THE ROLE OF BACTERIA
IN
PARALYTIC SHELLFISH POISONING

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For when the One Great Scorer comes to write against your name.
He marks - not that you won or lost - but how you played the game.

Grant LandRice
1880 - 1954

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ABBREVIATIONS

A. affine	= *Alexandrium affine*
A. lusitanicum	= *Alexandrium lusitanicum*
A. tamarense	= *Alexandrium tamarense*
ASP	= Amnesic shellfish poisoning
bp	= base pairs (referring to DNA fragment sizes)
cAMP	= cyclic adenosine monophosphate
CCMP	= Culture Collection of Marine Phytoplankton
CE-MS	= capillary electrophoresis mass spectroscopy
CFB	= *Cytophaga/Flavobacter/Bacteroides* phylum
CFU	= colony forming units
DAPI	= 4'-6-diamidino-2-phenylidole
dc	= decarbamoyl toxin
DGGE	= denaturing gradient gel electrophoresis
dH_2O	= double deionised water
DNA	= deoxyribonucleic acid
dsDNA	= double stranded DNA
DSP	= Diarretic shellfish poisoning
EDTA	= ethylenediamine-tetraacetic acid
fmol	= femtomol
g	= grams
GDE	= genetic database environment
GTX	= gonyautoxin
h	= hours
HPLC	= high performance liquid chromatography
kb	= kilobase (referring to molecular markers)
LMP agarose	= low melting point agarose
mM	= millimolar
mnb	= mouse neuroblastoma assay
MU	= mouse unit
NEPCC	= North East Pacific Culture Collection

ng	= nanograms
nm	= nanometers (wavelength)
nM	= nanomolar (toxicity)
NeoSTX	= neosaxitoxin
NRC	= National Research Council (Canada)
NSP	= Neurotoxic shellfish poisoning
OD	= optical density
osc	= oscilliation
oub	= ouabain
PCC	= Plymouth Culture Collection
PCR	= Polymerase Chain Reaction
pen	= penicillin
P. lima	= *Prorocentrum lima*
pmol	= picomoles
ppt	= parts per thousand
PST	= paralytic shellfish toxin
PSP	= paralytic shellfish poison or paralytic shellfish poisoning
RDP	= ribosomal database project
RFLP	= restriction fragment length polymorphism
RNA	= ribonucleic acid
rDNA	= ribosomal DNA
rRNA	= ribosomal ribonucleic acid
SCB	= sodium channel blocking
sd	= standard deviation
SDS	= sodium dodecyl sulphate
SEM	= standard error of the mean
S. trochoidea	= *Scrippsiella trochoidea*
SSU	= small subunit (referring to 16S rDNA)
strep	= streptomycin
STX	= saxitoxin
TAE	= tris-acetate EDTA buffer

Taq	= *Thermus aquaticus*
TE	= tris EDTA buffer
TEM	= transmisson electron microscope
TEMED	= N, N, N',N' - Tetraethylethylenediamine
T_m	= melting temperature of DNA
TTX	= Tetrodotoxin
U	= units (referring to restriction endonuclease *Hae* III and Taq polymerase)
u.v.	= Ultraviolet
UW	= University of Westminster Culture Collection
V3	= variable region three of the 16S genome
ver	= veratridine
vol vol^{-1}	= volume per volume
wt vol^{-1}	= weight per volume

SUMMARY

Historically the production of paralytic shellfish toxins (PST), has been attributed to dinoflagellates. However, in the last decade, increasing evidence has been presented to indicate the involvement of a wide range of bacterial species including cyanobacteria and heterotrophic bacteria (Gallacher *et al.*, 1997). Several studies investigating bacteria capable of PST production, have identified bacteria associated with dinoflagellates are capable of autonomous PST production (Gallacher *et al.*, 1997). However, more recent research has focussed on the effects of these bacteria on toxin production by dinoflagellates, for which the production of bacterial-free (axenic) cultures is essential to identify whether dinoflagellates are capable of autonomous toxin production, in the absence of bacteria.

Many different methods to produce axenic algal cultures have been published, including washing methods and the addition of bacteriolytic compounds (Guillard, 1973; Singh *et al.*, 1982; Kim *et al.*, 1993; Doucette and Powell, 1998). However, efforts to generate axenic dinoflagellate cultures, have been hampered not only by difficulties in removing associated bacteria, but also by the lack of effective methods for assessing the presence of certain bacteria. Traditionally, the absence of bacterial growth on marine media was considered acceptable proof for axenic status. However, as the numbers of bacteria determined by culture methods falls short of numbers detected using microscopy (Akagi *et al.*, 1977), culture methods alone have been deemed inadequate to determine the axenic status of algal cultures.

In this study, the production of an axenic dinoflagellate culture was vital, firstly, to assess the effect on dinoflagellate toxin production following removal of all associated bacteria, and secondly, to identify whether original toxicity was restored when the microflora was replaced. Methods to assess the axenic nature of cultures combined traditional methods of culturing, with epifluorescence microscopy, the method now frequently relied upon for axenic confirmation. However, molecular techniques were also included, which allowed the axenic status of dinoflagellate cultures to be confidently determined.

The availability of molecular techniques also enabled an assessment of the bacterial diversity associated with original dinoflagellate cultures to be conducted, with culture-based and non culture-based identification systems adopted. This investigation indicated that a diverse range of bacteria were associated with cultures, although discrepancies between the two detection methods were noted.

Results from the assessment of axenic dinoflagellate cultures confirmed the need for molecular methods, as bacterial DNA was identified in cultures which were considered axenic cultures using media assessment and epifluorescence microscopy. Nevertheless, an axenic dinoflagellate culture was generated allowing further studies to investigate the influence of bacteria on dinoflagellate PST profiles. Previous data indicated two different theories exist regarding bacterial influence on dinoflagellate toxicity, with data indicating bacteria influenced the production of PST by dinoflagellates (Kodama, 1990; Doucette, 1995; Franca *et al.*, 1995; Gallacher *et al.*, 1997). Although other researchers including Ishida *et al.*, (1997) and Dantzer and Levin, (1997) concluded no bacterial involvement in dinoflagellate PST production, with PST production being an inherited characteristic of dinoflagellate cells.

Therefore, investigations attempting to assess the influence of bacteria on dinoflagellate PST production, were conducted which compared the growth and toxin profiles of original and axenic cultures, and also assessed these parameters following the introduction of various bacterial microflora. These investigations indicated bacteria do play a role in influencing dinoflagellate toxicity, with different toxin profiles detected in axenic cultures compared to original cultures. However, re-introduction studies also indicated toxin profiles were altered whether bacteria from a toxic or non toxic dinoflagellate were introduced.

As mentioned above, evidence for bacterial production of PST and their ability to affect dinoflagellate toxin profiles already exists. However, the role of these bacteria in shellfish toxicity remains unelucidated even though reports of shellfish toxicity in areas devoid of toxin-producing dinoflagellates exist, indicating another source of

PST can be responsible for shellfish toxicity (Kodama and Ogata, 1988).

Nevertheless, only one study has been published attempting to identify the ability of shellfish to assimilate PST-producing bacteria (Gallacher and Birkbeck, 1993). This experiment showed *Mytilus edulis* became toxic following exposure to SCB-producing bacteria, with toxicity detected within three hours. However, the investigation left certain issues unaddressed including the minimum length of exposure time required before toxicity was detected, and whether varying the SCB-producing strain and level of inoculum had an effect on the levels of toxicity attainable.

Therefore, experiments addressing these issues were carried out. Results indicated toxicity could be detected in *Mytilus edulis* following only one hour exposure time to bacteria, with the quantities of bacteria used having a marked effect on the levels of toxicity detected. However, the levels of toxicity were below those commonly detected in the environment. Nevertheless, the study indicated bacteria could be a potential source of toxicity during PSP outbreaks, and should be investigated further.

G.L. Hold, 1999

CHAPTER 1 : INTRODUCTION

PARALYTIC SHELLFISH POISONING

Historical background

Reports of food poisoning caused by the consumption of shellfish have been documented for centuries, with one of the earliest events recorded in the writings of Captain George Vancouver (1793). One of his crew died and several others became ill after eating toxic mussels (Waldichuk, 1990), with the description of symptoms presented by sufferers typifying those of marine biotoxins, specifically paralytic shellfish poisoning (Kao, 1986).

The marine biotoxins, collectively known as phycotoxins, are a diverse group of biologically active compounds which accumulate in filter-feeding shellfish. The four toxin groups currently recognised are: amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP) and paralytic shellfish poisoning (PSP). PSP is the most potent, causing problems worldwide.

Structure of PSP toxins

Paralytic shellfish toxins (PST), consist of more than 20 naturally occurring derivatives (Fig. 1.1; Franco and Fernandez-Vila, 1993) of which saxitoxin (STX) is the parent toxin (Hall *et al.*, 1990; Levin, 1991). The toxins can be separated, according to their structure, into 3 groups with carbamate toxins being the most potent, ranging from 2234 - 673MU[A1] $\mu mole^{-1}$, the sulfocarbamoyl toxins being the least toxic, ranging from 350 - 18MU $\mu mole^{-1}$, with the decarbamoyl derivatives having intermediate toxicity (Levin, 1991; Kao, 1993; Table 1.1). The main structural difference between toxin groups occurs at the R4 position, with each toxin

[A1] 1Mouse unit of saxitoxin (STX) = "amount of toxin which will kill a 20g mouse in 15 minutes when 1ml of acidified mussel tissue extract is injected intraperitoneally." 1MU = 200ng STX (Fileman, 1988) or 180ng STX (Waldock *et al.*, 1991).

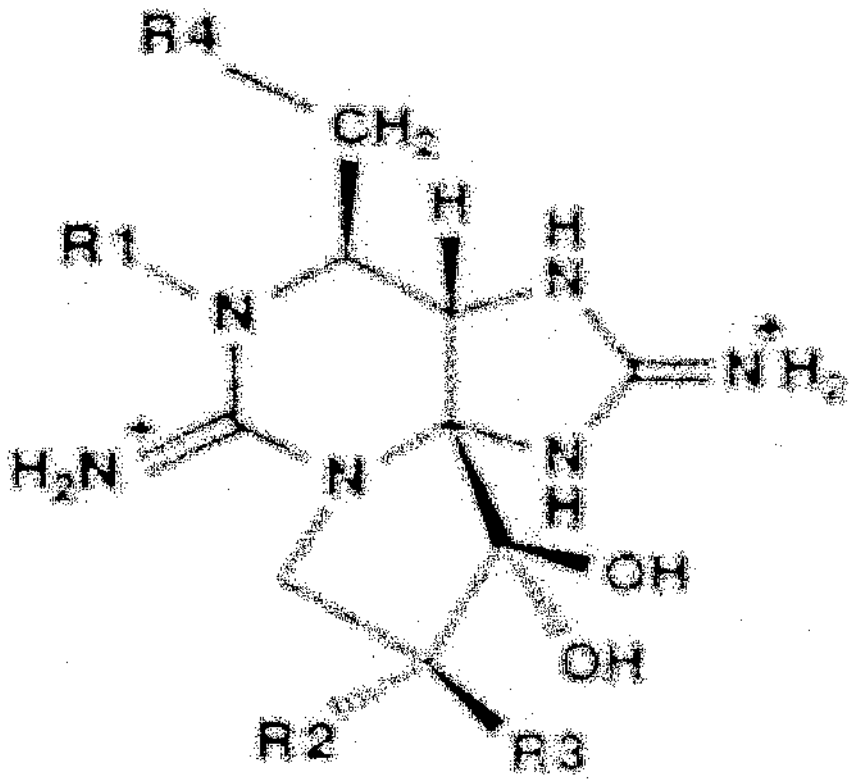


Figure 1.1 Structure of STX backbone. Natural derivatives have substitutions at R1 - R4. Substitution considerably modifies the individual potency of each toxin (see Table 1.1). Adapted from Franco and Fernandez-Vila (1993).

Toxins	Toxicity	Nature of substituent group:			
	(MU $\mu mole^{-1}$)	R1	R2	R3	R4
Carbamate					
STX	2045	H	H	H	$CONH_2$
neoSTX	1038	OH	H	H	$CONH_2$
GTX 1	1638	OH	H	OSO_3	$CONH_2$
GTX 2	793	H	H	OSO_3	$CONH_2$
GTX 3	2234	H	OSO_3	H	$CONH_2$
GTX 4	673	OH	OSO_3	H	$CONH_2$
Sulfocarbamoyl					
GTX 5	350	H	H	H	$CONHSO_3$
GTX 6	180	OH	H	H	$CONHSO_3$
C 3	18	OH	H	OSO_3	$CONHSO_3$
C 1	16	H	H	OSO_3	$CONHSO_3$
GTX 8	43	H	OSO_3	H	$CONHSO_3$
C 4	57	OH	OSO_3	H	$CONHSO_3$
Decarbamoyl					
dcSTX	1220	H	H	H	H
dcneoSTX	-*	OH	H	H	H
dcGTX 1	-	OH	H	OSO_3	H
dcGTX 2	530	H	H	OSO_3	H
dcGTX 3	990	H	OSO_3	H	H
dcGTX 4	-	OH	OSO_3	H	H

Table 1.1 Structure and specific toxicity of Paralytic Shellfish Toxins. Extracted and adapted from Franco and Fernandez-Vila, (1993).

* - = not determined

within groups differing at positions R1, R2 and R3. Saxitoxin has an LD_{50} of 10µg kg^{-1} in mice (intraperitoneal injection; Kao, 1986), which has been extrapolated to suggest a concentration of 120µg saxitoxin equivalents kg^{-1} body weight causing human illness and a lethal dose of 400-1060 µg kg^{-1} (Shumway, 1995).

In humans, PST interfere with voltage-gated sodium channels by binding to receptor sites, blocking the uptake of sodium ions into cells (Baden and Trainer, 1993). Sodium channels operate by depolarisation of cell membranes which leads to a conformational change in the molecule allowing sodium ions entry into cells. By binding to sodium channels, PST prevent the influx of ions therefore decreasing the cell's action potential, which in turn interferes with neural and muscular functions (Hall *et al.*, 1990). The difference in potency of the toxins is due to variation in their affinity for the receptor in the sodium channel α subunit transmembrane protein (Catterall, 1985), with substitution of N-1 or C-11 resulting in a lower toxicity, e.g. with sulfocarbamoyl toxins (Table 1.1; Shimizu, 1987).

As a result of their pharmacological action, PST can be described as sodium channel blocking (SCB) toxins and are grouped along with another group of SCB toxins, tetrodotoxin (TTX) and related compounds, which are responsible for puffer fish (*Tetradontiformes*) poisoning. Shellfish are not affected by PST, as nerves and muscles of shellfish are operated by voltage-gated calcium channels (Kao, 1993).

DINOFLAGELLATES AS A SOURCE OF TOXINS

Historically, as a result of work which suggested that mussels became toxic upon feeding on nearby dinoflagellates (Sommer *et al.*, 1937), PST in shellfish was attributed to the presence of these organisms. Subsequently, outbreaks of PSP were and still are commonly associated with blooms of toxic dinoflagellates.

In most temperate and cold waters, the dinoflagellates associated with PSP outbreaks belong to the genus *Alexandrium* (previously known as *Protogonyaulax* or

Gonyaulax; Shumway *et al.*, 1995). However, species of other genera, including *Gymnodinium* and *Pyrodinium*, have also been implicated. In recent years, the frequency of occurrence and geographic locations of these dinoflagellate blooms around coastal areas have increased dramatically. This has led to theories of changing environmental conditions, e.g. increased marine pollution and global warming triggering their production (Anderson, 1989; Iwasaki, 1989; Sinderman, 1990; Levin, 1991). However, non-toxic dinoflagellates of the same species as toxic isolates, e.g. *A. tamarense*, also occur and the presence of such dinoflagellates *per se* does not indicate a public health risk.

Toxin profiles of dinoflagellates

Saxitoxin was the first of the PST to be detected in dinoflagellates (Schantz, 1986) but it is usually a minor component in most *Alexandrium* species, with gonyautoxins 2 and 3 (GTX 2 and GTX 3) predominantly present (Shimizu, 1987). These sulphate esters are epimers and interchange freely between the two forms (Laycock *et al.*, 1994). However, GTX 3 is the more stable and toxic form, having a specific activity equivalent to saxitoxin (Table 1.1; Boyer *et al.*, 1987). The other gonyautoxins commonly found are GTX 1 and 4, which are also epimers, with GTX 1 being the more toxic (Table 1.1).

Individual dinoflagellate species are not associated with all of the PST, the combination and potency varies depending on the geographic site and environmental conditions. The more potent carbamate toxins predominate in dinoflagellates from northern latitudes, whilst sulphamate toxins are present in the highest proportions in southern strains (Oshima *et al.*, 1990). It has been suggested that differences in toxin profiles may be due to higher temperatures in southern regions allowing the conversion of carbamate toxins to the less potent sulphamate derivatives. (Anderson *et al.*, 1990). However, previous work by Hall and Reighart (1984) indicated that sulfocarbamoyl derivatives were easily transformed to carbamate toxins when heated at low pH.

It is recognised that dinoflagellate toxicity varies both between different isolates of a species and, as mentioned previously, both toxic and non-toxic isolates of the same species can co-exist (Maranda *et al.*, 1985; Cembella *et al.*, 1988). However, differences have also been detected between individual isolates of the same species, under varying growth conditions (Anderson *et al.*, 1990; Cembella *et al.*, 1990). It has also been reported that sub-clones of *A. tamarense* derived from a single clonal cell differ in toxicity (Ogata *et al.*, 1987). It was therefore, suggested that PST production was not a hereditary characteristic (Ogata *et al.*, 1987).

Relationship between dinoflagellate and shellfish toxin profiles

The toxin profile of shellfish collected from bloom areas often differs from that of the dominant dinoflagellate species present in the surrounding seawater. Reasons suggested for this include:-
i) selective excretion by the shellfish (Hall, 1985); ii) selective concentration within certain tissues (Oshima *et al.*, 1990) and iii) chemical conversion, possibly due to altered conditions such as pH, temperature, enzymatic transformation, or the presence of bacteria (Oshima, 1995).

Oshima and co-workers (1995) incubated GTX 2 and GTX 3 with crude enzyme extracts from *A. tamarense*, which resulted in the transformation of these toxins to GTX 1 and GTX 4, which they suggested indicated the presence of an oxidase in the extract (Oshima, 1995). This conversion was not detected in another dinoflagellate species, *Gymnodinium catenatum* but when C1 and C2 toxins were incubated with *G. catenatum*, conversion to the more potent GTX 2 and GTX 3 was noted, which was not apparent when *A. tamarense* extract was tested.

The conversion of toxins has also been reported in contaminated scallops, mussels and oysters (Oshima *et al.*, 1990). Similar conversions by bacteria, identified as *Vibrio* and *Pseudomonas* spp., isolated from the tissues of turbot (*Scophthalmus maximus)* and coral reef crabs, (*Atergatis floridus*) have also been reported (Kotaki

et al., 1985a,b). Interestingly, strains of the above genera have also been shown to be capable of TTX production (Noguchi *et al.*, 1986; Hwang *et al.*, 1990; Tamplin, 1990).

THE ASSOCIATION BETWEEN ALGAE AND BACTERIA

Bacteria are universally associated with algae in seawater and laboratory cultures (Berland *et al.*, 1970; Bell & Mitchell, 1972; Tostesen *et al.*, 1989), with algae having the ability to exert an influence over their bacterial population (Sieburth, 1968; Cole, 1982). This ability is thought to be possible due to the presence of cyclic adenosine monophosphate (cAMP), which may act as a metabolic cue in algal/bacterial interactions (Ammerman & Azam, 1981). The production and release of substances in the form of mucilaginous exudate from algae, capable of selectively supporting or eliminating bacterial species has been noted (Sieburth, 1968; Cole, 1982). However, bacterial remineralization, synthesising additional compounds beneficial to algal growth, including vitamin B_{12}, also represents a major supply of nutrients for algae, indicating the association is a two-way process (Pringsheim, 1912; Ericson and Lewis, 1953; Golterman, 1972; Haines and Guillard, 1974; Bloesh *et al.*, 1977; Singh *et al.*, 1982; Tostesen *et al.*, 1989).

Differences have also been noted in the expression of certain microbiological characteristics and cellular functions by bacteria, when attached to particles such as algae. For instance, progeny from attached bacteria, have been shown to be unable to release hydrolytic enzymes into the environment until becoming attached to a surface (Azam & Cho, 1987; Cooksey & Wigglesworth-Cooksey, 1995; Rath *et al.*, 1998).

There are several reports detailing bacterial diversity under natural algal bloom conditions, with Bell *et al.* (1974), and Romalde *et al.* (1990a,b), showing diversity and abundance to be low early in bloom development. Bell *et al.* (1974), also reported an increase in certain bacterial groups later in the bloom, indicating that

bacterial diversity changed within the environment. Although, there are reports which show that bacterial numbers increase through the course of a bloom (Zobell, 1963; Wood, 1963; Vaccaro *et al.*, 1968; Bell & Mitchell, 1972; Buck & Pierce, 1989; Romalde *et al.*, 1990a,b), other researchers have reported that the numbers decrease (Riquelme *et al.*, 1987,1989; Romalde *et al.*, 1990a). However, these apparently conflicting results were compiled using different algal species.

Along with environmental studies investigating microflora numbers, a few studies detailing changes in algal microflora have also been done in the laboratory. Findings by Nelinda *et al.* (1985) showed that the bacterial species composition of toxin producing dinoflagellates in culture varied as the level of toxicity produced by individual strains altered. Unfortunately, identification of the bacterial species present was limited to assessment of morphological criteria. However, Nelinda *et al.* (1985) also showed that *Alexandrium* cultures could influence their associated bacterial microflora, with this ability differing from one culture to another in a manner apparently unrelated to PST production.

This ability of different isolates of the same dinoflagellate species to allow different bacteria to predominate was also noted in laboratory cultures of *Ostreopsis lenticularis* and *Gamberdiscus toxicus* (Tostesen *et al.*, 1989), and with *Alexandrium tamarense* (Gallacher *et al.*, in preparation). Tostesen and co-workers used biochemical methods to classify associated bacteria, with Gallacher *et al.* combining biochemical analysis with 16S rDNA sequence data to identify the bacterial species present.

The use of molecular methods to infer phylogenetic diversity within a bacterial community

Until recently, studies of microbial communities associated with PST-producing dinoflagellates, have relied on morphology and/or biochemical techniques to infer diversity (Nelinda *et al.*, 1985; Tostesen *et al.*, 1989; Romalde *et al.*, 1990a,b).

However, this, in many cases, has not allowed the actual bacterial species composition of algal cultures to be determined. Reasons for this include the limited species-specific morphological variety amongst prokaryotes (Rappé *et al.*, 1998), and difficulties in identifying conditions for proliferation of isolates, in order to carry out techniques capable of inferring diversity (Ferguson *et al.*, 1984; Parkes *et al.*, 1990; Ward *et al.*, 1992; Wagner *et al.*, 1993 Amann *et al.*, 1995; Rappé *et al.*, 1998).

Several molecular biology techniques, which do not require isolation of bacterial strains, have become increasingly popular in community analysis, providing powerful adjuncts to culture-dependent techniques, and are now frequently used to detect and characterise natural communities (Muyzer and Ramsing, 1995). One such approach, coupling polymerase chain reaction (PCR) with rRNA-based phylogeny, has become effective in the exploration of microbial environments and the identification of uncultured organisms. Studies applying this approach have shown that gene sequences amplified directly from environmental DNA do not correspond to the genes of cultured isolates (Suzuki *et al.*, 1997). These results, support the hypothesis that the most abundant marine bacteria are not readily culturable by commonly used methods, and would therefore not be identified using traditional techniques. However, another explanation suggesting that marine bacteria can be easily cultured, but are not well represented in sequence databases, has also been put forward. This would indicate that microbial cultivation has not yet been employed exhaustively for determining taxonomic identities and distributions of bacteria.

Nevertheless, recognition of the biases associated with microbiological cultivation techniques (Rosswall and Kvilner, 1978; Brock, 1987; Wayne *et al.*, 1987; Ward *et al.*, 1990), has seen a marked shift to reliance on PCR-amplification of samples. This is usually followed by cloning or direct sequencing of 16S genes from naturally occurring microbial assemblages as a means of assessing diversity (Olsen *et al.*, 1986; Ward *et al.*, 1992; Giovannoni *et al.*, 1995). However, it is accepted that molecular techniques also suffer from biases and these are discussed later in this chapter.

The use of rRNA genes for characterising bacterial communities

Although many genes may be used as a genetic marker, rRNA genes offer distinct advantages. The extensive use of the 16S rRNA for studies of microbial systematics and evolution has resulted in large computer data bases such as the Ribosomal Database Project (RDP; Maidek *et al.*, 1994). Also, as rRNA genes are a mosaic of conserved and variable regions, they can be used to examine distant phylogenetic relationships with accuracy and also allow specific target sites for probes and PCR primers, to be designed (Britschgi and Giovannoni, 1991; Lane, 1991; Muyzer and Ramsing, 1995; Wheeler *et al.*, 1996).

Among the first environments to be studied using such molecular methods were oceanic habitats. Studies characterising bacterioplankton populations from many different locations (Delong *et al.*, 1993; Fuhrman *et al.*, 1993), led to two general conclusions:- 1) the vast majority of 16S rDNAs retrieved from natural, mixed population samples did not correspond to gene sequences obtained from cultured bacteria (see above, Giovannoni *et al.*, 1995); 2) although phylogenetically diverse, most sequences fell into a few distinct phylogenetic groups (Fuhrman *et al.*, 1993; Mullins *et al.*, 1995; Giovannoni *et al.*, 1996).

However, as with studies relying on culture, PCR-based studies of phylogenetic diversity are also subject to inherent errors, biases and artifacts (Liesack *et al.*, 1991; Reysenbach *et al.*, 1992; Amann *et al.*, 1995; Suzuki and Giovannoni, 1996; Wang and Wang, 1996). Biases include the potential creation of chimeric molecules during amplification (Liesack *et al.*, 1991; Robinson-Cox *et al.*, 1995; Wang and Wang, 1996), over representation of specific groups as a function of increasing PCR cycle number (Suzuki and Giovannoni, 1996), and under representation due to primer mismatches. Despite these problems, the usefulness of PCR for microbial diversity studies is still apparent (Giovannoni *et al.*, 1990; Fuhrman *et al.*, 1992). However, although the biases associated with molecular methods are not yet completely understood, they appear less limiting than those associated with culture-based

methods (Ward *et al.*, 1992; Giovannoni *et al.*, 1995).

Cloning techniques were initially used to determine genetic diversity of microbial communities and to identify uncultured microorganisms (Giovannoni *et al.*, 1990; Ward *et al.*, 1990; Britschgi *et al.*, 1991; Weller *et al.*, 1991). Research using this technique has examined DNA from various environments including hot spring cyanobacterial mats, and seawater samples, (Giovannoni *et al.*, 1990; Ward *et al.*, 1990). However, these initial techniques proved costly, labour intensive, and time consuming, allowing only limited samples to be analysed completely, and were potentially biased due to limitations including differences in clone libraries obtained from identical target DNA when experimental parameters were altered. Another technique, using probes for dot-blot hybridisation of extracted rRNA, was also used, however, this method only generated information on species specifically targeted by the probe sequence from within the community. It was, therefore, important to develop a method for analysing multiple samples by resolving the diversity of amplified products in a single electrophoretic profile. Of these methods, denaturing gradient gel electrophoresis (DGGE; Muyzer *et al.*, 1993; 1995; Wawer and Muyzer 1995) and restriction fragment length polymorphism based analyses (RFLP; Martinez-Murcia *et al.*, 1995) have been used successfully and are relatively straightforward.

The use of restriction fragment length polymorphism analysis for characterising culturable isolates from a community

RFLP analysis of cultured bacterial isolates, has been used previously to group representatives from marine environments (Suzuki *et al.*, 1997). The method described by Suzuki, utilises PCR to amplify a region of the 16S gene, which is then subjected to digestion using a restriction endonuclease such as *Hae* III, with subsequent RFLP profiles resolved using electrophoresis. It is an effective way of combining colony morphology with limited molecular information, in order to reduce the number of isolates requiring further identification using 16S rDNA sequencing.

However, it is not a method used frequently for such analysis, as usually researchers do not pay particular attention to individual isolates within a community, and are more interested in the major bacterial subclasses present. Nevertheless, Ishida and co-workers used RFLP and 16S rRNA sequencing to characterise bacteria which were isolated from the rapidophyte *Heterosigma carterae* (Ishida *et al.*, 1997). Analysis of sequence data indicated bacteria belonging to the *Cytophaga* class and γ-proteobacteria subdivision were present.

The use of denaturing gradient gel electrophoresis in community analysis

Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene segments is a rapid and reproducible genetic fingerprinting technique, which has been used to profile complex microbial communities (Muyzer *et al.*, 1993; Kowalchuk *et al.*, 1997; Ovreas *et al.*, 1997). The system has also been used to infer the phylogenetic affiliation of community members (Muyzer & de Waal, 1994; Muyzer *et al.*, 1995). The most commonly investigated regions of DNA to date, have been 16S gene sequences, although more recently, functional gene sequences including analysis of [NiFe] hydrogenase gene fragments have been utilised (Wawer and Muyzer, 1995).

Initially, the system used PCR to amplify a region of DNA from a mixed population. This allowed 50% of all sequence variation to be detected when normal length primers were used. However, the sensitivity of the detection system was increased, by the addition of a GC-rich sequence (GC clamp) to the 5' end of one of the primers, to impart melting stability to PCR products within complex samples, allowing almost 100% of sequence variations to be detected (Myers *et al.*, 1985). During the analysis, individual double-stranded DNA (dsDNA) molecules from a mixed sample denature along their length (adjacent to the GC clamp) according to their melting characteristics. This allows the complex sample to separate into discrete bands during electrophoresis through an acrylamide gel containing an increasing linear gradient of denaturant, so different PCR products can be excised and their nucleotide

sequences determined (Muyzer & de Waal, 1994; Muyzer *et al.*, 1995).

The system has been used successfully for many diverse applications including determination of genetic diversity of hydrothermal vent microbial communities (Muyzer *et al.*, 1995), biodegraded wall paintings (Rölleke *et al.*, 1995), and sulphate-reducing bacteria (Teske *et al.*, 1995). However, use of DGGE to characterise marine environments is not well documented, and no published reports using the system to identify bacteria associated with algae exist. Recently, Murray *et al.* (1996), used DGGE of PCR-amplified 16S rDNA fragments, to compare the phylogenetic diversity of bacteria from two estuaries. The two environments were found to be different in composition by comparison of DGGE profiles, although identification of individual morphotypes was not attempted.

However, DGGE analysis is not without limitations, as the degree of separation between PCR products can vary. This has already been shown to be a problem in complex bacterial communities in soil (Torsvik *et al.*, 1990). Furthermore, only limited sequence information can be obtained using DGGE, as separation of fragments longer than 500 base pairs is not currently possible (Muyzer and Ramsing, 1995). Therefore, techniques using cloning, or relying on culture, which allow greater sequence information to be generated, cannot be considered obsolete, although restrictions and biases due to each system must be acknowledged.

THE ROLE OF BACTERIA IN DINOFLAGELLATE TOXIN PRODUCTION

The issue of bacterial involvement in dinoflagellate toxin production was first proposed over three decades ago (Silva, 1962). Two different arguments have been proposed regarding bacterial involvement. These are, firstly, autonomous bacterial synthesis of PST and, secondly, the ability of bacteria to affect (directly or indirectly) levels of toxicity associated with dinoflagellate cells.

Bacterial production of PST

A bacterial origin of PST production was first suggested by Silva (1979, 1982), when bacteria-like particles were detected within toxin-producing dinoflagellate cells. Silva also indicated that bacteria isolated from toxin-producing cells, whilst not producing detectable levels of toxin, were able to elicit toxicity when introduced into previously non-toxic dinoflagellate cultures. Silva concluded that "the intracellular bacteria interfered with the dinoflagellate metabolism and must therefore be the main cause of their toxicity." Confirmation of some of Silva's work has been reported by Kodama *et al.* (1989) who used transmission electron microscopy (TEM) to demonstrate the presence of rod-shaped bacteria within strongly-toxic dinoflagellate cells, but not within non-toxic cultures. These results were later discredited by Taylor (1990), who indicated that the TEM results did not clearly show the presence of bacteria within the cells. Intensive research by other groups has also been unable to demonstrate bacteria within cells of toxic *Alexandrium* spp. (Rees and Hallegraff, 1991; Franca *et al.*, 1993), with earlier findings by Nelinda *et al.* (1985), showing that bacteria appeared to be "quite common in the nucleus of non-toxic dinoflagellates."

Kodama *et al.*, (1988) subsequently claimed to have detected PST in intracellular bacteria from toxic dinoflagellates including *A. tamarense*. The bacteria isolated by Kodama, were shown to produce PST, with STX being the main toxin found, although the dinoflagellate culture from which it was isolated produced predominantly GTXs. However, an increase in toxin production by the bacteria was found when grown in nutrient-depleted media, with an altered toxin profile also detected comprising mainly GTXs.

Increasing evidence showing that heterotrophic bacteria, both free living and associated with dinoflagellates, are capable of autonomous production of PST has been published by several groups (Kodama & Ogata, 1988; Kodama, 1990; Kodama *et al.*, 1990a; Doucette, 1995; Franca *et al.*, 1995; Gallacher *et al.*, 1997). The idea that dinoflagellate toxicity is due to symbiotic bacteria has been used to explain a

variety of circumstances, including why different blooms of the same species produce different combinations of toxins, why variations in toxicity have been observed within a single geographical region, and why considerable variation in toxicity exists between clonal and subclonal cultures grown under the same conditions (Ogata *et al.*, 1987; Kodama *et al.*, 1990b).

Bacterial effects on algal toxicity

Several workers have investigated the role of dinoflagellate-associated bacteria in toxin production. Kodama & Ogata (1988) found that the toxicity of *A. tamarense* and *Pseudonitzschia brevis* increased when cultured under axenic (bacterial-free) conditions, whereas Tostesen *et al.* (1989) reported the toxicity of *Gymnodinium veneficum* to reduce. Singh *et al.* (1982), and Boczar *et al.* (1988), showed axenic *A. tamarense* cultures to produce normal levels of PST, thus concluding that bacteria have no direct involvement in PST production. However, the methods used by Singh *et al.* (1982) and Boczar *et al.* (1988), to assess the bacterial status of these axenic cultures were very limited. Singh *et al.* (1982), also noted that axenic cultures grew more slowly, never reaching the density of untreated cultures. Even after compensating for reduced cell density, it took twice the time for axenic cultures to reach the stationary phase of growth. This retarded growth and density of the axenic cultures, Singh noted to be due to a nutrient deficiency in the media, usually compensated for by bacteria. Conversely, Douglas *et al.* (1993) showed that non-axenic domoic-acid-producing *Pseudonitzschia* cultures remained viable for 2 - 3 weeks longer than axenic cultures, generated using antibiotics, with lower toxin levels detected in axenic cultures compared to normal cultures. However, growth of the axenic cultures in the presence of Tris buffer produced domoic acid levels comparable with non-axenic cultures. Growing the normal culture in the presence of Tris buffer had no effect on the levels of toxicity produced until stationary phase after which time an increased domoic acid level was detected.

The effects of re-introducing bacteria to axenic dinoflagellate cultures

The majority of work investigating the role of bacteria in toxin production has concentrated on removing bacteria from toxin-producing diatom and dinoflagellate cultures, with only limited investigations monitoring changes in toxicity when bacteria are re-introduced to axenic cultures.

Only one bacterial re-introduction investigation, using *Alexandrium* cultures, has been published. Doucette and Powell (1998) introduced bacteria, isolated from a toxic *A. tamarense* culture, to axenic cultures of a toxic *A. lusitanicum* isolate, and noted toxicity levels were restored to those detected in the original culture. This ability to alter toxin levels in dinoflagellate cultures was explained by bacterial adhesion, a phenomena which also seemed to be species specific. Using diatom cultures, Bates *et al.* (1993) re-introduced bacteria to toxic *Pseudonitzschia* cultures, which had previously been treated with antibiotics to remove associated bacteria. Although no comparison was made between toxicity of the original culture and the axenic culture, the toxicity of the axenic culture increased 2 to 95 fold after introduction of bacteria, with no substantial effects on division rates or cell densities. Bates also introduced bacteria from a non-toxic diatom *Chaetoceros* species to the axenic *Pseudonitzschia* culture; this also increased toxin production dramatically, indicating that toxin production in bacteria, and their ability to influence their host's toxicity, were two mutually exclusive functions.

THE PRODUCTION OF AXENIC ALGAL CULTURES

The use of physical dissociation methods to produce axenic cultures

As stated above, several researchers claim to have produced axenic cultures and there are numerous methods published for producing bacteria-free algal cultures (Table 1.2). Early attempts tended to use physical dissociation techniques, including dilution-to-extinction of bacteria (Allen and Nelson, 1910), washing methods

TREATMENT	STERILITY TESTS USED	REFERENCE
Washing with diluent	limited range of media	Pringsheim, 1921, 1936, 1937; Chu, 1942; Droop, 1954; Singh *et al.*, 1982
Washing with diluent and dilution series	limited range of media, epifluorescence	Sako *et al.*, 1992; Kim *et al.*, 1993
Dilution to extinction	limited range of media	Allen and Nelson, 1910
Ultrasonication	microscopy	Brown and Bischoff, 1962
Heat and u.v. light	limited range of media, microscopy	Zobell and Long, 1938
Utilisation of vertical migration and phototactic behaviour	limited range of media, epifluorescence microscopy	Imai and Yamaguchi, 1994
Potassium tellurite and sonication	limited range of media, microscopy	Zobell and Long, 1938
Antibiotics	limited range of media	Spencer, 1952; Hoshaw and Rosowoski, 1973; Guillard, 1973
Antibiotics	limited range of media, epifluorescence microscopy	Douglas *et al.*, 1993
Washing with sterile seawater followed by SDS and lysozyme	limited range of media, epifluorescence microscopy	Doucette and Powell, 1997

Table 1.2 Summary of published methods for the production of bacteria-free algal cultures

(Pringsheim, 1921; 1936; 1937; Chu 1942), and ultrasonication (Brown and Bischoff, 1962). Work by Zobell and Long (1938), also investigated methods using heat and u.v. light, although without success. This work was instigated following earlier findings of Zobell & Allen (1935), which indicated that more than half of bacteria in seawater were attached to particles such as algae, and resisted removal by washing. Thus, for the first time, the effectiveness of attempts to produce axenic cultures was questioned.

Some of the early methods were also later criticised by Spencer (1952), who suggested that washing procedures would probably remove free-living bacteria, but not attached species. Droop (1954), also cast doubt on the dilution method (Allen and Nelson, 1910), by recognising that bacteria usually occur in higher numbers than algae, therefore, it would be impossible to dilute bacteria to extinction before losing all algal cells. However, Droop (1954) did have more success when he modified the washing method of Pringsheim (1946), but again indicated that algae must be free from attached bacteria before the washing method was successful. It has also been noted that certain algal species, including armoured dinoflagellate species, are irreversibly damaged by physical methods used to reduce bacterial contamination (J. Lewis, pers, comm.), thus requiring other techniques to achieve axenicity.

The use of chemicals in the production of axenic cultures

The first work involving bactericidal chemicals (Zobell and Long, 1938), used potassium tellurite to remove bacteria from algal cultures. However, success was limited, due to both the resistance of certain bacterial species and also the sensitivity of a number of algal cultures used to the chemical.

Antibiotics have been used routinely to remove bacteria from algal cultures since the 1950's, and this has become a standard technique for purifying algal cultures. The advantage of antibiotic treatments over washing methods is the ease of application, coupled with their effectiveness in removing bacteria from mucilagenous algal species

which cannot easily be separated from their accompanying microflora (Droop, 1967; Coler & Gunner, 1969).

Spencer (1952), used a combination of penicillin and streptomycin to remove bacteria from algal cultures and noted that the combination was effective in reducing most bacterial numbers, but not in removing moulds and bacterial species such as *Actinomycetes*. However, this combination is still widely used by researchers, with Douglas *et al.* (1993) using the same combination in studies of toxin production by marine diatoms. However, one of his cultures also required an additional antibiotic - gentamycin - to remove bacteria completely. Other workers have also used combinations of broad-spectrum antibiotics to produce axenic algal cultures (Hoshaw and Rosowski 1973; Guillard 1973). Both groups agreed with the original comments of Droop (1967), that different algal cultures require different combinations of antibiotics for effective removal of all associated bacteria. The most recently published data regarding the use of antibiotics to generate bacterial free toxin-producing dinoflagellate cultures (Dantzer and Levin, 1997) used high concentrations of penicillin to remove external bacteria from *Alexandrium* cultures. The results showed that penicillin successfully removed the associated bacteria, with subsequent lysis of antibiotic-treated dinoflagellate cultures not producing any bacterial growth when plated on agar medium.

The uncertainty of effects caused by the addition of antibiotics, and the ability of bacteria to become antibiotic resistant, led to the most recently published method for producing bacteria-free toxin-producing dinoflagellate cultures. Doucette and Powell (1998) exposed toxin-producing dinoflagellate cultures to a washing regime, followed by incubation with EDTA, lysozyme and sodium dodecyl sulphate (SDS), before a second washing stage. The bacterial status of cultures was assessed using epifluorescence microscopy and culture on marine media encompassing a range of nutrient levels.

One of the main criticisms of the techniques mentioned above is not the actual

methodology used to perform the treatments, but limitations of the methods used to assess their effectiveness. Initially, success of treatments was assessed by inoculating algal cultures on to media, with the absence of growth indicating an axenic culture. Unfortunately, the composition of media usually employed for this purpose was often selective for certain bacterial types, with concentrations of nutrients often unrealistically high compared to natural conditions (Azam & Ammerman, 1984; Nissen, Nissen & Azam, 1984).

Epifluorescence microscopy has become an invaluable tool for assessing the bacterial status of algal cultures, and has been included with added frequency in experiments since the 1970's. Bolch & Blackburn (1995) stated that the technique was more effective for assessing the axenic nature of algal cultures than the traditional plating methods. Epifluorescence analysis of algal cultures initially used DAPI and acridine orange was used to visualise bacteria associated with cultures (Hobbie *et al.*, 1977; Porter and Feig, 1980; Ferguson *et al.*, 1984). However, problems associated with autofluorescence of background detritus, and lack of clarity following sample preparation meant images were often not ideal for assessment of fine detail. Recently, more effective fluorescent stains which are capable of penetrating live algae and bacteria without the need to fix samples prior to analysis, such as Sybr Green 1 (Molecular Probes), have become available. This allows bacteria to be stained whilst still associated with live algal cells, rather than following the addition of a fixative such as formalin, which has been shown to alter cell structure (J. Lewis, pers. comm.).

Although molecular biology techniques have been used to detect and identify microorganisms within complex communities and to identify shifts in community structure due to external factors (Olsen *et al.*, 1986; Amann *et al.*, 1995), there are no reports of their use in assessing the axenic nature of algal cultures.

EVIDENCE FOR BACTERIA CONTRIBUTING TO SHELLFISH TOXICITY

Convincing evidence now exists to support bacterial production of PST (Gallacher *et al.*, 1997), however, their involvement with shellfish toxicity has not been elucidated.

MYTILUS EDULIS

Mytilis edulis, also known as the blue mussel, is a suspension feeding Lamellibranch, belonging to the family *Mytilidae* (Pelseneer, 1906). The anatomy of *Mytilis edulis* was first described by de Heide (1683), who demonstrated a ciliary system within the gill structure. Poli (1795) presented a more detailed dissection of the mussel, describing the passage of foodstuff through the gut. Later work showed that suspension-feeding bivalves such as *Mytilus edulis* obtain their food by retaining suspended organic particles from surrounding water. Inhalant currents brought about by lateral cilia beating across the length of the gill filament, allowing the movement of the food particles across the gills (White, 1937). The amount of food available to the bivalves is partly determined by the volume of water transported through the gills, and also by the efficiency with which particles are retained (Mohlenberg and Riisgard, 1978). Collected food particles such as phytoplankton, bacteria and detritus are bound in mucus strings, directed towards the food groove, onto the mouth and finally via the stomach to the digestive gland (Bernard, 1972). Large or unwanted particles are dropped onto the mantle surface and eliminated as pseudofaeces without entering the digestive system (White, 1937).

Factors affecting the filter feeding ability of *Mytilus edulis*

Many factors have been identified as having the ability to affect bivalve filter feeding efficiency. The age and size of bivalve shellfish appears to have a marked effect on filtration rates, with several authors showing that pumping rates are related to body

weight or shell length in many species, including *Mytilus edulis*, with smaller individuals pumping more rapidly per unit weight or length than larger individuals (Tsujii & Ohnishi, 1957; Rice & Smith, 1958; Theede, 1963; Coughlan & Ansell, 1964; Morton, 1971; Vahl, 1973b; Winter, 1969, 1973,1976, 1977).

Temperature is another factor which affects filtration (Theede, 1963; Winter, 1969; Dame, 1972; Widdows, 1973; Wilson & Seed, 1974; Schulte, 1975; Bayne *et al*., 1976), with filtration rates in *Mytilus edulis* increasing as temperature rises to an optimum level of 18°C. Salinity also has an effect, with Renzoni (1963) and Theede (1963) concluding the optimum salinity for bivalve filtration was that of their natural habitat. However, an optimum salinity of 34ppt was recorded by Wilson & Seed (1974), with no filtration seen below 15ppt or above 50ppt.

The sexual stage of the animal may also have a great effect on its ability to remove particulate matter from suspension. Dodgson (1928) observed that under similar temperature conditions, mussels cleared water more rapidly in September-October than in February-March and considered this may be due to gonad development which usually occurs in spring. However, the relative quantities of food present in seawater during the two periods were not taken into consideration. Conversely, Theede (1963) showed that filtration rates in *Mytilus edulis* were higher in spring than in late summer at comparable temperatures, although more particulate matter was present in the summer water.

The actual presence or absence of a food source is also a limiting factor for filtration, with investigations indicating a mechanical response to the presence of particles by *Mytilus edulis*, with filtration increasing with concentration (Wilson & Seed, 1974).

Ability of bivalve shellfish to remove bacteria-sized particles from the water column

In natural seawater, smaller particles constitute an important fraction in terms of volume (Haven & Morales-Alamo, 1970; Vahl, 1972a), and as noted by Jorgensen (1966), the nano- and ultraplankton (less than 5µm, including bacteria) in coastal waters constitute the larger part of the particulate matter, with bacteria being present in suspension, or attached to particles and fixed substrates (Amouroux, 1986).

Early investigations suggested that suspension feeding bivalves were able to retain particles of a few microns diameter with high efficiency, although it was demonstrated that the porosity of the *Mytilus edulis* gill had the ability to affect efficient retention of small particles (Zobell & Landon, 1937; Jorgensen, 1949; Jorgensen & Goldberg, 1953; Ballentine and Morton, 1956; Blake, 1961). Berry & Schleyer (1983) showed the ability of the mussel *Perna perna* to remove latex particles, of 0.46µm diameter, which corresponded approximately to the mean diameter of free-living bacteria (Azam and Cho, 1987), from suspension. Several other workers demonstrated that particles smaller than 5µm, including bacteria, played an important role in the nutritional strategy of *Mytilis edulis* (Zobell & Landon, 1937; Jorgensen & Goldberg, 1953; Ballentine and Morton, 1956; Blake, 1961; Jorgensen, 1949, 1966; Haven & Morales-Alamo, 1970; Vahl, 1972a). Vahl (1972a) and Jorgensen (1975) found that *Mytilus edulis* almost completely retained particles down to about 3µm, but below this retention efficiency reduced dramatically (Mohlenberg & Riisgard (1978), with other researchers showing that the size limit for complete retention of particles varied between bivalve species and was dependent upon the integrated activity of the gill ciliary system (Davids, 1964; Haven & Morales-Alamo, 1970; Vahl, 1972a, b; Vahl, 1973a; Bernard, 1972, 1974). However, evidence is also available that the same species of animal maintained in different habitats can have a 50% difference in ingestion rate (Kiorboe *et al.*, 1980).

Research by Field (1911) into the diet of *Mytilus edulis* indicated that only 50% of food bulk taken in could be identified as phytoplankton species, indicating the use of a food source other than phytoplankton. This work was further confirmed by Zobell & Landon (1937) and Zobell & Feltham (1938), who showed that bacteria could act as a food source for adult mussels by demonstrating growth of mussels fed solely on bacteria. The weight gained by these animals was greater than 16% compared to unfed mussels whose weight reduced by 12% over the same period. These experiments by Zobell and Landon (1937) also detected enzymes in shellfish capable of digesting bacteria. This was investigated further by McHenery *et al.* (1979), who detected high levels of the bacteriolytic enzyme lysozyme in the digestive system of *Mytilus edulis* and suggested that the enzyme's purpose was nutritional rather than as a host defense factor. Later, Birkbeck & McHenery (1982) showed that bacteria were degraded by *Mytilus edulis* with selected polymers being retained. These experiments by Birkbeck and McHenery also showed that 90% of bacteria were removed in less than 2h when *Mytilus edulis* were exposed to a range of bacteria at 10^6 bacterial cells ml^{-1}, but indicated that certain species were not removed as successfully as others. McHenery & Birkbeck (1986) later showed clearance rates of >90%, when a *Pseudomonas* sp. was introduced at the higher initial inoculum of 10^7cfu ml^{-1}. These clearance figures are similar to rates reported by Amouroux (1986), who showed that the clam *Venus verrucosa* cleared 95% of a bacterial suspension in 2h. Amouroux considered that bacteria contributed a significant component to the bivalve diet.

Work by Lucas *et al.* (1987), showed that after 6 h, 65% of natural marine bacterioplankton, calculated as 10^6cfu ml^{-1}, were removed from seawater by *Mytilus edulis*, concluding that it was unnecessary to overload an experimental system with a high inoculum to achieve removal of bacteria. Plusquellec *et al.* (1990) also studied uptake of bacteria by *Mytilus edulis* under natural conditions in the laboratory and showed the concentration of bacteria utilised by mussels to be influenced by bacterial species, particle density and season. This agreed with earlier data of Famme & Kofoed (1983), who indicated that the spectrum of particles retained by *Mytilus*

edulis appeared to be an adaptation by the animals to exploit the local resource available within the water column.

The uptake of SCB-producing bacteria by *Mytilus edulis*

It has been suggested that PST-producing bacteria may be the source of PST during times of high shellfish toxicity, with maximum bivalve toxicity noted when *A. tamarense* was no longer detectable within an environment.

Kodama *et al.*, (1993) examined different particle size fractions within the marine environment in relation to PST production and reported that free-living bacteria co-existed with dinoflagellates and were associated with bivalve toxicity. Analysis of toxicity from the dinoflagellate size fraction (>20μm) did not correlate with dinoflagellate abundance, with a large variation in toxicity over time also seen in the smallest (0.45-5µm) size fraction, although highest toxicity in the small size fraction was recorded during periods of dinoflagellate abundance. Toxicity was also recorded within the middle size fraction but there was no marked change in toxin levels over time. Scallops present in the area showed toxicity during the same time period, with high toxicity levels occurring during times of dinoflagellate abundance, although, scallop toxicity was also seen during a period when no dinoflagellates were observed. This suggested that the increase in scallop toxicity was due to PST-producing bacteria occurring in seawater in association with dinoflagellates.

Levasseur *et al.* (1995) showed that bacteria were capable of autonomous PST production when isolated from similar size fractions to those used by Kodama *et al.* (1993), indicating that particles smaller than dinoflagellates were producing PST. However, Levasseur also incubated the different size fractions in the dark and noted significant PST production in all size fractions, indicating non-photosynthetic organisms were capable of producing PST. Although bacteria attached to the dinoflagellates may have been responsible for PST production in the larger fractions, it was probable that free-living bacteria were responsible for toxin production in the

0.45-5μm size fraction.

Gallacher and Birkbeck (1993) also investigated the possibility of free-living SCB toxin producing bacteria and reported that 37% of bacterial isolates from seawater from Ardtoe, Scotland, obtained over a 1 year period were SCB toxin producers. The peak number of toxin producing bacteria coincided with a PSP outbreak in the area indicating a possible role of bacteria in the outbreak.

Studies on the uptake of SCB-producing bacteria by Gallacher & Birkbeck, (1993) demonstrated that 95% of an initial 10^7cells ml^{-1} inoculum of *Alteromonas tetraodonis* strain GFC was removed within 3h, with SCB activity subsequently detected in shellfish samples using the mouse neuroblastoma assay (Gallacher and Birkbeck, 1992).

G.L. Hold, 1999

OBJECT OF RESEARCH

The objectives of this work were two-fold. The first was to elucidate the role of bacteria in dinoflagellate toxicity. This objective comprised several aspects of investigation, beginning with characterisation of the bacterial species associated with paralytic shellfish toxin-producing dinoflagellates (concentrating on *Alexandrium* species), and to identify whether the microflora differed in different dinoflagellate cultures, both *Alexandrium* and non-*Alexandrium*, which did not produce paralytic shellfish toxins. A further aspect of this work involved producing a bacteria-free 'axenic' *Alexandrium* culture to assess the effects on growth and toxin production following removal of the associated microflora. The final stage of the first objective investigated the effects on toxicity and growth of the axenic culture when bacteria were re-introduced, in order to assess the ability of dinoflagellate cultures to sustain different microbial populations.

The second objective was to investigate the ability of SCB-producing bacteria to invoke toxicity in *Mytilus edulis*, with toxicity detected using the mouse neuroblastoma assay. This required adjustments to the existing methodology, in order to detect the low levels of toxicity expected in samples. Once optimised, the assay was used to assess levels of SCB activity generated during shellfish feeding experiments.

G.L. Hold, 1999

CHAPTER 2 : CHARACTERISATION OF THE MICROFLORA ASSOCIATED WITH DINOFLAGELLATE CULTURES

Introduction

The association between bacteria and algae is well documented (Berland *et al.*, 1970; Bell and Mitchell, 1972; Tostesen *et al.*, 1989). Traditionally, however, attempts to identify the actual bacterial species associated with algae were limited as they relied mainly morphological and/or biochemical techniques to infer diversity (Nelinda *et al.*, 1985; Tostesen *et al.*, 1989, Romalde *et al.*, 1990a; b). The use of molecular methods to identify bacteria within complex communities, including oceanic habitats, has shown that bacterial sequences determined using non-culture based methods do not correspond to gene sequences obtained from the cultured isolates (Suzuki *et al.*, 1997), emphasising the need to combine traditional and molecular approaches to obtain a complete picture of the composition of a microbial community. Previous studies using molecular techniques to identify bacteria associated with dinoflagellate cultures have concentrated on identifying specific bacterial isolates (Doucette, 1995; Gallacher *et al.*, 1997; Ishida *et al.*, 1997). However, only limited attempts to characterise *in situ* bacterial diversity have been documented. A recent study by Prokic *et al.* (1998) investigated the diversity of bacteria associated with the toxic dinoflagellate *Prorocentrum lima* by cloning and sequencing 16S rRNA genes. This study showed that different microflora were associated with cultures derived from the same original culture, but maintained in different laboratories. One of the cultures contained only α-Proteobacteria while isolates belonging to four bacterial phyla/subphyla were detected in the other culture. Nevertheless, *Roseobacter* related isolates were dominant in both cultures.

The *Roseobacter* genus was originally comprised of two species, *Roseobacter denitrificans* and *R. litoralis*, both of which were isolated from marine algae. However, two additional species have been included within the genus: *R. algicola*, isolated from *Prorocentrum lima* (Lafay *et al.*, 1995), and *R. gallaeciensis* isolated from the scallop *Pecten maximus* (Ruiz-Ponte *et al.*, 1998). These two species, although clearly related to the original *Roseobacter* species, do not produce bacteriochlorophyll a. In the last few years, isolates belonging to the *Roseobacter*

genus have been identified from many geographically distinct marine environments.

The aim of this study was to characterise the bacterial flora of two PST-producing *Alexandrium* species, using both culture-based, and non-culture-based techniques. Two other dinoflagellate cultures, a non toxin-producing *Alexandrium* culture, and a dinoflagellate (*S. trochoidea* NEPCC 15), of similar morphology, but not associated with PST production were also analysed, to identify differences in bacterial populations associated with different dinoflagellate cultures.

In the culture-based system bacteria were isolated on marine agar, from dinoflagellate cultures, and were subsequently grouped using morphology characteristics and RFLP analysis. Representative isolates from each group were then subsequently identified using 16S rDNA sequencing. The non-culture-based method utilised PCR amplification of bacterial sequences directly from dinoflagellate cultures, with DGGE analysis used to separate the individual amplification products, which were also sequenced to infer phylogenetic affiliation.

This investigation was carried out at the three dinoflagellate growth phases, to assess whether the bacterial flora altered over the course of the growth cycle. To our knowledge, this is the first such study to combine different molecular approaches to identify the bacteria associated with dinoflagellate cultures.

G.L. Hold, 1999

MATERIALS AND METHODS

Dinoflagellate maintenance and growth regime

Unless otherwise stated, all dinoflagellate cultures were maintained using Guillard's f/2 seawater enrichment media (Sigma). Reference to other growth media is made for sustaining some treated dinoflagellate cultures in Chapter 3, (See Appendix 1 for growth media formulations). All dinoflagellate strain information is detailed in Table 2.1. All dinoflagellate culture was conducted in a laminar flow cabinet. Seawater (collected 3 months previous to use), was autoclaved at 110°C for 30 min in borosilicate wide neck flasks with non-absorbent cotton-wool bungs, prior to the addition of f/2 (20ml l^{-1}, Sigma). Cultures were transferred into fresh medium every 14-21 days to keep stock cultures in the exponential phase of growth. Cultures were maintained at 15°C with a 14h:10h light:dark cycle (irradiance level 0.5 - 1.5 x 10^{16} quanta sec^{-1} cm^2). See Chapter 4 for growth curve analyses.

Isolation of bacteria from dinoflagellate cultures

Samples of dinoflagellate cultures *A. tamarense* NEPCC 407 and PCC 173a, *A. lusitanicum* NEPCC 253 and *S. trochoidea* NEPCC 15 from the three growth phases, were subjected to a ten-fold dilution series using sterile seawater. Each dilution was subsequently spread (100µl) in triplicate on marine agar plates, which were incubated for 14 days at 20°C. Following incubation, the dilution plate containing between 50 and 100 colonies was analysed further, by picking and replating each of the isolates present to obtain pure cultures. Isolation of some bacterial strains was done in conjunction with technical staff at the Marine Laboratory.

Strain name	Origin	Toxicity recorded in literature (pg STX equiv cell^{-1})	Original reference	Culture collection	Axenic status
Alexandrium lusitanicum NEPCC 253	Laguna Obidos, Portugal	0.43	Cembella *et al.* (1987)	North East Pacific Culture Collection (NEPCC)	non-axenic
A. tamarense NEPCC 407	Jericho Beach, Vancouver, Canada	1.32	Cembella *et al.* (1987)	NEPCC	non-axenic
A. tamarense CCMP 117	Ipswich Bay, USA	20.5	Alam *et al.* (1979) Schmidt & Loeblich (1979)	Culture Collection for Marine Phytoplankton (CCMP)	axenic
A. tamarense CCMP 1771	Plymouth, UK	non-toxic	Lebour (1925)	CCMP	axenic
A. tamarense PCC 173a	Plymouth, UK	non-toxic	Lebour (1925)	Plymouth Culture Collection (PCC)	non-axenic
A. tamarense UW4	Ardtoe, Scotland, UK	not defined	J. Lewis pers comm	University of Westminster Culture Collection (UW)	non-axenic
A. tamarense UW2C	Ardtoe, Scotland, UK	not defined	J. Lewis pers comm	UW	non-axenic
A. affine NEPCC 667	Vancouver, Canada	not detected	Cembella *et al.* (1987)	NEPCC	non-axenic
Scrippsiella trochoidea NEPCC 15	Pacific Point, British Columbia, Canada	not associated with toxicity		NEPCC	non-axenic

Table 2.1 Dinoflagellate strain information

Preparation of bacterial DNA for restriction fragment length polymorphism (RFLP) analysis

Nucleic acid extraction and PCR amplification

All chemical formulations for molecular procedures are included in Appendix 2. RFLP analysis of *S. trochoidea* NEPCC 15 bacterial isolates was done in collaboration with Dr. E. Smith.

Individual colonies from bacteria isolated from dinoflagellate cultures were placed in microcentrifuge tubes containing TE buffer (100µl; 10mM Tris, 10mM EDTA pH8; Sigma), and boiled for 5 min to release the DNA. Each crude DNA preparation, was subjected to PCR amplification using eubacterial primers 27F and 1522R (Fig. 2.1; supplied by Bioline), following the method described by Suzuki *et al.*, (1997). Amplification products were visualised by electrophoresis using a 2% agarose gel in 1 x TAE containing ethidium bromide (0.5µg ml^{-1}), with a marker (1KB; Gibco) and DNA quantification standards (Gibco), included on the gel for reference.

Restriction digests of PCR products

To generate RFLP patterns, 700ng of each PCR product was digested with 5 U of restriction endonuclease *Hae* III (Promega) for 3 h. Reactions were stopped by the addition of EDTA, with fragments resolved by gel electrophoresis in 2.5% low melting point (LMP) agarose in 1 X TAE stained with ethidium bromide (0.5µg ml^{-1}).

Preparation of bacterial isolates for 16S rDNA sequencing

Representative isolates from all dinoflagellate cultures possessing particular RFLP patterns were selected for identification by sequencing. PCR products were purified

27F	5'-AGAGTTTGATCMTGGCTCAG-3'
1522R	5'-AAGGAGGTGATCCANCCRCA-3'
Primer 1	5'-CCTACGGGAGGCAGCAG-3'
Primer 2	5'-ATTACCGCGGCTGCTGG-3'
Primer 3	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG-3'

Figure 2.1 Synthetic oligonucleotides used for PCR amplification in RFLP and DGGE analysis

on Qiaquick-spin columns (Qiagen) and samples subjected to bi-directional 16S rDNA sequencing.

Preparation of dinoflagellate culture DNA for denaturing gradient gel electrophoresis (DGGE)

Nucleic acid extraction

All buffers, beads and plastics used were either disposable/gamma sterilised or UV irradiated for 15 mins before use.

Dinoflagellate cultures *A. tamarense* NEPCC 407, CCMP 117, UW4, and UW2C, *A. affine* NEPCC 667, *A. lusitanicum* NEPCC 253 and *S. trochoidea* NEPCC 15 (See Table 2.1 for strain information), were harvested in the stationary phase of their growth cycle, for initial DGGE experiments. Further DGGE experiments used samples from the three growth phases of *A. tamarense* NEPCC 407 and PCC 173a, *A. lusitanicum* NEPCC 253 and *S. trochoidea* NEPCC 15. Each growth phase sample (1000ml) was centrifuged (10,000g x 10 min) and the supernatant decanted. The pellet was resuspended in 1ml TE buffer and stored at -20°C overnight. Following thawing, glass beads (0.5g) of 0.16 - 1.17 mm diameter (Sigma), were added to samples, which were alternately vortexed for 60 sec and placed on ice for 60 sec until visual signs of lysis were seen. 50µl of lysozyme solution (50mg ml^{-1} in distilled water, Sigma) was added to each tube and samples incubated at room temperature for 30 min with vortex mixing at 5-10 min intervals. Sodium dodecyl sulphate (SDS) solution (100µl of 10% wt vol^{-1} in distilled water, Sigma), was thoroughly mixed with each sample, followed by addition of proteinase K (50µl of 20 mg ml^{-1} in distilled water, Sigma) and a further incubation at room temperature for 30-60 min.

Sample DNA was cleaned up by adding 0.5ml TE-saturated phenol (Rathburn Chemicals), vortexing and the liquid placed in to an eppendorf tube and centrifuged

(10 min, 13,000 x *g*). The supernatant was removed to a clean eppendorf tube and 0.5ml saturated phenol added, the mixture vortexed and centrifuged again. This procedure was repeated until no interface could be seen. The 'clean' supernatant was placed in another clean eppendorf and 0.5ml chloroform (Rathburn Chemicals) added, the tube vortexed and centrifuged (10 min, 13,000 x *g*). The supernatant was concentrated using a Microcon 100 column (Amicon), and the concentrated DNA run on a 1% wt vol^{-1} low melting point (LMP) agarose gel containing ethidium bromide (0.5µg ml^{-1}) alongside a marker (1Kb, Gibco) at 75mV. When the bromophenol blue loading dye was close to the end of the gel, DNA above 8Kb was excised from the gel, to be used as template for future PCR reactions. All gel sections were stored at 4°C until required.

PCR amplification of dinoflagellate samples for DGGE analysis

Primers

Primers selected for PCR amplification of bacteria associated with dinoflagellate cultures were those described by Muyzer *et al.* (1993; Primers 1 - 3, Fig. 2.1). They amplify the variable V3 region of the 16S rDNA corresponding to positions 341 to 534 in *E. coli*. Primer 3 contains the same sequence as primer 1, but has at its 5' end an additional 40 nucleotide GC rich region (GC clamp). A combination of primers 2 and 3 was used to amplify samples for DGGE analysis, but primers 1 and 2 were used to amplify samples for 16S sequencing following DGGE analysis, as the GC clamp would interfere with sequencing reactions. Primers were synthesised by Bioline and stored at -20°C prior to use.

PCR method

Incubations were carried out in 0.5ml microfuge tubes. The reaction mixture contained 10x PCR buffer (100mM Tris-HCl, pH 9.0; 15mM $MgCl_2$; 500mM KCl; 0.1% [wt vol^{-1}] gelatin; 1% [vol vol^{-1}] Triton X-100)(Bioline); primers, 50pmol of

each, dATP, dTTP, dCTP, dGTP, final concentrations of 200μM each (Bioline) and template DNA (250ng) in a final volume of 100μl.

Samples were first incubated for 5 min at 95°C, to denature the template DNA, after which *Taq* DNA polymerase (0.2 unit, Bioline) was added to each reaction tube; these were then subjected to the following sequence of incubations:

1. Denaturation at 94°C for 30 sec
2. Primer extension at 55°C for 45 sec
3. Product extension at 72°C for 30 sec

The incubations were repeated for 30 cycles, after which product extension at 72°C was maintained for 10 min. Thermocycling was carried out using a programmable dri-block (Techne Genius).

Analysis of PCR products

PCR products were viewed under u.v. light following electrophoresis through 2% agarose gel containing ethidium bromide (0.5μg ml^{-1}). Specificity and yield of each reaction was assessed by comparison with a marker (100bp ladder; Gibco) and DNA quantitation standards (Gibco) which were included on the gel. Any remaining reaction mixture was stored at -20°C until analysed by DGGE.

Denaturing Gradient Gel Electrophoresis

DGGE (see Myers *et al.*, 1988) is a gel system which separates DNA fragments according to their melting properties. When a DNA fragment is electrophoresed through a linearly increasing gradient of denaturant (urea/formamide), the fragment remains double stranded until it reaches the concentration of denaturant equivalent to a melting temperature (T_m) that causes the lower temperature melting domains of the fragment to melt. At this point, branching of the molecule occurs, sharply reducing the mobility of the fragment in the gel to approximately 20% of the helical molecule (Myers *et al.*, 1987). Fragment separation on a gel can be caused by as

little as a single base difference.

To allow good resolution of fragments within a sample, it is necessary to distinguish between the effects of electrophoretic mobility and denaturant concentration. This is done using a perpendicular gradient gel. This relationship can be displayed as a sigmoidal curve in a gel, where migration occurs at right angles to the denaturant gradient (usually 0 - 100%), such that each molecule travels in a path of constant denaturant concentration. DNA molecules at the left side of the gel (low denaturant), will migrate as double stranded DNA (dsDNA), but at the other side of the gel, where the concentration of denaturant is high, the molecules melt into branched forms as soon as they enter the gel, and therefore halt. At intermediate concentrations of denaturant, the molecules have different degrees of melting which allows different mobilities. A steep transition in mobility occurs at the denaturant concentration corresponding to T_m. Calculation of the gradient range which encompasses the T_m and allows a 10 - 20% gradient either side, allows subsequent analysis of samples using the parallel gradient gel, which allows the examination of a large number of samples within one gel containing the optimised gradient. The parallel gel allows samples to be loaded in adjacent lanes, so molecules travel through an ascending denaturant concentration until reaching the gradient level where continued migration is slow. Separation of the different fragments within a sample corresponding to different PCR products, results in short bands at differing positions throughout the gradient, and these can subsequently be excised and identified.

Perpendicular gradient gel

All apparatus and chemicals used were part of the Dcode™ Universal Mutation Detection System (BIORAD), unless otherwise stated. 16 x 16cm glass plates were assembled with 0.1mm grooved spacers and single well comb which was tilted to the appropriate position using the casting stand and tilt rod. The gel was poured as follows: a solution containing 0% denaturant was placed in a syringe attached to the gradient delivery system (Lo density side). The solution contained 14.5ml of 0%

denaturant solution (see Appendix 2 for recipe), 150µl ammonium persulfate (10% wt vol^{-1} in distilled water) and 12µl TEMED. A second solution containing 100% denaturant was placed in a syringe attached to the gradient delivery system (Hi density side). This solution contained 14.5ml of 100% denaturant solution (see Appendix 2 for recipe), 150µl ammonium persulfate (10% wt vol^{-1} in distilled water) and 12µl TEMED. Syringes were connected to the gel sandwich assembly and the gel poured by rotating the cam wheel of the gradient delivery system. The gel was left to polymerise for 1 hour after which the gel sandwich was attached to the system core, the comb removed and the well rinsed with preheated 1X TAE. The core and attached gel assemblies were placed in the electrophoresis tank containing the running buffer (1X TAE) preheated to 65°C and left to equilibrate for 30 min before samples were loaded.

The samples were warmed to 37°C prior to loading and mixed with an equal volume of 2X loading dye (see Appendix 2 for recipe). A potential difference of 130 volts was applied to the Dcode system at a constant tank temperature of 65°C for four hours or until the dye was close to the end of the gel. Following electrophoresis, one glass plate was removed and the gel stained for 15 min in running buffer containing ethidium bromide (50µg ml^{-1}). The gel was destained using running buffer before examination under u.v. illumination. To determine the T_m of the sample, the width of the gel is divided by 100 to allow an indication of the distance within which a 1% change in denaturant had occurred. To identify the denaturant percentage of the T_m, the midpoint of the slope was measured and this figure divided by the distance allowing the 1% change in denaturant.

Parallel gradient gel

All samples were prepared as described previously using PCR amplification and 16 x 16cm glass plates assembled as before, but with ungrooved 1.0mm spacers. The denaturant solutions identified from the perpendicular gradient gel were prepared (see Appendix 2 for recipes) and placed in syringes attached to the gradient delivery

system. Both denaturant solutions had 150µl ammonium persulfate (10% wt vol^{-1} in distilled water), and 12µl TEMED added prior to placing in syringes. Syringes were connected to a 19 gauge needle which was attached to the top-centre of the gel sandwich, the gel poured and a toothed combed inserted. The gel was allowed to polymerise for 1 hour after which the comb was removed and the wells rinsed with running buffer (1X TAE) to remove unpolymerised material. Sample preparation prior to loading, run conditions and ethidium bromide staining following electrophoresis, were as described for the perpendicular gel system, with samples visualised using u.v. illumination following destaining.

