Development of clade- (*Roseobacter* and *Alteromonas*) and taxon-specific oligonucleotide probes to study interactions between toxic dinoflagellates and their associated bacteria

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Bacteria associated with toxic algae in culture have been implicated in the enhancement of algal toxin production and auto-toxigenesis. Toxigenic and non-toxigenic bacterial isolates from *Alexandrium* spp. have been determined to belong predominantly to the α- and γ-subclasses of the class Proteobacteria. Within these subclasses, the isolates were further classified into two clades that were phylogenetically affiliated with (1) the genus *Roseobacter*, a newly identified group that is of major significance within the α-*Proteobacteria* and (2) the genus *Alteromonas*. Specific 16S rRNA sequence signatures were identified for these clades as well as for three pairs of isolates and for one subcluster within the *Roseobacter* clade and for three clusters representing eight isolates in the *Alteromonas* clade. Oligonucleotide probes complementary to these regions were designed and their specificities were tested using dot-blot and whole-cell *in situ* hybridizations of target and non-target strains. The empirically determined dissociation temperatures of the probes ranged between 55 and 65 making them applicable as a set for screening environmental samples. *In situ* hybridization of bacteria in cultures of *Alexandrium* spp. with the clade-specific probes showed a loose association of these bacteria within the phycosphere. In addition, the fluorescent signal of the probes was bright and easily distinguishable from autofluorescent bacteria and the dinoflagellates.

Key words: *Alexandrium*, *Alteromonas*, bacteria, FISH, oligonucleotide probes, *Roseobacter*, toxic algae

Introduction

The association of bacteria with toxic algal species in culture has been frequently documented (Silva, 1962; Tosteson *et al.*, 1989; Rausch de Traubenberg & Lassus, 1991; Doucette, 1995; Doucette & Trick, 1995; Lafay *et al.*, 1995; Franca *et al.*, 1996; Kopp *et al.*, 1997; Stewart *et al.*, 1997; Babinchak *et al.*, 1998; Provic *et al.*, 1998). However, the exact role of bacteria in the association has not been determined. Autonomous production of paralytic shellfish poison (PSP)-like toxins by bacteria associated with toxic algae in culture and enhancement of algal toxigenesis by bacteria has also been reported independently by several researchers (Kodama *et al.*, 1988, 1990; Ogata *et al.*, 1990; Gallacher & Birkbeck, 1993, 1995; González *et al.*, 1995; Gallacher *et al.*, 1996, 1997; Doucette & Powell, 1998; Maas *et al.*, 1998). Taxon specificity and/or spatial proximity (attached, endosymbiotic or free-living in the phycosphere) has been demonstrated to be important to the association of toxigenic bacteria with toxic algae in a few cases (Doucette & Powell, 1998), whereas in others taxon specificity was not an issue and any bacterium re-introduced into the culture increased the toxicity of the algae (Bates *et al.*, 1995). Light and transmission electron microscope observations of bacteria associated with toxic algae have provided some information on spatial proximity (Franca *et al.*, 1996), however, results have been inconsistent and localization of bacteria known to be toxigenic within the algal cells has been problematic (Doucette *et al.*, 1998). Field studies of harmful algal blooms (HABs) and their bacterial flora have focused primarily on successional composition of and possible inhibition or stimulation of blooms by associated bacteria (Buck & Pierce, 1989; Romalde *et al.*, 1990; Fukami *et al.*, 1991*a*, *b*; Onji *et al.*, 1995; Ishida *et al.*, 1997). Qualitative composition of bacteria in these studies was determined by isolation, followed by morphological, physiological and biochemical tests and, more recently, RFLP analyses of 16S rDNA. Despite the intensive efforts of these methods, an ecological 'snapshot' providing simultaneous data of species composition, abundance and interaction of bacteria with toxic algae during a bloom event is not yet available.

Since the pioneering papers of Giovannoni *et al.* (1988), DeLong *et al.* (1989), Amann *et al.* (1990, 1992), and Manz *et al.* (1992), the application of whole-cell *in situ*

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hybridization to the study of microbial distribution (Glöckner *et al*., 1996; Ramsing *et al*., 1996) and succession of populations (Bruns & Berthe-Corte, 1998; Pernthaler *et al*., 1998) in the environment has increased rapidly. 16S rDNA oligonucleotide probing of bacteria in toxic algal cultures and HABs would provide qualitative as well as quantitative information necessary to understand bacterial/toxic algal associations and HAB ecology. Questions of taxon specificity, localization and environmental distribution of bacteria associated with HABs can be readily answered with oligonucleotide probing. In one of the first applications of *in situ* hybridization probing of toxic algal cultures, Babinchak *et al*. (1998) used fluorescently labelled, universal and class-specific 16S rDNA oligonucleotide probes to examine bacteria in cultures of *Alexandrium* spp. The most dominant classes of bacteria found were from the α- and γ-subclasses of the Proteobacteria.

In the current study, 16S rDNA oligonucleotide probes have been developed to detect and localize toxigenic and non-toxigenic bacteria in cultures of *Alexandrium* spp. The bacterial strains examined were isolated from cultures *of Alexandrium tamarense*, *A. lusitanicum* and *A. affine* by Gallacher *et al*. (1997). Based on 16S rRNA gene sequence analyses, isolated bacteria predominantly represented two distinct phylogenetic clades related to the genera *Roseobacter* (α-subclass Proteobacteria) and *Alteromonas* (γ-subclass Proteobacteria) (S. Gallacher, E. Mass, E. Moore, & G. Hold, unpublished data). Clade-, subclade- and isolate-specific probes were identified. As part of a continuing project to determine the localization of bacterial cells associated within the phycosphere of toxic algae and to determine the abundance and distribution of these bacteria in HABs, the presence of these bacteria throughout one PSP season will be presented in an accompanying paper (Töbe *et al*., in press). This paper presents development and specificity testing of these probes.

Materials and methods

Oligodeoxynucleotide probes

E. Moore (GBF, Germany) provided 16S rRNA gene sequences of the bacterial strains used in this study. Sequences were manually aligned in a bacterial database containing over 600 published and unpublished bacterial sequences representing a subset of the bacterial alignment provided by the Ribosomal Database Project (RDP; Maidak *et al*., 1999) using maximum primary and secondary structural similarity with the Olsen sequence editor (Larsen *et al*., 1993). This subset of the RDP alignment contained one or two representatives from each of the taxonomic entries provided in the bacterial taxonomic list from the RDP. Data sets of 29 and 45 species were produced from this alignment for phylogenetic analyses of the *Alteromonas* and *Roseobacter* clades, respectively. Masks of 1399 and 1076 nucleotides were used for the *Alteromonas* and *Roseobacter* data sets, respectively, using *Escherichia coli* as the outgroup for the *Alteromonas* data set and the 16S rRNA sequence *from Azospirillum lipoferum* (Z29619), *Rhodovibrio salinarum* (M59069) and *Rhodocista centenaria* (D12701) for the *Roseobacter* data set. Maximum likelihood analyses were performed using the fastDNAml program (v. 1·0) with a transition/transversion ratio generated from each of the data sets individually by the fastDNAml program (Larsen *et al*., 1993) and random taxon addition. Introduced gaps were treated as missing data. Distance analyses were performed using the PHYLIP computer program package (Felsenstein, 1993). Dissimilarity values (Fitch & Margoliash, 1967), based on pairwise comparisons of sequences, were transformed into distances using the Kimura two-parameter model (Kimura, 1980). Distance matrices were converted into dendograms using the neighbour-joining method (Felsenstein, 1993). Stability of monophyletic groups in distance trees was estimated with a bootstrap analysis (500 replicates) (Felsenstein, 1985).

