Aspects of the interaction between the marine bacterium *Alcanivorax* DG881 and the toxic dinoflagellate *Gymnodinium catenatum*

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Abstract

The presence of a bacterial community is vital to the germination and growth of the toxic dinoflagellate *Gymnodinium catenatum*. Previous research has shown that the bacterium *Alcanivorax* DG881 is an important stimulatory member of the dinoflagellate-associated bacterial community, however the nature of the interaction between the two organisms, and the substances and mechanism involved in growth stimulation are unknown. This thesis uses a uni-bacterial *G. catenatum* experimental culture model to investigate elements of the interaction between the marine bacterium *Alcanivorax* DG881 with the dinoflagellate *G. catenatum*.

In the first experiment, three treatments were used to determine whether the *G. catenatum* growth stimulating substances produced by *Alcanivorax* DG881 were extracellular or intracellular substances, and whether these substances need to be continuously provided to *G. catenatum* to support growth. Addition of extracellular filtrates from cultures of *G. catenatum* and it's associated bacteria showed increased growth stimulating activity in resting cyst germination experiments compared to treatments containing intracellular substances from *Alcanivorax* DG881 in absence of

live *Alcanivorax* DG881 cells. Repeated addition of extracellular filtrates sustained *G. catenatum* growth after germination for a significantly longer period and to higher cell concentrations than a single addition of extracellular filtrate. These results indicated that the *G. catenatum* did not obtain growth stimulating substances by ingesting bacteria but requires one or more extracellular dissolved products produced by *Alcanivorax* DG881. The patterns of growth suggest that the products were either labile or utilised by the dinoflagellate during growth.

It has been proposed that dinoflagellate-associated *Alcanivorax* DG881 benefits from the utilization of dissolved organic carbon (DOC) exuded from the dinoflagellate cell. To examine this idea, the single carbon utilization profile of *Alcanivorax* DG881 was compared with the closely related but no-stimulatory strain *Alcanivorax borkumensis* SK2 using the BIOLOG GN2 plate assay system. *Alcanivorax* DG881 was able to use a much wider range of carbon compounds for growth than *Alcanivorax borkumensis* SK2, particular a wider range of amino acids, known as an important component of the DOC exuded from algal cells. The data here suggest that *Alcanivorax* DG881 is relatively better adapted to a life associated with algal cells than *Alcanivorax borkumensis* SK2.

Detection and sequence characterization of putative saxitoxin synthesis gene

homologues was attempted. Degenerate PCR primers designed from sequence of three putative saxitoxin biosynthesis (Sxt) genes from cyanobacteria was used to screen *G. catenatum* total DNA extracts. PCR products of expected length were obtained for three Sxt genes and two products were sequenced and compared to the putative cyanobacterial homolog and other published DNA sequences available on Genbank. The putative *G. catenatum* SxtN gene sequence showed highest similarity with sulfurtransferase of bacteria *Francisella philomiragia subsp. Philomiragia* (84% similarity), and with a hypothetical protein the *Arabidopsis thaliana* (81% similarity). The putative *G. catenatum* SxtU gene sequence showed low similarity with a hypothetical protein of *Peptostreptococcus micros* (46% similarity) and hypothetical proteins from the fungi *Aspergillus oryzae* (46% similarity). Phylogenetic comparisons of the partial sequences of both candidate genes suggested that they were of bacterial rather than dinoflagellate origin, and bacteria associated with *G. catenatum* not involved in saxitoxin synthesis directly or indirectly.

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Chapter 1

Introduction

1-1 Dinoflagellates in the marine system

About 90% of known species of dinoflagellates are found in sea water. Although most species are planktonic, some are benthic, some symbiotic, and others parasitic (Taylor 1995). Most dinoflagellates are globular, single-celled species, although a few species are filamentous, with membrane bound organelles and flagella (Suthers and Rissik 2008). The pellicle (cell wall) often contains thin plates of cellulose within the alveoli. A unique decay-resistant chemical called dinosporin is associated with the cellulose plates, especially in dormant stages of the life cycle found in sediments.

Dinoflagellates reproduce asexually by simple cell division into two cells, at rates of up to one division per day. Each daughter cell of armoured dinoflagellates must replace the cellulose plates of the missing half after fission (Nybakken and Bertness 2005). Sexual reproduction is known in a number of species and occurs by fusion of morphologically and functionally similar (isogametic) or morphologically different gemetes (anisogametic). The sexual lifecycle of *Gymnodinium catenatum* is shown in Fig.1.1. Life histories often include the production of cysts (Fig. 1.2), dormant stages resist decay (by the presence of dinosporin) and to environmental stress. Cyst formation may follow seasonal sexual reproduction and is otherwise associated with the onset of unfavourable environmental conditions such as reduced light, temperature, and nutrients.

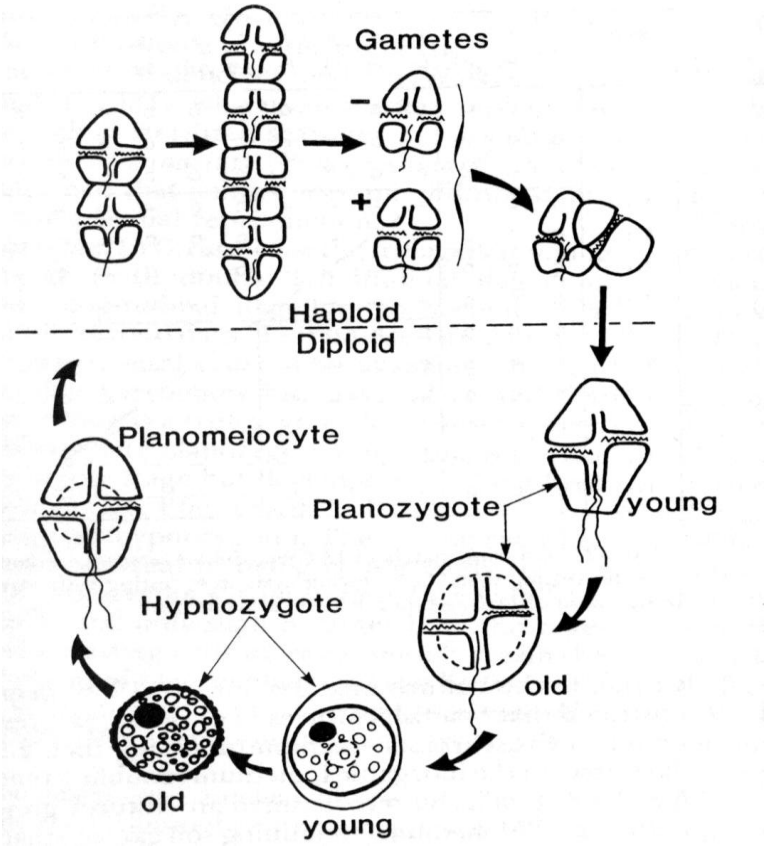

Figure1.1: Life cycle of *G. catenatum* (From Blackburn *et al*. 1989)

(a) (b) (c)

Figure 1.2: *G. catenatum* cyst and cells. (a) LM image of *G. catenatum* resting cyst; (b) *G. catenatum* 4-cell chain (LM) (c) *G. catenatum* cell chain (SEM). (Images by Dr C. Bolch)

Dinoflagellates obtain energy by several means. The plastid of photosynthetic dinoflagellates contains chlorophylls a and c and the accessory photosynthetic pigments beta-carotene and peridinin. The latter pigment, a carotenoid, gives dinoflagellates their typical golden-brown colour. They store food as starch of the same composition as that of green plants. Many photosynthetic dinoflagellates are mixotrophic, which means that supplement photosynthesis by either osmotrophy (absorbing nutrients) or phagotrophy (engulfing nutrients). Only half the species of dinoflagellate are photosynthetic; the other half live mainly by a combination of phagotrophy and osmotrophy.

Dinoflagellates play an important role in marine ecosystems. Dinoflagellates, diatom, and cocolithophores are producer in marine ecosystem and provide food for many marine species. Their flagella of dinoflagellates, allow them to swim vertically in the marine water, and absence of external skeleton of silicon give dinoflagellates an ecological advantage over diatoms. Diatoms are more tolerant than dinoflagellates of turbulence. Some dinoflagellates are parasitic and live in the intestine of marine crustaceans (Nybakken and Bertness 2005). The zooxanthellae are photosynthetic and provide food for their host organisms, and carbon dioxide, nutrients essential for growth, and shelter to zooxanthellae (Karleskint 1998).

1-2 Harmful algal blooms

Some marine dinoflagellates, such as the genera *Protogonyaulax*, *Gonyaulax* and *Gymnodinium* are responsible for the phenomenon known as harmful algal blooms (HABs, formerly called red tides). HABs occur when photosynthetic dinoflagellates (or

other primary producers) undergo population explosion (Ferrier *et al*. 2002). The most common type of HABs are referred to "Red Tides" because the bloom discolors the water, making it appear red. However, HABs may also be yellow, orange, brown, green, white, or pink, depending on which one of the three primary types of phytoplankton are responsible for the problem; dinoflagellate, diatoms, or cyanobacteria. Many HABs, produce vividly colored blooms of cells that accumulate on surface water. These high biomass blooms can cause hypoxia, can contribute to toxicity of fish and shellfish, and can cause other environmental problems.

Over past decades, the occurrence of HABs has increased both in frequency and global extent. Shipping movements across the globe have been implicated as the cause of several species of phytoplankton arriving where they have not previously been known to occur (Hallegraeff 1998). For example, in the 1970s HABs of *Alexandrium tamaranse* and *Alexandrium catenella* were only known in Europe, North America, and Japan. However, in 1990 HABs were reported throughout the Southern Hemisphere including South Africa, Australia, New Zealand, India, Thailand, Brunei, Sabah, the Philippines, and Papua New Guinea (Hallegraeff *et al*. 2003). Planktonic taxa are transported in the ballast tanks of ship, having been pumped into ballast tanks in a port and then pumped out of the tanks once they reach their destination (Hallegraeff 1998).

1-3 Shellfish poisoning

There are approximately 100 micro-algae that are known to produce specific toxins (Fogg 2002). Dinoflagellates have the largest number of harmful species (around 40

species). They can produce toxic compounds that accumulate in filter-feeding bivalves and commercially important crustaceans and finfish. These toxins can be extremely toxic and many of them are effective at low dosages. On a global scale, marine algal toxins are responsible for more than 60,000 intoxication incidents every year, with an overall mortality rate of 1.5 %. (Van Dolah 2000) Consumption of seafood contaminated by algal toxins results in various seafood poisoning syndromes: paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP), diarrheic shellfish poisoning (DSP), and ciguatera fish poisoning (CFP) (Table 1.1). Most of these poisonings are caused by neurotoxins with highly specific effects on the nervous system of animals, including humans, the neurotoxins interfering with nerve impulse transmission (Wang 2008). In addition to their human health effects, algal toxins are responsible for extensive die-offs of fish and shellfish and have been implicated in the episodic mortalities of marine mammals, birds, and other animals dependent on the marine food web (Pomeroy 1974; 1979) (Table 1.1).

Table 1.1: Major poisoning syndromes and toxin producing microalgae.

Type of Poisoning	Toxin group	Producer	Effect
Paralytic Shellfish Poisoning (PSP)	Saxitoxin Gonyautoxins	*Alexandrium catenella* *A. tamaranse* *Gymnodinium catenatum*	Tingling sensation Numbness around lips Headache Dizziness
Diarrheic Shellfish Poisoning (DSP)	Okadaic acid	*Dinophysis acuminate* *Prorocentrum lima*	Diarrhoea, Nausea, Vomiting Abdominal pain
Amnesic Shellfish Poisoning (ASP)	Domoic acid	*Pseudo-nitchia australis* *Pseudo-nitchia seriata*	Numesea Vomiting Diarrhoes Abdominal cramps
Neurotoxic Shellfish Poisoning (NSP)	Brevetoxin	*Karenia brevis* *K. bigiditata*	Nausea Diarrhoea Vomiting Muscle and joint pain
Ciguatera Fish Poisoning (CFP)	Ciguatoxin Maitotoxin Scaritoxin Palytoxin	*Gambierdiscus toxicus*	Nausea Vomiting Diarrhoea Headaches Abdominal pain

Paralytic shellfish toxins are comprised of saxitoxin (STX) and at least 20 other chemically related derivatives (Ohshima *et al*. 1993), which block sodium channels in mammalian nerve cells, thus preventing conductance of signals along the neuron (Baden and Trainer 1993; Kao 1993). The individual toxins vary in potency, with those containing a carbamate group (e.g., STX) being the most toxic and those with a sulfocarbamoyl group (e.g., C toxins) being the least toxic (Sullivan 1993). The proportions and concentrations of these toxins, are associated with dinoflagellates, such as *Alexandrium tamarense* (Steidinger 1993). It has been suggested that heterotrophic

bacteria responsible for toxin synthesis in these organisms (Kodama *et al*. 1988; Kodama *et al*. 1990; Silva 1962; Silva 1990). The original evidence to support this propositions electron micrographs depicting bacteria inside dinoflagellate cells, the subsequent isolation of these bacteria, and determinations of their toxicities (Kodama *et al*. 1990; Silva 1962; Silva 1990). However, other workers failed to detect intracellular bacteria in toxic dinoflagellates and were skeptical of claims that bacteria produced PST (Nelinda 1985). Controversy has surrounded this issue, with discussions focusing on whether or not bacteria exist within the dinoflagellate cell and what, if any, could be their role in dinoflagellate toxicity. The controversy seemed to distract researchers from an important point, testing whether or not the bacteria are capable of autonomous production of these toxins. Uribe and Espejo (2003) reported that axenic cultures of *Alexandrium minutum* were able to produce PSP toxins however, the toxicity was lower than for non axenic cultures (Uribe and Espejo 2003). Kodama *et al*. (1988) first reported PST-production by pure cultures of bacteria isolated from the dinoflagellate *Alexandrium tamarense*, however, more recent studies suggest that there are some compounds in dinoflagellate associated bacteria that have similar biological effects in cell-culture assays, but are structurally unrelated to saxitoxin toxicity (Baker *et al*. 2003).

1-4 Interactions between phytoplankton and bacteria

Bacteria and microalgae are ubiquitous and abundant in the aquatic environment and specific bacterial communities appear to be associated with dinoflagellate blooms (Furuki and Kobayashi 1991). Similar bacterial communities are also associated with

laboratory cultures (e.g. Green *et al*. 2004; Jasti *et al*. 2005). Phytoplankton cells release a significant proportion of photosynthetically-derived dissolved organic carbon (DOC) into the surrounding seawater that can be utilized very rapidly by the surrounding bacterial community (Myklestad 2000). While it seems clear that many marine bacteria benefit from using the DOC for growth, it is less clear which components of the phytoplankton DOC are utilized and by particular bacterial types or groups.

