Characterisation of bacterial communities associated with toxic and non-toxic dinoflagellates: *Alexandrium* spp. and *Scrippsiella trochoidea*

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**Abstract**

Several dinoflagellate species have been shown to produce potent neurotoxins known as paralytic shellfish toxins. Evidence is also accumulating that marine bacteria associated with dinoflagellates play a role in the accumulation of paralytic shellfish toxins. In this study, the diversity of bacteria in cultures of both toxic and non-toxic dinoflagellates, *Alexandrium* spp. and *Scrippsiella trochoidea*, were compared using colony morphology, restriction fragment length polymorphisms, denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes and, ultimately, sequence determination of the 16S rRNA genes. The results suggest that a number of different bacterial species are associated with dinoflagellates, some of which are common to each of the dinoflagellate cultures examined, whereas others appear to be unique to a particular dinoflagellate. The phylogenetic diversity of the bacteria observed was limited to two bacterial phyla, the Proteobacteria and the Cytophaga-Flavobacter-Bacteroides (CFB). Although phylum level diversity was limited, many distinct phylogenetic clades were recovered, including members of both the α- and γ-subclasses of the Proteobacteria. Additionally, several of the bacterial phylotypes isolated were not closely related to any published bacterial species but, rather, were identical to isolates characterised from *Alexandrium* cultures 4 years earlier. Finally, many of the bacteria isolated from the dinoflagellate cultures were related to microorganisms with known surface-associated life histories (e.g. the CFB phylum, *Hyphomonas*, *Caulobacter* and some members of the *Roseobacter* clade including *Ruegeria algicola*). © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Dinoflagellate; Marine bacterium; Paralytic shellfish toxin; Polymerase chain reaction-restriction fragment length polymorphism; Polymerase chain reaction-denaturing gradient gel electrophoresis

1. **Introduction**

Interactions between algae and bacteria are commonly observed in both freshwater and marine ecosystems with bacteria increasingly cited as potentially important regulators in processes of algal bloom initiation, maintenance and decline [1]. Bacteria are an inherent part of the physical environment of dinoflagellates, both in vitro and in vivo and, as such, may be considered dinoflagellate symbionts [2,3]. However, the ecological significance of most naturally occurring bacterial–algal associations is unclear.
and, in most cases, the bacterial species involved have not been identified. This is particularly the case with algae associated with the occurrence of toxins in shellfish, such as the paralytic shellfish toxin (PST) producing *Alexandrium* spp.

Although the value of 16S rRNA gene sequence data for identifying bacteria is rarely disputed, this information is severely limited for bacteria associated with toxic marine dinoflagellates. A recent study by Prokic et al. [4] investigated the diversity of bacteria associated with *Prorocentrum lima*, a known diarrhoeic shellfish poison producing dinoflagellate, via microbial cultivation and 16S rRNA gene cloning and sequencing. The authors found Roseobacter-related microorganisms of the α-Proteobacteria to dominate the *P. lima* microflora. Babichak et al. [5] used fluorescently labelled 16S rRNA targeted oligonucleotide probes to identify bacteria from toxic *Alexandrium* spp. as members of the α- and γ-Proteobacteria. However, resolution in this study was low, and a more detailed characterisation would be required to resolve any differences between the bacterial populations of toxic and non-toxic dinoflagellates. Several authors have also reported that bacteria isolated from *Alexandrium* spp. cultures are capable of producing PST but, again, limited information on their identity in the form of 16S rRNA gene sequence data is available [6–9].

The issue of bacterial PST toxigenesis is currently not resolved [3]. However, previous studies in our laboratory have indicated that the bacteria associated with cultures of the dinoflagellates *Alexandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 influence the toxicity of these dinoflagellates [10]. In order to elucidate the contribution of particular bacterial species to dinoflagellate/shellfish toxicity and bloom dynamics, it is essential that the ecology of bacteria associated with marine dinoflagellates such as *Alexandrium* spp. be studied. As a prelude to investigating algal–bacterial dynamics in the environment, it is necessary to establish which bacterial taxa are involved, initially requiring high resolution characterisation of the bacteria associated with dinoflagellate cultures. To this aim, using a combination of phenotypic (colony morphology) and molecular-based techniques (16S rDNA restriction fragment length polymorphisms (RFLP), denaturing gradient gel electrophoresis (DGGE) and, ultimately, sequence determination of PCR-amplified 16S rRNA genes), comparisons of the diversity of bacteria associated with PST producing dinoflagellate strains, *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 [11–13] and those of *A. tamarense* PCC 173a and Scrippsiella trochoidea NEPCC 15 which have no previously reported link with toxicity [14], were made. Furthermore comparisons were made with bacterial isolates obtained from *Alexandrium* spp. cultures 4 years prior to this investigation [6].

### 2. Materials and methods

#### 2.1. Dinoflagellate cultures

The dinoflagellates *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407, *A. tamarense* PCC 173a and *S. trochoidea* NEPCC 15 were cultured in Guilliards’ f/2 without silicate (Sigma, Dorset, UK) enriched seawater [15]. Cultures were maintained at 15°C with a 14:10 h, light:dark cycle (irradiance level 0.5–1.5×10^6 quanta s⁻¹ cm⁻²). Culture development was monitored daily by triplicate cell counts using a Sedgwick–Rafter counting chamber and visualised by the addition of Lugol’s iodine [16]. NEPCC strains were supplied by the North East Pacific Culture Collection, British Columbia, Canada and *A. tamarense* PCC 173a was from the Plymouth Culture Collection, Plymouth, UK.