Clade- and taxon-specific oligonucleotide probes were designed from a database containing 16S rRNA sequences imported from RDP as described and unpublished sequences using the PROBE-DESIGN tool of ARB (Strunk *et al*., 1998). Secondary structure predictions (i.e. self-complementation and exergonic properties) were then calculated using the DNASIS (version 2.1, Hitachi Software Engineering) and OLIGO programs (version 4.0, Hitachi, Japan). Probes exhibiting minimal (two GC bonds or fewer) self-complementation were subsequently analysed for specificity using the PROBE MATCH tool of the RDP (Maidak *et al*., 1999), Advanced BLAST (Altschul *et al*., 1990) and a database containing over 10 000 published and unpublished bacterial 16S rRNA sequences (M. Rappé, unpublished data). Additional strain sequences with up to five mismatches to the probe sequence, which were highlighted in our probe searches, were downloaded from GenBank (Benson *et al*., 1999) and entered into our database for further comparison.

C. Lazarus (University of Bristol, UK) synthesized unlabelled probes which were digoxigenin DIG-dUTP labelled in our laboratory using the DIG Oligonucleotide Tailing Kit (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Fluorescein and CY3 (5′) labelled probes were synthesized and HPLC-purified by Interactiva (Ulm, Germany). In addition, the universal eubacterial probe EUB338R was used as a positive control and the nonsense probe NON338 as a negative control (Manz *et al*., 1992). Probe names followed the format: bacterial strain number/*E. coli* position number/R, except for the two clade probes, for which we also could provide a probe name following the oligonucleotide probe database protocol (Alm *et al*., 1996).

Bacteria and growth conditions

The bacterial strains used in this study for probe testing were isolated by Gallacher *et al*. (1997) from toxic strains

of *A. tamarense* (NEPCC 407 and UW2C = toxic UK Scottish strain from Dr Jane Lewis, University of Westminster), *A. lusitanicum* (NEPCC253) and *A. affine* (NEPCC667). Bacterial strains used for specificity testing were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and are listed in Tables 1 and 2. Additional strains used for testing but not listed in Tables 1 and 2 are *Ruegeria algicola* (formerly *Roseobacter algicola*; Uchino *et al*., 1998) DSM10251, *Roseobacter denitrificans* DSM7001 and *Roseobacter litoralis* DSM6996. Strains, with the exception of *Clostridium proteolyticum*, were grown in ASG medium (Haygood & Nealson, 1985) at 25 °C or under culture conditions as recommended by the DSMZ catalogue.

DNA extraction and cell fixation

Cells were harvested at the exponential growth phase for DNA extraction and for fixation prior to *in situ* hybridization. Nucleic acids were extracted using a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle & Doyle, 1990). Nucleic acids of *C. proteolyticum* were extracted directly from the lyophilized pellet supplied by the DSMZ. Cells harvested for *in situ* hybridization were washed with 4 °C, 0·2 μm filtered, 1 × phosphate-buffered saline (PBS) and resuspended in 4 °C, 0·2 μm filtered, 4% paraformaldehyde in 1 × PBS (Stahl & Amann, 1991). Cells were fixed overnight at 4 °C, washed and resuspended in 1 × PBS. We found that overnight fixation rather than the generally practised 2–4 h fixation significantly enhanced signal of the fluorescently labelled oligonucleotide probes. For long-term storage (up to 6 months) at −20 °C, cells were resuspended in 1:1, 1 × PBS:96% ethanol. No noticeable deterioration of fluorescent signal was observed (Stahl & Amann, 1991). Samples of *Alexandrium* spp. cultures were similarly fixed with paraformaldehyde and subsequently stored at 4 °C in 80% ethanol (repeatedly refreshed) for several days to extract the autofluorescent chlorophyll.

PCR and dot-blot hybridizations

PCR (Saiki *et al*., 1988), with the bacterial-specific amplification primer pair 8F and 1542R, was used to amplify double-stranded rDNA (Edwards *et al*., 1989) using the conditions defined in Kopp *et al*. (1997). PCR-amplified genes were checked for purity and length by electrophoresis in 0·75% agarose gels. PCR products, 1 μl (*c*. 100 ng), were applied to positively charged nylon membrane-filters (Boehringer-Mannheim), and hybridized with 0·2 pmol of 5′-digoxygenin-labelled probe at the appropriate mid-point dissociation temperature (T_d) for each probe as previously described by Simon *et al*. (1997). T_d was estimated *a priori* using the Lathe (1985) formula (Ramsing *et al*., 1993). Optimal T_d for probe specificity was determined empirically. Hybridized probes were detected with chemiluminescence (DIG luminescent detection kit for nucleic acids with CSPD as a substrate, Boehringer-Mannheim) according to the manufacturer's instructions. Membrane filters were exposed to X-ray films (Amersham, Arlington Heights, IL) for 2–5 h to visualize the chemiluminescent reactions. For data presentation purposes, exposed X-ray films were scanned with a trans-illuminating scanner and edited with Adobe PhotoShop 4·0.

Fluorescent in situ hybridization (FISH)

Fixed-cell suspensions (10 μl) containing either a single bacterial strain or several strains in combination were applied to Teflon-coated hybridization slides (Paul Marienfeld, Bad Mergentheim, Germany) that had been pretreated with gelatin (Stahl & Amann, 1991). Suspensions, applied to the centre of each hybridization well, were spread with a pipette tip, air-dried and dehydrated in an ethanol series (50%, 80% and 96% aqueous ethanol, v/v) (Stahl & Amann, 1991). Hybridizations were performed in 15 μl volumes by application of hybridization buffer (HB) directly to cell smears. Formamide concentration in the HB was estimated *a priori* using the Lathe (1985) formula modified for formamide addition (Ramsing *et al*., 1993). Optimal formamide concentration was determined empirically. Probe (1·5 μl) hybridizations (2 h, 50 °C) and washes (10 min at 50 °C) were performed as previously described by Stahl & Amann (1991). Slides were air-dried in the dark, treated with DAPI (0·001 mg ml^{-1} final concentration) and mounted in Citifluor (Citifluor, Canterbury, UK). Sealed slides were observed with an Axioscope 20 epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with filter sets 09 and 14, for viewing fluorescein and CY3, respectively. Photographs were taken under × 1000 enlargement with 1600 ISO Fuji colour print film following 15–30 s exposure for epifluorescence. Slides were stored at -20 °C for up to 3 weeks with no noticeable reduction in fluorescent probe signal.