Algal-bacteria interactions are now also recognised as important factors influencing the ecology of marine phytoplankton (Kopp *et al*. 1997). In addition, there is mounting evidence shows that the bacterial community has a significant influence on the development growth and decline of harmful algal blooms (Doucette 1995; Doucette *et al*. 1998; Mayali and Azam 2004) and both stimulative and inhibitory effects are known (Furuki and Kobayashi 1991; Fukami *et al*. 1991). Bacterial influence on algal cell toxicity has also been described by many researchers. Associated bacteria are reported to increase algal toxicity (Bates *et al*. 1995; Hold *et al*. 2001). However, conflicting reports exist (Rausch de Traubenberg 1995; Danzer and Levin 1997). The algicidal ability of bacteria has also been widely studied (Imai *et al*. 1993; Lovejoy *et al*. 1998; Doucette *et al*. 1999), and understandably, due to public health risks and the cost to shellfish aquaculture and fisheries, research to date has concentrated on interactions with toxic algal species. Recent studies show that some bacteria have significant stimulatory effects on the growth of microalgae (Ferrier *et al*. 2002; Ashton *et al*. 2003; Uribe and Espejo 2003) and may also participate in mutualistic relationships with algal cells (Amin *et al*. 2009). However, we currently know little of the mechanisms or mediating factors by which bacteria interact with phytoplankton cells.

1-5 Thesis Aims

The aim of this study and resulting thesis was to investigate aspects of the relationship between growth stimulatory bacteria and the toxic dinoflagellate *Gymnodinium catenatum* and their influence on saxitoxin production by the dinoflagellate. Specifically this work aims to:

1) Investigate aspects of dinoflagellate growth stimulation by the dinoflagellate-associated bacterium *Alcanivorax* DG881.
2) Examine the organic carbon utilization of *Alcanivorax* DG881 compared with *Alcanivorax borkumensis* SK2.
3) Determine the potential source of toxin production by isolating genes involved in saxitoxin synthesis from *G. catenatum* and its associated bacterial community.

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Chapter 2

Growth stimulating activity of the marine bacterium *Alcanivorax* DG881 on the dinoflagellate *Gymnodinium catenatum*

2-1 Introduction

In the marine environment, phytoplankton and bacteria are fundamental microorganisms and the physical association has been demonstrated (Gonzalez and Bashan 2000; Smith *et al*. 2002, Uribe and Espejo 2003; Uronen *et al*. 2007; Oberhaus *et al*. 2008; Kim *et al*. 2009). Bacteria associated with phytoplankton can be attached to the surface of phytoplankton cells or intracellular (Kogure *et al*. 1982; Silva 1990), or free living, and may have stimulative or inhibitory/algicidal effects on the growth of phytoplankton cells, some bacteria also may control algal blooms (Cole 1982; Adachi *et al*. 2003). *Azospirillum brasilense* increases the microalgal population (Gonzalez *et al*. 2000), whereas some *Pseudomonas* species have antagonistic activity against several cyanobacteria (Kodani *et al*. 2002). *Pseudoalteromonas haloplanktis* AFMB-008041 is also able to regulate the harmful algal bloom of *Prorocentrum minimum* (Kim *et al*. 2009). The biomass of *Prymnesium parvum* and *Rhodomonas salina* are reported to increase significantly when both cultures are mixed with bacteria (Uronen *et al*. 2007). For some dinoflagellates, such as *Alexandrium* species and *Gymnodinium catenatum*, it has also been suggested intracellular and attached bacteria can stimulate dinoflagellate growth (Sakami *et al*. 1999; Alavi *et al*. 2001; Ferrier *et al*. 2002; Vincent 2003), affect or induce cyst formation (Adachi *et al*. 2003), produce of algicidal factor (Doucette *et al*. 1999), or may be involved directly or indirectly in the production of saxitoxin (Silva *et al*. 1982, Kodama *et al* 1990; Dantzer and Levin 1997).

It is not clear how bacteria stimulate algal growth or which compounds in bacteria are required for algal growth. Bacteria may stimulate algal growth by the release of various products such as vitamins and organic chelating agents (Cole 1982), production of assimilable nitrogen derivatives from the organic matter, production of CO_2 or other compounds approaching the completely oxidized state of carbon (Liebson *et al*. 1995), by influencing the pH and the redox potential, or finally by the release of inorganic nutrients (Croft *et al*. 2005).

The toxic dinoflagellate *Gymnodinium catenatum* is the only non-thecate species known to produce paralytic shellfish toxins (PST) both in freshwater and seawater. This species is becoming more serious concern in many countries, especially in Spain, Japan, Korea, and Australia. The first *G. catenatum* bloom reported in Australia was in the mid 1980's introduced by ballast water from Japanese ship (McMinn *et al*. 1997). As part of its sexual life cycle, *G. catenatum* produces benthic resting cysts (Anderson *et al*. 1988) which can be easily manipulated. Earlier studies have shown that *G. catenatum* cysts germinated without bacteria did not grow beyond a few days (Bolch *et al*. 2002; Vincent 2003; Bolch *et al*. 2004; Subramanian 2008). Experiments with antibiotic sensitive and resistant bacteria have also clearly demonstrated that *G. catenatum* cannot grow in laboratory cultures without the presence of particular marine bacteria (Bolch *et al*. 2011). However, the mechanism by which *G. catenatum* associated-bacteria stimulate *G. catenatum* growth is unknown.

This chapter aimed to examine whether *G. catenatum* growth stimulating activity of the bacterium *Alcanivorax* DG881 is maintained by dissolved factors, or requires the

presence of living bacteria cells. Cultures were established from surface-sterilized resting cysts and various cell lysates/fitrates from *Alcanivorax* DG881 cultures added to determine whether growth of *G. catenatum* can be maintained in the absence of live *Alcanivorax* DG881 cells.

2-2 Materials and methods

2-2-1 Dinoflagellate culture

The *Gymnodinium catenatum* strains GCHU11 and GCDE08 used in this study were isolated originaly from the Huon Estuary, and Derwent Estuary Tasmania, Australia and obtained from the CSIRO Collection of Living Microalgae (Hobart, Australia). GCHU11 and GCDE08 were maintained in GSe medium (Blackburn *et al*. 1989; see Apendix 1) in 150 mL Erlenmeyer flask at 18 ^{o}C ($\pm$2^{o}C) under 90$\pm$10 μmol photons m^{-2} s^{-1} of cool white fluorescent light with 12:12 h light:dark cycle.

2-2-2 Bacterial culture

The growth of *G. catenatum* can be maintained by co-culture with the marine bacterium *Alcanivorax* DG881 in the absence of other bacteria (Bolch *et al*. 2004, Subramanian 2008). *Alcanivorax* DG881 was cultured and maintained on modified Zobell's marine agar plate (ZM1) (Green *et al*. 2004) containing 5 gL^{-1} of bacterial peptone, 1 gL^{-1} of yeast extract, and 10 gL^{-1} of sodium acetate as a carbon source with 1.5 % w/v of Difco-Bacto agar. Additional trace elements and vitamins were filter-sterilized and added to the sterile medium after autoclaving and the medium (Green *et al*., 2004; see Appendix 2).

2-2-3 Cyst production

Late log-phase cultures of GCHU11 and GCDE08 were crossed to produce sexual resting cysts using the methods described by Blackburn *et al*. (1989). One mL of each culture was added to sterile 36 mm polystyrene petri dishes and 8 mL of GSe medium

(without added nitrate and phosphate) was added. The petri dishes were sealed with parafilm and incubated at 18 ^{o}C (±2 ^{o}C) under a light intensity of 90±10 μmol photons m^{-2} s^{-1} with a 12:12 L:D photoperiod, for 3-5 weeks until sufficient resting cysts (>100 per dish) were obtained for experiments.

2-2-4 Surface sterilization of cysts

Resting cysts that resulted from crossing the two cultures were located by examination with a Leica MZ9.5 bright-field/dark-field transmitted light stereomicroscope. A glass micropipette was used to isolate the cysts. The cysts were washed twice with sterile GSe medium. After washing, these cysts were transferred to a sterile 1.5mL tube and sterile GSe medium was added to a total volume of 0.5 mL. To sterilize the surface of resting cysts, 50 μL of 6 % of H_2O_2 (Orion Laboratories Pty Ltd, Australia) was added to achieve a final concentration of 0.55 % (v/v) of H_2O_2, and the tubes covered with aluminium foil and left at room temperature for 10 minutes. Tubes were centrifuged at 17,200 g for 30 s to pellet the cysts, and the supernatant removed with a sterile pipette and cysts resuspended in 0.5 mL of sterile GSe medium and centrifuged as above. This washing step was repeated twice and the washed cysts resuspended in 0.5 mL of sterile GSe medium. Cyst sterility was checked by spreading 100 μL of the cyst suspension on to a ZM1 agar plate followed by incubation at 25 ^{o}C for 3 days. Plates were inspected for bacterial colonies and if bacterial colonies were seen, the corresponding treatments were discarded and replaced treatments with freshly sterilized resting cysts.

2-2-5 Germination and growth stimulation experiments

Groups of 25-30 surface sterilized cysts were placed by micro-pipette into a series of 36

mm petri dishes and the treatments described in Table 2.1, 2.2, and 2.3 were added. GSe medium was also added to each treatment to make a final volume of 2 mL. The dishes were sealed with parafilm^{TM} and incubated at 18 ^{o}C ($\pm$2 ^{o}C) under 90$\pm$10 μmol photons m^{-2} s^{-1} with a 12:12 L:D photoperiod. The number of germinated cysts and dinoflagellate cells was recorded daily using a stereomicroscope (at 60 x mag). All germination and growth experiment treatments were carried out in parallel and the series of treatments replicated three times (replication in time).

Preparation of treatments:

Experiment 1: *Growth of G. catenatum without living bacteria.* (Table 2.1).

Treatment 1: Co-cultures of *G. catenatum* and mixed bacterial community filtrate were used. Culture medium from cyst production dishes was filtered through 0.45 μm filters (Millipore) and 0.2 ml of filtrate was added to 36 mm petri-dishes containing surface-sterilized cysts.

Treatment 2: *Alcanivorax* DG881 was cultured for three days in 7 mL Zobell's marine (ZM) broth the cells pelleted by centrifugation at 17,200 g for one minute. The supernatant was removed and the pellet was resuspended with 800 μL of GSe. This step was repeated three times to remove ZM medium. The bacterial cell pellet was resuspended in five mL of GSe medium and cultured for one day to allow bacteria to acclimatize to algal media. Bacteria cells were counted using hemocytometer (Strober 2001). This culture was diluted with GSe medium to 10^{-5} cells mL^{-1} and filtered through a 0.22 μm filter and 0.2 mL of filtrate was added to petri-dishes containing

surface-sterilized cysts.

Control: *Alcanivorax* DG881 was cultured for three days in 7 mL ZM broth then placed in five mL of GSe medium for one day to allow bacteria to acclimatize to algal media. This culture was diluted with GSe medium to 10^{-5} cells mL^{-1} to use as a control. A volume of 0.2 mL of *Alcanivorax* DG881 was added to petri-dishes containing surface-sterilized cysts.

Table 2.1: Description of treatments and control used in experiment 1. All treatments and control consisted of triplicate 36 mm petri dish, each dish contain 25-30 sterilized *G. catenatum* resting cysts and treatment described below.

Treatment	Details of treatment	Rational
Treatment 1	0.2 mL of 0.22 μm filtrate from a GCHU11/DE08 crossed culture	Examine whether dissolved products from the culture-associated mixed bacterial community stimulate the growth of *G. catenatum* ?
Treatment 2	0.2 mL of 0.22 μm filtrate from a culture of *Alcanivorax* DG881	Examine whether dissolved products from a pure culture of *Alcanivorax* DG881 culture stimulate growth of *G. catenatum*?
Positive Control	*Alcanivorax* DG881 with final concentration of 10^{-5} cells mL^{-1}	Control to determine whether cysts/bacteria model is functioning as expected

Experiment 2: *Stimulation of G. catenatum by intracellular substances from Alcanivorax* DG881. (Table 2.2).

Three different lysis methods were used to release intracellular material from *Alcanivorax* DG881 cells. In this thesis we use the term labile to mean unstable and/or likely to be degraded by physical or chemical factors, as opposed to being taken-up and utilized by the dinoflagellate or its associated bacteria. Firstly, freeze/thaw was used because it is a physical disruption process unlikely to inactive or denature labile biologically active growth factors. Secondly, Lysozyme-lysis was used as it weakens gram-negative bacterial cell walls to release intra-cellular material without the addition of detergent-like compounds that could inactivate or denature biologically active growth factors. Thirdly, Triton-X was used as this detergent lyses gram-negative cells very efficiently and assists the solubility of a range of proteins and other non-polar compounds that could be responsible for growth stimulation of the dinoflagellate cells

Treatment 1: *Alcanivorax* DG881 was cultured for three days in 7 mL ZM broth then placed in five mL of GSe medium for one day to allow bacteria to acclimatize to algal media. This culture was diluted with GSe medium to 10^{-5} cells mL^{-1}. The bacterial cells were lysed by addition of 0.1 % (w/v) of lysozyme and incubated for 30 min. A volume of 0.2 mL of the cell lysate was added to petri-dishes containing surface-sterilized cysts.

Treatment 2: *Alcanivorax* DG881 was cultured for three days in 7 mL ZM broth then placed in five mL of GSe medium for one day to allow bacteria to acclimatize to algal media. This culture was diluted with GSe medium to 10^{-5} cells mL^{-1}. This culture was lysed by addition of Triton-X (1 % w/v) (Amresco, USA) for 30 min. A volume of 0.2 mL of the lysate was added to petri-dishes containing surface-sterilized cysts.

Treatment 3: *Alcanivorax* DG881 was cultured for three days in 7 mL ZM broth then

placed in five mL of GSe medium for one day to allow bacteria to acclimatize to algal media. This culture was diluted with GSe medium to 10^{-5} cells mL^{-1}. This culture was lysed by three cycles of freeze/thaw and 0.2 mL of the cell lysated added to petri-dishes containing surface-sterilized cysts.

Control: *Alcanivorax* DG881 was cultured for three days in 7 mL ZM broth then placed in five mL of GSe medium for one day to allow bacteria to acclimatize to algal media. This culture was diluted with GSe medium to 10^{-5} cells mL^{-1}. A volume of 0.2 mL of the cell suspension was added to petri-dishes containing surface-sterilized cysts.

Table 2.2: Description of treatments and control used in experiment 2. All treatments and control consisted of triplicate 36 mm petri dish, each dish contain 25-30 sterilized resting cysts and treatment described below.

Treatment	Details of treatment	Rational
Treatment1	0.2 mL of lysed *Alcanivorax* DG881 cells	Examine whether dissolved products from the culture-associated mixed bacterial community stimulate the growth of *G. catenatum*
Treatment2	0.2 mL of *Alcanivorax* DG881 lysed using Triton-X	
Treatment3	0.2 mL of *Alcanivorax* DG881 lysed by repeated freeze and thaw	
Positive Control	*Alcanivorax* DG881 with final concentration of 10^{-5} cells mL^{-1}	Control to determine whether cysts/bacterial model is working as expected

Experiment 3: *Stability of growth stimulating substances from Alcanivorax DG881* (Table 2.3).

Treatments 1 and 2: Mid log phase of GCHU11 and GCDE08 were filtered through 0.22 μm filters (Millipore), the filtrates-mixed at a 1:1 ratio, and then 0.2 mL of filtrate added to sterilized cysts in 36 mm petri-dishes, as single addition at germination (treatment 2) or as repeated addition on day 0, 21 and 42 (Treatment 1). A volume 0.2 mL GSe medium was added on day 21 and 42 to Treatment 2.

Control: *Alcanivorax* DG881 was cultured for three days in 7 mL ZM broth then placed in five mL of GSe medium for one day to allow bacteria to acclimatize to algal media. This culture was diluted with GSe medium to 10^{-5} cells mL^{-1} to use as a control. A volume of 0.2 mL of *Alcanivorax* DG881 was added to petri-dishes containing surface-sterilized cysts.

