogen peroxide (Gauthier, 1976a; b). Currently there are no reports of anti-diatom activity in *P. rubra* and either of the known bioactive molecules or a new molecule may be responsible for the antidiatom characteristic of the bacterium. Isolates U7 and U11 had respectively 99% and 100% sequence similarity to *Pseudoalteromonas* sp. Moreover, phylogenetic analysis showed that the two isolates are possibly closely related (Figure 8.3; Appendix III).

Two of the bacterial isolates (U13 and U14) were identified as members of the genus *Vibrio* (Table 2.3; Figure 8.5, Appendix III). Phylogenetic analysis showed that the two isolates may be closely related (Figure 8.5; Appendix III). Isolate U13 had closest sequence similarity (99%) to *Vibrio* sp. A356, Genbank accession DQ005876. This bacterium was originally isolated from the surface of coralline algae. The isolate induced larval settlement in the common Australian sea urchin *Heliocidaris erythrogramma* (Hugget *et al.*, 2006). In another study, a *Vibrio* sp. isolated from
Ulva reticulata, growing in Hong Kong waters inhibited settlement and metamorphosis of the polychaete Hydroides elegans larvae (Dobretsov and Qian, 2002). Such findings suggest that Vibrio spp. live in close association with algal surfaces and may either play an inhibitive or inductive role. The results of this study correlate with findings of Dobretsov and Qian (2002), highlighting inhibitory characteristics of Vibrio spp. Prior to the results presented here, antidiatom activity in Vibrio spp. have not been reported.

Isolate U3 had activity against C. fusiformis and was identified as having closest sequence similarity (99%) to Bacillus sp. (Table 2.3; Figure 8.1, Appendix III). The general understanding of marine bacterial diversity gained from planktonic and epiphytic communities suggest that the majority of isolates are Gram-negative (Farmer and Hickman-Brenner, 1992). Also, recent culture-independent studies of bacterial diversity on the surface of Ulva spp. have not reported the presence of Bacillus sp. (Tujula, 2006; Longford et al., 2007). However, there is now an increased awareness of the presence of true marine Gram-positive bacteria, with a large number being isolated from marine sediments. Recently, Gontang et al., (2007) isolated 1,624 diverse Gram-positive bacteria spanning 22 families with 66% belonging to the class Actinobacteria and the remaining 34% being members of the class Bacilli. Gram-positive bacteria of the genus Bacillus have been reported in another study where B. firmus and B. mojavensis were isolated from the marine environment (Gontang et al., 2007; Ivanova et al., 1999; Ortega-Morales et al., 2008). Furthermore, members of the genus are known to produce bioactive lipopeptides that are responsible for the antibacterial activity observed (Ortega-Morales et al., 2008). This study has highlighted anti-diatom characteristics within Bacillus, which has not been reported previously. The mode of inhibition remains to be explored.
2.5 Conclusion

This chapter highlights the presence of surface associated bacteria on *Ulva* that may be responsible for the algae remaining unfouled in an environment prone to biofouling. Both antibacterial and anti-diatom activities are common characteristics of the epiphytic bacteria, with anti-diatom activity being a more widely prevalent feature. The epiphytic anti-fouling bacterial groups identified include the genera *Shewanella, Vibrio, Bacillus* and *Pseudoalteromonas*. The study further highlights anti-diatom activity in *Bacillus* and *Vibrio*, groups where such action has not previously been recognised. The close association between *Pseudoalteromonas* and algal surfaces has gained a lot of attention recently. This has mainly been due to its significant antifouling properties and success as surface colonizers. This study also suggests a close affiliation between *Pseudoalteromonas* and *Ulva* since the strains isolated from the algae displayed both antibacterial and anti-diatom properties.

The microfloral similarity on *Ulva* isolated from temperate and tropical regions is highlighted. It is evident that *Ulva* supports growth of many epiphytic bacteria that have potential as antifoulants. The abundance of *Ulva*, combined with warm tropical conditions may support high bacterial diversity, leading to the discovery of novel antifoulants. Together with advances in studies of microbial mediated defence systems, its application will be highly beneficial for the development of antifoulants.
Chapter 3: Anti-diatom properties of *Pseudoalteromonas* and *Roseobacter* strains

3.1 Introduction

Diatoms, like bacteria are primary colonizers of marine surfaces. Upon adhesion, through their characteristic secretion of adhesive mucilage, diatoms play an important role in early film formation. As the biofilm grows, adhesive exudates are released, trapping additional particles and microorganisms, progressively leading to the formation of a mature biofilm community (Silva-Aciares and Riquelme, 2007).

Once mature biofilms have formed on vessels, they are difficult to remove. The development of silicone-fouling release coatings have decreased the adhesion strength of attached organisms, which are removed as the vessel moves through water. Although macroalgae and some hard foulers such as barnacles detach relatively easily, diatom slimes, oysters and tube worms are attached tenaciously and are not easily removed, even at high speed (Callow and Callow, 2002). Due to the need for more efficient diatom control measures researchers are now exploring natural products for more effective alternatives (Silva-Aciares and Riquelme, 2007).

Living surfaces in the marine environment have developed efficient means of keeping their surface free of diatoms. Dobretsov *et al.* (2005) assessed the settlement response of diatoms to crude extracts of several sponge species from Hong Kong waters. Experiments showed that 6 out of 7 sponge extracts inhibited growth and caused mortality of the pennate diatom *Nitzschia paleacea* at tissue level concentration. Similarly, a study explored the anti-diatom strategy of the blue mussel, *Mytilus edulis* (Bers *et al.*, 2006). It was found that attachment of the benthic diatom *Amphora coffeaeformis* was significantly reduced by dichloromethane extracts, whereas ethyl acetate and diethyl ether fractions slowed diatom growth. These results provide evidence that living surfaces in the marine environment may moderate surface colonization of diatoms.
Chapter 3: Anti-diatom properties of *Pseudoalteromonas* and *Roseobacter* strains

*Pseudoalteromonas tunicata* and *Ph. gallaeciensis* are regarded as model epiphytic bacteria living in close association with marine algae. Both species are often isolated from the surface of *Ulva* spp. and are known specifically to prevent the settlement of common fouling organisms (Bowman, 2007; Brinkhoff et al., 2008). With the success of *P. tunicata* and *Ph. gallaeciensis* as surface colonisers, it is predicted that other members of these genera may also express antifouling activities. Additionally, anti-diatom activity is a characteristic largely unexplored in *Pseudoalteromonas* and *Roseobacter* spp. Such inhibitory activities may involve novel bioactive compounds. The study aimed to explore and compare the prevalence of anti-diatom activity across *Pseudoalteromonas* and *Roseobacter* spp. Since relatively little is known about the defence strategies of epiphytic bacteria against diatoms, the study further aimed to provide an insight into the mechanism. Information on gene identity and the transposon Tn10 were used as tools to manipulate the genome of the model epibiont, *P. tunicata*. Finally, a hypothetical model for the expression of anti-diatom activity in *P. tunicata* was proposed.