#### 2.2. Isolation of bacteria from dinoflagellate cultures

Samples (1 ml) of dinoflagellate cultures from early, late exponential and stationary growth phases were serially diluted (10-fold dilutions; neat – 10⁻³) in sterile seawater. Aliquots (100 μl) of each dilution were spread, in triplicate, onto marine agar medium (Difco 2216, Michigan, USA) and subsequently incubated for 14 days at 20°C. Bacteria from the dilution containing between 50 and 100 colonies were isolated from one of the replicate plates and replated individually onto marine agar, to obtain pure cultures. Resultant bacterial isolates were categorised by colony morphology and examined microscopically for their reaction to Gram stain. Isolates were stored in marine broth plus 10% (v/v) glycerol at −70°C, prior to RFLP investigation.

#### 2.3. RFLP analysis

Bacteria isolated from dinoflagellate cultures were resuspended in ATL buffer (Qiagen, Dorking, UK) and genomic DNA from each isolate was extracted following the QIAamp tissue protocol (Qiagen, Dorking, UK). DNA was stored at −20°C prior to PCR amplification. Small subunit (SSU) 16S rDNA was PCR-amplified from total genomic DNA, using the universal eubacterial primers 27F and 1522R (Bioline, London, UK) [17]. In a final volume of 50 μl, the reaction mixture contained 100 pmol of each amplification primer, 100 μM each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 10 ng template and 5 U of Taq DNA polymerase (Bioline, London, UK), which was added after a pre-cycling step in which the reaction mixture was heated to 95°C for 5 min. Amplification conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, for a total of 35 cycles followed by 72°C for 10 min [17]. Amplification products were visualised by electrophoresis using a 2% (w/v) agarose gel (Sigma, Dorset, UK).
containing ethidium bromide (0.5 mg ml$^{-1}$), with a 1-kb marker (Gibco, Glasgow, UK) included on the gel for reference.

To generate RFLP patterns, approx. 700 ng of each PCR product was digested with 5 units of the restriction endonuclease HaeIII (Promega, Southampton, UK), following the manufacturer’s specifications. Reactions were stopped by the addition of EDTA, and fragments were resolved by gel electrophoresis in 2.5% low melting point agarose gel (Gibco, Glasgow, UK), containing ethidium bromide (0.5 g ml$^{-1}$). DNA fragments were visualised under UV illumination with RFLP patterns grouped using Phoretix ID v 4 computer software (Phoretix International, Newcastle upon Tyne, UK).

2.4. DGGE analysis of 16S rDNA from dinoflagellate cultures

Cell pellets from 1-l cultures, at early, late exponential and stationary growth phases, of *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a and *S. trochoidea* NEPCC 15 were obtained by centrifugation at 10,000×g for 10 min. The cell pellet was resuspended in TE buffer (1 ml) and stored at −20°C until extraction of DNA. Following thawing, glass beads (0.5 g) of 0.16−1.17 mM diameter (Sigma, Dorset, UK) were added to the samples, which were alternately vortexed and placed on ice for 60 s, until visual signs of dinoflagellate cell lysis were observed. Following addition of 50μl lysozyme (50 mg ml$^{-1}$ in distilled water) (Sigma, Dorset, UK), samples were incubated for 30 min at room temperature. Sodium dodecyl sulfate (SDS) (100 μl, 10% (w/v) in distilled water) (Sigma, Dorset, UK) was mixed with each sample, followed by addition of 50 μl proteinase K (20 mg ml$^{-1}$ in distilled water) (Sigma, Dorset, UK) and further incubation at room temperature for 30−60 min before phenol/chloroform extraction [18].

Bacterial 16S rDNA was amplified using primers described by Muyzer et al. [19] which generated amplification products corresponding to nucleotide positions 341−534 in *Escherichia coli*. Amplification conditions were 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s, for 30 cycles followed by 72°C for 10 min. Amplification products were visualised by electrophoresis using a 2% (w/v) agarose gel (Sigma, Dorset, UK) containing ethidium bromide (0.5 mg ml$^{-1}$), with a 100-bp marker (Gibco, Glasgow, UK) included on the gel for reference.

DGGE was performed with a D-code 16/16 cm gel system using a 1.0 mm gel width (Bio-Rad, Herts, UK) maintained at a constant temperature of 65°C in 6 l of 1×TAE buffer (20 mM Tris acetate, 0.5 mM EDTA, pH 8.0). Gradients were formed between 20 and 60% denaturant (with 100% denaturant defined as 7 M urea and 40% (v/v) formamide). Gels were run at 130 V for 4−6 h and stained in 1×TAE containing ethidium bromide (0.5 mg l$^{-1}$) before destaining in distilled water for 15 min. Profiles were inspected under UV illumination.

DGGE bands were excised using a sterile razor blade and DNA eluted overnight in 100 μl of TE buffer. DNA was recovered using the Wizard PCR prep DNA purification system (Promega, Southampton, UK), with 5-μl aliquots of DNA from each excised band used as template in PCR amplifications as described above. PCR products were purified using Qiagen PCR spin columns (Qiagen, Dorking, UK).