Nucleotide sequence accession numbers

The GenBank accession numbers for the sequences of the organisms used to construct the phylogenetic trees are as follows: *Staleya guttiformis*, Y16427; *Roseobacter* sp. Shippagan, AF100168; *Sulfitobacter pontiacus*, Y13155; *Roseobacter denitrificans*, M59063; *Roseobacter litoralis*, X78312; *Roseobacter* sp. *Prionitis lanceolata* symbiont, U37762; *Roseobacter* sp. strain QSSC9-8, AF170751; *Ruegeria gelatinovorans*, D88523; *Roseobacter* sp. str. DSS-8, AF098493; *Octadecabacter antarcticus*, U14583; *Octadecabacter arcticus*, U73725; *Roseobacter* sp. str. DSS-1, AF098492; *Ruegeria atlantica* str. IAM 14464, D88527; *Silicibacter lacuscaerulensis*, U77644; *Ruegeria atlantica* str. WNA2, AF124521; *Roseobacter gallaeciensis*, Y13244; *Roseobacter* sp. str. 303 from *Loligo pealei*, AF022392; *Roseobacter* sp. str. J8W, AF026463; *Roseovarius tolerans*, Y11551; *Ruegeria algicola*, X78314; *Antarctobacter helio-*

Table 1. *Roseobacter* clade- and taxon/cluster-specific, 16S rRNA oligonucleotide probes and their specificity parameters

Probe name	OPD name[a]	Target species with zero mismatches	Probe sequence 5′–3′	*E. coli* pos.	Dot-blot T_d (°C)	% FA *in situ*[b]	Non-target species with 5 or fewer mismatches used for specificity testing (no. of mismatches)
ROSEO536R	S-*-ROSEO-0536-a-A-18	*Roseobacter* clade	CAACGCTAACCCCCTCCG	536–553	65	15–18	C116-18 * (1), *Paracoccus aminophilus*, DSM8538 (1), *Paracoccus denitrificans*, DSM65 (1), *Caulobacter subvibrioides*, DSM4735 (2), *Erythrobacter longus*, DSM6997 (2), *Erythrobacter litoralis*, DSM8509 (2), 4VaS17* (3), 407-2 and 4αVS3* (4)
ROSEOC536R	S-*-ROSEOC-0536-a-A-18		CAACGCTAGCCCCCTCCG	536–553	65	15–18	*Roseobacter* competitor probe matches non-target bacterial strains with 1 mismatch to the ROSEO536R probe
407-20/1446R	N/A	Isolates 407-20 and 667-2	GTCCGCTGCCTCAAAAGTT	1446–1466	60	10	*Paracoccus aminophilus*, DSM8538 (3)
667-12/191R	N/A	667-12 cluster	GGGCTAATCCTTCCTTCCCC	191–235	65	20	*Paracoccus denitrificans*, DSM65 (4)
667-12/994R	N/A	Isolates 667-9 and 667-12	GGAGCGACGACAAGTATGT	994–1011	60	20	*Marinilabilia salmonicolor*, DSM6580 (2)
253-11/1423R	N/A	Isolates 253-11 and 253-13	ACCGTCGTCGGGTAGACC	1423–1440	60	0	*Paracoccus aminophilus*, DSM8538 (2), *Paracoccus denitrificans*, DSM65 (2), 667-4 and 6, 667-19* (3) 667-9 and 12* (4)

[a] The Oligonucleotide Probe Database (Alm *et al.*, 1996).
[b] Percentage formamide in whole-cell *in situ* hybridization buffer, incubation 50 °C.
* Isolates from this study. N/A = not applicable

Table 2. *Alteromonas* clade- and taxon-specific 16S rRNA oligonucleotide probes and their specificity parameters

Probe Name	OPD name[a]	Target species with zero mismatches	Probe sequence 5′–3′	*E. coli* pos.	Dot-blot T_d (°C)	% FA *in situ*[b]	Non-target species with 5 or fewer mismatches used for specificity testing (no. of mismatches)
AC137R	S-*-AMAC-0137-a-A-18	*Alteromonas* clade	TGTTATCCCCCTCGCAAA	137–154	60	10	*Pseudoalteromonas denitrificans*, DSM6059 (4), *Shewanella baltica*, DSM9439 (5)
407-2/209R	N/A	Isolates 407-2, 407-5 and 407-6	CTTTGCGTGGGAGCCGG	209–227	65	10	*Alteromonas macleodii*, DSM6062 (2), 253-19 and 20* (3)
4αVS3/210R	N/A	Isolates 4αVS3, 2C3 and 2C6	TCTCTTTGCGCCAGAGCT	210–230	60	10	*Alteromonas macleodii*, DSM6062 (2), 253-19 and 20* (2), 407-2, 5, and 6* (4)
253-19/175R	N/A	Isolates 253-19 and 253-20	CAAGTGCACATTATGCGG	175–188	55	10	*Clostridium proteolyticum*, DSM3090 (4)

[a] The Oligonucleotide Probe Database (Alm *et al.*, 1996).
[b] Percentage formamide in whole-cell *in situ* hybridization buffer, incubation 50 °C.
* Isolates from this study. N/A, not applicable.

thermus, Y11552; *Sagittula stellata*, U58356; *Roseobacter* sp. str. LFR, L15345; *Roseobacter* sp. str. QSSC9-5, AF170750; *Marinosulfonomonas methylotropha*, U62894; *Paracoccus denitrificans*, X69159; *Paracoccus aminophilus*, D32239; *Rhodobacter capsulatus*, D16428; *Rhodobacter sphaeroides*, X53854; *Rhodovulum iodosum*, Y15011; *Maricaulis* sp. str. MCS 10, M83807; *Shewanella putrefaciens* ATCC 8071, X82133; *Shewanella putrefaciens* LMG 26268T, X81623; *Shewanella* sp., AF005269; *Pseudoalteromonas denitrificans*, X82138; *Vibrio marinus*, X82142; *Pseudoalteromonas haloplanktis*, X67024; *Pseudoalteromonas nigrifaciens*, X82146; *Pseudoalteromonas* sp., U85859; *Pseudoalteromonas atlantica*, X82134; *Colwellia psychroerythraea*, AF001375; *Alteromonas macleodii* str. CH-460, Y18233; *Alteromonas macleodii*, clone 1B161, Y18231; *Alteromonas macleodii*, clone 17B161, Y18229; *Alteromonas macleodii*, str. CH-516, Y18234; *Alteromonas macleodii*, X82145; unidentified gamma *Proteobacterium*, str. HTC032, AB010857; unidentified gamma *Proteobacterium*, str. HTC036, AB010855; unidentified gamma *Proteobacterium*, str. HTC034, AB010844; unidentified gamma *Proteobacterium*, str. HTC035, AB010856; *Alteromonas macleodii*, str. DSM6062, Y18228. Accession numbers for the bacterial isolates from dinoflagellates tested in this study (S. Gallacher, E. Mass, E. Moore & G. Hold, unpublished data) are: 407-20, AJ294353; 667-2, AJ294353; 667-19, AJ294355; 667-4, AJ294354; 667-6, AJ294354; 253-16, AJ294352; 667-12, AJ294356; 667-9, AJ294356; 253-13, AJ294351; 253-11, AJ294351; C116-18, AJ294357; 253-20, AJ294362; 253-19, AJ294362; 4αVS3, AJ294361; 2C3, AJ294361; 2C6, AJ294361; 407-6, AJ294360; 407-2, AJ294360; 407-5, AJ294360; and 4αVS17, AJ294358. Identical strains have the same accession number.