3.2 Materials and methods

3.2.1 Screening *Pseudoalteromonas* and *Roseobacter* strains for anti-diatom activity

Eight *Pseudoalteromonas* species strains and sixteen *Roseobacter* clade strain members (*Pseudoalteromonas aurantia*, *Pseudoalteromonas citrea*, *Pseudoalteromonas piscicida*, *Pseudoalteromonas undina*, *Pseudoalteromonas ulvae*, *Pseudoalteromonas haloplanktis*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas tunicata*, *Rhodobacter sphaeroides* 2.4.1, *Rhodobacter sphaeroides* 17025, *Rhodobacter sphaeroides* 17029, *Roseovarius nubinhibens* ISM, *Sulfitobacter* sp. EE-36, *Roseovarius nubinhibens* ISM, *Sulfitobacter* sp. NAS-14.1, *Sagittula stellata* E-37, *Silicibacter pomeroyi* DSS-3, *Dinoroseobacter shibae* DFL 12, *Maricaulis maris* MCS10, *Jannaschia* sp. CCS1, *Roseobacter* sp. CCS2, *Ruegeria R11*, *Phaeobacter gallaciensis* sp. 2.10 and *Phaeobacter gallaciensis* BS107) were obtained from the culture collection at Centre for Marine Bio-innovation, University of New South Wales, Australia. To screen for anti-diatom properties, the previously described anti-diatom bioassay (section 2.2.3) was used.
3.2.2 Analysis of anti-diatom strategy of *P. tunicata*

Transposons are discrete DNA segments that can repeatedly insert at sites in a genome and are therefore useful tools for genetic manipulation. In transposon mutagenesis, random insertions occur within a specific gene and result in the loss of function of that gene. One such system is a modified version of the transposon Tn10 known as mini-Tn10 (Herrero *et al*., 1990). This transposon carries a kanamycin-resistance marker that allows for easy selection of mutants. In addition, the transposase gene is outside of the mobile element, which allows for a stable insertion because of the loss of the transposase gene during the transfer. A transposon mutant library was created for *P. tunicata*. The library was screened for mutants lacking in anti-diatom activity and genetic analysis of transposon insertion sites in mutants were used to identify the affected genes.

3.2.2.1 Transposon mutagenesis

The transposon mutagenesis protocol established by James (1998) with modifications was used to generate non anti-diatom mutants of *P. tunicata*. Overnight cultures of both donor *E. coli* Sm 10 (containing pLOF mini-Tn 10 system) and the streptomycin resistant recipient *P. tunicata* (SmR) were prepared. The *E. coli* strain was grown with shaking at 37°C in LB10 medium (Appendix I) containing 85 μg/ml kanamycin and 100 μg/ml ampicillin. The *P. tunicata* strain was grown with shaking at room temperature in marine broth containing 200 μg/ml streptomycin.

The overnight cultures were washed twice to remove residual antibiotics. This was done by spinning the cultures at 5400 × g for 2 min, then resuspending in fresh media lacking antibiotics. After washing, the cells were resuspended in 1 volume of sterile 10 mM magnesium sulphate and gently mixed. To allow conjugation, a sterile 0.22 μm (2.5 cm diameter) membrane filter was placed onto LB15 agar plate (Appendix I) containing 0.3 mM isopropyl-β-D-thiogalactoside (IPTG). Donor and recipient cultures were mixed on the membrane filter in a volume ratio of 1:3 (50 μl *E. coli* + 150 μl *P. tunicata*) and incubated for 4 hr at 30°C. Following incubation, cells were removed from filters by gently rolling it up and placing into microcentrifuge tubes containing 1 ml marine broth and vortexed for 1 min. A 100 μl aliquot of the cell
suspension was plated onto marine agar (Difco marine broth solidified with 1.5% agar) containing 85 μg/ml kanamycin and 200 μg/ml streptomycin to select for recipient *P. tunicata* strains carrying the mini-Tn10 transposon. Plates were incubated for 48 hr at 30°C. Colonies were transferred from agar plates to 96-well plates containing 150 μl of marine broth containing 85 μg/ml kanamycin and 200 μg/ml streptomycin. Plates were incubated with shaking at 30°C for 48 hr. A 65 μl aliquot of glycerol was added to each well after the incubation period and plates stored at -80°C.

### 3.2.2.2 Screening for *P. tunicata* mutants lacking anti-diatom property

Screening for mutant phenotype was performed on 12 inch square agar plates. A 2 ml aliquot of diatom culture was spread plated onto each plate. An ethanol sterilized 96-pin replicator was used to transfer mutants from 96-well plates to the agar surface. Plates were incubated inverted at 20°C in a photoperiod of 16 hr light: 8 hr dark. Light was provided both from above and below the plates. Growth was monitored over 5 days. Loss of anti-diatom activity was inferred if a mutant failed to produce an inhibition zone. Plates inoculated with *P. tunicata* wild type served as controls. Confirmatory tests were performed on mutant strains by re-testing for the loss of anti-diatom activity as described above.

### 3.2.2.3 Growth rates of mutants

A comparison of the growth rates of transposon mutants and wild-type *P. tunicata* was performed. Strains were grown in 500 ml conical flasks containing 200 ml of growth media. The wild type *P. tunicata* was grown in marine broth whereas the non anti-diatom transposon mutants, DM1, DM2, DM3 and DM4 were grown in marine broth containing 85 μg/ml kanamycin and 200 μg/ml streptomycin. A 2 ml aliquot of an overnight culture was inoculated into an appropriate flask and incubated shaking at 23°C. Growth was monitored by absorbance readings at 610 nm over a 24 hr period. The experiment was carried out in duplicate and mean absorbance values were used to plot the growth curve of tested strains.
3.2.2.4 Genomic DNA extraction of non anti-diatom mutants

Genomic DNA was extracted from mutant cultures using the XS-buffer method (Tillett and Neilan, 2000), outlined in section 2.2.4.1. The extracted DNA was visualized by electrophoresis on 1% (w/v) agarose gel using λ-DNA digested with EcoRI/HindIII molecular weight marker for size and concentration estimation.

3.2.2.5 Generation of adaptor ligated DNA for panhandle PCR

A suppression PCR (Siebert et al., 1995) method termed pan-handle PCR can be used to walk from a known region into an unknown region in genomic DNA. The DNA sequence of the mini-Tn10 transposon is known. Hence, it is possible to use the panhandle-PCR method to obtain sequence information for the genes disrupted by the transposon. Due to the presence of inverted terminal repeats in adaptor molecules, PCR amplification of fragments with adaptor sequence at both ends will result in the ends of individual DNA strands forming “panhandle” structures following every denaturation step. Since these structures are more stable than the primer-template hybrid, exponential amplification is suppressed (Siebert et al., 1995). In contrast, PCR products formed by gene-specific primer and adaptor primer combinations cannot form panhandle structures, allowing PCR amplification to continue.

Genomic DNA extracted from mutant cultures was used for restriction digestion and ligation of adaptor molecules in a one step process. Each reaction mixture contained 1 μg of genomic DNA, 10 pmol/μl adaptor 1 (Appendix II), 10 pmol/μl adaptor 2 (Appendix II), 20 mM ATP, 2.5 units of T4 ligase, 0.5 units of blunt-end restriction enzyme (various, see Table 3.1), 1 × respective restriction enzyme buffer and deionised water to give a final reaction volume of 20 μl. Reactions were incubated at 20°C for 16 hr, after which the reaction was deactivated by heating to 68°C for 10 min.

The DNA was then precipitated using ethanol. Briefly, a 1/10th volume of 3 M sodium acetate (pH 5.2) was added and mixed well. Exactly 2.5 volumes of ice-cold absolute ethanol was then added and again mixed well. Tubes were chilled at -20°C for 60 min. After incubation, DNA was pelleted by centrifugation at 21 000 × g and
Chapter 3: Anti-diatom properties of *Pseudoalteromonas* and *Roseobacter* strains

4°C for 15 min. The supernatant was discarded and the pellet was washed in 70% (v/v) ethanol to remove salt. The tubes were inverted to dry the pellet, following which DNA was resuspended in 50 μl of sterile deionised water. This solution served as the template DNA for the PCR reactions described below.