2.5. 16S rDNA sequence determination and phylogenetic analysis

Representative bacterial isolates, from dinoflagellate cultures, possessing unique colony morphologies or RFLP patterns and bacteria previously isolated from dinoflagellate strains *A. tamarense* UW2c, *A. tamarense* UW4, *A. tamarense* NEPCC 407, *A. lusitanicum* NEPCC 253 and *Alexandrium affine* NEPCC 667 [6] were selected for DNA sequence analysis. Several bacterial isolates from each group were partially sequenced in order to confirm colony morphology and RFLP analyses were effective at classifying the cultured isolates. However, only one representative sequence from each group, spanning nearly the entire 16S rRNA gene, was submitted to the database. 16S rRNA gene PCR amplicons were purified using Qiaquick-spin columns (Qiagen, Dorking, UK) and subsequently sequenced bidirectionally, using universal 16S rRNA primers [20]. PCR amplicons obtained from DGGE profiles were also sequenced bidirectionally, using primers described by Muyzer et al. [19], on a 373A automated DNA sequencer using *Taq* polymerase initiated cycle sequencing with fluorescent dye-labelled dideoxynucleotides with protocols recommended by the manufacturer (Perkin Elmer, Bucks, UK). Nucleotide sequence fragments generated were assembled into consensus sequences using the GCG package v 7.0-UNIX [21].

The automatic alignment function of the ARB sequence analysis software package [22] was used initially to align the 16S rRNA gene sequences with reference sequences obtained from GenBank [23], the Ribosomal Database Project (RDP) [24], and the ARB database (Department of Microbiology, Technical University of Munich, Munich, Germany). The Genetic Data Environment (GDE) v 2.2 [25] sequence analysis software package was used subsequently to refine the alignment generated by ARB manually. Pairwise sequence similarities were generated, using GDE, and individual sequence masks employed for each pair of sequences analysed.

2.6. Nucleotide sequence accession numbers

Nucleotide sequences from the cultured bacteria were
3. Results

3.1. Diversity of culturable bacteria

Table 1 lists the 16S rDNA sequences most closely related to those of the 12 bacterial strains previously isolated from stationary phase batch cultures of *Alexandrium* spp. by Gallacher et al. [6]. All strains examined were clearly placed in the α- and γ-Proteobacteria subphyla, with the exception of strain 667-16, which was Gram-positive and most closely related to *Micrococcus luteus*. Other bacteria isolated from the weakly toxic *A. affine* NEPCC 667 belonged to the *Roseobacter* clade. Some bacteria isolated from *A. lusitanicum* NEPCC 253 were also associated with the *Roseobacter* clade and showed high sequence similarities with bacterial 16S rRNA gene clones recovered from a culture of the toxic dinoflagellate *P. lima* [4]. However, these strains were found to be related only distantly to the original *Roseobacter algicola* (now *Ruegeria algicola*) isolated from the phycosphere of *P. lima* (data not...
The 16S rDNA sequences of other bacterial strains isolated from *A. lusitanicum*, *A. tamarense* NEPCC 407 and UW2c, possessed high sequence similarities with those of the *Q*-Proteobacterium *Alteromonas macleodii*, a common inhabitant of coastal water and open oceans [26], and *Marinobacter aquaeolei*, originally isolated from the North Sea [27].

All bacteria isolated from *Alexandrium* spp. and *S. trochoidea* cultures in this investigation were identified as Gram-negative rods, with the majority of isolates (90%) forming distinct non-pigmented colonies; other bacteria exhibited pink, brown and yellow pigmentation following growth on marine agar (Table 2). Using colony morphology and RFLP profiles, the cultured bacteria were categorised into 26 groups (Fig. 1, Table 2). Based on 16S rDNA sequence analysis of representative isolates from each RFLP group, bacteria were identified as belonging to the *K*-Proteobacteria (with many sequences falling within the *Roseobacter* clade), *Q*-Proteobacteria and the Cytophaga-Flavobacter-Bacteroides (CFB) phylum (Table 2).
Table 2
Characterisation of bacteria isolated on marine agar, from the four dinoflagellate cultures *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407, *A. tamarense* PCC 173a and *S. trochoidea* NEPCC15