Negative control: A volume of 0.2 mL of GSe medium was added to petri-dishes containing surface-sterilized cysts.

Table 2.3: Description of treatments and controls used in experiment 3. All treatments and controls consisted of triplicate 36 mm petri dish, each dish contain 25-30 sterilized resting cysts and treatment described below.

Treatment	Details of treatment	Rational
Treatment 1	0.2 mL of 0.45 µm *G. catenatum* (GCHU11+GCDE08) culture filtrate added at days 0, 21 and 42	Examine whether the dissolved growth stimulating substance from *Alcanivorax* DG881 lose activity over time.
Treatment 2	0.2 mL of 0.45 µm *G. catenatum* (GCHU11+GCDE08) culture filtrate added at day 0	
Positive control	*Alcanivorax* DG881 with final concentration of 10^{-5} cells mL^{-1}	To determine whether the experimental model is working
Negative control	0.2 mL of GSe medium	To confirm no growth stimulation in absence of bacteria or filtrate

2-2-6 Statistical analysis

SPSS ver. 17.0 was used for statistical analysis of experiments 1, 2 and 3 (LEAD technologies, Chicago, USA). Significant differences among the treatments were tested using repeated measure ANOVA with time-blocking, Tukey's LSD post-hoc tests. For experiment 1, maximum *G. catenatum* cell numbers on day 19 were compared by one-way ANOVA with Tukey's LSD post-hoc tests. Also for experiment 3, maximum *G. catenatum* cell numbers in each treatment were compared by one-way ANOVA with Tukey's LSD post-hoc tests.

2-3 Results

2-3-1. Experiment 1: Growth of G. catenatum without living bacteria

Repeated measure ANOVA suggested that *G. catenatum* cell number within per dish were not significantly different (t=3.909; df=3 ; P=0.082) between treatments. However addition of dissolved products from *G. catenatum* and the mixed bacterial community showed the highest stimulatory activity among three treatments. Addition of *Alcanivorax* DG881 culture in the filtrate failed to support living *G. catenatum* cells after day 27, however addition of *G. catenatum*/bacterial filtrate and live *Alcanivorax* DG881 cells supported living *G. catenatum* cells until day 35 in all replicates (Fig 2.1).

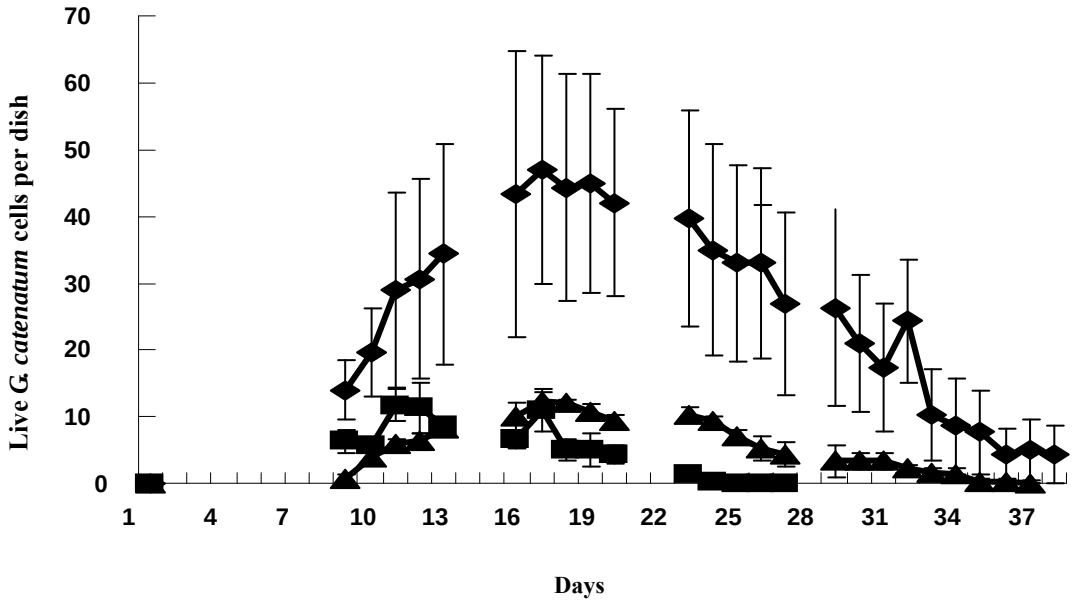

Fig. 2.1: Mean number of live *G. catenatum* cells (n=3; ±standard error) in the presence of algal and/or bacterial dissolved substances. Treatments are indicated as follow ◆, GCDE08/GCHU11 mixed bacterial culture filtrate; ■, *Alcanivorax* DG881 filtrate; ▲, *Alcanivorax* DG881 live cells. Breaks in date indicate where data was not collected

The maximum cell concentration of *G. catenatum* at day 17 (resulting from a combination of germination and cell division) was significantly higher in treatments with a mixed bacterial community added (t=15.561; df=3; *P*=0.004) than the treatments containing either live *Alcanivorax* DG881, or 0.2 μm filtrates of *Alcanivorax* DG881 culture (Fig 2.2).

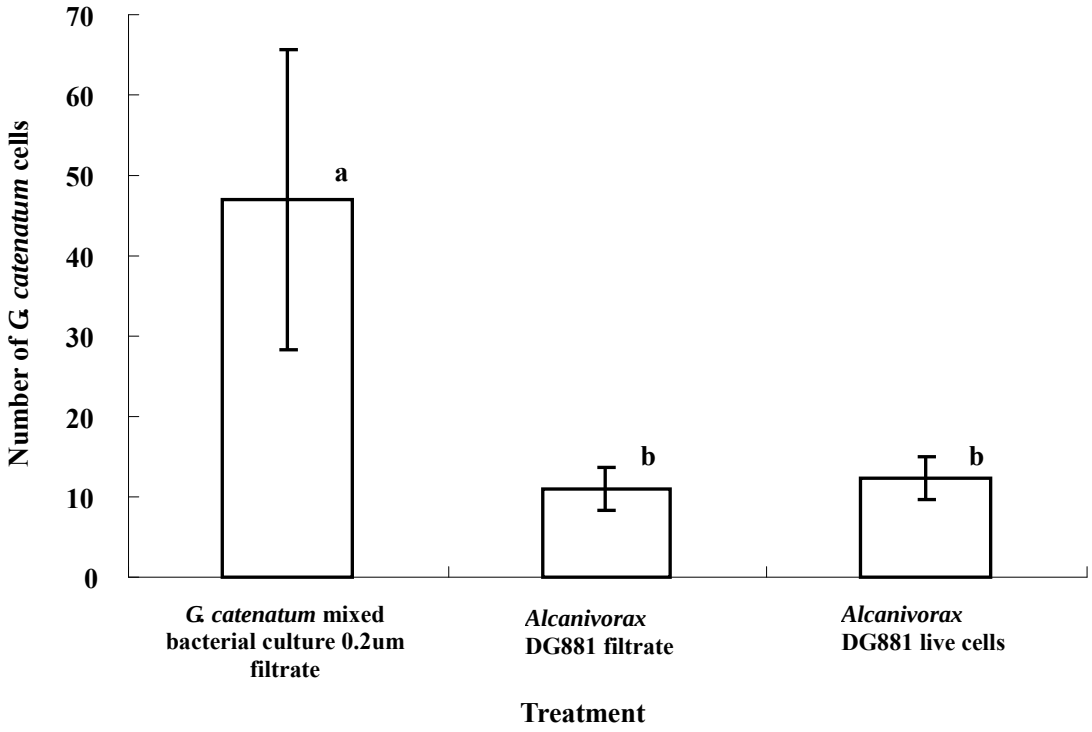

Fig. 2.2: Maximum cell concentration (±standard error) of *G. catenatum* grown with addition of algal and/or bacterial dissolved substances (day 17). Superscripts indicate significant differences (*P*≤0.05).

2-3-2. Experiment 2: Do intracellular substances produced by *Alcanivorax* DG881 stimulate growth of *G. catenatum.*

All *Alcanivorax* DG881 lysates demonstrated limited growth stimulating activity of *G. catenatum* calls (Fig.2.3). Repeated measure ANOVA suggested that *G. catenatum* cell

number per well did not differ among treatments (t=1.09; df=3; P=0.407). All three treatments with *Alcanivorax* DG881 lysates showed no *G. catenatum* cells after day 21, however addition of live *Alcanivorax* DG881 contained live *G. catenatum* cells up to day 25.

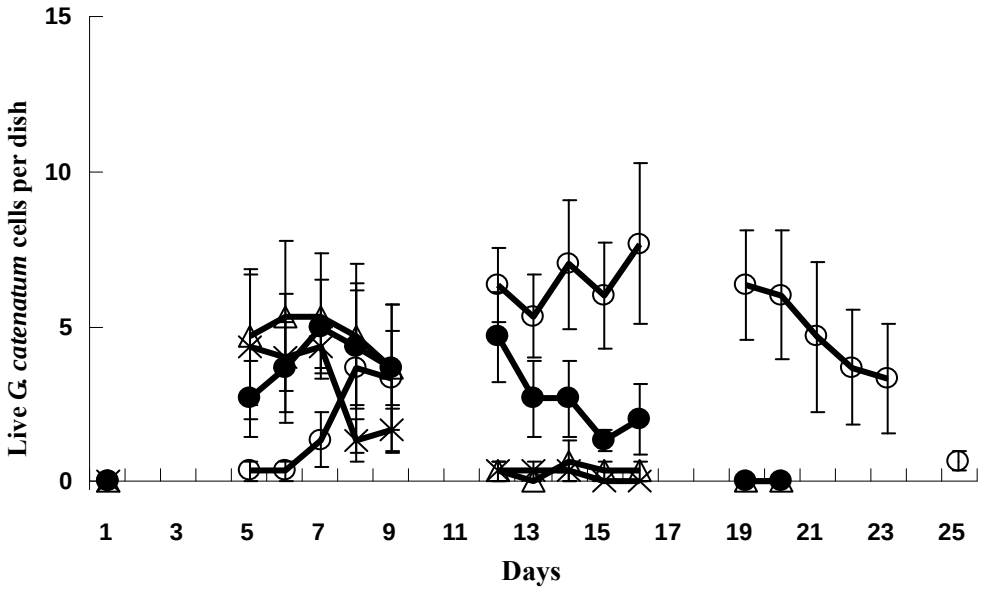

Fig. 2.3: Mean number of live *G. catenatum* cells (n=3; ±standard error) in the presence of bacterial intracellular substances. Treatments are indicated as follow, ✕, Lysozyme treated *Alcanivorax* DG881 cells; △, Triton-X treated *Alcanivorax* DG881 cells; ●, Freeze and thaw lysed *Alcanivorax* DG881 cells; ○, Control: live *Alcanivorax* DG881. Breaks in date indicate where data was not collected

2-3-3. Experiment 3: Does the growth stimulating substance from *Alcanivorax* DG881 lose activity overtime.

Repeated measures ANOVA suggested that *G. catenatum* cell number in the treatment of multiple addition of *G. catenatum* / mixed bacteria filtrate was significantly higher than the other treatments (t = 22.009; df = 3; *P* = 0.000) (Fig. 2.4). Resting cyst of *G. catenatum* germinated and grown with repeated addition of *G. catenatum* / mixed

bacteria filtrate showed increasing cell numbers over the course of the experiment. Cysts of *G. catenatum* grown with mixed bacteria filtrate peaked at Day 10 but declined in cell number after Day 16. Positive control (with live *Alcanivorax* DG881 cells) also declined after Day 10 and negative controls failed to survive beyond Day 14. *G. catenatum* cell number in multiple addition treatment started decrease at day 21, and day 34, however after filtrate of *G. catenatum* / mixed bacteria were added the cell number started increase again.

The maximum cell numbers achieved in the treatment of multiple addition of *G. catenatum* / mixed bacteria filtrate was significantly higher than the other treatments ($t = 14.216$; $df = 3$; $P = 0.001$) (Fig. 2.5).

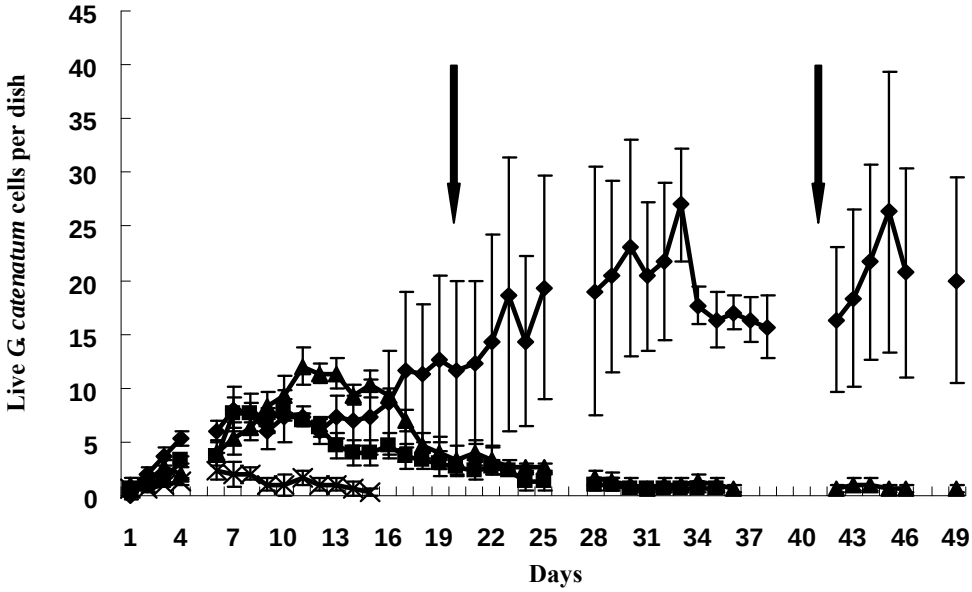

Fig. 2.4: Mean number of live *G. catenatum* cells (n=3; ±standard error) in the presence of algal and bacterial filtrate or live bacteria cell. Arrows indicate the days when additional filtrate was added in treatment 1 Treatments are indicated as follows: —◆—, Multiple addition of *G. catenatum*/bacteria culture filtrate; —■—, Single addition of *G. catenatum*/bacteria culture filtrate; —▲—, Positive control: *Alcanivorax* DG881 cells; —✕—, Negative control: no bacteria. Breaks in date indicate where data was not collected

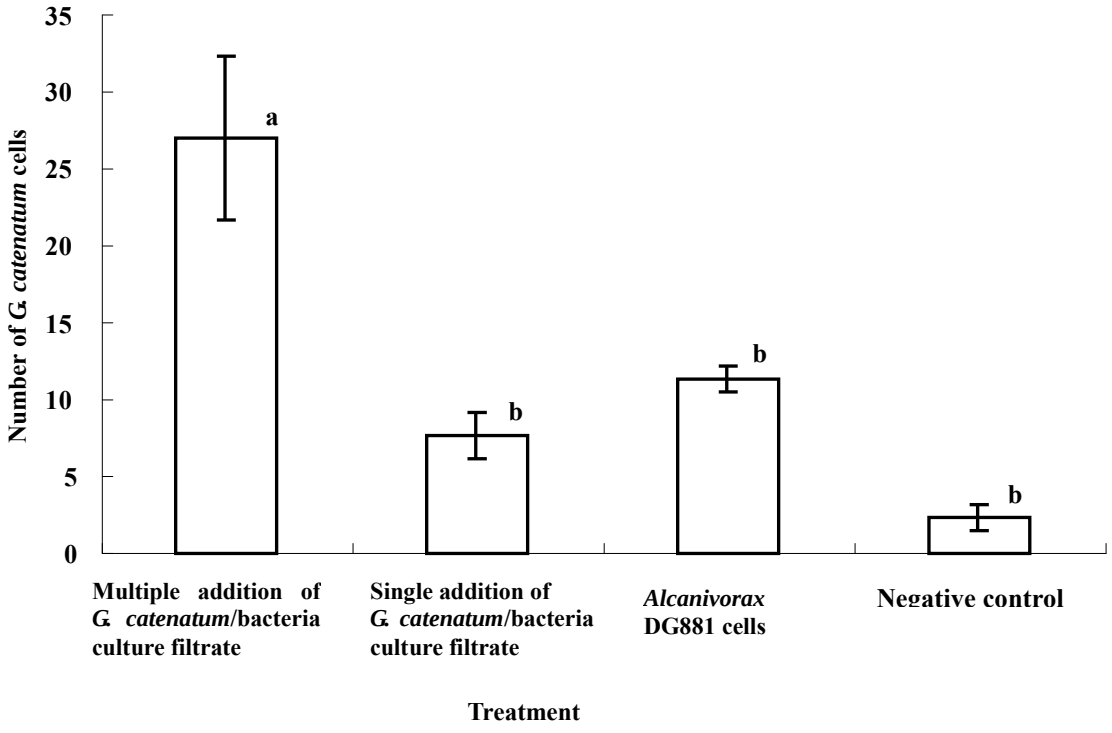

Fig. 2.5: Maximum cell concentration over course of experiment (±standard error) of *G. catenatum* grown with algal and bacterial filtrate or live bacteria cells. Superscripts indicate significant differences ($P \leq 0.05$).