Preparation of bands for sequencing

Bands from parallel gradient gels were excised with a sterile razor blade and the small blocks of acrylamide containing each individual band were placed in sterile 1.5ml tubes containing 100µl TE buffer (Sigma). Tubes were incubated at 37°C overnight to allow passive elution of DNA from gel fragments. Sample DNA was recovered using Wizard™ PCR Prep DNA purification system (Promega) as follows: following overnight incubation, the aqueous phase from each tube was transferred to fresh tubes, 1ml of purification resin added and tubes were vortexed for 20 seconds. Each sample was drawn through a minicolumn using a vacuum manifold, and subsequently washed with isopropanol (2ml, 80%, Sigma) drawn through the column. Each minicolumn was placed in a 1.5ml microcentrifuge tube and spun (2 min, 10,000g) to remove residual isopropanol, before columns were transferred to new tubes and 50µl TE buffer applied to each column and left for 1 min before briefly centrifuging (10,000g) to elute bound DNA. Samples were re-amplified using PCR conditions as before, but with primer 1 instead of primer 3. Following PCR, samples were again inspected using u.v. illumination of ethidium bromide stained agarose to confirm successful reamplification. The samples were then subjected to a cleanup process (Qiaquick PCR spin column) to remove excess primers and dNTP's which would inhibit subsequent PCR reactions. Samples were identified using bi-directional 16S rDNA sequencing.

Gene sequencing and phylogenetic analyses

Sequencing was carried out by Dr. P. Carter, University of Aberdeen, with sequence analysis performed under the supervision of Dr. M. Rappé, CNRS, Roscoff. Full length sequences of RFLP characterised bacterial strains were analysed using PCR products generated for RFLP analysis. Two internal primer sets were also included to allow the whole PCR product to be analysed.

DGGE bands and representative RFLP pattern isolates were sequenced using an ABI model 373A automated sequencer (Applied Biosystems Inc., Foster City, California). Bi-directional sequence data from the SSU rDNA gene fragments were manually aligned with bacterial sequences obtained from Genbank, the Ribosomal Database Project (RDP) (Maidak *et al.*, 1994), and the ARB sequence databases (Ludwig & Strunk, 1997) using the ARB and Genetic Data Environment (GDE) v2.2 (Steve Smith, Millipore, Bedford, Mass.) sequence analysis software packages. Raw sequence similarities were calculated without distance correction by using the program DNADIST available with the Phylogeny Inference Package (PHYLIP v3.5; Felsenstein, 1989). Sequence similarities were performed on partial sequences using conservative phylogenetic masks, which only included regions of umambiguous alignment.

For further identification of RFLP pattern representatives, evolutionary distances were calculated using the programme DNADIST and Kimura 2-parameter model for nucleotide change, with a transition/transversion ratio of 2.0. Random resampling of sequences (bootstrapping) to check the consistency of resulting trees was performed, with trees generated representing a consensus of 100 trees for each group of related sequences.

G.L. Hold, 1999

RESULTS

RFLP analysis of the bacterial flora of dinoflagellate cultures at three phases of the growth cycle

Bacteria cultured on marine agar from dinoflagellates *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, and *S. trochoidea* NEPCC 15, were identified as gram-negative rods. Approximately 500 bacterial isolates were categorised into 24 groups using a detailed colony morphology scheme (Collins and Lynes, 1984; Table 2.2). The majority of the isolates formed distinct non-pigmented colonies, with a small number of strains producing coloured pigments.

All of the strains were further analysed using restriction fragment length polymorphism (RFLP; Suzuki *et al.*, 1997), with the exception of yellow pigmented colonies from *A. lusitanicum* NEPCC 253 (not included in Table 2.2), which were not viable on sub-culture. Table 2.3 lists the number of RFLP patterns obtained for bacteria from each dinoflagellate with an example of the RFLP patterns generated shown in Figure 2.2. A total of 25 RFLP patterns were detected which corresponded to individual colony morphotypes (Table 2.2), with the exception of isolates described as circular, convex and cream with a rose centre, which gave two separate RFLP patterns (Figure 2.2; patterns 2 and 3).

Figure 2.3 shows the number of bacterial colony forming units ml^{-1}, for each RFLP pattern, from the four dinoflagellate cultures, at the three growth phases. Each bacterial group contained between 10^4 - 10^6 bacteria ml^{-1} of dinoflagellate culture, with numbers more dependent on the bacterial strain than dinoflagellate growth phase. However, the number of bacteria isolated from *S. trochoidea* NEPCC 15 was generally lower than those from the other dinoflagellates.

Representative isolates from each banding pattern, except patterns 18, 19, 20 and 24 (due to sample contamination), were further analysed using 16S rDNA sequencing (see Appendix 3 for individual sequences). The sequences were submitted to the SIMILARITY_RANK (Simrank) program of the Ribosomal Database Project (RDP),

RFLP pattern	Colony morphology (on MA)	RDP (Nearest phylogenetic neighbour)	RDP S_AB value
1	circular, smooth surface, umbonate, cream	*Thiobacillus* sp. str. TH1051	0.69
2	circular, convex, cream with rose centre	*Roseobacter algicola* ATCC 51440	0.871
3	circular, convex, cream with rose centre	*Roseobacter algicola* ATCC 51442	0.80
4	irregular, with lobate margin, beige	*Rhizobium loti* IAM 13588	0.75
5	circular, smooth, raised, cream	*Rhizobium* sp. str. H152	0.92
6	circular, smooth, gelatinous, flat, cream	*Altermonas macleodii* IAM 12920	0.89
7	irregular with an undulate margin, smooth, convex, dark brown	*Roseobacter littoralis* ATCC 49566	0.82
8	irregular with lobate margin, convex, smooth, brown centre	*Roseobacter littoralis* ATCC 49566	0.73
		Sulfitobacter sp. EE-36	0.94
9 a	circular with entire margin, smooth, cream	*Roseobacter algicola* ATCC 51440	0.91
9 b	circular with erose margin, granular, umbonate with a brown centre and pale margin	*Sulfitobacter* sp. EE-36	0.82
10	circular, smooth, viscous, raised, cream	*Sagittula stellata*	0.74
11	circular, mucoid, raised, bright yellow	*Cytophaga lytica* ATCC 23178	0.65
12	circular, flat, yellow	*Roseobacter algicola* ATCC 51442	0.78
13	circular, convex, viscous, yellow	*Altermonas macleodii* IAM 12920	0.9
14	circular mucoid, viscous, large cream	*Pseudoaltermonas haloplanktis*	0.95
15	punctiform, mucoid and viscous, pink	*Roseobacter algicola* ATCC 51442	0.77
16	irregular with undulate margin, convex, brown centre with cream margin	Lignin enrichment culture L-87	0.88
17	circular, entire, convex, smooth, brown centre with cream margin	*Hyphomonas* sp.. MHS3	0.92
18	irregular with lobate margin, white centre with pale margin	+	-
19	circular, entire, convex, smooth with brown centre and white margin	+	-
20	circular, umbonate, mucoid, with orange centre	+	-
21	circular, pulvinate, entire, smooth, white centre with cream margin	*Hyphomonas* sp. MHS3	0.53
22	circular, convex, smooth, cream	*Cytophaga latercula*	0.67
23	circular, pulvinate, smooth, cream	*Caulobacter crescentus* CB2A	0.70
24	circular, erose margin, brown	+	-
25	irregular, lobate, flat, yellow	*Pseudomonas stutzeri* AN11	0.96

Table 2.2 RFLP, colony morphology and nearest phylogenetic neighbour of bacteria isolated on marine agar from four dinoflagellate cultures at three growth phases. Representative isolates were obtained from: ALUS_253, *A. lusitanicum* NEPCC 253; ATAM_407, *A. tamarense* NEPCC 407; ATAM_173a, *A. tamarense* PCC 173a; SCRIPPS, *S. trochoidea* NEPCC 15.

[+] No rDNA sequence data available

Dinoflagellate	Number of isolates analysed by RFLP	Number of RFLP patterns detected
A. lusitanicum NEPCC 253	77	4
A. tamarense NEPCC 407	90	7
A. tamarense PCC 173a	130	8
S. trochoidea NEPCC 15	200	12

Table 2.3 Number of RFLP patterns from bacteria isolated from dinoflagellate cultures: *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407, *A. tamarense* PCC 173a and *S. trochoidea* NEPCC 15.

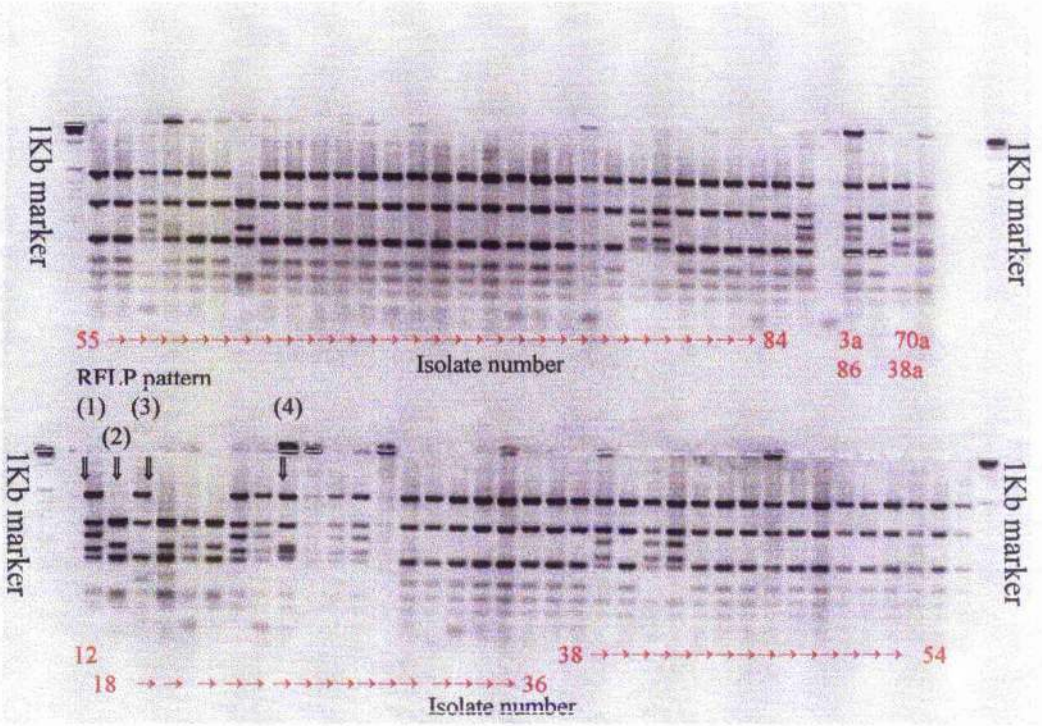


Figure 2.2 Restriction fragment length polymorphism (RFLP) patterns for representative bacterial isolates from 3 growth phases of *Alexandrium lusitanicum* NEPCC 253.

Numbers marked in red (12, 18 - 86, 3a, 38a, 70a), indicate different isolates, with representatives from each RFLP pattern (patterns (1) - (4)), subjected to sequence analysis for further identification.

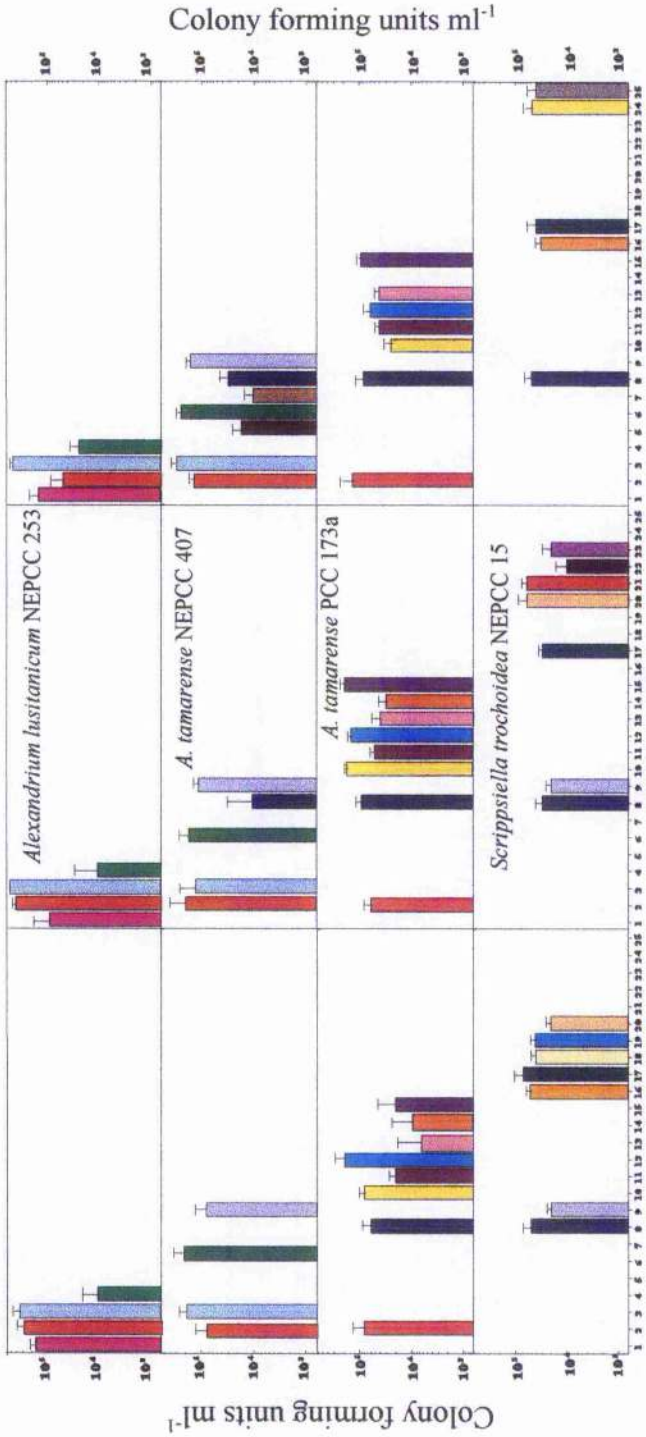


Figure 2.3 The number of bacteria (cfu $ml^{-1} \pm$ sem, n = 3) from each RFLP pattern (1 - 25), at three growth phases from dinoflagellate cultures.

and a preliminary list of closest phylogenetic neighbours determined (Table 2.2; Maidek, 1994). Based on Simrank values, expressed as S_AB values, sequences were classified according to phylum and sub-phylum affiliations. Fifteen of the RFLP patterns were identified as α-Proteobacterial, with eight of these patterns being *Roseobacter*-related sequences. γ-Proteobacterial isolates were classified as closest neighbours to four of the RFLP patterns, with a further two patterns identified as *Cytophaga/Flavobacter/Bacteroides* (CFB) related sequences. Four RFLP patterns gave S_AB values below 0.7 (patterns 1, 11, 20 and 22), indicating that these bacteria did not possess close sequence similarity to other isolates deposited within the database. However, a strong enough association was present to be able to infer that these isolates were α-Proteobacteria and CFB isolates. RDP separated the strains into the same groupings as RFLP analysis, with the exception of band 9 which was further divided into 9a and 9b, based on differences in sequence (Table 2.2).

Sequences representative of each RFLP pattern were subsequently aligned using the ARB programme. Phylogenetic trees (Figs. 2.4 - 2.7) were calculated with the neighbour-joining algorithm using the programme NEIGHBOUR of PHYLIP version 3.5 (Felsenstein, 1989). Bacterial sequences from dinoflagellates and other marine environments which have not yet been deposited in sequence databases (S. Gallacher, M. Rappé and L. Medlin pers. comm.), were included in the phylogenetic analysis, along with sequences available from Genbank, in order to provide the most complete comparison of strains.

All inferences associating RFLP pattern affiliation to bacterial classes determined by RDP, were confirmed by the further phylogenetic analysis. However, more closely related neighbours were identified in some instances, due to the inclusion of undeposited sequences. Of the isolates sequenced, most were categorised as α-Proteobacteria with the majority of these belonging to the *Roseobacter* clade. Of the latter, only strains from three representative RFLP patterns were associated with designated species: *Octadectabacter arcticus*, *Antarctobacter heliothermus* and *Roseobacter gallaenciesis* (Fig. 2.5; RFLP patterns 7, 10 and 9 respectively), with

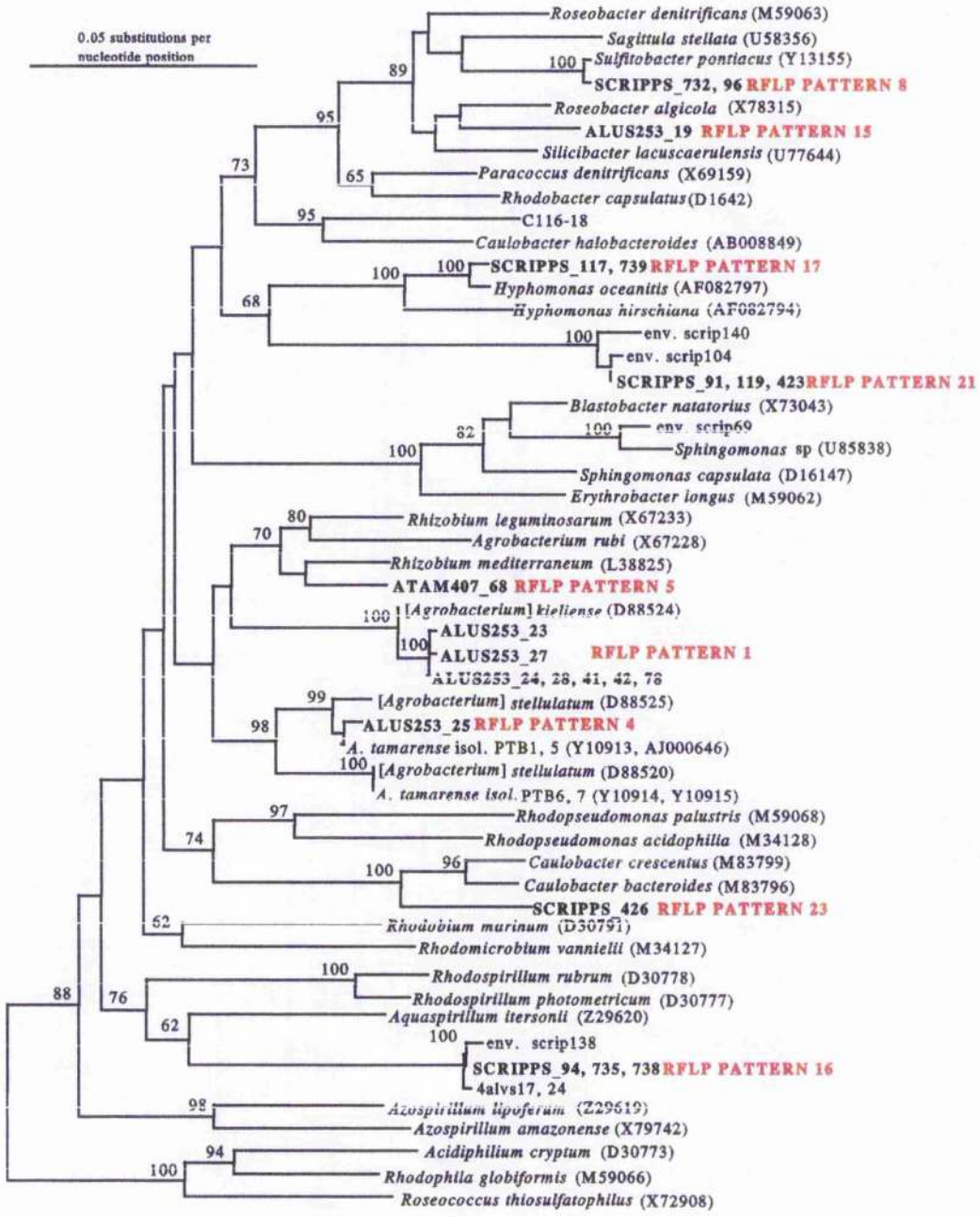


Figure 2.4 Phylogenetic affiliations of α-proteobacterial strains (16S rRNA genes) isolated from dinoflagellates ALUS_253, *A. lusitanicum* NEPCC 253; ATAM_407, *A. tamarense* NEPCC 407; ATAM_173a, *A. tamarense* PCC 173a; SCRIPPS, *S. trochoidea* NEPCC 15.

Phylogenetic trees were generated by the neighbour-joining method from a mask of 340 nucleotide positions; the tree is rooted to the γ and β-proteobacteria. Bootstrap values (n = 100 replicates) are indicated for each of the branches. Affiliations based on RFLP patterns are shown following isolate numbers.

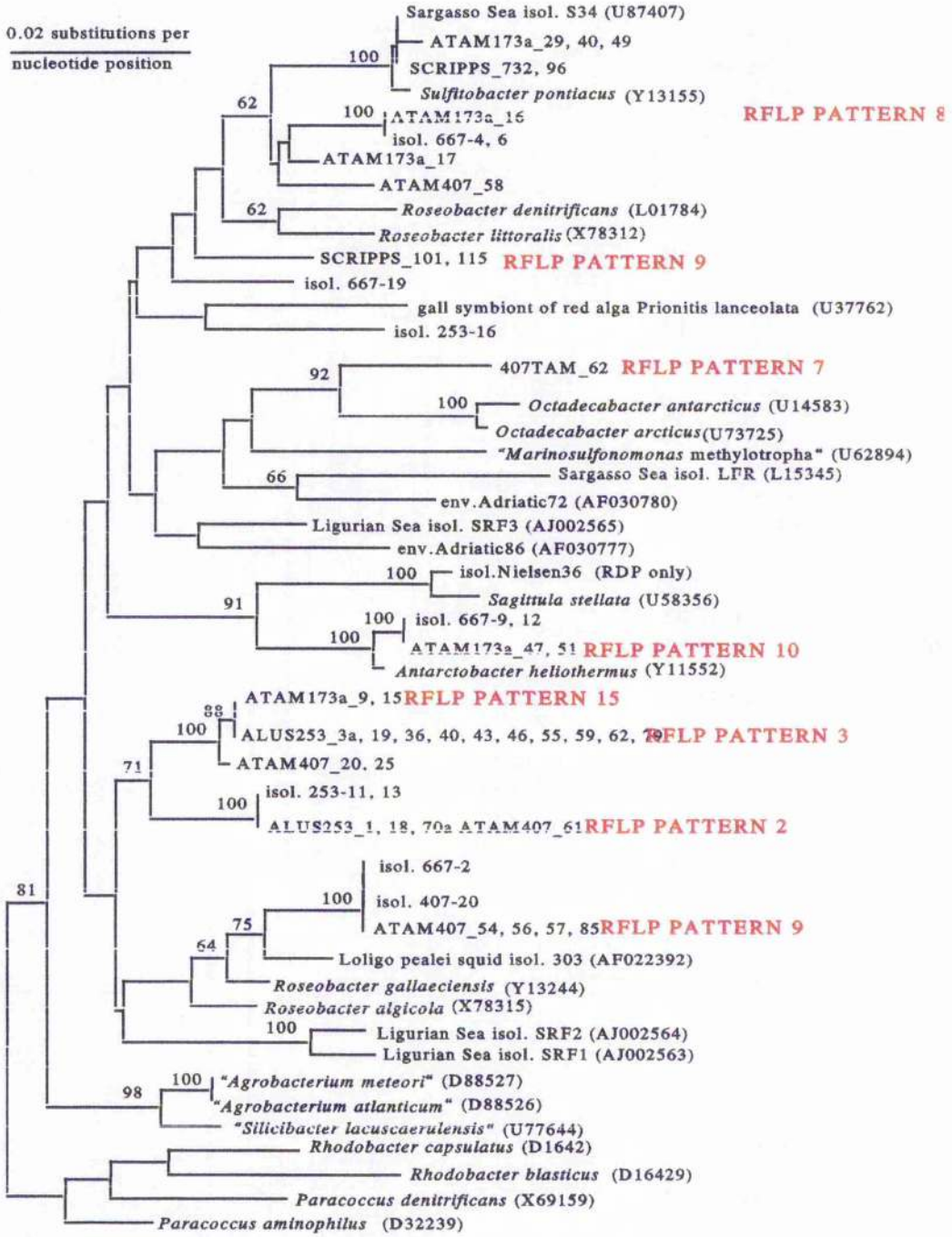


Figure 2.5 Phylogenetic affiliations of α-proteobacterial strains within the *Roseobacter* clade (16S rRNA genes) isolated from dinoflagellates ALUS_253, *A. lusitanicum* NEPCC 253; ATAM_407, *A. tamarense* NEPCC 407; ATAM_173a, *A. tamarense* PCC 173a; SCRIPPS, *S. trochoidea* NEPCC 15.

Phylogenetic trees were generated by the neighbour-joining method from a mask of 350 nucleotide positions; the tree is rooted to the γ and β-proteobacteria. Bootstrap values (n = 100 replicates) are indicated for each of the branches. Affiliations based on RFLP patterns are shown following isolate numbers.

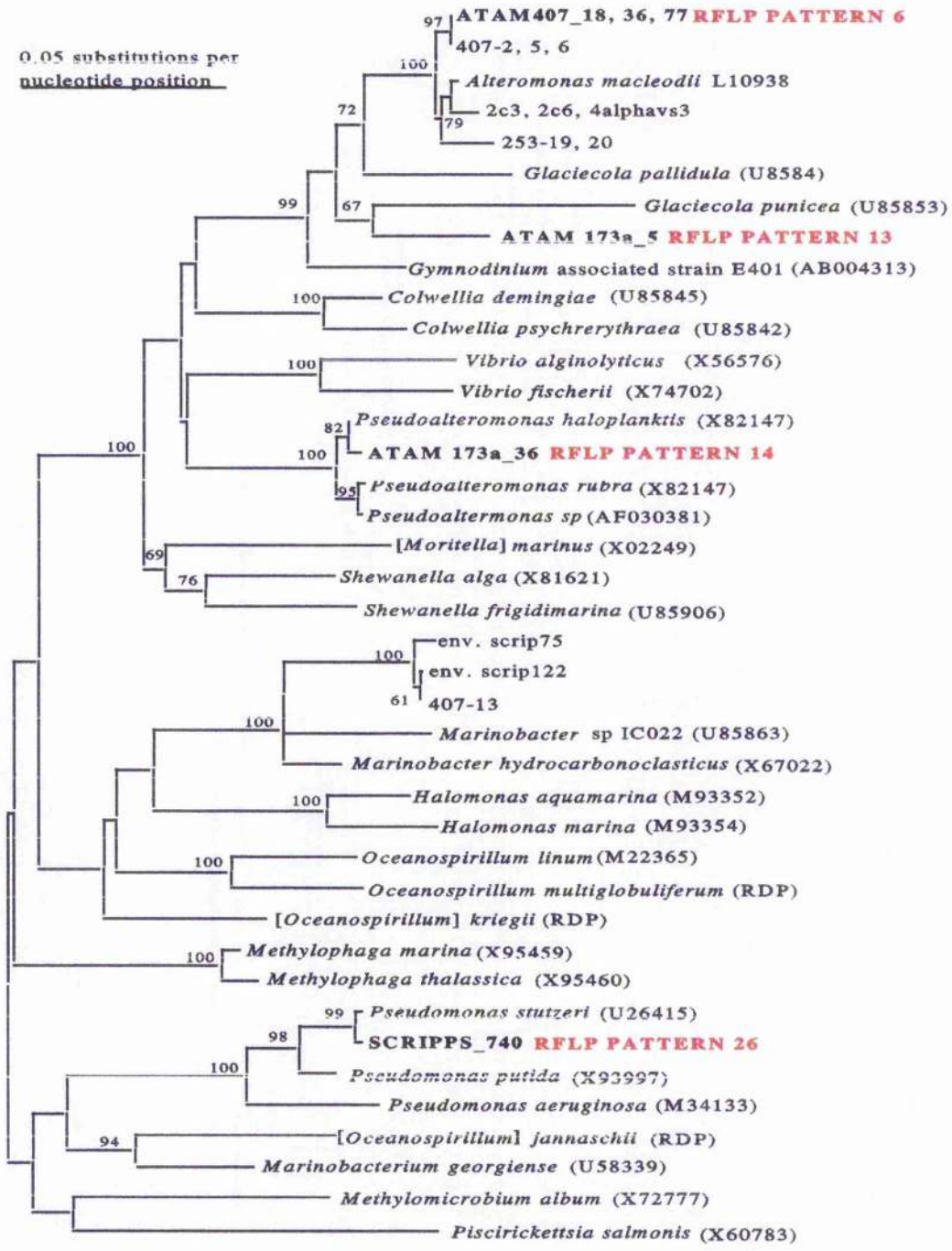


Figure 2.6 Phylogenetic affiliations of γ-proteobacterial strains (16S rRNA genes) isolated from dinoflagellates ALUS_253, *A. lusitanicum* NEPCC 253; ATAM_407, *A. tamarense* NEPCC 407; ATAM_173a, *A. tamarense* PCC 173a; SCRIPPS, *S. trochoidea* NEPCC 15.

Phylogenetic trees were generated by the neighbour-joining method from a mask of 440 nucleotide positions; the tree is rooted to β-proteobacteria. Bootstrap values (n = 100 replicates) are indicated for each of the branches. Affiliations based on RFLP patterns are shown following isolate numbers.

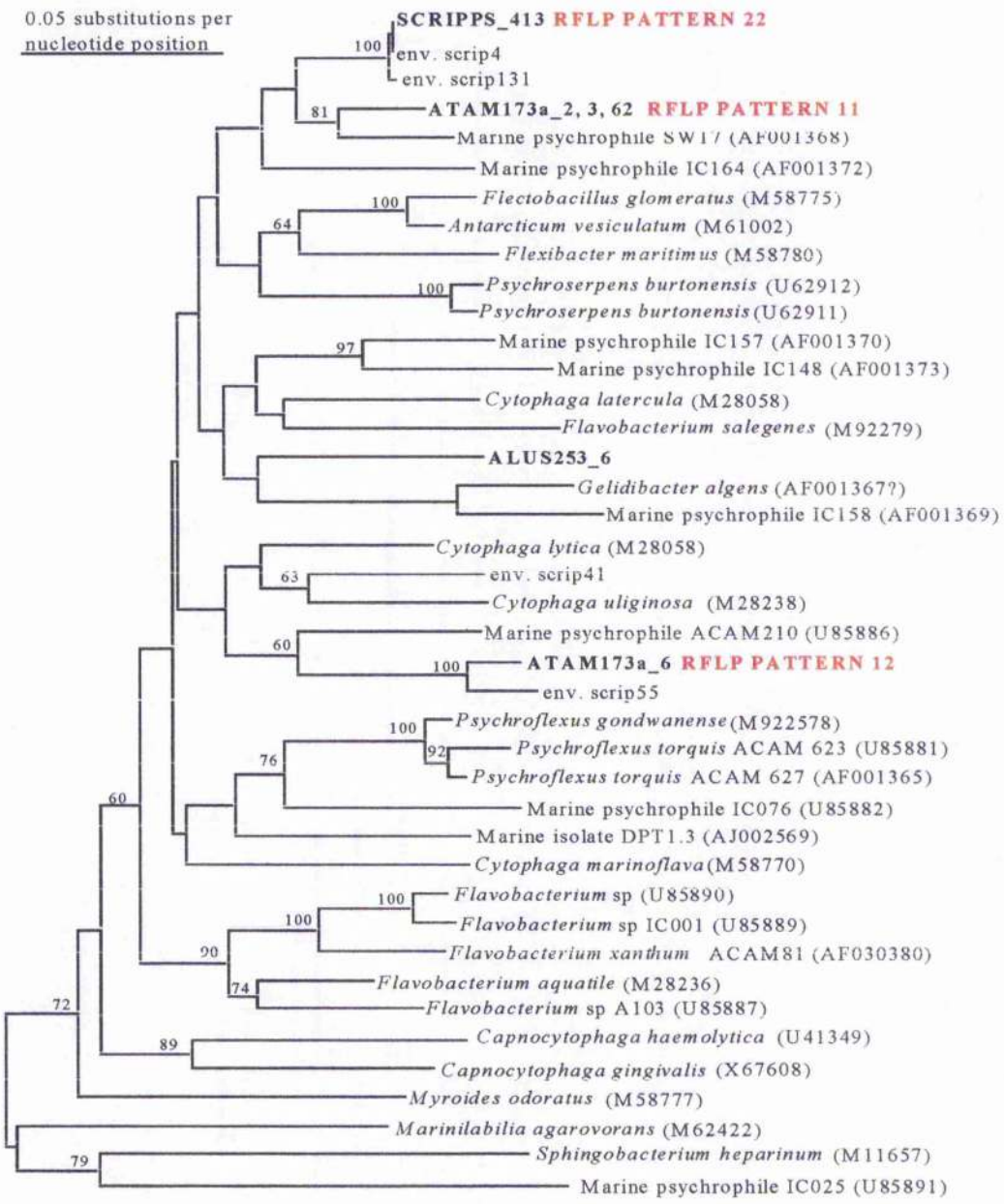


Figure 2.7 Phylogenetic affiliations of *Cytophaga* bacterial strains (16S rRNA genes) isolated from dinoflagellates ALUS_253, *A. lusitanicum* NEPCC 253; ATAM_407, *A. tamarense* NEPCC 407; ATAM_173a, *A. tamarense* PCC 173a; SCRIPPS, *S. trochoidea* NEPCC 15.

Phylogenetic trees were generated by the neighbour-joining method from a mask of 390 nucleotide positions; the tree is rooted to the *Bacteroides* genus. Bootstrap values (n = 100 replicates) are indicated for each of the branches. Affiliations based on RFLP patterns are shown following isolate numbers.

the remaining sequences having no closely related designated species (Fig. 2.5). Of seven RFLP patterns identified as α-Proteobacterial outwith the *Roseobacter* clade (Fig. 2.4), five were associated with designated species: *Hyphomonas oceanitis*, *Rhizobium mediterraneum*, *Agrobacterium kieliense*, *Agrobacterium stellulatum* and *Caulobacter bacteroides.*

The four patterns defined as γ-Proteobacteria (Fig. 2.6) were closely related to defined species: *Alteromonas macleodii*, *Glaciecola punicea*, *Pseudoalteromonas haloplanktis* and *Pseudomonas stutzeri.* A further four patterns were classified as *CFB* phylum isolates within the *Cytophaga* family (Fig. 2.7), however, not closely associated with any defined species. Within the groupings discussed above, several of the strains showed 100% similiarity to bacteria isolated from the same dinoflagellate culture a number of years previously, e.g ATAM407_54 and 407-20 (Fig. 2.5), indicating that some bacterial/dinoflagellate associations have remained stable over time.

Summarising the bacterial flora of each dinoflagellate, indicated that although certain bacterial groups were common to all cultures, different bacterial populations were maintained by each dinoflagellate culture (Tables 2.4 - 2.7). *A. lusitanicum* NEPCC 253, contained four different bacteria groups, which were detected in all phases; these were comprised of α-Proteobacteria, marine *Agrobacterium* related isolates (patterns 1 and 4; Fig 2.3; Fig. 2.4 and Table 2.4), and bacteria of the *Roseobacter* clade with no closely associated designated species (patterns 2 and 3; Fig 2.3; Fig. 2.5 and Table 2.5). Bacteria grouped as RFLP pattern 2, were also detected in all phases of *A. tamarense* NEPCC 407 and PCC 173a (Table 2.3), although pattern 3 was only detected in *A. tamarense* NEPCC 407.

A. lusitanicum NEPCC 253 also contained yellow pigmented bacteria which lost viability on sub-culture. Efforts were made to re-isolate this colony type by sub-culturing the isolate after two days as opposed to the fourteen days previously used. The strain, which was present at 1.56×10^6 cfu ml^{-1} at lag phase, 6.6×10^5 cfu ml^{-1} at

Dinoflagellate	phase of growth	α-proteobacteria isolates (excluding *Roseobacter* strains)						
		RFLP Pattern						
		17	21	5	1	4	23	16
		Related/Not related to defined species						
		Hyphomonas oceanitis	not related	*Rhizobium mediterraneum*	[*Agrobacterium*] kieliense	[*Agrobacterium*] stellulatum	*Caulobacter* species	not related
A. lusitanicum NEPCC 253	lag				X	X		
	log				X	X		
	stat				X	X		
A. tamarense NEPCC 407	lag							
	log							
	stat			X				
A. tamarense PCC173a	lag							
	log							
	stat							
S. trochoidea NEPCC 15	lag	X						X
	log	X	X				X	
	stat	X						X

Table 2.4 Summary of α-proteobacterial strains outwith the *Roseobacter* clade, from each dinoflagellate growth phase,depicted as RFLP patterns, with related defined species indicated where possible.
Stat = stationary

Dinoflagellate	phase of growth	α-proteobacteria *Roseobacter* Clade isolates							
		RFLP Pattern							
		8	9b	7	10	15	3	2	9a
		Related/Not related to defined species							
		not related	not related	*Octadecabacter arcticus*	*Sagittula stellata*	not related	not related	not related	*Roseobacter gallaeciensis*
A.lusitanicum NEPCC253	lag						X	X	
	log						X	X	
	stat						X	X	
A. tamarense NEPCC407	lag						X	X	X
	log	X					X	X	X
	stat	X		X			X	X	X
A. tamarense PCC173a	lag	X			X	X		X	
	log	X			X	X		X	
	stat	X			X	X		X	
S. trochoidea NEPCC 15	lag	X	X						
	log	X	X						
	stat	X							

Table 2.5 Summary of *Roseobacter* related strains from each dinoflagellate growth phase,depicted as RFLP patterns, with related defined species indicated where possible. stat = stationary

Dinoflagellate	phase of growth	γ-proteobacteria isolates			
		RFLP Pattern			
		6	13	14	25
		Related/Not related to defined species			
		Alteromonas species	*Glaciecola punicea*	*Pseudoalteromonas haloplanktis*	*Pseudomonas stutzeri*
A. lusitanicum NEPCC 253	lag				
	log				
	stat				
A. tamarense NEPCC407	lag	X			
	log	X			
	stat	X			
A. tamarense PCC 173a	lag		X	X	
	log		X	X	
	stat		X		
S. trochoidea NEPCC 15	lag				
	log				
	stat				X

Table 2.6 Summary of γ-proteobacteria related strains detected from each dinoflagellate growth phase, depicted as RFLP patterns, with related defined species indicated where possible.
stat = stationary

Dinoflagellate	phase of growth	*CFB* isolates			
		RFLP Pattern			
		22	11	no pattern	12
		Related/Not related to defined species			
		not related	not related	not related	not related
A. lusitanicum NEPCC 253	lag			X	
	log			X	
	stat			X	
A. tamarense NEPCC407	lag				
	log				
	stat				
A. tamarense PCC 173a	lag		X		X
	log		X		X
	stat		X		X
S. trochoidea NEPCC 15	lag				
	log	X			
	stat				

Table 2.7 Summary of CFB related strains detected from each dinoflagellate growth phase,depicted as RFLP patterns, with related defined species indicated where possible.
Stat = stationary

log phase and 1.03 x 10^6 cfu ml^{-1} at stationary phase, again lost viability after four sub-cultures but sufficent DNA was obtained to allow sequence information to be obtained. The isolate (ALUS253_6), was classified as a CFB phylum isolate, most closely associated with *Gelidibacter algens* (Fig. 2.7; Table 2.7), and was only obtained from *A. lusitanicum* NEPCC 253.

Bacteria associated with *A. tamarense* NEPCC 407 produced four banding patterns in lag phase (Fig. 2.3, patterns 2, 3, 6 and 9a). Patterns 2 and 3 were undefined *Roseobacter* related species as discussed above for *A. lusitanicum* NEPCC 253, although pattern 9a isolates were also *Roseobacter* clade isolates which were closely related to the newly defined species *Roseobacter gallaeciensis* (Fig. 2.3; Fig. 2.5; Table 2.5). Pattern 6 strains belonged to the γ-Proteobacteria subclass and were closely related to *Alteromonas* species (Fig. 2.6; Table 2.6), with an additional pattern (pattern 8) identified at log phase, which belonged to the *Roseobacter* clade, but not related to a defined species. This strain was also detected in all three growth phases of *A. tamarense* PCC 173a and *S. trochoidea* NEPCC 15. Two more patterns unique to *A. tamarense* NEPCC 407 (Fig. 2.3; patterns 5 and 7) were detected at stationary phase, both were α-Proteobacteria with pattern 7 most closely associated with *Octadecabacter arcticus* of the *Roseobacter* clade and pattern 5 related to *Rhizobium mediterraneum* (Fig. 2.4; Table 2.4).

The RFLP profile for bacteria from *A. tamarense* PCC 173a, although diverse, remained constant throughout the growth cycle, with the exception of isolates expressing pattern 14. This was a γ-proteobacterium, closely related to *Pseudoalteromonas haloplanktis*, and was unique to this dinoflagellate (Fig. 2.6; Table 2.6). *A. tamarense* PCC 173a shared two bacterial isolates with other dinoflagellates (Fig. 2.3; patterns 2 and 8), and contained a further five unique banding patterns (Fig. 2.3; patterns 10, 11, 12, 13, 15). Two of these belonged to the *Roseobacter* clade, one closely related to *Sagittula stellata* (pattern 10), and the other not related to any defined species (pattern 15; Fig. 2.5; Table 2.5). Two of the remaining isolates belonged to the *Cytophaga* class (patterns 11 and 12; Fig. 2.7;

Table 2.7), whereas the remaining strain a γ-Proteobacterium was closely related to *Glaciecola punicea* (pattern 13; Fig. 2.6; Table 2.6).

Identification of bacteria from *S. trochoidea* NEPCC 15 showed that the culture possessed seven RFLP patterns in lag and log phases, with only 5 patterns detected at stationary phase. This culture had the most transient bacterial population, for example, lag phase contained pattern 18 which subsequently fell below detectable levels, however, pattern 25, not detected in lag and log phases, appeared in stationary phase (Fig. 2.3). The majority of patterns associated with *S. trochoidea* NEPCC 15 were unique to this dinoflagellate with the exception of one strain belonging to the *Roseobacter* clade (patterns 8) previously detected in *A. tamarense* PCC 173a (Fig. 2.5; Table 2.5). Of the remaining eleven unique strains, five were α-Proteobacteria, with two not related to defined species (Fig. 2.4; Table 2.4; patterns 16 and 21). The remainder associated with *Hyphomonas oceanitis* (Fig. 2.4; pattern 17) and *Caulobacter* spp. (Fig. 2.4; pattern 23), with one strain belonging to the *Cytophaga* family (Fig. 2.7; Table 2.7; pattern 22); the final strain, a γ-proteobacterium was closely related to *Pseudomonas stutzeri* (pattern 25; Fig. 2.6; Table 2.6). Unfortunately, as mentioned previously, no sequence data could be obtained from bacteria identified by RFLP analysis as patterns 18, 19, 20 and 24.

Use of DGGE to identify the bacterial flora associated with dinoflagellates

Denaturing gradient gel electrophoresis (DGGE), has been widely used to detect different species present in bacterial communities (Muyzer *et al.*, 1993). In this study, it was used for two purposes; firstly, to identify bacterial strains associated with dinoflagellates without relying on culture and, secondly, to identify any remaining bacteria present in dinoflagellate cultures following treatment to produce axenic cultures (described in Chapter 3).

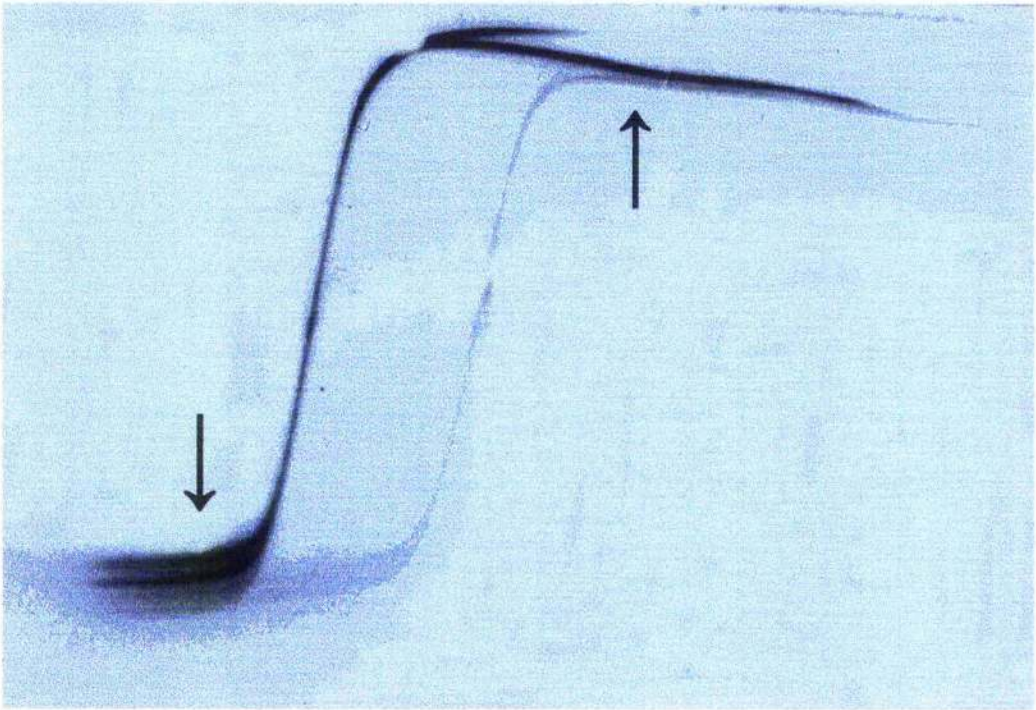
In order to use DGGE for identifying bacteria associated with dinoflagellate cultures, it was necessary to optimise the denaturant gradient to allow good separation of PCR

products. This was achieved by running perpendicular denaturing gels for products from *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, and *S. trochoidea* NEPCC 15, with DNA extracted and amplified from each growth phase.

Figure 2.8, indicates the perpendicular melt curve generated by dinoflagellate *A. lusitanicum* NEPCC 253, when a sample comprising lag, log and stationary phase PCR products was run through a 0 - 100% denaturant gradient. The figure demonstrates the majority of PCR products were denatured by a similar denaturant concentration (prominent sigmoidal curve), but that an additional group required a higher gradient (less prominent sigmoidal curve). The required gradient for analysis of all PCR products from *A. lusitanicum* NEPCC 253 was ascertained using the formula described in the Materials and Methods section, which identified a gradient of 20 - 60% as being required. Similar gradients were determined for the three other dinoflagellates, although each of these cultures only produced one sigmoidal curve.

In initial experiments using DGGE bacterial diversity was investigated at the stationary phase of growth, from six non-axenic dinoflagellates, namely - *A. tamarense* NEPCC 407, UW4, and UW2C, *A. lusitanicum* NEPCC 253 and *A. affine* NEPCC 667 and *S. trochoidea* NEPCC 15. All these dinoflagellates are known PST producers (Cembella *et al.*, 1987; J. Lewis pers comm; Cembella, 1987; Gallacher *et al.* 1997), with the exception of *A. affine* NEPCC 667 whose toxicity has been debated. *S. trochoidea* NEPCC 15, a dinoflagellate of similar size and morphology to *Alexandrium* species, but not previously associated with PST production, was included as a control (see Table 2.1 for strain details). A supposedly 'axenic' culture provided by CCMP - *A. tamarense* CCMP 117, was also examined.

DNA extracted from all cultures including the 'axenic' *A. tamarense* CCMP 117, generated a PCR product after amplification using eubacterial primers 341F and 534R (Primers 2 + 3; Fig. 2.1). PCR products were subsequently analysed by DGGE using the 20 - 60% gradient. Bands were detected at different locations within the denaturant gel, indicating 12 different PCR amplification products were present, most



0%→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→→100%

Figure 2.8 Perpendicular denaturing gradient gel electrophoresis of DNA from *A. lusitanicum* NEPCC 253.

Vertical arrows indicate the positions within the sample identifying the lower and higher limits of denaturant concentration, used for analysis of samples on parallel gels.

of the bands being detected in more than one dinoflagellate culture. Representative bands from each location were excised and subjected to 16S rDNA sequencing (see Appendix 4 for individual DNA sequences). Table 2.8, indicates the dinoflagellate cultures possessing particular bands.

Identification of DGGE bands

Following bi-directional 16S rDNA sequencing, Simrank S_AB values (RDP) were used to classify the twelve DGGE bands. Bacteria from three phylogenetic groups, the α and γ- Proteobacteria, and the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum were detected (Table 2.9; Exp. 1). All dinoflagellates contained α-Proteobacterial isolates related to the *Roseobacter* clade, although, *S. trochoidea* NEPCC 15 was unique in showing another α-Proteobacterial sequence unrelated to *Roseobacter*. γ-Proteobacteria sequences were detected in *A. tamarense* NEPCC 407 and UW4, but not in other cultures, with CFB phylum sequences only seen in *A. lusitanicum* NEPCC 253 and *S. trochoidea* NEPCC 15.

Subsequent phylogenetic analyses were performed on each group of related sequences, using conservative phylogenetic masks which included only regions of unambiguous alignment. Identification of bands 1, 2, 4, 5, 6 & 12 was achieved by comparison with *Roseobacter*-related reference sequences. Phylogenetic analysis was performed using a 141 nucleotide mask to produce a similarity matrix (Table 2.10). Table 2.11 contains a list of abbreviations used within Table 2.10, Table 2.12 and 2.13. The matrix (Table 2.10), indicated bands 1, 2, 5, 6 and 12 were more than 99% similar, differing in only one or two base pair positions within the sequence mask. Bands 1 and 2 were identical within the mask and to *Roseobacter litoralis*, with both bands present in dinoflagellate cultures, *A. tamarense* UW4 and 2C. Although these bands were identical using phylogenetic analysis of the 141 nucleotides, the bands (approx. 200 nucleotides) appeared far enough apart on the gel to be considered different. This would indicate that they differ by a few base pairs within their entire sequences. Bands 5, 6 and 12 were identical to strain 667-12, a bacterium isolated

Dinoflagellate	Bands identified											
	1	2	3	4	5	6	7	8	9	10	11	12
A. tamarense NEPCC 407		x	x	x	x	x		x	x			x
A. tamarense UW4	x	x						x	x			
A. tamarense UW2C	x	x										
A. tamarense CCMP 117				x								
A. lusitanicum NEPCC 253	x									x		
A. affine NEPCC 667		x		x	x	x						x
S. trochoidea NEPCC 15			x	x		x	x				x	

Table 2.8 DGGE bands associated with different dinoflagellates at the stationary phase of growth.

Dinoflagellate	Bacterial sequences detected							
	Roseobacter related bacteria		α-Proteobacteria outwith the *Roseobacter* clade		γ-Proteobacteria		CFB phylum bacteria	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
A. tamarense NEPCC 407	*	*		*	*	*		*
A. tamarense UW4	*	nt		nt	*	nt		nt
A. tamarense UW2C	*	nt		nt		nt		nt
A. tamarense CCMP 117	*	nt		nt		nt		nt
A. lusitanicum NEPCC 253	*	*		*			*	*
A. affine NEPCC 667	*	nt		nt		nt		nt
S. trochoidea NEPCC 15	*	*	*	*		*	*	*

Table 2.9 Summary of bacterial sequences detected in dinoflagellate cultures, at stationary phase by DGGE. Experiment 1 depicts data from the initial study using stationary phase cultures of *A. tamarense* NEPCC 407, UW4, UW2C and CCMP 117, *A. lusitanicum* NEPCC 253, *A. affine* NEPCC 667 and *S. trochoidea* NEPCC 15. Experiment 2 shows results from the more detailed experiment using *A. tamarense* NEPCC 407 and CCMP 117, *A. lusitanicum* NEPCC 253 and *S. trochoidea* NEPCC 15.
* = representatives detected
nt = not tested

Band 1															
Band 2.	100.0														
Band 4.	97.2	97.2													
Band 5.	99.3	99.3	97.9												
Band 6.	99.3	99.3	97.9	100.0											
Band 12	99.3	99.3	97.9	100.0	100.0										
S34	97.2	97.2	97.2	97.9	97.9	97.9									
E-37	99.3	99.3	96.5	98.6	98.6	98.6	96.5								
RosDeni	99.3	99.3	96.5	98.6	98.6	98.6	96.5	98.6							
RosLitor	100.0	100.0	97.2	99.3	99.3	99.3	97.2	99.3	99.3						
RosAlgoc	98.6	98.6	97.2	99.3	99.3	99.3	97.2	97.9	97.9	98.6					
UniAlph2	96.5	96.5	96.5	95.7	95.7	95.7	95.0	95.7	95.7	96.5	95.0				
223Lance	99.3	99.3	96.5	98.6	98.6	98.6	96.5	98.6	98.6	99.3	97.9	95.7			
Str36	98.6	98.6	97.2	99.3	99.3	99.3	97.2	99.3	97.9	98.6	98.6	95.0	97.9		
407-20	98.6	98.6	97.2	99.3	99.3	99.3	97.2	97.9	97.9	98.6	100.0	95.0	97.9	98.6	
667-12	99.3	99.3	97.9	100.0	100.0	100.0	97.9	98.6	98.6	99.3	99.3	95.7	98.6	99.3	99.3

Table 2.10 Similarity matrix for DGGE bands subsequently identified as *Roseobacter* related α-proteobacterial species isolated from dinoflagellates *A. lusitanicum* NEPCC 253, *A.* affine NEPCC 667, *S. trochoidea* NEPCC 15, *A. tamarense* UW4, UW2C, CCMP 117 and NEPCC 407 harvested in stationary phase.

For abbreviations, see Table 2.11

Abbreviation used in similarity matrix	Isolate identification
S34	Sargasso Sea isolate S34
E-37	environmental gene clone E37
RosDeni	*Roseobacter denitrificans*
RosLitor	*Roseobacter litoralis*
RosAlgoc	*Roseobacter algicola*
UniAlph2	*Octadecabacter antarcticus* sp. 307
223Lance	gall symbiont of red alga *Prionitis lanceolata* U37762
Str36	isolate Nielsen 36 (RDP only)
407-20	*A. tamarense* NEPCC 407 isolate 407-20
667-12	*A. affine* NEPCC 667 isolate 667-12
R.rubrum3	*Rhodospirillum rubrum*
R.phmetric	*Rhodospirillum photometricum*
Aqsp.iters	*Aquaspirillim itersonii*
Azs.lipofe	*Azospirillum lipoferum*
Azs.brazil	*Azospirillum brasilense*
Azs.halprf	*Azospirillum haloproferens*
env.MC77	environmental gene clone MC77
R.centenum	*Rhodocista cetenaria*
Rhc.sp1	*Rhodocistra* sp. MT-SP-2
Azs.sp4	*Azospirillum* sp. DSM4834
Azs.sp1	*Azospirillum* sp. DSM4835
Azs.amazon	*Azospirillum amazonia*
R.molischi	*Rhodospirillum molischianum*
R.fulvum	*Rhodospirillum fulvum*
Mag.gryphi	*Magnetospirillum gryphiswaldense*
Mag.magne2	*Magnetospirillum magnetotacticum*
R.sodomens	*Rhodospirillum sodomense*
R.salinarm	*Rhodospirillum salinarum*
scripp131	*S. trochoidea* NEPCC 15 clone library number 131

Abbreviation used in similarity matrix	Isolate identification
scripp4	*S. trochoidea* NEPCC 15 clone library number 4
F.aquatile	*Flavobacterium aquatile*
Cy.succini	*Cytophaga succinicans*
Cy.aquatil	*Cytophaga aquatile*
Cy.marina	*Cytophaga marinoflava* ATCC 19326
Flc.glomer	*Flectobacillus glomeratus*
Flx.marit2	*Flexibacter maritimus*
Ves.antarc	*Antarcticum vesiculatum*
Cap.canim	*Capnocytophaga canimatus*
OM271	environmental gene clone OM271
Cy.lytica	*Cytophaga lytica*
C.uligino	*Cytophaga uliginosa*
C.marino	*Cytophaga marinoflava* M58770
C.marino2	*Cytophaga marinoflava* NCIMB 397
env.agg13	environmental gene clone AGG13
Cy.latercu	*Cytophaga latercula*
F.salegens	*Flavobacterium salegenes*

Table 2.11 Abbreviations used in Tables 2.10, 2.12 and 2.13

previously from *A. affine* NEPCC 667, which grouped closely with *Sagittula stellata* and *Antarctobacter heliothermus* (Fig. 2.5). Again bands were considered identical using phylogenetic analysis, but they appeared at different positions within the gel.

Band 4 was closely related to other bands within the *Roseobacter* clade (96 - 98%) and was detected in *A. tamarense* NEPCC 407, *A. affine* NEPCC 667 and *S. trochoidea* NEPCC 15 (Table 2.8). This band was also detected in *A. tamarense* CCMP 117 which was provided by the CCMP as an axenic culture. Band 3 (not included within the analysis), was not sequenced successfully with both primers, hence, it was only possible to identify it as a member of the *Roseobacter* clade.

Band 7 was also identified as an α-Proteobacterium, detected only in *S. trochoidea* NEPCC 15 (Table 2.8). It was classified as belonging to the *Rhodospirillum* assemblage, most closely, but not strongly associated, with *Aquaspirillum itersonii* (94%; Table 2.12).

Bands 10 & 11 were identified as members of the CFB phylum of bacteria, specifically associated with the *Cytophaga* subgroup. Searches for related isolates indicated that those from the *Scrippsiella* unculturable clone library (Rappé *et al.*, in prep), were most similar, with no previously defined strains being closely associated (Table 2.13). Excluding ambiguous nucleotides, bands 10 and 11 were 95% similar with 144 bases being included in the analysis. The sequences differed in 7 nucleotide positions within the masked sequence, with band 11 being most similar to *Scrippsiella* clones.

Bands 8 & 9 were identified as γ-Proteobacteria, showing 97% similarity to each other and ca. 95% similarity to members of the *Methylomonas, Oceanospirillum*, and *Pseudomonas* groups of bacteria. With the short 200bp fragment of 16S rRNA gene sequence analysed by DGGE, a single closely related group of sequences could not be identified for these bands.

Band 7.																		
R.rubrum3	91.1																	
R.phmetric	90.4	99.3																
Aq.sp.iters	93.8	91.8	91.1															
Azs.lipofe	91.1	89.0	88.4	94.5														
Azs.brazil	91.1	90.4	89.7	93.2	98.6													
Azs.halprf	91.1	89.0	88.4	94.5	100.0	98.6												
env.MC77	91.1	90.4	89.7	95.9	95.2	93.8	95.2											
R.centenum	91.8	89.7	89.7	95.2	97.9	96.6	97.9	93.2										
*Rhc.*sp1	91.8	89.7	89.7	95.2	97.9	96.6	97.9	93.2	100.0									
*Azs.*sp4	90.4	88.4	88.4	93.8	96.6	95.2	96.6	91.8	98.6	98.6								
*Azs.*sp1	91.1	89.0	88.4	94.5	97.3	95.9	97.3	92.5	97.9	97.9	99.3							
Azs.amazon	91.1	91.8	91.1	93.2	97.3	98.6	97.3	93.8	95.2	95.2	93.8	94.5						
R.molischi	91.1	89.7	89.0	95.2	94.5	93.2	94.5	97.9	93.8	93.8	92.5	93.2	93.2					
R.fulvum	91.1	89.7	89.0	95.2	94.5	93.2	94.5	97.9	93.8	93.8	92.5	93.2	93.2	100.0				
Mag.gryphi	91.1	89.7	89.0	95.2	94.5	93.2	94.5	97.9	93.8	93.8	92.5	93.2	93.2	100.0	100.0			
Mag.magne2	87.7	87.0	87.7	92.5	95.2	93.8	95.2	93.2	95.2	95.2	96.6	96.6	92.5	93.8	93.8	93.8		
R.sodomens	93.2	92.5	91.8	92.5	90.4	91.8	90.4	95.2	89.7	89.7	88.4	89.0	91.8	94.5	94.5	94.5	89.7	
R.salinarm	93.2	92.5	91.8	92.5	90.4	91.8	90.4	95.2	89.7	89.7	88.4	89.0	91.8	94.5	94.5	94.5	89.7	100

Table 2.12 Similarity matrix for DGGE band 7 subsequently identified as an α-proteobacteria outwith the *Roseobacter* clade, isolated from *S. trochoidea* NEPCC 15 harvested in stationary phase.

For abbreviations, see Table 2.11

Band 11.																			
Band 10.	95.1																		
scripp131	90.3	88.2																	
scripp4	90.3	88.2	100.0																
F.aquatile	87.5	84.7	94.4	94.4															
Cy.succini	86.1	82.6	93.1	93.1	95.8														
Cy.aquatil	88.2	84.7	93.8	93.8	94.4	97.2													
Cy.marina	87.5	85.4	93.1	93.1	90.3	88.9	88.9												
Flc.glomer	90.3	86.8	91.7	91.7	89.6	88.2	91.0	91.0											
Flx.marit2	87.5	85.4	93.1	93.1	90.3	88.9	88.9	100.0	91.0										
Ves.antarc	90.3	86.8	94.4	94.4	92.4	91.0	93.8	91.0	97.2	91.0									
Cap.canim	85.4	85.3	90.3	90.3	88.2	87.5	88.2	88.9	84.0	88.9	86.1								
OM271	88.2	87.5	93.8	93.8	93.1	91.0	91.0	94.4	87.5	94.4	90.3	92.4							
Cy.lytica	86.8	85.4	91.0	91.0	90.3	88.9	89.6	89.6	88.9	89.6	88.9	91.7	92.4						
C.uliginо	87.5	86.1	91.7	91.7	88.2	86.8	88.2	90.3	89.6	90.3	90.3	89.6	90.3	93.8					
C.marino	85.4	82.6	91.0	91.0	88.2	86.8	86.8	91.7	86.1	91.7	88.9	93.1	91.7	93.1	94.4				
C.marino2	86.1	84.7	90.3	90.3	89.6	88.2	88.9	88.9	88.2	88.9	88.2	91.0	91.7	99.3	93.1	92.4			
env.agg13	88.2	87.5	96.5	96.5	91.0	89.6	90.3	92.4	88.2	92.4	91.0	91.7	93.1	91.7	93.1	91.7	91.0		
Cy.latercu	88.2	88.9	91.7	91.7	91.0	90.3	88.2	88.9	87.5	88.9	90.3	87.5	91.0	87.5	88.9	87.5	86.8	93.1	
F.solegens	88.9	88.2	90.3	90.3	91.0	90.3	87.5	87.5	88.2	87.5	88.2	84.7	90.3	87.5	86.8	85.4	86.8	88.2	93.1

Table 2.13 Similarity matrix for DGGE bands subsequently identified as belonging to the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum of bacteria, specifically asociated with the *Cytophaga* subgroup, isolated from dinoflagellates *A. lusitanicum* NEPCC 253 and *S. trochoidea* NEPCC15, harvested in stationary phase.

For abbreviations, see Table 2.11

To determine if the bacteria identified above were present at other stages of the dinoflagellate growth phase, and if the original data was reproducible, cultures of dinoflagellates *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and *S. trochoidea* NEPCC 15 were examined in more detail. For additional control purposes a non-toxic strain, *A. tamarense* PCC 173a, was also included.

Figure 2.9, is an example of the DGGE patterns generated from the three growth phases of dinoflagellate cultures *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407. Different profiles were detected from each dinoflagellate, although profiles remained constant throughout the growth cycle. Unique profiles were also determined for *A. tamarense* PCC 173a and *S. trochoidea* NEPCC 15. It was noted, however, that certain bands appearing at identical positions within different phase samples of each dinoflagellate varied in intensity, e.g. bands 4, 13 and 22 in *A. tamarense* NEPCC 407 (Fig. 2.9).

Sequence analysis of excised bands using RDP again identified the presence of bacteria from the CFB phylum, with α and γ-Proteobacteria sub-phylum isolates (particularly *Roseobacter* species). Phylogenetic analyses were again performed using conservative masks, but these were created for each individual DGGE sequence to eliminate areas of uncertain alignment and ambiguous nucleotide positions, whilst maximising the quantity of data available for each sequence. The length of most masks varied between 122 and 200 nucleotide positions, with identification of closest reference sequences to each of the excised bands presented in Tables 2.14 - 2.17. However, three sequences 173a/16, 173a/14 and SCRIPP/27 generated mask lengths of 73, 76 and 75 respectively indicating that little significance could be attributed from these sequence similarities.

DGGE analysis of *A. lusitanicum* NEPCC 253 indicated the presence of *Roseobacter* related species (253/33, 34, 35 and 52; Table 2.14) and 2 distinct α-Proteobacterial species (*Hyphomonas* 253/29 and 31; Table 2.15). Bands 253/34 and 35 gave 100% sequence similarity to isolates identified using the culture-based technique (RFLP

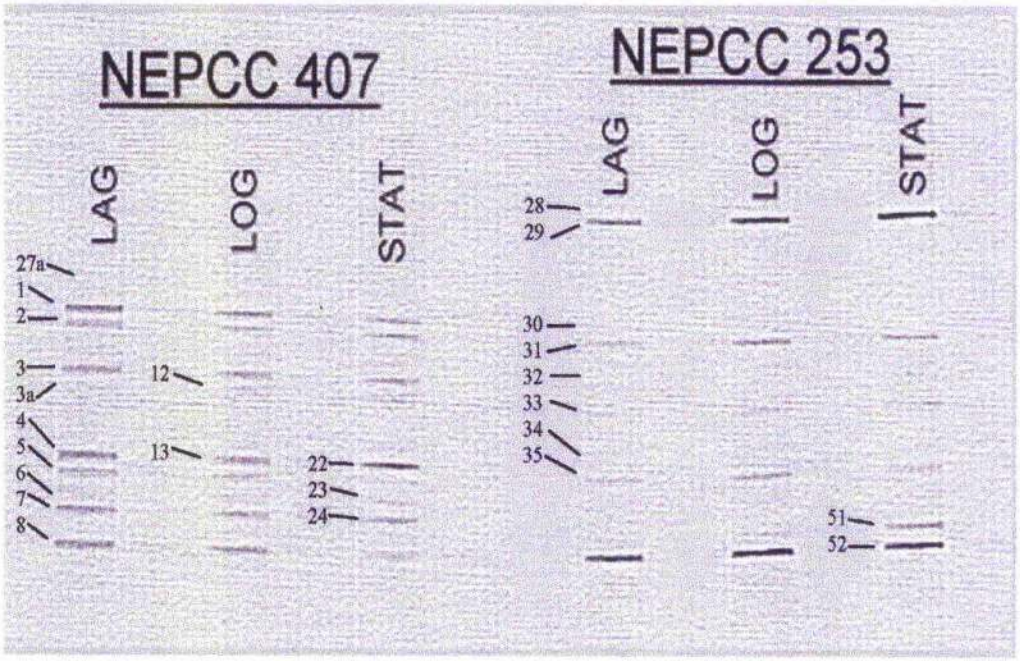


Figure 2.9 Parallel denaturing gradient gel of PCR amplified DNA from each growth phase from dinoflagellate cultures *A. tamarense* NEPCC 407 and *A. lusitanicum* NEPCC 253. Numbers correspond to the different bands which were excised and sequenced.

Dinoflagellate	Band Number	% Similarity	Reference sequence
A. lusitanicum NEPCC 253	253/33	95.4	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*, *Sagittula stellata*, isol. Nielsen 36, *Marinosulfunomonas* sp.
	253/34 + 35	100	No defined species (RFLP pattern 8).
	253/52	99.2	No defined species (RFLP pattern 3)
A. tamarense NEPCC 407	407/3a + 5	99.2	No defined species (RFLP pattern 8)
	407/4	91	*Roseobacter algicola*
	407/6	98.4	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*, *Sagittula stellata*, isol. Nielsen 36, *Marinosulfunomonas* sp.
	407/7	100	253-16
	407/8	100	No defined species (RFLP pattern 3)
	407/12 + 22	100	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*
	407/13	94.3	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*, *Sagittula stellata*, isol. Nielsen 36, *Marinosulfunomonas* sp.
	407/24	99.2	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*, *Sagittula stellata*, isol. Nielsen 36, *Marinosulfunomonas* sp.
	407/27a	100	*A. heliothermus* related strains (RFLP pattern 10), *Marinosulfunomonas* sp.
A. tamarense PCC 173a	173a/1	94.3	*A. heliothermus* related strains (RFLP pattern 10), *Marinosulfunomonas* sp.
	173a/2 + 9	100	No defined species (RFLP pattern 2)
	173a/3	92.7	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*, *Sagittula stellata*, isol. Nielsen 36, *Marinosulfunomonas* sp.

Dinoflagellate	Band Number	% Similarity	Reference sequence
A. tamarense PCC 173a	173a/4	100	*A. heliothermus* related strains (RFLP pattern 10), *Marinosulfunomonas* sp.
	173a/7	93.3	*A. heliothermus* related strains (RFLP pattern 10), *Marinosulfunomonas* sp.
	173a/8	97.6	*A. heliothermus* related strains (RFLP pattern 10), *Marinosulfunomonas* sp., isol. Nielsen 36, *Sagittula stellata*, SRF3, *Marinosulfunomonas* sp.
	173a/15	96.6	*R. gallaeciensis* related strains (RFLP pattern 9)
	173a/16		poor sequence
	173a/17	94.2	*R. gallaeciensis* related strains (RFLP pattern 9)
	173a/18	100	No defined species (RFLP pattern 2), *R. gallaeciensis* related strains (RFLP pattern 9)
	173a/19	100	*A. heliothermus* related strains (RFLP pattern 10)
	173a/20	99	*A. heliothermus* related strains (RFLP pattern 10), *R. gallaeciensis* related strains (RFLP pattern 9), No defined species (RFLP pattern 8)
	173a/23	98.4	No defined species (RFLP pattern 2)
	173a/24	87.2	*Roseobacter* sp.
	173a/25	95.3	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*, *Sagittula stellata*, isol. Nielsen 36, *Marinosulfunomonas* sp.
S. trochoidea NEPCC 15	scripp/26	96.2	*R. gallaeciensis* related strains (RFLP pattern 9), *A. heliothermus* related strains (RFLP pattern 10), *R. algicola*, *Sagittula stellata*, isol. Nielsen 36, *Marinosulfunomonas* sp.

Table 2.14 Degree of similarity of denaturing gradient gel electrophoresis (DGGE) bands to the *Roseobacter* clade from dinoflagellates *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, and *S. trochoidea* NEPCC 15, when compared to reference sequences.

Dinoflagellate	Band Number	% Similarity	Reference sequence
A.lusitanicum NEPCC 253	253/29b	93.9	*Hyphomonas oceanitis* related isolates (RFLP pattern 17)
	253/31	100	*Hyphomonas oceanitis* related isolates (RFLP pattern 17)
A. tamarense NEPCC 407	407/2	97.7	*Hyphomonas oceanitis* related isolates (RFLP pattern 17)
A. tamarense PCC 173a	173a/21	97.9	No defined species (RFLP pattern 16)
	173a/22	95	*Sphingomonas* sp. SW54
	173a/25	100	*Blastobacter natatorius*
S. trochoidea NEPCC 15	SCRIPP/31	91.3	*Agrobacterium stellulatum* related isolates (RFLP pattern 4)

Table 2.15. Degree of similarity of denaturing gradient gel electrophoresis (DGGE) bands grouped as α-Proteobacteria related sequences, but outwith the *Roseobacter* clade from dinoflagellates *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, and *S. trochoidea* NEPCC 15 when compared to reference sequences.

Dinoflagellate	Band Number	% Similarity	Reference sequence
A. tamarense NEPCC 407	407/3		not classified, poor sequence
A. tamarense PCC 173a	173a/11	97.2	*Rahnella aquatilis*
S. trochoidea NEPCC 15	SCRIPP/28	94	*Pseudomonas stutzeri*
	SCRIPP/29	96	*Pseudomonas syringae* ATCC 19310

Table 2.16. Degree of similarity of denaturing gradient gel electrophoresis (DGGE) bands grouped as γ-Proteobacteria related sequences from dinoflagellates *A. tamarense* NEPCC 407and PCC 173a, and *S. trochoidea* NEPCC 15 when compared to reference sequences.