<table>
<thead>
<tr>
<th>RFLP pattern</th>
<th>Colony morphology</th>
<th>Representative isolate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of similar bacteria sequenced</th>
<th>Most closely related sequence from a validly described bacterial species (GenBank accession No.)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sequence similarity (%)</th>
<th>Most closely related database sequence (GenBank accession No.)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Sequence similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Proteobacteria</td>
<td>circular, smooth surface, umbonate, cream</td>
<td>ALUS253_28</td>
<td>7</td>
<td><em>Ahrensia kielense</em> (D88524)</td>
<td>98.5</td>
<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>irregular, with lobate margin, cream</td>
<td>ALUS253_25</td>
<td>1</td>
<td><em>Stappia stellulata</em> (D88525)</td>
<td>98.6</td>
<td>PTB1 (Y10913)</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td>circular, smooth, raised, cream</td>
<td>ATAM407_68</td>
<td>1</td>
<td><em>Phyllobacterium myrsinacearum</em> (D12789)</td>
<td>96.8</td>
<td>PTB5 (AJ000646) env.RSHD13510 (AF190214) gene clone from <em>Pfiesteria</em> dinoflagellate culture CCMP 1829 env.HNSS21 from marine sponge (Z85872) endocytic bacterium Noc17 (AF262750)</td>
<td>99.4 97.9 97.0 99.5</td>
</tr>
<tr>
<td></td>
<td>irregular with undulate margin, convex, brown centre with cream margin</td>
<td>SCRIPPS_94</td>
<td>3</td>
<td><em>Aquaspirillum stertori</em> (Z29620)</td>
<td>90.9 env.RSHD3S10 (AF190214) gene clone from <em>Pfiesteria</em> dinoflagellate culture CCMP 1829</td>
<td>97.9 97.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>circular, entire, convex, smooth, brown centre with cream margin</td>
<td>SCRIPPS_739</td>
<td>2</td>
<td><em>Hyphomonas johnsonii</em> (AF082791)</td>
<td>98.7</td>
<td>env.PRLISY03 (Y15348) gene clone recovered from <em>Prorocentrum lima</em> env.kpc 34 (AF194397) gene clone recovered from <em>Aureococcus anophagefferens</em></td>
<td>99.7 99.9</td>
</tr>
<tr>
<td></td>
<td>circular, pulvinate, entire, smooth, white centre with cream margin</td>
<td>SCRIPPS_423</td>
<td>2</td>
<td><em>Hyphomonas oceanitis</em> (AF082797)</td>
<td>90.9</td>
<td>uncultured bacterium BURTON-39 env. <em>Kps</em> 34 (AF194397) gene clone recovered from <em>Aureococcus anophagefferens</em></td>
<td>96.3</td>
</tr>
<tr>
<td></td>
<td>circular, pulvinate, smooth, cream</td>
<td>SCRIPPS_426</td>
<td>1</td>
<td><em>Caudobacter vibrioides</em> (AJ227754)</td>
<td>93.7</td>
<td>uncultured <em>Caudobacter</em> SC2 (AF245033) env.D049 (AF177568) gene clone recovered from seawater env.EK018 (AF142857) gene clone recovered from seawater env.MC-1 (AF188164) gene clone from <em>Pfiesteria</em> sp.</td>
<td>94.8 95.5 95.4</td>
</tr>
<tr>
<td></td>
<td>circular, convex, cream with light pink centre</td>
<td>ALUS253_1</td>
<td>3</td>
<td><em>Ruegeria algicola</em> (X78313)</td>
<td>94.5</td>
<td>env.PR LICY03 (Y15348) gene clone recovered from <em>Prorocentrum lima</em> env.kpc 34 (AF194397) gene clone recovered from <em>Aureococcus anophagefferens</em></td>
<td>99.7 99.9</td>
</tr>
<tr>
<td></td>
<td>circular, convex, cream with light pink centre</td>
<td>ATAM407_61</td>
<td>2</td>
<td><em>Roseovaria tolerans</em> (Y11551)</td>
<td>96.5</td>
<td>env.PR LICY03 (Y15348) gene clone recovered from <em>Prorocentrum lima</em> env.kpc 34 (AF194397) gene clone recovered from <em>Aureococcus anophagefferens</em></td>
<td>99.7 99.9</td>
</tr>
<tr>
<td></td>
<td>circular, convex, cream with light pink centre</td>
<td>ALUS253_43</td>
<td>10</td>
<td><em>Roseovaria tolerans</em> (Y11551)</td>
<td>99.6</td>
<td>env.D048 (AF177568) gene clone recovered from seawater env.EK1018 (AF142900) gene clone recovered from seawater env.MC-1 (AF188164) gene clone from <em>Pfiesteria</em> sp.</td>
<td>95.5 95.4 95.4</td>
</tr>
<tr>
<td></td>
<td>irregular with an undulate margin, smooth, convex, dark brown</td>
<td>ATAM407_25</td>
<td>4</td>
<td>as above</td>
<td>94.3</td>
<td>env.D049 (AF177568) gene clone recovered from seawater env.EK1018 (AF142900) gene clone recovered from seawater env.MC-1 (AF188164) gene clone from <em>Pfiesteria</em> sp.</td>
<td>95.5 95.4 95.4</td>
</tr>
<tr>
<td></td>
<td>irregular with lobate margin, convex, smooth, brown centre</td>
<td>ATAM407_58</td>
<td>1</td>
<td><em>Sulfobacter mediterraneus</em> (Y17387)</td>
<td>98.7</td>
<td>env.D049 (AF177568) gene clone recovered from seawater env.EK1018 (AF142900) gene clone recovered from seawater env.MC-1 (AF188164) gene clone from <em>Pfiesteria</em> sp.</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>irregular with lobate margin, convex, smooth, brown centre</td>
<td>ATAM173a_16</td>
<td>1</td>
<td><em>Staekia gutiformis</em> (Y16427)</td>
<td>99.2</td>
<td>667-4 (see Table 1)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>irregular with lobate margin, convex, smooth, brown centre</td>
<td>ATAM173a_17</td>
<td>1</td>
<td><em>Sulfobacter mediterraneus</em> (Y17387)</td>
<td>99.7</td>
<td>–</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>irregular with lobate margin, convex, smooth, brown centre</td>
<td>ATAM173a_49</td>
<td>3</td>
<td><em>Sulfobacter pontiacus</em> (Y13155)</td>
<td>96</td>
<td>–</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>irregular with lobate margin, convex, smooth, brown centre</td>
<td>SCRIPPS_732</td>
<td>2</td>
<td><em>Sulfobacter pontiacus</em> (Y13155)</td>
<td>99.8</td>
<td>–</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> ALUS: Alga Laboratory Use Sample NEPCC: National Environmental Protection Council Culture Collection NEPCC: National Environmental Protection Council Culture Collection

<sup>b</sup> Sequence similarity to the most closely related sequence from a validly described bacterial species

<sup>c</sup> Sequence similarity to the most closely related database sequence from a validly described bacterial species
Table 2 (continued)