2-4 Discussion

Previous studies have shown that *Alcanivorax* DG881 and closely related *Alcanivorax* strains are associated with (and can be isolated from) a range of *G. catenatum* cultures from Australian, Korean and Japanese strains (Green *et al*. 2004, 2010). Related studies using the *G. catenatum* model based on cysts produced from crosses of different strains (GCDE06 and GCLV01) have also shown similar responses to that described here (Bolch *et al*. 2011, Albinsson 2011, unpublished data). Therefore the responses of *G. catenatum* documented in these experiments are likely to be indicative of the species more generally

Cell filtrates from co-cultures of *Gymnodinium catenatum* and mixed bacteria achieved the highest number of *G. catenatum* cells per dish suggesting that stimulation of growth of *G. catenatum* is through the release of dissolved substances from the bacteria community and/or the dinoflagellate cell. Previous studies have shown that *Alcanivorax* DG881 and closely related *Alcanivorax* strains are associated with (and can be isolated from) a range of *G. catenatum* cultures from Australian, Korean and Japanese strains (Green *et al*. 2004, 2010). Related studies using the *G. catenatum* model based on cysts produced from crosses of different strains (GCDE06 and GCLV01) have also shown similar responses to that deascribed here (Bolch *et al*. 2011, Albinsson 2011, unpublished data). Therefore the responses of *G. catenatum* documented in these experiments are likely to be indicative of the species more generally.

However, there is another possibility that *G. catenatum* growth stimulating substance

might have released from *G. catenatum* itself, not from the bacteria. Some researchers have suggested that *G. catenatum* and other mixotrophic species, obtain nutrients by ingesting bacteria, cyanobacteria, and small phytoplankton (Stoecker 1999, Skovgaard 2000, Jeong *et al*. 2005), however in this study treatment with live bacteria cell showed poor *G. catenatum* growth. Growth stimulation of microalgae by bacteria has been previously reported through a number a mechanisms, including production of vitamins, ncreasing the availability of iron, or the production of stimulatory organic carbon molecules (Luyen *et al*. 2007; Hardie *et al*. 1983), also NaCl, KCl, MgCl, and Na_2SiO_3 also highly influenced on the growth rate of microalgae (Jeong *et al*. 2001). For example, levoglucosan (1,6-anhydro-β-D-glucopyranose) produced by the green seaweed *Monostroma nitidum* enhanced microalgal cell growth and the growth rate of eight microalgal species by approximately 150 % in a range of algal culture media (Luyen *et al*. 2007).

In experiment 2, the *Alcanivorax* DG881 lysate showed no significant *G. catenatum* growth stimulation compared with live *Alcanivorax* DG881, which indicates that the growth stimulating substance produced by *Alcanivorax* DG881 is not an intracellular substance, or is not present at sufficiently high concentration to stimulate growth of the dinoflagellate. However the positive control, treatment added live *Alcanivorax* DG881 cells, also showed limited growth in experiment 2. This might have been caused by medium evaporation across both controls and treatments in this experiment, leading to increased salinity in the experiment model cultures that may have reduced the growth and survival of *G. catenatum* cells in the experiment. Additional studies in larger scale systems (e.g.150 mL flasks would potentially solve some of the problems experienced

during longer experiments (>2-3 weeks) in the low volumes used here. Alternatively, GSe medium has limited organic carbon to support growth of *Alcanivorax* DG881. Therefore, if *G. catenatum* growth was compromised by high salt concentration accumulated early in the experiment, then *Alcanivorax* DG881 (and any stimulatory products it produces) would also not reach sufficient concentrations to support additional growth of the dinoflagellate in the experimental model. The limited growth in *Alcanivorax* DG881 cell treatment also indicated that *G. catenatum* did not ingest bacteria to obtain nutrition for growth this result support the result from experiment 1. Alternatively, *Alcanivorax* DG881 may have failed to produce stimulating substances in this experiment.

The result of experiment 3, multiple addition of co-culture filtrate, showed that dissolved substance from *Alcanivorax* DG881 or possibly from actively growing *G. catenatum* cells, stimulated growth of germinated of *G. catenatum* cells. This supports the outcome of experiment 1 and also suggested that the growth stimulating substance from *Alcanivorax* DG881 was degraded or used by *G. catenatum* during growth.

The cultures used in this study were derived from surface-sterilized resting cysts and are assumed to be free of other contaminating bacteria. While this study did not confirm the absence of other bacteria in the experimental cultures, published studies using the same *G. catenatum* culture models indicate that the presence of other contaminating bacteria is uncommon, and that their effect on *G. catenatum* survival and growth is limited (Bolch et al. 2011).

Studies by Subramanian (2008), have shown that *G. catenatum* growth can deteriorate over extended culture in the uni-bacterial co-culture models used here. If growth stimulating ability of *Alcanivorax* DG881 is unstable, it may be lost after extended culture in the laboratory, explaining the failure of the bacterium to support *G. catenatum* growth in some of the experiments in this chapter.

A consistent finding in this chapter is that *G. catenatum* cannot germinate/grow in the absence of either actively growing bacteria or the dissolved products produced by actively growing *G. catenatum* cultured with a bacterial community. There are a wide range of reports of axenic (bacteria) culture of dinoflagellates, including culture of *Gymnodinium catenatum* (e.g. Boczar *et al*. 1988; Dantzer and Levin 1997; Alavi *et al*. 2001; Vincent 2003; Bolch *et al*. 2004). However, some of these previous studies provide limited information about how the bacteria-free status was determined. Bolch *et al*. (2011) showed that *G. catenatum* has an obligate requirement for marine bacteria for long term survival. Antibiotic treatment showed that the decline or removal of the bacteria affected *G. catenatum* growth (Bolch *et al*. 2011). While there is no guarantee that bacteria were absent from bacteria-free treatments in this study, if present, they were incapable of supporting *G. catenatum* growth.

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Chapter 3

Single carbon source usage by the dinoflagellate-associated bacterium *Alcanivorax* DG881 and *Alcanivorax borkumensis* SK2

3-1 Introduction

Many studies are carried out to examine the interaction between marine bacteria and microalgae. Bacteria exhibit stimulatory or inhibitory effect on algal cells, produce algicidal substances, and promote or interfere with sexual reproduction/cyst production (Cole 1982; Kodani *et al*. 2002; Uribe and Espejo 2003). On the other hand, microalgae produce dissolved organic for bacterial utilization, increase area for surface attachment, and produce anti-bacterial compounds that selects for particular groups (Cole 1982; Simon *et al*. 2002; Gonzalez and Bashan 2000). Previous studies have shown that some marine bacteria have significant effect on dinoflagellate growth. The marine bacteria *Alcanivorax* sp, *Marinobacter*, and *Brachybacterium* appear to play an important role in growth of dinoflagellate *Gymnodinium catenatum* (Bolch *et al*. 2002; Vincent 2003; Subramanian 2008). However, the metabolic capacity of *G. catenatum*-associated bacteria has not been studied.

Microalgae release extracellular dissolved organic carbon (DOC) in the natural environment during photosynthesis (Fogg 1966; Storch and Saunders 1978; Mague *et al*. 1980; Fogg 1983; Goto *et al*. 1999). According to Fogg (1966), the DOC release from phytoplankton during photosynthesis can be up to 50 % or more of total photosynthetic carbon production. The DOC released by phytoplankton is utilized very rapidly by the

bacterial community. (Smith and Wiebe 1976; Larsson and Hagstron 1979; Mague *et al*. 1980; Jensen 1983; Sondergaard *et al*. 1985; Sundh 1989; Gomes *et al*. 1991; Obernosterer and Herndl 1995). For example, Larsson and Hagstrom (1979) used $^{14}CO_2$ gas to demonstrate that the DOC released from phytoplankton is utilized by bacteria almost instantly after it is released from the phytoplankton.

Bacteria of the genus *Alcanivorax* are hydrocarbon-degrading bacteria (Golyshin et al. 2003) typically associated with marine oil-spills, but they are also known to be associated with a range of dinoflagellates and other chromophyte algae. Strain *Alcanivorax* DG881, isolated from a culture of the dinoflagellate *G. catenatum*, has been shown to stimulate *G. catenatum* germination and growth in uni-bacterial culture (Bolch *et al*. 2002; Vincent 2003; Subramanian 2008), however, the mechanisms and/or growth stimulating substances produced by strain DG881 are currently unknown. The closely related (99.6 % rRNA gene similarity) bacterium *Alcanivorax borkumensis* SK2, isolated from marine sediment (Golyshin *et al*. 2003), and does not appear to be capable of supporting growth of *G. catenatum* in uni-bacterial cultures (Bolch *et al*. 2004, Bolch *et al*. 2011). Given the different environments from which they were isolated, it is possible that these two strains may have distinct differences in the types of carbon they can use for growth. Organic carbon utilization by bacteria can be compared using the BIOLOG GN2 MicroPlate system which containing 95 single carbon sources. The BIOLOG system was originally designed for identification and characterization of a wide range of aerobic gram-negative bacteria. However, this assay plate have also been successfully used to assess functional diversity of microorganisms from environmental samples, based the pattern of single carbon sources utilization (Matsui *et al*. 2001;

O'Connell and Garland 2002; Stefanowicz 2006).

This chapter compares the carbon utilization of the *G. catenatum*-associated growth-stimulating bacterium *Alcanivorax* DG881 with the closely related non-stimulatory bacterium *A. borkumensis* SK2.

3-2 Materials and methods

3-2-1 Bacterial culture

Alcanivorax DG881 and *Alcanivorax borkumensis* SK2 were maintained on modified Zobell's marine agar plate (ZM1) (Green *et al.* 2004) containing 5 gL^{-1} of bacterial peptone, 1 gL^{-1} of yeast extract, 10 gL^{-1} of sodium acetate as a carbon source and was solidified with 1.5 % w/v of Difco-Bacto agar. Marine supplements containing filter-sterilized trace elements and vitamins were added to the sterile medium after autoclaving before pouring plates (Appendix 1).

3-2-2 Carbon source utilization

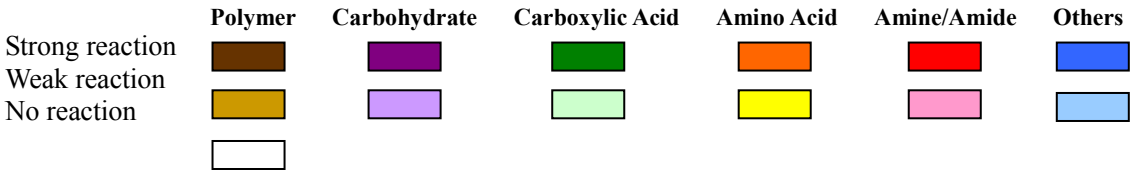
Alcanivorax DG881 and *A. borkumensis* SK2 carbon utilization was determined using BIOLOG GN2 plate assay method (Appendix 5). *Alcanivorax* DG881 and *A. borkumensis* SK2 colony was removed from ZM1 plate, cultured in 7 mL Zobell's marine (ZM) broth for three days, and the cells harvested by centrifugation at 17,200 g for one minute. The supernatant was removed and the pellet was resuspended in 800 µL of GSe algal culture medium (Blackburn *et al*. 1989). This step was repeated three times to remove ZM medium and the bacterial cell pellet was resuspended in 10 mL of GSe medium. Each suspension was diluted with GSe medium to 10^{-5} cells mL^{-1}. Each well of triplicate BIOLOG GN2 plates were inoculated with 150 µl of the bacterial suspension and incubated at 22 ^{o}C in the dark. Absorbance of wells at a wavelength 590 nm (OD_{590}) was also measured at 24, 48 and 72 h using a TECAN Genios microplate reader. To confirm that the absorbance was not a result of culture turbidity, the colour reaction was also observed visually at 72 h and categorized into: no, weak, or strong

colour development Comparison of OD_{590} at 48 h and 72 h was used to determine that colour development was generally complete by 48 h. Significant differences in the 48 h OD_{590} between *Alcanivorax* DG881 and *A. borkumensis* SK2 were compared using student's t-test implemented in the software package SPSS ver. 17.0 (LEAD technologies, Chicago, USA).

3-3 Results

Alcanivorax DG881 was capable of using significantly more (54 of the 95) BIOLOG GN2 carbon sources than *Alcanivorax borkumensis* SK2 (34 of 95) ($\chi^2 = 8.47$, $P<0.001$) (Fig. 3.1). Twenty-three carbon sources were utilized by both *Alcanivorax* DG881 and *A. borkumensis* SK2.

When single carbon sources were compared by compound group, *Alcanivorax* DG881 utilized significantly more amino acids ($\chi^2 = 4.14$, $P<0.037$) but differences in the number of other compound groups utilized were not significant (Fig. 3.2).