Dinoflagellate	Band Number	% Similarity	Reference sequence
A.lusitanicum NEPCC 253	253/30	100	*Gelidibacter algens* related isolates
A. tamarense NEPCC 407	407/1	95.1	Marine psychrophile IC076
A. tamarense PCC 173a	173a/10	99.1	No defined species (RFLP pattern 12)
	173a/14		poor sequence
S. trochoidea NEPCC 15	SCRIPP/27		poor sequence

Table 2.17. Degree of similarity of denaturing gradient gel electrophoresis (DGGE) bands grouped as *Cytophaga/Flavobacter/Bacteroides* related sequences from dinoflagellates *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, and *S. trochoidea* NEPCC 15 when compared to reference sequences.

pattern 8; Fig. 2.5), with band 253/52 differing by one or two nucleotides within the phylogenetic mask from the other group of cultured *Roseobacter* isolates which were identified (RFLP pattern 3; Fig. 2.5). A CFB phylum-related species (253/30; Table 2.17) was also detected in *A. lusitanicum* NEPCC 253, most closely related to *Gelidibacter algens*, with 100% sequence similarity to sequences identified in culture-based experiments (Fig. 2.7; ALUS253_6). An α-Proteobacterial sequence outwith the *Roseobacter* clade, classified potentially as a *Hyphomonas* species, was also detected which was not identified in previous DGGE experiments or RFLP analyses.

Analysis of DGGE sequences from *A. tamarense* NEPCC 407 showed that *Roseobacter* related sequences isolated from consecutive positions on the gel had high sequence similarity to previously detected cultured isolates (407/3a - 8; Fig. 2.9; Table 2.14). Nevertheless, a wide gradient separated the *Roseobacter* related sequences (25%), indicating the sensitivity of the system in detecting minimal differences in sequence. Identification of like-position bands from different growth phases (407/4, 13 & 22; Fig. 2.9; Table 2.14), confirmed the consistency of the banding position, although there was not 100% similarity between sequences. This is probably due to errors in sequence alignment caused by unresolvable bases, rather than actual differences in sequences. α-Proteobacteria sequences related to *Scrippsiella* unculturable clone library isolates and *Hyphomonas* species were also detected in *A. tamarense* NEPCC 407 (407/2; Table 2.15). A γ-Proteobacteria related sequence was also identified, although similarity to known reference sequences was low due to the quality of the sequence (407/3; Table 2.16). A band identified as belonging to the CFB phylum, not detected previously, was most closely related to the marine psychrophile IC076 (a member of the *Flavobacter* subgroup; 407/1; Table 2.17).

Sequences obtained from *A. tamarense* PCC 173a again identified several *Roseobacter* related sequences, previously detected in *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407, spanning a large distance through the gel (173a/1-4,

7-9, 15-20, 23-25; Table 2.14). Several other α-Proteobacterial species, including a *Scrippsiella* unculturable clone library isolate, similar to that identified in *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407, were identified. Two similar isolates, *Blastobacter natatorius* and *Sphingomonas* sp SW.54, both seawater isolates, were also identified in *A. tamarense* PCC 173a and not detected in other cultures (173a/22 and 25; Table 2.15). A γ-related sequence most closely associated with *Rahnella aquatilis* was detected (173a/11; Table 2.16), with a CFB related isolate most similar to a marine psychrophile, positioned within the *Cytophaga* subgroup also identified, with good sequence similarity (173a/10; Table 2.17).

Bands excised from *S. trochoidea* NEPCC 15 indicated a vastly different microflora compared to the other dinoflagellates, with only one band related to the *Roseobacter* clade (SCRIPP/26; Table 2.14). Another α-Proteobacterial sequence belonging to the *Agrobacterium* genus, grouping with certain bacteria isolated previously from a toxic *A. tamarense* (Kopp *et al.*, 1997; PTB1 and PTB2), but with low similarity (91%), was also identified (SCRIPP/31; Table 2.15). Two γ-Proteobacteria sequences were detected in the *Scrippsiella* culture, closely related to *Pseudomonas stutzeri* and *Pseudomonas syringae* (SCRIPP/28 and 29; Table 2.16), although not detected in initial DGGE experiments. Identification of CFB phyla isolates was also apparent in *S. trochoidea* NEPCC 15, (SCRIPP/27; Table 2.17), although the quality of the sequences were poor making identification of reference sequences impossible.

Comparison of DGGE results generated from stationary growth phase dinoflagellate samples from both experiments (Table 2.9), indicated that all bacterial classes and sub-classes detected in Experiment 1 were confirmed in Experiment 2. However, the second experiment identified some bacterial sequences not previously detected in the initial experiment. *A. lusitanicum* NEPCC 253 was previously not found to contain α-Proteobacterial sequences other than *Roseobacter* related isolates. However, both DGGE and RFLP analyses performed during Experiment 2 confirmed their presence, with *Roseobacter* related sequences and CFB isolates also reaffirmed. *A. tamarense* NEPCC 407 was initially reported to contain *Roseobacter* and γ-Proteobacterial

sequences, although Experiment 2 also identified the presence of α-Proteobacteria outwith the *Roseobacter* clade and CFB phylum isolates. RFLP also confirmed the presence of α-Proteobacterial isolates, but not the presence of CFB isolates.

S. trochoidea NEPCC 15 did not appear to contain γ-Proteobacteria in Experiment 1, although these were identified in the subsequent DGGE analysis, with RFLP confirming all identifications, except for CFB phylum isolates.

Table 2.18 summarises the results gained from dinoflagellates *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, and *S. trochoidea* NEPCC 15, when culture-based and non culture-based identification systems (RFLP and DGGE) were compared. In the majority of cases, the systems complemented each other, with all *A. lusitanicum* NEPCC 253 identifications detected by both systems and confirmation of *Roseobacter* related sequences in all dinoflagellates at the three growth phases. However, certain anomalies were apparent, where DGGE identified the presence of isolates, whereas RFLP did not. RFLP failed to detect α-Proteobacteria outwith the *Roseobacter* clade in lag and log phase samples of *A. tamarense* NEPCC 407, and in all phases of *A. tamarense* PCC 173a. CFB phylum isolates were also not detected in any growth phase of *A. tamarense* NEPCC 407, or in lag and stationary phases of *S. trochoidea* NEPCC 15, using RFLP. DGGE also identified γ-Proteobacteria in all phases of *S. trochoidea* NEPCC 15, with RFLP only identifying such isolates at stationary phase.

Dinoflagellate		*Roseobacter* related bacteria		α-Proteobacteria outwith the *Roseobacter* clade		γ-Proteobacteria		CFB phylum bacteria	
		RFLP	DGGE	RFLP	DGGE	RFLP	DGGE	RFLP	DGGE
A.lusitanicum NEPCC 253	lag	*	*	*	*	nd	nd	*	*
	log	*	*	*	*	nd	nd	*	*
	stat	*	*	*	*	nd	nd	*	*
A. tamarense NEPCC 407	lag	*	*	nd	*	*	*	nd	*
	log	*	*	nd	*	*	*	nd	*
	stat	*	*	*	*	*	*	nd	*
A. tamarense PCC 173a	lag	*	*	nd	*	*	*	*	*
	log	*	*	nd	*	*	*	*	*
	stat	*	*	nd	*	*	*	*	*
S. trochoidea NEPCC 15	lag	*	*	*	*	nd	*	nd	*
	log	*	*	*	*	nd	*	*	*
	stat	*	*	*	*	*	*	nd	*

Table 2.18 A comparison of the bacterial classes detected using RFLP and DGGE from dinoflagellate cultures *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, and *S. trochoidea* NEPCC 15.
* = detected
nd = not detected

DISCUSSION

Molecular methods have been used extensively to determine the phylogenetic diversity of bacterial species present within marine environments. However, the use of both culture-based and non culture-based techniques in one investigation within a community is not well documented. The object of this study was to investigate bacterial community diversity in toxin-producing dinoflagellate cultures, with the aim of detecting differences between the microflora of toxic and non-toxic dinoflagellate cultures.

Previous attempts to identify bacteria associated with dinoflagellate cultures have investigated both toxic *Alexandrium* and *Prorocentrum* species (Lafay *et al.*, 1995; Doucette, 1995; Gallacher *et al.*, 1997; Kopp *et al.*, 1997; Prokic *et al.*, 1998). Recently, Prokic *et al.* (1998) isolated bacteria and used cloning and sequencing to identify the microflora associated with cultures of *Prorocentrum lima*, derived from the same original culture but maintained in different laboratories. Their study indicated that the two cultures did not retain the same bacterial microflora, although bacteria related to the *Roseobacter* genus in the α-Proteobacteria were dominant in both cultures. One culture appeared to contain only α-Proteobacteria, while the other culture sustained a more complex flora comprising α-Proteobacteria, γ-Proteobacteria, *Cytophaga-Flavobacter-Bacteroides* phylum isolates and low G+C Gram-positive isolates. They identified bacterial isolates from both these cultures with significant sequence homology to *Roseobacter algicola*, a bacterium initially identified from the original dinoflagellate culture from which these secondary cultures were derived. However, cultures also contained bacteria which were phylogenetically affiliated with the *Roseobacter* clade, but were distinct from this original isolate. Prokic *et al.* concluded that toxic *P. lima* could maintain a large spectrum of bacteria, although no explanation was offered as to why the microflora of the two cultures had not remained similar.

The ability of other toxic dinoflagellates, in the form of *Alexandrium* strains, to sustain a diverse microflora with *Roseobacter* related species being dominant, was indicated in the current study, with at least two bacterial phyla or subphyla detected

in both cultures tested. The non-toxic *Alexandrium* culture was found to have different bacteria present within the microflora; again, *Roseobacter* related species predominated. Only the dinoflagellate species not associated with PST production, *S. trochoidea* NEPCC 15, appeared not to harbour large numbers of *Roseobacter* related isolates. This may indicate that a species-specific association exists between *Roseobacter* related strains and certain algal species, including *Prorocentrum* and *Alexandrium* species.

All *Alexandrium* cultures contained several *Roseobacter* related isolates, with certain strains isolated from toxic and non-toxic *Alexandrium* cultures showing 100% sequence similarity. Bacterial isolates from *S. trochoidea* NEPCC 15 within the *Roseobacter* clade did not group closely with these isolates. However, certain bacterial isolates from the non-toxic *Alexandrium* culture were grouped along with these isolates. Examination of α-Proteobacteria outside the *Roseobacter* clade indicated that all bacteria from toxic dinoflagellates clustered together, distinct from non-toxic bacterial isolates, and grouped with isolates from a previous study identifying bacteria from toxic *Alexandrium* cultures (Kopp *et al.*, 1997). All γ-Proteobacteria and CFB isolates were distinct, with no overlap of bacteria from toxic and non-toxic dinoflagellate strains.

The use of colony morphology and RFLP to assess the microflora of dinoflagellates

The 500 cellular clones isolated following serial dilution from the four dinoflagellate cultures in the current study were streamlined into manageable groups to allow group representatives to be further characterised. Differences in colony morphology was initially considered as selection criteria. However, as reports indicate, variability within morphotypes is uncertain and probably under-estimated, especially in common morphotypes (LeBaron *et al.*, 1998), it was decided that colony morphology was not specific enough to categorise the isolates. Since successful reports combining colony morphology and RFLP analysis of PCR-amplified rRNA genes to classify bacterial

isolates have been published (Suzuki *et al.* 1997; LeBaron *et al.* 1998), it was decided to investigate this approach to characterise our clones. Suzuki *et al.* (1997) used the tetrameric restriction endonuclease *Hae III* on PCR-amplified 16S rRNA genes to categorise bacteria isolated from seawater and obtained sufficient information from single enzyme digests of full length 16S rDNA sequences to classify bacterial isolates successfully.

In the current study, 500 isolates were divided into 25 groups based on RFLP and colony morphology and representatives of each group were further characterised using 16S sequencing. The inability of colony morphology alone to categorise bacteria was noted within the study. Although, a single colony morphology gave two different RFLP patterns, subsequent sequence analysis identified both as *Roseobacter* related isolates with relatively close sequence similarity. This indicated the potential of RFLP analysis to discriminate between closely related bacteria. However, RFLP analysis also grouped together two dissimilar colony morphotypes which on sequence analysis were also classified as *Roseobacter* related strains, but not closely associated. This indicated that restricting our RFLP analysis to one enzyme was not ideal, and that the use of three or more tetrameric restriction enzymes as proposed previously by Moyer *et al.* (1996) may be necessary to distinguish definitively between groups of closely related bacteria.

Within the current study, eight different RFLP patterns were subsequently classified as *Roseobacter*-clade related isolates, with most patterns dissimilar to known species. This provides strong evidence that these bacterial isolates are new strains belonging to a different genus, although further phenotypic and genotypic tests would be required to confirm this. The study by Prokic *et al.* (1998) on *P. lima* confirmed that previously identified bacteria were still present within cultures up to three years later. The current study also confirms the presence of certain bacteria which were isolated previously (Gallacher *et al.*, in prep.). In the study by Gallacher *et al.* bacteria were isolated from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 and their capability for sodium channel blocking toxin production tested as well as subsequent

identification of isolates using 16S rDNA sequencing. The current study shows that the majority of previously isolated strains are still present within dinoflagellate cultures, with the exception of 253-16, 253-19/253-20 and 407-13. This indicates that the association between these isolates and the dinoflagellate cultures was not stable or that levels of isolates fell below a detectable limit. Of the bacteria characterised previously by Gallacher *et al.* many were shown to produce PST (confirmed using HPLC and CE-MS) including isolates 407-2 and 253-11 (Gallacher *et al.*, 1997). Interestingly, certain bacteria from the non-toxic *A. tamarense* PCC 173a also clustered with these toxic strains. However, this was not the case for *S. trochoidea* NEPCC 15 bacterial isolates.

The use of DGGE to assess the microflora of dinoflagellates

Several researchers have used DGGE to estimate the genetic diversity of microbial communities in natural habitats, by taking the complexity of the DGGE 'fingerprint' as a measurement of community diversity (Muyzer *et al.*, 1993; 1994; Wawer and Muyzer, 1995; Ferris *et al.*, 1996; Teske *et al.*, 1996; Vallaeys *et al.*, 1997). In addition, the subsequent use of DNA sequencing of individual fragments to infer phylogenetic relationships within microbial communities allowing DGGE bands to be grouped alongside reference sequences, is well documented (Muyzer *et al.*, 1995; 1995; Kowalchuk *et al.*, 1997; Teske *et al.*, 1998). In the current study, bands excised from DGGE gels allowed identification of bacteria from the four phyla/subphyla previously shown to be present from bacterial culture and RFLP analysis. However, although DGGE confirmed the presence of bacteria from α-Proteobacteria outside the *Roseobacter* clade and within the γ-Proteobacteria, sequence similarities to cultured isolates were not conclusive.

This must, however, be put into context; due to the length of the sequence fragments obtained from DGGE analyses, as there is a restricted ability to identify isolates which is an accepted drawback of the system. Therefore, it was expected that DGGE percentage similarities would be lower than values obtained from RFLP sequences.

Although probably due to the length of the sequences, it is also possibly due to the primers selected, as these were designed to encompass a hypervariable region of the rRNA gene in order to identify as much sequence variation as possible. This is a known shortfall when using DGGE, therefore, it would be anticipated that the sequence similarities shown here would be artificially low compared to similarities of full sequences had these been available.

It would be beneficial for future work using DGGE to use longer fragments, nearer the separation limit of approximately 500bp (Myers *et al.* 1985; Muyzer *et al.* 1994). This would generate more sequence information than in this study and should be considered in future investigations using this technique. The use of a larger fragment in subsequent analyses by Vallaeys *et al.* (1997) indicated that certain DGGE profiles of small DNA fragments could not be suitably resolved. This indicated the limits of DGGE analysis in the measure of diversity in complex microbial populations when using universal primers to amplify small 16S rRNA gene fragments. Vallaeys *et al.* stated that the number of fragments visualised on a gel may underestimate the actual diversity of a community, and should be considered as a lower limit of estimation of the total numbers of species present. Although these shortfalls were recognised before the present study was instigated, it was decided to pursue work with the primer set which generated the 200bp fragment, as the method has been successfully used within a range of habitats (Muyzer *et al.*, 1993; 1994; Wawer and Muyzer, 1995; Ferris *et al.*, 1996; Teske *et al.*, 1996; Vallaeys *et al.*, 1997).

In this current study, it was also noted that bands excised from like positions on the DGGE gel from different growth phases, did not generate the anticipated 100% similarity to each other. This was probably due to errors in sequence alignment caused by unresolvable bases, rather than actual sequence differences. However, differently positioned bands were identified as 100% similar to each other. This is always a problem when large numbers of sequences require identification. If sequences cannot be analysed together as this will dramatically reduce the quantity of reference sequences included in the analyses. Therefore, all sequences were

compared separately to related reference sequences, resulting in different size masks being generated between isolates and allowing slightly different sequence similarities to be generated.

DGGE also failed to detect γ-Proteobacteria from *A. tamarense* NEPCC 407 and PCC 173a, although they were detected using cell isolation and RFLP analysis. Assessment of the ability of PCR coupled with DGGE primers to amplify these isolates was investigated by attempting to amplify the *Alteromonas macleodii* type strain and γ-Proteobacteria isolates classified as closely related to *Alteromonas macleodii* identified in this study. DGGE primers successfully amplified these isolates indicating that the only explanation for the previous lack of identification was due to difficulties in amplifying γ-Proteobacterial sequences from complex samples. Nevertheless, DGGE bands from *A. tamarense* NEPCC 407 and PCC 173a, classified as γ-Proteobacterial sequences, were detected during the initial DGGE analysis, although further classification was impossible due to sequence quality. Therefore, it is possible that RFLP-identifiable isolates were amplified during DGGE, although without further analysis this is pure speculation.

Interestingly, all DGGE sequence similarities for γ-Proteobacteria appear consistently low (94 - 97%) compared to results seen for the other bacterial classes, which could again be attributed to difficulty in sequencing this subphyla in this region. Ferris *et al.* (1995) also noted the absence of certain bacterial species when using DGGE compared to cultivation, indicating that this was possibly due to primer bias.

Although the results appear to highlight limitations of DGGE, there are many possible reasons why, although expected, populations may not have been detected by this method. These include sampling technique and PCR biases, with both explanations offered previously (Ward *et al.*, 1992). It must be noted, however, that DGGE identified certain bacterial phyla which the culture-based approach failed to detect, including the CFB phylum isolates from *A. tamarense* NEPCC 407 and α-proteobacterium isolates outwith the *Roseobacter* clade in *A. tamarense* PCC 173a.

This indicates that DGGE, although having limitations, still offers a rapid means of detecting potentially dominant populations. The system also eliminates any further biases generated by using other systems such as cloning (Rainey *et al.*, 1994) and, as shown in this study, can provide data not available using a culture-based approach. Information from DGGE could also be used to provide a starting point for culture-dependent microbiological investigations and as a guideline to identify and subsequently isolate specific microorganisms from natural bacterial communities (Kane *et al.*, 1993).

If it is assumed that non culture-based techniques provide more information on community structure than methods dependent on culture, explanations as to why the culture-based method failed to uncover certain bacteria in the current study must be addressed in order to improve the system for future use. Previous attempts to enumerate bacteria associated with dinoflagellate cultures assumed that marine agar was a suitable media for the growth of most marine bacteria (Romalde *et al.* 1990a, b). However, growing evidence indicates that marine oligotrophic bacteria require low levels of nutrients in order to grow (Akagi *et al.* 1977). In addition it has been shown that the numbers of bacteria isolated on solid marine media can be two orders of magnitude lower than numbers observed using microscopy (Kogure *et al.* 1977). This indicates that a considerable number of bacteria present may have been ignored by limiting the media used.

Limited success of cultivation in the laboratory was also the explanation forwarded for discrepancies detected when complex bacterial communities were compared using culture-based and non culture-based methods (Dunbar *et al.*, 1999). The work indicated that although similar results were detected using the two methods, culturing failed to identify the diversity detected using the non culture-based method. Therefore, in future comparisons of culture-based and non culture-based techniques, the use of several marine media which encompass a range of nutrient concentrations should be considered. Different agar formulations such as malt extract, seawater agar and 1/100 strength marine agar, have already been shown to enumerate bacterial

isolates from dinoflagellate cultures which are unable to grow on marine agar (S. Gallacher pers. comm.). This indicates that certain bacterial isolates were under-represented within the current study. Further culturing work is crucial, as only limited numbers of 16S rDNA sequences from cultured isolates are thought to have been deposited in sequence databases such as RDP and Genbank (Suzuki *et al.* 1997). However, consideration of the increased workload that including a more extensive media range would entail must be appreciated. Nevertheless, the information generated from microbial isolation marine agar in this study must be considered one of the most extensive investigations to date of culturable bacteria associated with toxic and non-toxic dinoflagellate cultures.

The application of molecular techniques to identify bacteria present within complex communities has been shown to be of great value. However, in consideration of the biases and limitations of both techniques, a more complete understanding can only be obtained by combining molecular and cultivation-based methods to thoroughly characterise a given habitat. Therefore, in conclusion, the results from this study emphasise the value and complementarity of both molecular and classic cultivation-based microbial isolation methods.

G.L. Hold, 1999

CHAPTER 3 : THE PRODUCTION OF AN AXENIC DINOFLAGELLATE CULTURE

Introduction

Although historically the production of PST was attributed to dinoflagellates including *Alexandrium* species, strong evidence now exists to show the autonomous production of PST by bacteria associated with dinoflagellate cultures (Kodama & Ogata, 1988; Kodama, 1990; Kodama *et al.*, 1990a; Doucette, 1995; Franca *et al.*, 1995; Gallacher *et al.*, 1997). However, the autonomous production of PST by dinoflagellates is still open to question due to scepticism regarding the generation of axenic cultures. Several researchers have claimed success in producing axenic dinoflagellate cultures (Singh *et al.*, 1982; Sako *et al.*, 1992; Imai and Yamaguchi, 1994), with numerous methods published. Traditional methods mainly rely on physical dissociation techniques such as sterile washing, dilution series, ultrasonication and centrifugation, however, more recently, methods requiring the addition of chemicals such as bacteriostatic compounds and antibiotics have been used.

Although all published methods indicate that bacteria-free cultures were generated, the truly axenic status of currently available cultures is open to question due to methods used to determine the absence of bacteria. The main method used for assessment of axenic cultures is media plating, generally with one or two media formulations, and occasionally microscopy. Unfortunately, the majority of the formulations utilised were nutrient-rich, leading to underestimates of bacterial numbers because many marine isolates fail to grow in high nutrient conditions (Buck, 1974; Akagi *et al.*, 1977; Ishida *et al.*, 1986). Therefore, more stringent methods for assessing the effectiveness of axenic protocols including epifluorescence microscopy, are required.

The production of axenic *Alexandrium* cultures has been reported by several researchers (Guillard, 1973; Singh *et al.*, 1982; Sako *et al.*, 1992; Imai and Yamaguchi, 1994; Doucette and Powell, 1998), with axenic cultures being produced by both physical dissociation methods and by the addition of chemicals to cultures.

Therefore, the aims of this investigation were to assess previous methods used to produce axenic algal cultures, paying particular attention to methods published using *Alexandrium* cultures, and to produce axenic dinoflagellate cultures from the strains under study. It was considered important to use more stringent methods to assess the bacterial status of the cultures, therefore molecular techniques were adopted alongside the traditional methods of media plating and epifluorescence microscopy.

G.L. Hold, 1999

MATERIALS AND METHODS

Dinoflagellate strains

The strains used were *A. lusitanicum* NEPCC 253, and *A. tamarense* NEPCC 407, CCMP 117 and 1771. The first two strains were chosen as they are PST-producing dinoflagellates, with *A. lusitanicum* NEPCC 253 being less toxic than *A. tamarense* NEPCC 407 (Franca *et al.*1995; Hummert *et al.* 1997), and because the bacterial microflora had been characterised (Chapter 2). The third strain, *A. tamarense* CCMP 117, was selected as it produces high levels of PST, and had been maintained as an 'axenic' culture for over five years by the Culture Collection for Marine Phytoplankton (CCMP, Bigelow Harbor, Maine). The last strain, *A. tamarense* CCMP 1771, was included as an 'axenic' non PST-producing culture, maintained by the culture collection as 'axenic' for less than two years.

Removal of bacteria from dinoflagellate cultures by a washing technique

The washing procedure for removing associated bacteria was adapted from Singh *et al.* (1982). Stationary phase cultures (100ml) from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 were centrifuged (10min, 2,000 x *g*) and the supernatant decanted. The cells were resuspended in sterile seawater (10ml) and centrifuged as before. This procedure was repeated a further three times, after which serial dilutions of the cell suspension in sterile seawater were made. The dilution series was plated in triplicate onto marine agar plates and incubated for 14 days at 20°C. Control samples, i.e. those not subjected to the sterile washing regime, were also serially diluted, plated in triplicate and incubated.

Removal of bacteria from dinoflagellate cultures following dinoflagellate cell lysis

A second set of samples (200ml), from the two cultures were centrifuged (10min, 2,000 x *g*), and glass beads (1g, 0.16 - 1.17mm - gamma sterilised, Sigma), added, with samples vortexed for 5 periods of 1 minute to lyse the dinoflagellate cells

(checked using microscopy). Following disruption, samples were subjected to the washing regime, serial dilution and plating as before. Control samples, i.e. those not subjected to washing, but lysed by the addition of glass beads and vortexing, were also serially diluted, plated in triplicate and incubated.

Antibiotic profiling of bacteria isolated from dinoflagellate cultures

Bacteria previously isolated and characterised from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 (See Chapter 2), were inoculated into marine broth (10ml, Difco), and incubated for 24h in a shaking incubator (20°C, 120 osc min^{-1}). Each bacterial suspension (200µl) was inoculated onto marine agar plates, spread evenly and allowed to dry, before antibiotic sensitivity discs (Oxoid), were placed evenly across the inoculum. Plates were subsequently inverted and incubated at 20°C for 48h before results were recorded. Antibiotic sensitivity was identified by production of a zone of inhibition of at least 5mm width around the disc.

Antibiotic treatment of dinoflagellate cultures

An antibiotic cocktail (ciprofloxacin, gentamycin, streptomycin and penicillin G at concentrations of 46 µg ml^{-1}, 240 µg ml^{-1}, 25 µg ml^{-1} and 20 units ml^{-1} respectively; see Table 2.2), deemed to be effective for the two dinoflagellate cultures from antibiotic profiles determinations of the individual bacteria, was added to flasks containing exponential phase dinoflagellate cultures. Flasks containing the above antibiotic cocktail with the omission of penicillin were included, with a half strength combination also added to a set of flasks. As bacteria remained in both cultures following addition of these combinations, surviving isolates were tested for sensivity to additional antibiotics to be included in further treatments. Penicillin was effective, therefore, a further treatment of control cultures by adding ciprofloxacin, gentamycin, streptomycin and penicillin G, at levels of 46 µg ml^{-1}, 240 µg ml^{-1}, 25 µg ml^{-1} and 20 units ml^{-1}, was used. All flasks were re-incubated under normal growth conditions (See Chapter 2), for a further 12 days before subculture.

Antibiotic	Stock solution	Concentration required	Dilution required
Streptomycin	25, 000μg ml^{-1}	25μg ml^{-1}	100μl stock solution added to 100ml dinoflagellate culture
Ciprofloxacin	2,000μg ml^{-1}	46μg ml^{-1}	2.3ml stock solution added to 100ml dinoflagellate culture
Gentamycin	40,000μg ml^{-1}	120μg ml^{-1}	0.3ml stock solution added to 100ml dinoflagellate culture
Penicillin G	100,000 units ml^{-1}	20 units ml^{-1}	20μl stock solution added to 100ml dinoflagellate culture

Table 3.1 Antibiotic concentrations used for production of axenic dinoflagellate cultures

Untreated dinoflagellate flasks were also maintained under the same regime and subcultured accordingly. Following the addition of antibiotics, treated cultures were allowed to go through three normal subculture patterns (in order to dilute out any effects due to the antibiotics) prior to assessment of bacteriological status.

Use of different marine culture media to assess the presence of bacteria in antibiotic-treated dinoflagellate cultures.

To detect the presence of any remaining culturable bacteria within antibiotic treated cultures, 17 different marine media (See Appendix 5 for formulations), were used. The two dinoflagellate cultures purchased and maintained as "axenic" i.e. *A. tamarense* CCMP 117 and 1771, were also included within the experiment. The original untreated *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 cultures were also included as positive controls. Cultures were inoculated (100µl) into 5 tubes and 3 plates of each medium and incubated at 20°C for 1 month.

EPIFLUORESCENCE MICROSCOPY

Preparation of Sybr Green 1 nucleic acid stain

Sybr Green 1 nucleic acid stain (Molecular Probes Inc.), was bought as a commercial stock solution, a 10,000-fold concentrate of the recommended working concentration. 10µl of the stock solution was added to 10ml of TE buffer (10mM Tris, 10mM EDTA pH8; Sigma) to give a solution ten times the recommended strength. This solution was then added to samples at a ratio of one part stain to nine parts sample, to give the correct final working stock concentration.

Preparation of dinoflagellate samples for epifluorescence microscopy

Samples from all dinoflagellate cultures were taken in a laminar flow cabinet and diluted in sterile seawater to give approximately 10^5 cells ml^{-1}. The previously

prepared Sybr Green 1 stain was added to samples, which were subsequently incubated in the dark at room temperature for 5 minutes before placing in a filter column and drawing slowly (<150mm Hg vacuum) onto a black polycarbonate membrane (0.2µm, Poretics Inc.). The membrane was fixed onto a microscope slide by addition of a drop of immersion oil above and below the membrane, and a coverslip added. Slides were examined immediately by epifluorescence microscopy (Zeiss, Axiovert 10) using oil immersion at an excitation wavelengh of 460nm.

Determination of PCR sensitivity limits for the detection of bacterial strains associated with *A. lusitanicum* NEPCC 253

Bacterial isolates from *A. lusitanicum* NEPCC 253, corresponding to RFLP patterns 1, 3 and 4 (Chapter 2; Fig. 2.2), and *Alteromonas macleodii* type strain, were inoculated into marine broth (100ml, Difco), and incubated at 20°C for 24h. The concentration of bacteria in the inoculum was determined using a Thoma counting chamber (Gibco). Samples were subjected to five freeze/thaw cycles, followed by boiling for 5 min, before serial dilutions were performed using sterile seawater to generate samples containing 10^3 to 10^8 cfu ml^{-1}.

Aliquots (5µl) of each dilution were used as templates for PCR reactions (performed as for DGGE analysis in Chapter 2), with amplification products concentrated using Prep-a-Gene (Biorad), before complete PCR reactions were visualised using ethidium bromide (50µl ml^{-1}) stained agarose (2% gel). A 100bp marker standard (Gibco), was also included on the gel for reference.

The use of PCR for checking the bacteriological status of dinoflagellate cultures

Antibiotic-treated dinoflagellate cultures of *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407, CCMP 117 and 1771, were subjected to PCR analysis in all phases of growth. Dinoflagellate cultures were sampled and DNA extracted as described in Chapter 2, with each growth phase sample subjected to two sets of PCR

reactions: set 1 eubacterial primers, Primers 2 and 3 (Fig. 2.1), used previously in Chapter 2 for DGGE analysis, and set 2 eubacterial primers 27F and 1522R (Fig. 2.1), as used previously in Chapter 2 for RFLP analysis. Each set of samples was amplified using the conditions specific for the primers used as described in Chapter 2.

Prior to visualisation of PCR products, samples were concentrated using Prep-a-Gene (Biorad), which allowed the concentration of 100µl PCR reaction volumes to 10µl. Concentrated PCR products (10µl), were inspected using electrophoresis (2% agarose gel in 1 X TAE containing ethidium bromide (0.5µg ml^{-1})), with markers (100bp and 1KB; Gibco) included on the gel for reference.

The use of DGGE to analyse PCR products generated from "axenic" dinoflagellate cultures

Following the visualisation of a PCR product in *A. tamarense* CCMP 117 using primers 2 and 3, DGGE analysis of the sample using experimental conditions determined in Chapter 2, identified the presence of one DGGE band, which was subsequently excised from the DGGE gel, the DNA eluted, recovered, re-amplified, cleaned and identified using bi-directional 16S rDNA sequencing as described previously in Chapter 2.

RESULTS

The use of a washing technique to produce an axenic dinoflagellate culture

The effectiveness of a washing technique in removing the microflora of dinoflagellate cultures was investigated by comparing bacteria from untreated dinoflagellate cells plated directly onto marine agar and washed dinoflagellate cells. Figure 3.1 summarises the morphotypes present in the untreated *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 cultures and those remaining after the sterile washing procedure. The procedure was effective at reducing bacterial numbers in *A. lusitanicum* NEPCC 253, with numbers of three morphotypes reduced by approximately 90%, with the other morphotype (the small cream) eliminated, which may indicate that this small cream isolate had a loose attachment with the dinoflagellate cells compared to the other morphotypes.

In earlier experiments using *A. tamarense* NEPCC 407, the culture exhibited four morphotypes which were characterised as detailed in Chapter 2. However, three additional morphotypes were identified during this experiment; the raised pink, small orange, and chalky yellow isolates, although they comprised a small percentage of the total bacteria present. Washing of the dinoflagellate cells did not result in removal of any morphotypes, with the numbers of the small orange isolate actually increasing on washing. The reduction in numbers of the other bacterial morphotypes in *A. tamarense* NEPCC 407 was not seen to the same extent as in *A. lusitanicum* NEPCC 253.

Therefore, washing appeared unsuccessful at removing bacteria associated with the dinoflagellate cells, which indicated that a tight association may exist between dinoflagellate cells and bacteria. An investigation assessing the level of attachment was instigated to determine the effectiveness of other physical dissociation techniques. Dinoflagellate cells were subjected to lysis and washing prior to plating onto marine agar, with quantities and types of bacteria compared to the untreated culture with bacterial numbers resulting from both treatments expressed as a percentage of the original culture. Results from *A. lusitanicum* NEPCC 253 (Fig.

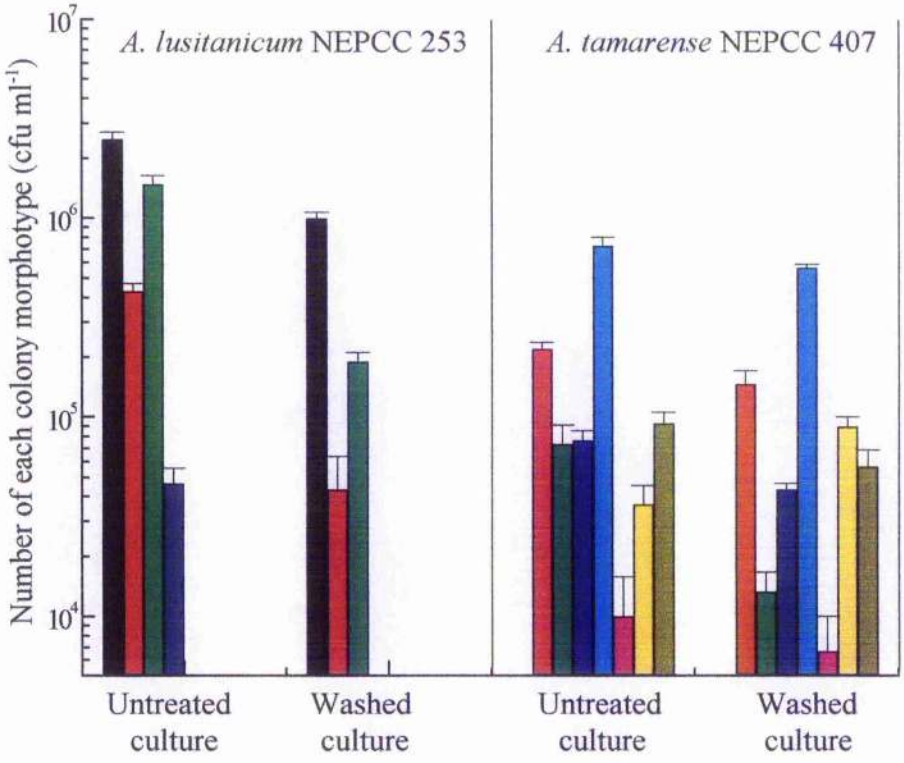


Figure 3.1 Concentration of each colony morphotype of bacteria in dinoflagellate cultures *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407, following washing, compared to untreated cultures.

The detection limit was 10cfu ml^{-1}.

■ = yellow/orange mucoid isolate, ■ = large cream isolate, ■ = small rose rough isolate, ■ = beige flat isolate, ■ = small cream isolate, ■ = raised pink isolate, ■ = small orange isolate, ■ = small chalky yellow isolate.

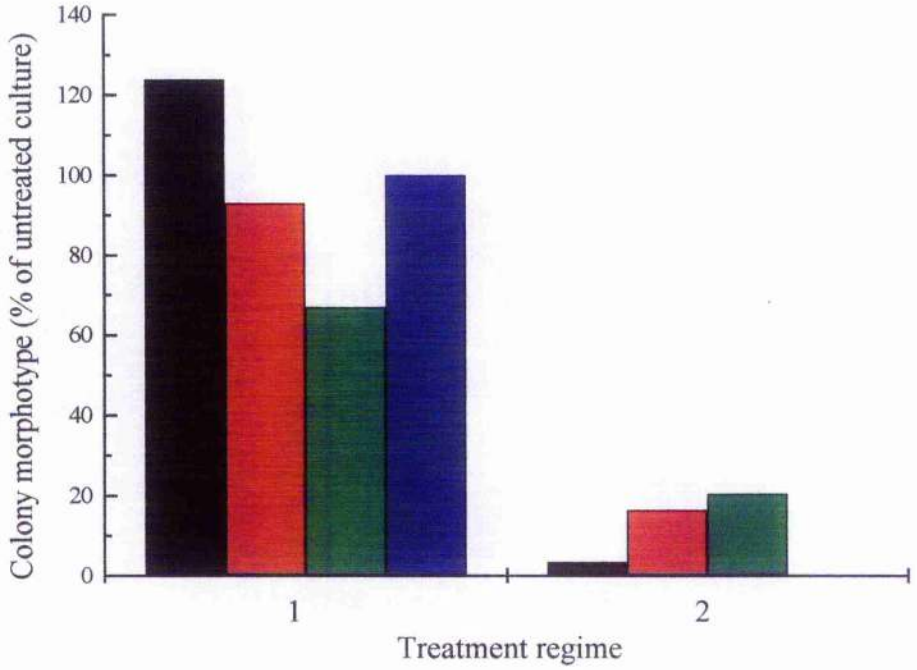


Figure 3.2 Colony morphotypes from stationary phase *A. lusitanicum* NEPCC 253, expressed as a percentage of the untreated culture, after being subjected to different bacterial dissociation methods. (1) dinoflagellate culture subjected to disruption by vortex mixing with the addition of glass beads to lyse dinoflagellate cells. (2) dinoflagellate cells subjected to disruption an in (1), but with the addition of sterile seawater washing after lysis.

■ = yellow/orange mucoid isolate, ■ = large cream isolate, ■ = small rose rough isolate, ■ = beige flat isolate.

3.2) indicate that lysing the culture gave 25% more yellow/orange isolates and 35% less rose coloured isolates, although the numbers of cream and beige morphotypes remained comparable to the original culture. Washing the lysed cells resulted in a large reduction of all bacterial types, with the beige isolate totally removed as seen during washing of whole cells. The difference between numbers of the large cream and rose coloured isolates when the two washing treatments were compared (Fig 3.1 and Fig 3.2) was negligible, although yellow/orange isolates were further reduced in the washed cell lysate compared to the washed whole cells.

Figure 3.3 depicts the results generated from *A. tamarense* NEPCC 407 after similar lysis treatment to *A. lusitanicum* NEPCC 253. Disruption of the dinoflagellate culture removed one of the less dominant morphotypes, the chalky yellow isolate and reduced the numbers of rose coloured and large cream isolates by 80% and 50% respectively. However, lysis of the culture also caused an increase in the numbers of the small cream, raised pink, and small orange isolates by 40%, 350%, and 80% respectively, with the beige isolate remaining largely unaffected. As with washing of the whole cell culture, washing of the cell lysate vastly reduced bacterial numbers, although another of the less dominant morphotypes, the small orange isolate was removed when the lysed culture was washed. This could indicate the morphotype to have been of intracellular origin. Interestingly, washing of the whole cells saw a large decrease in the numbers of the rose coloured isolate, however, examination of the washed lysate indicated the presence of more rose coloured isolates than were present in the original culture. Numbers of the small cream and raised pink morphotypes were reduced following washing of the cell lysate, with the large cream and medium beige morphotypes largely unaffected compared to whole cell numbers.

These investigations indicated the tight degree of association between the bacteria and the dinoflagellate cells, with even complete disruption of the algal cells not causing removal of the majority of morphotypes. Therefore it was accepted that

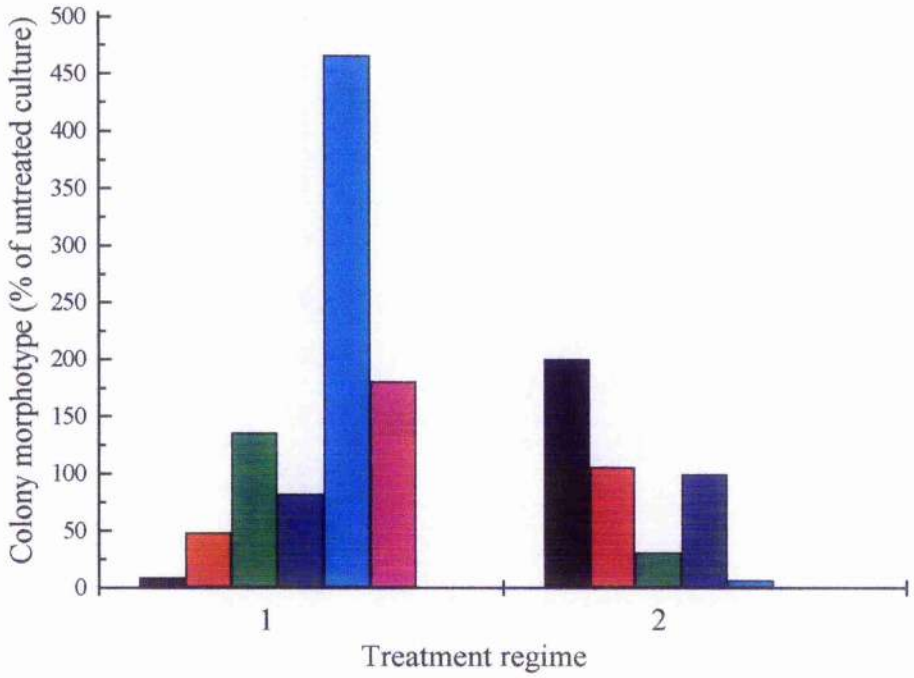


Figure 3.3 Colony morphotypes from stationary phase *A. tamarense* NEPCC 407, expressed as percentage of the untreated culture, after being subjected to different bacterial dissociation methods. (1) dinoflagellate culture subjected to disruption by vortex mixing with the addition of glass beads to lyse dinoflagellate cells. (2) dinoflagellate cells subjected to disruption an in (1), but with the addition of sterile seawater washing after lysis.

■ = rose coloured isolate, ■ = large cream isolate , ■ = small cream isolate, ■ = medium beige isolate, ■ = raised pink isolate, ■ = small orange isolate.

physical dissociation methods would not produce axenic cultures of the dinoflagellates present within the current study. Therefore another approach was required.

Identification of an effective antibiotic cocktail to produce an axenic dinoflagellate culture

The efficiency of antibiotics in removing the bacterial flora of *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 was assessed. Initially, bacteria were exposed to a range of antibiotic sensitivity discs (Oxoid) to ascertain which antibiotics were effective against the culturable bacteria described previously in Chapter 2.

Large cream isolates from *A. lusitanicum* NEPCC 253 were effectively removed by streptomycin and novobiocin, whilst *A. tamarense* NEPCC 407 isolates were resistant to these antibiotics, but sensitive to penicillin, gentamycin and ciprofloxacin. A similar response was detected with the rose coloured isolates from both dinoflagellate cultures, with all isolates responding to penicillin, streptomycin, gentamycin and ciprofloxacin. This combination, with the inclusion of kanamycin, was also effective against yellow/orange isolates in *A. lusitanicum* NEPCC 253. Ciprofloxacin was the only antibiotic tested which inhibited growth of beige isolates from *A. lusitanicum* NEPCC 253, whilst the beige isolates from *A. tamarense* NEPCC 407, although sensitive to ciprofloxacin, also responded to streptomycin and novobiocin.

From the above, it appeared that a combination of streptomycin, ciprofloxacin and gentamycin would be effective against all strains. Therefore, a cocktail of these antibiotics was used to treat dinoflagellate cultures, together with a second antibiotic cocktail half the strength of the first, in case dinoflagellate cells were sensitive to high doses of the antibiotics. Dinoflagellate cultures were subsequently exposed to these cocktails for 12 days, after which they were subcultured into fresh media free of antibiotics. As shown in Table 3.2 combinations 1 and 2 were both effective in

reducing the concentration of all bacterial morphotypes from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407, with combination 1 completely removing three bacterial morphotypes from each culture.

The addition of penicillin to combination 1 (Table 3.2 combination 3), removed all morphotypes from both dinoflagellate cultures without adversely affecting the growth of *A. lusitanicum* NEPCC 253 (See Chapter 4). However, it was noted that there was an adverse effect on the growth rate of *A. tamarense* NEPCC 407 under these conditions. This was rectified by altering growth conditions, allowing the culture to remain in continuous light for a 2 week period rather than the usual 14h:10h light:dark cycle. Following the altered light phase period, growth of the treated culture appeared stable which allowed the assessment of growth rates and toxicity profiles (See Chapter 4).

However, *A. tamarense* NEPCC 407 still contained a contaminant in the form of a fungus, which was not detected in the original culture. The fungal culture was identified by Dr S. Moss, University of Southampton, as a *Botrytis* sp., a genus known to contain many plant-pathogenic species but not usually associated with marine environments (Coley-Smith, 1980).

Use of a range of marine media to assess the effectiveness of antibiotic treatments

Following antibiotic treatments, dinoflagellate cultures were maintained through three growth cycles, to remove any potential influence the antibiotics may have had on growth of the cultures. Although antibiotic treated cultures were free of bacterial growth on marine agar, the possible presence of bacteria capable of growth in other media was investigated. Initially this involved inoculating 0.1ml of the treated dinoflagellate cultures onto seventeen different media, which were incubated at 20°C

Table 3.2a

Colony morphology	Antibiotic combination		
	1	2	3
yellow/orange	100	50	100
large cream	85	20	100
small rose	100	45	100
flat beige	100	75	100

Table 3.2b

Colony morphology	Antibiotic combination		
	1	2	3
rose	100	55	100
large cream	100	30	100
small cream	80	15	100
medium beige	100	35	100

Table 3.2 Percentage of each bacterial morphotype removed from cultures of *A. lusitanicum* NEPCC 253 (Table 3.2a), and *A. tamarense* NEPCC 407 (Table 3.2b), by various antibiotic cocktails.

Combination 1 = ciprofloxacin (46μg ml^{-1}), gentamycin (240μg ml^{-1}), streptomycin (25μg ml^{-1})

Combination 2 = ciprofloxacin (23μg ml^{-1}), gentamycin (120μg ml^{-1}), streptomycin (12.5μg ml^{-1})

Combination 3 = ciprofloxacin (46μg ml^{-1}), gentamycin (240μg ml^{-1}), streptomycin (25μg ml^{-1}) and penicillin G (20 units ml^{-1})

for 30 days, after which time no bacterial colonies were detected. As the detection limit for bacteria on an agar plate was 10 cfu ml^{-1}, it could be stated that there were no culturable bacteria present above this level. However, the *Botrytis* sp. was still detected in *A. tamarense* NEPCC 407 using most agar formulations, with the exception of the nutrient-poor media, including 1/100 strength marine agar, seawater media and $ST10^{-4}$. The probability of bacteria being present at < 10 cfu ml^{-1}, was assessed by inoculating 1ml of each dinoflagellate culture into 5 replicate tubes containing broth of each media formulation. After 30 days incubation, no turbidy was detected, although *Botrytis* was again present in the higher nutrient formulations.

From this work, using solid and liquid media, comprising a wide nutrient spectrum, it was concluded that no culturable bacteria remained in either dinoflagellate culture.

Epifluorescence microscopy of dinoflagellate cultures

Figure 3.4a shows the dinoflagellate culture *A. lusitanicum* NEPCC 253 prior to antibiotic treatment, stained using Sybr green 1 and examined using epifluorescence microscopy. The nucleus of the cell is visible as the large green fluorescing mass, surrounded by the rest of the dinoflagellate cell, which autofluoresces faintly red due to the presence of chlorophyll. The remainder of the green fluorescence, in the form of small particles, is due to bacterial cells present within the culture. Following successful antibiotic treatment, no fluorescence could be attributed to the presence of bacteria, either attached to the dinoflagellate cell, or free-living in the culture media (Fig. 3.4b).

Figure 3.4a *A. lusitanicum* NEPCC 253 prior to antibiotic treatment, stained using Sybr Green (x 400 magnification).

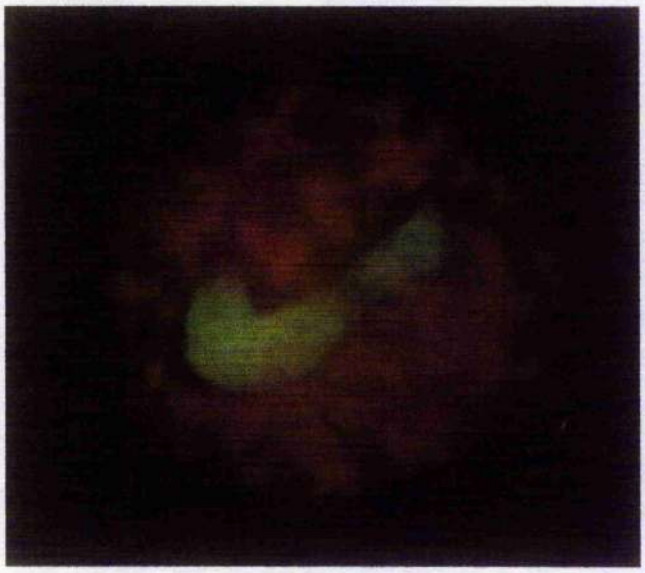


Figure 3.4b *A. lusitanicum* NEPCC 253 following antibiotic treatment, stained using Sybr Green (x 400 magnification).

Use of molecular techniques to confirm the axenic status of dinoflagellate cultures

The culture technique and epifluorescence microscopy described above confirmed that dinoflagellates were free of culturable bacteria. However, the possibility that unculturable bacteria remained was assessed using PCR analysis with two eubacterial primer sets (Chapter 2; Fig. 2.1). The primer sets used amplified different length fragments of the 16S gene, with primer set 1 targetting the hypervariable V3 region of the 16S gene corresponding to nucleotide positions 341 - 534 in *E. coli*, whilst primer set 2 corresponding to nucleotide positions 8 - 1522, amplified the whole 16S gene generating a 1500 base pair fragment. Prior to use, both primer sets were investigated for the spectrum of bacteria which, theoretically, would be targetted. The majority of bacteria, excluding certain β-proteobacteria and the *Planctomycetes*, would be amplified by primer set 1, and only a limited number of bacteria, including certain *Vibrio* species would be unaffected by primer set 2.

Prior to examining the dinoflagellate cultures, the sensitivity limit of the PCR reaction was investigated. Serial dilutions of three bacterial groups isolated from *A. lusitanicum* NEPCC 253 (RFLP patterns 1, 3 and 4; see Chapter 2) and *Alteromonas macleodii* type strain, were subjected to PCR amplification using primer set 1. Clearly visible products were detected with all bacteria in samples containing 10^1 - 10^7 cfu per reaction, indicating a detection limit of ≤ 10 cfu per reaction volume in all bacterial groups.

Following PCR amplification of dinoflagellate samples, PCR products were concentrated to allow the contents of the whole reaction volume to be visualised in ethidium bromide stained agarose. No PCR products were detected in either antibiotic treated culture following assessment with both primer sets. Therefore, cultures were subsequently considered bacteria-free.

Assessment of the bacteriological status of culture collection axenic dinoflagellates

The bacteriological status of the two "axenic" *A. tamarense* cultures obtained from the culture collection, CCMP 117 and 1771, was also investigated. The culture collection deemed these cultures to be bacteria-free as judged by the absence of bacteria by epifluorescence microscopy and inoculation into liquid media. Analysis using the seventeen different media formulations and epifluorescence microscopy described above, did not show the presence of bacteria in either culture. However, PCR amplification analysis with primer set 1, revealed a product from all growth phases of *A. tamarense* CCMP 117. Examination of these samples by DGGE showed there to be one PCR fragment. This was excised from the acrylamide gel, and subsequently identified (see Chapter 2) as an α-Proteobacterium related to the *Roseobacter* clade (see Appendix 4 for sequence information). PCR amplification of *A. tamarense* CCMP 117 using primer set 2, failed to generate a PCR product.

DISCUSSION

One of the aims of this project was to assess the influence of bacteria on dinoflagellate toxicity, with removal of the dinoflagellate microflora and determining the subsequent effect on toxicity being one approach that can be taken to address this issue. Several researchers including Singh *et al.*, (1982), Sako *et al.*, (1992), Imai & Yamaguchi, (1994) and Doucette and Powell, (1998), have claimed success in producing axenic dinoflagellate cultures using various methods, including washing techniques, dilution series, physical dissociation and addition of bacteriolytic compounds. Assessment of their success was determined using various media and occasionally epifluorescence microscopy.

Initial work in this study investigated the efficiency of a washing technique reported by Singh *et al.* (1982) to be effective in producing axenic *Alexandrium* cultures. Bacteria-free status of cultures was concluded by the lack of bacterial growth on three media formulations including a non-marine formula. However, although strains used in the current study were also *Alexandrium* species, the method was found to be ineffective in completely removing the majority of isolates, although all morphotypes were reduced. The ability of bacteria to remain in algal cultures following washing was also demonstrated by Spencer (1952), who showed that washing removed free-living bacteria, but not firmly attached isolates, from algal cultures. However, the effectiveness of this procedure was not demonstrated on dinoflagellate cultures. Lysis of dinoflagellate cells to remove bacteria in the current study also failed to remove all isolates, indicating that some bacteria are tightly associated with dinoflagellate cells.

Since the washing of the dinoflagellate cells was unsuccessful in producing bacteria-free cultures, the use of antibiotics was assessed. Several workers have claimed success using antibiotics (Spencer, 1952; Hoshaw and Rosowski, 1973; Guillard, 1973; Bates *et al.*, 1993), with broad-spectrum antibiotic cocktails being added to cultures, and subsequent assessment of remaining bacteria in cultures dictating further treatments. However, in the current study, a more methodical approach was adopted, in that antibiotic sensitivies of all culturable bacteria from the dinoflagellate

cultures were determined. This resulted in precise information for each dinoflagellate culture, allowing an effective cocktail to be generated which was subsequently adjusted to allow removal of all culturable bacteria without adversely affecting the dinoflagellate culture.

Using information from antibiotic sensitivity profiles, the antibiotic combination initially used for treatment of the two dinoflagellate cultures in this study included streptomycin, gentamycin, ciprofloxacin. However, this combination although effective against all previously cultured isolates did not produce bacterial free cultures. Therefore, subsequent investigations of antibiotics effective against remaining isolates indicated the addition of penicillin would produce bacterial free cultures. The combination of all four antibiotics was used recently to produce axenic *Alexandrium* cultures in New Zealand (E. Maas pers. comm.). However, due to the use of different *Alexandrium* species and culturing regimes, it was anticipated that the bacterial flora from the dinoflagellates used in this study would probably differ from cultures investigated by Maas, and therefore, the combination may not necessarily be effective. However, antibiotic treatments were repeated using the four antibiotics on previously untreated dinoflagellate cultures, with subsequent assessment using marine agar indicating no bacterial growth.

Investigation of published methods for assessing the axenic nature of algal cultures

One of the main criticisms of previously published protocols for producing axenic algal cultures, has been the lack of methods used to assess the sterility of cultures. The majority of published methods utilise limited media plating, containing mainly nutrient-rich formulations, although not necessarily suitable for marine organisms. However, as the detection limit of media plating is ten cfu ml^{-1}, it is possible for low concentrations of bacteria to have gone undetected and remained within cultures. Therefore, the second aim of the current study was to devise a more comprehensive method for assessing the bacterial status of supposedly axenic dinoflagellate cultures.

Bacteriological status of antibiotic treated dinoflagellate cultures

The use of seventeen different media formulations in the current study gave a high probability of enumerating bacterial species which may have remained following antibiotic treatment. The different media encompassed a wide variety of nutrient composition, with both general and selective formulae included. Several low nutrient formulations were employed as viable cell counts have often been shown to increase when low nutrient media are used, because most marine bacteria, under natural conditions, are not routinely exposed to high substrate concentrations (Azam and Ammerman, 1984; Nissen, Nissen and Azam 1984). This could indicate the presence of bacterial species which are not routinely detected using traditional high nutrient marine media such as marine agar (Buck, 1974; Akagi *et al.*, 1977; Ishida *et al.*, 1986).

It was also important to address the detection limit of methods to assess the bacterial free status of dinoflagellate cultures. As the detection limit of media plating was ten cfu ml^{-1} inoculations of broth were also used to confirm media plating results, and achieve even greater detection sensitivity.

The success of antibiotic treatment methods was shown by the lack of culturable bacteria in all seventeen different media preparations, both in agar and broths. However, following antibiotic treatment of *A. tamarense* NEPCC 407 a fungus subsequently identified as a *Botrytis* sp., was detected. It is possible that removal of bacteria allowed the *Botrytis* sp. to colonise, whereas previously its growth had been suppressed by the presence of bacteria or their extracellular products. The lack of fungal growth in four of the media f/2, $ST10^{-4}$, seawater media and 1/100 strength marine medium may reflect the high nutrient requirements for fungal growth. Although not detected in *A. tamarense* NEPCC 407 whilst bacteria were present, the ability of this fungal species to persist in the axenic culture for the duration of the study indicate its ability to survive in marine conditions. However, *Botrytis* sp. have not been previously detected in marine environments (Coley-Smith, 1980) and it was

not observed in any of the other axenic dinoflagellates, maintained under the same regime. Therefore, its presence is unlikely to be as a contaminant introduced during maintenance. Future work to produce an axenic culture of *A. tamarense* NEPCC 407, should also include a treatment capable of eliminating the fungal contamination.

The use of epifluorescence microscopy to determine the bacteriological status of treated dinoflagellate cultures

The majority of recently published studies on the production of axenic cultures rely on epifluorescence microscopy to confirm the axenic status of cultures. Thus, many believe that epifluorescence microscopy is a better method for assessing the axenic nature of cultures than traditional plating method (Bolch and Blackburn, 1995). In the current study, epifluorescence microscopy was initially done using DAPI, a stain commonly used to analyse algal/bacterial associations (Porter and Feig, 1980; Kim *et al.*, 1993). However, the results lacked clarity due to autofluorescence from background detritus within samples and this made images very difficult to interpret. This problem was eliminated by the use of Sybr Green 1, a nucleic acid stain developed initially to stain electrophoresis gels, although, it has been used successfully to stain picoplankton and other algae for flow cytometry analysis (Marie *et al.*, 1997), producing excellent results using both live or fixed samples.

Assessment of current literature indicates that DAPI is still the stain of choice for assessing the axenic nature of algal cultures. However, in the current study Sybr Green 1 was a much more effective stain for assessing algal/bacterial interactions, due to its ability to penetrate dinoflagellate cells without requiring sample fixation, unlike stains such as DAPI. Fixation is the main problem encountered in using epifluoresence microscopy to determine the axenic status of dinoflagellate cultures, as the introduction of glutaldehyde or formalin alters the structure of the mucilagenous layer surrounding dinoflagellate cells (J. Lewis, pers. comm.), causing difficulties when investigating the association between bacteria and dinoflagellate cells.

difficulties when investigating the association between bacteria and dinoflagellate cells.

Other microscopy techniques such as confocal microscopy or transmission electron microscopy (TEM) are now more frequently used for analysis of algal samples. These techniques allow section-by-section analysis of cells, which can be recombined to generate a 3-dimensional composite image. However, these techniques require a long sample preparation time and costly reagents and equipment to process samples; these methods were not available in this project. However, as these techniques are gaining favour for analysis of supposedly axenic cultures, future work must consider their possible use.

Molecular assessment of antibiotic treated cultures

Molecular techniques have been used routinely to detect the presence of bacteria within a community (Olsen *et al.*, 1986; Ward *et al.*, 1992; Amann *et al.*, 1995; Suzuki *et al* 1997). However, in the current study, molecular techniques were adopted to confirm the absence of bacteria. A PCR sensitivity investigation indicated a detection limit of less than one colony forming unit per 10ml of dinoflagellate culture, for several different bacterial morphotypes, indicating the system to be ten times more sensitive than traditional plate counts, not subject to the constraints of cultivation methods, and less time consuming for multiple samples than epifluorescence microscopy.

PCR amplification of antibiotic treated cultures using two universal eubacterial primer sets did not generate PCR products, indicating the presence of < 0.1 cfu ml^{-1} of dinoflagellate culture at all points of the growth cycle, equivalent to < 100 colony forming units per litre of culture. This roughly equates to one bacterium per ten dinoflagellate cells in lag phase, with 1 bacterium per 1000 dinoflagellate cells in stationary phase samples.

Axenic status of culture collection 'axenic' dinoflagellate cultures

Dinoflagellate cultures *A. tamarense* CCMP 117 and 1771 were bought as 'axenic' cultures from the culture collection (CCMP), and the axenic nature of these cultures had been regularly checked using broth inoculation and epifluorescence microscopy, whilst maintained at the culture collection. Subsequent assessment using the seventeen media, and epifluorescence microscopy using Sybr Green 1 in the current study also failed to detect any bacterial contamination. Although molecular analysis of *A. tamarense* CCMP 1771 failed to generate a PCR product from any growth phase, this was not the case for *A. tamarense* CCMP 117, with molecular analysis detecting a *Roseobacter* sp., a common marine bacterial species (Sorokin, 1995). Although the detection limit of the PCR reaction was lower than the plating method, more than 100 colonies would have been present within the whole culture for the positive PCR reaction to have been generated. It must be emphasised however, that the molecular technique would also have detected bacteria remaining unculturable by current plating methods.

The detection of such a readily culturable isolate (Sorokin, 1995), must question the viability of the bacteria within the culture, as both epifluorescence microscopy and media plating failed to detects its presence. Several possibilities can be offered to explain the detection of bacteria in A. *tamarense* CCMP 117 using molecular analysis. Firstly, contamination of samples could have occurred during processing. However, all samples from antibiotic treated cultures, *A. tamarense* CCMP 117 and 1771 were processed at the same time, with each growth phase sample being treated individually. Therefore, it is scarcely conceivable that all three *A. tamarense* CCMP 117 samples became contaminated when none of the other samples produced positive PCR reactions. The second possible reason for detection of bacterial DNA could be due to carry over of DNA during subculture. However, *A. tamarense* CCMP 117 had been maintained as axenic (determined by media plating and epifluorescence microscopy) in the current study for six months prior to molecular analysis, and previously maintained in the culture collection for five years. Therefore, it had been

repeatedly subcultured, which would have greatly reduced any bacterial DNA remaining following antibiotic treatment. This is further confirmed by negative PCR reactions from the dinoflagellate cultures treated with antibiotics during the current study, which did not generate PCR products although maintained for a much shorter time than *A. tamarense* CCMP 117 before analysis.

In conclusion, no definite answer can be offered as to why molecular analysis identified the presence of bacteria in *A. tamarense* CCMP 117, when other methods failed. But, this indicates that the molecular method used within the current study offers the most sensitive system to date for assessing the axenic nature of dinoflagellate cultures. The method provides an accurate method for identifying the presence of bacterial contamination in cultures, with a sensitivity level at least 100 times greater than traditional plating methods for those bacteria which could be cultured. Therefore, the use of molecular techniques must be considered essential for future experiments requiring definitive proof of the axenic nature of dinoflagellate cultures.

G.L. Hold, 1999

CHAPTER 4: INVESTIGATION OF BACTERIAL INFLUENCE ON DINOFLAGELLATE GROWTH AND TOXIN PRODUCTION

Introduction

The ability of axenic *Alexandrium* cultures to produce PST has been widely reported (Singh *et al.*, 1982; Boyer *et al.*, 1987; Boczar *et al.*, 1988; Doucette and Powell, 1998), although comparison of the levels of toxins produced by axenic cultures compared to original cultures varies. Singh *et al.*, (1982) and Dantzer and Levin, (1997), noted higher levels of toxin production in axenic cultures compared to original cultures, however, Sako *et al.*, (1992) noted levels to be comparable to original cultures, whilst Doucette and Powell, (1998) showed toxin production from axenic cultures was half that of original cultures. However, it is suggested by some researchers that the axenic cultures used within these studies were possibly not truly bacteria-free.

The majority of work investigating the ability of bacteria to alter *Alexandrium* sp. toxin profiles, has been concerned with effects observed when bacteria were removed, with only one study investigating the effect of re-introducing bacteria to dinoflagellate cultures (Doucette and Powell, 1998), although a parallel study was attempted in a toxin producing diatom culture (Bates *et al.*, 1993). Results from the two studies generated different findings, with re-introduction of bacteria to the diatom cultures causing an increase in toxicity compared to the original culture, although results from introducing bacteria to the axenic dinoflagellate culture appeared to merely restore the original toxicity of the culture.

In this study, effects on the growth and toxicity profiles of dinoflagellate cultures were investigated following the production of an axenic culture (as detailed in Chapter 3), with particular attention paid to effects on toxicity following the re-introduction of bacteria to axenic cultures. Toxicity following re-introduction of bacteria was seen to increase compared to both axenic and original cultures, with effects due to the introduction of a non-toxin producing dinoflagellate culture's microflora also assessed, however this did not appear to have such a dramatic effect on toxicity. Attempts were also made to assess the ability of the axenic dinoflagellate

culture to sustain different bacterial floras using molecular techniques detailed previously in Chapter 2.

G.L. Hold, 1999

MATERIALS AND METHODS

Dinoflagellate maintenance and cell counting

Cultures were maintained following protocols described in Chapter 2. Cell counts were obtained using flasks set aside purely for growth curve analysis, and maintained under the same conditions as experimental flasks. Aliquots of culture (5ml) from growth curve flasks were aseptically removed every second day following gentle swirling of flasks to generate a uniform suspension. Lugol's iodine (10µl) was added to each culture aliquot to fix the sample, which was then counted using a Sedgewick-rafter slide. Thirty six fields of vision were counted to give statistically viable results, with dense cultures serially diluted in sterile seawater to leave between 10 and 20 cells per field of vision.

PST extraction from dinoflagellate samples

Dinoflagellate cultures were harvested for PST analysis in lag, log and stationary growth phases. Aliquots of cultures (5ml) were set aside for growth curve analysis before the majority of the culture (1000ml) was collected by centrifugation (2000 x g, 10 min), the supernate decanted, the resulting cell pellet resuspended in 1ml acetic acid (0.05M), and frozen at -20°C overnight. Following thawing of samples, cells were disrupted by the addition of 25% w/v glass beads (0.16 - 1.17mm - gamma sterilised, Sigma), and vortex mixing for 3 min. Microscopic examination of the cell debris revealed cells had been completely disrupted. Samples were centrifuged briefly (13,000 x *g*, 30 sec), with the supernatant carefully removed and filtered through 0.45µm syringe filters prior to storage at -20°C for subsequent toxin analysis. Comparison between cell counts generated from growth curve analyses and flasks sacrificed for toxin analysis indicated negligible differences between the two data sets.

HPLC analysis of dinoflagellate samples

HPLC analysis of dinoflagellate samples followed the method described by Franco and Fernandez-Vila (1993), with the following amendments:

1) A silica based reverse phase column was used (C18; 250 x 4mm id, Purospher Merck), held at constant temperature (35°C), with a mobile phase flow rate of 1.0ml min^{-1}.

2) External calibration standards (NRC, Canada) were included before sample analysis and after every fourth run to monitor the performance of the system. Toxin composition profiles were determined from triplicate analyses.

3) Confirmation of toxin peaks was determined by the inclusion of internal toxin standards within samples.

Detection of SCB activity in bacterial isolates from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407

Examination of bacteria for SCB activity was carried out using the mouse neuroblastoma assay described by Gallacher and Birkbeck (1992), with the following amendments:-

1) All bacterial supernates were tested at 1/10 dilution.

2) The ouabain and veratridine concentrations were optimised to take into account matrix effects from the marine broth, and were used at concentrations of 0.6mM ouabain and 0.025mM veratridine.

3) Saxitoxin standards were diluted in 1/10 marine broth.

4) A saxitoxin standard curve was incorporated onto each sample plate.

Preparation of bacterial supernatants for determination of SCB activity

Bacterial isolates from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 were inoculated into 50ml flasks containing marine broth (30ml) and incubated in a rotary incubator (20°C, 100 osc min^{-1}). After 24h, bacterial suspensions were centrifuged (12,000 x *g*, 10min), before supernatants were carefully removed and aliquoted before freezing (-20°C), prior to analysis. Frozen supernatants were subsequently defrosted and diluted 1/10 in tissue culture dilution medium (RPMI + penicillin/streptomycin (2%)) before analysis using the MNB assay.

Optimisation of Ouabain and Veratridine Concentrations

The effect of marine broth on the ability of ouabain and veratridine to cause cell death was investigated by a ouabain/veratridine titration. This incorporated a combination of six ouabain and three veratridine concentrations in a chequerboard pattern, as described by Gallacher and Birkbeck (1992). Each well contained 50µl of the required concentration of ouabain and veratridine along with 100µl of either dilution medium, or marine broth diluted 1/10 in dilution medium. Plates were seeded as described by Gallacher and Birkbeck (1992), before incubation at 37°C for 24h. The response of the cells to ouabain and veratridine was assessed as stated in Gallacher and Birkbeck (1992).

Serial dilution of Saxitoxin Standard

A saxitoxin standard curve was constructed and included in each sample plate where sample quantification was required. STX dilutions were made up in 1/10 marine broth diluted in dilution medium. SCB activity percentages were calculated and dose response curves generated as described by Gallacher and Birkbeck (1992), to which sample SCB activity could be compared and converted to toxicity measurements.

Assessment of bacterial flora associated with dinoflagellate cultures *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a using colony morphology, RFLP and DGGE analysis

Dinoflagellate cultures *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a were subjected to microflora analysis prior to re-introduction experiments.

Samples of each culture from the three growth phases were subjected to serial dilution and plated onto marine agar as described previously in Chapter 2. All colonies from the dilution plate containing between 50 and 100 colonies were analysed further by picking and replating each isolate to obtain pure cultures.

Following classification using colony morphology (described previously in Chapter 2), isolates were also subjected to RFLP analysis (as described in Chapter 2) to further group the bacteria.

Samples from the three growth phases of each dinoflagellate culture were also subjected to analysis using DGGE. Samples were collected and analysed as described in Chapter 2, with corresponding samples from microflora analysis (see Chapter 2) included on the acrylamide gel to act as reference.