Results

Probe design

Phylogenetic analyses of 16S rRNA sequences (Olsen, 1988) revealed that most of the bacterial strains isolated from toxic *Alexandrium* spp. could be classified into two distinct clades: *Roseobacter* (α-subclass Proteobacteria) or *Alteromonas* (γ-subclass Proteobacteria) (S. Gallacher, E. Mass, E. Moore & G. Hold, unpublished data). 16S rRNA gene sequences from bacterial strains having 90% or greater similarity to either *Roseobacter denitrificans* or *Alteromonas macleodii* were downloaded from GenBank and used to define these clades further for probe design. Clade-specific oligodeoxynucleotide probes were designed for the *Roseobacter* and *Alteromonas* clades, respectively. Additionally, taxon/cluster-specific probes were designed for each of the three pairs of bacterial strains plus one subcluster determined to be within the *Roseobacter* clade and three taxon-specific probes were designed for bacterial strains represented by eight isolates falling within the *Alteromonas* clade (Figs 1, 2). Probe sequences are presented in Tables 1 and 2. Probe ROSEO536R showed 100% similarity to 117 of 124 known 16S rRNA sequences determined to be in the *Roseobacter* clade. 16S rRNA sequences of the *Roseobacter* clade bacteria that did not show 100% similarity to the ROSEO536R probe include (1) *Roseobacter* sp. Shippagan (1C/U mismatch, helix bond maintained) (AF100168), (2) *Sulfitobacter pontiacus* (1 G/U mismatch, helix bond maintained, non-canonical pairing) (Sorokin 1995; Y13155), (3) *Roseobacter* sp. DSS-1 (1 G/A mismatch, helix bond not maintained) (González *et al.*, 1999; AF098492), (4) *Roseobacter* sp. QSSC9-8 (1 G/A mismatch, helix bond not maintained) (AF170751), (5) *Roseobacter* sp. J8W (2 mismatches, C/G, G/A, helix bond not maintained) (Kaufman *et al.*, unpublished data; AF026463), (6) uncultured bacterium Taynaya-15 (1 G/A mm, helix not affected) (AF142961) and (7) *Prionitis lanceolata* gall symbiont (1 N/G mismatch, undetermined base) (Ashen & Goff, 1996; U37762).

Probe ROSEO536R also showed 100% similarity to the 16S rRNA sequence of *Rhodovulum iodosum* (Straub *et al.*, 1999; IY15011), which is not associated with the *Roseobacter* clade. At least one mismatch to the ROSEO536R probe sequence was found after searching all non-target eubacterial and archaebacterial 16S and 23S sequence data accessible through the RDP (Maidak *et al.*, 1999) and GenBank internet databases. A competitor probe (ROSEOC536R) recognizing this single and consistent mismatch was used in all hybridization experiments in conjunction with probe ROSEO536R to maximize probe specificity of ROSEO536R by blocking this binding site of those species containing the single mismatch (Amann *et al.*, 1990 and references therein).

Probe AMAC137R showed 100% similarity to all 16S rRNA sequences determined to be in the *Alteromonas* clade. A minimum of four mismatches were found to all accessible non-target eubacterial and archaebacterial 16S and 23S sequence data. Sequence similarity to 16S rRNA sequences was 100% for species/cluster-specific probes 407-20/1446R, 667-12/191R, 667-12/994R, 253-11/1423R, 407-2/209R, 4αVS3/210R and 253-19/175R and their target bacterial clusters. From 0 to 5 mismatches were found to accessible non-target eubacterial and archaebacterial 16S and 23S sequence data for these probes. Target and non-target strains with 0 to 5 mismatches in their 16S rRNA sequence which were used for specificity testing are listed in Tables 1 and 2. Probe specificity is indicated on the phylogenetic trees (Figs 1, 2).

Dot-blot hybridizations

Dot-blot analyses were conducted first to evaluate stringency conditions required for whole-cell *in situ* hybridization testing of probe specificity. Optimal T_d values of probes, empirically determined, ranged from 55 to 65 °C (Tables 1, 2). The specificity of DIG-labelled probes was interpreted as annealing of probe to target rDNA only. Dot-blots were exposed to X-ray film for