**Table 3.1: Restriction enzymes used for panhandle PCR**

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dra</em>I</td>
<td>TTT↓AAA</td>
</tr>
<tr>
<td><em>EcoRV</em></td>
<td>GAT↓ATC</td>
</tr>
<tr>
<td><em>HincII</em></td>
<td>GT(T,C)↓(A,G)AC</td>
</tr>
<tr>
<td><em>HpaI</em></td>
<td>GTT↓AAC</td>
</tr>
<tr>
<td><em>PvuII</em></td>
<td>CAG↓CTG</td>
</tr>
<tr>
<td><em>RsaI</em></td>
<td>GT↓AC</td>
</tr>
<tr>
<td><em>ScaI</em></td>
<td>ACT↓ACT</td>
</tr>
<tr>
<td><em>SspI</em></td>
<td>AAT↓ATT</td>
</tr>
<tr>
<td><em>XmnI</em></td>
<td>GAANN↓NNTTC</td>
</tr>
</tbody>
</table>

1All enzymes were purchased either from BioLabs, Promega or Roche

**3.2.2.6 Panhandle PCR**

PCR was performed in 20 μl reaction volumes using 1 μl of DNA solution (as described above), 10 mM dNTPs, 10 pmol of adaptor primer 1 (Appendix II), 10 pmol of sequence specific primer (Tn10C or Tn10D, Appendix II), 1 × Taq buffer, 2.5 mM MgCl₂ and 0.05 unit of Taq polymerase added after a hot start (95°C for 2 min). The cycle parameters were denaturation at 95°C for 30 sec and annealing/extension at 65°C for 7 min for 25 cycles. A final extension was performed at 65°C for 1 min and samples were held at 4°C.

PCR product concentration was estimated by agarose gel electrophoresis as described in section 2.2.4.2. Single band products were purified using QIAquick PCR.
Chapter 3: Anti-diatom properties of *Pseudoalteromonas* and *Roseobacter* strains

Purification Kit as per the manufacturer’s instructions. When more than one band was present, the band of interest was excised and DNA extracted from the gel slice using Invitrogen Quick Gel Extraction Kit following the manufacturer’s instructions. The purified product was examined by visualizing on agarose gel once again.

### 3.2.2.7 Sequencing

The purified PCR products were sequenced independently using the corresponding transposon-specific and adaptor primers. Reaction mixtures contained 15-20 pmol of either of Tn10C, Tn10D, AP1 or AP2 primers (Appendix II), 50-100 ng of DNA template, 5 × CSA sequencing buffer, 1 unit of BigDye™ terminator cycle sequencing reaction mix v.3.1 (Applied Biosystems) and sterile deionised water in a final volume of 20 μl. Cycle sequencing was conducted using the following thermoprofile: 94°C for 10 sec, 50°C for 5 sec and extension at 60°C for 4 min in 99 cycles. Extension products were purified by ethanol precipitation as described in section 2.2.4.4. Separation of sequencing products was performed on an ABI 3730 DNA sequencing system at the Automated Sequencing Facility, UNSW. Sequences obtained were compared to protein sequences available in the NCBI BLAST 2.0 (Altschul *et al.* 1990) and IMG 2.41 (Markowitz *et al.*, 2008) databases in order to identify genes disrupted by the transposon.

### 3.3 Results

#### 3.3.1 Growth inhibition of diatoms

All *Pseudoalteromonas* strains tested showed some degree of diatom growth inhibition (Figure 3.1). *Pseudoalteromonas aurantia*, *P. undina* and *P. haloplanktis* were the most effective while *P. citrea* and *P. ulvae* were the least inhibitory. Additionally, replicated assay plates showed varying sizes of growth inhibiton zones produced by *P. nigrifaciens*. The activity of *Roseobacter* strains against growth of *C. fusiformis* is illustrated in Figure 3.2. Approximately 44% of the *Roseobacter* clade species tested displayed anti-diatom activity. *Jannaschia* sp. CCS1 and *Phaeobacter gallaeciensis* 2.10 were the most effective, with replicated plates showing variation in
the level of activity. Nine strains of the *Roseobacter* clade did not inhibit diatom growth.

![Graph showing diatom growth inhibition by *Pseudoalteromonas* spp.](image)

**Figure 3.1:** Diatom growth inhibition by *Pseudoalteromonas* spp. The negative control plates showed no growth inhibition with uniform growth of diatoms across the plates (data not shown). Data used are mean and standard deviation of 5 replicates.
Figure 3.2: Diatom growth inhibition by members of the *Roseobacter* clade. The negative control plates showed no growth inhibition with uniform growth of diatoms across the plates (data not shown). Data used are mean and standard deviation of 5 replicates.
3.3.2 Mutants lacking in anti-diatom activity

Using mini-Tn10 and the suicide vector pLOF for delivery (Way et al., 1984), transposon mutants lacking the ability to inhibit diatom growth were successfully generated in *P. tunicata*. The transposon bank consisted of approximately 1000 transconjugants. After screening it was found that 4% of the transconjugants had lost activity. Four of the mutants (designated DM1, DM2, DM3 and DM4) were randomly selected for re-testing. Of these, 3 were used for further genotypic analysis. Figure 3.3 illustrates the presence of a zone of growth inhibition when wild type *P. tunicata* was tested against *C. fusiformis* and its absence when mutants of *P. tunicata* were used.

![Figure 3.3: Anti-diatom activity of wild type *P. tunicata* and non-antidiatom mutants (DM1, DM2, DM3 and DM4). Test bacterial culture was inoculated in small circles on plates previously inoculated with target diatom, *C. fusiformis*.](image-url)

*Figure 3.3*: Anti-diatom activity of wild type *P. tunicata* and non-antidiatom mutants (DM1, DM2, DM3 and DM4). Test bacterial culture was inoculated in small circles on plates previously inoculated with target diatom, *C. fusiformis*.
3.3.3 Growth curve of wild type and mutants of *P. tunicata*

To ensure that the loss of anti-diatom activity was not due to a mutation in an essential pathway that would lead to decreased cell activity and growth, an overnight growth experiment was performed. The wild type *P. tunicata* and the transposon generated anti-diatom mutants showed no significant difference in general growth pattern or rate. Cells entered logarithmic growth phase after approximately 2 hr and reached stationary phase after approximately 22 hr. A graph of optical density versus time is shown in Figure 3.4.

![Growth curve graph](image)

**Figure 3.4:** Growth curve of wild type and mutant strains of *P. tunicata*. Duplicate cultures (A and B) were inoculated as indicated in text (section 3.2.2.3) and growth was monitored by absorbance readings at 610 nm over a 24 hr period.

3.3.4 Panhandle PCR and DNA sequencing

Using panhandle PCR, the sites within the *P. tunicata* genome that had been disrupted by insertion of the mini-Tn10 transposon were amplified (Figure 3.5). Amplified regions were further used for sequencing to determine the transposon insertion sites.
Chapter 3: Anti-diatom properties of *Pseudoalteromonas* and *Roseobacter* strains

3.3.5 Genotype characterization of the non anti-diatom mutants

The genomic DNA regions flanking the transposon insertion sites in each of the three mutants were analysed by sequencing. Sequencing of the 3 mutants (DM1, DM2 and DM3) indicated different transposon insertion sites. Descriptions of each of the insertion sites are as follows:

3.3.5.1 DNA regions flanking the transposon insertion site in DM1

Sequencing results of the analysis of DM1 showed that the transposon had inserted into a gene, IMG locus tag PTD2_12754, with homology to a cation/multidrug efflux pump, AcrB/AcrD/AcrF family protein. The insertion had occurred at position 227 of the 3075 bp open reading frame (ORF) (Figure 9.1, Appendix IV). A schematic representation of the position of the cation/multidrug efflux pump gene within the *P. tunicata* genome is shown in Figure 3.6.

**Figure 3.5**: 1% agarose gel showing the results from a typical panhandle-PCR. Genomic DNA templates from DM3 were used. Lane 1: 250 ng molecular weight marker (λ EcoRI/HindIII digest); Lane 2: DraI digest and Tn10C primer; Lane 3: EcoRV digest and Tn10C primer, Lane 4: HincII digest and Tn10C primer; Lane 5: HpaI digest and Tn10C primer.
Chapter 3: Anti-diatom properties of *Pseudoalteromonas* and *Roseobacter* strains

### Inhibition of primary colonizers by marine surface-associated bacteria

3.3.5.2 DNA regions flanking the transposon insertion site in DM2

In mutant DM2, the two ORFs affected were IMG locus tags PTD2_01386 (homology to a beta-hexosaminidase) and PTD2_01391 (homology to a RTX toxin and related Ca$^{2+}$ binding protein). The gene positions are schematically represented in Figure 3.7. The sequence obtained indicated that the transposon inserted at the end of PTD2_01386 (position 2248 in the 2280 bp ORF), extended into the intergenic region and partially into locus PTD2_01391 (Figure 9.2, Appendix IV).