Assessment of the axenic status of *A. lusitanicum* NEPCC 253 prior to re-introduction experiments

Prior to re-introduction experiments, the bacterial status of the axenic culture was assessed following the protocols described in Chapter 3. Samples from each growth phase of the culture were subjected to investigation using the seventeen different media. Epifluorescence microscopy analysis and PCR were also performed on all growth phase samples.

Preparation of axenic dinoflagellate cultures for re-introduction experiments

Experiments allowing the re-introduction of bacteria to axenic dinoflagellate culture *A. lusitanicum* NEPCC 253 required a variety of samples to be collected from each growth phase. Large quantites of culture were required for HPLC and DGGE analysis (1000ml per analysis), therefore, each dinoflagellate culture required two flasks (1500ml cultures) per growth phase. Eight flasks of freshly subcultured axenic *A. lusitanicum* NEPCC 253 were required for re-introducing the bacterial flora of *A. lusitanicum* NEPCC 253, another eight flasks for re-introducing the microflora of *A. tamarense* PCC 173a, and a final eight flasks were required as axenic control flasks. Eight flasks of original *A. lusitanicum* NEPCC 253, were also included as a control culture. The number of flasks for each set of bacterial introductions included a single flask for growth curve analysis, and also a final flask for subsequent culture which

remained untouched during the analysis.

Disruption of dinoflagellate cultures *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a to allow the introduction of their microflora into axenic cultures of *A. lusitanicum* NEPCC 253.

Aliquots (2000ml) of stationary phase dinoflagellate cultures *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a were centrifuged (10,000 x *g*, 10 min), with the supernatant decanted and the resultant cell pellet resuspended in sterile seawater (10ml). This suspension was subjected to disruption by the addition of glass beads, followed by vortex mixing as described in Chapter 3, with microscopy used to confirm lysis of dinoflagellate cells.

Following complete lysis of dinoflagellate cells, 1ml of the suspension was subjected to a ten-fold dilution series using sterile seawater, with each dilution added (100µl) in triplicate to marine agar plates which were then incubated for 14 days at 20°C. Following incubation, the dilution plate containing between 50 and 100 colonies was analysed further, with bacteria isolated and classified as described previously in Chapter 2. Previously unidentified bacteria were subsequently identified using bi-directional 16S rDNA sequencing and phylogenetic analysis as described in Chapter 2.

Introduction of the microflora from *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a to axenic cultures of *A. lusitanicum* NEPCC 253.

The remaining disrupted cell suspension from *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a cultures were each added (500µl) to eight freshly subcultured flasks of axenic *A. lusitanicum* NEPCC 253. Flasks were swirled to generate a uniform suspension before being maintained under normal growth conditions described in Chapter 2 alongside control cultures, with growth curve data collected every second day from all cultures.

Sampling of growth phase cultures

All culture regimes were analysed at the three growth phases. Samples for HPLC analysis were collected and extracted for toxin analysis, as described previously. DGGE samples were collected and prepared for DGGE analysis as described in Chapter 2. Samples for identification using colony morphology and RFLP analysis were also taken following protocols described in Chapter 2.

RESULTS

As results from Chapter 3 demonstrated that antibiotic treated dinoflagellate cultures were bacterial-free, these cultures, *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407, along with the culture collection axenic *A. tamarense* CCMP 1771, will henceforth, be referred to as axenic cultures, whereas dinoflagellate cultures maintained with a normal bacterial flora will be referred to as control cultures. The supposedly axenic *A. tamarense* CCMP 117, will be referred to as 'axenic', when included in comparisons with truly axenic cultures. All references to lag phase cultures refer to newly subcultured flasks exhibiting minimal cell division.

The effect of removing the microflora on dinoflagellate growth curves and toxin production

Growth curves

The effects on growth and toxin production of dinoflagellate cultures following removal of bacteria (See Chapter 3), were investigated, by comparing control cultures of *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 to axenic cultures. Growth and toxicity of the axenic *A. tamarense* CCMP 1771 compared to its control culture PCC 173a were also investigated. The toxicity of *A. tamarense* CCMP 117, the dinoflagellate culture deemed 'axenic' by the culture collection was also assessed.

Figures 4.1a and b, show growth curves generated from dinoflagellate cultures *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 over a 30 day period. Respective control and axenic cultures showed no change in growth rate during the first 25 days, however, after this time, the axenic *A. lusitanicum* NEPCC 253, continued to increase in cell density, although cell numbers in the control culture began to decline. This was not observed with *A. tamarense* NEPCC 407. Further investigations into the decline in growth rate of *A. lusitanicum* NEPCC 253 control culture compared to the axenic culture were conducted. Figure 4.2 depicts the growth of *A. lusitanicum* NEPCC 253 cultures over 65 days, with the analysis

Figure 4.1a *A. lusitanicum* NEPCC 253

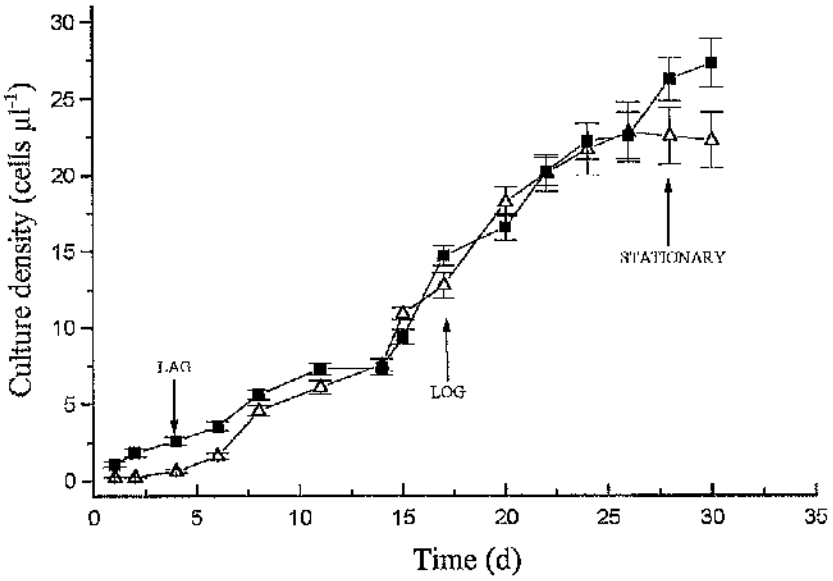


Figure 4.1b *A. tamarense* NEPCC 407

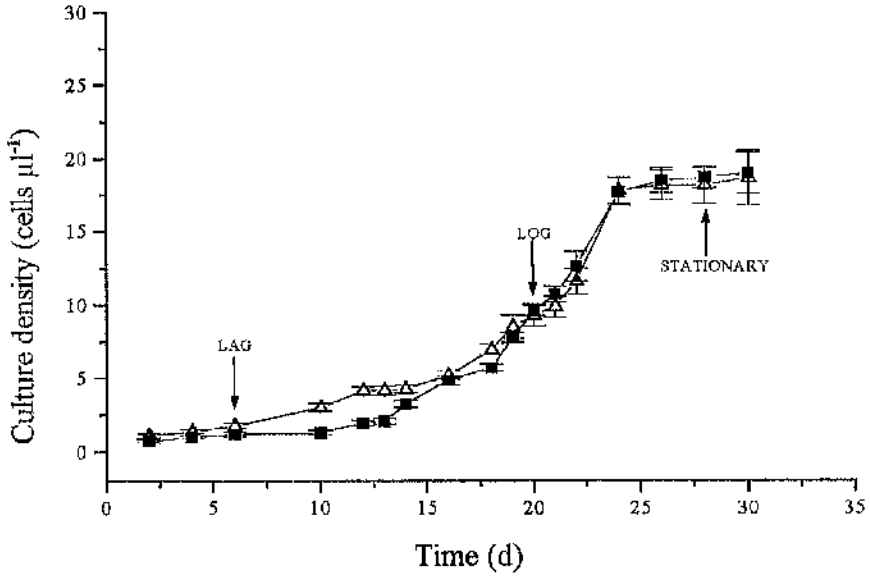


Figure 4.1 Growth curve (n = 3 ± sem) over 30 days for *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 in the presence/absence of bacteria. Arrows indicate cell sampling points for HPLC analysis.

△ = dinoflagellate culture with bacteria (control)
■ = dinoflagellate culture without bacteria (axenic)

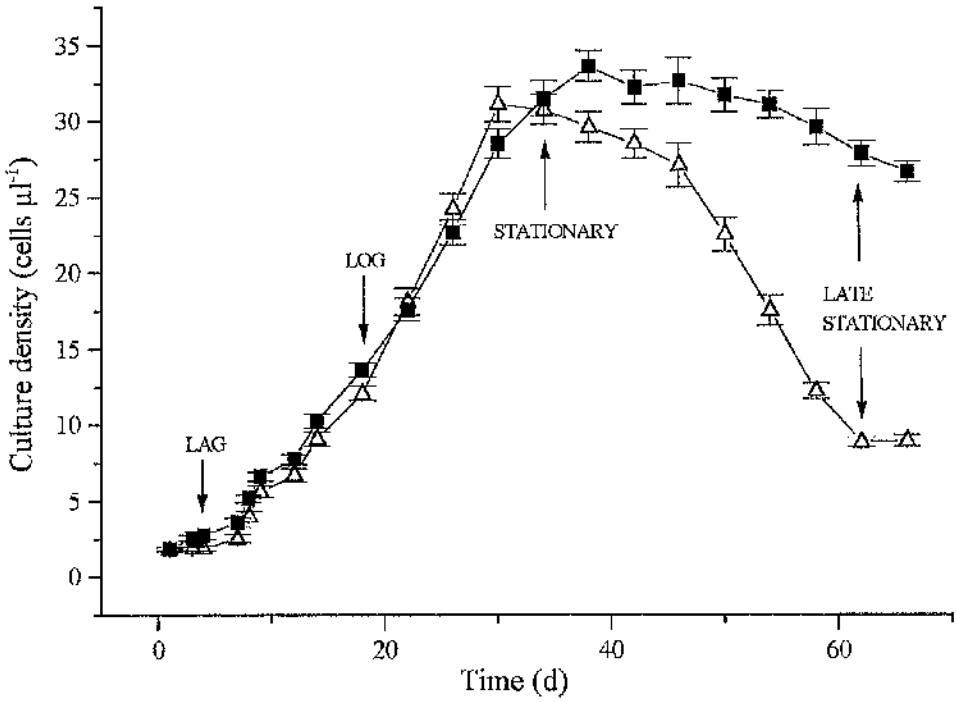


Figure 4.2 Growth curve (n = 3 ± sem) over 65 days for *A. lusitanicum* NEPCC 253 in the presence/absence of bacteria. Arrows indicate cell sampling points for HPLC analysis.

△ = dinoflagellate culture with bacteria (control)
■ = dinoflagellate culture without bacteria (axenic)

instigated following subculture of exponential cells from Fig 4.1a into fresh media. A change in growth rate was again detected between cultures, although it occurred after 30 days compared to 25 days in the previous analysis. Also, four times the number of cells were present in the axenic culture at 60 days, compared to the control culture.

Analysis of the growth cycle of *A. tamarense* CCMP 117 over a 35 day period (Fig. 4.3a), indicated the growth curve, although showing typical characteristics, had a lower cell density than other cultures. A maximum of seven thousand cells per litre of culture was noted in stationary growth phase, compared to greater than twenty thousand cell per litre of stationary growth phase detected in all other cultures. Similar patterns in growth curves for *A. tamarense* PCC 173a and *A. tamarense* CCMP 1771 over 35 days (Fig. 4.3b), were obtained in the early stages of the cycle. However, the curves started to bifurcate after 32 days, although both cultures continued to increase in cell density, with the control culture showing more cells compared to the axenic culture. This is a reversal of the pattern seen in *A. lusitanicum* NEPCC 253, where the original culture reduced in cell density compared to the axenic culture.

Effects on toxin production following antibiotic treatment of dinoflagellate cultures

All growth curve investigations, were coupled with toxin profile analysis at the three growth phases of each dinoflagellate culture. This allowed the toxin profile of *A. lusitanicum* NEPCC 253, to be assessed in two consecutive growth cycles, allowing information to be gathered on the stability of the toxin profile through subculture.

HPLC analysis was performed in triplicate for each sample, with negligible differences in values between replicate runs detected.

Figure 4.3a *A. tamarense* CCMP 117

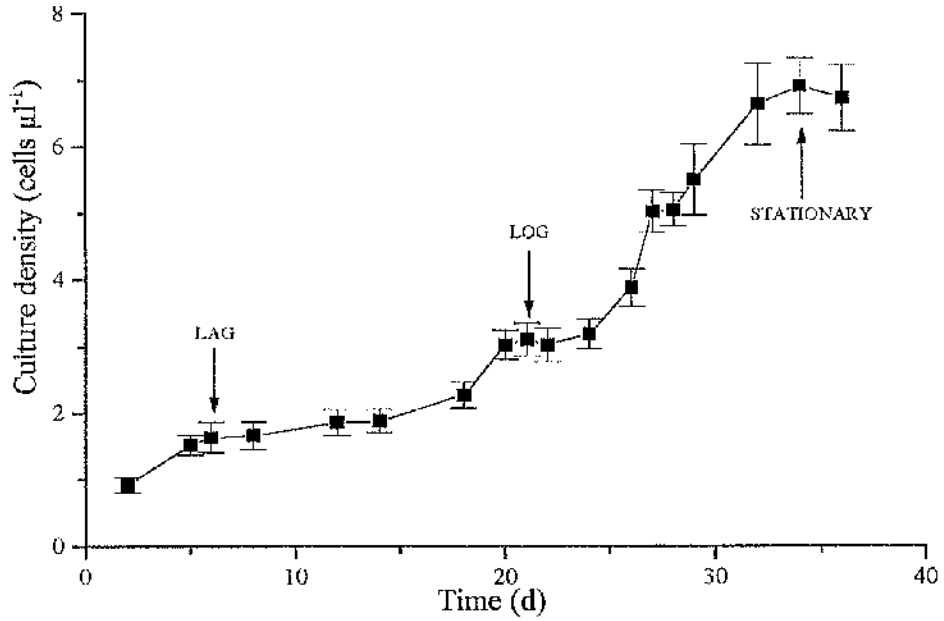


Figure 4.3b *A. tamarense* CCMP 1771 + PCC 173a

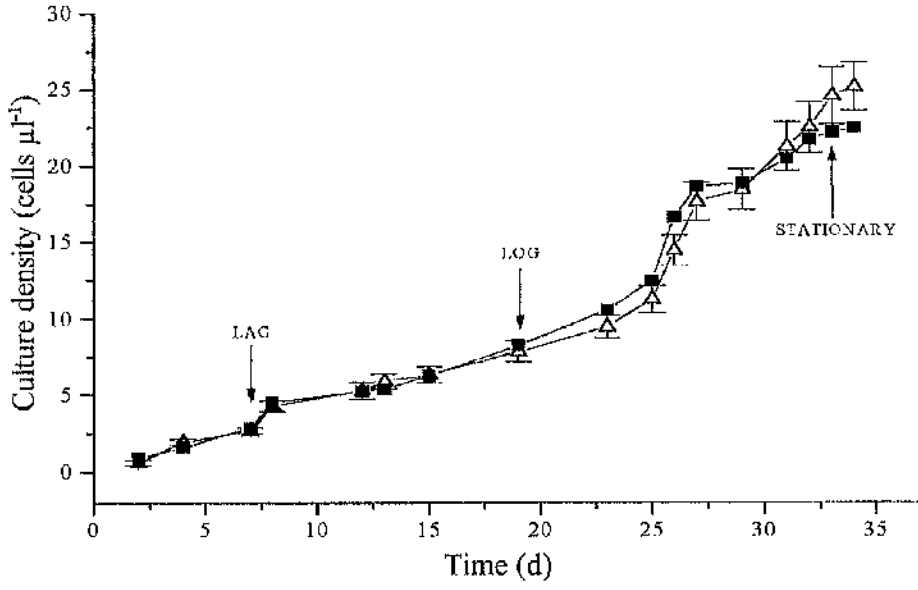


Figure 4.3 Growth curve for *A. tamarense* CCMP 117 and *A. tamarense* PCC 173a /CCMP 1771. Arrows indicate cell sampling points for HPLC analysis.

△ = dinoflagellate culture with bacteria (control)
■ = dinoflagellate culture without bacteria ('axenic')

Minimum and maximum potential toxicity values were generated using minimum and maximum cell counts obtained to take into account variation in toxicity values resulting from cell counts in a particular sample. These values are included on all toxicity graphs as error bars of toxicity values obtained from mean cell counts. Figure 4.4 demonstrates typical HPLC traces for PST standards GTX 1 - 4. GTX 1/4 standard also contained small quantities of GTX 2/3, with the standard for GTX 2/3 also containing trace quantities of dcSTX which is the small peak detected at 20 minutes. Figure 4.5 shows typical traces from *A. lusitanicum* NEPCC 253. The control culture (Fig. 4.5 A), contained five peaks, with the first peak at ca. 4.5 minutes initially thought to correspond to C toxins, however subsequent analysis indicated this was not the case. The two peaks (a and b), corresponded in retention time to GTX 4 and 1 respectively, with peaks (c and d) at 25 and 29.5 minutes possibly attributed to small quantities of GTX 3 and 2. Examination of the chromatogram from the axenic culture (Fig. 4.5B), indicated peaks a and b were again present, but at a different ratio to the control culture. Peaks c and d were also detected but in much higher quantities, with the peak at 4.5 minutes again observed. As peaks present in the sample traces corresponded in retention time to GTX 4, 1, 3 and 2 respectively, sample spiking with known concentrations of toxin standards was performed. Figure 4.6 shows the HPLC traces seen in Figure 4.5, when PST standard GTX 1/4 was added to the control culture sample (Fig. 4.6A) and GTX 2/3 was added to the axenic culture sample (Fig. 4.6B) prior to HPLC analysis. This strongly indicates the presence of GTX 1 - 4 within the samples, as the peaks in question increased when toxin standards were added.

Sample spiking is currently the strongest HPLC evidence available for confirmation of PST profiles, therefore all future reference to dinoflagellate toxin profiles in this chapter are based on this method.

Both control and axenic cultures of *A. lusitanicum* NEPCC 253 contained carbamate toxins GTX 1- 4 (Fig. 4.7 and Fig. 4.8). Non-quantifiable traces of GTX 2 were detected in the lag phase of the control culture, and also in log phase of both control

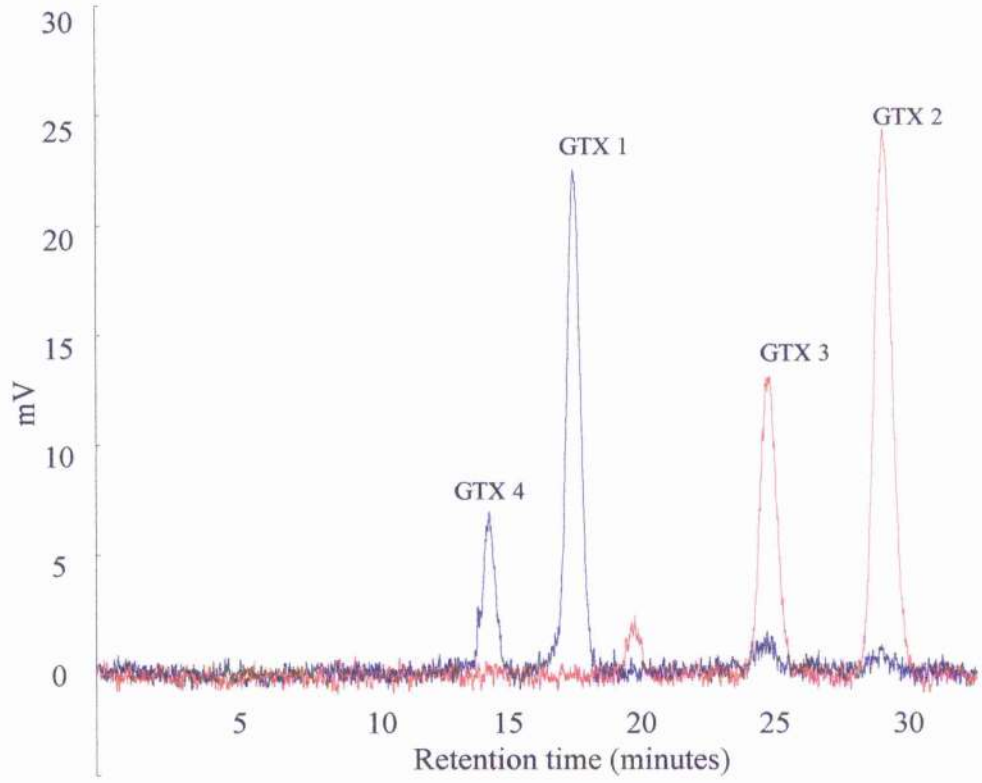


Figure 4.4 HPLC analysis of GTX standards

---- = GTX 1/4 standard

---- = GTX 2/3 standard

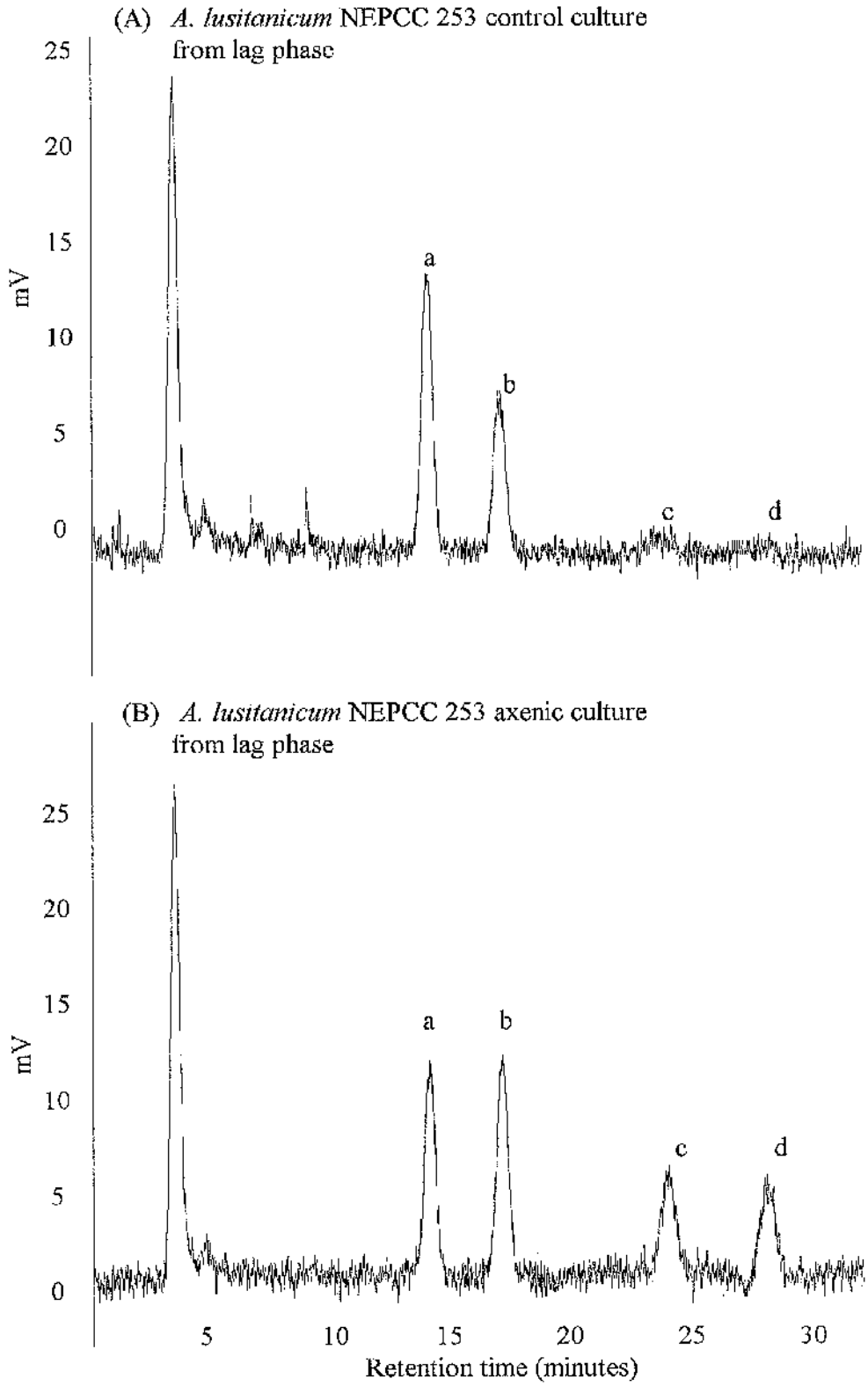


Figure 4.5 HPLC analysis of *A. lusitanicum* NEPCC 253 culture. (A) control culture, (B) axenic culture. Peaks a - d are referred to in the text.

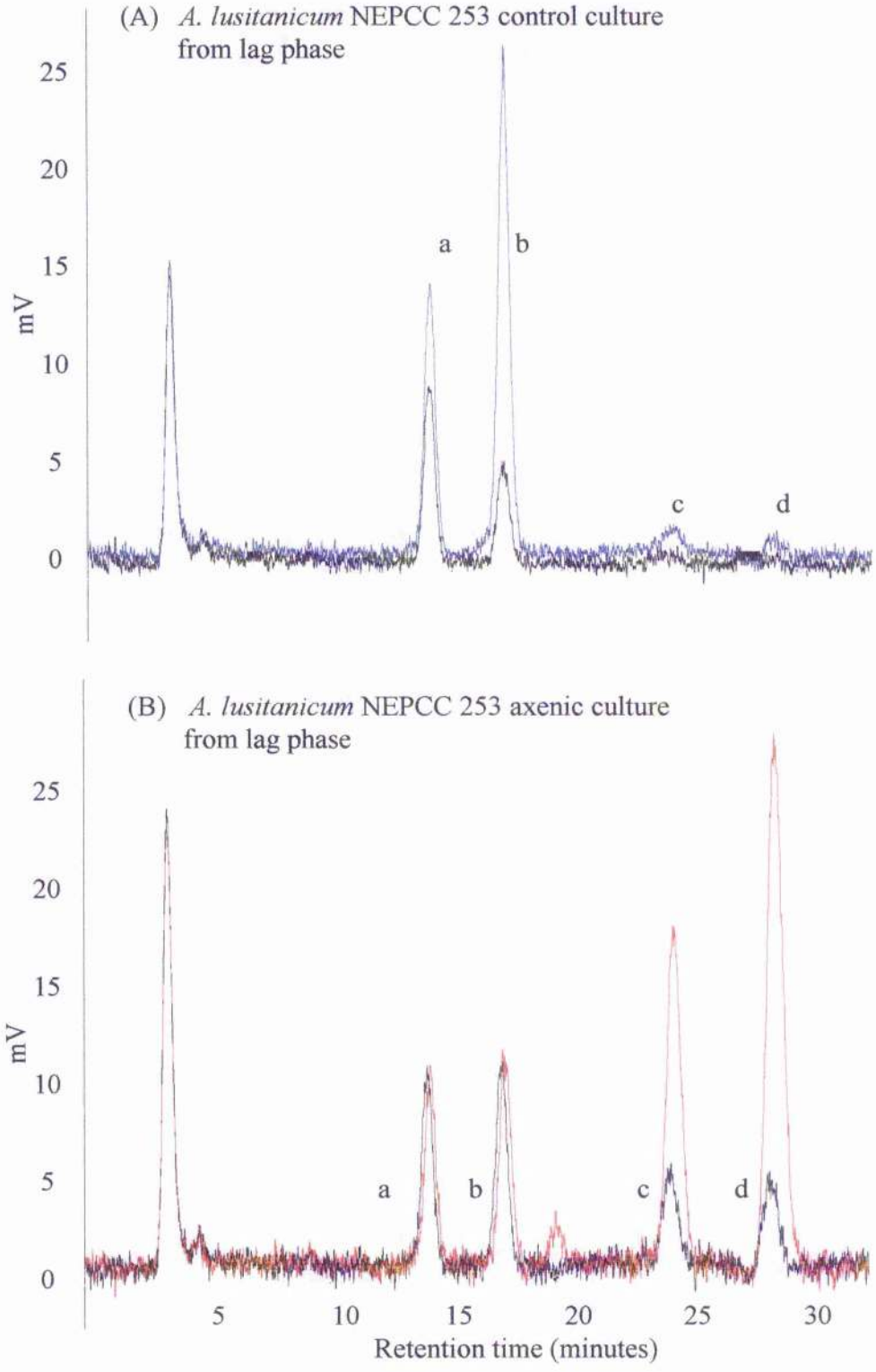


Figure 4.6 HPLC analysis of control and axenic *A. lusitanicum* NEPCC 253 cultures spiked with GTX 1 - 4. A = Control culture spiked with GTX 1/4; B = Axenic culture spiked with GTX 2/3. Black traces represent dinoflagellate sample traces, coloured traces (blue and red) represent samples spiked with GTX standards.

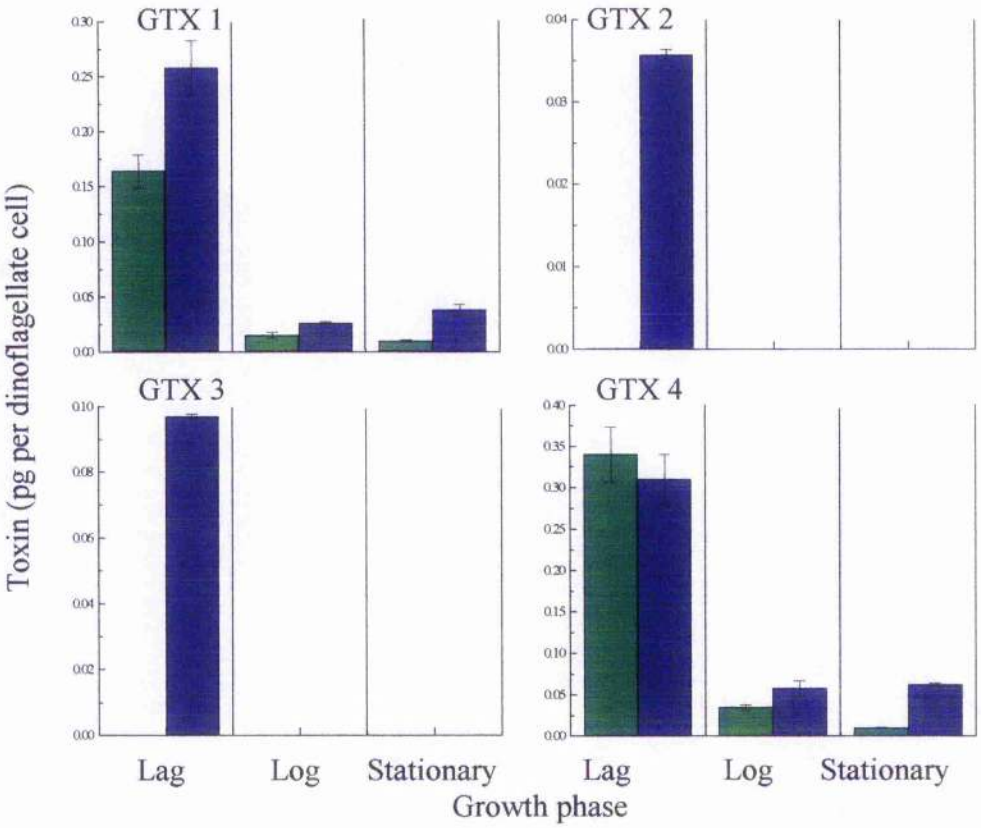


Figure 4.7 Effect of bacteria on PST production by *A. lusitanicum* NEPCC 253, grown in batch culture over 30 days. Results calculated from HPLC data (n = 3), with error bars indicating the maximum and minimum toxicity values, when variance in dinoflagellate cell counts are considered.

■ = control culture
■ = axenic culture

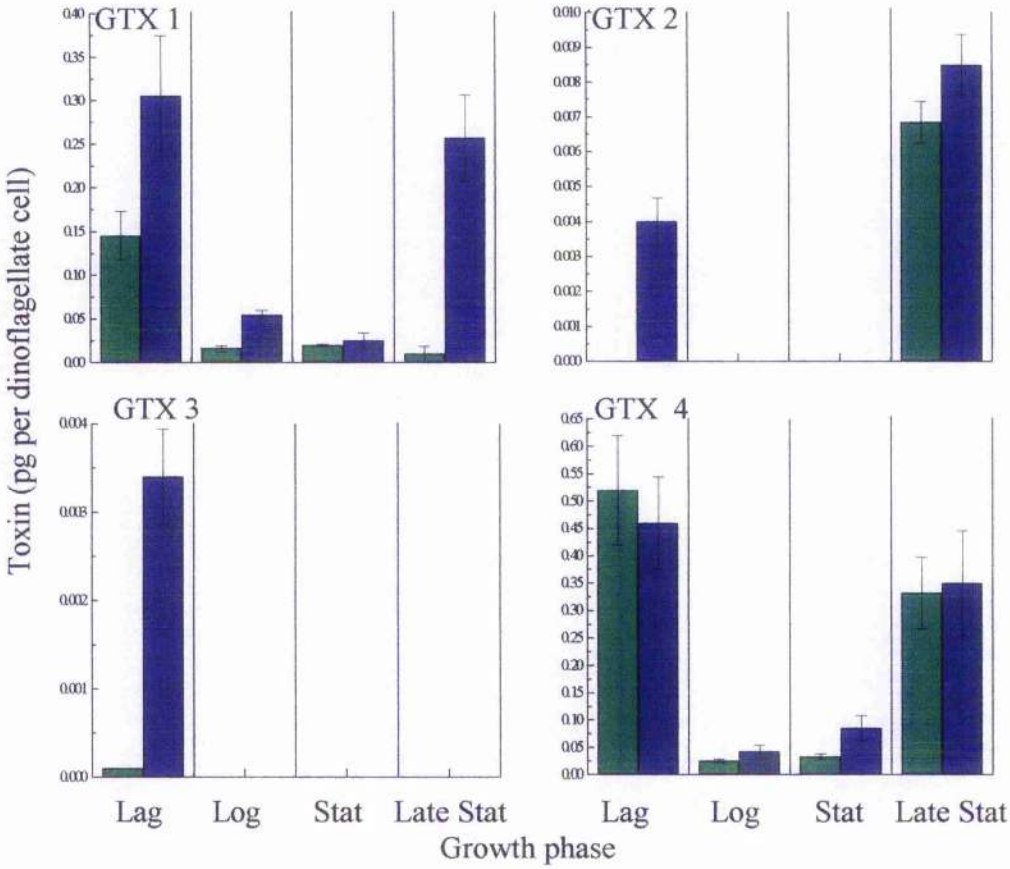


Figure 4.8 Effect of bacteria on PST production by *A. lusitanicum* NEPCC 253, grown in batch culture over 65 days. Results calculated from HPLC data (n = 3), with error bars indicating the maximum and minimum toxicity values, when variance in dinoflagellate cell counts are considered.

■ = control culture
■ = axenic culture

and axenic cultures. This was also the case with GTX 3, with the exception of lag phase in the 65 day analysis (Fig. 4.8), where levels of GTX 3 although small, were quantifiable. The axenic culture produced the most of each individual toxin in all growth phases, with the exception of GTX 4 during lag and late stationary phase (Figs. 4.7 and 4.8), where no significant difference was detected between the control and axenic cultures. The majority of toxicity in both cultures was detected in lag phase (Fig. 4.7 and Fig. 4.8), and late stationary phases (Fig. 4.8) of growth.

Toxin analysis of *A. tamarense* NEPCC 407 (Fig. 4.9), indicated a more diverse toxin profile than *A. lusitanicum* NEPCC 253, with six quantifiable toxin groups comprising of both carbamate and sulfocarbamoyl toxins. A further toxin - dcGTX 2, a decarbamoyl toxin, was also suspected in all phases of *A. tamarense* NEPCC 407. However, no calibrated standard was present, therefore quantification was not possible.

The most limited profile of both *A. tamarense* NEPCC 407 cultures, was detected in lag phase, with only three quantifiable toxins present in the control culture and two quantifiable toxins present in the axenic culture. However, trace amounts of other toxins were present; with GTX 1 detected in the control culture and neoSTX detected in the axenic culture. C 1 toxin was also present in trace amounts in the log phase of the axenic culture.

In general, the toxin profiles of *A. tamarense* NEPCC 407 cultures were similar, although, quantities varied greatly. Interestingly, distinctly different trends noted between cultures was seen in the production of GTX 4, with the control culture decreasing in toxicity over the growth cycle, while the axenic culture showed an increase in production as the culture matured. The detection of large quantities of GTX 4 in the axenic culture at stationary phase, coincided with the production of large quantities of C 4, the sulfocarbamoyl toxin formed following acidic derivatisation of GTX 4, whose presence was not detected in any control culture growth phases even when high levels of GTX 4 were present. This elevated level of

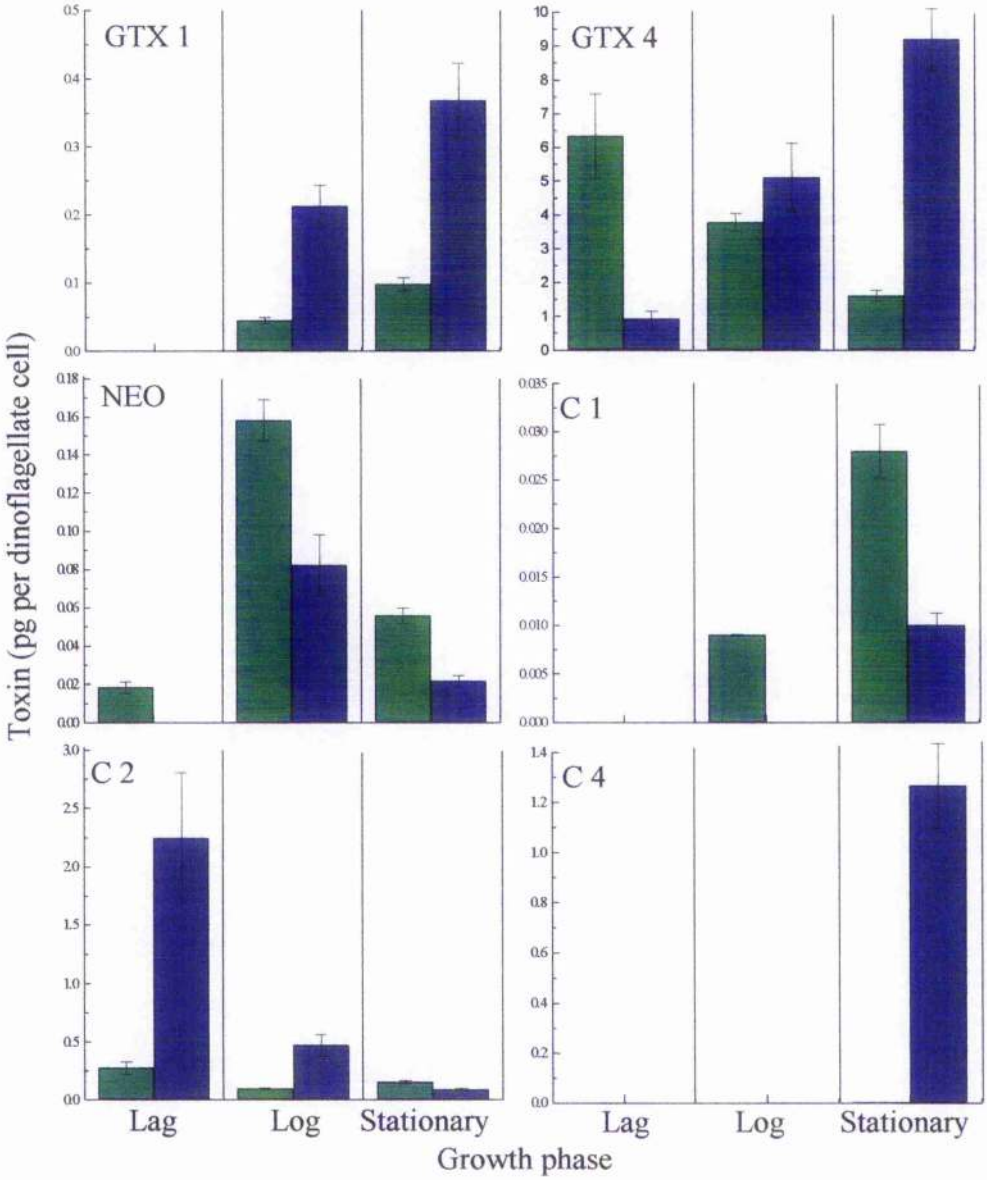


Figure 4.9 Effect of bacteria on PST production by *A. tamarense* NEPCC 407, grown in batch culture over 30 days. Results calculated from HPLC data (n = 3), with error bars indicating the maximum and minimum toxicity values, when variance in cell counts are considered.

■ = control culture
■ = axenic culture

GTX 4 also coincided with an increase in its isomer GTX 1.

Toxin profiles for the 'axenic' *A. tamarense* CCMP 117 (Figure 4.10), also showed a diverse profile of seven quantifiable toxins from the carbamate and sulfocarbamoyl toxin groups, with the presence of dcGTX 2 also suspected in all growth phases. Similar to *A. tamarense* NEPCC 407 cultures, the most limited toxin profile occurred during lag phase. GTX 1 and 2 were not present in this phase, although their level increased sharply throughout the rest of the growth cycle, in opposition to C 2, which decreased sharply over the growth cycle. The other toxins detected (GTX 3, GTX 4, NeoSTX and C 1) did not exhibit such clear trends, with highest toxicity values of GTX 3 and 4 detected in lag phase, whilst highest levels of NeoSTX and C 1 were present at stationary phase.

As expected toxicity was not detected in dinoflagellate cultures *A. tamarense* PCC 173a and CCMP 1771.

Identification of SCB production from bacteria previously isolated from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407

Bacterial isolates from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407, as detailed in Chapter 2, were assessed for their ability to produce SCB toxins using the mouse neuroblastoma assay. Potentially positive isolates, were re-tested on at least two subsequent occasions, with results indicating 37% of isolates from *A. lusitanicum* NEPCC 253 produced SCB activity. These isolates were from three RFLP patterns groups (patterns 1, 2 + 3) within the α-Proteobacterial subclass with approximately 50% of RFLP pattern 1 and 3 isolates exhibiting SCB activity, whereas only 20% of RFLP pattern 2 isolates tested positive (Chapter 2, Fig. 2.4 + 2.5) . SCB activity was also detected in 55% of *A. tamarense* NEPCC 407 bacterial isolates, with SCB producers comprising approximately 33% of the three major RFLP pattern groups (patterns 2, 3 +6) from the α and γ-Proteobacterial subclasses (Chapter 2 Fig. 2.4, 2.5 + 2.6). However, all toxic isolates produced SCB activity

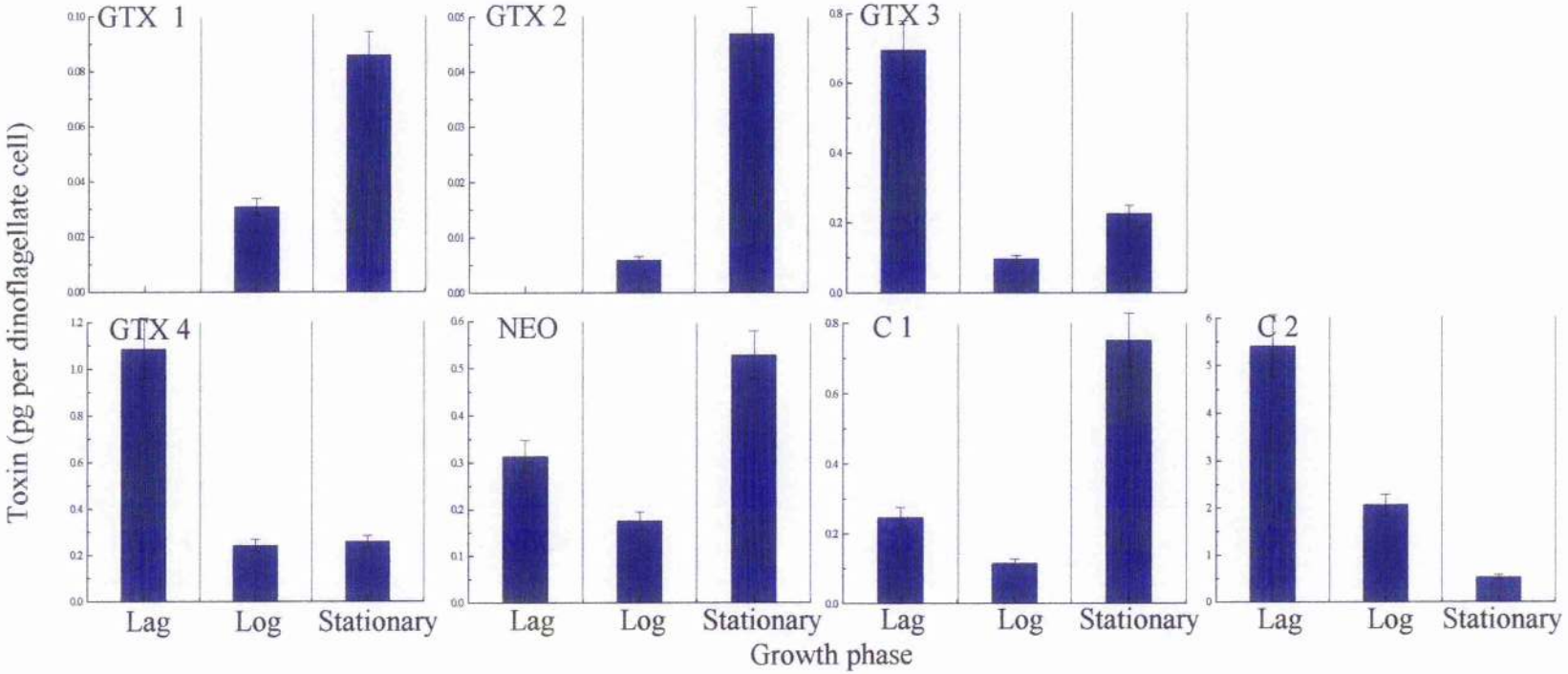


Figure 4.10 Toxicity profile from *A. tamarense* CCMP 117 grown in batch culture over 35 days. Results calculated from HPLC data (n = 3), with error bars indicating the maximum and minimum toxicity value, when variance in cell counts are considered.

of less than 25fmol STX equivalents per bacterial cell.

Assessment of the microflora from *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a prior to addition to axenic cultures

The microflora of *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a cultures, was assessed prior to introduction to axenic *A. lusitanicum* NEPCC 253 cultures, by comparison with results from Chapter 2 (Fig. 4.11 + Fig. 4.12 data sets 1 + 2). Both cultures possessed the same bacterial morphotypes as described previously, with these similarities confirmed using RFLP analysis (Chapter 2; Table 2.2). Numbers of each morphotype remained constant between the two analyses, although 16 months apart, with the exception of RFLP pattern 11 and 12 isolates from *A. tamarense* PCC 173a (Fig. 4.12), whose numbers had slightly increased and decreased respectively. In order to introduce bacteria to axenic cultures, control cultures were subjected to disruption to lyse dinoflagellate cells (described previously in Chapter 3). Comparison of the microflora from cultures following lysis (Fig 4.11 + 4.12 data set 3), indicated the four morphotypes of *A. lusitanicum* NEPCC 253 (Fig. 4.11 data set 3) and the seven from *A. tamarense* PCC 173a (Fig. 4.12 data set 3) remained comparable with bacterial numbers of unlysed cultures.

However, lysis also identified the presence of two new bacterial morphotypes in *A. lusitanicum* NEPCC 253 and a further two morphotypes in *A. tamarense* PCC 173a (Table 4.1 + Table 4.2). These isolates were subsequently examined using RFLP analysis (See Chapter 2), which grouped one of the strains from *A. lusitanicum* NEPCC 253, the translucent pinprick colony (Table 4.1), as RFLP pattern 21 (Chapter 2; Fig. 2.4; Table 2.4). This pattern was previously generated by a *S. trochoidea* NEPCC 15 bacterial isolate, subsequently identified as a *Hyphomonas* related species. However, colony morphology between the original RFLP pattern 21 isolate (smooth cream with white centre) and the newly isolated *A. lusitanicum* NEPCC 253 strain were not compatible. The other isolate from *A. lusitanicum* NEPCC 253, the small pink pinprick morphotype, generated an RFLP pattern not

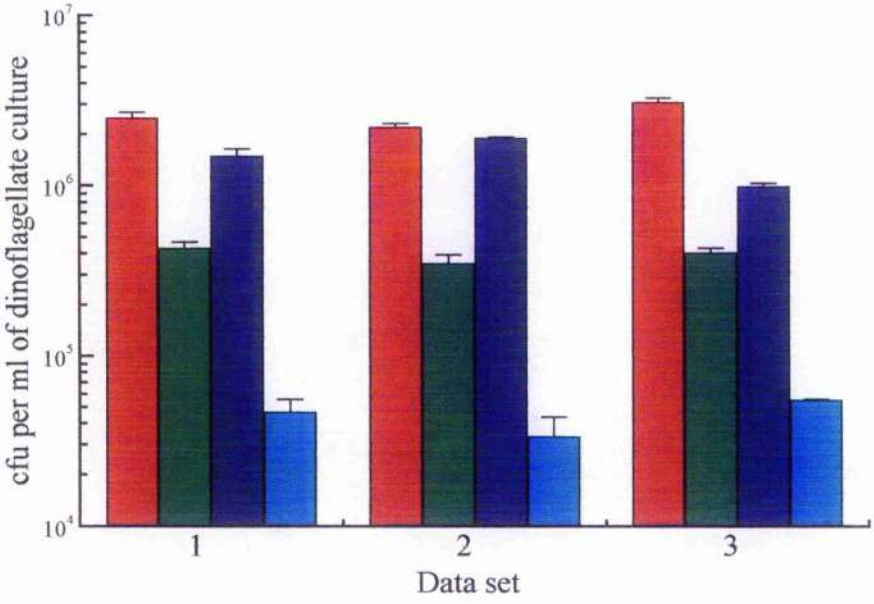


Figure 4.11 Comparison of bacterial numbers present on marine agar, per ml of *A. lusitanicum* NEPCC 253 culture, expressed as RFLP patterns, based on criteria determined in Chapter 2. ■= yellow/orange isolates with no RFLP pattern; ■= RFLP pattern 1 isolates; ■ = RFLP patterns 2 and 3 isolates; ■ = RFLP pattern 4 isolates.

Dataset 1 = control culture Chapter 2,
Dataset 2 = control culture 16 months later prior to re-introduction experiments,
Dataset 3 = dinoflagellate culture subjected to cell lysis during re-introduction experiments.

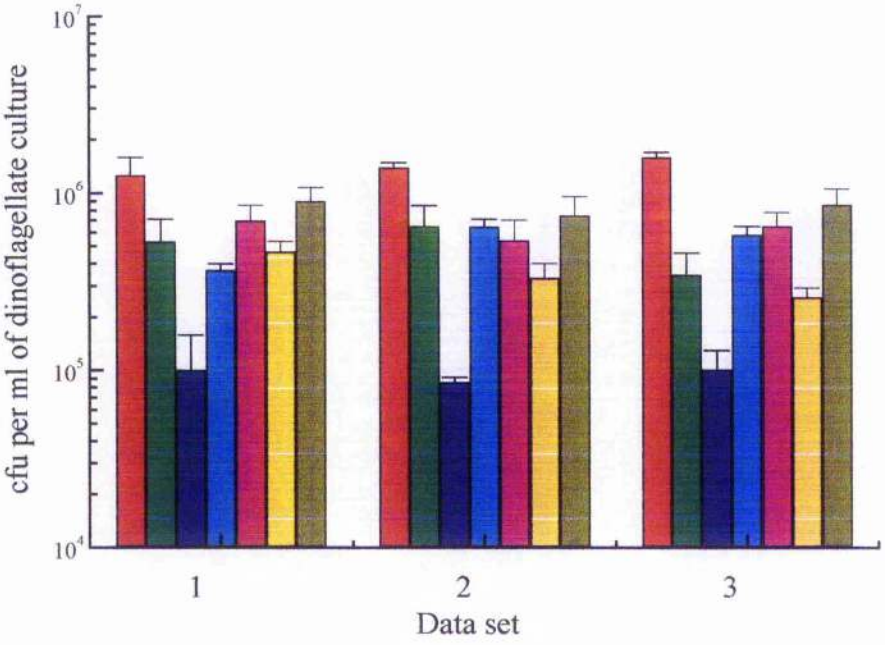


Figure 4.12 Comparison of bacterial numbers present on marine agar, per ml of *A. tamarense* PCC 173a culture, expressed as RFLP patterns, based on criteria determined in Chapter 2. ■ = RFLP pattern 2 isolates, ■ = RFLP pattern 8 isolates, ■ = RFLP patterns 10 isolates, ■ = RFLP pattern 11 isolates, ■ = RFLP pattern 12 isolates, ■ = RFLP pattern 13 isolates, ■ = RFLP pattern 15 isolates.

Dataset 1 = control culture Chapter 2,
Dataset 2 = control culture 16 months later prior to re-introduction experiments,
Dataset 3 = dinoflagellate culture subjected to cell lysis during re-introduction experiments

Morphotypes	RFLP patterns*	Identification*
yellow/orange mucoid	no pattern	*Gelidibacter algens* related isolates
large cream	pattern 1	*Agrobacterium kieliense* related isolates
convex cream with rose centre	patterns 2 and 3	*Roseobacter* clade, no closely defined species
flat irregular beige	pattern 4	*Agrobacterium stellulatum* related isolates
translucent pinprick	pattern 21	*Hyphomonas sp.*
small pinprick pink	not previously identified	*Sphingomonas sp.*

Table 4.1 Colony morphotypes isolated from lysed *A. lusitanicum* NEPCC 253 cells, prior to re-introduction to axenic cultures.

* Refer to Chapter 2

Morphotypes	RFLP patterns*	Identification*
convex cream with rose centre	pattern 2	*Roseobacter* clade, no closely defined species
convex smooth brown	pattern 8	*Roseobacter* clade, no closely defined species
smooth raised cream	pattern 10	*Antarctobacter heliothermus* related isolates
mucoid raised yellow	pattern 11	*Cytophaga* isolates, with no closely defined species
circular flat yellow	pattern 12	*Cytophaga* isolates, with no closely defined species
convex viscous yellow	pattern 13	*Glaciecola punicea* related isolates
punctiform mucoid pink	pattern 15	*Roseobacter* clade, no closely defined species
small pinprick pink	not previously identified	*Sphingomonas sp.*
irregular flat beige	not previously identified	*Sinorhizobium fredii* related isolates

Table 4.2 Colony morphotypes isolated from lysed *A. tamarense* PCC 173a cells, prior to re-introduction to axenic cultures.

* Refer to Chapter 2

detected previously during microflora characterisation in Chapter 2. However, the pattern was also generated by one of the newly identified *A. tamarense* PCC 173a isolates (Table 4.2), with colony morphology confirming similarity. The final isolate from *A. tamarense* PCC 173a, the irregular flat beige isolate also generated a previously undetected RFLP pattern. All four isolates, were subjected to 16S rDNA sequencing and phylogenetic analysis as described in Chapter 2 (See Appendix 3 for individual DNA sequences). Briefly, sequences were submitted to RDP to obtain a preliminary list of closest phylogenetic neighbours, with S_AB values generated from RDP allowing isolates to be classified according to phylum and sub-phylum affiliations, and subsequently aligned using ARB. Phylogenetic analysis identified the newly isolated bacteria from *A. lusitanicum* NEPCC 253 exhibiting RFLP pattern 21, was also a *Hyphomonas* related isolate, with an S_AB value of 0.7. The small pink pinprick isolates from *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a, were both classified as *Sphingomonas* related isolates with S_AB values of between 0.62 and 0.65 respectively. The beige bacterial isolate from *A. tamarense* PCC 173a, was classified as a *Sinorhizobium fredii* related isolate, associated with *Rhizobium* isolates with an S_AB value of 0.77.

DGGE analysis of control cultures prior to reintroduction studies, generated identical profiles to those generated during microflora characterisation experiments 16 months previous (See Chapter 2; Fig. 2.8; Tables 2.13 - 2.16). Therefore, further identification of the bacterial species present was not required.

However, as further bacterial morphotypes had been generated by culture lysis (described above), a re-examination of bacterial species identified previously using DGGE in Chapter 2 was instigated. This indicated DGGE analysis had previously identified bacterial sequences in *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a that were not confirmed by RFLP analysis. DGGE analysis of *A. lusitanicum* NEPCC 253 in Chapter 2 identified a *Hyphomonas* related species, not confirmed by culturing, with DGGE analysis of *A. tamarense* PCC 173a detecting a *Sphingomonas* related species which was not identified by culture methods. It is

therefore possible that these new isolates identified following dinoflagellate cell lysis, although culturable, were not detected due to competition, or they were able to grow on marine agar during microflora characterisation experiments in Chapter 2. However, lysis of cultures allowed these previously uncultured isolates to grow indicating the possibility of intracellular origin.

Effects on microflora stability, growth and toxicity following re-introduction of bacteria to axenic *A. lusitanicum* NEPCC 253

Effects on microflora stability, growth and toxicity upon re-introducing bacteria to axenic cultures of *A. lusitanicum* NEPCC 253 were assessed by introducing the original microflora of *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a. Introduction of *A. tamarense* PCC 173a microflora allowed effects on *A. lusitanicum* NEPCC 253 to be determined in the presence of a different bacterial microflora.

Microflora stability

RFLP and DGGE were used to assess the fate of the re-introduced microflora throughout the growth cycle, by comparison to control cultures of *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a. These control cultures were maintained alongside re-introduction flasks, with comparisons made at the three growth phases. Table 4.3a and b shows RFLP analysis results, with the RFLP patterns present in control cultures throughout the growth cycle, compared to the RFLP patterns present in re-introduction cultures. Both tables indicate that the microflora of the re-introduction cultures behaves the same as the microflora of the control cultures.

This was expected with *A. lusitanicum* NEPCC 253, as all morphotypes were detected throughout the growth cycle. However, with the microflora of *A. tamarense* PCC 173a being more complex, with certain morphotypes only detected at selected growth stages and with the microflora being introduced into a new dinoflagellate host culture, it was not expected that 100% similarity would exist.

RFLP pattern	Tentative ID (related strain)	Detected in control culture			Present on re-introduction	Detected after re-introduction of original microflora		
		Lag	Log	Stat		Lag	Log	Stat
1	*Agrobacterium*	x	x	x	x	x	x	x
2	*Roseobacter*	x	x	x	x	x	x	x
3	*Roseobacter*	x	x	x	x	x	x	x
4	*Agrobacterium*	x	x	x	x	x	x	x

Table 4.3a Presence of *A. lusitanicum* NEPCC 253 related bacteria in the control culture compared to re-introduction cultures.

RFLP pattern	Tentative ID (related strain)	Detected in control culture			Present on re-introduction	Detected after re-introduction of original microflora		
		Lag	Log	Stat		Lag	Log	Stat
2	*Roseobacter*	x	x	x	x	x	x	x
8	*Roseobacter*	x	x	x	x	x	x	x
10	*Antarctobacter*	x	x	x	x	x	x	x
11	*Cytophaga*	x	x	x	x	x	x	x
12	*Cytophaga*	x	x	x	x	x	x	x
13	*Glaciecola*	x	x	x	x	x	x	x
14	*Pseudomonas*	x	x	-	-	x	x	-
15	*Roseobacter*	x	x	x	x	x	x	x

Table 4.3b Presence of *A. tamarense* PCC 173a related bacteria in the control culture compared to re-introduction cultures.

x = detected

- = not detected

However, this was apparent, with certain morphotypes detected in later growth phases, which were not present on re-introduction due to the addition of the microflora from a lag phase culture. This would appear to indicate that the microflora of both dinoflagellate cultures were stable, with the microflora of *A. tamarense* PCC 173a capable of survival in a foreign host environment, but also the ability of *A. lusitanicum* NEPCC 253 cells to sustain a very different bacterial population to its usual microflora.

Stability of the microflora in re-introduction experiments throughout the growth phase was also confirmed by DGGE analysis, with identical profiles generated from control and re-introduction cultures.

Growth curves and toxicity assessment

Effects on growth following addition of the different bacterial floras, were compared both to control and axenic cultures (Fig. 4.13), with both new cultures generating growth curves comparable to the control culture, with a rapid drop in culture density noted on reaching stationary phase (Fig. 4.13 A, C + D). The axenic culture also appeared to drop in cell density on reaching stationary phase, although this effect was not as acute as in cultures containing bacteria. Toxin analysis of corresponding samples (Fig. 4.14), indicated profiles remained constant between cultures, with GTX 1 - 4 detected in samples at all phases, although the quantities of each toxin differed.

The quantity of toxins produced by the culture containing the re-introduced microflora of *A. lusitanicum* NEPCC 253 (Fig. 4.14), was particularly high in lag phase, with at least twice the quantity of each toxin present compared to all other cultures. This effect was drastically reduced in log phase. Stationary phase appeared to be the least toxic of the three growth phases, with the control culture producing the most GTX 2 and 3, with the axenic culture producing the most GTX 1 and 4.

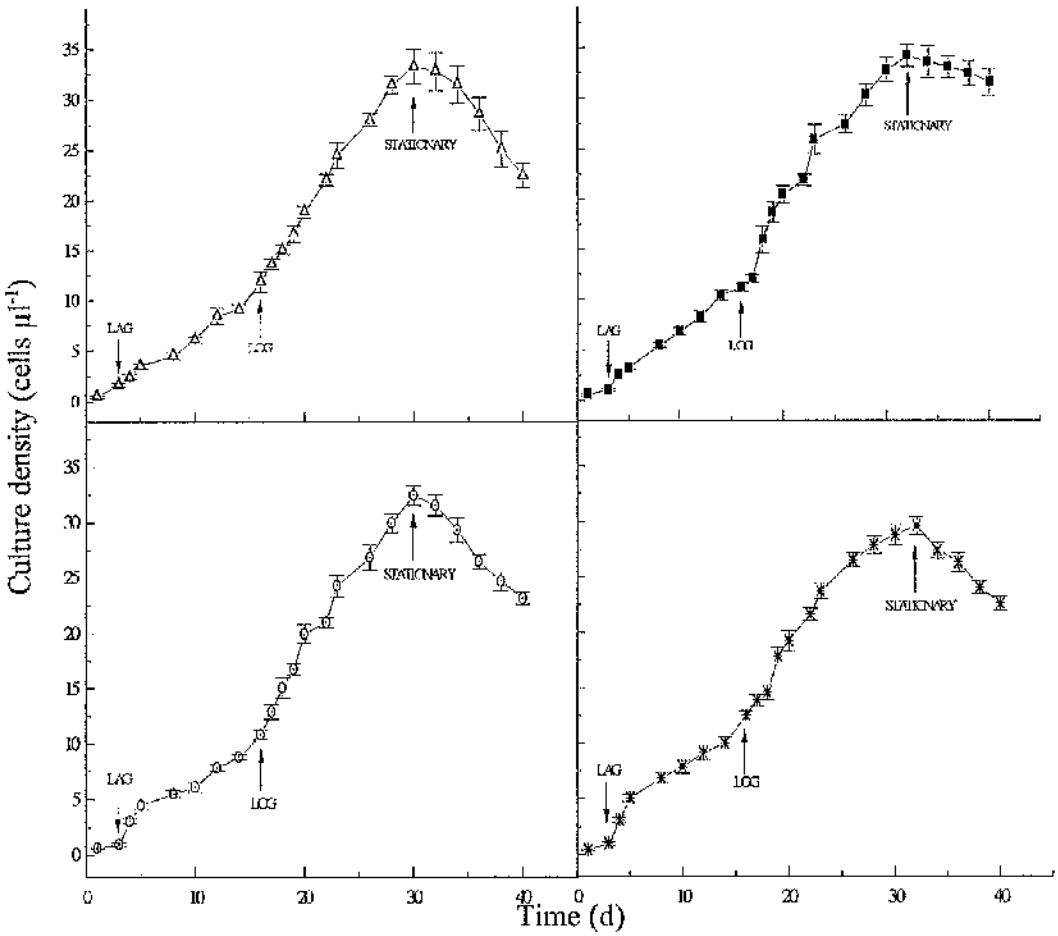


Figure 4.13 Growth curves (n = 3 ⊥ sem) for *A. lusitanicum* NEPCC 253. Arrows indicate cell sampling points for HPLC analysis. (△) control culture, (■) axenic culture, (○) axenic culture with original bacterial flora re-introduced, (✳) axenic culture with the bacterial flora from *A. tamarense* PCC 173a introduced.

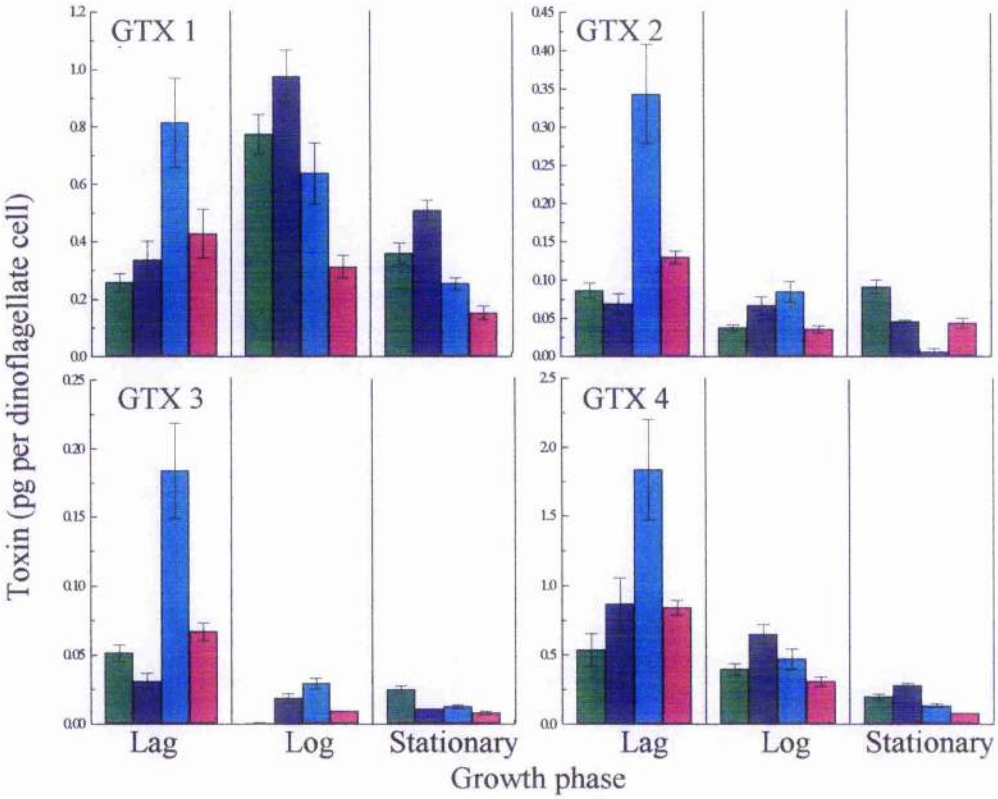


Figure 4.14 Effect of bacterial re-introduction on PST production by *A. lusitanicum* NEPCC 253, grown in batch culture over 35 days. Results calculated from HPLC data (n = 3), with error bars indicating the maximum and minimum toxicity values detectable, when variance in cell counts are considered.

■ = control bacterialised culture, ■ = axenic culture, ■ = axenic culture with original microflora re-introduced, ■ = axenic culture with microflora from *A. tamarense* PCC 173a introduced.

Generally the profile of the culture containing the *A. tamarense* PCC 173a microflora did not appear particularly similar to the axenic culture profile indicating the non-toxic dinoflagellate microflora was exerting some effect over dinoflagellate toxicity.

Comparison of control and axenic toxin profiles throughout the duration of the study

During the course of the current study which lasted 16 months, three growth and toxicity profiles were generated for control and axenic *A. lusitanicum* NEPCC 253 cultures, with the three toxin profiles presented in Fig. 4.15. Data sets 1 and 2 were generated from consecutive subcultures in months one and two, with data set 3 collected 16 months after data set 1. Comparison of growth curves (Fig. 4.1a; Fig. 4.2; Fig. 4.13 A + B), indicated similar trends for each culture throughout the experimental period, although the reduction in cell numbers seen in control cultures during stationary phase, was more pronounced in months 2 and 16 compared to month 1. However, the initial subculture for month 1, was less dense than the other two experiments, and cell densities reached during stationary phase were much lower. Therefore, it is possible this affected resultant growth curves.

Comparison of axenic culture growth curves also indicated lower cell densities in stationary phase of month 1, than the other two data sets, with again a lower cell inoculum added to initial flasks. However, growth trends in all axenic cultures were consistent with cell densities remaining constently higher than control cultures on reaching stationary phase.

Assessment of toxicity profiles from control and axenic cultures at months 1 and 2 of the investigation (Fig. 4.15 data sets 1 + 2), indicate limited differences between toxicity profiles, with values directly comparable as expressed per cell basis. However comparison of these toxin profiles, with profiles generated from cultures 16 months later (Fig. 4.15 data set 3), indicate toxin profiles in both control and

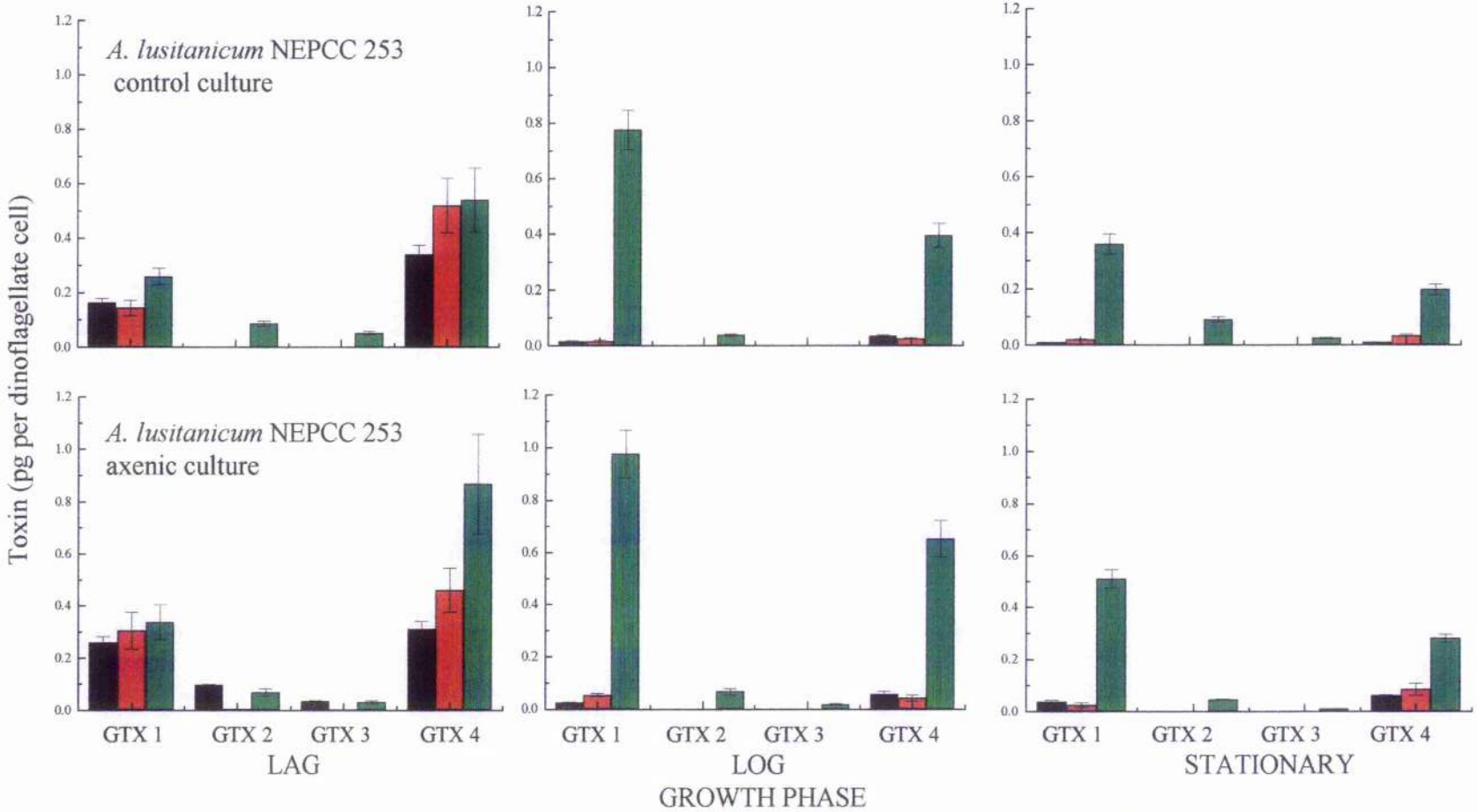


Figure 4.15 Comparison of toxin profiles over a 16 month period for control and axenic cultures of *A. lusitanicum* NEPCC 253, grown in batch culture over 30 days. Results calculated from HPLC data (n = 3), with error bars indicating the maximum and minimum toxicity values, when variance in dinoflagellate cell counts are considered. ■ = quantity of toxin from month 1; ■ = quantity of toxin from month 2; ■ = quantity of toxin from month 16.

axenic cultures have altered over the period. Data sets from months 1 + 2 show GTX 1 reduces dramatically through the growth cycle in both control and axenic cultures. However, 16 months later (data set 3), GTX 1 increases between lag and log phases.