A

	1	2	3	4	5	6	7	8	9	10	11	12
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G												
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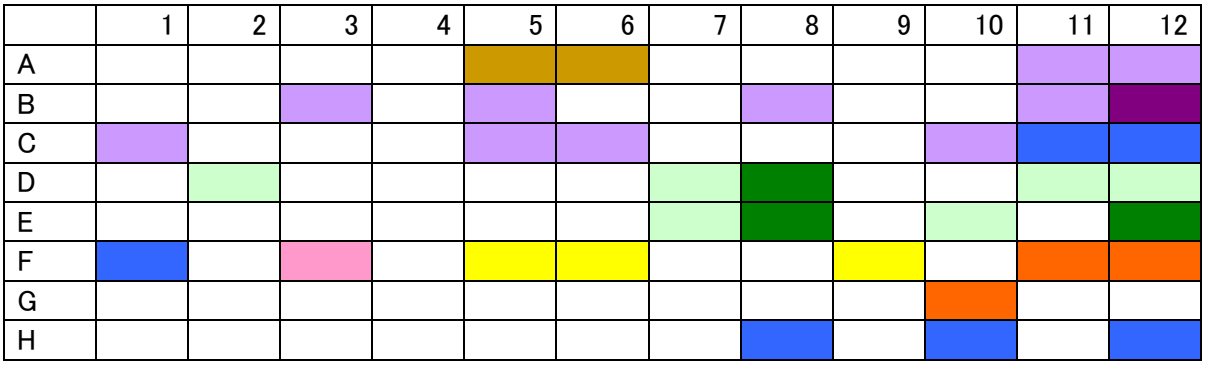
B



	1	2	3	4	5	6	7	8	9	10	11	12
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Fig. 3.1: Single carbon source usage by *Alcanivorax* bacteria assessed using BIOLOG GN2 plate determined after 72 h of incubation. A. *Alcanivorax* DG881; B. *Alcanivorax borkumensis* SK2.

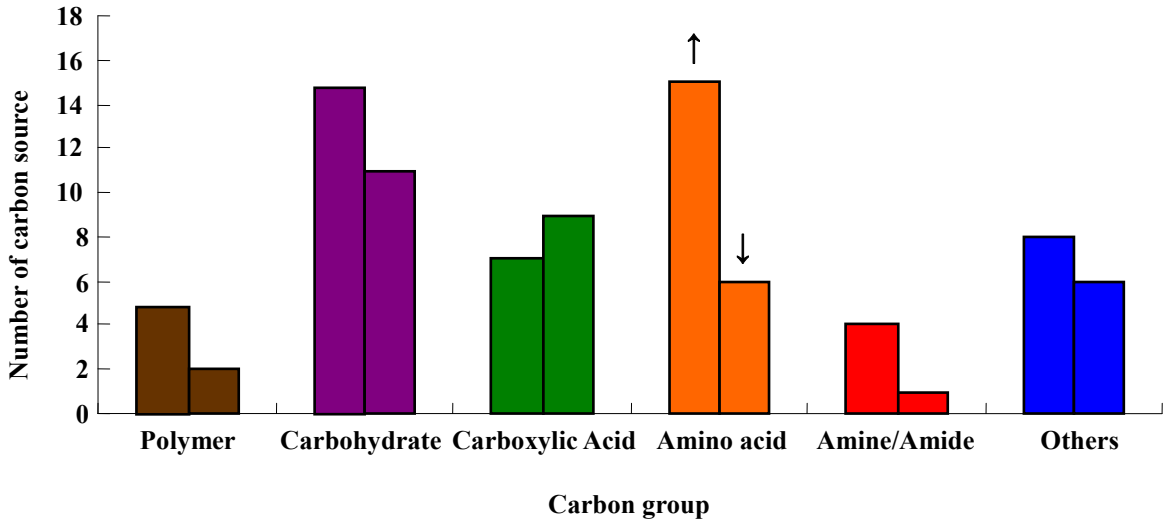

Fig. 3.2: Comparison of number of positive reactions for each organic compound group inoculated with DG881 or SK2 (left and right hand columns, respectively) on BIOLOG GN2 plate. Arrows indicate significantly higher/lower comparisons.

Comparison of the mean OD_{590} values indicated that *Alcanivorax* DG881 utilized 9 carbon significantly more effectively than *A. borkumensis* SK2: α-cyclodextrin (cyclic oligo saccharide), glycogen (polysaccharide), i-erythitol (sugar alchohol), α-D-lactose (disaccharide), α-keto-valeric acid (alkly carboxylic acid), L-leucine (amino acid), D,L-carnitine (ammonium compound), glycerol (polyol alcohol), and α-D-glucose-1-phosphate (cori ester) were significantly higher in *Alcanivorax* DG881 than *A. borkumensis* SK2 (Fig. 3.3). On the other hand, utilization of only 2 carbon sources was significantly higher in *A. borkumensis* SK2 than *Alcanivorax* DG881 (Fig. 3.4): succinic acid mono-methyl-ester and succinic acid (dicarboxylic acid).

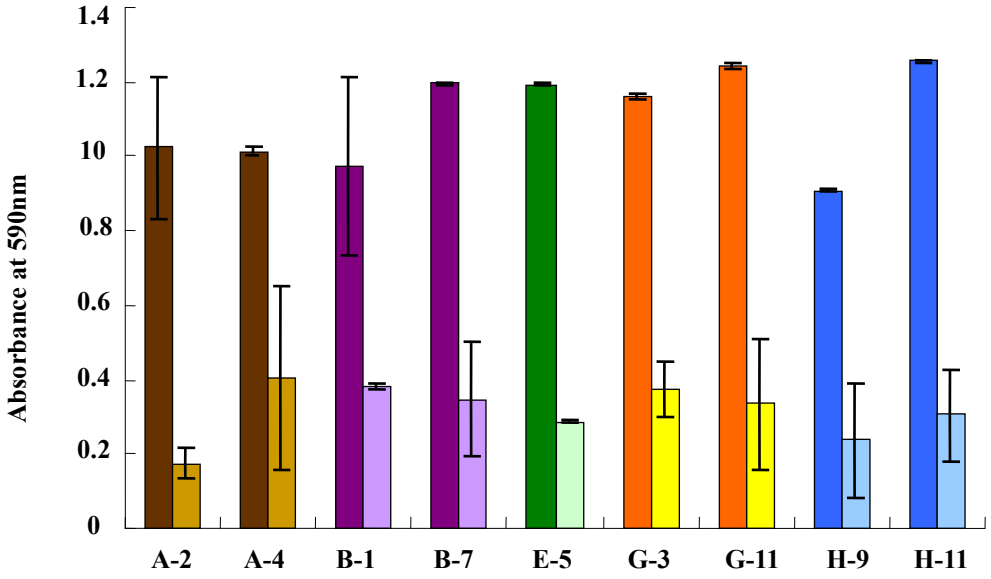

Fig. 3.3 Carbon sources with significantly higher utilization (absorbance at 590nm) by *Alcanivorax* DG881. A-2: α-Cyclodextrin, A-4: Glycogen, B-1: i-Erythitol, B-7: α-D-Lactose, E-5: α-keto-Valeric Acid, G-3: L-Leucine, G-11: D,L-Carnitine,

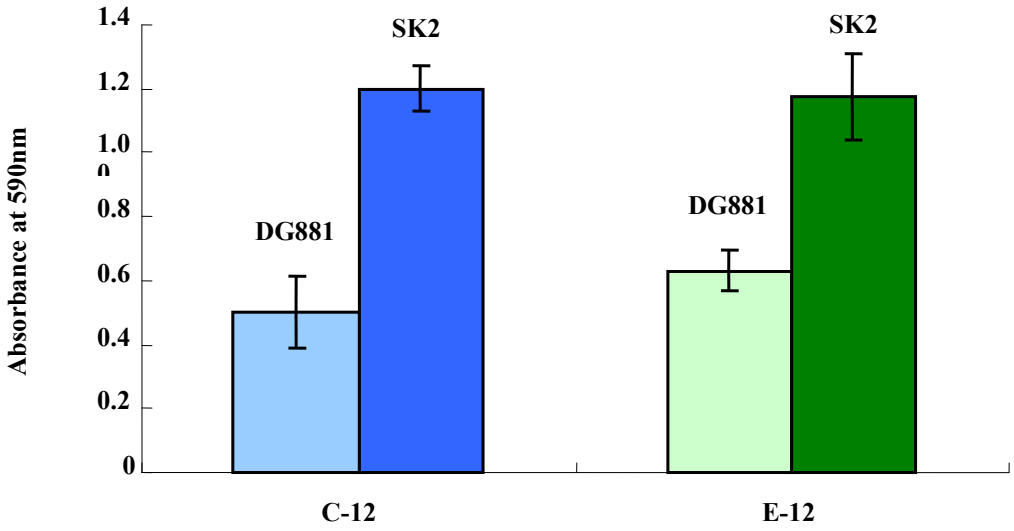

Fig. 3.4 Carbon sources with significantly higher utilization (absorbance at 590nm) by *Alcanivorax borkumensis* SK2. C-12: Succinic Acid Mono-Methyl-Ester, E-12: Succinic Acid.

3-4 Discussion

The most studied DOC from phytoplankton is glycolate (glycolic acid) and uptake by bacteria is also well known (Wright 1975; Wright and Shah 1975; Wright and Shah 1977; Edenborn and Litchfield 1987; Lau and Armbrust 2006; Lau *et al*. 2007). Many bacteria in both marine and fresh water are able to metabolize glycolate, however, only some bacteria are able to use glycolate as a sole carbon source in pure culture (Wright 1975; Wright and Shah 1975). Lue and Armbrust (2006) suggest that glycolate oxidase is an important enzyme for glycolate utilization and found that glycolate oxidase gene *glcD* from cultured and environmental marine bacteria. Further study by the same authors indicates that, the glycolate oxidase gene *glcD* are present in wide range of bacterial phyla. Quantitative reverse transcriptase PCR with *glcD* primers also indicate that bacteria respond to the glycolate release by phytoplankton is higher in the day time than at night (Lau *et al*. 2007).

The BIOLOG GN2 plate assays showed that *Alcanivorax* DG881 is capable of using a much wider range of organic carbon than more *Alcanivorax borkumensis* SK2. This was particularly evident when considering amino acid utilization. According to Myklestad (2000), carbohydrate (mono- and polysaccharides) are the dominant carbon compounds among the DOC released from phytoplankton, followed by proteins and amino acids. Other extracellular substances released from phytoplankton are organic acids, sugar alcohols, lipids, and fatty acids, vitamins. Strain *Alcanivorax* DG881 and its close relatives are typically found in association with algal cells strain DG881 was isolated from a culture of the dinoflagellate *Gymnodinium catenatum*. In comparison, *A.*

borkumensis SK2 was isolated from marine sediment and is considered to be primarily a hydrocarbon degrader with relatively limited capacity to utilize other carbon sources (Golyshin *et al*. 2003). The data here suggest that *Alcanivorax* DG881 would benefit from its relationship with dinoflagellate cells by utilization of carbohydrate and amino acids from the dinoflagellate cell released to the natural environment during photosynthesis and growth (Sundh 1989).

Romanenco (1977) suggested that bacteria decompose DOC released from dead algal cells and it is possible that the saccharides and amino acids are derived primarily from dead/dying cells rather than actively growing cells. However, there is a strong correlation of *Alcanivorax* DG881 numbers with dinoflagellate cell concentration in *G. catenatum*/*Alcanivorax* uni-bacterial co-culture during the early- and mid-logarithmic algal growth phases (Bolch *et al*. 2011). Growth rate of the co-cultured bacteria are approximately 20-fold lower than growth rates of similar bacteria grown in pure culture on single carbon sources (Bolch *et al*. 2011). This indicates that bacterial growth is primarily limited by release of DOC from actively growing dinoflagellate cells. Bacteria growth rate increases substantially at the beginning of dinoflagellate stationary phase (Subramanian 2008) when an increase in cell lysis and exuded DOC might be expected, therefore, it appears that the source may shift in stationary phase to DOC predominantly derived from dead/lysed dinoflagellate cells.

The broad range of saccharide and amino-acid utilization indicates that the *Alcanivorax* DG881 – *G. catenatum* relationship may be mutualistic. *Alcanivorax* DG881 likely utilizes DOC exuded by the dinoflagellate, and in turn produces dissolved growth

factors that stimulate and support the growth of the dinoflagellate cells (Chapter 2, Bolch *et al*. 2011). This relationship is thus similar to the carbon – iron mutualistic relationship proposed by Amin *et al*. (2009) between *Marinobacter* sp. and the dinoflagellate *Scrippsiella trochoidea*. While the growth factors remain unknown for *Alcanivorax* – *G. catenatum* relationship, a similar siderophore- mediated uptake of iron by the dinoflagellate may be involved as well as other additional factors (Bolch *et al*. 2011).

The two *Alcanivorax* strains examined here also differ in their capacity to support the growth of the dinoflagellate *G. catenatum* in uni-bacterial culture (Bolch *et al*. 2004, 2011). While *Alcanivorax* DG881 supports the growth of *G. catenatum*, *A. borkumensis* SK2 does not (Bolch *et al*. 2004; Subramanian 2008; Bolch *et al*. 2011). The wider amino acid and simple carbohydrate utilization profile of *Alcanivorax* DG881 suggests that this strain is adapted to the algal-cell exudates that would be available in algal-cell associated environment it inhabits. These additional metabolic capacities may be related to *G. catenatum* growth stimulating capability of *Alcanivorax* DG881.

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Chapter 4

Screening of *Gymnodinium catenatum* culture for homologues of the cyanobacterial saxitoxin synthesis genes *sxtN* and *sxtU*

4-1 Introduction

Saxitoxin (STX) is a neurotoxin naturally produced during HAB events by certain species of marine dinoflagellates *Alexandrium* sp., *Gymnodinium* sp., and *Pyrodinium* sp., and freshwater cyanobacteria *Anabeana* sp., *Cylindrospermopsis* sp., and *Lyngbya* sp. Saxitoxin selectively blocks voltage-gated Na^+ and Ca channels of nerve cells, preventing normal cellular function and leading to paralysis (Hartshorne *et al*. 1980). Symptoms of PSP normally occur within 10-30 minutes after ingesting a toxic food, begin with a tingling sensation of the lips, tongue and face. This tingling sensation will spread to arms and legs, and progress to muscular weakness, loss of motion activity, moreover PSP sometime lead to death (Clark *et al*. 1999).

Some cyanobacteria (blue green algae) also produce STX and form extensive blooms that lead to mass kills of fish and livestock mortalities (Negri *et al*. 1995). The occurrence of STX produced by blooms of *Anabaena circinalis* was reported in Australia in 1991 when an *A. circinalis* bloom covered over 1,000 km of the Darling River in Australia (Bowling and Baker 1996). The putative pathway of STX synthesis candidate gene cluster (Figure 4.1) has been recently described from *Cylindrospermopsis. raciborskii* T3 (Kellmann *et al*. 2008), a genus more commonly known for producing the alkaloid neurotoxin called cylindrospermopsin.