**Figure 3.6:** Genomic location of PTD2_12754 (shown in red), homologous to AcrB/AcrD/AcrF family protein. Positions of neighbouring genes are also shown. The base pair size is indicated along the top of the scale bar.

**Figure 3.7:** Genomic location of PTD2_01386 (indicated in red) and PTD2_01391 (indicated in blue, located downstream of PTD2_01386), homologous to beta-hexosaminidase and RTX toxin respectively. Positions of neighbouring genes are also shown. The base pair size is indicated along the top of the scale bar.
3.3.5.3 DNA regions flanking the transposon insertion site in DM3

Sequence analysis of DM3 showed that the transposon had inserted into a 702 bp ORF (Figure 9.3, Appendix IV), IMG locus tag PTD2_02946, a protein with homology to the HemeO protein family. PTD2_02946 is clustered with several other ORFs including a long chain fatty acid (LCFA)-coA ligase and a short chain alcohol dehydrogenase-like protein. A schematic representation of the position of PTD2_02946 is provided in Figure 3.8.

![Figure 3.8](image)

**Figure 3.8:** Genomic location of PTD2_02946, a HemeO protein family (indicated in red). Located upstream, next to PTD2_02946 is PTD2_02941, a short chain alcohol dehydrogenase-like protein (indicated in gold). Downstream of PTD2_02946 is PTD2_02951, a LCFA-coA ligase (indicated in violet). The base pair size is indicated along the top of the scale bar.

3.4 Discussion

3.4.1 Anti-diatom activity of Pseudoalteromonas spp. and Roseobacter clade

Since all the Pseudoalteromonas strains tested inhibited diatom growth, it is obvious that this characteristic is prevalent within the genus. Pseudoalteromonas aurantia, P. undina and P. haloplanktis were the most active against growth of C. fusiformis (Figure 3.1). Previously, P. aurantia has been isolated from the surface of U. australis and shown to produce unknown compounds that inhibit settlement of fouling organisms (Gauthier and Breittmayer, 1979; Bowman, 2007). Additionally, P. haloplanktis produces diketopiperazines, a probiotic beneficial to shellfish. Currently there are no reports of bioactive compounds from P. undina (Bowman, 2007).
During a study conducted to observe the range of antifouling activities expressed by *Pseudoalteromonas* spp., members of the genus produced a variety of bioactive compounds (Holmstrom *et al*., 2002). Most strains inhibited bacterial and fungal growth, algal spore germination and invertebrate larvae settlement. However, *P. nigrifaciens* displayed negligible activity in most bioassays. Interestingly, in this study *P. nigrifaciens* inhibited the growth of *C. fusiformis*. The different patterns of diatom growth inhibition by *Pseudoalteromonas* strains suggest diversity within the genus. The prevalence of anti-diatom activity may be an adaptation that allows *Pseudoalteromonas* to colonize a wide range of habitats.

Strains of the *Roseobacter* clade tested for activity against the growth of *C. fusiformis* also produced varying results. The greatest diatom growth inhibition was caused by *Jannaschia* sp. CCS1, followed by *Phaeobacter gallaeciensis* 2.10 and *Dinoroseobacter shibae* DFL 12 (Figure 3.2). Although anti-diatom activity by members of the *Roseobacter* clade has not been reported previously, their antibacterial activity has been investigated in several studies (Brinkhoff *et al*., 2004; Bruhn *et al*., 2005; Ruiz-Ponte *et al*., 1998). The latter characteristic is an advantage that may contribute to the dominance of the clade in alga-associated bacterial communities. There may be different reasons for variation in bioactive compound production. Certainly some strains may lack the ability to produce bioactive compounds. For example, *Ph*. strain 27-4 only produces antibiotic when grown in liquid nutrient medium under static conditions, which also facilitates rosette and biofilm formation (Bruhn *et al*., 2006; Bruhn *et al*., 2005). Such results suggest that culture conditions influence the production of antibacterial compounds.

Approximately half (56%) of the *Roseobacter* strains tested did not inhibit diatom growth. It has been suggested that the differing physiological characteristics reflect adaptation to the diverse ecological niches that *Roseobacter* occupies (Brinkhoff *et al*., 2008). The lack of activity against diatoms may therefore indicate that anti-diatom activity is not conferring a specific advantage. Members of the *Roseobacter* clade are found in temperate as well as polar oceans (Brinkhoff, *et al*., 2008) and dominate among marine alga-associated bacteria (Alavi *et al*., 2001; Buchan *et al*., 2005; Gonzalez *et al*., 2000). It is reported that 1 in 10 bacterial cells is a member of the *Roseobacter* group (Giovannoni and Rappe, 2000). Since the first description of
Roseobacter sp. in 1991, 38 affiliated and validated genera have been described (Brinkhoff, et al., 2008). In comparison, the Pseudoalteromonas group is smaller with as few as 35 species and 2 sub-species known to date (http://www.bacterio.cict.fr/p/pseudoalteromonas.html). As the members of the Roseobacter clade tested were only a small representation of the actual group, comparison of the prevalence of anti-diatom activity to the the much smaller Pseudoalteromonas group may be biased. Additionally, these results may suggest that the defence mechanisms of the two groups of bacteria differ. In recent years, members of Roseobacter have gained recognition for their antifouling properties (Brinkhoff, et al., 2008; Rao et al., 2006). In particular, Ph. gallaeciensis is known to be a more competitive biofilm-forming bacterium than P. tunicata (Rao et al., 2006). With such strong anti-diatom properties and competitive biofilm-forming characteristics, this species of Phaeobacter is worth further investigating for its potential in preventing biofouling.

3.4.2 Analysis of transposon insertion sites within the P. tunicata genome

To study the anti-diatom strategy of P. tunicata, transposon insertion sites in non anti-diatom mutants were analysed. In the first mutant, DM1, the transposon had inserted into a gene homologous to the cation/multidrug efflux pump (Figure 3.6). The cation/multidrug efflux pump (AcrB/AcrD/AcrF family protein) is an extremely conserved gene across many phyla. The protein is synonymous to acriflavin (a common tropical antiseptic) resistance protein in Pseudoalteromonas atlantica T6c (66% identity). In Escherichia coli, the AcrB genes encode a multi-drug efflux system, believed to protect the bacterium against hydrophobic inhibitors (Ma et al., 1993). The system is energized by proton-motive force and shows the widest substrate specificity amongst known multidrug pumps, including antibiotics, disinfectants, dyes, detergents and solvents.

ABC transporters are generally involved in transporting ions, carbohydrates, amino acids, antibiotics, polysaccharides and proteins (Saurin et al., 1999). ABC importers in particular, are involved in transporting ferri-siderophore complexes across the periplasmic space and cytoplasmic membrane back into the cells (Andrews et al., 2003). Whether an ABC system imports or exports molecule depends upon the
presence or absence of a periplasmic binding protein associated to the coding sequences for the ABC and transmembrane domains (Linton and Higgins, 1998).

The ABC-transporters of \textit{P. tunicata} have been suggested to be involved in iron transport (Evans \textit{et al.}, 2007). Analysis of the conserved domains of AcrB to determine protein function suggests a wide range of roles. This includes defence and transport mechanisms with a weak homology to SecD. The membrane protein SecD is a preprotein translocase subunit which is involved in intracellular trafficking and secretion. It may be speculated that the cation/multidrug efflux pump disrupted in DM1 is involved either in the secretion of molecules or putative toxins responsible for the anti-diatom property of \textit{P. tunicata}.