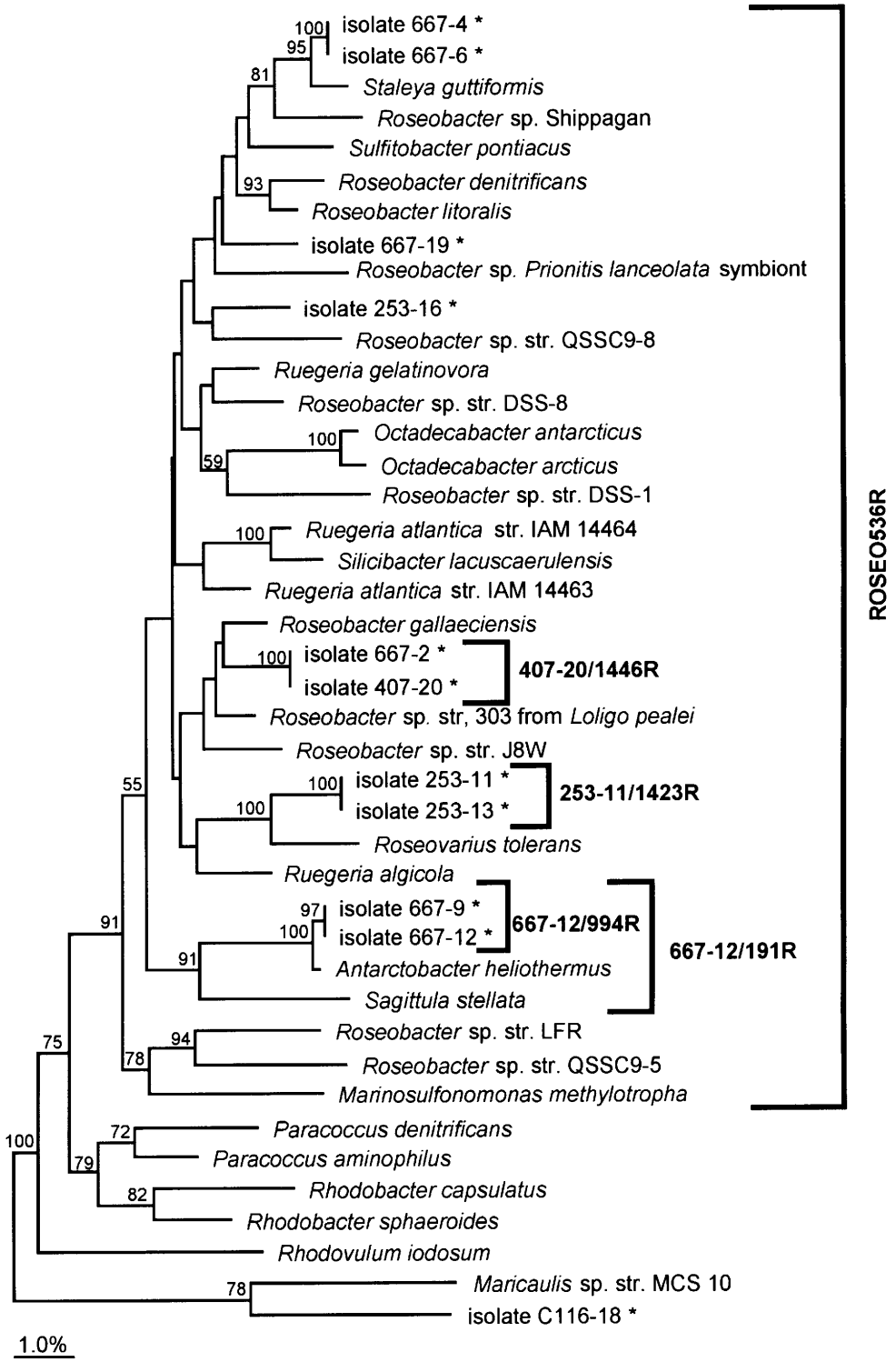


Fig. 1. Molecular phylogenetic tree inferred from 16S rRNA gene sequences representing the *Roseobacter* clade (α-Proteobacteria) using evolutionary distance methods. Asterisks indicate bacterial strains isolated from *Alexandrium* spp. by Gallacher *et al.* (unpublished). Numbers above nodes represent percentages > 50 from 500 bootstrap repetitions from the neighbour joining analysis (Felsenstein, 1985), and oligonucleotide probes identified and developed in this study are indicated at brackets. Outgroups were pruned from the tree.

1–5 h to confirm that no annealing of probe to non-target rDNA had occurred. No quantification of signal intensity was made.

Clade probe ROSEO536R and taxon/cluster-specific probes 407-20/1446R, 667-12/191R, 667-12/994R and 253-11/1423R were tested against *Roseobacter* clade strains (clade neighbours) and non-target strains outside the clade containing five or fewer mismatches in their 16S rRNA sequences (Figs 1, 3). Cluster-specific probe 667-12/191R was not tested against *Antarctobacter heliothermus* or *Sagittula stellata*. The ratio of ROSEO536R to its competitor, ROSEOC536R, was tested in a range of degreed intervals between 1:5 and 5:1, maintaining a constant concentration of 0·2 pmol. Optimal probe: competitor ratio was determined to be 1:1 and exhibited high specificity for all *Roseobacter* clade strains tested (Fig.

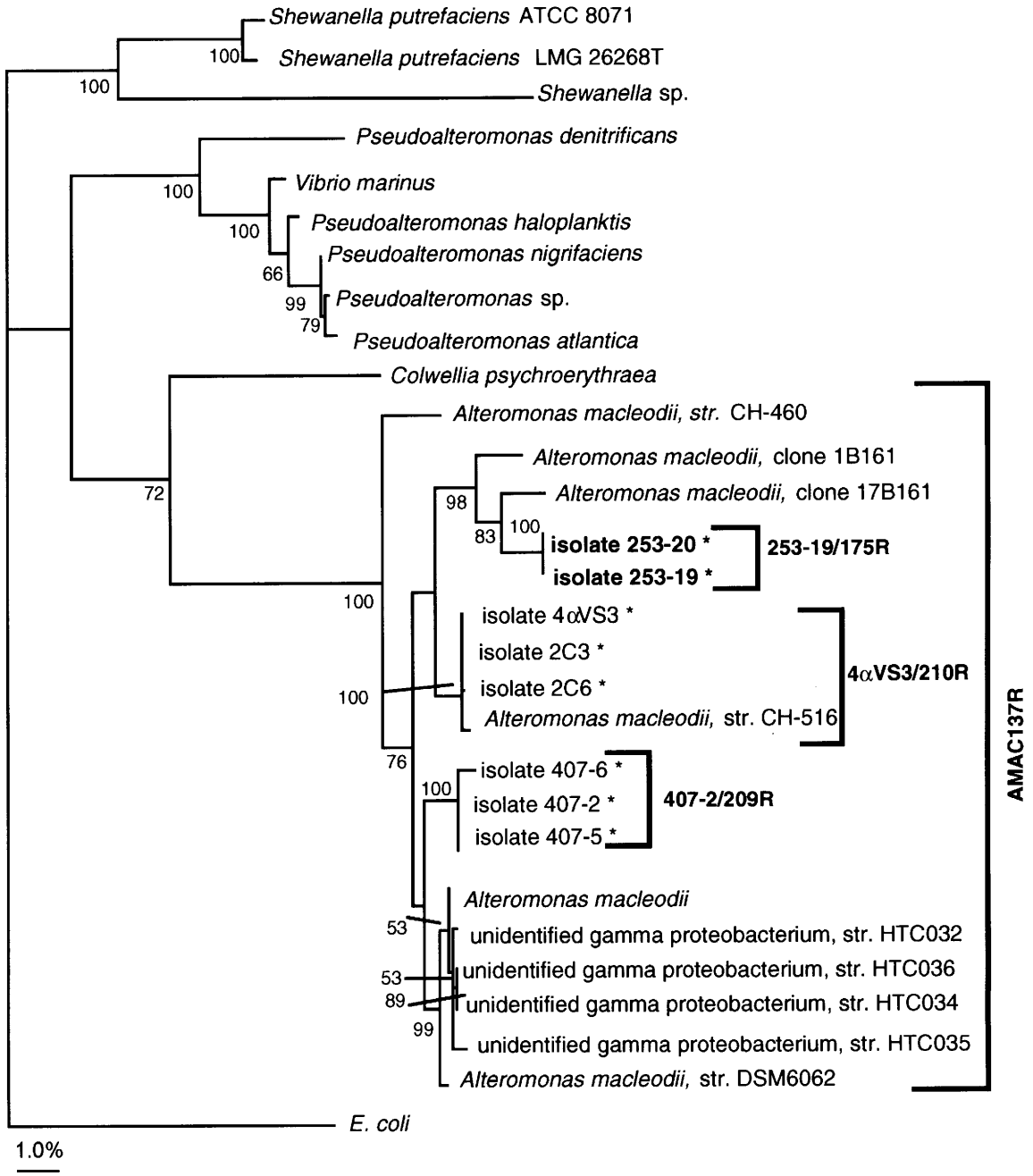


Fig. 2. Molecular phylogenetic tree inferred from 16S rRNA gene sequences representing the *Alteromonas* clade (γ-Proteobacteria) using the maximum likelihood method. Asterisks indicate bacterial strains isolated from *Alexandrium* spp. by Gallacher *et al.* (unpublished). Numbers above nodes represent percentages > 50 from 500 bootstrap repetitions from a neighbour joining analysis (Felsenstein, 1985), and oligonucleotide probes identified and developed in this study are indicated at brackets.