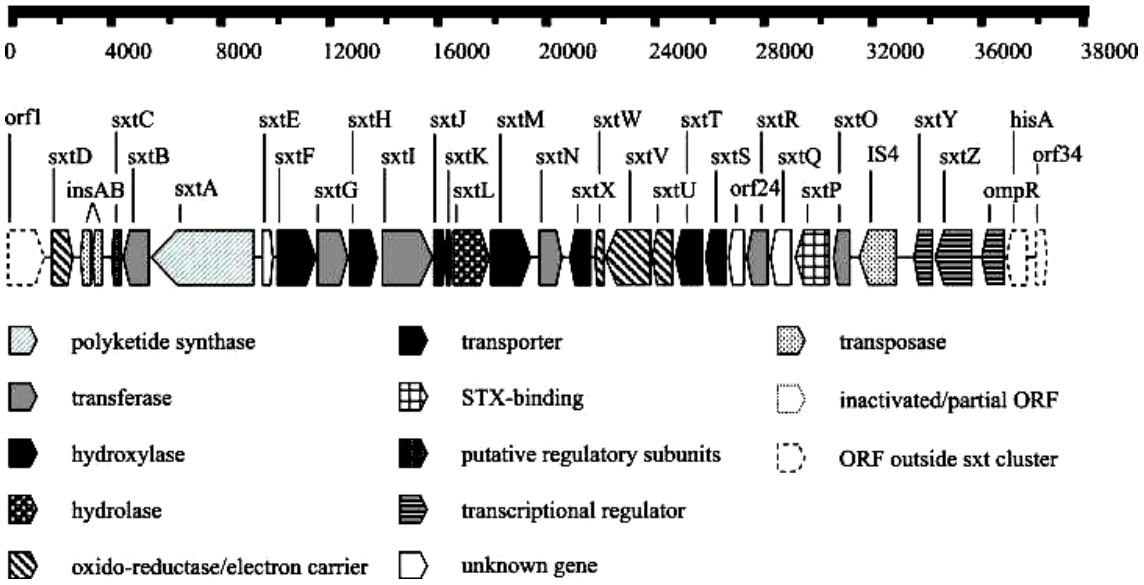


Fig. 4.1 STX synthesis candidate gene cluster (Kellmann *et al*. 2008)

It is likely that dinoflagellates produce STX by the same biosynthetic pathway as cyanobacteria (Shimizu 1993) and dinoflagellate and cyanobacterial genes share some sequence homology.

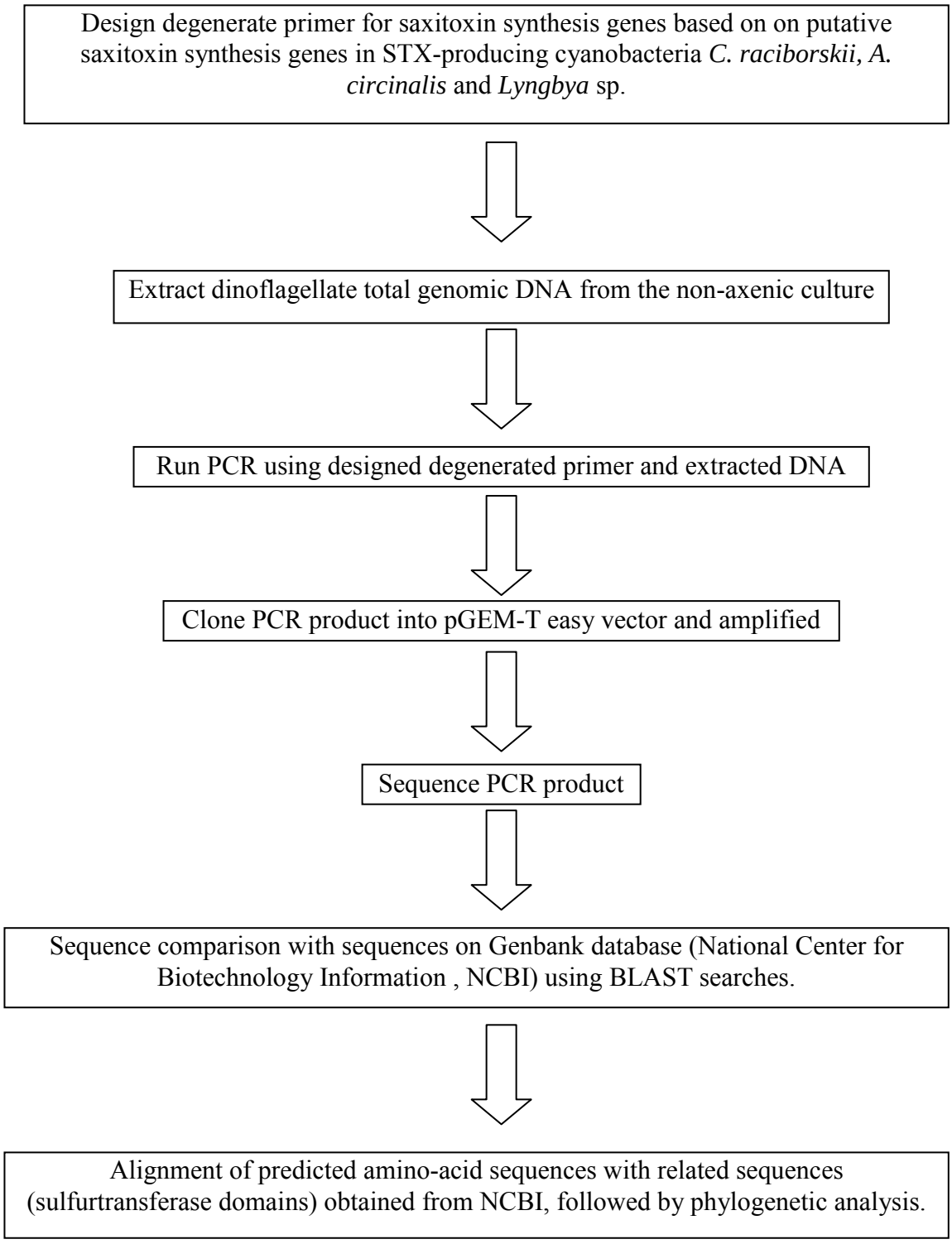
Gymnodinium catenatum is one of the toxic dinoflagellates that produce paralytic shellfish toxins (PSTs) and it is becoming a big issue in many countries, especially in Spain, Japan, Korea, and Australia. Shellfish, such as oysters, mussels and scallops, can accumulate and concentrate the toxin through filter-feeding on toxic dinoflagellates and the toxins in shellfish can be lethal to human (Hallegraeff 2003). The first PSP was reported in Australia in 1986 when *G. catenatum* appeared in Huon and Derwent estuaries, Tasmania (Hallegraeff *et al*. 1988; McMinn *et al*. 1997). Two major recurrences were reported in 1987 and 1991 in Tasmania and affected shellfish industry

(Hallegaeff 1993).

Aim of this study was to isolate key genes involved in saxitoxin synthesis from cultures of the dinoflagellate *G. catenatum* using primers designed from putative saxitoxin synthesis genes known from STX- producing cyanobacteria.

4-2 Materials and methods

Chapter 4 flow chart



4-2-1 Dinoflagellate and bacteria culture

Two non-axenic Strains of *G. catenatum* (GCHU11 and GCDE08) were grown in GSe medium (Blackburn *et al*. 1989; see Appendix 1) in 150 mL Erlenmeyer flask at 18 ^{o}C (±2 ^{o}C) under 90±10 μmol photons m^{-2} s^{-1} of cool white fluorescent light with 12:12 h light:dark photoperiod.

Alcanivorax DG881 was cultured on modified Zobell's marine agar plate (ZM1) (Green *et al*. 2004) containing 5 gL^{-1} of bacterial peptone, 1 gL^{-1} of yeast extract, 10 gL^{-1} of sodium acetate as a carbon source and was solidified with 1.5 % w/v of Difco-Bacto agar. Marine supplements containing filter-sterilized trace elements and vitamins were added to the sterile medium after autoclaving before pouring plates (Appendix 2). *Marinobacter* was cultured on ZM1 agar plates containing 5 gL^{-1} of bacterial peptone, 1 gL^{-1} of yeast extract, and solidified with 1.5 % (w/v) of Difco-Bacto^{TM} agar. Marine supplements containing filter-sterilized trace elements and vitamins were added to the sterile medium after autoclaving before pouring plates

4-2-2 DNA extraction

Dinoflagellate total genomic DNA from the non-axenic culture was extracted from 5-10 mL of dense mid-log phase culture harvested by centrifugation. The pellet was resuspended in 400 μL of MQ water and 50 μL of 10 % SDS and 50 μL of 10x STE buffer (100 mM Tris, 100 mM EDTA, 100 mM NaCl, pH 8.0) added. Then 500 mL of Tris-buffered phenol was added and mixed by inversion followed by centrifugation for 10 minutes at 17,200 g. The aqueous phase was transferred to another tube and the extraction process repeated twice with 500 μL of phenol:chloroform:isoamyl alcohol

(24:24:1) and once with 500 μL chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to another tube, precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.0) and two volumes of cold 100 % ethanol, and centrifugation at 13000 rpm for 20 minutes at 4 ^{o}C. The pellet was washed with 70 % of ethanol, dried and then suspended in 50 μL TE buffer (Appendix 3). The DNA concentration was determined using a Turner TBS 380 DNA fluorometer (Turner Designs, USA) according to manufacturer's standard protocols (available at: www.topac.com/hoechest33258.html). The extracted DNA was kept at -20 ^{o}C until needed.

Alcanivorax DG881 DNA was extracted from culture plate. A streak of cell was taken from agar plate and suspend in TE buffer. 30 μL 10 % SDS was added and mixed thoroughly by shaking then 3 μL 20 mg mL^{-1} Proteinase K was added and incubate at 56 ^{o}C for 60 min. 100 μl of 5 M NaCl was added to the solution then 80 μL of CTAB/NaCl was added and mixed well and incubate at 65 ^{o}C for 30 min. An equal volume of Chloroform:isoamyl alcohol (24:1) was added and mixed by inversion followed by centrifugation for 5 minutes at 17,200 g. The aqueous phase was transferred to another tube and the extraction process repeated twice with equal volume of phenol:chloroform:isoamyl alcohol (24:24:1). The aqueous phase was transferred to another tube, precipitated by addition of 1:1 volume cold isopropanol alcohol. The pellet was washed with 70 % of ethanol, dried and then suspended in 50 μL TE buffer (Appendix 4).

4-2-3 Primer design for saxitoxin synthesis genes

The terminology used in the thesis for a gene or the protein that it encodes follow standard biochemical convention. A gene is referred to in lower case italics with an upper case gene identifier (*sxtN*) whereas the protein product of the gene is referred to with a capital letter and non-italics (SxtN). The abbreviation STX refers to the chemical compound (saxitoxin) produced by the entire biosynthetic pathway.

Degenerate (mixed base sequences) PCR primers (Table 4.1) for saxitoxin synthesis genes were designed based on putative saxitoxin synthesis genes discovered in STX-producing cyanobacteria *C. raciborskii, A. circinalis* and *Lyngbya* sp. Conserved regions were determined from sequence alignments using ClustalX and bases that varied between species included in the degenerate primer mixture.

Table 4.1: Degenerate PCR primer sets used to screen saxitoxin syntheses gene from *G. catenatum*

Gene	Primer	Sequence	Target gene
sxtD	Forward	GANTGGACNGTNATHTTHT	Sterol desaturase
	Reverse	TCRAARTTYCNGGRAANTG	
sxtN	Forward	AAYAARCAYATHGCNATCTCC	Sulfotransferase
	Reverse	GGNGGRTTYTTDTADTACCT	
sxtU	Forward	GTNAAYAAYGCNGGNATHGG	Alcohol
	Reverse	ATYTCRTTNACRAANACRA	dehydrogenase

4-2-4 PCR and electrophoresis

Temperature gradient PCR was performed to test the Sxt primers shown in Table 3.1 using *G. catenatum* and *Alcanivorax* DG881 extracted DNA. The 50 μL reactions contained 10-25 ng of DNA, 1x Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 10 pmol of each primer and 1 unit of Taq polymerase (BioTaq, Bioline, UK). PCR reaction was initiated with a denaturing temperature of 95 ^oC for 5 minutes followed by 30 cycles of 95 ^oC for 30 seconds, 55 ^oC to 45 ^oC degradation temperatures for 45 seconds, 72 ^oC for 1 minute, then final extension at 72 ^oC for 7 minutes. Amplified DNA was separated by electrophoresis using 1 % agarose gel in TBE (0.89 M Tris-borate, 20 mM EDTA, pH 8.3) buffer and visualized by UV transiluminater. *C. raciborskii* T3 DNA was used as positive control. LSU-rDNA primers were used to determine *G. catenatum* DNA quality.

PCR were performed in 20 μL reaction volume containing 1x Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 10 pmol forward and reverse primer, 0.2 units of Taq polymerase, and between 10 to 100 ng of genomic DNA template. The degenerate primer set used in this study are listed in Table 3.1. PCR cycle start with a denaturing at 95 ^oC for 5 minutes followed by 30 cycle of denaturing at 95 ^oC for 30 seconds, annealing at 42 ^oC for 45 seconds, extension at 72 ^oC for 1minutes, an extra extension at 72 ^oC for 7 minutes. DNA was separated by electrophoresis using 1 % agarose gel in TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 7.8), and visualized by UV transilumination after staining in ethidium bromide (0.5 μg/mL).

4-2-5 Cloning and Sequencing

The amplified DNA fragments from PCR reaction were purified from the agarose gel using QIAGEN gel extraction kit. The extracted DNA was ligated into pGEM-T easy vector (Promega) then transferred to competent cell DH5α according to manufacturer's instructions. Colony PCR was used for amplification and sequencing insert DNA using vector primer MpF and MpR. The colony PCR was performed in 20 μL reaction volume containing 1x Taq polymerase buffer 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 10 pmol forward and reverse primers, and 0.2 units of Taq polymerase. A colony containing the plasmid with an insert was used as template. A small amount of colony was picked by gently using 10 μL pipette tip, then placed into a PCR tube containing 20 μLof reaction mix and mixed gently few times.

The PCR cycle started with a denaturing at 95 ^{o}C for 5 minutes followed by 30 cycle of denaturing at 95 ^{o}C for 30 seconds, annealing at 42 ^{o}C for 45 seconds, extension at 72 ^{o}C for 1 minute, an extra extension at 72 ^{o}C for 7 minute. DNA was separated by electrophoresis using 1 % agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8), and visualized by UV transilumination after staining in ethidium bromide (0.5 μg/mL). Sequence data was analysed using ABI Prism-Autoassemble software and similarity and identity to existing sequence were determined using BLAST.

4-2-6 Multiple sequence alignment and phylogenetic analysis

Putative *sxt* gene sequences obtained from *G. catenatum* and its bacterial community (*sxtN* and *sxtU*) were translated in to amino acid sequence an open reading flame was found. The *sxtN* and *sxtU* were converted to deduced amino acid sequences and

compared with National Center for Biotechnology Information (NCBI) database by conducting BLAST search and available homologues were obtained. The amino acid sequence datasets obtained from NCBI were aligned using Geneious Pro version 3.8.5. and manually adjusted if necessary. Several sulfortransferase sequences are also obtained from NCBI database and aligned with SxtN from *G. catenatum*. Initial comparisons indicated that sequences of putative SxtN gene homologues obtained from *G. catenatum* were related to Rhodanase/sulfurtranferase family of proteins. Amino acid sequences of sulfurtransferase domains were obtained from NCBI and each domain was aligned by profile alignment program in Geneious Pro version 3.8.5 and exported to the program PAUP 4.0* (Swofford 1998) for phylogenetic analysis. Phylogenetic trees of known sulfurtransferase domains or sulfotransferase were constructed using the neighbour joining algorithim (Saitou and Nei 1987) and displayed using the program TreeView (Page 2001). A single domain sulfurtransferase, GlpE from the gamma-proteobacterium *Pseudomonas aeruginosa* was selected as as outgroup to root the analyses because: 1) The GlpE protein domain is the most closely related rhodanase family sulfurtransferase to the YceA domain which the putative *sxtN* sequence was most closely related; and 2) *P. aeruginosa* is the most closely related bacterium from which the GlpE domain sequence is known.