In mutant DM2 two ORFs are affected by transposon insertion (Figure 3.7). The transposon inserted at the end of a beta-hexosaminidase homologue that in \textit{Pseudoalteromonas} sp. strain S91 is involved in chitin degradation (Techkarnjanaruk and Goodman, 1999). Chitin is an important component of diatom cells. A study investigated the effects of two commercial chitin synthesis inhibitors, dimilin and polyoxin D, on chitin fiber formation and cell sedimentation in the diatoms \textit{Thalassiosira fluviatilis} and \textit{Cyclotella cryptica} (Morin \textit{et al.}, 1986). While dimilin treated diatoms were indistinguishable from controls, the polyoxin D treated cells of both diatom species completely lacked the characteristic chitin fibers. The polyoxin D cultures were also characterized by significantly lower population densities, increased sedimentation rates and strong tendency to clump in comparison to control and dimilin treatments (Morin \textit{et al.}, 1986). One function of chitin is in the formation of spines for diatom cell buoyancy. When the spines are removed by physical shearing or digestion by chitinase, the otherwise intact diatom cells lose their buoyancy, settling 1.7 times faster (Walsby and Xypolyta, 1977; Smucker, 1991). These results highlight the importance of chitin fiber formation in diatoms and suggest the likely impact of chitin-degrading enzymes (such as beta-hexosaminidase) produced by \textit{P. tunicata} on diatoms.

To test whether a disruption at the beta-hexosaminidase gene homologue is responsible for loss of production of a chitin degrading enzyme further studies are required. An experiment of the mutant’s growth on media with chitin as the sole
carbon source is suggested. Growth could be monitored by measuring optical density over a period of 48 hr. A standard logarithmic (S-shaped) graph would indicate the mutant’s ability to degrade chitin whereas decreased growth would indicate a disruption that results in the loss of production of the chitin-degrading enzyme.

Located downstream of the transposon insertion site is a RTX toxin-like gene (Figure 3.7). RTX toxins are pore-forming, soluble, secreted proteins produced by a broad range of pathogenic Gram-negative bacteria. In vitro, these most often exhibit cytotoxic and hemolytic activities. They are particularly common in the Pasteurellaceae which are disease causing in animals and humans (Joachim and Peter, 2002). The RTX gene cluster has also been identified in *Vibrio cholerae*, where it produces proteins with haemolytic activity but also has roles in biofilm formation and cell-cell adherence (Chatterjee *et al.*, 2008; Lin *et al.*, 1999). Although the function of the RTX toxin-like genes in *P. tunicata* is still unknown, since it is a Gammaproteobacteria like *Vibrio*, it seems likely that there would be some degree of functional similarity between these two bacteria. This is supported by the presence of both cadherin/sarcoglycan-homologous domains and a secretory signal peptide in the *P. tunicata* (PTD2_01391) gene. These suggest a secreted protein with roles in adhesion or cell wall stabilisation. Functional similarity of *Vibrio* and *Pseudoalteromonas* proteins has been noted previously (Egan *et al.*, 2002a).

The sequence obtained through Panhandle PCR and sequencing overlapped mostly in gene PTD2_01391 (homology to RTX toxin). It is unclear if the RTX toxin gene was disrupted which led to any change in anti-diatom activity. However, it may also be that the intergenic region upstream of the RTX toxin gene contains regulatory regions, the disruption of which affected the expression of RTX toxin (or other gene) leading to a loss in anti-diatom property. Research has shown that the intergenic region between the divergently transcribed niiA and niaD genes of *Aspergillus nidulans* contains multiple NirA binding sites, which act bidirectionally (Punt *et al.*, 1995). Apparently, the insertion of an unrelated upstream activating sequence into the intergenic region strongly affected the expression of both genes, irrespective of the orientation in which the element was inserted. Also, located downstream of the RTX toxin is PTD2_01396 (Figure 3.7), which is a hypothetical protein, the function of
which is not yet known. However, analysis of the conserved domains predicts the protein to be a peptidase or a periplasmic protease.

Located upstream of PTD2_01386, are two Ton-B receptor proteins (PTD2_01376 and PTD2_01381) with roles in iron transport. Faraldo-Gomez and Sansom (2003) also suggest TonB-dependent receptors to be responsible for the transport of large extracellular molecules, such as vitamin B$_{12}$ and iron carriers (siderophores) into the bacterial cells. The TonB proteins in _E. coli_ interact with outer membrane receptor proteins that bind and take up specific substrates into the periplasmic space (Chimento _et al._, 2003). In the absence of TonB, the receptors bind their substrates but do not carry out active transport (Koebnik, 2005). The TonB complex senses signals from outside the bacterial cell and transmits them via two membranes into the cytoplasm, leading to transcriptional activation of target genes.

In _P. tunicata_, gene expression of proteins involved in iron acquisition and uptake, including TonB are controlled by WmpR (a ToxR-like regulator, that also controls expression of bioactive compounds, type IV pili and biofilm formation; Stelzer _et al._, 2006). Recent work by Evans _et al._ (2007) links iron transport by TonB receptors to a type-II secretion pathway. A disruption of the type-II secretion machinery in _P. tunicata_ (wmpD mutant) results in the loss of pigment production and loss of bioactive compounds against all target organisms. In addition, the upregulation of TonB system biopolymer-transport proteins in the _wmpD_ mutant suggests a role in transport and acquisition of iron. It has thus been suggested that the type-II secretion pathway may be responsible for the transport of extracellular enzymes that obtain precursor molecules for pigments and other bioactive compounds.

In mutant DM3 the transposon had inserted into PTD2_02946, a hypothetical protein with homology to the HemeO protein family (Figure 3.8). Analysis of the conserved domains demonstrates that the protein has heme-binding capacity. Acquisition of heme from the environment is often for the purpose of obtaining iron (Wilks, 2002). _Neisseria meningitides_ and _Pseudomonas aeruginosa_ are pathogens known to utilize the host’s heme as an iron source. These pathogens depend on heme oxygenase for the release of iron (Ratliff _et al._, 2001; Zhu _et al._, 2000a; b). In living organisms, including diatoms, heme biosynthesis represents an essential metabolic pathway that
provides the precursors for cytochrome prosthetic groups, photosynthetic pigments, and vitamin B-12 (Obornik and Green, 2005).

These results imply that a mutation in PTD2_02946, may prevent iron acquisition, which by some means impairs anti-diatom activity. It may be that *P. tunicata* acquires iron from diatom heme, an interaction that disrupts an essential metabolic pathway in diatoms. However, this remains to be studied. At this stage we can only speculate that iron is important either indirectly (such as in the form of a precursor of a regulatory protein) or directly for virulence against diatoms.

Analysis of the different transposon insertion sites and corresponding loss in anti-diatom activity can be used to propose a mechanism for anti-diatom activity in *P. tunicata*. Iron is suggested to be essential for the expression of anti-diatom activity. The HemeO homologue participates in the acquisition of iron from the environment or directly from diatoms. Type II-secretion pathway regulates the secretion of TonB, which would be needed to bind and transport the iron. Acquired iron is then involved either directly or indirectly in the regulation and expression of the RTX toxin-like gene. A multidrug efflux system is involved in pumping toxic proteins out of the bacterial cell which lead to diatom growth inhibition. This is clearly a complex system involving a number of steps, disruption of any one leading to loss of activity. Further study is needed to confirm this general mechanism and fill in critical remaining details.
3.5 Conclusion

Anti-diatom activity is a characteristic feature of many *Pseudoalteromonas* and members of the *Roseobacter* clade. These current findings emphasize the success of these groups as marine surface colonizers and the diversity of these groups. With the search on for more effective means of controlling biofouling, the genus *Pseudoalteromonas* and the *Roseobacter* clade need to be studied in much greater detail as possible sources of antifoulants.