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<th>Most closely related database sequence (GenBank accession No.)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Sequence similarity (%)</th>
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<tbody>
<tr>
<td>12</td>
<td>circular with entire margin, smooth, cream</td>
<td>ATAM407_56 7</td>
<td>Roseobacter gallaeciensis (Y13244)</td>
<td>96.9</td>
<td>667-2 (see Table 1)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>circular with erose margin, granular, umbonate with a brown centre and pale margin</td>
<td>SCRIPPS_101 2</td>
<td>Sulfitobacter brevis (Y16425)</td>
<td>97.2</td>
<td>Sulfitobacter sp. DSS-2 (AF098490)</td>
<td>97.3</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>circular, smooth, gelatinous, raised, cream</td>
<td>ATAM173a_51 2</td>
<td>Antarcobacter heliothermus (Y11552)</td>
<td>99.6</td>
<td>667-12 (see Table 1)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>punctiform, gelatinous, pink</td>
<td>ATAM173a_9 2</td>
<td>Roseovarius tolerans (Y11551)</td>
<td>96.3</td>
<td>env.PR.LISY03 (Y15348) gene clone recovered from Protroncentrum lima</td>
<td>98.3</td>
<td></td>
</tr>
</tbody>
</table>

γ-Proteobacteria

| 15 | circular, smooth, gelatinous, flat, cream | ATAM407_18 3 | Alteromonas macleodii (X82145) | 98.9 | 407-2 (see Table 1) | 99.9 |

| 16 | circular, convex, gelatinous, yellow | ATAM173a_5 1 | Alteromonas macleodii (AF173965) | 93.7 | 2c3 (see Table 1) | 97.9 |
| 17 | circular mucoid, gelatinous, large cream | ATAM407_2035 2 | Marinobacter aquaeolei (AF173969) | 99.3 | 253-19 (see Table 1) | 97.7 |

| 18 | irregular, lobate, flat, yellow | SCRIPPS_740 1 | Pseudalteromonas haloplankis subsp. tetraodonis (AF214730) | 99.8 | polar gas vacuolate strain 214.6 (U73724) | 97.1 |

Cytophaga-Flavobacter-Bacteroides (CFB)

| 19 | circular, gelatinous, raised, bright yellow | ATAM173a_2 2 | Polaribacter igrenii (M61002) | 96.6 | EBAC34 (AF268227) gene clone isolated from seawater | 98.0 |
| 20 | circular, flat, yellow | ATAM173a_3 3 | Gelibacter algens (U62914) | 92.3 | marine psychrophile SW17 (AF001368) | 94.6 |

| 21 | circular, convex, smooth, cream | SCRIPPS_413 1 | Psychrosomonas bartonensis (U62913) | 93.1 | Cytophaga sp. KT02ds18 (AF235126) | 98.1 |
| 22 | circular, entire, raised, gelatinous, yellow | ALUS253_6 2 | Gelibacter algens (AF001367) | 92.0 | Flavobacterium sp. 5N-3 (AB032514) | 94.8 |

Pseudoalteromonas haloplankis subsp. tetraodonis (AF214730) | 94.6 |

<sup>a</sup> Indicates validly described sequence showed highest sequence similarity.

<sup>b</sup> 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.

<sup>c</sup> Most closely related sequence from a validly described bacterial species indicates the defined type strain with highest 16S rDNA sequence similarity to each particular isolate.

<sup>d</sup> Most closely related database sequence indicates the 16S rDNA sequence within the database which has highest 16S rDNA sequence similarity to each particular isolate.
In general, each colony morphotype was associated with a unique HaeIII RFLP pattern. Exceptions to this observation were bacterial isolates from *Alexandrium* cultures described as circular, convex and cream-coloured with a rose centre, which gave two distinct RFLP patterns (patterns 8 and 9) (Table 2). In contrast, examination of 16S rDNA sequence data from representative isolates from *A. tamarense* NEPCC 407 and *S. trochoidea* cultures, categorised as possessing RFLP pattern 12 but showing different colony morphologies, indicated diverse phylogenetic neighbours (Table 2). Representative sequences from RFLP patterns 11, 17 and 19 sharing the same colony characteristics also had distant phylogenetic neighbours.

### 3.2. Bacterial diversity associated with *A. lusitanicum* NEPCC 253

In this study, *A. lusitanicum* NEPCC 253 was observed to support the most limited culturable bacterial population, with bacterial isolates, characterised into five RFLP patterns (patterns 1, 2, 8, 9 and 22), being prominent at all stages of sampling (Fig. 1). Sequence analysis of 16S rDNA from representative isolates indicated that the majority of the bacteria associated with this dinoflagellate were α-Proteobacteria. RFLP pattern 8 and 9 isolates which grouped within the *Roseobacter* clade were closely related with only a 2.5% difference in their 16S rDNA sequence over the 1250 base pairs analysed. Bacteria exhibiting RFLP pattern 22 with strongest sequence similarity to *Gelidibacter algens* were the only isolates from this dinoflagellate to be located outside the α-Proteobacteria subphylum (Table 2).

### 3.3. Bacteria associated with *A. tamarense* NEPCC 407

As observed in *A. lusitanicum*, bacteria generating RFLP patterns 8 and 9 were present at all stages of growth in cultures of *A. tamarense* NEPCC 407 but at much lower numbers (Fig. 1). However, in this culture, bacteria possessing pattern 15, with strong sequence similarity to the previously isolated 407-2, 2c3 and 253-19 (Table 1), and RFLP pattern 12 isolates were also consistently present. Pattern 11 isolates were detected only at late exponential and stationary growth phases of this culture but at low levels (Fig. 1). Other α-Proteobacterial isolates possessing 16S rDNA sequence types forming RFLP patterns 3 and 10 only reached detectable levels in the stationary growth phase of *A. tamarense* NEPCC 407. γ-Proteobacterial isolates possessing 16S rDNA sequence types showing banding pattern 17 reached only low levels in late exponential samples (Fig. 1), with 16S rRNA gene sequences from these bacteria (ATAM407_2035) almost identical to that of isolate 407-13 (Table 1) and also showing high similarities to 16S rDNA sequences obtained from environmental clone libraries of *S. trochoidea* [28].