4-3 Result

4-3-1 PCR amplification

Temperature gradient PCR using cyanobacteria specific primer sets amplified clear single products of the expected size for all three genes were amplified from *C. raciborskii* T3, indicating that the primer and PCR conditions were suitable for amplification. However, no product was amplified from *G. catenatum* culture DNA using the primers SxtD genes while control primer set amplified expected size product from *G. catenatum* DNA. PCR products close to the expected length (525 bp) were obtained using primers for *sxtN* and *sxtU* while no product was obtained from *Alcanivorax* DG881 DNA. PCR products obtained using the primers for both *sxtN* and *sxtU* also resulted in poorly resolved sequence.

4-3-2 Analysis of candidate SxtN

The partial *sxtN* sequence fragment from cloned PCR product yielded a DNA sequence of 408 nucleotides which could be translated to a protein sequence of 136 amino acids. The candidate SxtN amino acid sequence showed high similarity with sulfurtransferase domain sequences derived from the gram-negative, halophilic coccobacillus, *Francisella philomiragia subsp. Philomiragia*, *Francisella tularensis subsp novicida*, *Psychrobacter arcticus* and a sulfurtransferase from an unidentified gamma Proteobacterium. The sequence also showed high similarity with a hypothetical protein of the higher plants *Arabidopsis thaliana* and *Oryza sativa* (Table 4.2).

The phylogenetic analysis of predicted amino-acids showed that SxtN from *G.*

catenatum did not cluster with sulfotransferase sequences (Table 4.3) however, instead grouped with YceA domain of sulfurtransferase gene cluster (Table 4.4).

Table 4.2: Sulfotransferase gene (*sxtN*) sequence similarity

BLAST similarity match	Enzyme family	% similarity
Francisella philomiragia subsp. Philomiragia	Sulfurtransferase	84 %
Francisella tularensis subsp novicida	Sulfurtransferase	83 %
Psychrobacter arcticus	Hypothetical protein	83 %
Gamma Proteobacterium	Sulfurtransferase	81 %
Arabidopsis thaliana	Unknown protein	81 %
Oryza sativa	Hypothetical protein	81 %

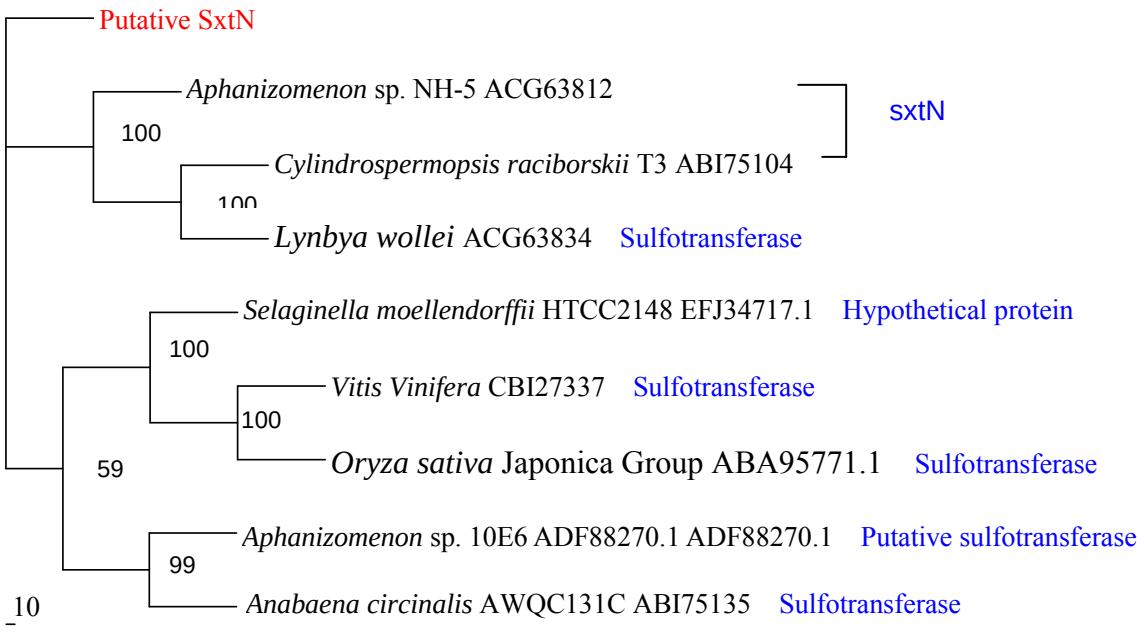


Fig 4.2 Phylogenetic tree of cyanobacterial SxtN predicted amino acid sequences and related plant sulfotransferase protein sequences. The tree was generated using neighbour-joining in the program PAUP 4.0* (Swofford 1998). Values at branch points represent bootstrap support values for clusters.

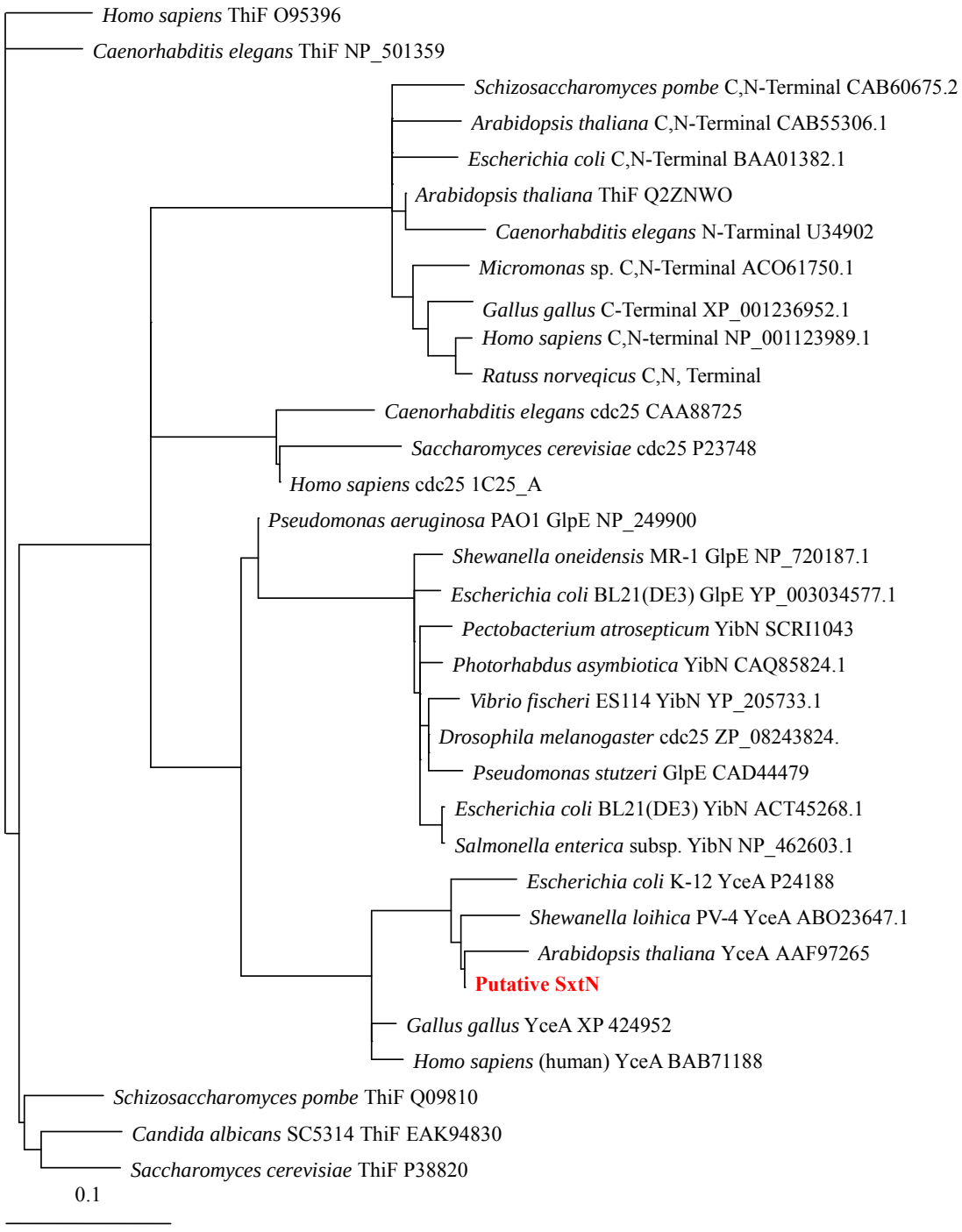


Fig 4.3. Neighbour-joining phylogenetic tree of the sulfurtransferase protein superfamily. Position of candidate *G. catenatum* SxtN (red text) among the YceA family of sulfurtransferases is indicated.

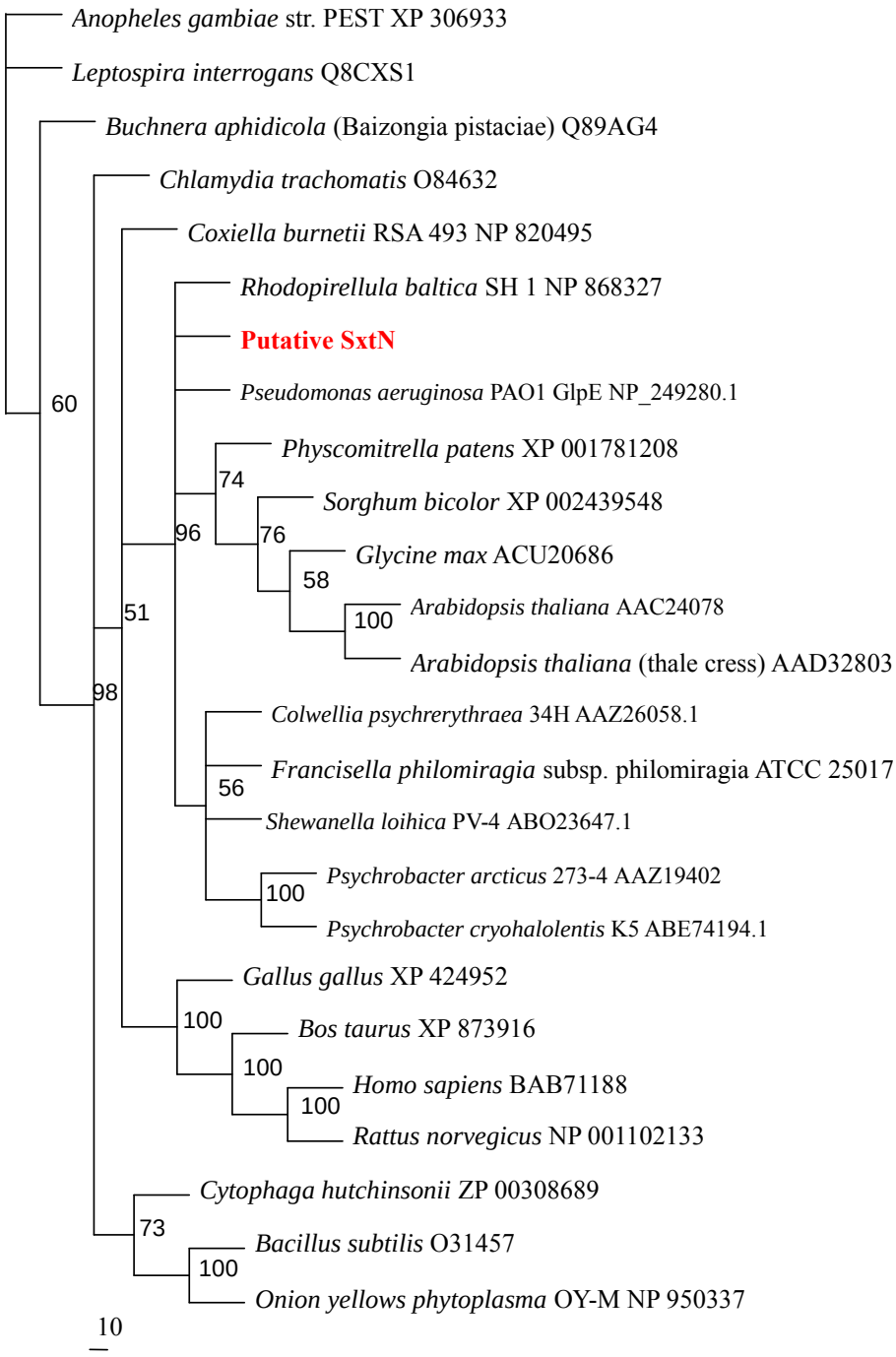


Fig. 4.4. Bootstrap consensus phylogenetic tree of the YceA subfamily of sulfurtransferases. Tree generated using the Neighbour-joining algorithm. Figures at branch points indicate bootstrap support values for clusters with >50 % bootstrap support.

4-3-3 Analysis of candidate SxtU

The partial *sxtU* fragment amplified and sequenced yielded a DNA sequence of 336 nucleotides which could be translated to a protein sequence of 112 amino acids without only low homology to any characterized protein. The candidate SxtU protein sequence showed low similarity with a hypothetical protein of the bacterium *Peptostreptococcus micros* and proteins from the fungi *Aspergillus oryzae*, and *Ustilago maydis*.

Table 4.3: Dehydrogenase (SxtU) gene sequence similarity

BLAST similarity match	Enzyme family	% similarity
Peptostreptococcus micros	Hypothetical protein	46 %
Aspergillus oryzae	Hypothetical protein	46 %
Ustilago mavdis	Hypothetical protein	42 %

4-4 Discussion

The deduced amino acid sequence of SxtN from *G. catenatum* showed high similarity (more than 80 %) with the (protein family) sulfurtransferase from several bacteria, indicating that the SxtN sequence obtained in this study is likely sulfurtransferase. The deduced amino acid sequence of SxtN also showed similarity with hypothetical protein from higher plants. On the NCBI database the gene coverage of sequence data from microalgae is very limited, therefore it difficult to determine whether the putative SxtN originated from the bacteria community associated with *G. catenatum*, or the dinoflagellate. As the similarity of the putative SxtN to bacterial sulfurtransferases is high, SxtN is most likely a sulfurtransferase of bacterial origin. The similarity with bacterial sulfurtranferases may however be due to the lack comparable microalgal of protein sequence data across a wide range of protein families.

The primers designed for the *sxtN* gene were targeted against a sulfotransferase however the sequence obtained in this study showed highest similarity with sulfurtransferases. Sulfurtransferases (E.C. 2.8.1.x) are found in all major evolutionary phyla, vertebrates, plant, and bacteria, yet their biological role is debated. The thiosulfate:cyanide sulfurtransferase/rhodanese family (E.C. 2.8.1.1) are a group of enzymes that catalyse the transfer atom from a suitable sulfur donor to a nucleophilic sulfur acceptor. The most well studied sulfurtransferase is bovine liver rhodanese (Ploegman *et al*. 1979, Westley *et al*. 1983, Gliubich *et al*. 1996). The active site of bovine rhodanese is characterized by the presence of a cysteine residue, which promotes formation of a persulfide intermediate during the catalytic cycle (Ploegman *et al*. 1979).