The ability of *P. tunicata* to inhibit diatom growth was studied in detail by generating transposon mutants lacking in anti-diatom activity. Four mutants were successfully generated, of which three were further analysed. Results suggest several possible mechanisms of expression of anti-diatom activity. Genes observed to be important in anti-diatom activity were predicted to have functions including a cation/multidrug efflux pump, a beta-hexosaminidase protein, a RTX toxin and a heme binding protein. The experiments conducted in this research will form the basis of future studies that will identify the mechanism of anti-diatom activity in *P. tunicata*. 
Chapter 4: General discussion

This thesis describes an investigation of the antifouling characteristics of marine epiphytic bacteria, against primary surface colonizers of *Ulva* spp. from Fiji. As initial colonizers, both bacteria and diatoms are crucial for the subsequent development of a mature biofouling community. The presence of epiphytic bacteria, which may have a role in regulating growth of bacteria and diatoms on the surface of *Ulva*, along with their identification, is addressed in Chapter 2. Anti-diatom properties of epiphytic bacteria, which remains an under-explored area in marine ecology, is studied in Chapter 3. This final chapter outlines and discusses the major findings presented in the thesis and suggests directions for future work.

4.1 Antifouling properties of surface-associated bacteria

Bacteria live symbiotically on algal hosts by limiting colonization of surface foulers in exchange for space and nutrients. Specifically, epibionts regulate biofilm formation which benefits the host by preventing the development of a mature biofouling community. To cooperate in such symbiotic interactions, bacteria often need adaptive responses such as the production of antifouling molecules. Such antifouling properties confer a competitive advantage to bacteria competing for resources.

The first two aims of this thesis were to, isolate and identify epibionts of Fijian *Ulva* with inhibitory properties. Both antibacterial and anti-diatom activities were found to be common to these bacteria. Approximately 60% of the isolates inhibited the target bacteria and 80% inhibited growth of the diatom, *C. fusiformis*. The level of growth inhibition varied widely. The red pigmented, isolate U15 (deposited under GenBank accession FJ235137) was the most effective. This observation is consistent with previous studies that have correlated pigmentation with natural product formation in *Pseudoalteromonas* spp. (Egan *et al.*, 2002b). Other isolates with inhibitory properties were identified as members of *Shewanella, Pseudoalteromonas*, *Vibrio* and *Bacillus*. The results were correlated with previous findings of antifouling activity within the respective genera. Interestingly, anti-diatom activity was demonstrated in both *Bacillus* and *Vibrio*, groups where such activity has not previously been
recognized. It is speculated that many epibiotic bacteria have antifouling potential but these remain unrecognized.

The third aim of the thesis was to screen *Pseudoalteromonas* and *Roseobacter* isolates for anti-diatom activity. Results showed that of the tested strains, all the *Pseudoalteromonas* spp. and 44% of the *Roseobacter* strains inhibited growth of *C. fusiformis*. The research highlights the prevalence of anti-diatom activity in *Pseudoalteromonas*. The lower occurrence of anti-diatom activity in *Roseobacter* may reflect the clade’s greater ecological diversity. It may be that anti-diatom activity does not provide specific advantages in all cases.

The study re-emphasizes the need to preserve bacterial biodiversity especially symbiotic forms. The technology available to assess bacterial symbioses with higher organisms is limited (Egan *et al*., 2008). Advances in techniques will not only assist in exploring existing microbial diversity for novel bioactive compounds but also contribute significantly towards the exploitation of microbial defence mechanisms.

### 4.2 Modelling anti-diatom mechanism in *P. tunicata*

The marine epiphyte, *P. tunicata* is a model organism for studies of surface-associations. The bacterium is believed to exhibit the broadest range of inhibitory activities including antibacterial, anti-fungal, anti-algal and anti-larval characteristics (Holmstrom *et al*., 2002). Although the antifouling capability of *P. tunicata* has been highlighted, its anti-diatom capacity has not been explored specifically.

The final aim of the study was to identify genes involved in anti-diatom activity in *P. tunicata* and suggest a model describing the mode of action. *Pseudoalteromonas tunicata* mutants lacking in anti-diatom activity were generated by transposon mutagenesis. These provided some insight into the organism’s anti-diatom strategies. Three mutants were chosen for study and DNA sequence analysis revealed transposon insertion at three different locations in the genome. These included a gene homologous to a cation/multidrug efflux pump, a beta-hexosaminidase gene, RTX toxin-like gene and a member of the HemeO protein family. Sequence analysis of DM1 showed that the transposon had disrupted a gene homologous to the
cation/multidrug efflux pump protein, belonging to the AcrB/AcrD/AcrF family. Analysis of the conserved domains of AcrB gene suggests a wide range of roles including defence, transport mechanisms, intracellular trafficking and secretion. The study suggests the gene may be involved in the secretion of toxin/s responsible for the anti-diatom activity.

The two ORFs found affected by transposon insertion in mutant DM2 were homologous to beta-hexosaminidase and a RTX toxin. Beta-hexosaminidase is a chitin-degrading enzyme which suggests that chitin fiber formation in diatoms may be targeted by *P. tunicata*. Located downstream of the beta-hexosaminidase gene is an RTX toxin gene. The RTX gene cluster is present in a range of pathogenic Gram-negative bacteria and exhibits cytotoxic and haemolytic activities (Joachim and Peter, 2002; Chatterjee et al., 2008; Lin et al., 1999). Conserved domain analysis of the RTX toxin gene suggests involvement in adhesion and cell wall stabilisation or calcium-binding capacity. The non-coding region between the genes may contain regulatory elements, the disruption of which could affect the expression of the RTX gene, leading to a loss in anti-diatom activity. Interestingly, located upstream of the beta-hexosaminidase gene are a pair of genes encoding TonB receptors, which are involved in iron transport. Recent work by Evans et al., (2007) links iron transport by TonB receptors to a type-II secretion pathway. Sequence analysis of this mutant reveals a complex system, the expression of which may be regulated by iron.

In the last mutant analysed the transposon inserted into an ORF homologous to a member of the HemeO protein family. Conserved domain analysis suggests a heme-binding capacity. Heme is critical for iron acquisition (Wilks, 2002). In diatoms, heme biosynthesis represents an essential metabolic pathway which provides the precursors for cytochrome prosthetic groups, photosynthetic pigments, and vitamin B-12 (Obornik and Green, 2005). As with results of DM2, analysis of this mutant suggests an important role for iron in the anti-diatom strategy of *P. tunicata*. Iron may be obtained from diatoms by the HemeO homologue.
Based on these results a preliminary model for the expression of anti-diatom activity is proposed (Figure 4.1). Briefly, iron is suggested to be essential for the expression of anti-diatom activity and is obtained by the HemeO homologue. The type II-secretion pathway likely relates to the secretion of TonB, resulting in the binding and transport of the acquired iron. Iron is also suggested to be involved either directly or indirectly in the expression of the RTX toxin. Finally, the multidrug efflux system likely pumps toxins out of the bacterial cell that inhibit diatom growth.

**Figure 4.1:** Hypothetical model for the regulation of anti-diatom activity in *P. tunicata*. The *P. tunicata* cell is represented. HemeO family protein is involved in the acquisition of iron from diatom cells. Type II pathway possibly secretes TonB, which binds and transports the iron acquired into the bacterial cell. Iron is used either in the regulation of chitinase-producing beta-hexosaminidase gene or the RTX toxin gene. The multidrug efflux system pumps out putative toxin(s).
4.3 Future directions and implications

Ecologically, antibacterial and anti-diatom properties give epibionts a competitive advantage over other surface colonizing microbes when competing for space and nutrients. Additionally, bacteria with inhibitory characteristics are often able to form symbioses with algae, providing a microbial-mediated defence system in return for nutrition. This study has highlighted the possibility of finding novel bioactive compounds from tropical epibionts. Future work might investigate the defence strategies of algal epibionts against specific target organisms. With appropriate modification, bacterial defence systems could be used as environment-friendly controls of biofouling.