A marked difference was also noted in quantities of GTX 2 and 3 over the time course of the experiment. Only trace amounts were detected by control cultures during months 1 and 2 (Fig. 4.15 data sets 1 + 2), however, data set 3 indicates quantifiable levels of both toxins in all phases with the exception of log phase, where only trace levels of GTX 3 remained. Levels of GTX 2 and 3 for the axenic culture also showed higher levels in data set 3 than previous data sets, with the exception of lag phase, where levels were comparable with the first data set. GTX 4 levels remain at detectable levels in all cultures at all phases, with each data set showing highest quantities per cell basis in lag phase. Generally each data set indicates a dramatic fall in GTX 4 during log phase compared to lag phase, with stationary phase containing comparable levels of GTX 4 to log phase.

In conclusion, both control and axenic cultures reported higher or comparable levels of all toxins per cell basis in re-introduction experiments (Fig. 4.15 data set 3), when compared to original experiments (Fig. 4.15 data sets 1 + 2), indicating although growth cycles may have remained relatively constant over the course of the investigation, along with numbers of associated microflora, toxin profiles have altered dramatically.

DISCUSSION

Toxin profiles from all control dinoflagellate cultures used in the current study had previously been determined, although, investigations were only carried out at stationary phase (Cembella *et al.*, 1987; Franca *et al.*, 1995; Hummert *et al.*, 1998). Similar profiles to previous published reports were reported in the current study, with the toxicity profile from *A. lusitanicum* NEPCC 253 identical to that reported by Franca *et al.*, (1995), who also indicated it had remained constant for the last 30 years. Franca *et al.*, (1995) also detected the presence of a large peak which was thought to be due to C toxins, as seen in the current study, however in both cases, this was later rejected.

Previous reports examining effects on growth following the generation of axenic cultures, generally indicate that algae grow more slowly in axenic culture. This reduced growth rate has been attributed to a reduction in nutrients within the growth medium, usually supplied by the associated bacteria (Singh *et al.*, 1982; Tostensen *et al.*, 1989). Investigations into toxin production following the generation of axenic cultures however, has yielded more controversial results than effects on growth, with reports of non-existent, lower, similar and higher levels of toxin production in axenic cultures compared to control cultures (Singh *et al.*, 1982; Tostensen *et al.*, 1989; Kim *et al.*, 1993; Doucette and Powell, 1998). There is only one report documenting the effects on toxicity following the re-introduction of bacteria to axenic cultures, however information on growth rates was not presented.

The aim of the current study was to identify changes in growth rates and toxin production following the generation of truly axenic dinoflagellate cultures (Chapter 3), with subsequent examination of these factors when different bacterial microflora were introduced. The latter part of the study also assessed the bacterial microflora, growth rates and toxin profiles of dinoflagellate cultures over a 16 month period, to determine whether these functions remained stable.

As mentioned previously, growth rates of axenic cultures in some published reports were lower than control cultures (Singh *et al.*, 1982; Tostensen *et al.*, 1989),

however, in the current study this was not always the case. Growth rates of all axenic cultures matched control cultures, until stationary phase, upon which time axenic *A. lusitanicum* NEPCC 253 growth rates exceeded those of the control culture in stationary phase. The lower growth rates in previous studies were attributed to the lack of bacterial exudate, however, an opposing theory attributing increased dinoflagellate cell numbers in axenic *A. lusitanicum* NEPCC 253 in stationary phase, due to a reduced nutrient utilisation of cultures following removal of the associated bacteria, must be proffered. It is possible to propose that the bacterial flora of *A. lusitanicum* NEPCC 253, was having a limiting effect on dinoflagellate growth rates in stationary phase of control cultures, as removal of the bacteria led to an increased division rate under identical conditions. However, as this change in growth rates between control and axenic cultures was not detected in *A. tamarense* NEPCC 407 cultures following removal of bacteria, this indicates different dinoflagellate cultures are probably affected in different ways by their associated microflora. Removal of bacteria from *A. tamarense* PCC 173a actually caused growth rates to drop on reaching stationary phase, indicating bacteria were possibly producing nutrients upon which dinoflagellate cultures were reliant for sustaining cell numbers in stationary phase.

These effects could be attributed to interactions between the microflora associated with particular dinoflagellate cultures. If this was the case, it would be expected that these microflora would exert similar effects when introduced to different cultures. However, on re-introduction of the microflora from *A. lusitanicum* NEPCC 253 and *A. tamarense* PCC 173a to axenic *A. lusitanicum* NEPCC 253, both re-introduced cultures showed growth profiles typical of the control *A. lusitanicum* NEPCC 253 culture, in that cell numbers reduced compared to the axenic culture on reaching stationary phase. This was not expected based on growth curve results generated by *A. tamarense* PCC 173a, which appeared to indicate the presence of its microflora caused dinoflagellate cell numbers to increase in stationary phase. This would indicate differences in growth rates of dinoflagellate cells following removal or addition of bacteria are dinoflagellate species specific and not dependent on the

composition of the microflora.

During the current study, levels of certain toxins in axenic cultures exceeded control cultures, with highest levels of most individual toxins being detected in lag and stationary phases, which contradicts previously published data by Boyer *et al.*, (1987). Boyer indicated toxicity was generally highest in log phase, with the low levels of toxicity detected in lag and stationary phases probably reflecting low CO_2 levels in the growth medium. Although contradicting Boyer by indicating highest levels of toxicity were detected in lag and stationary phases, the current study agrees with other published literature that toxin production varies between growth stages when grown in batch cultures (Prakash, 1967; Proctor *et al.*, 1975; White and Maranda, 1978; Oshima and Yasumoto, 1979; Schmidt and Loeblich, 1979). Nevertheless, these differences in toxicity between the current study and the results of Boyer, could be explained due to the use of different dinoflagellate strains and also the use of different culture medium.

It was apparent from the current study that removing bacteria from toxic dinoflagellate cultures caused many different effects on toxicity, with vastly differing quantities of specific toxins being seen in most axenic cultures compared to control cultures. Also in some cases, different toxins were detected. These findings would appear to strongly indicate bacterial involvement in dinoflagellate toxin production.

The ability of bacteria to transform PST, was first reported by Kotaki *et al.*, (1985a), who showed the conversion of GTX 1/4 to STX, with GTX 2 and 3 also detected. This ability to convert PST was identified in a wide range of bacteria indicating the enzymes required for such reactions were wide spread within the bacterial kingdom. Transformation of PST by bacteria could explain why different profiles are detected between control and axenic cultures and could also explain why toxins were present in certain phases and not in others. However, the ability of bacteria to transform PST was not shown in the current study, but should be the basis for future work in order to determine if the bacterial strains present in this study are capable of

biotransformation.

This ability of bacteria to interconvert PST could also potentially explain the differences in the total toxicity values detected between control and axenic cultures in published studies. However, many enzymes would be required to perform the various interconversions due to the different toxin groups, therefore, as bacteria may possess varying enzyme combinations, it is feasible that different microflora may produce different degrees of transformation. Therefore, future work assessing the role of associated bacteria in toxin transformation, must investigate which bacteria play a role in biotransformation and whether the numbers of these isolates at a particular growth phase can explain differences in toxicity profiles between control and axenic cultures.

Previous re-introduction studies by Doucette and Powell, (1998), assessing dinoflagellate toxicity, added a toxic *Pseudomonas stutzeri* isolate from *A. lusitanicum* NEPCC 253 back to the axenic culture. The axenic culture was initially seen to produce half the toxicity of the control culture, however, on introduction of the bacteria, toxicity levels were restored to those detected in control cultures. In subsequent experiments, bacteria contained within dialysis tubing, 300K molecular weight cut-off, were introduced to algal cultures, with no change in toxicity detected. This suggested bacterial influence on toxicity was dependent on the attachment between bacteria and dinoflagellate cells, although the mechanism was uncertain. Doucette and Powell, also introduced bacteria from other toxic dinoflagellates to axenic *A. lusitanicum* NEPCC 253 cultures, although no effect on toxicity was detected, which was attributed to the lack of recognition between host dinoflagellate cells and the bacteria.

In the current study, axenic cultures produced similar quantities of toxins compared to control cultures, with elevated levels of toxicity detected following re-introduction of *A. lusitanicum* NEPCC 253 bacteria to axenic cultures. However, the levels detected exceeded quantities present in control cultures, which does not correlate

with Doucette's results. The initial increase in toxicity in lag phase cultures seen on re-introduction of the host microflora could possibly be attributed to carry over of toxicity following introduction of the bacteria to the axenic culture. However, if this was the case, it would be predicted that levels of toxicity in cultures containing bacteria from *A. tamarense* PCC 173a, would remain similar to the axenic culture. However, this was not the case, with greater levels of toxicity detected in these cultures compared to axenic cultures. Interestingly, levels of toxicity in both re-introduction cultures appear to normalise as the growth cycle progressed, with results more comparable with control and axenic toxin levels detected in log and stationary phases. Possible reasons for this include conversion of toxins by bacteria to non-toxic compounds, or reduced production of toxins in later growth stages of re-introduction cultures as dinoflagellate cultures adjust to the presence of the bacteria.

Other studies investigating effects on toxicity following re-introduction of bacteria to algal cultures, were performed by Bates *et al* (1993). The experiments allowed bacteria from a toxic diatom to be re-introduced to the axenic culture, with large increases in toxicity detected, which were comparable to levels detected in the current study. Subsequent investigations by Bates, allowed bacteria from a non-toxic diatom species to be introduced to the axenic toxic diatom, which resulted in even higher levels of toxicity being detected, an event not comparable to the current study.

Although comparisons can be drawn between the findings presented in the current study and the published data, the present study offers the only re-introduction investigation to date which assesses the effects on toxin production when complete microflora are presented to dinoflagellate cultures. Previous studies have concentrated on assessing the effects of individual bacterial isolates. However, it is well recognised that bacteria exhibit different characteristics when maintained under different conditions (Azam and Ammerman, 1984), therefore it must be accepted that bacteria will probably not elicit usual responses when investigated individually. In order to assess true microflora effects all associated bacteria must be included within the analysis. Nevertheless, investigations using single isolates are still important in

order to identify whether particular enzymes are present, although conclusions about their actual contribution under normal conditions based on their effects in monoculture, must be extrapolated with care.

Production of SCB activity by bacterial isolates

The ability of bacterial isolates from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 to produce SCB toxins was previously investigated by Gallacher *et al.*, (1997). The study indicated 60% of *A. tamarense* NEPCC 407 and *A. lusitanicum* NEPCC 253 bacterial isolates isolated from stationary phase dinoflagellate cultures produced SCB toxins. Gallacher also noted bacterial SCB production to be 36 - 66 fmol STX equivalents per bacterial cell. A particular bacterial isolate (407-2), from *A. tamarense* NEPCC 407 analysed further using HPLC and CE-MS was seen to contain STX, neoSTX, GTX 1 - 4 and C toxins. This toxin profile is almost identical to the toxin profile detected from the *A. tamarense* NEPCC 407 culture in the current study and also the toxin profile published by Cembella *et al.*, (1987). As some SCB-producing bacteria isolated within the current study have been classified as being 100% identical to the 407-2 isolate using 16S rDNA sequencing, it is possible that this toxin profile existed in bacteria within the current study.

However, levels of toxicity produced by individual bacterial isolates, although comparable to levels detected previously from dinoflagellate associated bacteria, were previously indicated by Gallacher *et al.* as not able to explain the toxicity detected in dinoflagellate cultures (Gallacher *et al.*, 1997). However, Gallacher drew a parallel with bacterial production of TTX, where the quantity of toxin detected was not sufficient to explain the high levels detected in the animals of source. Therefore, it is feasible that the differences in toxicity detected *in vitro* during the current study, were due to the inability to reproduce *in-vivo* conditions, particularly in relation to changes in phenotypic expression which may occur upon surface attachment and upon exposure to exudate (Doucette, 1995).

Re-introduction studies allowing the original microflora and also the microflora of a non-toxin producing dinoflagellate provided strong evidence for bacterial involvement in dinoflagellate toxin production. These studies also tentatively indicated that bacterial involvement could be independent of a microfloras ability to produce SCB toxins as introduction of the microflora from the non-toxic dinoflagellate *A. tamarense* PCC 173a, altered the toxin profile of the axenic culture. Unfortunately, this is pure speculation at the present time as bacterial isolates from *A. tamarense* PCC 173a were not tested for SCB activity. If further time had been available, assessment of bacteria isolated from *A. tamarense* PCC 173a for SCB production would have been a priority. This would have determined whether the effects on toxicity seen by introducing the microflora of *A. tamarense* PCC 173a to axenic *A. lustanicum* NEPCC 253 could be attributed purely to bacterial influence on host toxicity rather than due to bacterial production of SCB toxins.

Nevertheless, different levels of effect were detected between the two re-introduction cultures, with addition of the original flora generating a larger increase in toxicity compared to addition of the microflora from *A. tamarense* PCC 173a. It is possible (as recognised by Doucette and Powell, 1998), that differences were due to the extent of host cell recognition of bacteria, which obviously would have been greater on introduction of the host microflora. Therefore it is possible to hypothesise that the lower although still elevated level of toxins produced by the non-toxic dinoflagellate microflora, compared to the control culture, was due to the lack of recognition of the 'foreign' microflora.

Stability of dinoflagellate cultures over a 16 month period

As the microflora and growth profiles of *A. lusitanicum* NEPCC 253 remained constant over the 16 month investigation, changes in toxin content of control cultures over the same time period cannot be attributed to these factors. Changes in toxicity were also detected in axenic cultures, which also cannot be explained by differing growth rates, with the change also seen to be independent of bacterial involvement.

No explanation can be offered to address the change in toxin profile that has occurred over the course of the study. However, at the outset, it was not thought necessary to assess the stability of dinoflagellate culture toxicity as this extent of change was not anticipated. Therefore, in hindsight, any future work must assess changes in the toxin producing capabilities of cultures, which would require assessment of toxin production over several consecutive growth cycles. Nevertheless, as indicated by Cembella *et al.*, (1987), it is more important to assess whether the same toxin profile is present over time, rather than the quantity of each toxin detected as environmental factors are known to influence production of individual toxins. The results from the current study indicate the same toxin profile was present over time.

In conclusion, the production of axenic cultures appears to have no effect on growth profiles until reaching stationary phase, where effects appear specific to the dinoflagellate culture under investigation. However, removal of bacteria from cultures dramatically alters toxin profiles, with axenic cultures retaining the ability to produce toxins. Re-introduction of bacteria to axenic cultures, whether from toxic or non-toxic dinoflagellate cultures also results in an elevation of toxin profiles. Indicating that bacteria do influence dinoflagellate toxin profiles and emphasises the need for further investigation in this field.

CHAPTER 5 : THE INFLUENCE OF PST-PRODUCING BACTERIA ON THE TOXICITY OF *MYTILUS EDULIS*

Introduction

There is now convincing evidence that certain bacteria produce PST (Gallacher *et al.*, 1997), and it has been suggested that PST-producing bacteria have been the source of PST during some episodes of shellfish toxicity (Kodama and Ogata, 1988). Filtration of seawater has shown the presence of PST in fractions containing particles of the approximate size of bacteria (Kodama *et al.*, 1993; Levasseur *et al.*, 1995). The detection of bacteria capable of SCB toxin production was also investigated by Gallacher and Birkbeck (1993) who found that more than 37% of bacteria isolated from seawater produced SCB activity, and that *Mytilus edulis* could filter such bacteria from suspension and accumulate SCB-toxicity within three hours of exposure. Due to the low levels of toxicity produced by these bacteria, it is currently impossible to identify the SCB activity as due to PST.

In this study, the uptake of SCB-producing bacteria by *Mytilus edulis*, and subsequent detection of toxicity in shellfish flesh were studied to confirm the original findings of Gallacher and Birkbeck (1993). Particular care was taken to optimise certain parameters in the tissue culture assay, to allow detection of SCB activity in low dilutions of shellfish flesh homogenate. The results confirmed that shellfish could accumulate SCB-toxins following exposure to SCB-producing bacteria, with toxicity detected within one hour of exposure to bacteria.

MATERIALS AND METHODS

Tissue culture

The mouse neuroblastoma assay was used as described by Gallacher and Birkbeck (1992), with the following amendments:-

1) The ouabain and veratridine concentrations were optimised to take into account matrix effects from shellfish flesh

2) Saxitoxin standards were diluted in negative control shellfish extract

3) A saxitoxin standard curve was incorporated onto each sample plate.

Optimisation of Ouabain and Veratridine Concentrations

The effects of the shellfish matrix on the ability of ouabain and veratridine to cause cell death was investigated by titration of various ouabain/veratridine concentrations as described for bacteria in Chapter 4. These incorporated a combination of 6 ouabain and 3 veratridine concentrations in a chequerboard pattern as described by Gallacher and Birkbeck (1992), but incorporating sample matrix controls. Each well contained 50µl of the required concentration of ouabain and veratridine along with 100µl of the respective assay medium (RPMI + penicillin/streptomycin, 2%), but for shellfish sample toxicity (tested at 1/8, 1/10 and 1/12 dilution of shellfish sample), shellfish homogenate diluted 1/8, 1/10, or 1/12 in dilution medium was incorporated. Plates were seeded as described by Gallacher and Birkbeck (1992) and combinations of ouabain, veratridine and respective assay medium were incorporated before incubation at 37°C for 24h. The response of the cells to ouabain and veratridine was assessed as stated in Gallacher and Birkbeck (1992).

Serial Dilution of Saxitoxin Standard

A saxitoxin standard curve was constructed and included in each sample plate when quantification of sample toxicity was required. Each set of STX dilutions was made up in the assay medium appropriate for the plate; for testing shellfish samples at 1/8, 1/10 and 1/12 strength, saxitoxin standards were diluted in shellfish homogenate at

the same concentrations. SCB activity percentages were calculated and dose response curves generated as described by Gallacher and Birkbeck (1992), to which sample SCB activity was compared and converted to saxitoxin equivalent concentrations.

SHELLFISH FEEDING

Bacterial Strains and Culture Conditions

The bacteria used are listed in Table 5.1. Strains, originally from -80°C glycerol stocks, were stored on marine agar plates (Difco) at 4°C and cultured in marine broth (Difco) at 20°C.

Bacterial growth curves

SCB producing bacterial strains were inoculated into 50ml flasks containing marine broth (30ml) and incubated in a rotary incubator (20°C, 100 osc min^{-1}). After 24h, 10ml was sub-cultured into 1000ml of marine broth and incubated as above. Samples were removed every 30 min to obtain viable cell counts by plating 0.1ml of ten-fold dilutions in triplicate, onto marine agar plates and incubating for 48h at 20°C. Optical density (OD) readings (600nm) using a spectrophotometer (Varian Cary 3E uv-visible spectrophotometer) were also obtained every 30 min and used to construct an optical density growth curve. This was subsequently compared with the growth curve generated from total viable count results, so that comparison of OD readings to previous total viable count growth curves allowed an immediate estimation of the numbers of bacteria being added to experimental jars. These OD results were confirmed by plate count results subsequently available after 2 days.

Bacterium	Strain	Source
Shewanella alga	OK1	Alga (*Jania* sp.; Simidu *et al.*, 1990)
unidentified isolate	A862	Seawater (Gallacher and Birkbeck, 1992)
Alteromonas sp.	407-2	Dinoflagellate (*A. tamarense* NEPCC 407; Gallacher *et al.*, 1996)

Table 5.1 SCB producing bacteria used in feeding studies with *Mytilus edulis*

Uptake of SCB producing bacteria by *Mytilus edulis*

The protocol for the uptake of bacteria by mussels was adapted from Birkbeck and McHenery (1982). The concentration of bacterial cells in 24h cultures of SCB-producing bacteria was determined by optical density measurements as detailed above. Samples were also taken for subsequent plate count analysis. From sample OD values and the OD/bacterial concentration conversion factor determined above for each bacterial strain, an appropriate volume of suspension was added to give 10^6cfu ml^{-1}, 10^7cfu ml^{-1}or 10 ěfu ml^{-1}in experimental jars containing 1000ml of aerated seawater and 10 mussels (*M. edulis*, 5-6cm length), or control jars containing seawater and bacteria. Control jars containing seawater and mussels were included to generate negative control shellfish samples for subsequent mouse neuroblastoma assays. Samples of seawater for total viable counts, and shellfish for SCB detection, were taken hourly for up to 6h (dependent on the time course of the experiment) and a final 24h sample taken.

Analysis of mussel extracts in a tissue culture assay

Shellfish were processed in accordance with the standard method for PSP analysis (AOAC, 1990). This involved combining the soft parts of mussels from each jar, and homogenising them with an equal volume of 0.1M HCl. The pH was adjusted to between pH 2.0 - 4.0 and the resultant mixture boiled gently for 5 minutes, after which it was allowed to cool to room temperature. The pH of the mixture was re-checked before centrifugation (12,000 x g, 10 min) to obtain a clear supernatant which was decanted and frozen in aliquots (-20°C) until required for testing in the tissue culture assay.

Calculation of sample toxicity

Sample toxicity was determined by calculating SCB activity from each sample dilution as described by Gallacher and Birkbeck (1992). The SCB activity from each

sample dilution was converted to saxitoxin equivalents (nM) by comparison with the corresponding saxitoxin dose response curve. Appropriate scaling factors for each sample were applied to normalise all dilutions used and final toxicity figures were expressed as µg per 100g of shellfish flesh. See below for details:-

$$\frac{\text{sample shellfish OD - reagent blank OD}}{\text{cell survival OD}} \times 100 = \%\ \text{cell survival of sample at 1/X dilution} \quad \text{(A)}$$

$$\frac{\text{negative control shellfish OD - reagent blank OD}}{\text{cell survival OD}} \times 100 = \%\ \text{cell survival of control at 1/X dilution} \quad \text{(B)}$$

$$\frac{A - B}{100 - B} \times 100 = \%\ \text{SCB activity in 100µl of sample at 1/X dilution} \quad \text{(C)}$$

Subsequent determination of STX equivalents (nM) in 100µl of sample at 1/X dilution were obtained by reading (C) from the corresponding STX dose response curve (D)

∴ D = STX equivalents (nM) in 100µl of sample at 1/X dilution

D x 10 = STX equivalents (nM) in 1000µl of sample at 1/X dilution (E)

E x 2 = STX equivalents (nM) in 1000µl of sample at 1/X dilution, scaled up by a factor of 2 to compensate for dilution during homogenisation (F)

F x X = STX equivalents (nM) in 1000µl of sample at neat concentration (G)

G x 2 = STX equivalents (nM) in 1000µl of neat sample scaled up by a factor of 2 to take into account dilution in the well (H)

$\frac{H}{1000}$ = STX equivalents (µM) in 1000µl shellfish homogenate (I)

I x 100 = STX equivalents µM/100g shellfish flesh (J)

$\frac{J}{300^*}$ = STX equivalents µg/100g shellfish flesh

* = molecular weight of saxitoxin

RESULTS

Optimisation of the mouse neuroblastoma assay

To determine the response of the MNB cells to sodium channel blocking compounds, it was first necessary to optimise the concentration of chemicals used to stimulate the influx of sodium ions, i.e. ouabain and veratridine. To obtain maximum sensitivity from the assay these chemicals must give maximum cell death when added in combination, and little or no cell death when used separately (Kogure *et al.*, 1988). Figure 5.1a demonstrates cell survival after exposure of MNB cells to various combinations of ouabain and veratridine in dilution medium (RPMI + penicillin/streptomycin. In dilution medium alone, concentrations of 0.4mM ouabain and 0.025mM veratridine gave 92% cell death (8% cell survival) when combined, but 100% cell survival when used individually. However, when cell survival was determined at these concentrations of ouabain and veratridine when diluted in shellfish extract (Fig. 5.1b - d), cell survival increased to 60% when a 1/8 dilution of shellfish extract was incorporated. Cell survival subsequently decreased as the concentration of shellfish extract was reduced to 1/12 dilution, but still remained higher than the ouabain and veratridine alone level (Fig. 5.2). Hence, if the appropriate dilution of shellfish extract were not used as a negative control, false positives would occur. Although differences in cell survival were apparent between the three dilutions of shellfish extract, a combination of 0.7mM ouabain and 0.025mM veratridine allowed the three shellfish extract dilutions to be tested on the same plate (Table 5.2), thus reducing the number of plates required.

Saxitoxin dose response curve

Figure 5.3 demonstrates the effect on saxitoxin response (expressed as SCB activity) when STX was serially diluted in either dilution medium or 1/12 shellfish extract in dilution medium, using ouabain and veratridine concentrations previously assessed as optimal for 1/12 shellfish extract. Little response was detected with STX

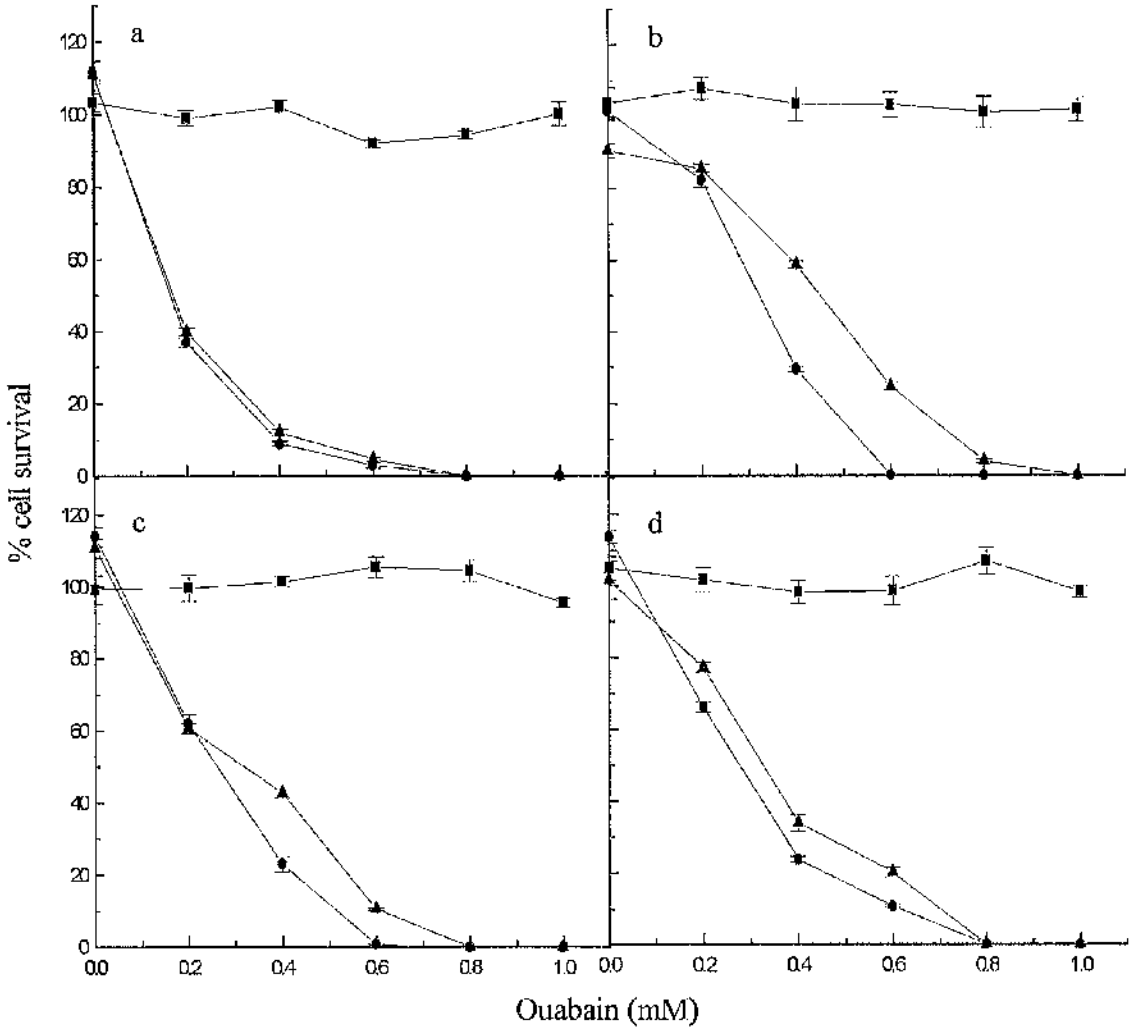


Figure 5.1 Survival of MNB cells after exposure to various concentrations of ouabain and veratridine in different concentrations of shellfish extract in dilution medium (mean ± sem, n = 3), (a) dilution media alone (b) shellfish extract diluted 1/8 in dilution medium (c) shellfish extract diluted 1/10 in dilution medium (d) shellfish extract diluted 1/12 in dilution medium.

(■) = 0mM veratridine, (▲) = 0.025mM veratridine, (●) = 0.05mM veratridine.

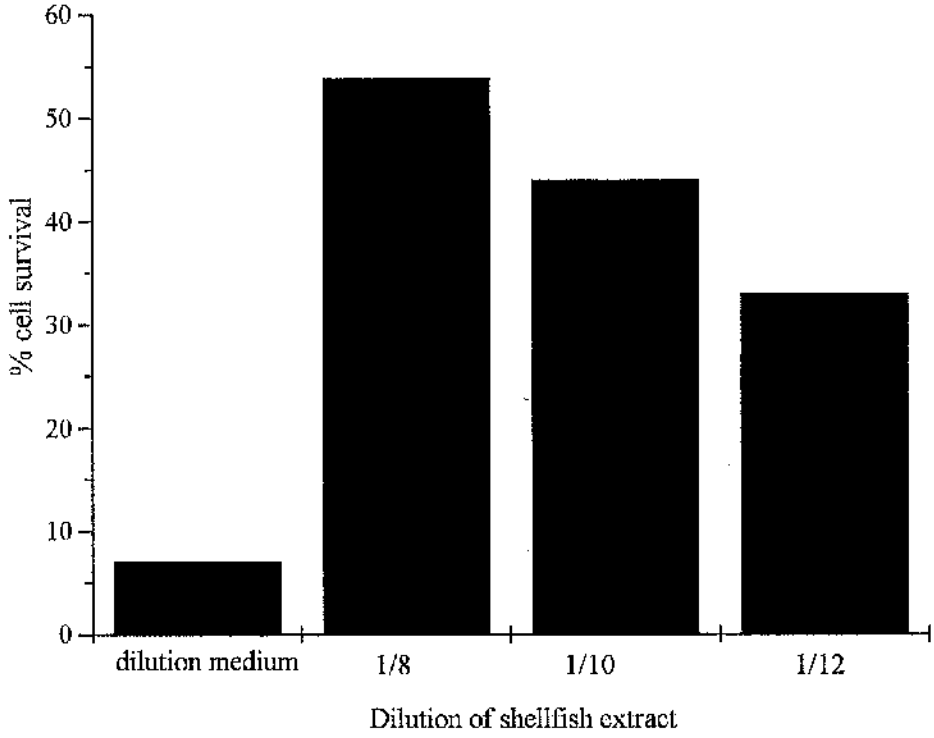


Figure 5.2 Mouse neuroblastoma cell survival after exposure to 0.4mM ouabain and 0.025 mM veratridine combined with dilution medium or different dilutions of shellfish extract in dilution medium.

	Ouabain [mM]	Veratridine [mM]
RPMI	0.4	0.025
1/8	0.7	0.025
1/10	0.7	0.025
1/12	0.7	0.025

Table 5.2 Concentrations of ouabain and veratridine which allowed the required cell survival range (10-20%) and therefore the levels used in experiments to determine SCB activity in shellfish samples.

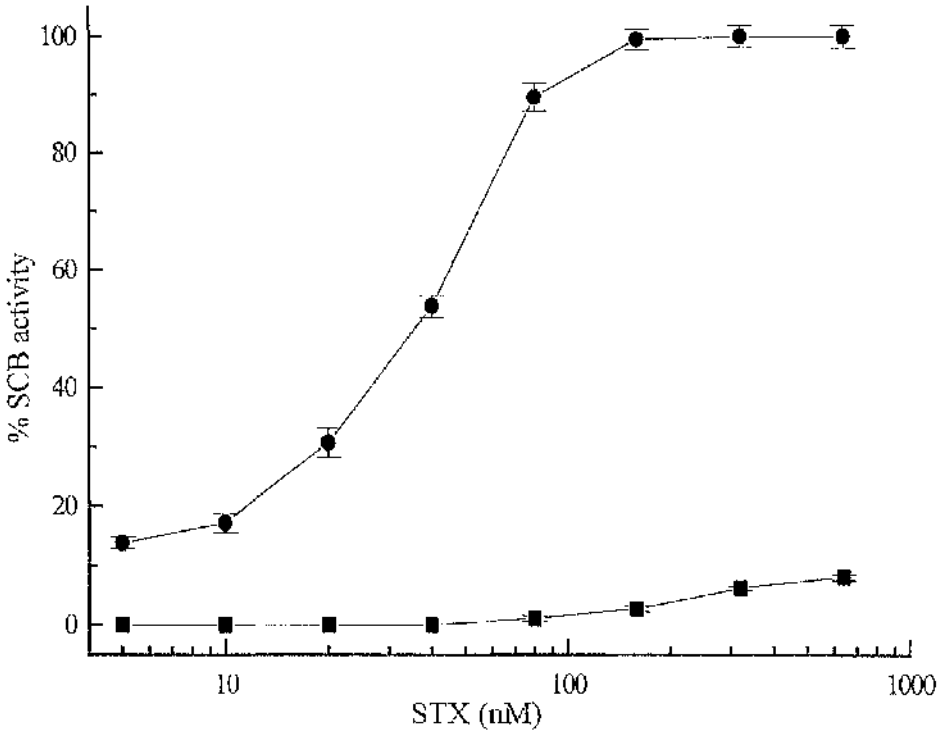


Figure 5.3 Comparison of cell survival, expressed as percentage sodium channel blocking (SCB) activity, when ouabain/veratridine concentrations were optimised for shellfish extract.

(■) = dilution medium (mean ± sem, n = 12), (●) = shellfish extract diluted 1/12 in dilution medium (mean ± sem, n = 6).

in dilution medium until 100nM, whereas a response with STX diluted 1/12 in shellfish extract was detected at 5nM. Table 5.3 details the detection limit and corresponding linear ranges for saxitoxin prepared in dilution medium, compared to shellfish extract diluted 1/8, 1/10 and 1/12 in dilution medium, when levels of ouabain and veratridine were optimised for shellfish samples. The results clearly show the importance of correctly ascertaining ouabain and veratridine concentrations.

Comparison of optical density and total viable counts for bacterial growth curves

So that optical density (OD) measurements could be used to determine the bacterial inoculum for shellfish feeding experiments, the relationship between OD and viable counts ml^{-1} was determined at various points in a 24h batch culture. Figure 5.4 shows the change in OD and viable counts of *Shewanella alga* during growth in marine broth over 24h. The trends of each data set are similar, with OD readings directly related to the viable count in the range 0.4 - 1.8.

Table 5.4 indicates the viable count determinations for 24h marine broth batch cultures of the three different bacterial isolates used in feeding studies. As OD readings for initial inocula were above the linear range upper limit for each bacterial strain accurate assessment of bacterial numbers was not possible. However, it was possible to ascertain from OD readings that each strain was in stationary phase. Therefore an assumption of maximal cell numbers present allowed the addition of bacteria to experimental jars, with the accurate determination of bacterial numbers added being determined later from viable counts.

	Detection limit [nM]	Linear range [nM]
RPMI	100	no linear range
1/8	5	10 - 30
1/10	5	10 - 40
1/12	5	20 - 80

Table 5.3 Detection limits and linear ranges of saxitoxin dose response curves for dilution media and diluted shellfish extract determined using ouabain and veratridine concentrations optimised for shellfish extracts.

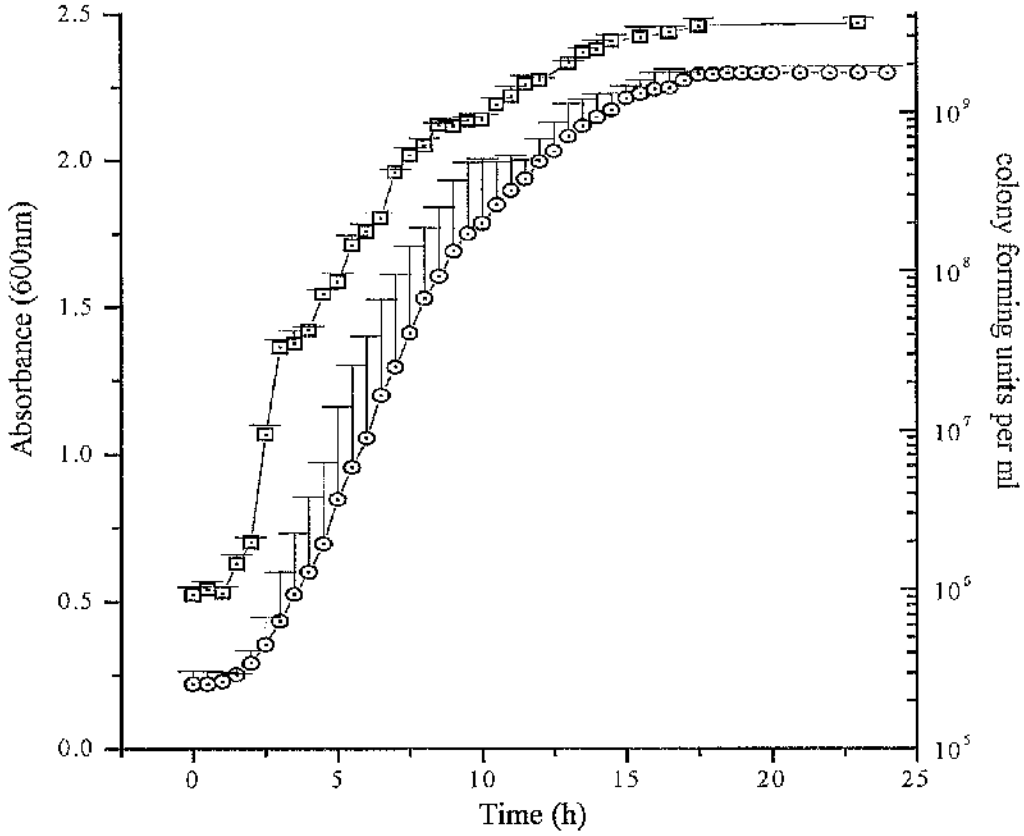


Figure 5.4 Growth of *Shewanella alga* in marine broth batch culture over a 24h period, as measured using spectrophotometry (OD) and viable counts.

(○) = optical density (600nm; mean ± sd, n = 2), (□) = colony forming units (cfu) per ml, (mean ± sd, n = 2).

Strain	Viable count per ml of 24h broth ($x10^9$)
Alteromonas sp. (407-2)	4.52 ± 0.02
unidentified isolate (A862)	3.83 ± 0.01
Shewanella alga (OK1)	8.33 ± 0.019

Table 5.4 Viable counts (mean ± sem, n = 3) for the 3 bacterial isolates grown for 24h in marine broth batch culture and used for feeding studies.

Removal of *Alteromonas* sp. 407-2 suspension by *Mytilus edulis*

Figure 5.5 shows the removal of *Alteromonas* sp. 407-2 from seawater over 24 hours. The culture added had a viable count of $4.52 \times 10^9 \pm 2.04 \times 10^7$ cfu ml^{-1} giving an initial density of 1.53×10^6 bacterial cells ml^{-1} of seawater in experimental jars. In the presence of *Mytilus edulis*, the bacterial concentration in seawater fell by 73% in the first hour, compared to 2% in control jars. A further 12% of bacteria were removed after 3h by the mussels, with less than 1% of the original suspension remaining after 24h, compared to 96% of the original inoculum remaining after 24h in jars without shellfish. No visible signs of pseudofaeces production by *Mytilus edulis* occurred, nor was settling or clumping of bacteria apparent, therefore, assimilation of the bacteria by *Mytilus edulis* was assumed. The rapid removal of bacteria in the first hour corresponded to detection of 0.84µg saxitoxin equivalents per 100g of shellfish tissue, with the highest level of toxicity (1.275 µg saxitoxin equivalents per 100g of shellfish tissue) detected at 4h (Fig. 5.5). Toxicity levels subsequently dropped to 0.4µg saxitoxin equivalents per 100g of shellfish tissue at 6h, with a rise in toxicity detected after 24h.

Bacterial uptake and SCB activity of *Mytilus edulis* after exposure to different inoculum densities of *Shewanella alga* and isolate A862.

Shewanella alga and A862 were inoculated at different concentrations into seawater containing *Mytilus edulis*, with removal of bacteria and subsequent SCB activity monitored over a 3h period. Table 5.5a summarises the removal of A862 from seawater in the presence of *Mytilus edulis*. With an initial inoculum of 10^6 cells ml^{-1}, less than 20% of bacteria were eliminated in 3h, with no toxicity detected in shellfish samples. Similarly with an initial loading of 10^8 cells ml^{-1}, only 44% of bacteria were removed after 3h, although in this case toxicity was detected. *Mytilus edulis* was more effective at clearing bacteria from the 10^7 cells ml^{-1} loading, with only 14% of bacteria remaining in seawater after 1h. SCB activity was detected

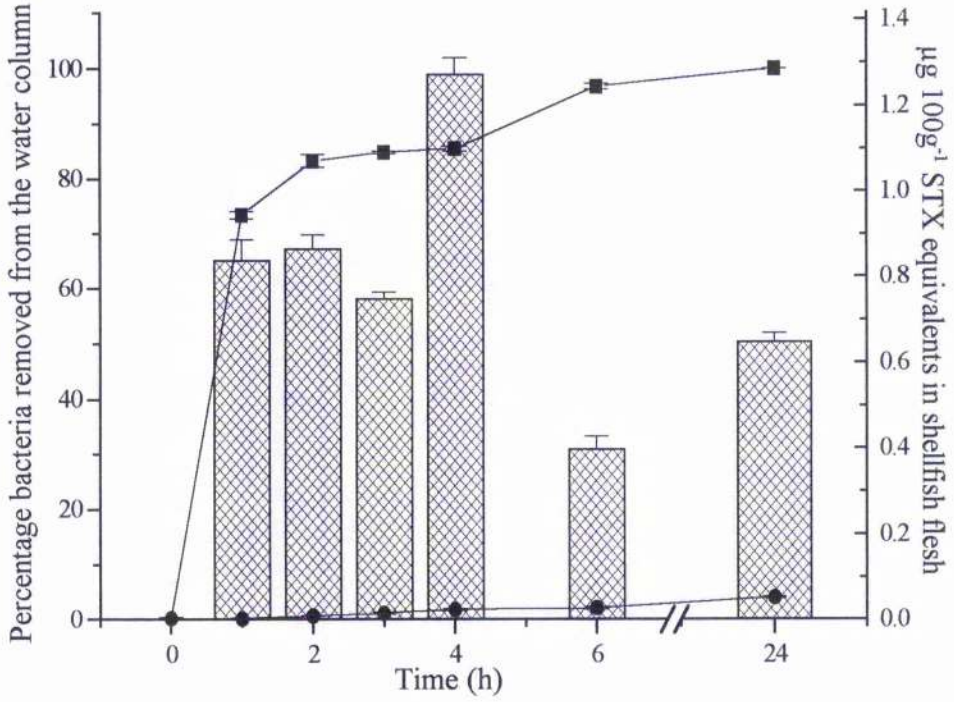


Figure 5.5 Removal of *Alteromonas* sp. 407-2 from jars containing *Mytilus edulis* and subsequent toxicity of shellfish samples over 24h. (⊠) = toxicity (STX equivalents µg $100g^{-1}$ of shellfish flesh; mean ± sd, n = 2); (■) = cfu ml^{-1} of seawater from tank containing *Mytilus edulis* (mean ± sem, n = 3); (●) = cfu ml^{-1} of seawater from control tank containing seawater and bacteria only (mean ± sem, n = 3).

Initial inoculum (ml^{-1})	Time (h)	(cfu ml^{-1} ± sd) $x10^8$	% bacteria removed	Toxicity
	0	0.08 ± 0.001	0	-ve
10^6	1	0.07 ± 0.004	3.8	-ve
	3	0.06 ± 0.006	17.8	-ve
	0	0.523 ± 0.064	0	-ve
10^7	1	0.073 ± 0.009	86.1	+ve
	3	0.062 ± 0.001	88.1	+ve
	0	8.13 ±0.131	0	-ve
10^8	1	7.40 + 0.7	9	-ve
	3	4.56 ± 1.25	44	+ve

Table 5.5a

Initial inoculum (ml^{-1})	Time (h)	(cfu ml^{-1} ± sd) $x10^8$	% bacteria removed	Toxicity
	0	0.064 ± 0.003	0	-ve
10^6	1	0.060 ± .0.025	5.8	-ve
	3	0.027 ± 0.006	56.9	+ve
	0	0.836 ± 0.086	0	-ve
10^7	1	0.18 ± 0.0235	78.5	+ve
	3	0.039 ± 0.005	95.3	+ve
	0	6.70 ±0.121	0	-ve
10^8	1	6.23 ± 0.45	7.1	-ve
	3	2.08 ± 0.286	69	+ve

Table 5.5b

Table 5.5 Number of bacteria (mean ± sd, n = 2) remaining following exposure of *Mytilus edulis* to varying inocula of a) A862, and b) *Shewanella alga* for a three hour period and resultant shellfish toxicity.

within 1h and was still present after 3h, with little change occurring in bacterial numbers.

With *Shewanella alga* (Table 5.5b), results were similar to those found for A862, with maximum removal of bacteria occurring with a 10^7 cfu ml^{-1} inoculum. However, *S. alga* was more effectively removed from seawater at 10^6 inoculum than A862, with almost 40% more bacteria having been removed after 3hr, with toxicity subsequently detected. Due to maximum removal of bacteria occurring with 10^7 cells ml^{-1} initial inoculum and the quickest detection of toxicity, a further experiment was undertaken using *S. alga* at an initial loading of 10^7 cells ml^{-1}.

Exposure of mussels to *Shewanella alga* at exposure levels of 10^7 cfu ml^{-1} over a 6 hour period.

Further investigations into *S. alga* uptake by *Mytilus edulis* over a six hour period (Fig 5.6), indicated 95% of the initial inoculum was removed within 1h, with SCB activity detected. However, maximum toxicity of 1.14µg saxitoxin equivalents per 100g shellfish flesh was not detected until 3h, although, bacterial levels remaining in seawater had hardly altered since 1h. Toxicity was still detected at 6h, although the concentration had dropped suggesting that some processing of toxin or depuration had occurred. A control consisting of seawater and bacteria determined the proportion of bacteria not removed by mussels, which indicated 18% of initial inoculum was lost over 6h when *Mytilus edulis* was absent compared to 99% when shellfish were present. It was therefore reasonable to deduce that *Mytilus edulis* were responsible for removal of the bulk of bacteria and that this resulted in the accumulation of SCB toxins in shellfish flesh.

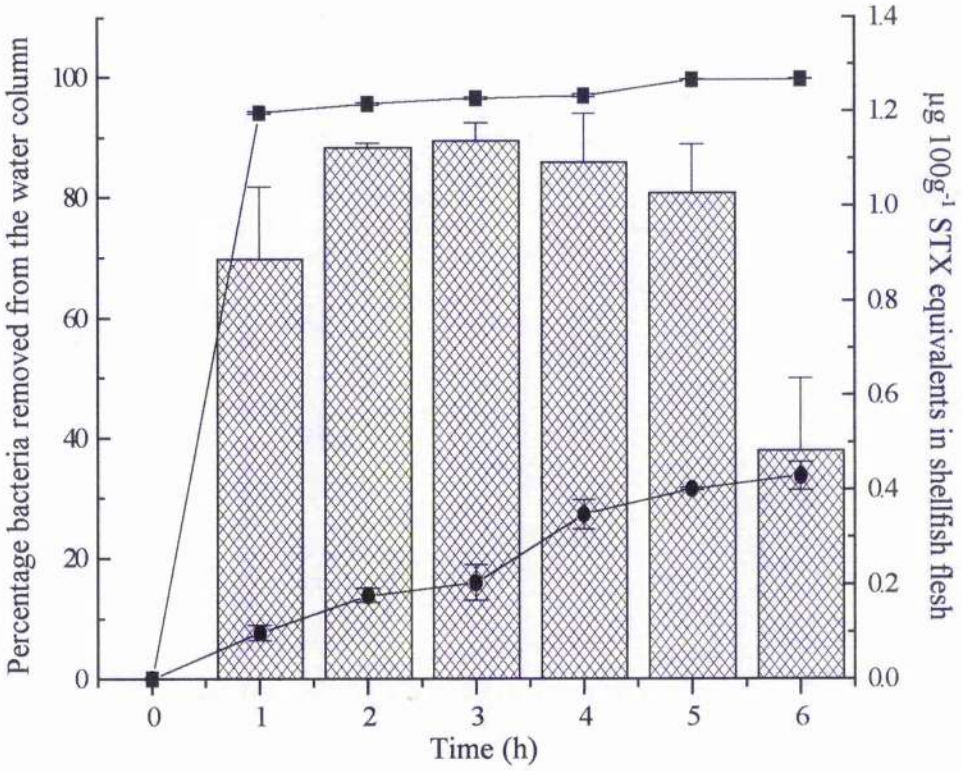


Figure 5.6 Percentage removal of *Shewanella alga* from jars containing *Mytilus edulis* and subsequent toxicity of shellfish samples. (⊠) = toxicity (STX equivalents µg $100g^{-1}$ of shellfish flesh) (mean ± sd, n = 2); (■) = cfu $ml^{-1}$of seawater from tank containing *Mytilus edulis* (mean ± sem, n = 2); (●) = cfu $ml^{-1}$of seawater from control tank containing seawater and bacteria only (mean ± sem, n = 3).

DISCUSSION

Uptake of bacteria by *Mytilus edulis*

Earlier experiments by Gallacher and Birkbeck (1993), indicated that sodium channel blocking activity could be detected in mussels (*Mytilus edulis*) exposed to *Alteromonas tetraodonis* strain GFC. However, several parameters, including matrix effects and the methodology were not optimised. Particular aspects of the methodology to consider were maximising bacterial removal to allow subsequent detection of toxicity within the shortest period of time. The objective of the current study was to confirm and expand on the above report by addressing these issues.

This study clearly showed that shellfish flesh altered the response of the cell line to ouabain and veratridine, by increasing cell survival. This may have been due to sodium ions present in shellfish extract, which at high concentrations, are known to compete with saxitoxin for binding to the sodium channel (Weigle and Barchi, 1978). Therefore, unless appropriate matrix controls for this increased cell survival were incorporated into the assay, false positives could result when testing low dilutions of shellfish flesh.

Once the mouse neuroblastoma assay was optimised, initial shellfish feeding studies were conducted with the PST-producing bacterium, *Alteromonas* species 407-2. Experiments showed 85% of bacteria, from a 10^7cfu ml^{-1} initial loading, were removed from suspension in 3h, with a corresponding SCB activity detection of 0.75µg saxitoxin equivalents per 100g of shellfish tissue. The experiment confirmed the work of Gallacher and Birkbeck (1993), in that mussels could remove *Alteromonas* sp. from seawater, with resultant SCB activity. However, further experiments utilising 407-2 were not possible as the strain formed large clumps, which prevented accurate assessment of bacterial numbers. Owing to time constraints and the availability of other SCB-producing bacterial strains, work on strain 407-2 was not continued and later work concentrated on exposing mussels to *Shewanella alga* and an unidentified seawater isolate A862. Initial experiments with these strains

investigated the effects of exposing mussels to different concentrations of bacteria, and subsequent removal of bacterial cells from suspension.

Previous reports on the removal rates of bacteria from seawater by mussels, utilised a variety of bacterial densities as the initial inoculum (Zobell, 1936; McHenery and Birkbeck, 1982; Gallacher and Birkbeck, 1993). Zobell (1936), exposed mussels to 10^8 bacterial cells per ml and showed less than 1% of the original inoculum remained after 3h. In the current investigation, with the same cell density, only 44% of strain A862 and 69% of *Shewanella alga* were removed after 3h. However, toxicity was not detected in these samples as quick as in lower initial inoculum samples. There are a multitude of possible reasons for these differences including the use of different bacterial strains (Birkbeck and McHenery, 1982), and experimental protocols, physical environmental considerations such as temperature and salinity reported by Theede (1963) and Renzoni (1963), shellfish physiological factors including gill porosity (Zobell and Landon, 1937; Jorgensen, 1949) and seasonal variance (Dodgson, 1928; Theede, 1963). Exposure of *Mytilus edulis* to different bacterial strains by Birkbeck and McHenery (1982), indicated rates of filtration could be altered depending on the bacterial species present. However, experiments in this study, using an initial density of 10^7cfu ml^{-1}, showed that more than 90% of bacteria were removed within 3h, which was equivalent to results reported by McHenery and Birkbeck (1982) and Gallacher and Birkbeck (1993). Therefore an inoculum of 10^7cfu ml^{-1} was deemed optimum for further experiments and was used to determine if *Shewanella alga* caused SCB activity in *Mytilus edulis*.

Detection of SCB activity in *Mytilus edulis*

Following exposure of mussels to *Shewanella alga*, at an initial inoculum of 10^7 cfu ml^{-1}, 95% of bacteria were removed within 1h, with 0.89µg STX equivalents per 100g detected in the shellfish flesh. After 3h, a further 2% of bacteria had been removed and SCB activity had increased to 1.14µg STX equivalents per 100g. This toxicity value is lower than the 2µg per 100g STX equivalents previously reported

by Gallacher & Birkbeck (1993). This may be due to the use of a different bacterial strain, or the fact that in the current study shellfish samples were not tested after 7h compared to single time point of 24h chosen by Gallacher and Birkbeck (1993). The latter point is important as this study showed that the amount of toxin detected in shellfish samples did vary over time, with maximum toxicity detected at 3h followed by a steady decrease to the final sampling point of 6h. Further investigations should focus on determining how quickly mussels commence depuration and whether the rise in toxicity detected at 24h in the initial experiment is reproducible. The most likely assumption to make regarding the increase in toxicity at 24h would be transformation of the toxins to more active forms, although to substantiate this theory, HPLC analysis would be required. Unfortunately, the levels of toxicity detected in shellfish samples were too low for HPLC analysis by current protocols (Franco *et al.*, 1993). Nevertheless, this study and the earlier one of Gallacher and Birkbeck (1993) have shown that SCB activity can be detected in mussels after exposure to SCB-producing bacteria and gives weight to the statement by Kodama & Ogata (1988) that toxification of bivalves in some areas may be due to bacteria.

Although levels detected in the current study are comparable with those previously detected by Gallacher and Birkbeck, the levels of SCB activity are still lower than those reported to occur naturally in the environment. If levels of toxicity detected in this study were extrapolated to take into account potentially available bacteria (10^9cfu l^{-1}; Amann, 1987) in natural seawater, a toxicity value of 1.2×10^5µg STX equivalents per 100g shellfish flesh could be achieved in 24h, based on mussels filtering up to 2l of seawater h^{-1} (Winter 1978) over this period, assuming all bacteria were toxic and the ability of mussels to filter and process this high quantity of bacteria. Birkbeck and McHenery (1982), calculated that mussels can process 10^9 bacteria h^{-1} indicating that high concentrations of SCB toxins could accumulate in mussels as suggested here. The efficiency of particle removal by mussels has previously been shown to vary depending on age, size and also food concentration (Winter, 1973; Wilson and Seed, 1974). However, experiments exposing mussels

continuously to single and mixed bacterial suspensions would address this issue and furthermore mimic a more natural situation and possibly enhance toxicity.

Other explanations as to why levels of toxicity detected in this study are lower than those routinely detected in the environment are also available. Previously, researchers have indicated that growth of bacteria in batch culture is not ideal for maximum toxin production (Gallacher and Birkbeck, 1993; Doucette, 1995). Doucette, (1995) and Gallacher *et al.* (1997), both discussed bacterial adhesion indicating that this could play a big factor in the ability of bacteria to produce PSTs in any great quantities. It is known that in a dinoflagellate culture, bacteria attach to the dinoflagellate cell wall (Nelinda *et al.*, 1985), and in the marine environment, bacteria can attach to phytoplankton and inorganic particles (Doucette *et al.*, 1998), most of which can be readily accumulated by animals such as bivalves (Vahl, 1973a). Therefore, future experiments which take into account adhesion could result in increased toxicity levels in shellfish, by reproducing the natural situation. Previous experiments investigating bacterial uptake from seawater when mussels were exposed to bacteria in the presence of algae, were reported by McHenery and Birkbeck (1985), who showed an increased uptake of bacteria in the presence of algae. Although these experiments did not use SCB producing bacteria, it could still be assumed that if the quantity of SCB producing bacteria accumulated by mussels can be increased by introducing algae into the experimental setup, then the resultant SCB activity detected in mussel flesh would be higher.

In conclusion, SCB activity could be detected in shellfish exposed to SCB producing bacteria and there is much scope for more detailed investigations within this research area.

G.L. Hold, 1999

CONCLUDING SUMMARY

This thesis set out to examine the role of bacteria in paralytic shellfish poisoning. Emphasis was placed on identifying differences between the microflora associated with toxic dinoflagellate strains and the microflora associated with non-toxic cultures. The work also attempted to further the current understanding of the production of PST by dinoflagellate strains, by assessing the ability of bacteria to alter these profiles. Finally, the detection of SCB activity in *Mytilus edulis* following exposure to SCB producing bacteria was investigated, with the aim of confirming whether bacteria could be identified as the sole source of toxicity. The findings from the above experiments were as follows:-

- Dinoflagellate sustain a diverse bacterial microflora, with bacteria identified from two or three phyla/subphyla present within each dinoflagellate culture studied.

- *Roseobacter* related isolates appear to be the dominant strains associated with *Alexandrium* cultures - independent of whether the dinoflagellate cultures produce PST.

- Several potentially new bacterial species were detected, however, further work is required to confirm these identifications.

- Most of the major phylogenetic groups detected within the study contained bacterial isolates which produced SCB toxins.

- The majority of α-Proteobacteria from *Alexandrium* cultures were closely related, regardless of whether cultures were PST producers. However, bacteria from *S. trochoidea* NEPCC 15, the non toxic, non-*Alexandrium* species were not related to any α-Proteobacteria from toxic dinoflagellate strains, but certain isolates did group with bacteria from the non toxic *Alexandrium* culture.

- Although the two molecular bacteria identification methods generated comparable results, more bacteria were identified by the non culture-based technique, indicating the limitations of relying on culture to infer diversity. However, stronger identifications were possible using the culture-based method, due to the longer length DNA sequences generated, indicating a combination of the two techniques is required to confidently attribute diversity with identification.

- Antibiotic treatments appear to be an effective method for completely removing bacteria associated with dinoflagellate cultures, with physical dissociation methods shown to be ineffective.

- The inclusion of molecular methods for assessing the bacterial status of axenic cultures proved essential, as traditional methods failed to detect the presence of bacteria. This indicates the need to adopt more stringent checking methods when assessing the bacteriological status of cultures, with the current study providing such a method.

- Limited effects on dinoflagellate growth profiles were detected following the production of axenic cultures, with the effects appearing to be species specific.

- Production of axenic dinoflagellate cultures altered toxin profiles, with differing quantities of certain toxins detected, however, in some cases previously undetected toxins were apparent.

- Re-introduction of bacteria to axenic cultures showed a change in dinoflagellate toxin profiles, with the introduction of the microflora from a non toxin-producing culture also causing changes to the toxin profile of the axenic culture, indicating the possibility that the ability

of bacteria to alter dinoflagellate toxin profiles could be independant from their ability to produce PST.

- The microflora of dinoflagellate cultures was shown to be stable over a 16 month period.

- The ability of SCB-producing bacteria to cause toxicity in *Mytilus edulis* was confirmed when shellfish were exposed to levels of bacteria comparable to levels present within the environment.

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APPENDICES

APPENDIX 1

Dinoflagellate Culture Media

1. f/2

f/2 Guillard's marine water enrichment solution without silicate. (Guillard 1975) Supplied by Sigma catalogue number G 0154. Requiring 20ml of the 50x solution made up to 1 litre with autoclaved seawater (110°C, 30 mins).

2. K minimum (Km) medium

Working stock solutions	Amount added per litre of seawater base (ml)
$NaNO_3$ (75g/l ddH_20)	1.0
$NaH_2PO_4.H_20$ (5g/l ddH_20)	1.0
Trace metals (see below)	1.0
Vitamins (see below)	0.5
Na_2SeO_3 (see below)	1.0

Working trace metal solution

For 1 litre stock	
Na_2EDTA ($2H_20$)	4.36g (4.57 of dihydrate)
$FeCl_3.6H_20$	3.15g

Dissolve each of the above separately in ultrapure distilled water. The EDTA may require 500ml for dissolution; dissolve the $FeCl_3$ in approx 100ml and then mix. Add 1ml of the following primary stock solutions

Trace metal **primary** stocks	g/100ml
$ZnSO_4.7H_20$	2.2
$CoCl_2.6H_20$	1.0
$MnCl_2.4H_20$	1.8
$NaMo0_42H_20$	0.63

Make up to 1 litre using ultrapure distilled water. This will generate a clear, pale yellow/brown solution containing no precipitate. Store in the dark at 4°C.

Vitamin stock solutions

Primary vitamin stocks

Cyanocobalamin (Vitamin B_{12} - Sigma V2876). Make up at 1mg ml^{-1} in ultrapure distilled water.

Biotin (Vitamin H - Sigma B4501). Make up at 0.1mg ml^{-1} in ultrapure distilled water.

Working vitamin stock
To 900ml ultrapure distilled water add 10ml of Biotin primary stock and 1ml Cyanocobalamin primary stock.
Weigh out 200mg Thiamine HCl (Vitamin B_1 - Sigma T4625) and dissolve in 50ml ultrapure distilled water. Add to the above solution and make up to 1 litre with ultrapure distilled water.

Selenite working stock
Dissolve 0.173g of Na_2SeO_3 in ultrapure distilled water (= 17.3mg ml^{-1}). Take 1ml of this solution and make up to 1 litre with ultrapure distilled water.

APPENDIX 2

Denaturing Gradient Gel Electrophoresis Reagents

All chemicals unless otherwise stated were supplied by BIORAD.

50X TAE Buffer

		Final concentration
Tris base	242.0g	2M
Acetic acid, glacial	57.1ml	1M
0.5M EDTA, pH 8.0	100ml	50mM
distilled water	To 1 litre	

Dissolve and autoclave at 121°C for 20-30 minutes.
Store at room temperature.

Acrylamide/bis needed for particular size range of fragment

Gel percentage	Base Pair Separation
6%	300-1000 bp
8%	200-400 bp
10%	100-300 bp

0% Denaturing Solution @ 10%

40% Acrylamide/Bis	25ml
50X TAE buffer	2ml
distilled water	73ml

Degas for 10-15 minutes. Filter through a 0.45µm filter.
Store at 4°C in a brown bottle for approximately 1 month.

100% Denaturing Solution @ 10%

40% Acrylamide/Bis	25ml
50X TAE buffer	2ml
Formamide (deionised)	40ml
Urea	42g
distilled water	Upto 100ml

Degas for 10-15 minutes. Filter through a 0.45µm filter.
Store at 4°C in a brown bottle for approximately 1 month.

For denaturing solutions of less than 100%, use the quantities of acrylamide, 50X TAE buffer and distilled water as for the 100% solution, with the following quantities of deionised formamide and urea.

Denaturing soln.	10%	20%	30%	40%	50%	60%	70%	80%	90%
Formamide (ml)	4	8	12	16	20	24	28	32	36
Urea (g)	4.2	8.4	12.6	16.8	21	25.2	29.4	33.6	37.8

10% Ammonium Persulfate

Ammonium persulfate	0.1g
distilled water	1.0ml

Make up fresh as required and store on ice.

2x gel loading dye

		Final concentration
2% Bromophenol blue	0.25ml	0.05%
2% Xylene cyanol	0.25ml	0.05%
100% glycerol	7.0ml	70%
distilled water	2.5ml	

Store at room temperature

1x TAE running buffer

50X TAE buffer	140ml
distilled water	6,860ml

APPENDIX 3.