Analysis of the structures of rhodanese proteins indicates that they display similar

three-dimensional conformations (Ploegman *et al*. 1978, Bordo and Bork 2002), but often low amino acid sequence similarity. The SxtN amino acid sequence obtained here, aligned most closely with a RHOD YceA domain within the rhodanese enzyme superfamily. YceA domains in *E. coli* are known to be composed of a rhodanese domain, a hypothetical domain, and a cysteine-rich domain (Cheng *et al*. 2008), but the function of this protein family is unknown. The YceA family is closely related to GlpE, a single-domain periplasmic rhodanese that accounts for 15 % of the sulfurtranferase activity in *E. coli*, the majority of activity can be attributed to PspE the major cytoplasmic rhodanese of *E. coli*, to (Cheng *et al*. 2008). The putative SxtN sequence isolated here is thus more likely a sulfurtransferase and may have been amplified due to non-specific primer binding to non-target sequences from either the dinoflagellate or bacterial DNA present in the DNA extracts screened. Recent research indicates that putative dinoflagellate homologs of SxtA are relatively divergent from their cyanobacterial counterparts (Dr S. Murray, University of New South Wales; personal communication), therefore SxtN homologues may be similarly divergent and difficult to amplify from dinoflagellate DNA using primers designed from cyanobacterial sequences.

There have been limited reports of sulfotransferase activity from dinoflagellates (Sako *et al*. 2001; Yoshida *et al*. 2002; Wang *et al*. 2007). Wang *et al*. (2007) prepared a crude enzyme extraction of a toxic dinoflagellate species, *Alexandrium tamarense* CI01, which can transfer a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to N-21 in the carbamoyl group of gonyautoxin 2/3 (GTX2/3) to produce C1/C2, but is inactive toward STX to produce GTX5. Moreover, the activity shows no difference

when various sulfur compounds was used as sulfate donor. These results suggested that the sulfotransferase specific to GTX2/3 is present in the cells of *A. tamarense* CI01 and is involved in PSP toxin biosynthesis. Yoshida *et al*. (2002) purified sulfotransferase from *G. catenatum* GC21V. In addition, the sulfotransferase from different dinoflagellates is species-specific, which may explain the variation in PSP toxin profiles of toxic dinoflagellates

The deduced amino acid sequence of SxtU showed a very low similarity with hypothetical proteins from both prokaryote and eukaryote sources. Moreover there are limited microalgal genes for comparison in gene/protein databases, therefore, it is difficult to determine whether the SxtU sequence originated from *G. catenatum* or the associated bacteria.

Given the level of sequence similarity to bacterial sulfurtransferases of the YceA family and that the dinoflagellate culture from which DNA was extracted was not axenic (included *G. catenatum*-associated bacteria), it is concluded that the putative SxtN sequence isolated here is of bacterial rather than dinoflagellate origin.

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Chapter 5

Summary and conclusion

5-1 *G. catenatum* grow without live bacteria

This study shows that substances in dinoflagellate/bacteria co-culture filtrate stimulate or maintain *G. catenatum* growth in the absence of bacteria and indicating that the growth stimulating is not the result of ingestion of bacteria by the dinoflagellate cell. The stimulating effect of *Alcanivorax* DG881 is likely due to the production of dissolved growth promoting substances and does not require the presence or contact with live bacteria. As multiple additions of dinoflagellate/bacteria co-culture filtrate supported dinoflagellate growth for longer periods, this indicates that the substances are either labile or taken up and used by *G. catenatum* for growth.

5-2 Carbon source usage of the dinoflagellate associated bacterium *Alcanivorax* DG881 and *A. borkumensis* SK2.

In this study carbon utilization by the *G. catenatum* growth stimulating bacterium *Alcanivorax* DG881 and non-stimulating *A. borkumensis* SK2 were compared. The dinoflagellate associated *Alcanivorax* DG881 showed a capacity to utilize a broader range of carbon compounds than the closely related non-stimulatory bacterium *A. borkumensis* SK2. This result indicated that *Alcanivorax* DG881 has a wider metabolic capacity to utilize different types of carbon, suited to that released by dinoflagellate cells and/or the associated bacteria.

5-3 Putative saxitoxin synthesis genes from *G. catenatum*

Two putative saxitoxin synthesis genes, *sxtN* and *sxtU*, were cloned and sequenced from *G. catenatum* and its associated bacteria, however both sequences did not show similarity with any known cyanobacterial STX synthesis genes. Putative SxtN showed high similarity with sulfurtransferase from several bacteria species, this indicated that this gene is more likely a sulfurtransferase of YceA family amplified from the *G. catenatum*-associated bacteria rather than of dinoflagellate origin.

5-4 Future research

A number of additional a lines of research could be pursued that would assist in clarifying the nature of the relationship between *Alcanivorax* DG881 and the dinoflagellate *Gymnodinium catenatum*.

1) The type and nature of the stimulating substances could be determined by combining column-fractionation of the dissolved fraction *G. catenatum-Alcanivorax* co-cultures. Fractions could then be assayed for growth stimulation of *G. catenatum* in experiments similar to chapter 2 of this thesis.
2) Re-design the PCR primers based on recently discovered STX synthesis genes from a range of dinoflagellates (Stuken et al. 2011).
3) Screening for other secondary product synthesis genes such as polyketide synthesis genes and hydroxylases from *G. catenatum.* Given the predominance of sulfocarbamoyl fractions (C-toxins) in the STX profile of *G. catenatum*, there may be some value in targeting proteins and genes involved in the production of these

particular compounds

4) Additional experiments could determine whether the DOC released from *G. catenatum* can be taken up *Alcanivorax* DG881 and the non-stimulatory bacterium *A. borkumensis* SK2 and utilised efficiently for growth.

5-5 Reference

Stuken, A., Orr, R. J. S., Kellmann, R., Murray, S. A., Neilan, B. A. and K. S. Jakobsen. (2011). Discovery of Nuclear-Encoded Genes for the Neurotoxin Saxitoxin in Dinoflagellates. *PLoS ONE*. **6**(5), e20096

GSe Medium Preparation

1. Sea water

Autoclave filtered seawater in one L Teflon bottle

2. Distilled water

Autoclave distilled water to sterilise

3. Stock Solution

1. KNO_3	100.0 gL^{-1} H_2O
2. K2HPO4	34.8 gL-1 H2O
3. Vitamins	
Biotin	0.2 mg 100 $mgL^{-1}H_2O$
Vitamin B12	0.1 mg 100 $mgL^{-1}H_2O$
Thiamine HCl	100.0 mg $mgL^{-1}H_2O$
4. PII Metal Mix	
Na2 EDTA	6.0 $gL^{-1}H_2O$
$FeCl_3 \cdot 6H_2O$	0.29 $gL^{-1}H_2O$
H_3BO_3	6.85 $gL^{-1}H_2O$
$MnCl_2 \cdot 4H_2O$	0.86 $gL^{-1}H_2O$
$ZnCl_2$	0.06 $gL^{-1}H_2O$
$CoCl_2 \cdot 6H_2O$	0.026 $gL^{-1}H_2O$
5. Selenium H_2SeO_3	1.29 $mgL^{-1}H_2O$

Make up each stock solution separately and add

4. Nutrient solution

To prepare GSe medium, solution of nutrients is made up for 100mL mix.

Nitrate stock	20 mL
Phosphate stock	10 mL
Vitamin stock	10 mL
PII Metal Mix	50 mL
Selenium stock	10 mL

Make up to 200 mL with distilled water

Nutrient solution was autoclaved in Schott, then filter sterilised using 0.22 μm filter

5. To prepare final GSe medium

The following were added in sterile one litre Teflon bottle

900 mL sterile filtered sea water (1)
100 mL sterile distilled water
20 mL nutrient solution (4)

Modification of GSe medium

GSe medium without extracted soil was used in culture flasks. GSe(-N-P) was also used in this study that contains same amount of nutrients but without nitrate and phosphate.

BIOLOG GN2 Plate – Carbon compounds

BIOLOG

Gram Negative Identification Test Panel

GN2 MicroPlate™

A1 Water	A2 α-Cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galactosamine	A8 N-Acetyl-D-Glucosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-Inositol	B8 α-D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β-Methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono Methyl Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxybutyric Acid	D11 β-Hydroxybutyric Acid	D12 γ-Hydroxybutyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α-Keto Butyric Acid	E4 α-Keto Glutaric Acid	E5 α-Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromosuccinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 Glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Ornithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 γ-Amino Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenylethyl-amine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α-Glycerol Phosphate	H11 α-D-Glucose-1-Phosphate	H12 D-Glucose-6-Phosphate

Bacterial growth media

Zobell marine agar (ZM1)

Bacto Peptone	5 g
Yeast extract (Difco)	1 g
Bacto-Agar (omit for broth)	15 g
Sodium acetate	1 % w/v
0.2mm filtered sea water	750 mL
MilliQ water	250 mL

5 mL 100x Marine Supplement (see below)

100x Marine Supplement

10x Trace elements	1 mL
Na_2SeO_3 (2 mgL^{-1})	10 mL
MilliQ water	84 mL

Prepare and autoclave the above stock. Once cool add 5 mL 0.2 mm filter sterile 2x vitamin stock. Store in the dark at 4 ^oC.

10x Trace elements (100mL)

Na_2EDTA	4.36 g
$FeCl_3 \cdot 6H_2O$	3.15 g
$ZnSO_4 \cdot 7H_2O$	0.022 g
$CoCl_2 \cdot 6H_2O$	0.01 g
$MnCl_2 \cdot 4H_2O$	0.18 g
$NaMoO_4 \cdot 2H_2O$	0.0063 g

Combine and filter sterile. Store in the dark at 4 ^oC.

2x vitamin mixture (100 mL)

Cyanocobalamin (B_{12})	0.0005 g
Thiamine HCl (B_1)	0.1 g
Biotin	0.0005 g

Combine and filter sterile. Store in the dark at 4 ^oC.

Genomic DNA isolation from *G. catenatum* cultures.

Lysis Buffer
100 mM Tris-HCl pH 8.0
150 mM NaCl
10 mM EDTA

Lysozyme
50 mg ml^{-1} in Lysis Buffer

Add lysozyme to a sterile 15 mL tube and dissolve in the appropriate amount of sterile lysis buffer. 0.2 μm filter sterile and dispense 500-1000 μl volumes and store at -20 ^{o}C. Stock can be freeze/thawed a couple of before it needs to be checked.

CTAB/NaCl
0.7 M NaCl
10 % CTAB

Prepare stock by adding 4.1 g NaCl to a 200 mL bottle and dissolve in 80 mL MQ-dH_2O. Add a magnetic stir-bar and autoclave. While the solution is still hot or pre-heat to *ca*. 60-70 ^{o}C, progressively add small quantities of the 10 g CTAB (*ca*. 1 g) to the solution with stirring and heating to dissolve. Autoclave. Warm to *ca*. 60 ^{o}C before use, as it is very viscous.

1. Pellet cells from 1-5 mL *G. catenatum* by centrifugation at 13,000 x rpm for 15-20 sec in a sterile 1.5 mL centrifuge tube. Immediately remove as much of the supernatant as possible. Repeat to pellet cells.

2. Resuspend the cell pellet in 470 μl of lysis buffer. Add *ca*. 52 μl 50 mg mL^{-1} lysozyme in lysis buffer. Mix and incubate for 30 min at 37 ^{o}C.

3. Add 28 μl 10 % SDS (0.5 % final concentration) add 10 μl 20 mg ml^{-1} proteinase K (in 10 mM Tris-HCl, pH 8.0; 374 μg mL^{-1}). Mix and incubate at 56 ^{o}C for 30 min.

4. Add 70 μl 5 M NaCl and mix thoroughly (to make solution 0.7 M NaCl final concentration). Add 70 μl CTAB/NaCl solution (to 1 % CTAB). Mix and incubate at 65 ^{o}C for 10 min. Final volume at this point should be 700 μl.

5. Add an equal volume of Chloroform:Isoamyl alchol (24:1), mix by repeated inversion for 30 sec and centrifuge for 5 min at 13,000 x rpm.

6. Remove the aqueous phase to a new tube (the interface can be removed with a toothpick if it interferes with removal of the aqueous phase).

7. Add a equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1), mix by 30 sec inversion and centrifuge for 5 min.

8. Remove the aqueous phase to a new tube and add 0.6 vol. 100 % isopropanol alcohol and mix by inversion and incubate at room temperature for 15-30 mis. Collect precipitate by centrifugation at 13,000 x rpm for 15min. Wash once with 70 % ethanol.

9. Resuspend DNA in dH_2O at approximate 20-50 µl. Store at -20 ^oC. Quantitative DNA by fluorometry.

10. For the PCR, use 1 µl per 50 µl reaction with 2.0 mM Mg^{2+}.

CTAB DNA extraction protocol for bacterial genomic DNA (1.5 mL)

Reagents

1. 1x TE buffer
   10 mM Tris-HCl pH8.0
   1m M EDTA
2. 10 % SDS
3. 20 mg mL^{-1} Proteinase K
   Store at -20 ^{o}C
4. 5 M NaCl
5. CTAB/NaCl
   0.7 M NaCl
   10 % CTAB

Prepare stock by adding 4.1 g NaCl to a 200 mL bottle and dissolve in 80 mL MQ-dH_2O. Add a magnetic stir-bar and autoclave While the solution is still hot or pre-heat to *ca*. 60-70 ^{o}C, progressively add small quantities of the 10 g CTAB (*ca*. 1 g) to the solution with stirring and heating to dissolve. Autoclave. Warm to *ca*. 60 ^{o}C before use, as it is very viscous.

6. Chloroform:Isoamyle alcohol (24:1)
7. Tris buffer phenol. Store in the dark at 4 ^{o}C
   *Buffered phenol more then 2 months old can damage DNA
8. 100 % isopropanol alcohol. Store at -20 ^{o}C
9. 70 % Ethanol. Store at -20 ^{o}C

Method

1. Lable tubes and add 567 µL 1x TE buffer. Using an inoculating loop under sterile condition (laminar flow chamber, flame, etc.) remove a good streak of cell material from agar plate and suspend in TE buffer. Vortex suspension thoroughly until no clumps can be seen in the solution.

2. Add 30 µL 10 % SDS and mix thoroughly by shaking. Add 3 µl 20 mg mL^{-1} Proteinase K. Mix thoroughly by shaking and incubate at 56 ^{o}C for 60 min.

3. Add 100 µl 5 M NaCl and mix thoroughly. Add 80 µl CTAB/NaCl solution, mix by shaking and incubate at 65 ^{o}C for 30 min.

----------Perform steps 4-9 in FUMEHOOD----------

4. Add an equal volume of Chloroform:Isoamyle alcohol (24:1), mix thoroughly, hold cap on with finger, and centrifuge for 5 min at 13,000 x rpm.

5. Remove the aqueous phase (should be the top layer) to a new tube making sure to get none of the interface of or organic phase.

6. Add an equal volume of this buffered phenol to the aqueous phase, mix thoroughly,

holding cap on with finger, and centrifuge for 5 min at 13,000 rpm.

7. Remove the aqueous phase (should be the top layer) to a new tube making sure to get none of the interface or organic phase.
   Repeat step 4

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8. Remove the aqueous phase to a new tube and add 1:1 volume cold isopropanol alcohol and mix thoroughly by repeated inversion. A visible precipitate should form.

9. Remove supernatant by tipping out and wash pellet by adding 400 μL 70 % ethanol, making sure not to dislodge pellet. Centrifuge for 5 min at 13,000 rpm.

10. Remove supernatant. Resuspend DNA in 20-100 μL x10 TE buffer.

Store at -20 ^{o}C. Extracted DNA can be quantified for concentration or dilute approximately 1:10 with MQ H_2O; 2-5 mL of diluted DNA can be used in a 25 mL PCR reaction.