Furthermore, the results of this research may also form the basis of future studies that explore the mechanisms of anti-diatom activity in surface-associated bacteria, especially *P. tunicata*. An in-depth study would help further develop the proposed model. The generation of knockouts in genes already identified as important may provide further insight into the role of the respective gene in anti-diatom activity. An improved understanding of the anti-diatom mechanisms will provide both environmental and economic benefits, by leading to improved methods for the control of tenacious biofilms.
References


References


Appendix I: Media and buffers

Media and buffers

**Luria Broth (LB) medium (per litre)**

**LB 10**
10 g NaCl,
10 g tryptone,
5 g yeast extract
For agar plates add 15 g agar before autoclaving

**LB 15**
15 g NaCl,
10 g tryptone,
5 g yeast extract
For agar plates add 15 g agar before autoclaving

**XS Buffer (per 50 ml)**

0.5 g potassium ethyl xanthogenate
5 ml 1M Tris-HCl, pH 7.4
2 ml 0.45M EDTA, pH 8
2.5 ml 20% sodium dodecylsulfate
10 ml 4M ammonium acetate
dH₂O up to 50 ml

**5 × TBE Buffer (per litre)**

54 g Tris base
27.5 g boric acid
20 ml 0.5M EDTA solution (pH 8.0)
Appendix I: Media and buffers

F/2 Medium (Guillard and Ryther, 1962; Guillard, 1975)

To 950 ml filtered seawater add:

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Compound</th>
<th>Stock Solution</th>
<th>Molar Concentration in Final Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml</td>
<td>NaNO₃</td>
<td>75 g/L dH₂O</td>
<td>8.83 × 10⁻⁴ M</td>
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<tr>
<td>1 ml</td>
<td>NaH₂PO₄·H₂O</td>
<td>5 g/L dH₂O</td>
<td>3.63 × 10⁻⁵ M</td>
</tr>
<tr>
<td>1 ml</td>
<td>Na₂SiO₃·9H₂O</td>
<td>30 g/L dH₂O</td>
<td>1.07 × 10⁻⁴ M</td>
</tr>
<tr>
<td>1 ml</td>
<td>f/2 trace metal solution</td>
<td>(see instructions below)</td>
<td>-</td>
</tr>
<tr>
<td>0.5 ml</td>
<td>f/2 vitamin solution</td>
<td>(see instructions below)</td>
<td>-</td>
</tr>
</tbody>
</table>

Make final volume up to around 1 L with filtered seawater and autoclave without adding f/2 vitamin solution. Allow to cool and add filter-sterilized f/2 vitamin solution. Sterile f/2 trace metal solution may be added after autoclaving.

F/2 Trace Metal Solution (Guillard and Ryther, 1962; Guillard, 1975)

To 950 ml filtered seawater add:

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Compound</th>
<th>Stock Solution</th>
<th>Molar Concentration in Final Medium</th>
</tr>
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<tr>
<td>3.15 g</td>
<td>FeCl₃·6H₂O</td>
<td>-</td>
<td>1 × 10⁻⁵ M</td>
</tr>
<tr>
<td>4.36 g</td>
<td>Na₂EDTA·2H₂O</td>
<td>-</td>
<td>1 × 10⁻³ M</td>
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<tr>
<td>1 ml</td>
<td>CuSO₄·5H₂O</td>
<td>9.8 g/L dH₂O</td>
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<td>1 ml</td>
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<td>6.3 g/L dH₂O</td>
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<td>CoCl₂·6H₂O</td>
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<tr>
<td>1 ml</td>
<td>MnCl₂·4H₂O</td>
<td>180.0 g/L dH₂O</td>
<td>9 × 10⁻⁸ M</td>
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Make final volume up to 1 L with dH₂O and autoclave.
**F/2 Vitamin Solution** (Guillard and Ryther, 1962; Guillard, 1975)

To 950 ml dH2O add:

<table>
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<th>Compound</th>
<th>Stock Solution</th>
<th>Molar Concentration in Final Medium</th>
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<tr>
<td>1 ml</td>
<td>Vitamin B12 (cyanocobalamin)</td>
<td>1.0 g/L dH2O</td>
<td>$1 \times 10^{-10} \text{ M}$</td>
</tr>
<tr>
<td>10 ml</td>
<td>Biotin</td>
<td>0.1 g/L dH2O</td>
<td>$2 \times 10^{-9} \text{ M}$</td>
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<tr>
<td>200 mg</td>
<td>Thiamine.HCl</td>
<td>-</td>
<td>$3 \times 10^{-7} \text{ M}$</td>
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</table>

Make final volume up to 1 L with dH2O and filter sterilize.
Appendix II

Primers (5’- 3’)

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<tr>
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<td>P- ACC TGC CC -NH2</td>
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<tr>
<td>Ap1</td>
<td>GGA TCC TAA TAC GAC TCA CTA TAG GGC</td>
</tr>
<tr>
<td>Ap2</td>
<td>AAT AGG GCT CGA GCG GC</td>
</tr>
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<td>GCT GAC TTG ACG GGA CGG CG</td>
</tr>
<tr>
<td>Tn10D</td>
<td>CCT CGA GCA AGA CGT TTC CCG</td>
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</table>
Appendix III: Phylogenetic relationship of algal isolates to bacteria on ARB Project database.

Figure 8.1: Phylogenetic relationship of isolate U3 to bacteria on ARB Project Database

Inhibition of primary colonizers by marine surface-associated bacteria
Appendix III: Phylogenetic relationship of algal isolates to bacteria on ARB Project database

**Figure 8.2:** Phylogenetic relationship of isolate U₄ to bacteria on ARB Project Database
Appendix III: Phylogenetic relationship of algal isolates to bacteria on ARB Project database

**Figure 8.3:** Phylogenetic relationship of isolate U₁ and U₂ to bacteria on ARB Project Database

Inhibition of primary colonizers by marine surface-associated bacteria
Figure 8.4: Phylogenetic relationship of isolate U₈ to bacteria on ARB Project Database

Inhibition of primary colonizers by marine surface-associated bacteria
Appendix III: Phylogenetic relationship of algal isolates to bacteria on ARB Project database

Figure 8.5: Phylogenetic relationship of isolate U13 and U14 to bacteria on ARB Project Database

Inhibition of primary colonizers by marine surface-associated bacteria
Appendix III: Phylogenetic relationship of algal isolates to bacteria on ARB Project database

**Figure 8.6:** Phylogenetic relationship of isolate U₁₅ to bacteria on ARB Project Database

Inhibition of primary colonizers by marine surface-associated bacteria
Appendix IV

Genome Sequence (PTD2_12754- IMG locus tag or ZP_01132901 Genbank accession)

>gi|88857803:493602-496676 Pseudoalteromonas tunicata D2 1099591001423, whole genome shotgun sequence

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tgggctgaaag tggcttgacc ttaccaatta aactaccaat ggtcggcaca
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Inhibition of primary colonizers by marine surface-associated bacteria
Appendix IV Transposon insertion sites in the P. tunicata genome

Figure 9.1: P. tunicata genome sequence section showing the point of insertion of Tn10 in DM1. The disrupted ORF is indicated in green and the point of insertion of the transposon is indicated with an inverted yellow arrowhead. The sequence obtained via panhandle PCR and sequencing is indicated in orange.

Gene Sequence (coding sequence 233041...578021)
Appendix IV Transposon insertion sites in the *P. tunicata* genome

>638341157.NZ_AAOH01000001 *Pseudoalteromonas tunicata* D2, whole genome shotgun sequence.