### 3.4. Bacteria associated with *A. tamarense* PCC 173a

Bacteria exhibiting RFLP patterns 8, 11, 13, 14, 16, 19 and 20 were consistently present at all growth points of *A. tamarense* PCC 173a, while pattern 17 isolates were detected only during the exponential phase (Fig. 1, Table 2). RFLP patterns 13, 16, 19 and 20 were characteristic of isolates from *A. tamarense* PCC 173a in the current study with the representative sequence from pattern 13 identified as an α-Proteobacterium with 100% sequence similarity to the previously isolated strain 667-12 (Table 1). The pattern 16 representative belonged to the γ-Proteobacteria subphyllum and patterns 19 and 20 clustered within the CFB phylum.

### 3.5. Bacteria associated with *S. trochoidea* NEPCC 15

Of the dinoflagellates studied, the culturable bacteria associated with *S. trochoidea* showed the highest degree of phylogenetic variation over the course of the dinoflagellate growth cycle (Fig. 1), with 12 different RFLP patterns identified. Following analysis of the sequence data from RFLP pattern representatives, isolates identified as pattern 12 were clearly distinguished from those bacteria categorised as pattern 12 from *A. tamarense* NEPCC 407. Some of the bacteria had 16S rDNA sequences similar or identical to those obtained from environmental clones of this dinoflagellate (patterns 4 and 21) [28] or isolate 4αs17, again previously obtained from *Alexandrium* spp. (pattern 4) (Tables 1 and 2).

On continued subculture, isolates showing RFLP patterns 23–26 could not be maintained and subsequently 16S rDNA phylogenetic analysis was not possible.

### 3.6. DGGE analysis of dinoflagellate cultures

Examination of PCR-amplified 16S rDNA DGGE profiles demonstrated that different profiles were detected for each dinoflagellate culture (Fig. 2). Each profile remained constant throughout the different growth phases although individual band intensities varied during growth. In all cases, more DGGE bands were identified than cultured isolates, as distinguished by RFLP patterns and colony morphologies (Figs. 1 and 2, Table 2). The PCR products derived from total genomic DNA from *A. lusitanicum* NEPCC 253 and *A. tamarense* NEPCC 407 each generated 10 distinct DGGE bands, while those of *A. tamarense* PCC 173a and *S. trochoidea* NEPCC 15 generated 16 bands (Fig. 2). 16S rDNA DGGE bands were excised for sequence analysis although some bands failed to re-amplify or contained mixed sequences, so further analysis was not possible. Thus, overall, DGGE profiling did not detect the same diversity of bacteria which could be cultured on marine agar. In *A. lusitanicum* NEPCC 253, *A. tamarense* NEPCC 407 and PCC 173a, identically positioned bands from the different growth points were excised...
Table 3
Comparison of the percentage of cultured isolates classified as being *Roseobacter* related, α-Proteobacteria (not including *Roseobacter*-related isolates), γ-Proteobacteria and CFB and non-cultured (DGGE) profiles

<table>
<thead>
<tr>
<th></th>
<th>A. lusitanicum NEPCC 253</th>
<th>A. tamarense PCC 407</th>
<th>A. tamarense PCC 173a</th>
<th>S. trochoidea NEPCC 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Proteobacteria (Roseobacter)</td>
<td>Cultured</td>
<td>66.7</td>
<td>52.3</td>
<td>30.8</td>
</tr>
<tr>
<td>DGGE</td>
<td>Cultured</td>
<td>16.7</td>
<td>4.1</td>
<td>30.8</td>
</tr>
<tr>
<td>γ-Proteobacteria</td>
<td>Cultured</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CFB</td>
<td>Cultured</td>
<td>16.6</td>
<td>47.3</td>
<td>38.4</td>
</tr>
<tr>
<td>DGGE</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* denotes the presence of bacterial 16S rDNA detected sequences by DGGE analysis; ND, not detected.

to assess reproducibility in the DGGE profiles from different growth samples. The sequences recovered from these co-migrating bands from the different sampling points (early and late exponential and stationary), were virtually identical with differences observed assumed to be artifacts due to single sequencing reactions.

3.7. 16S rDNA DGGE analysis of *A. lusitanicum* NEPCC 253

A representative cultured isolate from each of the five RFLP patterns obtained from *A. lusitanicum* NEPCC 253 was successfully amplified using the PCR conditions for DGGE analysis and demonstrated 100% sequence recognition with the primers. However, DGGE analysis of this dinoflagellate culture failed to identify the presence of all RFLP groups. 16S rDNA sequence analysis of excised DGGE bands in *A. lusitanicum* cultures demonstrated the presence of members of the *Roseobacter* clade (Fig. 2, bands 253/6, 7, 10) with strong sequence similarity to RFLP pattern 3 and other α-Proteobacteria most closely related to SCRIPPS_739 – a *Hyphomonas*-related isolate (Fig. 2, 253/2, 4). A further band (253/3) was related to the *Cytophagales* and possessed an identical 16S rDNA sequence to that of ALUS253_6 (RFLP pattern 22) and *G. algens* (AF001367). Proteobacteria from the γ-subphylum were not cultured from this dinoflagellate at any growth phase examined and, subsequently, 16S rDNA sequences belonging to this group were also not recovered from DGGE gels (Table 3).