16S rDNA sequences from RFLP analyses

NAME ALUS253_3a
LENGTH 843 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  GCGGACGGGT GAGTAACGCG TGGGAACGTA CCCTCTTCTG CGGAATAGCC
  51  ACTGGAAACG GTGAGTAATA CCGCATACGC CCTTCGGGGG AAAGATTTAT
 101  CGGAGGAGGA TCGGCCCGCG TTGGATTAGG TAGTTGGTGG GGTAATGGCC
 151  TACCAAGCCT ACGATCCATA GCTGGTTTTA GAGGATGATC AGCCACACTG
 201  GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATCTT
 251  AGACAATGGG CGCAAGCCTG ATCTAGCCAT GCCGCGTGTG TGACGAAGGC
 301  CTTAGGGTCG TAAAGCACTT TCGCCTGTGA TGATAATGAC AGTAGCAGGT
 351  AAAGAAACCC CGGCTAACTC CGTGCCAGCA GCCGCGGTAA TACGGAGGGG
 401  GTTAGCGTTG TTCGGAATTA CTGGGCGTAA AGCGCACGTA GGCGGACCAG
 451  AAAGTTGGGG GTGAAATCCC GGGGCTCAAC CCCGGAACTG CCTCCAAAAC
 501  TTCTGGTCTG GAGTTCGAGA GAGGTGAGTG GAATTCCGAG TGTAGAGGTG
 551  AAATTCGTAG ATATTCGGAG GAACACCAGT GGCGAAGGCG GCTCACTGGC
 601  TCGATACTGA CGCTGAGGTG CGAAAGTGTG GGGAGCAAAC AGGATTAGAT
 651  ACCCTGGTAG TCCACACCGT AAACGATGAA TGCCAGTCGT CGGCAAGCAT
 701  GCTTGTCGGT GACACACCTA ACGGATTAAG CATTCCGCCT GGGGAGTACG
 751  GTCGCAAGAT TAAAACTCAA AGGAATTGAC GGGGGCCCGC ACAAGCGGTG
 801  GAGCATGTGG TTTAATTCGA AGCAACGCGC AGAACCTTAC CAA
```

NAME ALUS253_6
LENGTH 1462 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

```
   1  GATCATGGCT CAGGATGAAC GCTAGCGGCA GGCTTAACAC ATGCAAGTCG
  51  AGGGGTAACA GGGTTTTCGG ACTGCTGACG ACCGGCGCAC GrrTGCGTAA
 101  cCGCGTATGA AACCTACCTT ATACAGGGGG ATAGCCCAGA GAAATTTGGA
 151  TTAATACCCC ATGGTACTGT GAATCTGCAT GGATTTATAG TTAAAGATTT
 201  ATCGGTATAA GATGGTCATG CGTTCTATTA GTTAGTTGGT AAGGTAACGG
 251  CTTACCAAGA CTGCGATAGA TAGGGGCCCT GAGAGGGGGA TCCCCCACAC
 301  TGGTACTGAG ACACGGACCA GACTCCTACG GGAGGCAGCA GTGAGGAATA
 351  TTGGACAATG GTGGAGACAC TGATCCAGCC ATGCCgCGTG TAgGAAGACT
 401  GcCCTATGGG TTGTAAACTA CTTTTATAGA GGAAGAAACG CAGATACGTG
 451  TATTTGTTTG ACGGTACTCT ACGAATAAGG ATCGGCTAAC TCCGTGCCAG
 501  CAGCCGCGGT AATACGGAGG ATCCAAGCGT TATCCGGAAy CaTTGGGTTT
 551  AAAGGGTCCG CAGGCGGwTG TTTAAGTCAG AGGTGAAAGT TTGCAGCTCA
 601  ACTGTAAAAT TGCCTTTGAT ACTGAATAAC TTGAGTTATA ATGAAGTGGT
 651  TAGAATATGT AGTGTAGCGG TGAAATGCAT AGATATTACA TAGAATACCG
 701  ATTGCGAAGG CAGATCACTA ATTATATACT GACGCTGAGG GACGAAAGCG
 751  TGGGGAGCGA ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG
 801  GTCACTAGCT GTTTGGACTT TTGTCTGAGT GGCTAAGCGA AAGTGATAAG
 851  TGACCCACCT GGGGAGTACG ATCGCAAGAT TGAAACTCAA AGGAATTGAC
 901  GGGGGCCCGC ACAAGCGGTG GAgCmTGTGG TwTAATTCGA TGATACGCGA
 951  GGAACCTTAC CAGGGCTTAA ATGTAGAGTG ACAGGGGTAG AGATACCTTT
1001  TTCTTCGGAC ACTTTACAAG GTGCTGCATG GTTGT,CGTC AGCTCGTGCC
1051  GTGAGGTGTC AGGTTAAGTC CTATAACGAG CGCAACCCCT GTTGTTAGTT
1101  ACCAGCACGT AGTGGTGGGG ACTCTAACAA GACTGCCGGT GCAAACCGTG
1151  AGGAAGGTGG GGATGACGTC AAATCATCAC GGCCCTTACG TCCTGGGCTA
1201  CACACGTGCT ACAATGGTAG GTACAGAGAG CAGCCACCTC GCAAGGGGGA
1251  GCGAATCTAC AAAACCTATC TCAGTTCGGA TCGGAGTCTG CAACTCGACT
1301  CCGTGAAGCT GGAATCGCTA GTAATCGGAT ATCArCCATG ATCCGGTGAA
1351  TACGTTCCCG GGCCTTGTAC ACACCGCCcG TCAAGCCATG GAAGCTGGGG
1401  GTACCTGAAG TCGGtGACCG TAAGGAGCTG CCTAGGGTAA AACTAGTAAC
1451  TGGGGCTAAG TC
```

NAME ALUS253_18
LENGTH 1281 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TGATCATGGC TCAGAACGAA CGCTGGCGGC AGGCCTAACA CATGCAAGTC
  51  GAGCGAACCT TCgGGTTAGC GGCGGACGGG TGAGTAACGC GTGGGAACGT
 101  ACCCTCTTCT GCGGGATAGC CACTGGAAAC GGTGAGTAAT AcCCGCATAC
 151  GCCCTTTGGG GGAAAGATTT ATCGGAGGAG GATCGGCCCG CGTTGGATTA
 201  GGTAGTTGGT GGGGTAATGG CCTACCAAGC CTACGATCCA TAGCTGGTTT
 251  TAGAGGATGA TCAGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG
 301  GGAGGCAGCA GTGGGGAATC TTAGACAATG GGCGCAAGCC TGATCTAGCC
 351  ATGCCGCGTG AGTGACGAAg GCCTTAGGGT CGTAAAGCTC tTTTCGCCAG
 401  AGATGATAAT GaCAGTATCT GGTAAAGAAA CCCCGGCTAA CTCCGTGCCA
 451  GCAGcCCGCG GTAATACGGA GGGGGTTAGC GTTGtTCG3a ATTaCTGGGC
 501  GTAAAGCCCa CGTAGGCGGA TTGGAAAGTT GGGGGTGAAA TCCCAGGGCT
 551  CAACCCTGGA ACGGCCTCCA AAACTCCCAG TCTAGAGTTC GAGAGAGGTG
 601  AGTGGAATTC CGAGTGTAGA GGTGAAATTC GTAGATATTC GGAGGAACAC
 651  CAGTGGCGAA GGCGGCTCAC TGGCTCGATA CTGACGCTGA GGTGCGAAAG
 701  TGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACA CCGTAAACGA
 751  TGAATGCCAG TCGTCGGCAA GCATGCTTGT CGGTgACACA CCTAACGGAT
 801  TAAGCATTCC GCCTGGGGAG TACGGTCGCA AGATTAAAAC TCAAAGGAAT
 851  TGACGGGGGC CCGCACAAGC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC
 901  GCGCAGAACC TTACCAACCC TTGACATGGA TATCGTAGTT ACCAGArATG
 951  GTTTCGTCAG TTCGGCTGGA TATCACACAG GTGCTGCATG GCTGTCGTCA
1001  GCTCGTGTCG TGAGATGTTC GGTTAAGTCC GGCAACGAGC GCAACCCACA
1051  TCCCTAGTTG CCAGCAGGTT AAGCTGGGCA CTCTATGGAA ACTGCCCGTG
1101  ATAAGCGGGA GGAAGGTGTG GATGACGTCA AGTCCTCATG GCCCTTACGG
1151  GTTGGGCTAC ACACGTGCTA CAATGGTGGT GACAATGGGT TAATCCCAAA
1201  AAGCCATCTC AGTTCGGATT GGGGTCTGCA ACTCGACCCC ATGAAGTCGG
1251  AATCGCTAGT AATCGCGTAA CAGCATGACG C
```

NAME ALUS253_19
LENGTH 1282 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TGATCATGGC TCAGAACGAA CGCTGGCGGC AGGCCTAACA CATGCAAGTC
  51  GAGCGAGACC TTCGGGTCTA GCGGCGGACG GGTGAGTAAC GCGTGGGAAC
 101  GTACCCTCTT CTGCGGAATA GCCACTGGAA ACGGTGAGTA ATACCGCATA
 151  CGCCCTTCGG GGGAAAGATT TATCGGAGGA GGATCGGCCC GCGTTGGATT
 201  AGGTAGTTGG TGGGGTAATG GCCTACCAAG CCTACGATCC ATAGCTGGTT
 251  TTAGAGGATG ATCAGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC
 301  GGGAGGCAGC AGTGGGGAAT CTTAGACAAT GGGCGCAAGC CTGATCTAGC
 351  CATGCCGCGT GTGTGACGAA gGCCTTAGGG TCGTAAAGCA CTTTCGCCTG
 401  TgATGATAAT gACAGTAGCA GGTAAAGAAA CCCCGGCTAA CTCCGTGCCA
 451  GCAGCCGCGG TAATACGGAG GGGGTTAGCG TTGTTCGGAA TTACTGCGCG
 501  TAAAGCGCAC GTAGGCGGAC CAGAAAGTTG GGGGTGAAAT CCCGGGGCTC
 551  AACCCCGGAA CTGCCTCCAA AACTTCTGGT CTGGAGTTCG AGAGAGGTGA
 601  GTGGAATTCC GAGTGTAGAG GTGAAATTCG TAGATATTCG GAGGAACACC
 651  AGTGGCGAAG GCGGCTCACT GGCTCGATAC TGACGCTGAG GTGCGAAAGT
 701  GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACAC CGTAAACGAT
 751  GAATGCCAGT CGTCGGCAAG CATGCTTGTC GGTGACACAC CTAACGGATT
 801  AAGCATTCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT
 851  GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG
 901  CGCAGAACCT TACCAACCCT TGACATCCTG ATCGCGGATC GCGGAGACGC
 951  TTTCCTTCAG TTCGGCTGGA TCAGTGACAG GTGCTGCATG GCTGTCGTCA
1001  GCTCGTGTCG TGAGATGTTC GGTTAAGTCC GGCAACGAGC GCAACCCACA
1051  TCCCTAGTTG CCAGCAGTTC GGCTGGGCAC TCTATGGAAA CTGCCCGTGA
1101  TAAGCGGGAG GAAGGTGTGG ATGACGTCAA GTCCTCATGG CCCTTACGGG
1151  TTGGGCTACA CACGTGCTAC AATGGTGGTG ACAATGGGTT AATCCCAAAA
1201  AACCATCTCA GTTCGGATTG GGGTCTGCAA CTCGACCCCA TGAAGTCGGA
1251  ATCGCTAGTA ATCGCGTAAC AGCATGACGC GG
```

NAME ALUS253_23
LENGTH 804 nucleotides
AFFILIATION α-proteobacteria

```
   1  GCTTAATGCG TTAACTGCGC CACCGAACAG TATACTGCCC GACGGCTAGC
```

```
 51  TTCCATCGTT TACGGCGTGG ACTACCAGGG TATCTAATCC TGTTTGCTCC
101  CCACGCTTTC GCACCTCAGC GTCAGTATCG AGCCAGTGAG CCGCCTTCGC
151  CACTGGTGTT CCTCCGAATA TCTACGAATT TCACCTCTAC ACTCGGAATT
201  CCACTCACCT CTCTCGATCT CTAGACTGAC AGTATTAAAG GCAGTTCCAG
251  GGTTGAGCCC TGGGATTTCA CCTCTAACTG ATCAATCCGC CTACGTGCGC
301  TTTACGCCCA GTAATTCCGA ACAACGCTAG CCCCCTTCGT ATTACCGCGG
351  CTGCTGGCAC GAAGTTAGCC GGGGCTTCTT CTATGGTTAC CG.CATTATC
401  TTcACCATTG AAAGTGCTTT ACAACCCTAA GGCCTTCATC ACACACGCGG
451  CATGGCTGGA TCAGGCTTTC GCCCATTGTC CAATATTCCC CACTGCTGCC
501  TCCCGTAGGA GTCTGGGCCG TGTCTCAGTC CCAGTGTGGC TGATCATCCT
551  CTCAGACCAG CTATAGATCG TCGCCATGGT AGGCCTTTAC CCCACCATCT
601  AGCTAATCTA ACGCGGGCTA ATCTATCAGC AATAAATCTT TCCCCCAAAG
651  GGCGTATACG GTATTAGCAG TCGTTTCCAA CTGTTGTTCC GTACTGATAG
701  GTATATTCCC ACGCGTTACT CACCCGTCTG CCACTGCCTC CGAAGAGACC
751  GTTCGACTTG CATGTGTTAA GCCTGCCGCC AGCGTTCGTT CTGAGCCATG
801  ATCA
```

NAME ALUS253_24
LENGTH 647 nucleotides
AFFILIATION α-proteobacteria

```
  1  CACATGCAAG TCGAACGGTC TCTTCGGAGG CAGTGGCAGA CGGGTGAGTA
 51  ACGCGTGGGA ATATACCTAT CAGTACGGAA CAACAGTTGG AAACGACTGC
101  TAATACCGTA TACGCCCTTT GGGGGAAAGA TTTATTGCTG ATAGATTAGC
151  CCGCGTTAGA TTAGCTAGAT GGTGCGGTAA AGGCCTACCA TGGCGACGAT
201  CTATAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC
251  CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGGACA ATGGGCGAAA
301  GCCTGATCCA GCCATGCCGC GTGTGTGATG AAGGCCTTAG GGTTGTAAAG
351  CACTTTCAAT GGTGAAGATA ATGACGGTAA CCATAGAAGA AGCCCCGGCT
401  AACTTCGTGC CAGCAGCCGC GGTAATACGA AGGGGGCTAG CGTTGTTCGG
451  AATTACTGGG CGTAAAGCGC ACGTAGGCGG ATTGATCAGT TAGAGGTGAA
501  ATCCCAGGGC TCAACCCTGG AACTGCCTTT AATACTGTCA GTCTAGAGAT
551  CGAGAGAGGT GAGTGGAATT CCGAGTGTAG AGGTGAAATT CGTAGATATT
601  CGGAGGAACA CCAGTGGCGA AGGCGGCTCA CTGGCTCGAT ACTGACG
```

NAME ALUS253_25
LENGTH 657 nucleotides
AFFILIATION α-proteobacteria

```
  1  CACATGCAAG TCGAACGGTC TCTTCGGAGG CAGTGGCAGA CGGCTCAGTA
 51  ACGCGTGGGA ACATACCTTT CGGTACGGAA CAACAGTTGG AAACGACTGC
101  TAATACCGTA TACGCCCTAT GGGGGAAAGA TTTATCGCCG AGAGATTGGC
151  CCGmGTTGGA TTAGCTAGTT GGTGGGGTAA TGGCCTACCA AGGCGACGAT
201  CCATAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC
251  CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGGACA ATGGGCGCAA
301  GCCTGATCCA GCCATGCCGC GTGAGTGATG AAGGCCTTAG GGTTGTAAAG
351  CTCTTTCGCC GGTGAAGATA ATGACGGTAA CCGGTAAAGA AGCCCCGGCT
401  AACTTCGTGC CAGCAGCCGC GGTAATACGA AGGGGGCTAG CGTTGTTCGG
451  AATTACTGGG CGTAAAGCGC ACGTAGGCTG ACTTTTAAGT CAGGGGTGAA
501  ATCCCGGGGC TCAACCTCGG AACTGCCTTT GATACTGGAA GTCTTGAGTC
551  CGAGAGAGGT GAGTGGAACT CCGAGTGTAG AGGTGAAATT CGTAGATATT
601  CGGAAGAACA CCAGTGGCGA AGGCGGCTCA CTGGCTCGGT ACTGACGCTG
651  AGGTGCG
```

NAME ALUS253_27
LENGTH 659 nucleotides
AFFILIATION α-proteobacteria

```
  1  CGCACCTCAG CGTCAGTATC GAGCCAGTGA GCCGCCTTCG CCACTGGTGT
 51  TCCTCCGAAT ATCTACGAAT TTCACCTCTA CACTCGGAAT TCCACTCACC
101  TCTCTCGATC TCTAGACTGA CAGTATTAAA GGCAGTTCCA GGGTTGAGCC
151  CTGGGATTTC ACCTCTAACT GATCAATCCG CCTACGTGCG CTCTACGCCC
201  AGTAATTCCG AACAACGCTA GCCCCCTTCG TATTACCGCG GCTGCTGGCA
251  CGAAGTTAGC CGGGGCTTCT TCTATGGTTA CCGTCATTAT CTTCACCATT
301  GAAAGTGCTT TACAACCCTA AGGCCTTCAT CACACACGCG GCATGGCTGG
351  ATCAGGCTTT CGCCCATTGT CCAATATTCC CCACTGCTGC CTCCCGTAGG
401  AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTCAGACCA
```

```
 451  GCTATAGATC GTCGCCATGG TAGGCCTTTA CCCCACCATC TAGCTAATCT
 501  AACGCGGGCT AATCTATCAG CAATAAATCT TTCCCCCAAA GGGCGTATAC
 551  GGTATTAGCA GTCGTTTCCA ACTGTTGTTC CGTACTGATA GGTATATTCC
 601  CACGCGTTAC TCACCCGTCT GCCACTGCCT CCGAAGAGAC CGTTCGACTT
 651  GCATGTGTT
```

NAME ALUS253_28
LENGTH 1304 nucleotides
AFFILIATION α-proteobacteria

```
   1  GATCATGGCT CAGAACGAAC GCTGGCGGCA GGCTTAACAC ATGCAAGTCG
  51  AACGGTCTCT TCGGAGGCAG TGGCAGACGG GTGAGTAACG CGTGGGAATA
 101  TACCTATCAG TACGGAACAA CAGTTGGAAA CGACTGCTAA TACCGTATAC
 151  GCCCTTTGGG GGAAAGATTT ATTGCTGATA GATTAGCCCG CGTTAGATTA
 201  GCTAGATGGT GGGGTAAAGG CCTACCATGG CGACGATCTA TAGCTGGTCT
 251  GAGAGGATGA TCAGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG
 301  GGAGGCAGCA GTGGGGAATA TTGGACAATG GGCGAAAGCC TGATCCAGCC
 351  ATGCCGCGTG TGTGATGAAG GCCTTAGGGT TGTAAAGCAC TTTCAATGGT
 401  GAAGATAATG aCGGTAACCA TAGAAGAAGC CCCGGCTAAC TTCGTGCCAG
 451  CAGcCGCGGT TAATACGAAG GGGGCTAGCG TTGTTCGGAA TTACTGGGCG
 501  TAAAGCGCAC GTAGGCGGAT TGATCAGTTA GAGGTGAAAT CCCAGGGCTC
 551  AACCCTGGAA CTGCCTTTAA TACTGTCAGT CTAGAGATCG AGAGAGGTGA
 601  GTGGAATTCC GAGTGTAGAG GTGAAATTCG TAGATATTCG GAGGAACACC
 651  AGTGGCGAAG GCGGCTCACT GGCTCGATAC TGACGCTGAG GTGCGAAAGC
 701  GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT
 751  GGAAGCTAGC CGTCGGGCAG TATACTGTTC GGTGGCCCAG TTAACGCATT
 801  AAGCTTCCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT
 851  GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG
 901  CGCAGAACCT TACCAGCCCT TGACATACCG ATCGCGGTAT CTGGAGACAG
 951  ATACCTTCAG TTAGGCTGGA TCGGATACAG GTGCTGCATG GCTGTCGTCA
1001  GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTCG
1051  CCTTTAGTTG CCAGCATTAA GTTGGGCACT CTAGAGGGAC TGCCGGTGAT
1101  AAGCCGGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC CCTTACGGGC
1151  TGGGCTACAC ACGTGCTACA ATGGTGGTGA CAGTGGGCAG CGAGACCGCG
1201  AGGTCGAGCT AATCTCCAAA AACCATCTCA GTTCGGATCG CACTCTGCAA
1251  CTCGAGTGCG TGAAGTTGGA ATCGCTAGTA ATCGTGGATC AGCATGCCAC
1301  GGTG
```

NAME ALUS253_36
LENGTH 912 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TGATGATAAT GCAGTAGCAG GTAAAGAAAC CCCGGCTAAC TCCGTGCCAG
  51  CAGCCGCGGT AATACGGAGG GGGTTAGCGT TGTTCGGAAT TACTGGGCGT
 101  AAAGCGCACG TAGGCGGACC AGAAAGTTGG GGGTGAAATC CCGGGGCTCA
 151  ACCCCCGAAC TGCCTCCAAA ACTTCTGGTC TGGAGTTCGA GAGAGGTGAG
 201  TGGAATTCCG AGTGTAGAGG TGAAATTCGT AGATATTCGG AGGAACACCA
 251  GTGGCGAAGG CGGCTCACTG GCTCGATACT GACGCTGAGG TGCGAAAGTG
 301  TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCCACAC CGTAAACGAT
 351  GAATGCCAGT CGTCGGCAAG CATGCTTGTC GGTGACACAC CTAACGGATT
 401  AAGCATTCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT
 451  GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG
 501  CGCAGAACCT TACCAACCCT TGACATCCTG ATCGCGGATC GCGGAGACGC
 551  TTTCCTTCAG TTCGGCTGGA TCAGTGACAG GTGCTGCATG GCTGTCGTCA
 601  GCTCGTGTCG TGAGATGTTC GGTTAAGTCC GGCAACGAGC GCAACCCACA
 651  TCCCTAGTTG CCAGCAGTTC GGCTGGGCAC TCTATGGAAA CTGCCCGTGA
 701  TAAGCGGGAG GAAGGTGTGG ATGACGTCAA GTCCTCATGG CCCTTACGGG
 751  TTGGGCTACA CACGTGCTAC AATGGTGGTG ACAATGCGTT AATCCCAAAA
 801  AACCATCTCA GTTCGGATTG CGGTCTGCAA CTCGACCCCA TGAAGTCGGA
 851  ATCGCTAGTA ATCGCGTAAC AGCATGACGC GGTGAATACG TTCCCGGGCC
 901  TTGTACACAC CG
```

NAME ALUS253_40
LENGTH 1282 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TCATGGCTCA GAACGAACGC TGGCGGCAGG CCTAACACAT GCAAGTCGAG
  51  CGAGACCTTC gGGTCTAGCG GCGGACGGGT GAGTAACGCG TGGGAACGTA
 101  CCCTCTTCTG CGGAATAGCC ACTGGAAACG GTGAGTAATA CCGCATACGC
 151  CCTTCGGGGG AAAGATTTAT CGGAGGAGGA TCGGCCCGCG TTGGATTAGG
 201  TAGTTGGTGG GGTAATGGCC TACCAAGCCT ACGATCCATA GCTGGTTTTA
 251  GAGGATGATC AGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
 301  AGGCAGCAGT GGGGAATCTT AGACAATGGG CGCAAGCCTG ATCTAGCCAT
 351  GCCGCGTGTG TGACGAAGGC CTTAGGGTCG TAAAGCACTT TCGCCTGTGA
 401  TGATAATGaC AGTAGCAGGT AAAGAAACCC CGGCTAACTC CGTGCCAGCA
 451  GcCCGCGGTA ATACGGAGGG GGTTAGCGTT GTTCGGAATT ACTGGGCGTA
 501  AAGCGCACGT AGGCGGACCA GAAAGTTGGG GGTGAAATCC CGGGGCTCAA
 551  CCCCGGAACT GCCTCCAAAA CTTCTGGTCT GGAGTTCGAG AGAGGTGAGT
 601  GGAATTCCGA GTGTAGAGGT GAAATTCGTA GATATTCGGA GGAACACCAG
 651  TGGCGAAGGC GGCTCACTGG CTCGATACTG ACGCTGAGGT GCGAAAGTGT
 701  GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACACCG TAAACGATGA
 751  ATGCCAGTCG TCGGCAAGCA TGCTTGTCGG TGACACACCT AACGGATTAA
 801  GCATTCCGCC TGGGGAGTAC GGTCGCAAGA TTAAAACTCA AAGGAATTGA
 851  CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG AAGCAACGCG
 901  CAGAACCTTA CCAACCCTTG ACATCCTGAT CGCGGATCGC GGAGACGCTT
 951  TCCTTCAGTT CGGCTGGATC AGTGACAGGT GCTGCATGGC TGTCGTCAGC
1001  TCGTGTCGTG AGATGTTCGG TTAAGTCCGG CAACGAGCGC AACCCACATC
1051  CCTAGTTGCC AGCAGTTCGG CTGGGCACTC TATGGAAACT GCCCGTGATA
1101  AGCGGGAGGA AGGTGTGGAT GACGTCAAGT CCTCATGGCC CTTACGGGTT
1151  GGGCTACACA CGTGCTACAA TGGTGGTGAC AATGGGTTAA TCCCAAAAAA
1201  CCATCTCAGT TCGGATTGGG GTCTGCAACT CGACCCCATG AAGTCGGAAT
1251  CGCTAGTAAT CGCGTAACAG CATGACGCGG TG
```

NAME ALUS253_41
LENGTH 1290 nucleotides
AFFILIATION α-proteobacteria

```
   1  TGATCATGGC TCAGAACGAA CGCTGGCGGC AGGCTTAACA CATGCAAGTC
  51  GAACGGTCTC TTCGGAGgCA GTGGCAGACG GGTGAGTAAC GCGTGGGAAT
 101  ATACCTATCA GTACGGAACA ACAGTTGGAA ACGACTGCTA ATACCGTATA
 151  CGCCCTTTCG GGGAAAGATT TATTGCTGAT AGATTAGCCC GCGTTAGATT
 201  AGCTAGATGG TGGGGTAAAG GCCTACCATG GCGACGATCT ATAGCTGGTC
 251  TGAGAGGATG ATCAGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC
 301  GGGAGGCAGC AGTGGGGAAT ATTGGACAAT GGGCGAAAGC CTGATCCAGC
 351  CATGCCGCGT GTGTGATGAA gGCCTTAGGG TTGTAAAGCA CTTTCAATGG
 401  TgAAGATAAT gACGGTAaCC ATAGAAGAAg CCCCGGCTAA CTTCGTGCCA
 451  GCAGcCGCGG GTAATACGAA GGGGGCTAGC GTTGTTCGGA ATTACTGGGC
 501  GTAAAGCGCA CGTAGGCGGA TTGATCAGTT AGAGGTGAAA TCCCAGGGCT
 551  CAACCCTGGA ACTGCCTTTA ATACTGTCAG TCTAGAGATC GAGAGAGGTG
 601  AGTGGAATTC CGAGTGTAGA GGTGAAATTC GTAGATATTC GGAGGAACAC
 651  CAGTGGCGAA GGCGGCTCAC TGGCTCGATA CTGACGCTGA GGTGCGAAAG
 701  CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA
 751  TGGAAGCTAG CCGTCGGGCA GTATACTGTT CGGTgGCGCA GTTAACGCAT
 801  TAAGCTTCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT
 851  GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG
 901  CGCAGAACCT TACCArCCCT TGACATACCG ATCGCGGTAT CTGGAGACAC
 951  ATaCCTTCAG TTAGGCTGGA TCGGATACAG GTGCTGCATG GCTGTCGTCA
1001  rCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTCG
1051  CCTTTAGTTG CCAGCATTAA GTTGGGCACT CTAGAGGGAC TGCCGGTGAT
1101  AAGCCGGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC CCTTACGGGC
1151  TGGGCTACAC ACGTGCTACA ATGGTGGTGA CAGTGGGCAG CGAGACCGCG
1201  AGGTCTAGCT AATCTCCAAA AACCATCTCA GTTCGGATCG CACTCTGCAA
1251  CTCGAGTGCG TGAAGTTGGA ATCGCTAGTA ATCGTGGATC
```

NAME ALUS253_42
LENGTH 663 nucleotides
AFFILIATION α-proteobacteria

```
   1  GCTTAACACA TGCAAGTCGA ACGGTCTCTT CGGAGGCAGT GGCAGACGGG
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```
  51  TGAGTAACGC GTGGGAATAT ACCTATCAGT ACGGAACAAC AGTTGGAAAC
 101  GACTGCTAAT ACCGTATACG CCCTTTGGGG GAAAGATTTA TTGCTGATAG
 151  ATTAGCCCGC GTTAGATTAG CTAGATGGTG GGGTAAAGGC CTACCATGGC
 201  GACGATCTAT AGCTGGTCTG AGAGGATGAT CAGCCACACT GGGACTGAGA
 251  CACGGCCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGGACAATGG
 301  GCGAAAGCCT GATCCAGCCA TGCCGCGTGT GTGATGAAGG CCTTAGGGTT
 351  GTAAAGCACT TTCAATGGTG AAGATAATGA CGGTAACCAT AGAAGAAGCC
 401  CCGGCTAACT TCGTGCCAGC AGCCGCGGTA ATACGAAGGG GGCTAGCGTT
 451  GTTCGGAATT ACTGGGCGTA AAGCGCACGT AGGCGGATTG ATCAGTTAGA
 501  GGTGAAATCC CAGGGCTCAA CCCTGGAACT GCCTTTAATA CTGTCAGTCT
 551  AGAGATCGAG AGAGGTGAGT GGAATTCCGA GTGTAGAGGT GAAATTCGTA
 601  GATATTCGGA CGAACACCAG TGGCGAAGGC GGCTCACTGG CTCGATACTG
 651  ACGCTGAGGT GCG
```

NAME ALUS253_43
LENGTH 1363 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TTGATCATGG CTCAGAACGA ACGCTGGCGG CAGGCCTAAC ACATGCAAGT
  51  CGAGCGAGAC CTTCkGGTCT AGCGGCGGAC GGGTGAGTAA CGCGTGGGAA
 101  CGTACCCTCT TCTGCGGAAT AGCCACTGGA AACGGTGAGT AATACCGCAT
 151  ACGCCCTTCG GGGGAAAGAT TTATCGCAGG ACGATCGGCC CGCGTTGGAT
 201  TAGGTAGTTG GTGGGGTAAT GGCCTACCAA GCCTACGATC CATAGCTGGT
 251  TTTAGAGGAT GATCAGCCAC ACTGGGACTG AgACACGGCC CAGACTCCTA
 301  CGGGAGGCAG CAGTGGGGAA TCTTAGACAA TGGGCGCAAG CCTGATCTAG
 351  CCATGCCGCG TGTGTGACkA AGGCCTTAGG GTCGTAAAGC ACTTTCGCCT
 401  GTGATGATAA TGACAGTAGC AGGTAAAGAA ACCCCGGCTA ACTCCGTGCC
 451  AGCAGCCGCG GTAATACGGA GGGGGTTAGC GTTGTTCGGA ATTACTGGGC
 501  GTAAAG.CGC ACGTAGGCGG ACCAGAAAGT TGGGGGTGAA ATCCCGGGGC
 551  TCAACCCCGC AACTGCCTCC AAAACTTCTG GTCTGGAGTT CGAGAGAGGT
 601  GAGTGGAATT CCGAGTGTAG AGGTGAAATT CGTAGATATT CGGAGGAACA
 651  CCAGTGGCGA AGGCGGCTCA CTGGCTCGAT ACTGACGCTG AGGTGCGAAA
 701  GTGTGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCCAC ACCGTAAACG
 751  ATGAATGCCA GTCGTCGGCA AGCATGCTTG TCGGTGACAC ACCTAACGGA
 801  TTAAGCATTC CGCCTGGGGA GTACGGTCGC AAGATTAAAA CTCAAAGGAA
 851  TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA
 901  CGCGCAGAAC CTTACCAACC CTTGACATCC TGATCGCGGA TCGCGGAGAC
 951  GCTTTCCTTC AGTTCGGCTG GATCAGTGAC AGGTGCTGCA TGGCTGTCGT
1001  CAGCTCGTGT CGTGAGATGT TCGGTTAAGT CCGGCAACGA GCGCAACCCA
1051  CATCCCTAGT TGCCAGCAGT TCGGCTGGGC ACTCTATGGA AACTGCCCGT
1101  GATAAGCGGG AGGAAGGTGT GCATGACGTC AAGTCCTCAT GGCCCTTACG
1151  GGTTGGGCTA CACACGTGCT ACAATGGTGG TGACAATGGG TTAATCCCAA
1201  AAAACCATCT CAGTTCGGAT TGGGGTCTGC AACTCGACCC CATGAAGTCG
1251  GAATCGCTAG TAATCGCGTA ACAGCATGAC GCGGTGAATA CGTTCCCGGG
1301  CCTTGTACAC ACCGcCCGTC ACACCATGGG AGTTGGGTTT ACCCGACGGG
1351  CCGTGCGCTA ACC
```

NAME ALUS253_46
LENGTH 1327 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  GGTTAGCGCA CGGCCGTCGG GTAAACCCAA CTCCCATGGT GTGACGGGCG
  51  GTGTGTACAA GGCCCGGGAA CGTATTCACC GCGTCATGCT GTTACGCGAT
 101  TACTAGCGAT TCCGACTTCA TGGGGTCGAG TTGCAGACCC CAATCCGAAC
 151  TGAGATGGTT TTTTGGGATT AACCCATTGT CACCACCATT GTAGCACGTG
 201  TGTAGCCCAA CCCGTAAGGG CCATGAGGAC TTGACGTCAT CCACACCTTC
 251  CTCCCGCTTA TCACGGGCAG TTTCCATAGA GTGCCCAGCC GAACTGCTGG
 301  CAACTAGGGA TGTGGGTTGC GCTCGTTGCC GGACTTAACC GAACATCTCA
 351  CGACACGAGC TGACGACAGC CATGCAgCAC CTGTCACTGA TCCAGCCGAA
 401  CTCAAGGAAA GCGTCTCCGC GATCCGCGAT CAGGATGTCA AGGGTTGGyA
 451  AGGTTCTGCG CGTTGCTTCG AATTAAACCA CATGCTCCAC CGCTTGTGCG
 501  GGCCCCCGTC AATTCCTTTG AGTTTCAATC TTGCGACCGT ACTCCCCAGG
 551  CGGAATGCTT AATCCGTTAG GTGTGTCACC GACAAGCATG CTTGCmGACG
 601  ACTGGCATTC ATCGTTTACG GTGTGGACTA CCAGGGTATC TAATCCTGTT
 651  TGCTCCCCAC ACTTTCGCAC CTCAGCGTCA GTATCGAGCC AGTGAGCCGC
 701  CTTCGCCACT GGTGTTCCTC CGAATATCTA CGAATTTCAC CTCTACACTC
 751  GGAATTCCAC TCACCTCTCT CGAACtCCAG ACCAGAAGTT TTGGAGGCAG
 801  TTCCGGGGTT GAGCCCCGCG ATTTCACCCC CAACTTTCTG GTCCGcCTAC
```

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 851  GTGCGCTTTA CGCCCAGTAA TTCCGAACAA CgGCTAACCC CCTCCGTATT
 901  ACCGCGGCTG CTGGCACGGA GTTAGCCGGG GTTTCTTTAC CTGCTACTGT
 951  CATTATCATC ACAGGCGAAA GTGCTTTACG ACCCTAAGGC TTCGTCACAC
1001  ACGCGGCATG GCTAGATCAG GCTTGCGCCC ATTGTCTAAG ATTCCCCACT
1051  GCTGCCTCCC GTAGGAGTCT GGGGCCGTGT CTCAGTCCCA GTGTGGCTGA
1101  TCATCCTCTA AAACCAGCTA TGGATCGTAG GCTTGGTAGG CCATTACCCC
1151  ACCAACTACa TAATCCAACG CGGGCCGATC CTCCTCCGAT AAATCTTTCC
1201  CCCGAAGCGC GTATGCGGTA TTACTCACCG TTCCCAGTGG CTATTCCGCA
1251  GAAGAGGGTA CGTTCCCACG CGTTACTCAC CCGTCTGCCG CTAGACCAkA
1301  AGGTCTCGCT CGACTTGCAT GTGTTAG
```

NAME ALUS253_55
LENGTH 963 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  AGGTTAGCGC ACGGCCGTCG GGTAAACCCA ACTCCCATGG TGTGACGGGC
  51  GGTGTGTACA AGGCCCGGGA ACGTATTCAC CGCGTCATGC TGTTACGCGA
 101  TTACTAGCGA TTCCGACTTC ATGGGGTCGA GTTGCAGACC CCAATCCGAA
 151  CTGAGATGGT TTTTTGGGAT TAACCCATTG TCACCACCAT TGTAGCACGT
 201  GTGTAGCCCA ACCCGTAAGG GCCATGAGGA CTTGACGTCA TCCACACCTT
 251  CCTCCCGCTT ATCACGGGCA GTTTCCATAG AGTGCCCAGC CGAACTGCTG
 301  GCAACTAGGG ATGTGGGTTG CGCTCGTTGC CGGACTTAAC CGAACATCTC
 351  ACGACACGAG CTGACGACAG CCATGCAGCA CCTGTCACTG ATCCAGCCGA
 401  ACTGAAGGAA AGCGTCTCCG CGATCCGCGA TCAGGATGTC AAGGGTTGGT
 451  AAGGTTCTGC GCGTTGCTTC GAATTAAACC ACATGCTCCA CCGCTTGTGC
 501  GGGCCCCCGT CAATTCCTTT GAGTTTTAAT CTTGCGACCG TACTCCCCAG
 551  GCGGAATGCT TAATCCGTTA GGTGTGTCAC CGACAAGCAT GCTTGCCGAC
 601  GACTGGCATT CATCGTTTAC GGTGTGGACT ACCAGGGTAT CTAATCCTGT
 651  TTGCTCCCCA CACTTTCGCA CCTCAGCGTC AGTATCGAGC CAGTGAGCCG
 701  CCTTCGCCAC TGGTGTTCCT CCGAATATCT ACGAATTTCA CCTCTACACT
 751  CGGAATTCCA CTCACCTCTC TCGAACTCCA GACCAGAAGT TTTGGAGGCA
 801  GTTCCGGGGT TGAGCCCCGG GATTTCACCC CCAACTTTCT GGTCCGCCTA
 851  CGTGCGCTTT ACGCCCAGTA ATTCCGAACA ACGCTAACCC CCTCCGTATT
 901  ACCGCGGCTG CTGGCACGGA GTTAGCCGGG GTTTCTTTAC CTGCTACTGT
 951  CATTATCATC ACA
```

NAME ALUS253_59
LENGTH 1363 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TAGAGTTTGA TCATGGCTCA GAACGAACGC TGCGGCAGGC CTAACACATG
  51  CAAGTCGAGC GAGACCTTCG GGTCTAGCGG CGGACGGGTG AGTAACGCGT
 101  GGGAACGTAC CCTCTTCTGC GGAATAGCCA CTGGAAACGG TGAGTAATAC
 151  CGCATACGCC CTTCGGGGGA AAGATTTATC GGAGGAGGAT CGGCCCGCGT
 201  TGGATTAGGT AGTTGGTGGG GTAATGGCCT ACCAAGCCTA CGATCCATAG
 251  CTGGTTTTAG AGGATGATCA GCCACACTGG GACTGAGACA CGGCCCAGAC
 301  TCCTACGGGA GGCAGCAGTG GGGAATCTTA GACAATGGGC GCAAGCCTGA
 351  TCTAGCCATG CCGCGTGTGT GACGAAGGCC TTAGGGTCGT AAAGCACTTT
 401  CGCCTGTGAT GATAATGACA GTAGCAGCTA AAGAAACCCC CGCTAACTCC
 451  GTGCCAGCAG CCGCGGTAAT ACGGAGGGGG TTAGcCGTTG TTCGGAATTA
 501  CTGGGCGTAA AGCGCACGTA GGCGGACCAG AAAGTTGGGG GTGAAATCCC
 551  GGGGCTCAAC CCCGGAACTG CCTCCAAAAC TTCTGGTCTG GAGTTCGAGA
 601  GAGGTGAGTG GAATTCCGAG TGTAGAGGTG AAATTCGTAG ATATTCGGAG
 651  GAACACCAGT GGCGAAGGCG GCTCACTGGC TCGATACTGA CGCTGAGGTG
 701  CGAAAGTGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACACCGT
 751  AAACGATGAA TGCCAGTCGT CGGCAAGCAT GCTTGTCGGT GACACACCTA
 801  ACGGATTAAC CATTCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA
 851  AGGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
 901  AGCAACGCGC AGAACCTTAC CAACCCTTGA CATCCTGATC GCGGATCGCG
 951  GAGACGCTTT CCTTCAGTTC GGCTGGATCA GTGACAGGTG CTGCATGGCT
1001  GTCGTCAGCT CGTGTCGTGA GATGTTCGGT TAAGTCCGGC AACGAGCGCA
1051  ACCCACATCC CTAGTTGCCA GCAGTTCGGC TGGGCACTCT ATGGAAACTG
1101  CCCGTGATAA GCGGGAGGAA GGTGTGGATG ACGTCAAGTC CTCATGGCCC
1151  TTACGGGTTG GGCTACACAC GTGCTACAAT GGTGGTGACA ATGGGTTAAT
1201  CCCAAAAAAC CATCTCAGTT CGGATTGGGG TCTGCAACTC GACCCCATGA
1251  AGTCGGAATC GCTAGTAATC GCGTAACAGC ATGACGCGGT GAATACGTTC
1301  CCGGGCCTTG TACACACCGC CCGTCACACC ATGGGAGTTG GGTTTACCCG
1351  ACGGGCCGTG CGC
```

NAME ALUS253_62
LENGTH 662 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  CGCACCTCAG CGTCAGTATC GAGCCAGTGA GCCGCCTTCG CCACTGGTGT
  51  TCCTCCGAAT ATCTACGAAT TTCACCTCTA CACTCGGAAT TCCACTCACC
 101  TCTCTCGAAC TCCAGACCAG AAGTTTTGGA GGCAGTTCCG GGGTTGAGCC
 151  CCGGGATTTC ACCCCCAACT TTCTGGTCCG CCTACGTGCG CTTTACGCCC
 201  AGTAATTCCG AACAACGCTA ACCCCCTCCG TATTACCGCG GCTGCTGGCA
 251  CGGAGTTAGC CGGGGTTTCT TTACCTGCTA CTGTCATTAT CATCAGAGGC
 301  GAAAGTGCTT TACGACCCTA AGGCCTTCGT CACACACGCG GCATGGCTAG
 351  ATCAGGCTTG CCCCCATTGT CTAAGATTCC CCACTGCTGC CTCCCGTAGG
 401  AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTAAAACCA
 451  GCTATGGATC GTAGGCTTGG TAGGCCATTA CCCCACCAAC TACCTAATCC
 501  AACGCGGGCC GATCCTCCTC CGATAAATCT TTCCCCCGAA GGGCGTATGC
 551  GGTATTACTC ACCGTTTCCA GTGGCTATTC CGCAGAAGAG GGTACGTTCC
 601  CACGCGTTAC TCACCCGTCC GCCGCTAGAC CCGAAGGTCT CGCTCGACTT
 651  GCATGTGTTA GG
```

NAME ALUS253_70a
LENGTH 459 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  GCCAGGTTAG CGCACCGTCG TCGGGTArAC CCAACTCCCA TGGTGTGACG
  51  GsCGGTGTGT ACAAGGCCCG GGAACGTATT CACCGCGTCA TGCTGTTACG
 101  CGATTACTAG CGATTCCGAC TTCATGGGGT CGAGTTGCAG ACCCCAATCC
 151  GAACTGAGAT GGCTTTTTGG GATTAACCCA TTGTCACCAC CATTGTAGCA
 201  CGTGTGTAGC CCAACCCGTA AGGGCCATGA GGACTTGACG TCATCCACAC
 251  CTTCCTCCCG CTTATCACGG GCAGTTTCCA TAGAGTGCCC AGCTTAACCT
 301  GCTGGCAACT AGGGATGTGG GTTGCGCTcG TTGCCGGACT TAACCGAACA
 351  TyTCACGACA CGAGCTGACG ACAGCCATGC AGCACCTGTG TsATATCCAG
 401  CCGAACTGAC GAAACCATCT CTGGTAACTA CGATATCCAT GTCAAGGGTT
 451  GGTAAGGTT
```

NAME ALUS253_78
LENGTH 1299 nucleotides
AFFILIATION α-proteobacteria

```
   1  ATCATGGCTC AGAACGAACG CTGGCGGCAG GCTTAACACA TGCAAGTCGA
  51  ACGGTCTCTT CGGAGGCAGT GGCAGACGGG TGAGTAACGC GTGGGAATAT
 101  ACCTATCAGT ACGGAACAAC AGTTGGAAAC GACTGCTAAT ACCGTATACG
 151  CCCTTTGGGG GAAAGATTTA TTGCTGATAG ATTAGCCCGC GTTAGATTAG
 201  CTAGATGGTG GGGTAAAGGC CTACCATGGC GACGATCTAT AGCTGGTCTG
 251  AGAGGATGAT CAGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG
 301  GAGGCAGCAG TGGGGAATAT TGGACAATGG GCGAAAGCCT GATCCAGCCA
 351  TGCCGCGTGT GTGATGAAGG CCTTAGGGTT GTAAAGCACT TTCAATGGTg
 401  AAGATAATgA CGGTAACCAT AGAAGAAgCC CCGGCTAACT TCGTGCCAGC
 451  AGcCCGCGGT AATACGAAGG GGGCTAGcCG TTGTTCGGAA TTACTgGGCG
 501  TAAAGCGCAC GTAGGCGGAT TGATCAGTTA GAGGTGAAAT CCCAGGGCTC
 551  AACCCTGGAA CTGCCTTTAA TACTGTCAGT CTAGAGATCG AGAGAGGTGA
 601  GTGGAATTCC GAGTGTAGAG GTGAAATTCG TAGATATTCG GAGGAACACC
 651  AGTGGCGAAG GCGGCTCACT GGCTCGATAC TGACGCTGAG GTGCGAAAGC
 701  GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT
 751  GGAAGCTAGC CGTCGGGCAG TATACTGTTC GGTGCCGCAG TTAACGCATT
 801  AAGCTTCCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT
 851  GACGGGGGCC CGCACAAGCG GTGkrmmTG TGGTTTAATT CGAAGCAACG
 901  CGCAGAACCT TACCAGCCCT TGACATACCG ATCGCGGTAT CTGGAGACAG
 951  ATACCTTCAG TTAGGCTGGA TCGGATACAG GTGCTGCATG GCTGTCGTCA
1001  GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTCG
1051  CCTTTAGTTG CCAGCATTAA GTTGGGCACT CTAGAGGGAC TGCCGGTGAT
1101  AAGCCGGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGn CCTTACGGGC
1151  TGGGCTACAC ACGTGCTACA ATGGTGGTGA CAGTGGGCAG CGAGACCGCG
1201  AGGTCGAGCT AATCTCCAAA AACCATCTCA GTTCGGATCG CACTCTGCAA
1251  CTCGAGTGCG TGAAGTTGGA ATCGCTAGTA ATCGTGGATC AGCATGCCA
```

NAME ALUS253_79
LENGTH 656 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  CGCACCTCAG CGTCAGTATC GAGCCAGTGA GCCGCCTTCG CCACTGGTGT
  51  TCCTCCGAAT ATCTACGAAT TTCACCTCTA CACTCGGAAT TCCACTCACC
 101  TCTCTCGAAC TCCAGACCAG AAGTTTTGGA GGCAGTTCCG GGGTTGAGCC
 151  CCGGGATTTC ACCCCCAACT TTCTGGTCCG CCTACGTGCG CTTTACGCCC
 201  AGTAATTCCG AACAACGCTA ACCCCCTCCG TATTACCGCG GCTGCTGGCA
 251  CGGAGTTAGC CGGGGTTTCT TTACCTGCTA CTGTCATTAT CATCACAGGC
 301  GAAAGTGCTT TACGACCCTA AGGCCTTCGT CACACACGCG GCATGGCTAG
 351  ATCAGGCTTG CGCCCATTGT CTAAGATTCC CCACTGCTGC CTCCCGTAGG
 401  AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTAAAACCA
 451  GCTATGGATC GTAGGCTTGG TAGGCCATTA CCCCACCAAC TACCTAATCC
 501  AACGCGGGCC GATCCTCCTC CGATAAATCT TTCCCCCGAA GGGCGTATGC
 551  GGTATTACTC ACCGTTTCCA GTGGCTATTC CGCAGAAGAG GGTACGTTCC
 601  CACGCGTTAC TCACCCGTCC GCCGCTAGAC CCGAAGGTCT CGCTCGACTT
 651  GCATGT
```

NAME ATAM407_1
LENGTH 1362 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  CGCACGGCCG TCGGGTAAAC CCAACTCCCA TGGTGTGACG GGCGGTGTGT
  51  ACAAGGCCCG GGAACGTATT CACCGCGTCA TGCTGTTACG CGATTACTAG
 101  CGATTCCGAC TTCATGGGGT CGAGTTGCAG ACCCCAATCC GAACTGAGAT
 151  GGTTTTTTGG GATTAACCCA TTGTCACCAC CATTGTAGCA CGTGTGTAGC
 201  CCAACCCGTA AGGGCCATGA GGACTTGACG TCATCCACAC CTTCCTCCCG
 251  CTTATCACGG GCAGTTTCCA TAGAGTGCCC AGCCGAACTG CTGGCAACTA
 301  GGGATGTGGG TTGCGCTCGT TGCCGGACTT AACCGAACAT CTCACGACAC
 351  GAGCTGACGA CAGCCATGCA GCACCTGTCA CTGATCCAGC CGAACTGAAG
 401  GAAAGCGTCT CCGCGATCCG CGATCAGGAT GTCAAGGGTT GGTAAGGTTC
 451  TGCGCGTTGC TTCGAATTAA ACCACATGCT CCACCGCTTG TGCGGGCCCC
 501  CGTCAATTCC TTTGAGTTTT AATCTTGCGA CCGTACTCCC CAGGCGGAAT
 551  GCTTAATCCG TTAGGTGTGT CACCGACAAG CATGCTTGCC GACGACTGGC
 601  ATTCATCGTT TACGGTGTGG ACTACCAGGG TATCTAATCC TGTTTGCTCC
 651  CCACACTTTC GCACCTCAGC GTCAGTATCG AGCCAGTGAG CCGCCTTCGC
 701  CACTGGTGTT CCTCCGAATA TCTACGAATT TCACCTCTAC ACTCGGAATT
 751  CCACTCACCT CTCTCGAACT CCAGACCAGA AGTTTTGGAG GCAGTTCCGG
 801  GGTTGAGCCC CGGGATTTCA CCCCCAACTT TCTGGTCCGC CTACGTGCGC
 851  TTTACGCCCA GTAATTCCGA ACAACGCTAA CCCCCTCCGT ATTACCGCGG
 901  CTGCTGGCAC GGAGTTAGCC GGGGTTTCTT TACCTGCTAC TGTCATTATC
 951  ATCACAGGCG AAAGTGCTTT ACGACCCTAA GGCyTTmGTC ACACACGCGG
1001  CATGGCTAGA TCAGGCTTGC GCCCATTGTC TAAGATTCCC CasTGsTGCC
1051  TCCyGTAGGA GTmTrGGCCG TGTmTCAGTC CCAGrGTGGs TGATCATCCT
1101  CTAAAaCCAC cTATGgATCG TAGGCTTGGT AGGCCATTAC CCCACCAACT
1151  ACsTAATCCA asGCGGGCCG ATCCTCCTCc gATAAATCTT TCCCCCGAAG
1201  rGCGTATGCG GTATwcACTC wCCGTTTCCA GTGGctATTC CGCAGAAGAG
1251  GGwACGTTCC CACGcGTTAC TCACCCGTCy GCCGCTAGaC CmgAAGGTCT
1301  CGCTCGACTT GCATGTGTTA GGCCTGCCGC CAGCGTTCGT TCTGAGCCAT
1351  GATCAAACTC TA
```

NAME ATAM407_11
LENGTH 398 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  CGCACCTCAG CGTCAGTATC GAGCCAGTGA GCCGCCTTCG CCACTGGTGT
  51  TCCTCCGAAT ATCTACGAAT TTCACCTCTA CACTCGGAAT TCCACTCACC
 101  TCTCTCGAAC TCCAGACCAG AAGTTTTGGA GGCAGTTCCG GGGTTGAGCC
 151  CCGGGATTTC ACCCCCAACT TTCTGGTCCG CCTACGTGCG CTTTACGCCC
 201  AGTAATTCCG AACAACGCTA ACCCCCTCCG TATTACCGCG GCTGCTGGCA
 251  CGGAGTTAGCC GGGGTTTCTT TACCTGCTAC TGTCATTATC ATCACAGGCG
 301 AAAGTGCTTTA CGACCCTAAG GCCTTCGTCA CACACGCGGC ATGGCTAGAT
 351  CAGGCTTGCG CCCATTGTCT AAGATTCCCC ACTGCTGCCT CCCGTA
```

NAME ATAM407_18
LENGTH 1422 nucleotides
AFFILIATION γ-proteobacteria

```
   1  TTGATCATGG CTCAGATTGA ACGCTGCGGC AGGCCTAACA CATGCAAGT
  51  GAACGGTAAC ATTTcTAGCT TGCTAGAAGA TGACGAGTGG CGGACGGGTG
 101  AGTAATGCTT GGGAACTTGC CTTTGCGAGG GGGATAACAG TTGGAAACGA
 151  CTGCTAATAC CGCATAATGT CTTCGGACCA AAGGGGGCTy cGGCTCCCAC
 201  GCAAAGAGAG GCCCAAGTGA GATTAGCTAG TTCGTrAGGT AAAGGCTCAC
 251  CAAGGCAACG ATCTCTAGCT GTTCTGAGAG GAAGATCAgC CACACTGGGA
 301  CTGAGACACG GCCCAzACTC CTACGGGAGG CAGCAGTGGG GAATATTGCA
 351  CAATGGGCGA AAGCCTGATG CAGCCATGCC GCGTGTGTGA AGAAGGCCTT
 401  CGGGTTGTAA AGCACTTTCA GTTGTGAGGA AAAGTTAGTA GTTAATACCT
 451  GCTAGCCGTG ACGTTAACAA CAGAwGAAGC ACCGGCTAAC TCCGTGCCAG
 501  CAGCCGCsGT AATACGGAGG GTGCGAGCGT TAATCGGAAT TACTGGGCGT
 551  AAAGCGCACG CAGGCGGTTT GTTAAGCTAG ATGTGAAAGC CCCGAGCTCA
 601  ACTTGGGATG GTCATTTAGA ACTGGCAGAC TACAGTCTTG GAGAGGGGAG
 651  TGGAATTCCA GGTGTAGCGG TGAAATGCGT AGATATCTGG AGGAACATCA
 701  GTGGCGAAGG CGACTCCCTG GCCAAAGACT GACGCTCATG TGCGAAAGTG
 751  TGGGTAGCGa AACAGGATTA GATACCCTGG TAGTCCACAC CGTAAACGCT
 801  GTCTACTAGC TGTGTGTGCC TTTAAGGCGT GCGTAGCGAA GCTAACGCGA
 851  TAAGTAGACC GCCTGGGGAG TACGGCCGCA AGGTTAAAAC TCAAATGAAT
 901  TGACGGGGGC CCGCACAAGC GGTGGAGCAT GTGGTTTAAT TCGATGCAAC
 951  GCGAAGAACC TTACCTACAC TTGACATGCA GAGAAGTTAC TAGAGATAGT
1001  TTCGTGCCTT CGGGAACTCT GACACAGGTG CTGCATGGCT GTCGTCAGCT
1051  CGTGTCGTGA GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGTCC
1101  TTAGTTGCCA GCATTAAGTT GGGCACTCTA AGGAGACTGC CGGTGACAAA
1151  CCGGAGGAAG GTGGGGACGA CGTCAAGTCA TCATGGCCCT TACGTGTAGG
1201  GCTACACACG TGCTACAATG GCATTTACAG AGGGAAGCGA GACAGTGATG
1251  TCGAGCGGAC CCCTTAAAGA ATGTCGTAGT CCGGATTGGA GTCTGCAACT
1301  CGACTCCATG AAGTCGGAAT CGCTAGTAAT CGCAGGTCAG AATACTGCGG
1351  TGAATACGTT CCCGGGCCTT GTACACACCG CCCGTCACAC CATGGGAGTG
1401  GGATGCAAAA GAAGTAGTTA GT
```

NAME ATAM407_20
LENGTH 877 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  AGAGTTTGAT CATGGCTCAG AACGAACGCT GCGGCAGGCC TAACACATGC
  51  wAGTCGAGCG AGACCTTCGG GTCTAGCGGC GGACGGGTGA GTAACGCGTG
 101  GGAACGTACC CTCTTCTGCG GAATAGCCAC TGGAAACGGT GAGTAATACC
 151  GCATACGCCC TTCGGGGGAA AGATTTATCG GAGGAGGATC GGCCCGCGTT
 201  GGATTAGGTA GTTGGTGGGG TAATGGCCTA CCAAGCCTAC GATCCATAGC
 251  TGGTTTTAGA GGATGATCAG CCACACTGGG ACTGAGACAC GGCCCAGACT
 301  CCTACGGGAG GCAGCAGTGG GGAATCTTAG ACAATGGGCG CAAGCCTGAT
 351  CTAGCCATGC CGCGTGTGTG ACGAAGGCCT TAGGGTCGTA AAGCACTTTC
 401  GCCTGTGATG ATAATGACAG TAGCAGGTAA AGAAACCCCG GCTAACTCCG
 451  TGCCAGCAGC CGCGGTAATA CGGAGGGGCT TAGCGTTGTT CGGAATTACT
 501  GGGCGTAAAG CGCACGTAGG CGGACCAGAA AGTTGGGGGT GAAATCCCGG
 551  GGCTCAACCC CGGAACTGCC TCCAAAACTT CTGGTCTGGA GTTCGAGAGA
 601  GGTGAGTGGA ATTCCGAGTC TAGAGGTGAA ATTCGTAGAT ATTCGGAGGA
 651  ACACCAGTGG CGAAGGCGGC TCACTGGCTC GATACTGACG CTGAGGTGCG
 701  AAAGTGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACACCGTAA
 751  ACGATgAATG CCAGTCGTCg GCAAGCATGC TTGtTCGGTG ACACACCTAA
 801  CGGATTAAGC ATTCCGCCTG GCGAGTACGG TCGTAAGATT AAAACTCACA
 851  GGAATTGACG GGGGCCCkCA CAAACGG
```

NAME ATAM407_25
LENGTH 1360 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TTGATCATGG CTCAGAACGA ACGCTGGCGG CAGGCCTAAC ACATGCAAGT
  51  CGAGCGAkAC CTTCGGGTct AGCGGCGGAC GGGTkAGTAA CGcGTGGGAA
 101  CgTACCCTCT TcTGCGGAAT AgCCtCTGGA AACGGTGAGT AATACCGCAT
 151  ACGCyCTTcG GGGGAAAGAT TTATCGGAGr AGGATCGGCC CGCGTTgGAT
 201  TAGgTAGTTG GTGGGGTAAT GGCCTACCAA GCCTACGATC CATAGCTGGT
 251  TTTAGAGGAT GATCAgCCAC ACTGGGACTG AGACACGGCC CAGACTCCTA
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 301  CGGGAGGCAs CAGTGGGGAA TCTTAGACAA TGGGCGCAAG CCTGATCTAG
 351  CCATGCCGCG TGwGTGAyGA AgGCCTTAGG GTCGTmAAGC aCTTTCGCCT
 401  GtGATGATAA TGACAGTAtC aGGTAAAGAA ACCCCGGCTA ACTCCGTGCC
 451  AGCAGCCGCG GTAATACGGA kGGGGTTAGC GTTGTTCGGA ATTACTGGGC
 501  GTAAAGCGCA CGTAGGCGGA CCAgAAAGTT rGGGGTGAAA TCCCGGGGCT
 551  CAACCCCGGA ACTGCCTCCA AAACTkCTGG TCTrGAGTTC GAGAGAGGTG
 601  AGTGGAATTC CGAGTGTAGA GGTGAAATTC GTAGATATTC GGAGGAACAC
 651  CAGTGGCGAA GGCGGCTCAC TGGCTCGATA CTGACGCTGA kGTGCGAAAG
 701  tGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACa CCGTAAACGA
 751  TGAATGCCAG TCGTCGGCAA GCATGCTyGT CGGTGACACA CCTAACGGAT
 801  TAAGCATTCC GCCTGGGGAG TACGGTCGCA AGATTAAAAC TCAAAGGAAT
 851  TGACGGGGGC CCGCACAAGC GGTGGAkCAT GTGGwTTAAT TCGAAGCAAC
 901  GCGCAGAACC TTACCAACCC TTGACATGCT GATCGCGGAT CGCGGAGACG
 951  CTTTCCTTCA GTTCGGCTGG ATCAGGwsAC AGGTGCTGCA TGGCTGTCGT
1001  CAGCTcGTGT CGTkAGATGT TCGGTTAAGT CmGkCAsCGA GCGCAAsCCA
1051  CATCCmTAGT TGCCAGCAGT TmGGCTGGGC ACTCTAtGgA AACTGCCCGT
1101  GATAAgCGGG AGGAAGGTGT GkATGACGTC AAGTCmTCAT GGCCCTTmCG
1151  GGTTGGGCTA CACACGTGCT ACAATGGTrG TGACAATGGG TTAATCCCaA
1201  AAAACCATcT CAGTTmGGAT TGgGGTCTGc AAcTmGACCC CATGAAGTcG
1251  GAATCGCTaA GTAATCGctG TAACAGCATG AmgCGGTgAA TACGTTCwkG
1301  GGCCTTGTAC rCACmGCCCG TCAsTCCATs GGAGTTAGGT TGATCCrCCG
1351  TGCCGTGCGC
```

NAME ATAM407_36
LENGTH 704 nucleotides
AFFILIATION γ-proteobacteria

```
   1  CATGCAAGTC GAACGGTAAC ATTTCTAGCT TGCTAGAAGA TGACGAGTGG
  51  CGGACGGGTG AGTAATGCTT GGGAACTTGC CTTTGCGAGG GGGATAACAG
 101  TTGGAAACGA CTGCTAATAC CGCATAATGT CTTCGGACCA AAGGGGGCTT
 151  CGGCTCCCAC GCAAAGAGAG GCCCAAGTGA GATTAGCTAG TTGGTGAkGT
 201  AAAGGCTTAC CAAGGCaACG ATCTCTAGCT GTTCTGAGAG GAAGATCAgC
 251  CACACTGGGA CTGAGACACG GcCCArACTC CTACGGGAGG CAGCAGTGGG
 301  GAATATTGCA CAATGGGCGA AAGCCTGATG CAGCCATGCC GCGTGTGTGA
 351  AGAAGGCCTT CGGGTTGTAA AGCACTTTCA GTTGTGAGGA AAAGTTAGTA
 401  GTTAATACCT GCTAGCCGTG ACGTTAACAA CAGAAGAAGC ACCGGCTAAC
 451  TCCGTGCCAG CAGCCGCGGT AATACGGAGG GTGCGAGCGT TAATCGGAAT
 501  TACTGGGCGT AAAGCGCACG CAGGCGGTTT GTTAAGCTAG ATGTGAAAGC
 551  CCCGAGCTCA ACTTGGGATG GTCATTTAGA ACTGGCAGAC TAGAGTCTTG
 601  GAGAGGGGAG TGGAATTCCA GGTGTAGCGG TGAAATGCGT AGATATCTGG
 651  AGGAACATCA GTGGCGAAGG CGACTCCCTG GCCAAAGACT GACGCTCATG
 701  TGCG
```

NAME ATAM407_48
LENGTH 618 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  GGTCTAGCGG CAGACGGGTG AGTAACGCGT GGGAACGTAC CCTCTTCTGC
  51  GGAATAGCCA CTGCAAACGG TGAGTAATAC CGCATACGCC CTTCGGGGGA
 101  AAGATTTATC GGAGGAGGAT CGGCCCGCGw TGGATTAGGT AGTTGGTGGG
 151  GTAATGGCCT ACCAAGCCTA CGATCCATAG CTGGTTTTAG AGGATGATCA
 201  GCCACACTGG GACTGAgACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTG
 251  GGGAATCTTA GACAATGGGC GCAAGCCTGA TCTAGCCATG CCGCGTGTGT
 301  GACGAAGGCC TTAGGGTCGT AAAGCACTTT CGCCTGTGAT GATAATGACA
 351  GTAGCAGGTA AAGAAACCCC GGCTAACTCC GTGCCAGCAG CCGCGGTAAT
 401  ACGGAGGGGG TTAGCgTTGT TCGGAATTAC TGGGCGTAAA GCGCACGTAG
 451  GCGGACCAGA AAGTTGGGGG TGAAATCCCG GGGCTCAACC CCGGAACTGC
 501  CTCCAAAACT TCTGGTCTGG AGTTCGAGAG AGGTGAGTGG AATTCCGAGT
 551  GTAGAGGTGA AATTCGTAGA TATTCGGAGG AACACCAGTG GCGAAGGCGG
 601  CTCACTGGCT CGATACTG
```

NAME ATAM407_54
LENGTH 959 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  GCCAGAGATG ATAATGACAG TATCTGGTAA AGAAACCCCG GCTAACTCCG
  51  TGCCAGCAGC CGCGGTAATA CGGAGGGGGT TAGCGTTGTT CGGAATTACT
```

```
101  GGGCGTAAAG CGCACGTAGG CGGATTGGAA AGTTGGGGGT GAAATCCCAG
151  GGCTCAACCC TGGAACGGCC TCCAAAACTC CCAGTCTAGA GTTCGAGAGA
201  GGTGAGTGGA ATTCCGAGTG TAGAGGTGAA ATTCGTAGAT ATTCGGAGGA
251  ACACCAGTGG CGAAGGCGGC TCACTGGCTC GATACTGACG CTGAGGTGCG
301  AAAGTGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACACCGTAA
351  ACGATGAATG CCAGTCGTCG GCAAGCATGC TTGTCGGTGA CACACCTAAC
401  GGATTAAGCA TTCCGCCTGG GGAGTACGGT CGCAAGATTA AAACTCAAAG
451  GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGAAG
501  CAACGCGCAG AACCTTACCA ACCCTTGACA TGGATATCGT AGTTACCAGA
551  GATGGTTTCG TCAGTTCGGC TGGATATCAC ACAGGTGCTG CATGGCTGTC
601  GTCAGCTCGT GTCGTGAGAT GTTCGGTTAA GTCCGGCAAC GAGCGCAACC
651  CACATCCCTA GTTGCCAGCA CGTTAAGCTG GGCACTCTAT GGAAACTGCC
701  CGTGATAAGC GGGAGGAAGG TGTGGATGAC GTCAAGTCCT CATGGCCCTT
751  ACGGGTTGGG CTACACACGT GCTACAATGG TGGTGACAAT GGGTTAATCC
801  CAAAAAGCCA TCTCAgTTCG GATTGGGGTC TGCAACTCGA CCCCATGAAG
851  TCkGAATCGC TAGTAATCGC GTAACAGCAT GACGCGGTGA ATACGTTCCC
901  GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTTGGG TCTACCCGAC
951  GACGGTGCG
```

NAME ATAM407_56
LENGTH 1348 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TTGATCATGG CTCAGAACGA ACGCTGGCGG CAGGCTTAAC ACATGCAAGT
  51  CGAGCGCACT CTTCGGAGTG AGCGGCGGAC GGGTTAGTAA CGCGTGGGAA
 101  CGTGCCCTTC TCTAAGGAAT AGCCACTGGA AACGGTGAGT AATACCTTAT
 151  ACGCCCTTCG GGGGAAAGAT TTATCGGAgA AGGATCGGCC CGCGTTaGAT
 201  TAGATAGTTG GTGGGGTAAT GGCCTACCAA GTCTACGATC TATAGCTGGT
 251  TTTAGAGGAT GATCAGCAAC ACTGGGACTG AGACACGGCC CAgACTCCTA
 301  CGGGAGGCAG CAGTGGGGAA TCTTGGACAA TGGGCGCAAG CCTGATCCAG
 351  CCATGCCGCG TGAGTGATGA AGGCCTTAGG GTCGTAAAGC TCTTTCGCCA
 401  GAGATGATAA TGACAGTATC TGGTAAAGAA ACCCCGGCTA ACTCCGTGCC
 451  AGCAGCCGCG GTAATACGGA GGGGGTTAGC GTTGTTCGGA ATTACTGGGC
 501  GTAAAGCGCA CGTAGGCGGA TCAgAAAGTA TAGGGTGAAA TCCCAGGGCT
 551  CAACCCTGGA ACTGCCTTGT AAACTCCTGG TCTTGAGTTC GAGAGAGGTG
 601  AGTGGAATTC CGAGTGTAGA GGTGAAATTC GTAGATATTC GGAGGAACAC
 651  CAGTGGCGAA GGCGGCTCAC TGGCTCGATA CTGACGCTGA GGTGCGAAAG
 701  TGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACA CCGTAAACGA
 751  TGAATGCCAG ACGTCAGCAA GCATGCTTGT TGGTGTCACA CCTAACGGAT
 801  TAAGCATTCC GCCTGGGGAG TACCGTCGCA AGATTAAAAC TCAAAGGAAT
 851  TGACGGGGGC CCGCACAAGC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC
 901  GCGCAGAACC TTACCAACCC TTGACATCCT TGGACCGCTA GAGAGATCTA
 951  GCTTTCTCGC AAGAGACCAA GTGACAGGTG CTGCATGGCT GTCGTCAGCT
1001  CGTGTCGTGA GATGTTCGGT TAAGTCCGGC AACGAGCGCA ACCCACATCC
1051  TTAGTTGCCA GCAGTTCGGC TGGGCACTCT AGGGAAACTG CCGGTGATAA
1101  GCGGGAGGAA GGTGTGGATG ACGTCAAGTC CTCATGGCCC TTACGGGTTG
1151  GGCTACACAC GTGCTACAAT GGCATCAACA ATGGGTTAAT CCCCAAAAGA
1201  TGTCTCAGTT CGGATTGGGG TCTGCAACTC GACCCCATGA AGTCGGAATC
1251  GCTAGTAATC GCGTAACAGC ATGACGCGGT GAATACGTTC CCGGGCCTTG
1301  TACACACCGC CCGTCACACC ATGGGAGTTG GATCTACCCG AAGGCCGT
```

NAME ATAM407_57
LENGTH 637 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  GTCGAGCGCA CTCTTCGGAG TGAGCGGCGG ACGGGTTAGT AACGCGTGGG
  51  AACGTGCCCT TCTCTAAGGA ATAGCCACTG GAAACGGTGA GTAATACCTT
 101  ATACGCCCTT CGGGGGAAAG ATTTATCGGA GAAGGATCGG CCCGCGTTAC
 151  ATTAGATAGT TGGTGGGGTA ATGGCCTACC AAGTCTACGA TCTATAGCTG
 201  GTTTTAGAGG ATGATCAGCA ACACTGGGAC TGAGACACGG CCCAGACTCC
 251  TACGGGAGGC AGCAGTGGGG AATCTTGGAC AATGGGCGCA AGCCTGATCC
 301  AGCCATGCCG CGTGAGTGAT GAAGGCCTTA GGGTCGTAAA GCTCTTTCGC
 351  CAGAGATGAT AATGACAGTA TCTGGTAAAG AAACCCCGGC TAACTCCGTG
 401  CCAGCAGCCG CGGTAATACG GAGGGGGTTA GcCGTTGTTC GGAATTACTG
 451  GGCGTAAAGC GCACGTAGGC GGATCAGAAA GTATAGGGTG AAATCCCAGG
 501  GCTCAACCCT GGAACTGCCT TGTAAACTCC TGGTCTTGAG TTCGAGAGAG
 551  GTGAGTGGAA TTCCGAGTGT AGAGGTGAAA TTCGTAGATA TTCGGAGGAA
 601  CACCAGTGGC GAAGGCGGCT CACTGGCTCG ATACTGA
```

NAME ATAM407_58
LENGTH 1350 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TGATCATGGC TCAGAACGAA CGCTGGCGGC AGGCCTAACA CATGCAAGTC
  51  GAGCGCACCT TCGGGTGAGC GGCGGACGGG TTAGTAACGC GTGGGAACGT
 101  ACCCTTTTCT ACGGAATAGC CTCGGGAAAC TGAGAGTAAT ACCGTATAAG
 151  CCCTTCGGGG GAAAGATTTA TCGGGAAAGG ATCGGCCCGC GTTAGATTAG
 201  ATAGTTGGTG GGGTAATGGC CTACCAAGTC TACGATCTAT AGCTGGTTTT
 251  AGAGGATGAT CAGCAACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG
 301  GAGGCAGCAG TGGGGAATCT TAgACAATGG GCGAAAGCCT GATCTAGCCA
 351  TGCCGCGTGT GTGATGAAGG CCCTAkGGTC GTAAAGCACT TTCGCCAGGG
 401  ATGATAATGA CAGTACCTGG TAAAGAAACC CCGGCTAACT CCGTGCCAGC
 451  AGCCGCGGTA ATACGGAGGG GGTTAGCGTT GTTCGGAATT ACTGGGCGTA
 501  AAGCGTACGT AGGCGGATCA GAAAGTAGGG GGTGAAATCC CGAGGCTCAA
 551  CCTCGCAACT GCCTCCTAAA CTCCTGGTCT TGAGTTCGAG AGAGGTGAGT
 601  GGAATTCCAA GTGTAGAGGT GAAATTCGTA GATATTTGGA GGAACACCAG
 651  TGGCGAAGGC GGCTCACTGG CTCGATACTG ACGCTGAGGT ACGAAAGTGT
 701  GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACACCG TAAACGATGA
 751  ATGCCAGTCG TCGGGCAGTA TACTGTTCGG TGACACACCT AACGGATTAA
 801  GCATTCCGCC TGGGGAGTAC GGTCGCAAGA TTAAAACTCA AAGGAATTGA
 851  CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG AAGCAACGCG
 901  CAGAACCTTA CCAACCCTTG ACATCCTGTG CTAACCCGAG AGATCGGGCG
 951  TTCACTTCGG TGACGCAGTG ACAGGTGCTG CATGGCTGTC GTCAGCTCGT
1001  GTCGTGAGAT GTTCGGTTAA GTCCGGCAAC GAGCGCAACC CACATCTTTA
1051  GTTGCCAGCA GTTCGGCTGG GCACTCTAAA GAAACTGCCC GTGATAAGCG
1101  GGAGGAAGGT GTGGATGACG TCAAGTCCTC ATGGCCCTTA CGGGTTGGGC
1151  TACACACGTG CTACAATGGT AGTGACAATG GGTTAATCCC AAAAAGCTAT
1201  CTCAGTTCGG ATTGGGGTCT GCAACTCGAC CCCATGAAGT CGGAATCGCT
1251  AGTAATCGCG TAACAGCATG ACGCGGTGAA TACGTTCCCG GGCCTTGTAC
1301  ACACCGCCCG TCACACCATG GGAGTTGGTT CTACCCGACG ACGCTGCGCT
```