233041 ataaccgagt taaaaaatcg gggtttttat atcgtcaact agggaaataa aatgtctttat
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catttttttt catttttttt catttttttt catttttttt catttttttt catttttttt catttttttt
catttttttt catttttttt catttttttt catttttttt catttttttt catttttttt catttttttt
catttttttt catttttttt catttttttt catttttttt catttttttt catttttttt catttttttt
catttttttt cattttt testosterone
Appendix IV Transposon insertion sites in the *P. tunicata* genome

Inhibition of primary colonizers by marine surface-associated bacteria

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235321 tattatattta ataatggcga tgtgtatatta accatgcacc attaggcga tcggttttca
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Inhibition of primary colonizers by marine surface-associated bacteria
Appendix IV Transposon insertion sites in the *P. tunicata* genome

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237841 gcgaatgcaaa taaacgccac acctccctgc tttgcgctag gtaaacagat agcagcttat  
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238981 gcgaacacttc ataaattcga gcagcgactat ctaaaaaaaa actatttttttt tgcgctcttt  
239041 tatcataagtc aacagtttcg ttttttttcc aacaatccca aacaccaact  
239101 tcgatcctaag ccgaaacctt cattttattt cttcagacgca atgtagctttt  
239161 acggctccga ccaactattc ctcggctact ccaaatggttt gctgttcttt acaatgttttt  
239221 aacgcccggt tgcgctactt cttctttttc cagctgttacct  
239281 tatatttttttc gcacacggtt ctaaaatttc cttctttttc cagctgttacct  
239341 ctttacagtc ctggctcagtt ttaaattttc tggctctcact gttttttagt  
239401 gatgtagatt cagcttcact gcacagctgct tgggttggtt gcctcttattc ccagctgctt  
239461 ggtcttattc tctactttcc cctttgctct gtcgagctat cgctatttttt cctaagttttt  
239521 atggagtttc ccttctactt cttgctctgg gccagatttt cagttttttc  
239581 ctaattctta ccggttttct cagctgttacct  
239641 taacatttcc tggctcttct tattttttttt cttctttttt cagctgttacct  
239701 ttcgcagtcc ttaaaattttt gcagctgttacct  
239761 aactccactc tgaagttgtc gccagctgct tgggttggtt gcctcttattc ccagctgctt  
239821 ctgggtgtcc tgcgctactt cttctttttc cagctgttacct  
239881 ccggtgtcttc cttttttttc cttctttttt cagctgttacct  
239941 ggtgcttcttc gcagctgttacct  
240001 gtggctactt ttaaatgctt cagctgttacct  
240061 tgactacggtc tgggttggtt gcctcttattc ccagctgctt  
240121 atacgctttc gcacacagtt tattttttttt cttctttttt cagctgttacct  
240181 atacagcaga tataattttt cttcagactt ataaatctta ctggctcttt atcagctttccttccggtattg
Appendix IV Transposon insertion sites in the *P. tunicata* genome

Inhibition of primary colonizers by marine surface-associated bacteria

**Figure 9.2:** *P. tunicata* genome sequence section showing the point of insertion of Tn10 in DM2 (indicated with an inverted yellow arrowhead). The two ORFs affected were locus tags PTD2_01386 (indicated in light green) and PTD2_01391 (indicated in brown). Sequence obtained through panhandle PCR and sequencing is indicated using 3 colours (depicting the different overlapping regions) whereby it starts at the end of locus PTD2_01386 (indicated in blue), follows into the intergenic region (indicated in orange) and partially into locus PTD2_01391 (indicated in pink). The ORF upstream of PTD2_01386 is PTD2_01381 (indicated in aqua) and the ORF downstream of PTD2_01391 is PTD2_01396 (indicated in dark green).

**Gene Sequence (PTD2_02946 IMG locus tag and ZP_01132126 Genbank accession)**
Appendix IV Transposon insertion sites in the *P. tunicata* genome

>gi|88857000:575410-576111 Pseudoalteromonas tunicata D2 1099591001414, whole genome shotgun sequence

574561 aaacaaaaac acgcagacac ccggctaatgt taaattttta tagaatttca tgtgggccac
574621 ctttatatttg tgttttcttt acttggtttca taatgactag tttttttggaa atagcggtaat
574681 caactaagct tggaaaaata ccattaagct tggcaaaaaa tcgctcagga aaataaatca
574741 caactggtgcc aatctcgtgta atctggttgg ttactagttg tttcttgcact tgtggccagac
574801 tatccatgtg atggccaga gcagatattc tagccccctt cagaacacc aaaaattttg
574861 tatcgtggtgc ccttgggcgca agataaagca catcaatcggg tgtctcattct aagctccagt
574921 taagcgcgctg cggataacca aacttactgc gccataacact cgttcagctgtaattggac
574981 gaaaaaccaat acgtgccaaac gcctagcggaa catgaccaat tgtgacactg tgttttttgtg
575041 ataatgtgcga caaaacagct tqaagttata acatctcgta caattgacct attttccaaag
575101 tttttctaggatatctcagcctcgatagc aaaatttggc atcttcttttac ggaccttggcac
575161 tgttaatag ggacagcgca cccatcattt caacttgctc ttccttttgaga ctgctcttcg
575221 cttcttcacgt ggttaaatcat gcggatattc atctgctttt ggttatcaaaa ctatgagtac
575281 gttgtggcacc tttttagttca tgtgagccca ccaataaca acgtataacct gtagcatgaa
575341 gttcgcagcc cattgctttgg ccaatcggcc acgtctgggcc agctatatcc ataaaggttg
575401 attttttagt atcataagcc cctgacwact gcagcttgtt gogttagtttg aagctcagcc
575461 gttgagcgcc gcctttggttc tggacttttca gctggaaaa cctgctcagga actacttctct
575521 tttaattagtc agtttctttg tcaattgcgt cgcttttttt ggtttctttta cctttttttt
575581 cttaacgctca attatttgct gacaaatcag ggaacgccat tgcattatct gctttttttt
575641 aagctgagga gcggtaagcg ccttgctttga cgcctgatct gcattttgagactacttctt
575701 tctgattcgc atttttttgc ggttatctt ggtttttttg cctttttttt cttttttttt
575761 cgcctatagt gcccttattc gcggacacaa cagccatttt gactttgtaactaattgcttt
575821 atccataccca caattccgca cctactctct gtcctttttt gtccttcttt ctcttttttt
575881 attatatttt ggaacttcag tctgcttctt ggtctttttt ctttcttttt cttttttttt
575941 agoecattttt taagcgttcg tattgctttc ataagctgtg gacgtgctgtg aaaaagggcc
576001 atacattcga atggcttaat gccccatcag aaaaacatg gttgataaat aatctttttt
576061 atattgtgctgc gactgctttgt tttttttttg cccctttttcga ctatcttttcttt
576121 aagccagagca ccaacagttc agcaccattg gtttatattgc atggtcataa cccatcagtt
576181 gcgctaaatt cgctgactgg ttcttgcttt gtttgctgtg ggtttttttt gaaatattttt
576241 tcgatggctcc ctcgatgtga ttcgtgcttt gcccttcttc gcagctgttcc ataccttttt
576301 attttgctat taacctttttt gattgtagc atctgtggcc ccttgctcttt gggctttttt
576361 gttcagcagtc aacaataata gtaatttttc tttttttttt tttttttttt cttttttttt
576421 aggcgggtgc cttgacataa gctttgcttc cattttgccc gatttttctg cttttttttt
576481 gataataattc gatattttttt cggattatc ctaatttttaa aacagtcttt ggttaattttt
576541 aatacttctcc ctttatggtgct cttcattcag gatgcttttg gttatccacc aacgtttttt
576601 tggcaatttgc cacagacaca atatgtattgt atctctttt aagttggttatt cctttttttt
576661 acctttctttc gcctgctttg gccttttctt ctttcttttt tttttttttt cttttttttt
576721 catttttttt cttttttttt cttttttttt cttttttttt cttttttttt cttttttttt

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Figure 9.3: *P. tunicata* genome sequence section showing the point of insertion of Tn10 in DM3. The disrupted ORF is indicated in blue and the point of insertion of the transposon is indicated with an inverted yellow arrowhead. The sequence obtained via panhandle PCR and sequencing is indicated in orange. Located upstream, next to locus PTD2_02946 is PTD2_02941 (ORF indicated in green) and downstream of the locus PTD2_02946 is PTD2_02951 (ORF indicated in pink).