3.8. 16S rDNA DGGE analysis of *A. tamarense* NEPCC 407

As with *A. lusitanicum* NEPCC 253, all cultured isolates showed 100% sequence similarity to DGGE primers, with representatives from each RFLP pattern identified from this dinoflagellate amplifying under the PCR conditions used for DGGE analysis, although again not all RFLP patterns were represented in the community profiles. 16S rDNA DGGE analysis of *A. tamarense* NEPCC 407 indicated the presence of many *Roseobacter*-related sequences with six bands excised from different positions in the DGGE profile showing greater than 98% sequence similarity to *Roseobacter*-related bacterial 16S rDNA sequences over the approx. 200 nucleotides analysed (Fig. 2). *Roseobacter*-related cultured isolates classified as belonging to RFLP patterns 9, 11 and 12 were all identified within the DGGE profile with >99% sequence similarity. Other bacterial 16S rDNA sequences identified from the DGGE profiles included a *Hyphomonas*-related sequence (isolate SCRIPPS_739 (97.7%)), within the α-Proteobacteria (band 407/3). However, α-Proteobacteria, excluding *Roseobacter* strains, were not isolated by culturing at early growth phases of this dinoflagellate. Other DGGE 16S rDNA bands possessed low similarities (<90%) to γ-Proteobacteria (407/4) and an unknown marine psychrophile within the CFB phylum (407/2), although CFB phylum bacteria were not detected from this dinoflagellate culture using enrichment techniques.

3.9. 16S rDNA DGGE analysis of *A. tamarense* PCC 173a

The majority of 16S rDNA DGGE bands excised were confirmed as *Roseobacter*-related sequences. This result corresponds with the observation of the high percentage of these bacteria cultured from this dinoflagellate on marine agar medium (Figs. 1 and 2). However, other α-proteobacterial sequences detected by DGGE were not observed in this culture by cultivation on marine agar, for example *Sphingomonas* sp., corresponding to band 173a/2 (Fig. 2, Table 3). Also, confirmation of the presence of
bacteria belonging to the CFB phylum was obtained from DGGE (Fig. 2, bands 173a/12 and 16). γ-Proteobacteria-related sequences were also detected (band 173a/13), with other bands possessing low sequence similarity values to database 16S rDNA sequences (Fig. 2, ATAM173a/7). Examination of the 16S rDNA sequences obtained from cultured isolates from _A. tamarense_ PCC 173a indicated that bacterial isolates grouped as RFLP patterns 14, 16, 17 and 19 did not have 100% sequence similarity with the DGGE primers and would therefore not be represented in the DGGE profiles. However, all other RFLP patterns from this algal culture were confirmed by DGGE analysis

3.10. 16S rDNA DGGE analysis of _S. trochoidea_ NEPCC 15

Sequence determination and analysis of DGGE bands excised from profiles from the PCR-amplified 16S rDNA of _S. trochoidea_ cultures was less successful, with only limited sequence information being generated. 16S rDNA DGGE profiles from _Scrippsiella_ cultures, however, along with _A. tamarense_ PCC 173a contained the highest number of bands (Fig. 2). As with _A. lusitanicum_ and _A. tamarense_ NEPCC 407, all cultured isolates showed 100% sequence similarity with the DGGE primers, although only limited RFLP patterns were confirmed in the DGGE analysis. The DGGE bands which could be sequenced were affiliated to all phyla in this alga detected by culturing (Table 3). However, culturing on marine agar failed to detect members of the γ-Proteobacteria in the exponential growth phase of the dinoflagellate culture, although two adjacent bands from within the DGGE profile possessed between 94 and 96% sequence similarity to γ-Proteobacteria belonging to the _Pseudomonas_ genus (Fig. 1, RFLP pattern 25; Fig. 2, SCRIPP/6 and 7).

In contrast, DGGE band SCRIPP/13 possessed high similarity with the 16S rDNA sequences of isolates forming RFLP pattern 12 (ATAM407_56 and 667-2), cultured from this dinoflagellate only in earlier growth phases.

4. Discussion

In this study, bacterial strains isolated 4 years previously from a range of dinoflagellate cultures were characterised by analysis of their 16S rRNA gene sequences (Table 1). Previous HPLC and CE-MS evidence suggested that bacterial isolate 667-2 belonging to the _Roseobacter_ clade, and isolates 407-2 and 253-19 both closely related to
Am. macleodii, may be capable of synthesising multiple PSP derivatives [6]. Other putatively toxigenic bacteria include isolate 253-13, also belonging to the Roseobacter clade, and isolate 2c3, again showing high similarity with Am. macleodii (Gallacher et al., unpublished). These strains were associated with stationary phase cultures of the dinoflagellate and it was considered to be of interest to determine the predominance of bacterial strains, particularly those which are considered toxic, at different stages of the dinoflagellate life cycle. Hence we have further extensively characterised and compared the bacterial consortia living with both toxic and non-toxic Alexandrium species and a dinoflagellate species not associated with toxicity (S. trochoidea). Toxin profiles of the dinoflagellates strains A. lusitanicum NEPCC 253 and A. tamarense NEPCC 407 used in this study have previously been investigated on several occasions [10–13] and prior to this study their toxicity was confirmed by HPLC (data not shown).

A combination of colony morphology and RFLP analysis of PCR-amplified 16S rRNA genes was used to sort the large number of cultivated isolates obtained from the dinoflagellates into groups, thereby reducing the extent of 16S rDNA sequence analysis. Previous work by Lebaron et al. [29] on marine bacteria demonstrated that colony morphology underestimated taxonomic diversity. It was suggested that, unless used in conjunction with other selection criteria, such as RFLP analysis of 16S rRNA genes, effective differentiation between isolates could not be achieved. In general, the majority of bacterial strains isolated in the current study were distinguishable from each other using detailed colony morphology and RFLP independently (Table 2) as bacterial strains sharing the same RFLP pattern gave identical sequences (Table 2). Combining both techniques provided a powerful tool which proved useful in identifying strains for further phylogenetic analysis.