NAME ATAM407_61
LENGTH 1377 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  AGTTTGATCA TGGCTCAGAA CGAACGCTGG CGGCAGGCCT AACACATGCA
  51  AGTCGAGCGA ACCTTCGGGT TAGCGGCGGA CGGGTGAGTA ACGCGTGGGA
 101  ACGTACCCTC TTCTGCGGGA TAGCCACTGG AAACGGTGAG TAATACCGCA
 151  TACGCCCTTT GGGGGAAAGA TTTATCGGAG GAGGATCGGC CCGCGTTGGA
 201  TTAGGTAGTT GGTGGGGTAA TGGCCTACCA AGCCTACGAT CCATAGCTGG
 251  TTTTAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
 301  ACGGGAGGCA GCAGTGGGGA ATCTTAGACA ATGGGCGCAA GCCTGATCTA
 351  GCCATGCCGC GTGAGTGACG AAGGCCTTAG GGTCGTAAAG CTCTTTCGCC
 401  AGAGATGATA ATGaCAGTAT CTGGTAAAGA AACCCCGGCT AACTCCGTGC
 451  CAGCAGCCGC GGTAATACGG AGGGGGTTAG CGTTGTTCGG AATTACTGGG
 501  CGTAAAGCGC ACGTAGGCGG ATTGGAAAGT TGGGGGTGAA ATCCCAGGGC
 551  TCAACCCTGG AACGGCCTCC AAAACTCCCA GTCTAGAGTT CGAGAGAGGT
 601  GAGTGGAATT CCGAGTGTAG AGGTGAAATT CGTAGATATT CGGAGGAACA
 651  CCAGTGGCGA AGGCGGCTCA CTGGCTCGAT ACTGACGCTG AGGTGCGAAA
 701  GTGTGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCCAC ACCGTAAACG
 751  ATGAATGCCA GTCGTCGGCA GCATGCTTTG TCGGTGACAC ACCTAACGGA
 801  TTAAGCATTC CGCCTGGGGA GTACGGTCGC AAGATTAAAA CTCAAAGGAA
 851  TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA
 901  CGCGCAGAAC CTTACCAACC CTTGACATGG ATATCGTAGT TACCAGAGAT
 951  CGTTTCGTCA GTTCGGCTGG ATATCACACA GGTGCTGCAT GGCTGTCGTC
1001  AGCTCGTGTC GTGAGATGTT CGGTTAAGTC CGGCAACGAG CGCAACCCAC
1051  ATCCCTAGTT GCCAGCAGGT TAAGCTGGGC ACTCTATGGA AACTGCCCGT
1101  GATAAGCGGG AGGAAGGTGT GGATGACGTC AAGTCCTCAT GGCCCTTACG
1151  GGTTGGGCTA CACACGTGCT ACAATGGTGG TGACAATGGG TTAATCCCAA
1201  AAAGCCATCT CAGTTCGGAT TGGGGTCTGC AACTCGACCC CATGAAGTCG
1251  GAATCGCTAG TAATCGCGTA ACAGCATGAC CCGGTGAATA CGTTCCCGGG
1301  CCTTGTACAC ACCGCCCGTC ACACCATGGG AGTTGGGTCT ACCCGACGAC
1351  GGTGCGCTAA CCTCGCAAGA GGAGGCA
```

NAME ATAM407_62
LENGTH 1355 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TGATCATGGC TCAGAACGAA CGCTGGCGGC ACGCCTAACA CATGCAAGTC
  51  GAGCGGcTACC TTCGGGTGGA GCGGCGGACG GGTkAGTAAC GCGTGGGAAC
 101  ATACCCTTTT CTACGGAATA GCCTCGGGAA ACTGAGAGTA ATACCGTATA
 151  CGCCCcTCGG GGGAAAGATT TATCGGTGAA GGATTGGCCC GCGTAAGATT
 201  AGATAGTTGG TGGGGTAATG GCCTACCAAG TCkACGATCT TTAGCTGGTT
 251  TGAGAGGATG ATCAGCAACA CTGGGACTGA GACACGGCCC AGACTCCTAC
 301  GGGAGGCAGC AGTGGGGAAT CTTAGACAAT GGGCGCAAGC CTGATCTAGC
 351  gATGCCGCGT GAGTGATGAA GGTCTTAGGA TCGTAAAGCT CTTTCGCCAG
 401  AGATGATAAT GACAGTATCT GGTAAAGAAA CCCCGGCTAA CTCCGTGCCA
 451  GCAGCCGCGG TAATACGGAG GGGGTTAGCG TTGTTCGGAA TTACTGGGCG
 501  TAAAGCGCAC GTAGGCGGAT TATTAAGTGA GGGGTGAAAT CCCGGGGCTC
 551  AACCCCGGAA CTGCCTCTCA TACTGGTAGT CTAGAGTTCG AGAGAGGTGA
 601  GTGGAATTCC GAGTGTAGAG GTGAAATTCG TAGATATTCG GAGGAACACC
 651  AGTGGCGAAG GCGGCTCACT GGCTCGATAC TGACGCTGAG GTGCGAAAGC
 701  GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACrC CGTAAACGAT
 751  GAATGCCAGA CGTCAGGGGG CTTGCCCTTT GGTGTCACAC CTAACGGATT
 801  AAGCATTCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT
 851  GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG
 901  CGCAGAACCT TACCAACCCT TGACATGTGT ATCGAGATTT CCCGAGAGGG
 951  ATTTCGTCAG TTCGGCTGGA TACAACACAG GTGmTGCATG GCTGTcGTCA
1001  GCTCGTGTCG TGAGATGTTC GGTTAAGTCC GGCAACGAGC GCAACCCACA
1051  TCTTTAGTTG CCAgCAGTTC GGCTGGGCAc TCTAkAGAAA CTGCCCGTGA
1101  TAAGcGGGAG GAAGGTGTGG ATGACGTCAA GTCCTCATGG CCCTTACGGG
1151  TTGGGcTACA CACGTGcTAC AATGGCATcT ACAGTGGGTT AATCCCCAAA
1201  AgATGTcTCA GTTCGGATTG GGGTCTGCAA CTCGACCCCA TGAAGTCGGA
1251  ATcGCTAGTA ATCGCGTAAC AGCATGACkC GGTGAATACG TTCCCGGGCC
1301  TTGTACACAC CGTCAGTCAC ACCATGGTAG TTGGTTCTAC CTGACGTCCG
1351  TGCGC
```

NAME ATAM407_68
LENGTH 920 nucleotides
AFFILIATION α-proteobacteria

```
   1  TAGAGTTTGA TCATGGCTCA GAACGAACGC TGGCGGCAGG CTTAACACAT
  51  GCAAGTCGAG CGCCCCGCAA GGGGAGCGGC AGACGGGTGA GTAACGCGTG
 101  GGAATCTACC CATCTCTACG GAATAACTCA GGGAAACTTG TGCTAATACC
 151  GTATACGCCC TTCGGGGGAA AGATTTATCG GAGATGGATG AGCCCGCGTT
 201  GGATTAGCTA GTTGGTGGGG TAAAGGCCTA CCAAGGCGAC GATCCATAGC
 251  TGGTCTGAGA GGATGATCAG CCACACTGGG ACTGAGACAC GGCCCAGACT
 301  CCTACGGGAG GCAGCAGTGG GGAATATTGG ACAATGGGCG CAAGCCTGAT
 351  CCAGCCATGC CGCGTGTGTG ATGAAGGCCC TAGGGTTGTA AAGCACTTTC
 401  AACGGTgAAG ATAATGACGG TAACCGTAGA AGAAGCCCCG GCTAACTTCG
 451  TGCCAGCAGC CGCGGTAATA CGAAGGGGGC TAGCGTTGTT CGGAATTACT
 501  GGGCGTAAAG CGCACGTAGG CGGATCGTTA AGTGAGGGGT GAAATCCCAG
 551  GGCTCAACCC TGGAACTGCC TTTCATACTG GCGATCTTGA GTTCGAGAGA
 601  GGTGAGTGGA ATTCCGAGTG TAGAGGTGAA ATTCGTAGAT ATTCGGAGGA
 651  ACACCAGTGG CGAAGGCGGC TCACTGGCTC GATACTGACG CTGAGGTGCG
 701  AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA
 751  ACGATGAATG TTAGCCGTCG GGCAGTTTAC TGTTCGGTGG CGCAGCTAAC
 801  GCATTAAACA TTCCGCCTGG GGAGTACGGT CGCAAGATTA AAACTCAAAG
 851  GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGAAG
 901  CAACGCGCAG AACCTTACCA
```

NAME ATAM407_77
LENGTH 695 nucleotides
AFFILIATION γ-proteobacteria

```
   1  CATGCAAGTC GAACGGTAAC ATTTCTAGCT TGCTAGAAGA TGACGAGTGG
  51  CGGACGGGTG AGTAATGCTT GGGAACTTGC CTTTGCGAGG GGGATAACAG
 101  TTGGAAACGA CTGCTAATAC CGCATAATGT CTTCGGACCA AAGGGGGCTT
 151  CGGCTCCCAC GCAAAGAGAG GCCCAAGTGA GATTAGCTAG TTGGTGAGGT
 201  AAAGGCTTAC CAAGGCAACG ATCTCTAGCT GTTCTGAGAG GAAGATCArC
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251  CACACTGGGA CTGAGACACG GyCCArACTC CTACGGGAGG CAGCAGTGGG
301  GAATATTGCA CAATGGGCGA AAGCCTGATG CAGCCATGCC GCGTGTGTGA
351  AGAAGGCCTT CGGGTTGTAA AGCACTTTCA GTTGTGAGGA AAAGTTAGTA
401  GTTAATACCT GCTAGCCGTG ACGTTAACAA CAGAAGAAGC ACCGGCTAAC
451  TCCGTGCCAG CAGCCGCGGT AATACGGAGG GTGCGAGCGT TAATCGGAAT
501  TACTGGGCGT AAAGCGCACG CAGGCGGTTT GTTAAGCTAG ATGTGAAAGC
551  CCCGAGCTCA ACTTGGGATG GTCATTTAGA ACTGGCAGAC TAGAGTCTTG
601  GAGAGGGGAG TGGAATTCCA GGTGTAGCGG TGAAATGCGT AGATATCTGG
651  AGGAACATCA GTGGCGAAGG CGACTCCCTG GCCAAAGACT GACGC
```

NAME ATAM407_85
LENGTH 902 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
  1  TAGAGTTTGA TCATGGCTCA GAACGAACGC TGGCGGCAGG CTTAACACAT
 51  GCAAGTCGAG CGCACTCTTC GGAGTGAGCG GCGGACGGGT TAGTAACGCG
101  TGGGAACGTG CCCTTCTCTA AGGAATAGCC ACTGGAAACG GTGAGTAATA
151  CCTTATACGC CCTTCGGGGG AAAGATTTAT CGGAGAAGGA TCGGCCCGCG
201  TTAGATTAGA TAGTTGGTGG GGTAATGGCC TACCAAGTCT ACGATCTATA
251  GCTGGTTTTA GAGGATGATC AGCAACACTG GGACTGAGAC ACGGCCCAGA
301  CTCCTACGGG AGGCAkCAGT GGGGAATCTT GGACAATGGG CGCAAGCCTG
351  ATCCAGCCAT GCCGCGTGAG TGATGAAkGC CTTAsGGTCG TAAAGCTCTT
401  TCGCCAGAGA TGATAATGAC AGTATCTGGT AAAGAAACCC CsGCTAACTC
451  CGTGCCAGCA GCCGCGGTAA TACGGAGGGG GTTAGCGTTG TTCGGAATTA
501  CTGGGCGTAA AGCGCACGTA GGCGGATCAG AAAGTATAGG GTGAAATCCC
551  AGGGCTCAAC CCTGGAACTG CCTTGTAAAC TCCTGGTCTT GAGTTCGAGA
601  GAGGTGAGTG GAATTCCGAG TGTAGAGGTG AAATTCGTAG ATATTCGGAG
651  GAACACCAGT GGCGAAGGCG GCTCACTGGC TCGATACTGA CGCTGAGGTG
701  CGAAAGTGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACACCGT
751  AAACGATGAA TGCCAGACGT CAGCAAGCAT GCTTGTTGGT GTCACACCTA
801  ACGGATTAAG CATTCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA
851  AGGAATmGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG GTTAATTCTA
901  AG
```

NAME ATAM173a_2
LENGTH 1427 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

```
   1  CGGCTCCTTG CGGTGACCGA CTTCAGGCAC TCCCAGCTTC CATGGCTTGA
  51  CGGGCGGTGT GTACAAGGCC CGGGAACGTA TTCACCGGAT CATGGCTGAT
 101  ATCCGATTAC TAGCGATTCC AGCTTCACGG AGTCGAGTTG CAGACTCCGA
 151  TCCGAACTGT GATATGGTTT ATAGATTTGC TCTCTGTTGC CAGATGGCTG
 201  CTCATTGTCC ATACCATTGT AGCACGTGTG TGGCCCAGGA CGTAAGGGCC
 251  GTGATGATTT GACGTCATCC CCACCTTCCT CGCGGTTTGC ACCGGCAGTC
 301  TCGCTAGAGT CCCCATCTTT ACATGCTGGC AACTAACGAC AAGGGTTGCG
 351  CTCGTTATAG GACTTAACCT GACACCTCAC GGCACGAGCT GACGACAACC
 401  ATGCAGCACC TTGTAATCTG TCCGAAGAAA ACTCTATCTC TAAAGCTGTC
 451  AGACTACATT TAAGCCCTGG TAAGGTTCCT cGCGTATCAT CGAATTAAAC
 501  CACATGyTCC ACCGCTTGTG CGGGCCCCCG TCAATTCCTT TGAGTTTCAG
 551  TCTTGCGACC GTACTCCCCA GGTGGGATAC TTATCACTTT CGCTTAGTCA
 601  CTGAGGTAAA CCCCAACAAC TAGTATCCAT CGTTTACGGC GTGGACTACC
 651  AGGGTATCTA ATCCTGTTCG CTCCCCACGC TTTCGTCCAT GAGCGTCAGT
 701  ACATACGTAG TAGACTGCCT TCGCAATCGG TATTCTGTGT AATATCTATG
 751  CATTTCACCG CTACACTACA CATTCTATCT ACTTCCATAT GACTCAAGTC
 801  AACCAGTATC AAAGGCAGTT CCATAGTTAA GCTATGGGAT TTCACCTCTG
 851  ACTTAATTGA CCGCCTGCGG ACCCTTTAAA CCCAATGATT CCGGATAACG
 901  CTTGGACCCT CCGTATTACC GCGGCTGCTG GCACGGAGTT AGCCGGTCCT
 951  TATTCTTACA GTACCGTCAA GCCGCTACAC GTAGCGGTGT TTCTTCCTGT
1001  ATAAAAGCAG TTTACAACCC ATAGGGCAGT CTTCCTGCAC GCGGCATGGC
1051  TGGGTCAGAG TTGCCTCCAT TGCCCAATAT TCCTcACTGC TGCCTCCCGT
1101  AGGAGTCTGG TCCGTGTCTC ArTACCAGTG TGGGGGATCC CCCTCTCAGG
1151  GCCCCTACCT ATCGTAGCCA TGGTAAGCCG TTACCTTACC ATCTAGCTAA
1201  TAGGACGCAT AGCCATCTTT TACCGATAAA TCTTTAATTA AAAACTGATG
1251  CCAGTTCTCA ATACTATGGG ATATTAATCT TCATTTCTAA AGGCTATCCC
1301  CCTGTAAAAG GTAAGTTCTA TACGCGTTAC GCACCCGTGC GCCGGTCGTC
1351  ATCTGTGCAA GCACAATGTT ACCCCTCGAC TTGCATGTGT TAAGCCTGCC
1401  GCTAGCGTTC ATCCTGAGCC ATGATCA
```

NAME ATAM173a_3
LENGTH 1398 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

```
   1  CACTCCCAGC TTCCATGGCT TGACGGGCGG TGTGTACAAG GCCCGGGAAC
  51  GTATTCACCG GATCATGGCT GATATsCGAT TACTAGCGAT TCCAGCTTCA
 101  CGGAGTCGAG TTGCAGACTC CGATCCGAAC TGTGATAGGG TTTATAGATT
 151  CGCTCCTGGT CACCCAGTGG CTGCTmTCTG TCCCTACCAT TGTAGCACGT
 201  GTGTAGCCCA GGACGTAAGG GCCGTGATGA TTTGACGTCA TCCCCACCTT
 251  CCTCGCAGTT TGCACTGGCA GTCTTGCTAG AGTTCCCATC TTTACATGCT
 301  GGCAACTAAC AACAGGGGTT GCGCTCGTTA TAGGACTTAA CCTGACACCT
 351  CACGGCACGA GCTGACGACA ACCATGCAGC ACCTTGTAAA TTGTCCGAAG
 401  AAAAGTCTAT CTCTAAACCT GTCAATCTAC ATTTAAGCCC TGGTAAGGTT
 451  CCTCGCGTAT CATCGAATTA AACCACATGC TCCACCGCTT GTGCGGGCCC
 501  CCGTCAATTC CTTTGAGTTT CATTCTTGCG AACGTACTCC CCAGGTGGGA
 551  TACTTATCAC TTTCGCTTAG CCaCTGAACC GAAGTCCAAC AGCTAGTATC
 601  CATCGTTTAC GGCGTAGACT ACCGGGGTAT CTAATCCCGT TCGCTACCTA
 651  CGCTTTCGTC CATCAGTGTC AATTGATTAT TAGTAATCTG CCTTCGCAAT
 701  TGGTATTCTA TGTAATATCT ATGCATTTCA CCGCTACACT ACATATTCTA
 751  ACTACTTCAT AATAATTCAA GATAACCAGT ATCAAAGGCA ATTCTACAGT
 801  TGAGCTGCAG ACTTTCACCT CTGACTTAAT TATCCACCTA CGGACCCTTT
 851  AAACCCAATG ATTCCGGATA ACGCTTGGAT CCTCCGTATT ACCGCGGCTG
 901  CTGGCACGGA GTTAGCCGAT CCTTATTCTT ACAGTACCGT CAAGCTCCCT
 951  GCTCGAGGGA GTGTTTCTTC CTGTATAAAA GCAGTTTACA ACCCATAGGG
1001  CAGTCTTCCT GCACGCGGCA TGGCTGGATC AGAGTTGCCT CCATTGTCCA
1051  ATATTCCTCA CTGCTGCCTC CCGTAGGAGT CTGGTCCGTG TCTCAGTACC
1101  AGTGTGGGGG ATCCCCCTCT CAGGGCCCCT ACCTATCGTT GCCTTGGTGT
1151  GCCGTTACCA CACCAACTAG CTAATAGGAC GCATACTCAT CTTTTGCCGT
1201  AACCTTTAAT ATAATGCTCA TGCGAGCTCT ATATACTATG CGGTATTAAT
1251  CCAAATTTCT CTGGGCTATC CCACAGCAAA AGGCAGATTG TATACGCGTT
1301  ACGCACCCGT GCGCCGGTCG TCGGCGGTGC AAGCACCCCG TTACCCCTCG
1351  ACTTGCATGT GTTAGGCCTG CCGCTAGCGT TCATCCTGAG CCATGATC
```

NAME ATAM173a_5
LENGTH 1463 nucleotides
AFFILIATION γ-proteobacteria

```
   1  CAGTCATGAA TCACAAAGTG GTAACCGTCC TCCCGAAGGT TAGACTAGCT
  51  ACTTCTTTTG CAACCCACTC CCATGGTGTG ACGGGCGGTG TGTACAAGGC
 101  CCGGGAACGT ATTCACCGCA ACATTCTGAT TTGCGATTAC TAGCGATTCC
 151  GACTTCATGG AGTCGAGTTG CAGACTCCAA TCCGGACTAC GACGAGCTTT
 201  AAGGGATCCG CTTACCCTCG CAGGTTCGCT TCCCTCTGTA CTCGCCATTG
 251  TAGCACGTGT GTAGCCCTAC TCGTAAGGGC CATGATGACT TGACGTCGTC
 301  CCCACCTTCC TCCGGTTTGT CACCGGCAGT CTCCTTAGAG TGCCCAACTT
 351  AAGGCTGGCA ACTAAGGACA AGGGTTGCGC TCGTTGCGGG ACTTAACCCA
 401  ACATCTCACG ACACGAGCTG ACGACAGCCA TGCAGCACCT GTATCTAGAT
 451  TCCCGAAGGC ACCAATTCAT CTCTGAAAAG TTTCTAGTAT GTCAAGAGTA
 501  GGTAAGGTTC TTCGCGTTGC ATCGAATTAA ACCACATGCT CCACCGCTTG
 551  TGCGGGCCCC CGTCAATTCA TTTGAGTTTT AACCTTGCGG CCGTACTCCC
 601  CAGGCGGTCT ACTTATCGCG TTAGCTTCGC TACTCACGGA TTAAATCCAC
 651  AAACAGCTAG TAGACAGCGT TTACGGTGTG GACTACCAGG GTATCTAATC
 701  CTGTTCGCTA CCCACACTTT CGCACATGAG CGTCAGTCTT TGGCCAGGGA
 751  GTCGCCTTCG CCACTGATGT TCCTTCTGAT ATCTACGCAT TTCACCGCTA
 801  CACCAGAAAT TCCACTCCCC TCTCCAAGAC TCTAGCCTGC CAGTTCTAAA
 851  TGCAATTCCC AGGTTGAGCC CGGGGCTTTC ACATCTAGCT TAACAAACCG
 901  CCTGCGTGCg GCTTTACGCC CAGTAATTCC GATTAACGCT CGCACCCTCC
 951  GTATTACCGC GGCTGCTGGC ACGGAGTTAG CCGGTGCTTC TTCTGTTGCT
1001  AACGTCACAT CTGATGGGTA TTAACCACCA AACTTTCCTC ACAACTGAAA
1051  GTGCTTTACA ACCCGAAGGC CTTCTTCACA CACGCGGCAT GGCTGCATCA
1101  GGGTTTCCCC CATTGTGCAA TATTCCCCAC TGCTGCCTcC CGTAGGAGTC
1151  TGGACCGTGT CTCAGTTCCA GTGTGGCTGA TCTTCCTCTC AGAACAGCTA
1201  GAGATCGTCG CCTTGGTGAG CCTTTACCTC ACCAACAAGC TAATCTCGCT
1251  TAGGCCACTC TCTGGGCGGG AGCCGAAGCC CCCTTTGGTC CGTAGACGTT
1301  ATGCGGTATT AGCCATCGTT TCCAATGGTT ATCCCCCACC CAAAGGCATG
1351  TTCCTAAGTA TTACTCACCC GTCCGCACTC GACATCATCT AGCAAGCTAG
1401  ACATGTTTCC GTTCGACTTG CATGTGTTAG GCCTGCCGCA GCGTTCAATC
1451  TGAGCCATGA TCA
```

NAME ATAM173a_6
LENGTH 1485 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

```
   1 TGATCATGGC TCAGGATGAA CGCTAGCGGC AGGCTTAACA CATGCAAGTC
  51 GAGGGGTAAC ATTTGTGCTT GCACAAGATG ACGACCGGCG CACGGGTGCG
 101 CACCGCGTAT GGAACCTGCC TTGTACAGGG GAATAGCCCA GGGAAACTTG
 151 GATTAATGCC CCGTAGTACC GCGACCCGGC ATCGGGATGC GGTTAAAGTC
 201 TTCGGGCGGT ACAAGATGGC CATGCGTCCC ATTAGCTAGT TGGTAAGGTA
 251 ACGGCTTACC AAGGCTACGA TGGGTAGGGG CCCTGAGAGG GGGATCCCCC
 301 ACACTGGTAC TGAGACACGG ACCAGACTCC TACGGGAGGC AGCAGTGAGG
 351 AATATTGGAC AATGGAGGAG ACTCTGATCC AGCCATG.CC GCGTGCAGGA
 401 AGAATGCCCT ATGGGTAGTA AACTGCTTTT ATACGGGAAG AAAAAGACCT
 451 ACGTGTAGCT TACTGACGGT ACCGTAAGAA TAAGGACCGC CTAACTCCGT
 501 GCCAGCAGCC GCGGTAATAC GGAGGGTCCG AGCGTTATCC GGAATTaTTG
 551 GGTTTAAAGG GTCCGTAGGC GGGCCcGATA AGTCAGGGGT GAAAGTCTGC
 601 CGCTCAACGG TAGAACTGCC TTTGATACTG TCGGTCTTGA GTTATAGTGA
 651 AGTTGCCGGA ATATGTAGTG TAGCGGTGAA ATGCATAGAT ATTACATAGA
 701 ACACCGATTG CGAAGGCAGG TGACTAACTA TATACTGACG CTGATGGACG
 751 AAAGCGTGGG GAGCGAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA
 801 ACGATGGATA CTAGCTGTCC GGTGCCTTGA GTACTGGGCG GCCAAGCGAA
 851 AGTGATAAGT ATCCCACCTG GGGAGTACGT TCGCAAGAAT GAAACTCAAA
 901 GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT TTAATTCGAT
 951 GATACGCGAG GAACCTTACC AGGGCTTAAA TGCATATTGA CAGGTCTAGA
1001 GATAGATTTT CCTTCGGGCA ATTTCCAACG TGCTGCATGG TTGTCGTCAG
1051 CTCGTGCCGT GAGGTGTCA. GGTtAAGTCC TATAACGAGC GCAaCCCCTA
1101 CC.GTTAGTT GCCAGCATGT CATGATGGGG ACTCTAACGG GACTGCCGGT
1151 GCAAACCGTG AGGAAGGTGG GGATGACGTC AAATCATCAC GGCCCTTACG
1201 TCCTGGGCCA CACACGTGCT ACAATGGCAG GTACAGAGAG CAGCCACGTC
1251 GCAAGGCGGA GCGAATCTAT AAAACCTGTC ACAGTTCGGA TCGGGGTCTG
1301 CAACTCGACC CCGTGAAGCT GGAATCGCTA GTAATCGGAT ATCAGCCATG
1351 ATCCGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCAAGCCATG
1401 GAAGCCGGGA GTGCCTGAAG TCCGTCACCG TAAGGAGCGG CcTAAGGCAA
1451 GATCGGTAAC TAGGGCTAAG TCGTAACAAC GTAGC
```

NAME ATAM173a_9
LENGTH 625 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
  1 TATCGAGCCA GTGAGCCGCC TTCGCCACTG GTGTTCCTCC AAATATCTAC
 51 GAATTTCACC TCTACACTTG GAATTCCACT CACCTCTCTC GAACTCTAGA
101 CTGATAGTTT ACAGGGCAGT TCCAGGGTTG AGCCCTGGGA TTTCACCCCA
151 TACTTTCCAA TCCGCCTACG TACGCTTTAC GCCCAGTAAT TCCGAACAAC
201 GCTAACCCCC TCmGTATTAC CGCGGCTGCT GGCACGGAGT TAGCCGGGGT
251 TTCTTTACCA GATACTGTCA TTATCATCTC TGGCGAAAGA GCTTTACGAC
301 CCTAAGGCCT TCGTCACTCA CGCGGCATGG CTAGATCAGG CTTGCGCCCA
351 TTGTCTAAGA TTCCCCACTG CTGCCTCCCG TAGGAGTCTG GGCCGTGTCT
401 CAGTCCCAGT GTTGCTGATC ATCCTCTCAA ACCAGCTAAA GATCGTAGAC
451 TTGGTAGGCC ATTACCCCAC CAACTATCTA ATCTTACGCG GGCCAATCCT
501 TCwCCGATAA ATCTTTCCCC CGAAGGGCGT ATACGGTATT ACTCTCAGTT
551 TCCCGAGGCT ATTCCGTAGA AAAGGGTATG TTCCCACGCG TTACTAACCC
601 GTCCGCCGCT CACTCCGAAG AGTGC
```

NAME ATAM173a_15
LENGTH 615 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
  1 CGTTAGTATC GAGCCAGTGA GCCGCCTTCG NCACTGGTGT TCCTCCGAAT
 51 ATCTACGAAT TTCACCTCTA CACTCGGAAT TCCACTCACC TCTCTNGAAC
101 TCTAGACTGG GAGTTTTGGA GGCCGTTCCA GGGTTGAGCC CTGGGATTTC
151 ACCCCCAACT TTCCAATCCG CCTACGTGCG CTTTACGCCC ACTAATTCCG
201 AACAACGCTA ACCCCCTCCG TATTACCGCG GCTGCTGGCA CGGAGTTAGC
251 CGGGGTTTCT TTACCAGATA CTGTCATTAT CATCTCTGGC GAAAGAGCTT
301 TACGACCCTA AGGCCTTCGT CACTCACGCG GCATGGCTAG ATCAGGCTTG
351 CGCCCATTGT CTAAGATTCC CCACTGCTG CCTCCCGTAG GAGTCTGGGC
401 CGTGTCTCAG TCCCAGTGTG GCTGATCATC CTCTAAAACC AGCTATGGAT
451 CGTAGGCTTG GTAGGCCATT ACCCCACCAA CTACCTAATC CAACGCGGGC
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501 CGATCCTCCT CCGATAAATC TTTCCCCCAA AGGGCGTATG CGGTATTACT
551 CACCGTTTCC AGTGGCTATC CCGCAGAAGA GGGTACGTTC CCACGCGTTA
601 CTCACCCGTC CGCCG
```

NAME ATAM173a_16
LENGTH 1339 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1 TAGAGTTTGA TCATGGCTCA GAACGAACGC TGGCGGCAGG CCTAACACAT
  51 GCAAGTCGAG CGCACCCTTC GGGGCGAGCG GCGGACGGGT TAGTAACGCG
 101 TGGGAACATA CCCTTTGGTA CGGAATAGCC TCGGGAAACT GAGAGTAATA
 151 CCGTATGAGC CCTTCGGGGG AAAGATTTAT CGCCAAAGGA TTGGCCCGCG
 201 TAAGATTAGA TAGTTGGTGG GGTAATGGCC TACCAAGTCT ACGATCTTTA
 251 GCTGGTTTTA GAGGATGATC AGCAACACTG GGACTGAGAC ACGGCCCAGA
 301 CTCCTACGGG AGGCAGCAGT GGGGAATCTT AGACAATGGG CGCAAGCCTG
 351 ATCTAGCCAT GCCGCGTGTG TGATGAAGGC CTTAGGGTCG TAAAGCACTT
 401 TCGCCAGGGA TGATAATGAC AGTACCTGGT AAAGAAACCC CGGCTAACTC
 451 CGTGCCAGCA GCCGCGGTAA TACGGAGGGG GTTAGCGTTG TTCGGAATTA
 501 CTGGGCGTAA AGCGTACGTA GgCGGATCAG AAAGTAAGGG GTGAAATCCC
 551 AGGGCTCAAC CCTGGAACTG CCTCTTAAAC TCCTGGTCTT GAGTTCGAGA
 601 GAGGTGAGTG GAATTCCGAG TGTAGAGGTG AAATTCGTAG ATATTCGGAG
 651 GAACACCAGT GGCGAAGGCG GCTCACTGGC TCGATACTGA CGCTGAGGTA
 701 CGAAAGTGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACACCGT
 751 AAACGATGAA TGCCAGTCGT CGGGCAGTAT ACTGTTCGGT GACACACCTA
 801 ACGGATTAAG CATTCCGCCt GGGGAGTACG GTCGCAAGAT TAAAACTCAA
 851 AGGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
 901 AGCAACGCGC AGAACCTTAC CAACCCTTGA CATCCTGTGC TAACCCGAGA
 951 GATCGGGCGT TCACTTCGGT GACGCAaGTG ACAGGTGCTG CATGGCTGTC
1001 GTCAGCTCGT GTCGTGAGAT GTTCGGTTAA GTCCGGCAAC GAGCGCAACC
1051 CACATCTTTA GTTGCCATCA TTTAGTTGGG CACTCTAAAG AAACTGCCCG
1101 TGATAAGCGG GAGGAAGGTG TGGATGACGT CAAGTCCTCA TGGCCCTTAC
1151 GGGTTGGGCT ACACACGTGC TACAATGGCA GTGACAATGG GTTAATCCCA
1201 AAAAGCTGTC TCAGTTCGGA TTGGGGTCTG CAACTCGACC CCATGAAGTC
1251 GGAATCGCTA GTAATCGCGT AACAGCATGA CGCGGTGAAT ACGTTCCCGG
1301 GCCTTGTACA CACCGCCCGT CACACCATGG GAGTTGGTT
```

NAME ATAM173a_17
LENGTH 1363 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1 TAGAGTTTGA TCATGGCTCA GAACGAACGC TGGCGGCAGG CCTAACACAT
  51 GCAAGTCGAG CGCACCCTTC GGGGCGAGCG GCGGACGGGT TAGTAACGCG
 101 TGGGAACATA CCCTTTTCTA CGGAATAGCC TCGGGAAACT GAGAGTAATA
 151 CCGTATACGC CCTTCGGGGG AAAGATTTAT CGGAGAAGGA TTGGCCCGCG
 201 TTAGATTAGA TAGTTGGTGG GGTAATGGCC TACCAAGTCT ACGATCTATA
 251 GCTGGTTTTA GAGGATGATC AGCAACACTG GGACTGAGAC ACGGCCCAGA
 301 CTCCTACGGG AGGCAGCAGT GGGGAATCTT AGACAATGGG CGCAAGCCTG
 351 ATCTAGCCAT GCCGCGTGTG TGATGAAGGC CTTAGGGTCG TAAAGCACTT
 401 TCGCCAGGGA TGATAATGAC AGTACCTGGT AAAGAAACCC CGGCTAACTC
 451 CGTGCCAGCA GCCGCGGTAA TACGGAGGGG GTTAGCGTTG TTCGGAATT
 501 ACTGGGCGTA AAGCGTACGT AGGCCGATCA GAAAGTAGGG GGTGAAATCC
 551 CAGGGCTCAA CCCTGGAACT GCCTCCTAAA CTCCTGGTCT TGAGTTCGAG
 601 AGAGGTGAGT GGAATTCCAA GTGTAGAGGT GAAATTCGTA GATATTTGGA
 651 GGAACACCAG TGGCGAAGGC GGCTCACTGG CTCGATACTG ACGCTGAGGT
 701 ACGAAAGTGT GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACACCG
 751 TAAACGATGA ATGCCAGTCG TCGGGCAGTA TACTGTTCGG TGACACACCT
 801 AACGGATTAA GCATTCCGCC TGGGGAGTAC GCTCGCAAGA TTAAAACTCA
 851 AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG
 901 AAGCAACGCG CAGAACCTTA CCAACCCTTG ACATCCTGTG CTAACCCGAG
 951 AGATCGCGCC TTCACTTCGG TGACGCAGTG ACAGGTGCTG CATGGCTGTC
1001 GTCAGCTCGT GTCGTGAGAT GTTCGGTTAA GTCCGGCAAC GAGCGCAACC
1051 CACATCTTTA GTTGCCATCA GTTCGGCTGG GCACTCTAAA GAAACTGCCC
1101 GTGATAAGCG GGAGGAAGGT GTGGATGACG TCAAGTCCTC ATGGCCCTTA
1151 CGGGTTGGGC TACACACGTG CTACAATGGC AGTGACAATG GGTTAATCCC
1201 AAAAAGCTGT CTCAGTTCGG ATTGGGGTCT GCAACTCGAC CCCATGAAGT
1251 CGGAATCGCT AGTAATCGCG TAACAGCATG ACGCGGTGAA TACGTTCCCG
1301 GGCCTTGTAC ACACCGCCCG TCACACCATG GGAGTTGGTT CTACCCGACG
1351 ACGCTGCGCT AAC
```

NAME ATAM173a_29
LENGTH 1395 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  CAGTCGCTGA TCCTACCGTG GCCGCCTGCC TCCCCGAAGG GTTAGCGCAG
  51  CGTCGTCGGG TAGAACCAAC TCCCATGGTG TGACGGGCGG TGTGTACAAG
 101  GCCCGGGAAC GTATTCACCG CGTCATGCTG TTACGCGATT ACTAGCGATT
 151  CCGACTTCAT GGGGTCGAGT TGCAGACCCC AATCCGAACT GAGACATCTT
 201  TTGGAGATTA ACTCACTGTA GATGCCATTG TAGCACGTGT GTAGCCCAAC
 251  CCGTAAGGGC CATGAGGACT TGACGTCATC CACACCTTCC TCCCGCTTAT
 301  CACGCGCAGT TTCCCTAGAG TGCCCAGCCG AACTGCTGGC AACTAAGGAT
 351  GTGGGTTGCG CTCGTTGCCG GACTTAACCG AACATCTCAC GACACGAGCT
 401  GACGACAGCC ATGCAGCACC TGTCACTCGG yCwCCGAAGT GGAAACCAGA
 451  TCTCTCTGGC GGTCCGAGGA TGTCAAGGGT TGGTAAGGTT CTGCGCGTTG
 501  CTTCGAATTA AACCACATGC TCCACCGCTT GTGCGGGCCC CCGTCAATTC
 551  CTTTGAGTTT TAATCTTGCG ACCGTACTCC CCAGGCCGAA TGCTTAATCC
 601  GTTAGGTGTG ACACCGAAGG GCAAGCCCCC CGACGTCTGG CATTCATCGT
 651  TTACGGTGTG GACTACCAGG GTATCTAATC CTGTTTGCTC CCCACACTTT
 701  CGTACCTCAG CGTCAGTATC GAGCCAGTGA GCCGCCTTCG CCACTGGTGT
 751  TCCTCCAAAT ATCTACGAAT TTCACCTCTA CACTTGGAAT TCCACTCACC
 801  TCTCTCGAAC TCTAGACTGA TAGTTTACAr GGCAGTTCCA GGGTTGAGCC
 851  CTGGGATTTC ACCCCATACT TTCCAATCCG CCTACGTACG CTTTACGCCC
 901  AGtAATTCCG AACAACGCTA ACCCCCTCCG TATTACCGCG GCTGCTGGCA
 951  CGGAGTTAGC CGGGGTTTCT TTACCAGATA CTGTCATTAT CATCTCTGGC
1001  CAAAGAGCTT TACGACCCTA AGGCCTTCGT CACTCACGCG GCATGGCTAG
1051  ATCAGGCTTG CGCCCATTGT CTAAGATTCC CCACTGCTGC CTCCCGTAGG
1101  AGTCTGGGCC GTGTCTCAGT CCCAGTGTTG CTGATCATCC TCTCAAACCA
1151  GCTAnAGATC GTAGACTTGG TAGGCCATTA CCCCACCAAC TATCTAATCT
1201  TACGCGGGCC AATCCTTCAC CGATAAATCT TTCCCCCGAA GGGCGTATAC
1251  GGTATTACTC TCAGTTTCCC GAGGCTATTC CGTAGAAAAG GGTATGTTCC
1301  CACGCGTTAC TAACCCGTCC GCCGCTCACT CCGAAGAGTG CGCTCGACTT
1351  GCATGTGTTA GGCCTGCCGC CAGCGTTCGT TCTGAGCCAT GATCA
```

NAME ATAM173a_36
LENGTH 1439 nucleotides
AFFILIATION γ-proteobacteria

```
   1  CGTCCTCCCG AGGGTTAGAC TATCTACTTC TGGAGCAACC CACTCCCATG
  51  GTGTGACGGs CGGTGTGTAC AAGGCCCGGG AACGTATTCA CCGCGTCATT
 101  CTGATACGCG ATTACTAGCG ATTCCGACTT CATGGAGTCG AGTTGCAGAC
 151  TCCAATCCGG ACTACGACGC ACTTTAAGTG ATTCGCTTAC TCTCGCArGT
 201  TCGCAGCACT CTGTATGCGC CATTGTAGCA CGTGTGTAGC CCTACACGTA
 251  AGGGCCATGA TGACTTGACG TCGTCCCCAC CTTCCTCCGG TTTATCACCG
 301  GCAGTCTCCT TAGAGTTCTC AGCATTACCT GCTAGCAACT AAGGATAGGG
 351  GTTGCGCTCG TTGCGGGACT TAACCCAACA TCTCACAACA CGAGCTGACG
 401  ACAGCCATGC AGCACCTGTA TCAGAGTTCC CGAAGGCACC AAACCATCTC
 451  TGGTAAGTTC TCTGTATGTC AAGTGTAGGT AAGGTTCTTC GCGTTGCATC
 501  GAATTAAACC ACATGCTCCA CCGCTTGTGC GGGCCCCCGT CAATTCATTT
 551  GAGTTTTAAC CTTGCGGCCG TACTCCCCAG GCGGTCTACT TAATGCGTTA
 601  GCTTTGAAAA ACAGAACCGA GGyTCCGAGC TTCTAGTAGA CATCGTTTAC
 651  GGCGTGGACT ACCAGGGTAT CTAATCCTGT TTGCTCCCCA CGCTTTCGTA
 701  CATGAGCGTC AGTGTTGACC CAGGTGGCTG CCTTCGCCAT CGGTATTCCT
 751  TCAGATCTCT ACGCATTTCA CCGCTACACC TGAAATTCTA CCACCCTCTA
 801  TCACACTCTA GTTTGCCAGT TCGAAATGCA GTTCCCAGGT TGAGCCCGGG
 851  GCTTTCACAT CTCGCTTAAC AAACCGCCTG CGTACGCTTT ACGCCCAGTA
 901  ATTCCGATTA ACGCTCGCAC CCTCCGTATT ACCGCGGCTG CTGGCACGGA
 951  GTTAGCCGGT GCTTCTTCTG TCAGTAACGT CACAGcTAGC cCGTATTAAC
1001  GACTAACcTT CCTCCCTGAC TGAAAGTGCT TTACAACCCG AAGGCCTTCT
1051  TCACACACGC GGCATGGCTG CATCAGGCTT GCGCCCATTG TGCAATATTC
1101  CCCACTGCTG CCTCCCGTAG GAGTCTGGAC CGTGTCTCAG TTCCAGTGTG
1151  GCTGATCATC CTCTCAAACC AGCTAGGGAT CGTTGCCTTG GTGAGCCATT
1201  ACCTCACCAA CTAGCTAATC CCACTTGGGC CAATCTAAAG GCGAGAGCCG
1251  AAGCCCCCTT TGGTCCGTAG ACATTATGCG GTATTAGCAG TCGTTTCCAA
1301  CTGTTGTCCC CCACCTCAAG GCATGTTCCC AAGCATTACT CACCCGTCCG
1351  CCGCTCGTCA TCTTCTAGCA AGCTAGAAAT GTTACCGCTC GACTTGCATG
1401  TGTTAGGCCT GCCGCCAGCG TTCAATCTGA GCCATGATC
```

NAME ATAM173a_40
LENGTH 1434 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TGATCATGGC TCAGAACGAA CGCTGCGGCA GGCCTAACAC ATGCAAGTCG
  51  AGCGCACTCT TCGGAGTGAG CGGCGGACGG GTTAGTAACG CGTGGGAACA
 101  TACCCTTTTC TACGGAATAG CCTCGGGAAA CTGAGAGTAA TACCGTATAC
 151  GCCCTTCGGG GGAAAGATTT ATCGGaGAAG GATTGGCCCG CGTTTGATTA
 201  gATAGTgwGG TGGGGTAATG GCCTACCAAG TCTACkATCA ATAGCTGGTT
 251  TGAGAGGATG ATCAGCAACA CTGGGACTGA kACACGGCCC AGACTCCTAC
 301  GGGAGGCAGC AgTGGGGAAT CTTAgACAAT GGGCGCAAGC CTGATCTAGC
 351  CATGCCgCGT GAGTGACgAA gGCCTTAGGG TCGTAAAGCT CTTTCGCCAG
 401  AGATGATaAT GACAGTATCT GGTAAAGAAA CCCCGGCTAA CTCCGTGCCA
 451  GCAGCCGCkG TAATACgGAg GGGGTTAGcC gTTGTTCgGA ATTACTGGGC
 501  gTAAAGCGTA CGTAGGCGGA TTAgAAAGTA gGGGGTGAAA TCCCAGGGCT
 551  CAACCCTGGA ACTGCCTCCT AAACTACTAG TCTAGAGTTC GAGAGAGGTG
 601  AGTGGAATTC CAAGTGTAGA GGTGAAATTC GTAGATATTT GGAGGAACAC
 651  CAGTGGCGAA GGCGGCTCAC TGGCTCGATA CTGACGCTGA GGTACGAAAC
 701  TGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACA CCGTAAACGA
 751  TGAATGCCAG ACGTCGGGGG GCTTGCCCTT CGGTGTCACA CCTAACGGAT
 801  TAAGCATTCC GCCTGGGGAG TACGGTCGCA AGATTAAAAC TCAAAGGAAT
 851  TGACGGGGGC CCGCACAAGC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC
 901  GCGCAGAACC TTACCAACCC TTGACATCCT CGGACCGCCA GAGAGATCTG
 951  GTTTCCACTT CGGTGGCCGA GTGACAGGTG CTGCATGGCT GTCGTCAGCT
1001  CGTGTCGTGA GATGTTCGGT TAAGTCCsGC AACGAGCGCA ACCCACATCC
1051  TTArTTGCCA gCAGTTCgGC TGGGCACTCT AGGGAAACTG CCCGTGATAA
1101  GCGGGAGGAA GGTGTGGATG ACGTCAAGTC CTCATGGCCC TTACGGGTTG
1151  GGCTACACAC GTGCTACAAT GGCATCTACA GTGAGTTAAT CTCCAAAAGA
1201  TGTCTCAGTT CGGATTGGGG TCTGCAACTC GACCCCATGA AGTCGGAATC
1251  GCTAGTAATC GCGTAACAGC ATGACGCGGT GAATACGTTC CCGGGCCTTG
1301  TACACACCGC CCGTCACACC ATGGGAGTTG GTTCTACCCG ACGACGCTGC
1351  GCTAACCCTT CGGGGAGGCA GGCGGCCACG GTAGGATCAG CGACTGGGGT
1401  GAAGTCGTAA CAAGGTAGCC CGTAGGGGAA CCTG
```

NAME ATAM173a_47
LENGTH 1247 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  AATATGCCCT TCTGTTGAGG ATAGCCCTGG GAAACTGGGA GTAATACTCC
  51  ATACGCCCTA CGGGGGAAGG AAGGATTAGC CCGCGTTAGA TTArGTAGTT
 101  GGTGAGGTAA TGGCTCACCA AGCCTACGAT CTATAGCTGG TTTTAGAGGA
 151  TGATCAGCCA CACTGGGACT GAGACACGGC CCAGACTCCT ACGGkAsGCA
 201  rCrsTGsGGA ATCTTAkACA ATGGGCgCAA GCCTGATCTA gCCATGCCGc
 251  GTGAGTGATG AAgGCCTTAg GGTCGTAAAG CTcTTTCGcC AGGGATGATA
 301  ATGACAGTAC CTGGTAAAGA AACCCCGGCT AACTCCGTCC CAGCAGCCGC
 351  GGTAATACGG AGGGGGTTAG CGTTGTTCGG AATTACTGGG CGTAAAGCGC
 401  GCGTAGGCGG ACCAGAAAGT ATGGGGTGAA ATCCCGGGGC TCAACCCCGG
 451  AACTGCCTCA TAAACTCCTG GTCTTGAGTT CGAGAGAGGT GAGTCGAATT
 501  CCGAGTGTAG AGGTGAAATT CGTAGATATT CGGAGGAACA CCAGTGGCGA
 551  AGGCGGCTCA CTGGCTCGAT ACTGACGCTG AGGTGCGAAA GTGTGGGGAG
 601  CAAACAGGAT TAGATACCCT GGTAGTCCAC ACCGTAAACG ATGAATGCCA
 651  GTCGTCGGCA AGCATGCTTG TCGGTGACAC ACCTAACGGA TTAAGCATTC
 701  CGCCTGGGGA GTACGGTCGC AAGATTAAAA CTCAAAGGAA TTGACGGGGC
 751  CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA CGCGCAGAAC
 801  CTTACCAACC CTTGACATAC TTGTCGTCGC TCCAGAGATG GAGCTTTCAG
 851  TTAGGCTGGA CAAGATACAG GTGCTGCATG GCTGTCGTCA GCTCGTGTCG
 901  TGAGATGTTC GGTTAAGTCC GGCAACGAGC GCAACCCACA TCTTCAGTTG
 951  CCAGCAGTTC GGCTGGGCAC TCTGGAGAAA CTGCCCGTGA TAAGCGGGAG
1001  GAAGGTGTGG ATGACGTCAA GTCCTCATGG CCCTTACGGG TTGGGCTACA
1051  CACGTGCTAC AATGGCATCT ACAATGGGTT AATCCCCAAA ACAATGGTCT CA
1101  GTTCGGATTG GGGTCTGCAA CTCGACCCCA TGAAGTCGGA ATCGCTAGTA
1151  ATCGCGTAAC AGCATGACGC GGTGAATACG TTCCCGGGCC TTGTACACAC
1201  CGCCGTCACA CCATGGGAGT TGGTTCTACC TGACGGCCGT GCGCTAA
```

NAME ATAM173a_49
LENGTH 1346 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  CGAACGCTGG CGGCAGGCGT AACACATGCA AGTCGArmGC ACCTTCGGGT
  51  GAGCGrCrGA CGGGTTAGTA ACGCGTGGGA ACATACCCTT TTmTACGGAA
 101  TAGCCTCGGG AAACTGAkAG TAATACgyGT ATAAGCCCTT CkGGGGAAAG
 151  ATTkATCGGG AAAGGATTGG CCCGCGTTAG ATTAGATAGT TGGTGGGGTA
 201  ATGGCmTACC AAGTCTACGA TCTATAGCTG GTTTTAGAGG ATGATCAGCA
 251  wCAmTGGkAC TGAGACACkG mCCAGACTCs TACGGkAGGC AGCAgTgGGG
 301  AATcTTAGAC AATGGGCGCa AGCcTGATCT AGCCATGCcG CGTGTgTGAT
 351  GAAGGTCTTA GGATCGTAAA GCACTTTCGC CAGGrATGAT AATGACAGTA
 401  CsTGGTAAAG AAACCCCGGC TAAyTCCGTG CCAGCAGCCG CGGTAATACs
 451  rAGGkGGTTA GC.GTTGTTC GGAATTaCTG GGCGTAAAGC GTACGTAGGC
 501  GGATTGGAAA GTTGGGGGTG AAATCCCAGG GCTCAACCCT GGAACTGCCT
 551  CCAAAACTAT CAGTCTAGAG TTCgAkArAG GTGAgTGGAA TTCCAAGTGT
 601  AsAGGTGAAA TTmGTAGATA TTTGkAGGAA CACCAGTGGC GAAGGCGGCT
 651  CACTGGCTCk ATACTGACGC TrAkGTACGA AAGTGTGGGG AGCAAACAGG
 701  ATTAGATACC CTGGTAGTCC ACACCGTAAA CGATGAATGC CAGTCGTCGG
 751  GCAGTATACT GTTCGGTGAC ACACCTAACG GATTAAGCAT TCCCCCTGGG
 801  GAGTACGGTC GCAAGATTAA AACTCAAAGG AATTGACGGG GGCCtsCACA
 851  AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGCAGA ACCTTACCAA
 901  CCCTTGACAT CCTGTGCTAA CCCGAGAGAT CGGGCGTTCA CTTCGGyGAC
 951  GCAGTGACAG GTGCTGCATG GCTGTCGTCA GCTCkTGTCG TGAtGATGTT
1001  CGGTTAAGTC CGGCAACGAG CGCAACCCAC ATCCTTAGTT GCCAGCAGTT
1051  CrGCTGGGCA CTCTAAGGAA ACTGCCCGTG ATAAGCGGGA GGAAGGTGTG
1101  GATGACGTCA AGTCCTCATG GCCCTTACGG GTTGGGCTAC ACACGTGCTA
1151  CAATGGCAGT GACAATGGGT TAATCCCAAA AAGCTGTCTC AGTTCGGATT
1201  GGGGTyTGCA ACTCGACCCC ATGAAGTCGG AATCGCTAGT AATCGCGTAA
1251  CAGCATaGAC rCGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC
1301  ACACCATGGG AGTTGGTTCT ACCCGACGAC GCTGCGCTAA CCTTCG
```

NAME ATAM173a_51
LENGTH 1373 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TTTGATCATG GCTCAGAACG AACGCTGCGG CAGGCCTAAC ACATGCAAGT
  51  CGTGCGCGCC CTTCGGGGTG AGCGGCGGAC GGGTGAGTAA CGCGTGGGAA
 101  TATGCCCTTC TGTTGAGGAT AGCCCTGGGA AACTGGGAGT AATACTCGAT
 151  ACGCCCTACG GGGGAAGGAA GGATTAGCCC GCGTTAGATT AGGTAGTTGG
 201  TGAGGTAATG GCTCACCAAG CCTACGATCT ATAGCTGGTT TTAGAGGATG
 251  ATCAGCCACA CTGGGAcTGA GACACGGCCC AGACTCCTAC gGGAGGCAgC
 301  AGTGGGGAAT CTTAGACAAT GGGCGCAAGC CTGATCTAGC CATGCCGCGT
 351  GAGTGATGAA GGCCTTAGGG TCGTAAAGCT CTTTCGCCAG GGATGATAAT
 401  GACAGTACCT GGTAAAGAAA CCCCGGCTAA CTCCGTGCCA GCAGCCGCGG
 451  TAATACsGAG GGGGTTAGCG TTGTTCGGAA TTaCTGGGCG TAAAGCGCGC
 501  GTAGGCGGAC CAGAAAGTAT GGGGTGAAAT CCCGGGGCTC AACCCCGGAA
 551  CTGCCTCATA AACTCCTGGT CTTGAGTTCG AGAGAGGTGA GTGGAATTCC
 601  GAGTGTAGAG GTGAAATTCG TAGATATTCG GAGGAACACC AGTGGCGAAG
 651  GCGGCTCACT GGCTCGATAC TGACGCTGAG GTGCGAAAGT GTGGGGAGCA
 701  AACAGGATTA GATACCCTGG TAGTCCACAC CGTAAACGAT GAATGCCAGT
 751  CGTCGGCAAG CATGCTTGTC GGTGACACAC CTAACGGATT AAGCATTCCG
 801  CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT GACGGGGGCC
 851  CGCaCAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG CGCAGAACCT
 901  TACCAACCCT TGACATACTT GTCGTCGCTC CAGAGATGGA gCTTTCAGTT
 951  AGGCTGGACA AGATACAGGT GCTGCATGGC TGTCGTCAGC TCGTGTCGTG
1001  AGATGTTCGG TTAAGTCCGC CAACGAGCGC AACCCACATC TTCAGTTGCC
1051  AGCAGTTCGG CTGGGCACTC TGGAGAAACT GCCCGTGATA AGCGGGAGGA
1101  AGGTGTGGAT GACGTCAAGT CCTCATGGCC CTTACGGGTT GGGCTACACA
1151  CGTGCTACAA TGGCATCTAC AATGGGTTAA TCCCCAAAAG ATGTCTCAGT
1201  TCGGATTGGG GTCTGCAACT CGACCCCATG AAGTCGGAAT CGCTAGTAAT
1251  CGCGTAACAG CATGACGCGG TGAATACGTT CCCGGGCCTT GTACACACCG
1301  CCCGTCACAC CATGGGAGTT GGTTCTACCT GACGGgCCGT GCGCTAACCT
1351  TCGGGAGGCA GCGGACCACG GTA
```

NAME ATAM173a_62
LENGTH 1431 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TGACGGACTT CAGGCACTCC CGGCTTCCAT GGCTTGACGG GCGGTGTGTA
  51  CAAGGCCCGG GAACGTATTC ACCGGATCAT GGCTGATATC CGATTACTAG
 101  CGATTCCAGC TTCACGGGGT CGAGTTGCAG ACCCCGATCC GAACTGTGAC
 151  AGGTTTTATA GATTCGCTCC GCCTTGCGAC GTGGCTGCTC TCTGTACCTG
 201  CCATTGTAGC ACGTGTGTGG CCCAGGACGT AAGGGCCGTG ATGATTTGAC
 251  GTCATCCCCA CCTTCCTCAC GGTTTGCACC GGCAGTCCCG TTAGAGTCCC
 301  CATCATGACA TGCTGGCAAC TAACGGTAGG GGTTGCGCTC GTTATAGGAC
 351  TTAACCTGAC ACCTCACGGC ACGAGCTGAC GACAACCATG CAGCACCTTG
 401  CAAATTGCCC GAAGGAAAAT CTATCTCTAG ACCTGTCAAT ATGCATTTAA
 451  GCCCTGGTAA GGTTCCTCGC GTATCATCGA ATTAAACCAC ATGCTCCACC
 501  GCTTGTGCGG GCCCCGTCA ATTCCTTTGA GTTTCATTCT TGCGAACGTA
 551  CTCCCCAGGT GGGATACTTA TCACTTTCGC TTGGCCGCCC AGTACTCAAG
 601  GCACCGGACA GCTAGTATCC ATCGTTTACG GCGTGGACTA CCAGGGTATC
 651  TAATCCTGTT CGCTCCCCAC GCTTTCGTCC ATCAGCGTCA GTATATAGTT
 701  AGTCACCTGC CTTCGCAATC GGTGTTCTAT GTAATATCTA TGCATTTCAC
 751  CGCTACACTA CATATTCCGG CAACTTCACT ATAACTCAAG ACCGACAGTA
 801  TCAAAGGCAG TTCTACCGTT GAGCGGCAGA CTTTCACCCC TGACTTATCG
 851  GgCCCGCCTA CGGACCCTTT AAACCCAATA ATTCCGGATA ACGCTCGGAC
 901  CCTCCGTATT ACCGCGGCTG CTGGCACGGA GTTAGCCGGT CCTTATTCTT
 951  ACGGTACCGT CAGTAAGCTA CACGTAGCTC TTTTTCTTCC CGTATAAAAG
1001  CAGTTTACTA CCCATAGGGC ATTCTTCsTG CACGCGGgCA TGGCTGGATC
1051  AGAGTCTCCT CCATTGTCCA ATATTCCTCA CTGCTGCCTC CCGTAGGAGT
1101  CTGGTCCGTG TCTCAGTACC AGTGTGGGGG ATCCCCCTCT CAGGGCCCCT
1151  ACCCATCGTA GCCTTGGTAA GCCGTTACCT TACCAACTAG CTAATGGGAC
1201  GCATGGCCAT CTTGTACCGC CCGAAGACTT TAACCGCATC CCGATGCCGG
1251  GTCGCGGTAC TACGGGGCAT TAATCCAAGT TTCCCTGGGC TATTCCCCTG
1301  TACAAGGCAG GTTCCATACG CGGTGCGCAC CCGTGCGCCG GTCGTCATCT
1351  TGTGCAAGCA CAAATGTTAC CCCTCGACTT GCATGTGTTA AGCCTGCCGC
1401  TAGCGTTCAT CCTGAGCCAT GATCAAACTn T
```

NAME SCRIPPS_91
LENGTH 1448 nucleotides
AFFILIATION α-proteobacteria

```
   1  TTAGAGTTTG ATCATGGCTC AGAACGAACG CTGGCGGCGT GCTTAATACA
  51  TGCAAGTCGA ACGAGACCCT GGTGCTTGCA CCAGGTGACA GTGGCAGACG
 101  GGTGAgTAAC GCGTGGCAAC CTACCCTTCA CTACGGGACA ACAGTTGGAA
 151  ACGACTGCTA ATACcGTATA CGTcCTCCGG gAGAAAGATT TATCGGTGAT
 201  GGATgGGGCC GCGTTAGATT AGCTAGATGG TGGGGTAATG GCCTACCATG
 251  GCGaCGATCT ATAGCGGGTC TGAGAGGATG ATCCGCCACA CTGGGACTGA
 301  GACACGGCCC AGACTCCTAC GGCAGGCAGC AGTAGGGAAT ATTGGACAAT
 351  GGGCGAAAGC CTGATCCAGC AACGCCGCGT GAATGATGAA GGCCTTAGGG
 401  TTGTAAAATT CTTTCGCTAG GGAAGATAAT GACTGTACCT AGTAAGAAG
 451  CCCCGGCTAA CTCCGTGCCA GCAGCCGCGG TAATACGGAG GGGGCTAGCG
 501  TTGTTCGGAA TTACTGGGCG TAAAGCGTGC GTAGGCGGAC TAGCAAGTAT
 551  AGCGTGAAAT CCCAGGGCTC AACCCTGGAA CTGCCTTATA AACTGCTAGT
 601  CTTGAGTTCT GGAGAGGTAA GTGGAATTCC TAGTGTAGAG GTGAAATTCG
 651  TAGATATTAG GAGGAACACC AGAGGCGAAG GCGCCTTACT GGACAGATAC
 701  TGACCCTGAG GCACGAAAGT GTGGGGAGCA AACAGGATTA GATACCCTGG
 751  TAGTCCACAC CGTAAACGAT GAGAGCTAGT TGTCTGCAAG CATGCTTGTA
 801  GGTGACGCAG CTAACGCATT AAGCTCTCCG CCTGGGGAGT ACGGTCGCAA
 851  GATTAAAACT CAAAGAAATT GACGGGGGCC CGCACAAGCG GTgGAGCATG
 901  TGGTTTAATT CGAAGCAACG CGCAgAACCT TACCTACCCT TGACATGCCG
 951  GTCGAGATTT CCAGAGATGG ATTTCGTCAA TTCGGTTGGA CCGTGCACAG
1001  GTGCTGCATG GCTGTCGTCA GCTCGTGTCG TGAGATGTTG GGTTAAGTCC
1051  CGCAACGAGC GCAACCCTCA CTTTTAGTTG CCAGCATTTA GTTGGGCACT
1101  CTAGAAGAAC TGCCGGTGCT AAGCCGGAGG AAGGTGGGGA TGACGTCAAG
1151  TCCTCATGGC CCTTACGGGT AGGGCTACAC ACGTGCTACA ATGGCGCTGA
1201  CAGAGGGTTA ATCCCTAAAA GGCGTCTCAG TTCGGATTGT CTTCTGCAAC
1251  TCGAAGACAT GAAGTTGGAA TCGCTAGTAA TCGTGGATCA GCATGCCACG
1301  GTGAATACGT TCCCGGGCCT TGTACACACC GCCCGTCACA CCATGGGAGT
1351  TGGTTCTACC CGAAGGCGCT GCGCTAACCA GTTTACTGGA GGCAGGCGAC
1401  CACGGTAGGG TCAAGCGACT GGCGTGAAGT CGTAACAAGG TAGCCGTA
```

NAME SCRIPPS_94
LENGTH 1463 nucleotides
AFFILIATION α-proteobacteria

```
   1  AGTTTGATCA TGGCTCAGAA CGAACGCTGG CGGCAGGCCT AACACATGCA
  51  AGTCGTACGA GAAGGTTCTT TCGGSAACTG GAGAGTGGCG CACGGGTGAG
 101  TAACGCGTGG GGACCTACCT CTTAGTGCGG GATAACGGTT GGAAACGACC
 151  GCTAATACCG CATACGCCCT TCGGGGGAAA GATTTATCGC TAAGAGATGG
 201  ACCCGCGTTG GATTAGATAG TTGGTGAGGT AATGGCTCAC CAAGTCGGCG
 251  ATCCATAGCT GGTTTGAGAG GATGATCAGC CACACTGGGA CTGAGACACG
 301  GCCCAGACTC CTACGGGAGG CAGCAGTGGG GAATATTGGA CAATgGGGGC
 351  AACCeTGATC CAGCCATGCC GCGTGAGTGA AGAAGGCCTT CGGGTTGTAA
 401  AGCTCTTTCA GATGCGAAGA TGATGACGGT AACATCAGAA gAAGCCCCGG
 451  CTAATTTCGT GCCAGCAGCC GCGGTAATAC GAAAGGGGCA AGCGTTGTTC
 501  GGATTTACTG GGCGTAAAGG GCACGCAGGC GGTCTTGCCA GTCAGGGGTG
 551  AAAGCCCGGG GCTCAACCCC GGAACTGCCT CTGATACTGC AAGACTAGAG
 601  ACTAGGAGAG GGTGGTGGAA TTCCCAGTGT AGAGGTGAAA TTCGTAGATA
 651  TTGGGAGGAA CACCAGAGGC GAAGGCGGCC ACCTGGACTA GATCTGACGC
 701  TCAGGTGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC
 751  ACGCCGTAAA CGATGAGTGC TAGTTGTCGG GACTTCGGTT TCGGTGACGC
 801  AGCTAACGCA TTAAGCACTC CGCCTGGGGA GTACGGTCGC AAGATTAAAA
 851  CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
 901  TTCGAAGCAA CGCGCAGAAC CTTACCAACC CTTGACATCC CTATCGCGAT
 951  TTCCAGAGAT GGATTTCATC AGTTCGGCTG GATAGGTGAC AGGTGCTGCA
1001  TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA
1051  GCGCAACCCC TATCCTTAGT TGCCAGCATT TAGTTGGGCA CTCTAGGGAG
1101  ACTGCCGGTG ACAAGCCgGA gGAAGGCGGG GATGACGTCA AGTCCTCATG
1151  GCCCTTACGG GTTGGGCTAC ACACGTGCTA CAATGGTAAC TACAGAGGGC
1201  TGCTTCTTGG CAACAAGTGG CGAATCCCAA AAAGTTATCT CAGTTCGGAT
1251  TGCACTCTGC AACTCGAGTG CATGAAGTTG GAATCGCTAG TAATCGTGGA
1301  TCAGCATGCC ACGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC
1351  ACACCATGGG AGTTGGTTTT ACCCGAAGAC GGTGGGCTAA CCAGATTTAT
1401  CTGGAGGCAG CCGGCCACGG TAAGGTCAGC GACTGGGGTG AAGTCGTAAC
1451  AAGGTAGCCC GTA
```

NAME SCRIPPS_96
LENGTH 669 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  AGGCCTAA CACATGCAAG TCGAGCGCAC CTTCGGGTGA GCGGCGGACG
  51  GGTTAGTAAC GCGTGGGAAC ATACCCTTTT CTACGGAATA GCCTCGGGAA
 101  ACTGAGAGTA ATACCGTATA AGCCCTTCgG GGGAAACATT TATCgGGAAA
 151  GGATTGGCCC GCGTTAGATT AGATAGTTGG TGGGGTAATG GCCTACCAAG
 201  TCTACGATCT ATAGCTGGTT TTAGAGGATG ATCAGCAACA CTGGGACTGA
 251  GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT CTTAGACAAT
 301  GGGCGCAAGC CTGATCTAGC CATGCCGCGT GTGTGATGAA GGTCTTAGGA
 351  TCGTAAAGCA CTTTCGCCAG GGATGATAAT GACAGTACCT GGTAAAGAAA
 401  CCCCGGCTAA CTCCGTGCCA GCAGCCGCGG TAATACGGAG GGGGTTAGcC
 451  GTTGTTCGGA ATTACTGGGC GTAAAGCGTA CGTAGGCGGA TTGGAAAGTT
 501  GGGGGTGAAA TCCCAGGGCT CAACCCTGGA ACTGCCTCCA AAACTATCAG
 551  TCTAGAGTTC GAGAGAGGTG ArTGGAATTC CAAGTGTAGA GGTGAAATTC
 601  GTAGATATTT GGAGGAACAC CArTGGCGAA GGCGGCTCAC TGGCTCGATA
 651  CTGACGCTGA GGTACGAAA
```

NAME SCRIPPS_101
LENGTH 1437 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  AGTTTGATCA TGGCTCAGAA CGAACGCTGG CGGCAGGCCT AACACATGCA
  51  AGTCGAGCGA GACCTTCGGG TCTAGCGGCG GACGGGTTAG TAACGCGTGG
 101  GAACGTGCCC TTCTCTGCGG AATAGCCACT GGAAACGGTG AGTAATACCG
 151  CATACGCCCT TCGGGGGAAA GATTTATCGG AGAAGGATCG GCCCCGCGTTA
 201  GATTAGATAG TTGGTGGGGT AATGGCCTAC CAAGTCTACG ATCTATAGCT
 251  GGTTTTAGAG GATGATCAGC AACACTGGGA CTGAGACACG GCCCAGACTC
 301  CTACGGGAGG CAGCAGTGGG GAATCTTAGA CAATGGGCGC AAGCCTGATC
 351  TAGCCATGCC GCGTGTGTGA TGAAGGTCTT AGGATCGTAA AGCACTTTCG
 401  CCAGGGATGA TAATGACAGT ACCTGGTAAA GAAACCCCGG CTAACTCCGT
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 451  GCCAGCAGCC GCGGTAATAC GGAGGGGGTT AGCGTTGTTC GGaATTaCTG
 501  GGCGTAAAGC GTACGTAGGC GGATCAGAAA GTAAGGGGTG AAATCCCAGG
 551  GCTCAACCCT GGAACTGCCT CTTAAACTCC TGGTCTTGAG TTCGAGAGAG
 601  GTGAGTGGAA TTCCAAGTGT AGAGGTGAAA TTCGTAGATA TTTGGAGGAA
 651  CACCAGTGGC GAAGGCGGCT CACTGGCTCG ATACTGACGC TGAGGTACGA
 701  AAGTGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACACCGTAAA
 751  CGATGAATGC CAGTCGTCGG GCAGTATACT GTTCGGTGAC ACACCTAACG
 801  GATTAAGCAT TCCGCCTGGG GAGTACGGTC GCAAGATTAA AACTCAAAGG
 851  AATTGACGGG GGCCCGCACA AGCGGtGGAG CATGTGGTTT AATTCGAAGC
 901  AACGCGCAGA ACCTTACCAA CCCTTGACAT CCTGTGCTAA CCCGAGAGAT
 951  CGGGCGTCCA CTTCGGTGGC GCAGTGACAG GTGCTGCATG GCTGTCGTCA
1001  GCTCGTGTCG TGAGATGTTC GGTTAAGTCC GGCAACGAGC GCAACCCACA
1051  TCCTTAGTTG CCAGCAGTTC GGCTGGGCAC TCTAAGGAAA CTGCCCGTGA
1101  TAAGCGGGAG GAAGGTGTGG ATGACGTCAA GTCCTCATGG CCCTTACGGG
1151  TTGGGCTACA CACGTGCTAC AATGGCAGTG ACAATGGGTT AATCCCCAAA
1201  AGCTGTCTCA GTTCGGATTG GGGTCTGCAA CTCGACCCCA TGAAGTCGGA
1251  ATCGCTAGTA ATCGTGGAAC AGCATGCCAC GGTGAATACG TTCCCGGGCC
1301  TTGTACACAC CGCCCGTCAC ACCATGGGAG TTGGTTCTAC CCGACgGGCC
1351  GTGCGCCAAC CTTTCGAGGA GGCAGCGGAC CACGGTAGGA TCAGCGACTG
1401  GGGTGAAGTC GTAACAAGGT ACCCGTAGGG GAACCTG
```

NAME SCRIPPS_115
LENGTH 1373 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  TTACAGTTTG ATCATGGCTC AGAACGAACG CTGGCGGCAG GCCTAACACA
  51  TGCAAGTCGA GCGAGACCTT CGGGTCTAGC GGCGGACGGG TTAGTAACGC
 101  GTGGGAACGT GCCCTTCTCT GCGGAATAGC CACTGGAAAC GGTGAGTAAT
 151  ACCGCATACG CCCTTCGGGG GAAAGATTTA TCGGAGAAGG ATCGGCCCsC
 201  sTywkATTAG ATAGcTTGGc TGGGGTAATG GCCTACCAag TmTACGATCT
 251  ATAGCTGGTT TTAGAGGATG ATCAGCAACA CTGGGACTGA GACACGGCCC
 301  AGACTCcTAC GGGAGGCAGC AGTGGGGAAT CTTAGACAAT GGGCGCAAGC
 351  CTGATCTAGC CATGCCGCGT GTGTGATGAA GGTCTTAGGA TCGTAAAGCA
 401  CTTTCGCCAG GGATGATAAT GaCAGTACCT GGTAAAGAAA CCCCGGCTAA
 451  CTCCGTGCCA GCAGCCGCGG TAATACGGAG GGGGTTAGCG TTGTTCGGAA
 501  TTaCTGGGCG TAAAGCGTAC GTAGGCGGAT CAagAAAGTA AGGGGTGAAA
 551  TCCCAGGGCT CAACCCTGGA ACTGCCTCTT AAACTCCTGG TCTTGAGTTC
 601  CAGAGAGGTG AGTGGAATTC CAAGTGTAGA GGTGAAATTC GTAGATATTT
 651  GGAGGAACAC CAGTGGCGAA GGCGGCTCAC TGGCTCGATA CTGACGCTGA
 701  GGTACGAAAG TGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACA
 751  CCGTAAACGA TGAATGCCAG TCGTCGGGCA GTATACTGTT CGGTGACACA
 801  CCTAACGGAT TAAGCATTCC GCcTGGGgAG TACGGTCGCA AGATTAAAAC
 851  TCAAAGGAAT TGACGGGGGC CCGCACAAGC GG.TGgAGCA TGTGGTTTAA
 901  TTCGAAGCAA CGCGCAGAaC CTTACCAACC CTTGACATCc TGTGCTAACC
 951  CGAGAGATCG GGCGTCCACT TCGGTGGCGC AGTGACAGGT GCTGCATGGC
1001  TGTCGTCAGC TCGTGTCGTG AGATGTTCGG TTAAGTCCGG CAACGAGCGC
1051  AACCCACATC CTTAGTTGCC AGCAGTTCGG CTGGGCACTC TAAGGAAACT
1101  GCCCGTGATA AGCGGGAaGA AGGTGTGGAT GACGTCAAGT CCTCATGGCC
1151  CTTACGGGTT GGGCTACACA CGTGCTACAA TGGCAGTGAC AATGGGTTAA
1201  TCCCCAAAAG CTGTCTCAGT TCGGATTGGG GTCTGCAACT CGACCCCATG
1251  AAGTCGGAAT CGCTACTAAT CGTGGAACAG CATGCCACGG TGAATACGTT
1301  CCCGGGCCTT GTACACACCG CCCGTCACAC CATGGGAGTT GGTTCTACCC
1351  GACGGGCCGT GCGCCAACCT TT
```