3, Column A). When tested against non-target strains containing one to three mismatches in their 16S rRNA sequences, stringent hybridization conditions of probe ROSEO536R were observed at 62 °C. However, it was necessary to increase the hybridization temperature to 65 °C to remove non-specific signal that occurred with non-target strains containing four and five mismatches (data not shown). Taxon-specific probe 407-20/1446R was specific for its target strains, 407-20 and 667-2 (Fig. 1) and exhibited no non-specific signal with clade neighbours or a non-target strain, *Paracoccus aminophilus*, containing three mismatches (Fig. 3, column B). Cluster-specific probe 667-12/191R was specific for its target strains, 667-9 and 667-12 (Fig. 1) and exhibited no non-specific signal with clade neighbours or non-target strain *Paracoccus denitrificans* with four mismatches (Fig. 3, column C). The signal from this probe was very weak due to the high hybridization stringency required for specificity (Table 1), which attenuated the signal. Probe 667-12/994R was specific for its target strains, 667-9 and 667-12 (Fig. 1) and exhibited no non-specific signal with clade neighbours or non-target strain *Marinilabilia salmonicolor* with two mismatches (Fig. 3, column D). Probe 253-11/1423R was specific for its target strains, 253-11 and 253-13 (Fig. 1) and exhibited no non-specific signal with clade neighbours containing three and four mismatches and non-target

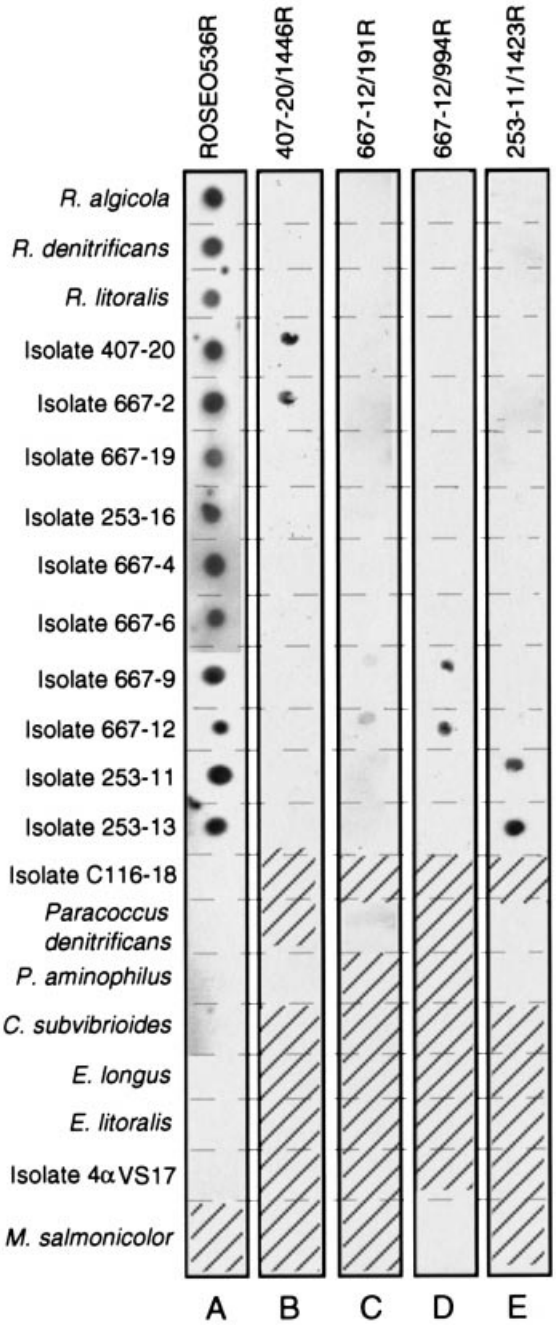


Fig. 3. Dot-blot hybridizations of PCR-amplified 16S rRNA genes from members of the *Roseobacter* clade and non-target taxa with digoxygenin-labelled oligonucleotide probes: ROSEO536R, 407-20/1446R, 667-12/191R, 667-12/994R and 253-11/1423R. Hatched blocks indicate blanks.

strains *Paracoccus denitrificans* and *P. aminophilus* with two mismatches (Fig. 3, column E).

Similarly, clade probe AMAC137R and taxon-specific probes 407-2/209R, 4αVS3/210R and 253-19/175R were tested against *Alteromonas* clade strains (clade neighbours) and non-target strains outside of the clade containing five or fewer mismatches in their 16S rRNA sequences (Figs 2, 4). Probe AMAC137R was highly specific for its target strains (Fig. 2) and exhibited no non-specific signal with non-target strains *Pseudoalteromonas denitrificans* and *Shewanella baltica* containing four and five mismatches, respectively (Fig. 4, column A). Taxon-

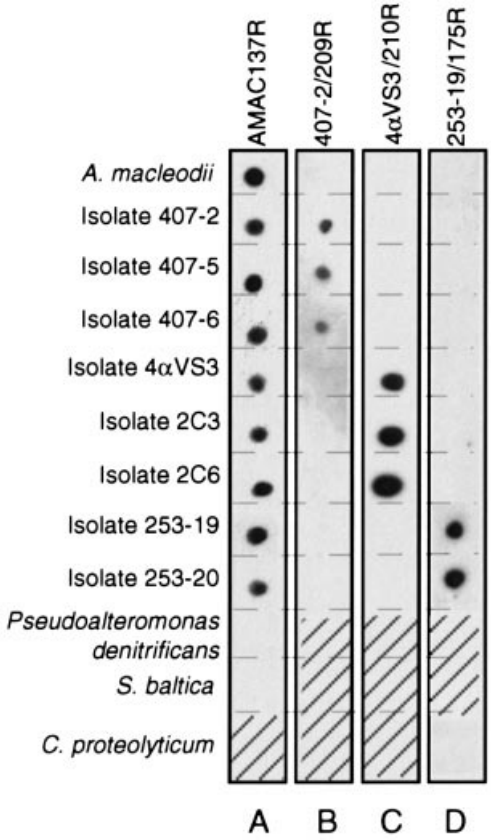
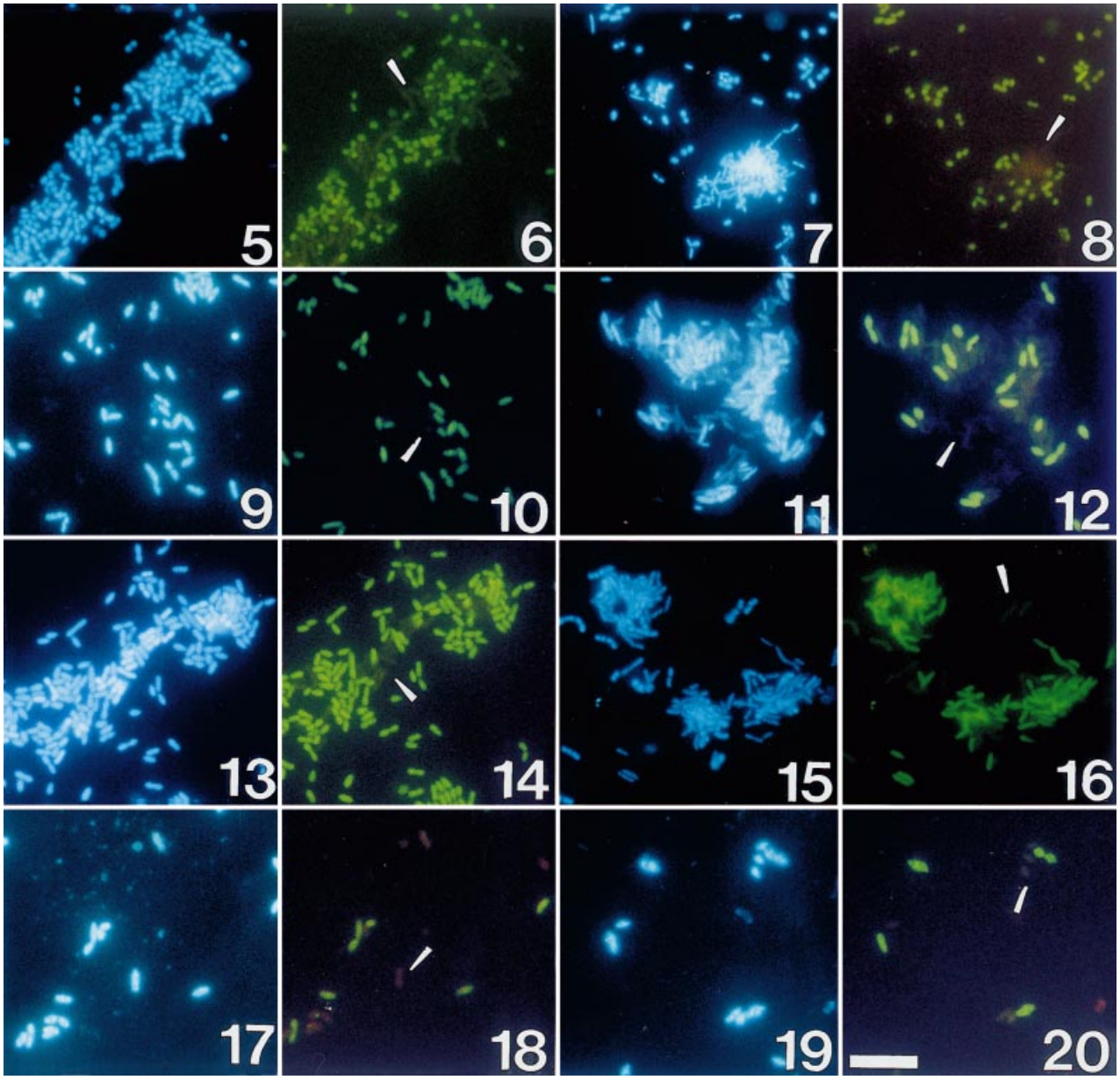