Overall, the phylogenetic diversity was limited to two bacterial phyla, the Proteobacteria and CFB, with the former restricted to the α- and γ-Proteobacteria subclasses. This is similar to the findings of Babinchak et al. [5] who, using oligonucleotide probes, found similar classes of bacteria in a range of dinoflagellates. Interestingly, comparative 16S rRNA gene sequence analyses revealed that many of our isolates showed close identity to clades of predominantly marine bacterial isolates that do not contain validly described representative taxa. This result is common in phylogenetic studies of naturally occurring marine bacterial communities [28], and indicates that novel microbial species can be relatively easily cultivated from marine dinoflagellate cultures using standard marine agar media.

In this study, the α-Proteobacteria, particularly those of the Roseobacter clade, dominated the microflora of all dinoflagellate cultures. One of these strains (ALUS253_25), isolated from A. lusitanicum, was found to be closely related to PTB1 and PTB5 strains previously isolated from a highly toxic Japanese A. tamarense clone [8]. Further, Roseobacter-related isolates from toxic A. lusitanicum and A. tamarense NEPCC 407 classified as RFLP pattern 8 demonstrated high sequence similarity with the toxigenic bacterium 253-13, while pattern 12 showed complete sequence identity with the sodium channel blocking isolate 667-2 obtained by Gallacher et al. [6].

Many of the bacteria were related to microorganisms with known surface-associated life histories (CFB phylum, Hyphomonas, Caulobacter [30] and some members of the Roseobacter clade, for example Roseobacter algicola (reclassified as Ruegeria algicola [31] and found in the phyco-sphere of P. lima [32]), Roseobacter litoralis and Roseobacter denitrificans, obtained from the surface of green seaweed [33]). This may be important in bacterial/dinoflagellate interactions in relation to bacterial effects on PST production. One of the few studies to take into account possible effects of bacterial adhesion on dinoflagellates demonstrated a decrease in culture toxicity when toxigenic bacteria were prevented from physical contact with an axenic A. lusitanicum culture [34].

Microbial cultivation also demonstrated that a dynamic system exists within dinoflagellate culture, with shifts in numbers and/or composition of the bacterial flora occurring throughout the dinoflagellate vegetative cell cycle. However, PCR-DGGE fingerprinting analyses of the microbial community showed consistent profiles from various stages of the growth cycle. One possible explanation for this discrepancy is that the active portion of the microbial community shifts throughout the dinoflagellate growth cycle, which is reflected in the types of bacteria we were able to culture at the different growth stages. Regardless of the reason, it is apparent that our study suffers from the same cultivation biases that have been documented for other studies employing both microbial cultivation and cultivation-independent molecular ecology approaches. The DGGE analysis identified some gene sequences which were not detected using the culture-based method, for example the identification of α-Proteobacteria outwith the Roseobacter clade from A. tamarense PCC 173a, thus highlighting the need to use both approaches (Table 3). In this study, the α-Proteobacteria, particularly those of the Roseobacter clade, dominated the culturable microflora of all dinoflagellate cultures. A predominance of Roseobacter-related sequences (with the exception of S. trochoidea) was also identified by DGGE analysis, with more than 50% of sequences from the three Alexandrium cultures being grouped within the clade.

Previously it was suggested that maximum bacterial numbers occurred during stationary phase of the dinoflagellate life cycle in batch culture, where dinoflagellate cell death could lead to increased availability of nutrients [3]. This study indicated that the bacterial numbers fluctuated according to cell type (as defined by RFLP) independently of the dinoflagellate growth phase.

Bacterial isolates from the non-toxin producing dinoflag-
gellates *S. trochoidea* and *A. tamarense* PCC 173a showed the most diverse populations with many of the strains unique to these particular cultures. This suggests that a species-specific association may exist between some bacteria and certain algal species and that differences exist in the microflora between toxic and non-toxic dinoflagellates. However, further work using more dinoflagellate cultures is required to determine if this is the case. Future studies should take into account the effect on the isolated microflora of using media other than marine agar and also the potential of bacteria to resist cultivation because of their interdependence with other microbes [35].

Further work is also needed to ascertain if these bacterial populations are stable amongst different subcultures of the same dinoflagellate strain. Spurious contamination of algal cultures in the laboratory cannot be discounted, but evidence demonstrating a functional significance of bacterial–algal associations (e.g. modulation of toxicity) would argue for a selective component to these relationships. This may indeed be the case as some bacterial strains, although isolated 4 years apart, were 99.9% similar based on 16S rDNA sequence identity. This includes isolates from the *Alphoromonas* clade which were identical to those previously reported as producing PST [6]. Also many of the bacterial gene sequences recovered from *S. trochoidea* using marine agar showed high phylogenetic similarity to environmental clone sequences obtained previously from the same culture [28].

In conclusion, this study has identified bacteria associated with three growth phases of PST-associated dinoflagellates, some of which are undefined species/genera. The study significantly adds to the limited information which is currently available within the sequence databases on bacteria of algal origin. The results can also be used to investigate the interaction of bacteria with dinoflagellates in culture and in the environment. Specifically, oligonucleotide probes for in situ hybridisation studies can now be designed to investigate the kinetics and physical association of these bacteria with dinoflagellates in relation to both toxicity and bloom dynamics.

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