NAME SCRIPPS_117
LENGTH 654 nucleotides
AFFILIATION α-proteobacteria

```
   1  TTAACACATG CAAGTCGAAC GATATAGTGG CAGACGGGTG AGTAACGCGT
  51  GGGAACGTAC CTTTCACTAC GGAATAGCTC TTGGAAACGA GTGGTAATAC
 101  CGTATACGCC CTTCGGGGGA AAGATTTATC GGTGAAAGAT CGGCCCGCGT
 151  TAGATTAGCT AGTTGGTAGG GTAATGGCCT ACCAAGGCGA CGATCTATAG
 201  CTGGTCTGAG AGGATGATCA GCCACACTGG GACTGAGACA CGGCCCAGAC
 251  TCCTACGGGA GGCAGCAGTG GGGAATCTTG CACAATGGGC GAAAGCCTGA
 301  TGCAGCCATG CCGCGTGAAT GATGAAGGCC TTAGGGTTGT AAAATTCTTT
 351  CGCTAGCGAT GACAATGACA GTACCTAGTA AAGAAGCCCC GGCTAACTTC
 401  GTGCCAGCAG CCGCGGTAAT ACGAAGGGGG CTAGCGTTGT TCGGAATTAC
 451  TGGGCGTAAA GCGCACGTAG GCGGACTTTT AAGTCAGATG TGAAATCCCG
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 501  GGGCTCAACC TCGGAACTGC ATTTGAAACT GGAAGTCTAG AGACCAGGAG
 551  AGGTTAGCGG AATACCGAGT GTAGAGGTGA AATTCGTAGA TATTCGGTGG
 601  AACACCAGTG GCGAAGGCGG CTAACTGGAC TGGTACTGAC GCTGAGGTGC
 651  GAAA
```

NAME SCRIPPS_119
LENGTH 684 nucleotides
AFFILIATION α-proteobacteria

```
   1  GGCGTGCTTA ATACATGCAA GTCGAACGAG ACCCTGGTGC TTGCACCAGG
  51  TGACAGTGGC AGACGGGTGA GTAACGCGTG GCAACCTACC CTTCACTACG
 101  GGACAACAGT TGGAAACGAC TGCTAATACC GTATACGTCC TCCGGGAGAA
 151  AGATTTATCG GTGATGGATG GGGCCGCGTT AGATTAGCTA GATGGTGGGG
 201  TAATGGCCTA CCATGGCGAC GATCTATAGC GGGTCTGAGA GGATGATCCG
 251  CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTAG
 301  GGAATATTGG ACAATGGGCG AAAGCCTGAT CCAGCAACGC CGCGTGAATG
 351  ATGAAGGCCT TAGGGTTGTA AAATTCTTTC GCTAGGGAAG ATAATGACTG
 401  TACCTAGTAA AGAAGCCCCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA
 451  CGGAGGGGGC TAGCGTTGT TCGGAATTAC TGGGCGTAAA GCGTGCGTAG
 501  GCGGACTAGC AAGTATAGGG TGAAATCCCA GGGCTCAACC CTGGAACTGC
 551  CTTATAAACT GCTAGTCTTG AGTTCTGGAG AGGTAAGTGG AATTCCTArT
 601  GTAGAGGTGA AATTCGTAGA TATTAGGAGG AACACCAGAG GCGAAGGCGG
 651  CTTACTGGAC AGATACTGAC GCTGAGGCAC GAAA
```

NAME SCRIPPS_413
LENGTH 1505 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

```
   1  TTAGAGTTTG ATCATGGCTC AGGATGAACG CTAGCGGCAG GCTTAACACA
  51  TGCAAGTCGA GGGGTAGCAG GAAAAAGCTT GCTTTTTGC TGACGACCGG
 101  CGCACGGGTG CGTAACGCGT ATGCAATCTA CCTTTTGCTG AGGGATAGCC
 151  CAGAGAAATT TGGATTAATA CCTCATAGTA TGATGACTTG GCATCAAGAT
 201  ATCATTAAAG GTTACGGCAA AAGATGAGCA TGCGTTCTAT TAGCTAGTTG
 251  GTGTGGTAAC GGCATACCAA GCCAACGATA GATAGGGGTC CTGAGAGGGA
 301  GATCCCCCAC ACTGGTACTG AGACACGGAC CAGACTCcTA CGGGAGGCAG
 351  CAGTGAGGAA TATTGGaCAA TGGAGGCAAC TCTGATCCAG CCATGCCGCG
 401  TGCAGGAAGA C.TGCCCTAT GGGTTGTAAA CTGCTTTTAT ACAGGAAGAA
 451  ACACCTCTAC GTGTAGAGGC TTGACGGTAC TGTAAGAATA AGGATCGGCT
 501  AACTCCGTGC CAGCAGCCGC GGTAATACGG AGGATCCAAG CGTTaTCCGG
 551  AATCaTTGGG TTTAAAGGGT CCGTAGGTGG ATAATTAAGT CAGAGGTGAA
 601  ATCCTGCAGC TTAACTGTAG AATTGCCTTT GATACTGGTT GTCTTGAGTT
 651  ATTATGAAGT GGTTAGAATA TGTAGTGTAG CGGTGAAATG CATAGATATT
 701  ACATAGAATA CCAATTGCGA AGGCAGATCA CTAATAATAT ACTGACACTG
 751  ATGGACGAAA GCGTGGGGAG CGAACAGGAT TAGATACCCT GGTAGTCCAC
 801  GCCGTAAACG ATGGTCACTA GCTGTTCGGA TTTCGGTCTG AGtGGCTAAG
 851  CGAAAGTgAT AAGTGACCCA CCTGGGGAGT ACGTTCGCAA GAATGAAACT
 901  CAAAGGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT
 951  CGATGATACG CGAGGAaCCT TACCAGGGCT TAAATGTGGT CTGA.CAGCT
1001  TTAGAGATAG AGTTTTCTTC GGACAGATCA CAAGGTGCTG CATGGTTGTC
1051  GTCAGCTCGT GCCGTGAGGT GTCAGGTTAA GTCCTATAAC GAGCGCAACC
1101  CCTGTTGTTA GTTGCCAGCG AGTAATGTCG GGAACTCTAG CAAGACTGCC
1151  GGTGCAAACC GTGAGGAAGG TGGGGATGAC GTCAAATCAT CACGGCCCTT
1201  ACGTCCTGGG CTACACACGT GCTACAATGG TAGGGACAGA GAGCAGCCAC
1251  TTCGCGAGAA GCAGCGAATC TATAAACCCT ATCACAGTTC GGATCGGAGT
1301  CTGCAACTCG ACTCCGTGAA GCTGGAATCG CTAGTAATCG CATATCAGCC
1351  ATGATGCGGT GAATACGTTC CCGGGCCTTG TACACACCGc CCGTCAAGCC
1401  ATGGAAGCTG GGAGTGCCTG AAGTCCGTCA CCGCAAGGAG CGGCCTAGGG
1451  TAAAATCGGT AACTAGGGCT AAGTCGTAAC AAGGTAGCCG TACCGGAAGG
1501  TGCGG
```

NAME SCRIPPS_423
LENGTH 1456 nucleotides
AFFILIATION α-proteobacteria

```
   1  TAGAGTTTGA TCATGGCTCA GAACGAACGC TGGCGGCGTG CTTAATACAT
  51  GCAAGTCGAA CGAGACCCTG GTGCTTGCAC CAGGTGACAG TGGCAGACGG
 101  GTGAGTAACG CGTGGCAACC TACCCTTCAC TACGGGACAA CAGTTGGAAA
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 151  CGACTGCTAA TACCGTATAC GTCCTCCGGG AGAAAGATTT ATCGGTGATG
 201  GATGGGGCCG CGTTAGATTA GCTAGATGGT GGGGTAATGG CCTACCATGG
 251  CGACGATCTA TAGCGGGTCT GAGAGGATGA TCCGCCACAC TGGGACTGAG
 301  ACACGGCCCA GACTCCTACG GGAGGCAGCA GTAGGGAATA TTGGACAATG
 351  GGCGAAAGCC TGATCCAGCA ACGCCGCGTG AATGATGAAG GCCTTAGGGT
 401  TGTAAAATTC TTTCGCTAGG GAAGATAATG ACTGTACCTA GTAAAGAAGC
 451  CCCGGCTAAC TCCGTGCCAG CAGCCGCGGT AATACGGAGG GGGCTAGCGT
 501  TGTTCGGAAT TACTGGGCGT AAAGCGTGCG TAGGCGGACT AGCAAGTATA
 551  GGGTGAAATC CCAGGGCTCA ACCCTGGAAC TGCCTTATAA ACTGCTAGTC
 601  TTGAGTTCTG GAGAGGTAAG TGGAATTCCT AGTGTAGAGG TGAAATTCGT
 651  AGATATTAGG AGGAACACCA GAGGCGAAGG CGGCTTACTG GACAGATACT
 701  GACGCTGAGG CACGAAAGTG TGGGGAGCAA ACAGGATTAG ATACCCTGGT
 751  AGTCCACACC GTAAACGATG AGAGCTAGTT GTCTGCAAGC ATGCTTGTAG
 801  GTGACGCAGC TAACGCATTA AGCTCTCCGC CTGGGGAGTA CGGTCGCAAG
 851  ATTAAAACTC AAAGAAATTG ACGGGGGCCC GCACAAGCGG TGGAGCATGT
 901  GGTTTAATTC GAAGCAACGC GCAGAACCTT ACCTACCCTT GACATGCCGG
 951  TCGAGATTTC CAGAGATGGA TTTCGTCAAT TCGGTTGGAC CGTGCACAGG
1001  TGCTGCATGG CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC
1051  GCAACGAGCG CAACCCTCAC TTTTAGTTGC CAGCATTTAG TTGGGCACTC
1101  TAGAAGAACT GCCGGTGCTA AGCCGGAGGA ACGTGGGGAT GACGTCAAGT
1151  CCTCATGGCC CTTACGGGTA GGGCTACACA CGTGCTACAA TGGCGCTGAC
1201  AGAGGGTTAA TCCCTAAAAG GCGTCTCAGT TCGGATTGTC TTCTGCAACT
1251  CGAAGACATG AAGTTGGAAT CGCTAGTAAT CGTGGATCAG CATGCCACGG
1301  TGAATACGTT CCCGGGCCTT GTACACACCG CCCGTCACAC CATGGGAGTT
1351  GGTTCTACCC GAAGGCGCTG CGCTAACCAG TTTACTGGAG GCAGGCGACC
1401  ACGCTAGGGT CAGCGACTGG GGTGAAGTCG TAACAAGGTA GCCGTAGGGG
1451  AACCTG
```

NAME SCRIPPS_426
LENGTH 656 nucleotides
AFFILIATION α-proteobacteria

```
   1  AATACATGCA AGTCGAGCGC ACCTTCGGGT GAGCGGCGCA CGGGTGAGTA
  51  ACGCGTGGGA ATATGCCCTA TGGTgCGGAA CAACTGAgGG AAACTTCAGC
 101  TAATACCGCA TGTGCCCTAC GGGGGAAAGA TTTATCGCCA TAGGAGTAGC
 151  CCGCGTTGGA TTAGTTTGTT GGTGAGGTAA TGGCTCACCA AGACTTCGAT
 201  CCATAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC
 251  CCAGACTCCT ACGGGAGGCA GCAGTAGGGA ATCTTGCGCA ATGGGCGAAA
 301  GCCTGACGCA GCCATGCCGC GTGAATGATG AAGGTCTTAG GATTGTAAAA
 351  TTCTTTCACC GGGGACGATA ATGACGGTAC CCGGAGAAGA AGCTCCGGCT
 401  AACTTCGTCC CAGCAGCCGC GGTAATACGA AGGGGGCTAG CGTTGCTCGG
 451  AATTACTGGG CGTAAAGGGC GCGTAGGCGG ACAGTTTAGT CAGAGGTGAA
 501  AGCCCAGGGC TCAACCTTGG AATAGCCTTT GATACTGACT GTCTTGAGTA
 551  CsGGAGArGT GTGTGGAACT CCGAGTGTAG ArGTGAAATT CGTAGATATT
 601  CGGAAGAACA CCAGTGGCGA AGGCGACACA CTGGCCCGTT ACTGACGCTG
 651  AGGCG
```

NAME SCRIPPS_732
LENGTH 1332 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

```
   1  GGCCTAACAC ATGCAAGTCG AGCGCACCTT CGGGTGAGCG GCGGACGGGT
  51  TAGTAACGCG TGGGAACATA CCCTTTTCTA CGGAATAGCC TCGGGAAACT
 101  GAGAGTAATA CCGTATAAGC CCTTCGGGGG AAAGATTTAT CGGGAAAGGA
 151  TTGGCCCGCG TTAGATTAGA TAGTTGGTGG GGTAATGGCC TACCAAGTCT
 201  ACGATCTATA GCTGGTTTTA GAGGATGATC AGCAACACTG GGACTGAGAC
 251  ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATCTT AGACAATGGG
 301  CGCAAGCCTG ATCTAGCCAT GCCGCGTGTG TGATGAAGGT CTTAGGATCG
 351  TAAAGCACTT TCGCCAGGGA TGATAATGAC AGTACCTGGT AAAGAAACCC
 401  CGGCTAACTC CGTGCCAGCA GCCGCGGTAA TACGGAGGGG GTTAGCGTTG
 451  TTCGGAATTA CTGGGCGTAA AGCGTACGTA GGCGGATTGG AAAGTTGGGG
 501  GTGAAATCCC AGGGCTCAAC CCTGGAACTG CCTCCAAAAC TATCAGTCTA
 551  GAGTTCGAGA GAGGTGAGTG GAATTCCAAG TGTAGAGGTG AAATTCGTAG
 601  ATATTTGGAG GAACACCAGT GGCGAAGGCG GCTCACTGGC TCGATACTGA
 651  CGCTGAGGTA CGAAAGTGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG
 701  TCCACACCGT AAACGATGAA TGCCAGTCGT CGGGCAGTAT ACTGTTCGGT
 751  GACACACCTA ACGGATTAAG CATTCCGCCT GGGGAGTACG GTCGCAAGAT
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 801  TAAAACTCAA AGGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG
 851  TTTAATTCGA AGCAACGCGC AGAACCTTAC CAACCCTTGA CATCCTGTGC
 901  TAACCCGAGA GATCGGGCGT TCACTTCGGT GACGCAGTGA CAGGTGCTGC
 951  ATGGCTGTCG TCAGCTCGTG TCGTGAGATG TTCGGTTAAG TCCGGCAACG
1001  AGCGCAACCC ACATCCTTAG TTGCCAGCAG TTCGGCTGGC CACTCTAAGG
1051  AAACTGCCCG TGATAAGCGG GAGGAAGGTG TGGATGACGT CAAGTCCTCA
1101  TGGCCCTTAC GGGTTGGGCT ACACACGTGC TACAATGGCA GTGACAATGG
1151  GTTAATCCCA AAAAGCTGTC TCAGTTCGGA TTGGGGTCTG CAACTCGACC
1201  CCATGAAGTC GGAATCGCTA GTAATCGCGT AACAGCATGA CGCGGTGAAT
1251  ACGTTCCCGG GCCTTGTACA CACCGCCCGT CACACCATGG GAGTTGGTTC
1301  TACCCGACGA CGCTGCGCTA ACCTTGGGGG GG
```

NAME SCRIPPS_735
LENGTH 676 nucleotides
AFFILIATION α-proteobacteria

```
   1  CTTACACATG CAAGTCGTAC GAGAAGGTTC TTTCGGGAAC TGGAGAGTGG
  51  CGCACGGGTG AGTAACGCGT GGGGACCTAC CTCTTAGTGG GGGATAACGG
 101  TTgGAAACGa CCGCTAATAC CGCATACGCC CTTCGGGGGA AAGATTTATC
 151  GCTAAGAGAT GGAcCCGCGG TTGGATTAGA TAGTTGGTGA GGTAATGGCT
 201  CACCAAGTCG GCGATCCATA GCTGGTTTGA GAGGATGATC AGCCACACTG
 251  GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT
 301  GGACAATGGG GGCAACCCTG ATCCAGCCAT GCCGCGTGAG TGAAGAAGGC
 351  CTTCGGGTTG TAAAGCTCTT TCAGATGCGA AGATGATGAC GGTAACATCA
 401  GAAGAAGCCC CGGCTAATTT CGTGCCAGCA GCCGCGGTAA TACGAAAGGG
 451  GCAAGCGTTG TTCGGATTTA CTGGGCGTAA AGGGCACGCA gGCGGTCTTG
 501  CCAGTCAGGG GTGAAAGCCC GGGGCTCAAC CCCGGAACTG CCTCTGATAC
 551  TGCAAGACTA GAGACTAgGA rArGGTGGTG GAATTCCCAG TGTAGAGGTG
 601  AAATTCGTAG ATATTGGGAG GAACACCAGA GGCGAAGGCG GGCCACCTGG
 651  ACTAGATCTG ACGCTCAGGT GCGAAA
```

NAME SCRIPPS_738
LENGTH 1417 nucleotides
AFFILIATION α-proteobacteria

```
   1  TAGAGTTTGA TCATGGCTCA GAACGAACGC TGCGGCAGGC CTAACACATG
  51  CAAGTCGTAC GAGAAGGTTC TTCGGGGAAC TGGAGAGTGG CGCACGCGTG
 101  AGTAACGCGT GGGGACCTAC CTCTTAGTGG GGGATAACGG TTGGAAACGA
 151  CCGCTAATAC CGCATACGCC CTTCGGGGGA AAGATTTATC GCTAAGAGAT
 201  GGACCCGCGT TGGATTAGAT AGTTGGTGAG GTAATGGCTC ACCAAGTCGG
 251  CGATCCATAG CTGGTTTGAG AGGATGATCA GCCACACTGG GACTGAGACA
 301  CGGCCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG GACAATGGGG
 351  GCAACCCTGA TCCAGCCATG CCGCGTGAGT GAAGAAGGcC TTCGGGTTGT
 401  AAAGCTCTTT CAGATGCGAA GATGATGACG GTAACATCAG AAGAAGCCCC
 451  GGCTAATTTC GTGCCAGCAG CCGCGGTAAT ACGAAAGGGg CAAGCGTTGT
 501  TCGGATTTAC TGGGCGTAAA GGGCACGCAg GCGGTCTTGC CAGTCAGGGG
 551  TGAAAGCCCG GGGCTCAACC CCGGAACTGC CTCTGATACT GCAAGACTAG
 601  AGACTAGGAG AGGGTGGTGG AATTCCCAGT GTAGAGGTGA AATTCGTAGA
 651  TATTGGGAGG AACACCAGAG GCGAAGGCGG CCACCTGGAC TAGATCTGAC
 701  GCTCAGGTGC GAAAGCGTGG GGAGCAAACA GGATTACATA CCCTGGTAGT
 751  CCACGCCGTA AACGATGAGT GCTAGTTGTC GGGACTTCGG TTTCGGTGAC
 801  GCAGCTAACG CATTAAGCAC TCCGCcTGGG GAGTACGGTC GCAAGATTAA
 851  AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
 901  AATTCGAAGC AACGCGCAGA ACCTTACCAA CCCTTGACAT CCCTATCGCG
 951  ATTTCCAGAG ATGGATTTCA TCAGTTCGGC TGGATAGGTG ACAGGTGCTG
1001  CATGGC.TGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA AGTCCCGCAA
1051  CGAGCGCAAC CCCTATCCTT AGTTGCCAGC ATTTAGTTGG GCACTCTAGG
1101  GAGACTGCCG GTGACAAGCC GGAGGAAGGC GGGGATGACG TCAAGTCCTC
1151  ATGGCCCTTA CGGGTTGGGC TACACACGTG CTACAATGGT AACTACAGAG
1201  GGCTGCTTCT TGGCAACAAG TGGCGAATCC CAAAAAGTTA TCTCAGTTCG
1251  GATTGCACTC TGCAACTCGA GTGCATGAAG TTGGAATCGC TAGTAATCGT
1301  GGATCAGCAT GCCACCGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC
1351  GTCACACCAT GGGAGTTGGT TTTACCCGAA GACGGTGGGC TAACCAGATT
1401  TATCTGGAGG CAGCCGG
```

NAME SCRIPPS_739
LENGTH 1375 nucleotides
AFFILIATION α-proteobacteria

```
   1 AGTTTGATCA TGGCTCAGAA CGAACGCTGG CGGCAGGCTT AACACATGCA
  51 AGTCGAACGA TATAGTGGCA GACGGGTGAG TAACGCGTGG GAACGTACCT
 101 TTCACTACGG AATAGCTCTT GGAAACGAGT GGTAATACCG TATACGCCCT
 151 TCGGGGGAAA GATTTATCGG TGAAAGATCG GCCCGCGTTA GATTAGCTAG
 201 TTGGTAGGGT AATGGCCTAC CAAGGCGACG ATCTATAGCT GGTCTGAGAG
 251 GATGATCAGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG
 301 CAGCAGTGGG GAATCTTGCA CAATGGGCGA AAGCCTGATG CAGCCATGCC
 351 GCGTGAATGA TGAAGGCCTT AGGGTTGTAA AATTCTTTCG CTAGGGATGA
 401 TAATGACAGT ACCTAGTAAA GAAGCCCCGG CTAACTTCGT GCCAGCAGCC
 451 GCGGTAATAC GAAGGGGGCT AGCGTTGTTC GGAATTACTG GGCGTAAAGC
 501 GCACGTAGGC GGACTTTTAA GTCAGATGTG AAATCCCGGG GCTCAACCTC
 551 GGAACTGCAT TTGAAACTGG AAGTCTAGAG ACCAGGAGAG GTTAGCGGAA
 601 TACCGAGTGT AGAGGTGAAA TTCGTAGATA TTCGGTGGAA CACCAGTGGC
 651 GAAGGCGGCT AACTGGACTG GTACTGACGC TGAGGTGCGA AAGTGTGGGG
 701 AGCAAACAGG ATTAGATACC CTGGTAGTCC ACACCGTAAA CGATGAGAGC
 751 TAGTTGTTGG CAGGCATGCC TGTCGGTGAC GCAGCTAACG CATTAAGCTC
 801 TCCGCCTGGG GAGTACGGTC GCAAGATTAA AACTCAAAGA AATTGACGGG
 851 GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGCAGA
 901 ACCTTACCTA CCCTTGACAT ACCGATCGCG GTTTCCAGAG ATGGATTCCT
 951 TCAGTTAGGC TGGATCGGAT ACAGGTGCTG CATGGCTGTC GTCAGCTCGT
1001 GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCATCCTTA
1051 GTTGCCATCA CGTTTGGGTG GGCACTCTAA GGAAACTGCC GGTGGTAAGC
1101 CGGAGGAAGG TGGGGATGAC GTCAAGTCCT CATGGCCCTT ACGGGTAGGG
1151 CTACACACGT GCTACAATGG CAGTGACAAT GGGTTAATCC CCAAAAACTG
1201 TCTCAGTTCG GATTGTCGTC TGCAACTCGA CGGCATGAAG GTGGAATCGC
1251 TAGTAATCGT GGATCAGCAT GCCACGGTGA ATACGTTCCC GGGCCTTGTA
1301 CACACCGCCC GTCACATCAT GGGAATTGGT TCTACCCGAA GACGCTGTGC
1351 TAACTTCGGA GGCAGGCGGC CACGG
```

NAME SCRIPPS_740
LENGTH 1482 nucleotides
AFFILIATION γ-proteobacteria

```
   1 AGTTTGATCA TGGCTCAGAT TGAACGCTGG CGGCAGGCCT AACACATGCA
  51 AGTCGAGCGG ATGAGTGGAG CTTGCTCCAT GATTCAGCGG CGGACGGGTG
 101 AGTAATGCCT AGGAATCTGC CTGGTAGTGG GGGACAACGT TTCGAAAGGA
 151 ACGCTAATAC CGCATACGTC CTACGGGAGA AAGCAGGGGA CCTTCGGGCC
 201 TTGCGCTATC AGATGAGCCT AGGTCGGATT AGCTAGTTGG TGAGGTAATG
 251 GCTCACCAAG GCGACGATCC GTAACTGGTC TGAGAGGATC ATCAGTCACA
 301 CTGGAACTGA GACACGGTCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT
 351 ATTGGACAAT GGGCGAAAGC CTGATCCAGC CATGCCGCGT GTGTGAAGAA
 401 GGTCTTcGGA TTGTAAAGCA CTTTAAGTTG GGAGGAAGGG CATTAATCTA
 451 ATACGTTAGT GTTTTGACGT TACCGACAGA ATAAGCACCG GCTAACTTCG
 501 TGCCAGCAGC CGCGGTAATA CGAAGGGTGC AAGCGTTAAT CGGAATTACT
 551 GGGCGTAAAG CGCGCGTAgG TGGTTTGTTA AGTTGAATGT GAAAGCCCCG
 601 GGCTCAACCT GGGAACTGCA TcCAAAACTG GCAAGCTAGA GTATGGCAGA
 651 GGGTGGTGGA ATTTCCTGTG TAGCGGTGAA ATGCGTAGAT ATAGGAAGGA
 701 ACACCAGTGG CGAAGGCGAC CACCTGGGCT AATACTGACA CTGAGGTGCG
 751 AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA
 801 ACGATGTCGA CTAGCCGTTG GGATCCTTGA GATCTTAGtG GCGCAGCTAA
 851 CGCATTAAGT CGACCGCCTG GGGAGTACGG CCGCAAGGTT AAAACTCAAA
 901 TGAATTGACG GGGGCCCGCA CAAGCGGTGr AGCATGTGGT TTAATTCGAA
 951 GCAACGCGAA GAaCCTTACC AGGCCTTGAC ATGCAGAGaA CTTTCCAGAG
1001 ATGGATTGGT GCCTTCGGGA GCTCTGACAC AGGTGCTGCA TGGCTGTCGT
1051 CAGCTCGTGT CGTGAGATGT TGGGTTAAgT CCCGTAACGA GCGCAACCCT
1101 TGTCCTTAGT TACCAGCACA TAATGGTGGG CACTCTAAGG AGACTGCCGG
1151 TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCATCA TGGCCCTTAC
1201 GGCCTGGGCT ACACACGTGC TACAATGGTC GGTACAAAGG GTTGCCAAGC
1251 CGCGAGGTGG AGCTAATCCC ATAAAACCGA TCGTAGTCCG GATCGCAGTC
1301 TGCAACTCGA CTGCGTGAAG TCGGAATCGC TAGTAATCGT GAATCAGAAT
1351 GTCACGGTGA ATACGTTCCC GGGCmTTGTA CACACCGcCC GTCACACCAT
1401 GGGAGTGGGT TGCTCCAGAA GTAGCTAGTC TAACCTTCGG GAGGACGGTT
1451 ACCACGGACT GATTCATGAC TGGGGTGAAG TC
```

NAME *A. lusitanicum* NEPCC 253 re-introduction bacteria 1
LENGTH 1330 nucleotides
AFFILIATION α-proteobacteria

```
   1 AGTTTGATCA TGGCTCAGAA CGAACGCTGG CGGCAGGCTT AACACATGCA
  51 AGTCGAACGA TATAGTGGCA GACGGGTGAG TAACGCGTGG GAACGTACCT
 101 TTCACTACGG AATAGCTCTT GGAAACGAGT GGTAATACCG TATACGCCCT
 151 TCGGGGGAAA GATTTATCGG TGAAAGATCG GCCCGCGTTA GATTAGCTAG
 201 TTGGTAGGGT AATGGCCTAC CAAGGCGACG ATCTATAGCT GGTCTGAGAG
 251 GATGATCAGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG
 301 CAGCAGTGGG GAATCTTGCA CAATGGGCGA AAGCCTGATG CAGCCATGCC
 351 GCGTGAATGA TGAAGGCCTT AGGGTTGTAA AATTCTTTCG CTAGGGATGA
 401 TAATGACAGT ACCTAGTAAA GAAGCCCCGG CTAACTTCGT GCCAGCAGCC
 451 GCGGTAATAC GAAGGGGGCT AGCGTTGTTC GGAATTACTG GGCGTAAAGC
 501 GCACGTAGGC GGACTTTTAA GTCAGATGTG AAATCCCGGG GCTCAACCTC
 551 GGAACTGCAT TTGAAACTGG AAGTCTAGAG ACCAGGAGAG GTTAGCGGAA
 601 TACCGAGTGT AGAGGTGAAA TTCGTAGATA TTCGGTGGAA CACCAGTGGC
 651 GAAGGCGGCT AACTGGACTG GTACTGACGC TGAGGTGCGA AAGTGTGGGG
 701 AGCAAACAGG ATTAGATACC CTGGTAGTCC ACACCGTAAA CGATGAGAGC
 751 TAGTTGTTGG CAGGCATGCC TGTCGGTGAC GCAGCTAACG CATTAAGCTC
 801 TCCGCCTGGG GAGTACGGTC GCAAGATTAA AACTCAAAGA AATTGACGGG
 851 GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGCAGA
 901 ACCTTACCTA CCCTTGACAT ACCGATCGCG GTTTCCAGAG ATGGATTCCT
 951 TCAGTTAGGC TGGATCGGAT ACAGGTGCTG CATGGCTGTC GTCAGCTCGT
1001 GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTCATCCTTA
1051 GTTGCCATCA CGTTTGGGTG GCACTCTAA GGAAACTGCC GGTGGTAAGC
1101 CGGAGGAAGG TGGGGATGAC GTCAAGTCCT CATGGCCCTT ACGGGTAGGG
1151 CTACACACGT GCTACAATGG CAGTGACAAT GGGTTAATCC CCAAAAACTG
1201 TCTCAGTTCG GATTGTCGTC TGCAACTCGA CGGCATGAAG GTGGAATCGC
1251 TAGTAATCGT GGATCAGCAT GCCACGGTGA ATACGTTCCC GGGCCTTGTA
1301 CACACCGCCC GTCACATCAT GGGAATTGGT
```

NAME *A. lusitanicum* NEPCC 253 re-introduction bacteria 2
LENGTH 948 nucleotides
AFFILIATION α-proteobacteria

```
  1 GGAATAACAG TTAGAAATGA CTGCTAATAC CGGATGATGT CTTCGGACCA
 51 AAGATTTATC GGCAAGGGAT CANCCCGCGT AGGATTAGGT AGTTGGTGGG
101 GTAAAGGCCT ACCAAGCCGA CGATCCTTAG CTGGTCTGAG AGGATCAGCC
151 ACACTGGGCT GAGACACGGC CCAGACTCCT ACGGGAGGCA GCAGTGGGGA
201 ATATTGGACA ATGGGCGCAA GCCTGATCCA GCAATGCCGC GTGAGTGAGA
251 AGGCCTTCGG GTCGTAAAGC TCTTTTACCA GGGATGATAA TGACAGTACC
301 TGGAGAATAA GCTCCGGCTA ACTCCGTGCC AGCAGCCGCG GTA ATACGG
351 AGGGAGCTAG CGTTGTTCGG AATTACTGGG CGTAAAGCGC ACGTAGGCGG
401 CTACTCAAGT CAGGAGGTCA AAGCCCGGGG CTCAACCCCG GAACTGCCTT
451 TGAAACTAGG TAGCTGGAAT CTTGGAGAGG TCAGTGGAAT TCCGAGTGTA
501 GAGGTGAAAT TCGTAGATAT TCGGAAGAAC ACCAGTGGGA AGGCGACTGA
551 CTGGACAAGT ATTGACGCTG AGGTGCGAAA GCGTGGGGAN CAAACAGGAT
601 TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGATAACTA GCTGTCCGGT
651 CACTTGGTGA TTGGGTGGCG CAGCTAACGC ATAAGTTATC GCCTGGGGAG
701 TACGGTCGCA AGATTAAAAC TCAAAGGAAT TGACGGGGGC CTCCACAAGC
751 GGTGGAGCAT GTGGTTTAAT TGGAAGCAAC GCGCAGAACC TTACCAGCGT
801 TTGACATCCG CGCTACTTCC AGAGATGGAA GGTTCCCTTC GGGGACGCGG
851 TGACAGGTNC TGCATGGCTG TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT
901 AAGTCCCGCA ACGAGCGCAA CCCTCGTCCT TAGTTGCCAT CAT TTAGT
```

NAME *A. tamarense* PCC 173a re-introduction bacteria 1
LENGTH 1037 nucleotides
AFFILIATION α-proteobacteria

```
   1 AGAACGAACG CTGGCGGCAT GCCTACACAT GCAAGTCGAA CGAGATCTTC
  51 GGATCTAGTG GCGCACGGGT GCGTAACGCG TGGGAATCTG CCCTTGGGTT
 101 CGGAATAATG AGAAATTACT GCTAATACCG GATGTCTTCG ACCAAAGATT
 151 TATCGCCCAG GGATGAGCCC GCGTAGGATT AGGTAGTTGG TGGGGTAATGG
 201 CCTACCAAGC CGACGATCCT TAGCTGGTCT GAGAGGATGA TCAGCCACAC
 251 TGGGACTGAG ACACGGCCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT
 301 TGGACAATGG GCGAAAGCCT GATCCAGCAA TGCCGCGTGA GGATGAAGGC
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 351 CTTAGGGTTG TAAAGCTCTT TTACCAGGGA TGATAATGAC AGTACCTGGA
 401 GAATAAGTCC GGCTAACTCC GTGCCAGCAG CCGCGGTAAA CGGAGGGAGC
 451 AGCGTTGTTC GGAATTACTG GGCGAAAGCG CGCGTAGGCG GTTACTCAAG
 501 TCAGAGGTGA AAGCCCCGGG CTCAACCCCG GAACTGCCTT TGAAACTAGG
 551 TGACTAGAAT CTTGGAGAGGT CAGTGGAATT CGAGTGTAGA GGTGAAATTC
 601 GTAGATATTC GGAAGAACAC CAGTGGCAAG GCGACTGACT GGACAAGTAT
 651 TGACGCTGAG GTGCGAAAGC GTGGGGAGCA ACAGGATTAG ATACCCTGGT
 701 AGTCCACGCC GTAAACGATG ATAACTAGCT GTCCGGGTAC TTGGTACTTG
 751 GGTGGCGCAG CTAACGCATA ATTATCCGCC TGGGGAGTAC GGTCGCAAGA
 801 TTAAAACTCA AAGAATTGAC GGGGGCCTCA CAAGCGGTGG AGCATGTGGT
 851 TTAATTCGAA GCAACGCGCA GAACCTTACC AGCGTT TGA CATGCCGGTC
 901 GCGGATTTGG GAGACCATTT CCTTCAGTTC GGCTGGACCG TGCACAGGTG
 951  CTGCATGGCT GTCGTCAGCT CGTCGTGAGA TGTTGGGTTA AGTCCCGCAA
1001  CGAGCGCAAC CCTCGTCCTT AGTTGCCAGC ATTTGGT
```

NAME *A. tamarense* PCC 173a re-introduction bacteria 2
LENGTH 837 nucleotides
AFFILIATION α-proteobacteria

```
   1  TAGAGTTTGA TCATGGCTCA GAACGAACGC TGGCGGCAGG CTTAACACAT
  51  GCAAGTCGAG CGCCCCGCAA GGGGAGCGGC AGACGGGTGA GTAACGCGTG
 101  GGAATCTACC CATCTCTACG GAATAACTCA GGGAAACTTG TGCTAATACC
 151  GTATACGCCC TTCGGGGGAA AGATTTATCG GAGATGGATG AGCCCGCGTT
 201  GGATTAGCTA GTTGGTGGGG TAAAGGCCTA CCAAGGCGAC GATCCATAGC
 251  TGGTCTGAGA GGATGATCAG CCACACTGGG ACTGAGACAC GGCCCAGACT
 301  CCTACGGGAG GCAGCAGTGG GGAATATTGG ACAATGGGCG CAAGCCTGAT
 351  CCAGCCATGC CGCGTGTGTG ATGAAGGCCC TAGGGTTGTA AAGCACTTTC
 401  AACGGTgAAG ATAATGACGG TAACCGTAGA AGAAGCCCCG GCTAACTTCG
 451  TGCCAGCAGC CGCGGTAATA CGAAGGGGGC TAGCGTTGTT CGGAATTACT
 501  GGGCGTAAAG CGCACGTAGG CGGATCGTTA AGTGAGGGGT GAAATCCCAG
 551  GGCTCAACCC TGGAACTGCC TTTCATACTG GCGATCTTGA GTTCGAGAGA
 601  GGTGAGTGGA ATTCCGAGTG TAGAGGTGAA ATTCGTAGAT ATTCGGAGGA
 651  ACACCAGTGG CGAAGGCGGC TCACTGGCTC GATACTGACG CTGAGGTGCG
 701  AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA
 751  ACGATGAATG TTAGCCGTCG GGCAGTTTAC TGTTCGGTGG CGCAGCTAAC
 801  GCATTAAACA TTCCGCCTGG GGAGTAC
```

APPENDIX 4

DNA sequences from DGGE analysis

NAME Band 1
LENGTH 175 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 GGGGATCTTG GACAATGGGC GAAANCTGAT CNAGCCATGC CGCGTGTGTG ANGAATGCCC
61 TATGGTNGTN AAGCTCTTTC GCCNGGGANG ATAATGACAN TACCTGGTAA ANAAACCCCG
121 GNTAACTCCA TGCCAGCAGC CGCGGTNNTA TTCCNTGNCC NCAGGNATTG TAATA

NAME Band 2
LENGTH 175 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 TGGGGATCTT GGAAATGGGC GAAAGCTGAT CNAGCCATGC CGCGTGTGTG ATGAATGCCC
61 TANGGTCGTA AAGCTCTTTC GCCNGNGATG ATAATGACAG TANCTGGTAA AGAAACCCCG
121 GNTAACTCCN TGCCAGCAGC CGCGGTAATA TTCCGTGNCN CNAGGNANTG TAATA

NAME Band 3
LENGTH 174 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 GGGGATTTGG ACAATGGGCG AAACCTGATC CAGCCATGCC GCGTGTGTGA AGANGCCCTA
61 TGGTNGTTAA NCTCTTTCGC CNGAGAAGAA AANGACAATA CCTGNTNAAG TAAACNCCGG
121 NNNCCTCCAT GCCAGATCNN GCGGTNCTAT GCCATCANCC GCGGTAATAT AATA

NAME Band 4
LENGTH 175 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 GGGGATCTTN GACAATGGGC GCAACCTGAT CTAGCCATGC CGCGTGTGTG ACGAATGCCT
61 TAGGGTCGTN AAGCNCTTTC GCCTGTGANG AAAATGACAG TACCTGGTAA AGAAACCCCG
121 GCTAACTCCA TGCCAGCAGC CGCGGTNATA TGCCATCANC CGCGGTAATA TAATA

NAME Band 5
LENGTH 169 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 GGGGATCTTG GACAATGGGC GCAACCTGAT CNAGCCATGC CGCGTGTGTG ATGAAGNCNT
61 AGGGTNGTNA AGCTCTTTCG CCNGAGAAGA NAATGACAGT ATCTGGTAAA GAAACCCCGG
121 CTAACTCCGT GCCAGCAGCC GCGGTAATAT GCCANNAGCC GCGGTATTA

NAME Band 6
LENGTH 174 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 TGGGGATTTG GACATGGGCG NAAGCCTGAT CNAGCCATGC CGCGTGAGTG ATGAAGCCTT
61 AGGGTCGTAA AGCTCTTTCG CCNGAGATGA TAATGACNGT ANCTGGTAAA GAAACCCCGG
121 CTAACTCCGT GCCAGCAGCC GCGGTAATAT TCCATGNCCN NAGGCGCNGT AATA

NAME Band 7
LENGTH 169 nucleotides
AFFILIATION α-proteobacteria

1 CCTACGGGAG GCAGCAGTGG GGAATATTGG ACAATGGGGG CAAGCCTGAT CCAGCCATGC
61 CGCGTGNGTG AAGAAGTCCT TcGGGTTGTA AAGCTCTTTC GCATGGGAAG AAGATGACGG
121 TACCnTcaGA AGAACCCCGG CTAATTccGT GCCAGCAGCC GCGGTAATA

NAME Band 8
LENGTH 195 nucleotides
AFFILIATION γ-proteobacteria

1 CCTACGGGAG GCAGCAGTGG GGAATATTGG ACAATGGGCG AAAGCCTGAT CCAGCCATGC
61 CGCGTGTGTG AAGAAGGnCT TCGGnTTGTA AAGCACTTTC aTCcGGGAAG AAGTGcnTcc
121 NGCTAATACC TgaGgTncAT GACGnTACCg AuAGAATAAG cACCGGCTAA CTCCGTGCCA
181 GCAGCCGCGG TAATA

NAME Band 9
LENGTH 195 nucleotides
AFFILIATION γ-proteobacteria

1 CCTACGGGAG GCAGCAGTGG GGAATATTGG ACAATGGGCG AAAGCCTGAT CCAGCCATGC
61 CGCGTGTGTG AAGAAGgcCT TCGGGTTGTA AAGCACTTTT ATTCGGGAAG AAaGGNCTCn
121 TGCTNATACC TGTGGknyAG GACGNTACCa AaAGAagAAg cACCGGCTAA CTCCGTGCCA
181 GCAGCCGCGG TAATA

NAME Band 10
LENGTH 187 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

1 CCTACGgGAC ACaGCAGTGa gGaATATTGG ACAATGGGCG AGAnNCTGAT CCAGCCATGC
61 CGCGTGTnTG AAGACTGCCC TmTGGgTnnT AAACTnnTTT TATAGAGGAA GAaannGCGA
121 TaCgTgTATc TgTTTGACgG TACTcTACCA ATAAnGATCG GcTnncTCCn nnCCATCnCC
181 CnCGGTA

NAME Band 11
LENGTH 188 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

1 CTACGgGACA CAGCAGTGag GnATaTTGGA CAATgGnngA gAnTCTGATC CAGCCaTGCC
61 GCGTGTGTGA AGAnTGCCCT ATGGGTGnTA AAcTncTTTT ATAGaGGAAg AaACgCTGAT
121 ACGTGTATcT gTTTGACgGT ACTgTAAGAA TAAgcATCGg cTaanTcCnT gCCATCnCCC
181 gCGGTAAT

NAME Band 12
LENGTH 160 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 GGGGATCTTN GACAATGGGC GAAAGCTGAT CTAGCCATGC CGCGTGNGTG ATGAATGCCN
61 TAGGGTCGTA AAGCTCTTTC GCCNGNGATG ATAATGACAG TANCTGGTAA AGAAACCCCG
121 GCTAACTCCG TGCCAGCAGC CGCGGTAATA TTCCNTNNCG

NAME 253/29b
LENGTH 164 nucleotides
AFFILIATION α-proteobacteria

1 CNNACNNTAG GCAACAGTGG NGAATATTAN ACAATGGGCG CGAGCcTAAT eCAGCCATgC
61 cGeGTGNATG ATGAAGGCNT TAGGGTTGTA aAACTCTTTC GCTNGGgATG ATAATGACAG
121 TACCTAGTAA AgAAGCCCeG GCTAacTeca Tgecageage cgcg

NAME 253/30
LENGTH 185 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

1 TCGGGAGGCA GCAGTGagGA AATTGGAcAA TGGTGGAgAC aCTGATCCAG CCaTGCCGCG
61 TGTAgGAAGA CTGCCCTATG GGTTGTAAAC TACTTTTATA GAGGAAGAAA CGCaGATACg
121 TGTATTTGaT TgACGGTACT cTaCgAATaA nGaTCGGCTA AaTCCnTGCC AGCAGCCGCG
181 nAATA

NAME 253/31
LENGTH 169 nucleotides
AFFILIATION α-proteobacteria

1 CCTACGGGAG GCAGCAgTGG GGAaTaTTGC aCAATGGGCG AAAGCCTGAT GCAGCCATGC
61 CGCGTGAATG ATGAAGGCCT TAGGGTTGTA AAATTCTTTC GCTAGGGATG ATAATGACAG
121 TACCTAGTAA AGAAGCCCeG geTAACTTCG TGCCAGCAGC CGCGnAATA

NAME 253/33
LENGTH 169 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGGAG GCAGCAGTGG GGaATaTTGG ACAaTGGGCG aAAGCCTGAT CeAGCCaTGC
61 CGCGTGnGTG ATGAAgGCCT TAGGGTTGTA AAGCTCTTTC accgGTGATG ATAATGACAG
121 TAcCgGanGA AGAAacCeCG GCTAacTnCG TGCCAGCAGC CGCGnTATA

NAME 253/34
LENGTH 166 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CGGGAGGCAG CAgTGGGGAA TCTTaGacAa TGGGCGCAAG CCTGATCTAG CCATGCCGCG
61 TGaGTGAcGA AGGCCTTAGG GTCGTAAAGC TCTTTCGCCn GaGATGATAA TGACAGTATC
121 nGGTAAAGAA ACCCcGgcTA ACTCCGTGCC AGCAGCCGCG nnnATA

NAME 253/35
LENGTH 165 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CGGGAGGCAG CAGTGGGGAA TCTTagACAa TGGGCGCAAG CCTGATCTAG CCATGCCGCG
61 TGAGTGAcGA AGGCCTTAGG GTCGTAAAGC TCTTTCGCCa GAGATGATAA TGACAGTATC
121 nGGTAAAGAA ACCcCgigCTA ACTCCGTGCC AGCAGCCGCG nTATA

NAME 253/51
LENGTH 208 nucleotides
AFFILIATION unidentified

1 CCCTACGGGA GGCAGCAGTG ACnAAnnnTn GnCAACAggg GGGAnccTTa aaAagGcagC
61 aaGaccGaac TaaacaTacC ccgTGTgTGa cgAAnaacgc aTgaTcgTTa aAGaaCacTc
121 gcccciTgaTg acaaTgacAc TacaacGTAa aaAaAaCCac ccgaaCTCCG TGCCAGCAGC
181 CGnGnnATAT GCCAnCAnCC GCGGAATA

NAME 253/52
LENGTH 169 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGnAG GCAGCAGTGG GGaATCTTaG acAATGGGCG CAAGCCTgAT CTAGCCATGC
61 CGCGTGTGTG ACGAAgGccT TAnGGTcgTA AAGCACTTTC GCCTGTgATg aTAATgacag
121 TaccaggTaa agAAACCCCG GCTAACTCCG TGCCAGCAGC CGnGnnATA

NAME 407/1
LENGTH 186 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

1 TACGGnAGGC AGCAGTGaGG AaTaTTgcaC AATgGacGCA AGTCTGaTcC AgCCATGCCG
61 cGTGCAggAA gAATgCCcTa TGGGTTGTAa aCTGcTTTaT aTGGGaAgTa AnCCTcTnAC
121 gTGTaGAGAG cTgacGGTAC CAnncgAATa anCacCGgcT AACTcCnTGC CAGCAGCCGC
181 GGAATA

NAME 407/2
LENGTH 162 nucleotides
AFFILIATION α-proteobacteria

1 TCCTaGGgAG GCAGCAGTGG GGAATATTGC aCAATGGGCG CAAGCCTGAT GCAGCcATGC
61 cGCGTGAATG ATGAAGGCCT TAgGGTTGTA aAAcTCTTTC GcTaGGGATg ATAATGaCaG
121 TACCTAgTaA AGAAgCCCcG GcTAACTCCa TgCCAgCAgC CG

NAME 407/3
LENGTH 194 nucleotides
AFFILIATION γ-proteobacteria

1 CCTACGGGAG GCAGCAGTGG GGAATaTTGC ACAATGcaCg caAGCCTgAT GCAGCCaTgC
61 cGcGTGaGTg AaGAaGGCcT TAGGGTTGTA aAGCTCTTTC aGcTGgnnnn nnnnnnnnn
121 nnnnnnnnCC TGcTAaCaGT GAcgTcTACA TCaCAACAAG CACCGGcTaA CTCCGTGCCA
181 GCAGCCGCGT AATA

NAME 407/3a
LENGTH 162 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 AGGCAGCAGT GnGGAATCTT acaCAATGGG CGCAAGCCTG ATCTAGCCAT GCCGCGTGAG
61 TGATGAAGGC cTTAGGGTCG TAAAGCTCTT TCGCCAGaGA TGATAATGAC aGTATCTGgT
121 aAAGAAaCCC CGGCTAACTC CGTGCCAgCA GcCGCGGTAa TA

NAME 407/4
LENGTH 142 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 TACTaTAGGC aAcAGTGCGA AATTTTGaAC aATgGacGCA AgCcTgATCc AGCCaTgCCg
61 CgTGagTGAT GAAGGCcTTA gGaTCgTAAA gCTnnnnCna cannnaTnaT AATGacagTa
121 ccTggTcAAg angCCCgGGC TA

NAME 407/5
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGnAG GCAGCAGTGn GaAATcTTAG ACAATGGGCG CAAGCCTGAT CTAGCCATgC
61 cGCGTGAGTG ATGAAGGCCT TAGGGTCGTA AAGCTcTTTC GcCaGaGATG ATAATGaCaG
121 TATCTGGTnA AgAAaCCCCG GCTAACTCCG TGCCAGCAGC CgCGGTnATA

NAME 407/6
LENGTH 162 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 AGGCAGCAGT GnGGAATCTT GGACaATGGG CGcAAGCCTG ATCCAGCCaT GCCGCGTGAg
61 TGATGAAGGC CTTAGGGTcG TAAAgCTCTT TCgcCAGGGA aGATAATGAC nGTACCTGGT
121 nAAGAAnCCC CGGCTAACTC CGTGCCAGCA GCCGCGGTAA TA

NAME 407/7
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGGAG GCAGCAGTGn GGAATCTTAG ACAATGGGCG CAAGCCTGAT CTAGCCATGC
61 CGCGTGAGTG ATGAAGGCCT TAGGGTCGTA AAGCTCTTTC GCCTGTGAAG ATAATGACnG
121 TAgCAGGTaA AGAAaCCCCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA

NAME 407/8
LENGTH 166 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 ACGGGAGGCA GCAGTGGGGA ATCTTAGACA ATGGGCGCAA GCCTGATCTA GCCATGCCGC
61 GTGTGTGACG AAGGccTTAG GGTCGTAAAG CACTTTCGCC TGTGATGATA ATGACAGTAG
121 CAGGTAAAGA AACCCCGGCT AACTCCGTGC CAGCAGCCGC GTAATA

NAME 407/12
LENGTH 166 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CGunAGGCAn CAgTGgGgAA TCTTggACAa TGGGCgCAAG CcTGATcnAG CCATGCCGCG
61 TGAGTGATGA AGGcCTTAGG GTCGTAAAgC TCTTTCgCCA GaGATGATAA TGACAGTATC
121 TGgTaAAGAA aCcCcGgcTA ACTCCGTGCC AuCAnCCGCG GTnnTA

NAME 407/13
LENGTH 138 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 AGTggGGaAT cTTGGACaAT GGgcgCaagc CTgATCcAGC CATCCcgCGT GaGTGATgAa
61 GGccTTATGg TCGTAAAGCT cTTTcncTac nTaTnaTaAT gacagTaccg GnTAATAAaC
121 CCcGcanAnC TCCnTGCC

NAME 407/22
LENGTH 169 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CnTACGGnAG GCAGCAGTGG GGAaTcTTGG ACAATGGGCG CAAGCCTGAT CCAGCCATgC
61 CGCGTGAGTG ATGAAgGccT TAgGGTCGTA AAgCTcTTTC GcCAGAGATG ATAATGaCAG
121 TATCTGGTAA aGaAAcCCCG GCTAACTCCG TGCCAGCAGC CnCGnnATA

NAME 407/24
LENGTH 160 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CGGGAGGCAG CAGTGGGgaA TCTTGGACaA TGGGCGCAaG CCTGATCcAG CCaTGCCGCG
61 TGAGTGATGA AgGCCTTAGG GTCGTAAAGC TCTTTCGCCA GaGATGATAA TGACaGTACC
121 TGGTaaAgaa acCccgGCTA ACTCCGTGCC AGCAGCCGCG

NAME 407/24A
LENGTH 163 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTCGGGAGG CAGCAGTGgG gAATCTTGGA cAaTGGGCGc AaGCCTGATC cAGCCaTGCC
61 GCGTGAgTGA TGAAGgCcTT AgGGTTGTAA AgCTCTTTCa cCAGGGAaGA TAATGACgGT
121 ACCTGgTGAA GAAnCCCcGg cTAACTCCGT GCCAGCAGCC GCG

NAME 407/27a
LENGTH 168 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTAGGGAGG CAGCAGTGGG GaaTcTTAGa CAATGggCGC AAGCCTGATC nAgCCATGCC
61 GCGTGAGTGA TGAAgGCCTT AgGGTCGTAA AGCTCTTTCG CCaGAGATGA TAATGACaGT
121 AcCTGGTAAA GaAaCCCcGG CTAACTCCGT GCCAGCAGCC GCGnnATA

NAME 173a/1
LENGTH 146 nucleotides
AFFILIATION α-proteobacteria related to Roseobacter clade

1 TACGnnAGGC AACAGTGnGG AATnTTgcac aaTGGunngCA AgeCTGATCc AGCCaTGCcG
61 CGTGcGTaAT GAAgGCcTTA gGGTcgTAAA gCTcTTTcnc cATgGATGAT AaTGACgGTA
121 CcTGTAnnAG AnnCCCCGGA TaACTC

NAME 173a/2
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGGAG GCAGCAGTGG GGAATCTTAG ACAATGGGCG CAAGCCTGAT CTAGCCATGC
61 CGCGTGAGTG ATGAAGGCCT TAGGGTCGTA AAGCTCTTTC GCCAGAGATG ATAATGACAG
121 TATCTGGTAA AGAAACCCCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA

NAME 173a/3
LENGTH 148 nucleotides
AFFILIATION α-proteobacteria related to Roseobacter clade

1 TCcTACGnGA GGCAnCAGTG nGnAATnTTg CacaaTGGgc gcAAgCCTGA TCnAGCCaTG
61 CcGcGTGaGT gATGAAgGcc TTAGGgTngT AAAgCTcTTT cTcTAcaGAT GATAATGACg
121 GTACcTGTnC AAGAATCCCC GGaTnACT

NAME 173a/4
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTAnnGGAG uCACCAGnGG GGAATCTTAn ACAATGGGCG CAAgCcTGAT CTAgCCATgC
61 CGCGTGaGTG ATGAAgGCCT TAgGGTCGTA AAGCTCTTTC nccAgGGATG ATAATGaCag
121 TACCTGGTAA AGAAACCCCG GnTAAnTCCn TnCCAGCACn CGnGGTnATA

NAME 173a/5
LENGTH 165 nucleotides
AFFILIATION unidentified

1 CCTACnGTAG GCAACAnnGC nGnATnTTnn ACTAnGCAnC AGnGCnnAAT CTnACCCAnC
61 CGTGTCTAGC AnnATnnATn nAnGCTGcGT GAGCaTaTgn nGGnTaTAAG GTCGTnCAGC
121 TCTTTnnCCn TGGAgTnAnA ATGACGGTAC nnTcgaGAAn AAGCA

NAME 173a/6
LENGTH 142 nucleotides
AFFILIATION unidentified

1 TTGAGTGAAG AnCGTCCTaT GGGTCGTACa TGCTCTTgAA TnGATGATGA TCCTgTACAT
61 GAnCTGgTCA ATGAAACCCC AGcGTAAngT GTGCCCCAAn ACGCGenGAT ATACCCCCTa
121 CCGGAAGCnn CAGTCATcAg CC

NAME 173a/7
LENGTH 127 nucleotides
AFFILIATION α-proteobacteria related to Roseobacter clade

1 CnTAcGGTAG GCAnCAGTGG GGAATCTTAG CCAATGGGCG CnAGCnTnAT CTAGCCAngC
61 CGCGTGAGTT ATGAAGGCCT TAGGGTCGTA TAGCTCCTTC GCCnTGTATn ATAATGACnG
121 TACCGGT

NAME 173a/8
LENGTH 167 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CcTnCGngAG GCAGCAGTGC GTAATCTnnc aCaaTGGGcG CAAgCcTGAT gnAgcCATGC
61 cGCGTGAGTG AcgaAGGCCT TAGGGTcGTA aAGCTCTTTC GCTAGAGATg ATAATGACaG
121 TAccTGgTnA AgAAACCCCG GnTAACTCCg TGCCAGCAgC CGCnGTA

NAME 173a/9
LENGTH 122 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGGAG GCAGCAGTGG GGAATCTTAG ACAATGGGCG CAAGCCTGAT CTAGCCATGC
61 CGCGTGAGTG ATGAAGGCCT TAGGGTCGTA AAGCTCTTTC GCCAGAGATG ATAATGACAG
121 TA

NAME 173/10
LENGTH 191 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

1 CCTACGGGAG GCAGCAGTGA GGAATATTnG nCAATGGanG aGAcTCTGAT CCAgCCATGC
61 CGcGTGCAgg aAgAATgCCC TaTGgGTAgT AaucTgTTTT TaTacggnnn nnnnnnnnnC
121 TacGTGTTGC TTAcaTGACG GTACCnnTnG AATnAnGACC GGgTAACTnn nTGCCAGCAG
181 CCGCGGTAAT A

NAME 173a/11
LENGTH 195 nucleotides
AFFILIATION γ-proteobacteria

1 CCTAnGGGAG GCAGCAGTnG GGAATnTTGc aCaaTGggCg cAAGCCTgAT CCAGCCATnC
61 CGCGTGTgTg AaGAagGCcT TAGGgTTGTa AAGCaCTTTn AGTAgnnAgG nnnnnnnnnn
121 nnnnnnnnnn nnnnannnTT GAcggTaCcT acagaATAAg CaCCGGCTnA CTCCGTGCCA
181 GCAGCCGCGG TAATA

NAME 173a/12
LENGTH 166 nucleotides
AFFILIATION unidentified

1 GCaCTTGGaC GCAAgTCTGA TCCAGCCATG CCGCGTGCTT GATGAATGCC CTATGGGTTG
61 nAAgCTGTTT cTATAnagAT GATAAcgnnn GTACCTGTTC nAGacTCCCC GGnTcAnTcC
121 nAnCCAGcAT CCGTTAnTTT ATTCCaACag CCCCGGTAAT aAAATA

NAME 173a/13
LENGTH 150 nucleotides
AFFILIATION unidentified

1 CcTACGngAG GCAGCAGTGC GTAATnTnGC CCAATGGGGT nnAnCnTGAT CnnGCnATGC
61 TGCGTGAGnG AnGAAGGCCT TAGGGTCGTA AAGCTCTTTC ACCnCCGACG ATAATGACGG
121 TACCGcAGAA GAAGCACGGC TAaTTCAAAG

NAME 173a/14
LENGTH 80 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

1 TGGACGCAAC TCTGATCCAG CCATGCCGCG TGcGTGAAGA ATGCCnTATG GTTGnAAAgC
61 TsTTTCTnnTA CAGATGAAAA

NAME 173a/15
LENGTH 127 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGnAG GCAGCAGTGn GnAATCTTGC AcaaTGGggT CAAgccTGAT CeAGCCATGC
61 cGCGTGaGTg ATGaAgGCCT TAnGGTcGTA AAgCTcTTTC TnTACAGATG AAAAannCnn
121 TAnnTGT

NAME 173a/16
LENGTH 82 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 ggnAATnTTG nCnAATGGGG TCAAnCCTGA TCGAGCCATG CnGCGTGAGT GnTGAnGGCC
61 TTAGGGTCGT AAAGCTCTnT TC

NAME 173a/17
LENGTH 143 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CGnAAnGCAn CAGnGCGTAA TnTTGCacTT GGGcgcaAgC cTGATgnaGC CATGccGcGT
61 gnGTgATgaa gGCCTTAgGG TcGTAa/AgCT CTTTcACCag GGaTgATAaT GACaGTATnT
121 GnTCCAGAnA CCnCnGATAA CTC

NAME 173a/18
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGGAG GCAGCAGTGG GGAATnTTnG ACAATGGGCG CAAGCCTGAT CTAGCCATGC
61 CGCGTGAGTG ATGAAGGCCT TAGGGTCGTA AAGCTCTTTC GCCAGaGATG ATAATGACAG
121 TATCTGgTAA AGAAACCCCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA

NAME 173a/19
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGGAG GCAGCAGTGG GGAATCTTnG ACAATGGGCG CAAGCCTGAT CnAGCCATGC
61 CGCGTGAGTG ATGAAGGCCT TAGGGTCGTA AAGCTCTTTC GCCAGGGATG ATAATGACAG
121 TACCTGGTAA AGAAACCCCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA

NAME 173a/20
LENGTH 140 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CCTACGGGAG GCAGCAGTGG GGAATnTTnG ACAATGGGCG CAAGCCTGAT CTAGCCATGC
61 CGCGTGTGTG ATGAAGGCCT TAGGGTCGTA AAGCnCTTTC GCCAGGGATG ATAATGACAG
121 TAcaggTAAA GnACCACGGC

NAME 173a/21
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria

1 CCTACGGGAG GCAGCAGTGG GGAATaTTGG ACAaTGGGGG CAAgCCTGAT CcAGCCaTGC
61 CGCGTGAGTG ATGAAGGCCT TAGGGTTGTA AAGCTCTTTC ACcTGCGATG ATaATGACgG
121 TAcCagcaGA AgAAACCCCG GCTAAnTnCG TGCCAGCAGC CGCGGTAATA

NAME 173a/22
LENGTH 134 nucleotides
AFFILIATION α-proteobacteria

1 TCcTACGGAA GGCAGCAGTG nGGAATATTG nACAATGGGG GCAAGCCTGA TCCAGCCATG
61 CCGCGTGAGT GATGAAGGCC TTAGGGTTGT AAAGCTCTTT CACCAGGGAT GATAATGACn
121 GTACCGGcAG AanA

NAME 173a/23
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CcTnCGnAAG GCAGCAGTGn GnAATCTTac acaaTGgggg CAAGCcTGAT gTAgCCATGC
61 cGcGTgAGTG aTGAAGGCCT TAGGGTnGTA AAGCTCTTTc gCCAGAGATG ATAATGACAG
121 TATCTGgTca agAAACCCCG GnTAACTCCG TGCCAGCAgC CGCnGTAnTA

NAME 173a/24
LENGTH 145 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 GCGTAATncC CaATgcTcGc aagccTGATG caTccATGaC GcgTgAGTga cgaAGGCcTT
61 agGGTcGTAA aGCTCTTTca nCagugATgA TggTgACaGT aTCTGGnCCn TAAACCCTnG
121 nTAACTCCnT GCCAgCAAnC nCCGT

NAME 173a/25
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria related to *Roseobacter* clade

1 CcTACGGnAG GCAGCAGTGn GGAATATTGn nCAATGGGCG cAAGCCTGAT CCAGCaaTGC
61 CGCGTGaGTG ATGAAGgCCT TAGGGTTGTA AAGCTCTTTT ACCAGGGATG ATAATGACAG
121 TACCTGGAGA ATAAaCnccg gCTAACTCCG TGCCAGCAgC CGCGGTAATA

NAME Scripp/26
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria

1 CcTACGGnAG GCAGCAGTnn GGAATATTGc acaaTGGGCG naAgCCTGAT CCAGCAATGC
61 CGCGTGAgTG ATGAAGgCCT TAGGGTTGTA AAgCTCTTTC gCTAGGGATG ATAATGACaG
121 TACCTnGTAA AGAAACCCCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA

NAME Scripp/27
LENGTH 157 nucleotides
AFFILIATION cytophaga-flavobacter-bacteroides phylum

1 ACGnAAmnCA GCAGTGnnGn ATAnTAGGCA AnAGAaCnGA aTCTgATCcA gCCnTgcCgc
61 GTgcgTgaag aATgCCcTaT GnGaTGTAna CTGTTTTTaT aTngGnAGAn nnngagCTAC
121 GTGTagCTTA aTGACGGTAC CggaccAATa cAGaaCG

NAME Scripp/28
LENGTH 193 nucleotides
AFFILIATION γ-proteobacteria

1 CCTACGGGAG GCAGCAGTGG GGAATnTTGG ACAATGnACg AAAgCCTGAT CCAGCCATGC
61 CGCGTgnGTG AAGAAGGTCT TnGGaTTGTA AAGCTCTTTa AGnnnnnnnn nnnnnnnnnn
121 nnTTaACCTT AcTGTCTnGA CgaTnCCAAc nCAGnAAGCA CCGGcTnACT TCgTGCCAgC
181 AGCCGCnGTA ATA

NAME Scripp/29
LENGTH 195 nucleotides
AFFILIATION γ-proteobacteria

1 cCCTACGGGA GGCAGCAGTG GGGAATnTTG CACAATGGGC nAAAGcCTGA TnCAgcCATG
61 CCGCGTGTGT gAAGAAGGTC TTcGGaTTGT AAAGCaCTTT AAGTGGaAgG AAnnnnnnnn
121 nnnnnnnnCT TgacaGTcTT GACGkTaCcT ACacAaTaAG CACCGGCTAA CTcagTGCCA
181 GCAGCCGCGG TAATA

NAME Scripp/30
LENGTH 193 nucleotides
AFFILIATION unidentified

1 CTACGGGAGG CAnCAGTncG GAATCTcacc aaaggaccCA AGCCTaATcc aacaCaagCC

61 GCGTgAgTcA TGAAGGTCTT nGGnTgGTAA AgCTCTgTAn AGgaAnGAAc AunTgTGcAc
121 naccaagnn cGTcTTGACG GTacCTAnnC AGAAAGCCCC GGCTAACTaC GTGCCAGCAG
181 CCGCGGTAAT AAA

NAME Scripp/31
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria

1 CCTAcGcAAn ACAGCAGTGG GGAATAnTnn ACAATGGGGg CTAgcCTgAT cCagaCATGc
61 CGCGTgAGTg ATgAaGCCCT TAGGGTTGTA AAgCTCTTTC nnCnGGgaCG ATaaTGACGG
121 TACCGGnnnA ATAAACCCCG GCTAACTTCG ngCCATCAnn CGCGGyAATA

NAME Scripp/32
LENGTH 192 nucleotides
AFFILIATION unidentified

1 CAccTACGGG aCGcTnnGnA AGnCAGCATT GGGGAATATT GCaCAATGGG CGgAAGCCTG
61 ATGCAGCaAC GCCGCgTgcG GcATGAcGGC TTCgGgTTGT AAACCGCTTT CgccTGggAc
121 gAAGCgTgAG TgACGGTAaG naTnAAgAgC ncCGnnTAAC TACGTGCCng CAnnCcAGGT
181 AATACGGTAA TA

NAME CCMP 117 band from "axenic" culture
LENGTH 170 nucleotides
AFFILIATION α-proteobacteria

1 TCCTACGGGA GGCAGCAgTG GgGAATaTTg GACAATGGGC GCAAGCcTGA TCcAgCCATG
61 CCGCGTGAGT GATGAAGGCC TTAGGgTnGT AAAGCTCTTT CACCaGGGAT GATAATgACA
121 GTACCTGGna AAgAAaCCCc GGCTAACTCc gTGCCAGCAG CCGCGGAATA

APPENDIX 5

Bacterial Culture Media

All chemicals unless otherwise stated were supplied by Sigma Chemicals.
All media formulations were adjusted to pH 7.6 prior to autoclaving.

Marine Agar

Bacto-Marine Agar 2216 (Difco code 0790)	55.1g/l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Marine Broth

Bacto-Marine Broth 2216 (Difco code 0791)	37.4g/l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

The recipes below are for broth cultures. For plates 15g/l technical agar (agar no. 3; Oxoid L13) was added prior to autoclaving.

$ST10^{0}$ medium

Trypticase	1g/l
Yeast extract	0.1g/l
Seawater	1 l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

$ST10^{-1}$ medium

Trypticase	0.1g/l
Yeast extract	0.01g/l
Seawater	1 l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

$ST10^{-4}$ medium

Trypticase	0.1mg/l
Yeast extract	0.01mg/l
Seawater	1 l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

1/100 strength Marine Broth

Bacto-Marine Broth 2216 (Difco code 0791)	0.374g/l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Seawater medium

Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Peptone Seawater A medium

Ferric Phosphate	0.1g/l
Bacto-peptone	5g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Peptone Seawater B medium

Peptone	1g/l
Ferric Phosphate	0.1g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Peptone Seawater B without Iron medium

Peptone	1g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Malt extract medium

Malt Extract	10g/l
Bacto-peptone	5g/l
Distilled water	250ml
Seawater	750ml

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Yeast extract medium

Yeast extract	0.1g/l
Ferric Phosphate	0.1g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Yeast extract without Iron medium

Yeast Extract	0.1g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

f/2 medium

f/2 Guillard's marine water enrichment solution without silicate. (Sigma catalogue number G 0154)	20ml/l
Seawater	980ml

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Casein Seawater medium

Bacto-peptone	0.5g/l
Soluble Casein	0.5g/l
Soluble Starch	0.5g/l
Glycerol	1ml/l
Dipotassium hydrogen phosphate	0.2g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Peptone Glucose medium

Glucose	1g/l
Bacto-peptone	1g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Peptone Yeast medium

Peptone	1g/l
Yeast Extract	1g/l
Ferric Phosphate	0.1g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

Peptone Yeast without Iron medium

Peptone	1g/l
Yeast Extract	1g/l
Seawater	1l

Boiled for 2 minutes and autoclaved at 121°C for 15 minutes.

APPENDIX 6

LIST OF SUPPLIERS

Applied Biosystems	Foster City, California, USA.
Amicon Ltd	Upper Mill, Stonehouse, Gloucestershire.
Bioline	16 The Edge Business Centre, Humber Road, London.
BIORAD	Biorad House, Maylands Avenue, Hemel Hempstead, Herts.
BDH	Hunter Boulevard, Magna Park, Lutterworth, Leics.
CCMP	Bigelow Laboratory, West Boothbay Harbor, Maine, USA.
Difco	Michigan, USA.
Gibco BRL	3 Washington Road, Paisley.
Merck	Burnfield Avenue, Thornliebank, Glasgow.
Millipore	The Boulevard, Blackmoor Lane, Watford, Herts.
Molecular Probes Inc	Molecular Probes Europe BV, PoortGebouw, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.
NRC-Canada	1411 Oxford Street, Halifax, Nova Scotia, Canada.
OSWEL DNA Service,	Department of Chemistry, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh.
PCC	Citadel Hill, Plymouth, PL1 2PB.
Pharmacia	Davy Avenue, Knowlhill, Milton Keynes.
Porvair Filtronics	Unit 6, Shepperton Business Park, Govett Avenue, Shepperton, Middlesex.
Promega	Epsilon House, Enterprise Road, Chilworth Research Centre, Southampton.
Qiagen	Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey.
Rathburn Chemicals Ltd	Caberston Road, Walkerburn, Peebles. Scotland. EH43 6AU.
Sigma Chemical Co.	Fancy Road, Poole, Dorset.
UW	University of Westminster.
Whatman Scientific	St. Leonard's Road, 20/20 Maidstone, Kent.