Fig. 4. Dot-blot hybridizations of PCR-amplified 16S rRNA genes from members of the *Alteromonas* clade and non-target taxa with digoxygenin-labelled oligonucleotide probes: AMAC137R, 407-2/209R, 4αVS3/210R and 253-19/175R. Hatched blocks indicate blanks.

specific probe 407-2/209R was specific for its target strains, 407-2, 407-5 and 407-6 (Fig. 2); however, probe signal was weak due to high stringency conditions (Table 2, Fig. 4, column B). Loss of non-specific signal with clade neighbours was achieved at 65 °C hybridization and by reducing the salt concentration in the second wash buffer (0·5a × SSC) (data not shown). Probe 4αVS3/210R was specific for its target strains, 4αVS3, 2C3, *Alteromonas macleodii* str. CH516 and 2C6 (Fig. 2) and exhibited no non-specific signal with clade neighbours containing two and four mismatches (Fig. 4, column C). Probe 253-19/175R was specific for its target strains, 253-19 and 253-20 (Fig. 2) and exhibited no non-specific signal with non-target strain *C. proteolyticum* containing four mismatches (Fig. 4, column D).

Whole-cell in situ *hybridization*

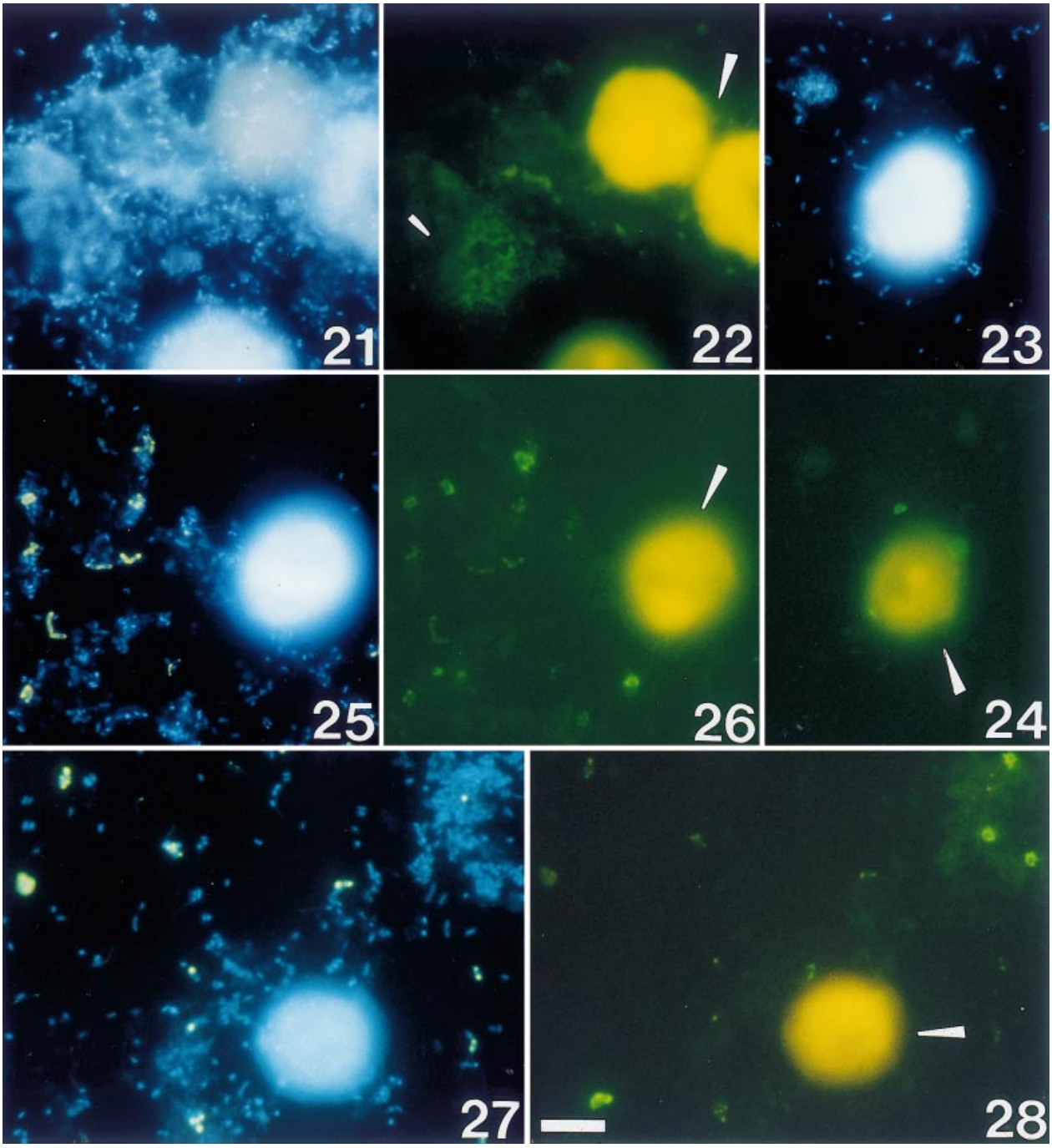
All hybridizations were performed at a uniform temperature. To maintain stringency and temperature hybridization uniformity, de-ionized formamide was added to the hybridization buffer to compensate for the required increase in hybridization temperature as determined by the T_d calculations used in the dot-blot experiments for each probe tested. After initial testing, several of the taxon-specific probes exhibited attenuation of fluorescent signal at the higher (> 10%) formamide



Figs 5–20. Whole-cell hybridization with fluorescein-labelled probes. Matched epifluorescence photosets, DAPI and probe-conferred fluorescein fluorescence from mixed bacterial cell preparations of target and non-target strains (indicated by arrowheads) are displayed for each field. Positive label of the fluorescein-labelled probe appears green. Figs 5, 6. Probe ROSEO536R hybridized with *Roseobacter denitrificans* and non-target *Paracoccus denitrificans* (one mismatch); Figs 7, 8. Probe ROSEO536R hybridized with *R. denitrificans* and non-target *E. longus* (two mismatches); Figs 9, 10. Probe AMAC137R hybridized with *A. macleodii* and non-target *Pseudoalteromonas denitrificans* (four mismatches); Figs 11, 12. Probe AMAC137R hybridized with *A. macleodii* and non-target *S. baltica* (five mismatches); Figs 13, 14. Probe 407-20/1446R hybridized with isolate 407-20 and non-target *P. aminophilus* (three mismatches); Figs 15, 16. Probe 667-12/191R hybridized with isolate 667-12 and non-target *Paracoccus denitrificans* (four mismatches). Figs 17, 18. Probe 253-19/175R hybridized with isolate 253-19 and non-target isolate 2C3 (> five mismatches). Figs 19, 20. Probe 4αVS3/210R hybridized with isolate 4αVS3 and non-target isolate 407-2 (four mismatches). Scale bar represents: Fig. 20, 10 μm and can be applied to all figures.

concentrations necessary to achieve optimal stringency at 46 °C. Based upon these results, dot-blot testing and *a priori* estimates (Ramsing *et al.*, 1993) of probe T_d, it was decided to increase the uniform whole-cell *in situ* hybridization incubation temperature from the widely used 46 °C (Stahl & Amann, 1991) to 50 °C. Increase in the hybridization incubation temperature was not detrimental to the bacterial cell integrity or probe fluorescent signal (Stahl & Amann, 1991). Optimal formamide concentration for probe specificity at a 50 °C hybridization temperature was determined by visualization of fluorescent signal brightness. Optimal formamide concentrations for probes are reported in Tables 1 and 2. All strains hybridized with the positive control probe EUB338 (CY3 label) and no signal was detected in hybridizations with the negative control probe NON338 (CY3 label) (data not shown). All other probes developed for this study were labelled with fluorescein.

Clade probe ROSE536R and taxon/cluster-specific probes 407-20/1446R, 667-12/191R, 667-12/994R and



Figs 21–28. Whole-cell hybridization with fluorescein-labelled probes. Matched epifluorescence photosets of DAPI and probe-conferred fluorescein fluorescence from bacteria associated with *Alexandrium* spp. (indicated by large arrowheads) in culture are displayed for each field. Positive label of the fluorescein probe appears green and autofluorescence of dinoflagellate cells and some bacteria is yellow. Figs 21, 22 and 23, 24. Probe ROSEO536R hybridized with bacteria associated with toxic dinoflagellates *A. tamarense* (NEPCC407) and *A. lusitanicum* (NEPCC253), respectively. Note in Fig. 22 the mass of bacteria labelled with the ROSEO536R probe. Figs 25, 26 and 27, 28. Probe AMAC137R hybridized with bacteria associated with the toxic dinoflagellates *A. tamarense* (NEPCC407) and *A. lusitanicum* (NEPCC253), respectively. Scale bar represents: Fig. 28, 10 μm and can be applied to all figures.

253-11/1423R were tested against *Roseobacter* clade strains (clade neighbours) and non-target strains outside the clade containing five or fewer mismatches in their 16S rRNA sequences. Cluster-specific probe 667-12/191R was not tested against *Antarctobacter heliothermus* or *Sagittula stellata*. Similarly, clade probe AMAC137R and taxon-specific probes 407-2/209R, 4αVS3/210R and 253-19/175R were tested against *Alteromonas* clade strains (clade neighbours) and non-target strains outside of the clade containing five or fewer mismatches in their 16S rRNA sequences. Probe ROSEO536R demonstrated high specificity with isolates determined to be *Roseobacter* clade strains (Fig. 1) (S. Gallacher, E. Mass, E. Moore & G. Hold, unpublished data) and the available known target *Roseobacter* sp. strains, *R. denitrificans*, *R. algicola* and *R. litoralis*. No hybridization of ROSEO536R was detectable with non-target strains tested having one to five mismatches including C116-18 (this study), *Paracoccus denitrificans*, *P.*

aminophilus, *Caulobacter subvibrioides*, *Erythrobacter longus*, *Erythrobacter litoralis*, 4αVS17, 407-2 and 4αVS3 (this study). Hybridization of ROSEO536R with target *R. denitrificans* in mixed bacterial cell preparations with non-target bacterial strains *Paracoccus denitrificans* (one mismatch) and *E. longus* (two mismatches) is demonstrated in Figs 5, 6 and 7, 8, respectively. The hybridization of ROSEO536R with other *Roseobacter* clade members and non-target strains is not shown. Probe AMAC137R was specific for isolates determined to be related to the genus *Alteromonas* (Fig. 2) (S. Gallacher, E. Mass, E. Moore & G. Hold, unpublished data) and related to *A. macleodii* strain DSM6062. Non-target bacterial strains *Pseudoalteromonas denitrificans* and *S. baltica*, having four and five mismatches, respectively, to AMAC137R, exhibited no detectable hybridization to the probe (Figs 9, 10 and 11, 12, respectively). The hybridization of AMAC137R with other *Alteromonas* sp. clade members and non-target strains is not shown. Taxon/cluster-specific probes, 407-20/1446R, 667-12/191R, 253-19/175R and 4αVS3/210R also demonstrated high specificity for target strains when tested with non-target strains in mixed bacterial cell preparations (Figs 13, 14; 15, 16; 17, 18; and 19, 20, respectively). No signal could be detected in hybridizations with probe 253-11/1423R, whereas probe 667-12/994R produced only a faint signal. In an attempt to remove possible protein blocking of target DNA, bacterial cell preparations were pre-treated with a 200 mM HCl solution (Boehringer Mannheim, 1996) for up to 30 min but no enhancement of probe signal was observed (data not shown). We have subsequently found that by increasing the length of hybridization to overnight, we can achieve strong hybridization signals with both probes (Töbe *et al.*, in press). The fluorescent signal of probe 407-2/209R was strong in target strains 407-2, 407-5 and 407-6, however, it exhibited a weak non-specific signal with non-target clade neighbours including *A. macleodii* (data not shown). The addition of formamide above a concentration of 10% in the HB resulted in dramatic attenuation of the target probe signal. The adjustment of pH and salt concentrations in the HB did not improve probe specificity (data not shown). Subsequent applications of this probe in overnight hybridizations have improved the specificity (Töbe *et al.*, in press). The specificity of probes ROSEO536R and AMAC137R was also tested at an incubation temperature of 46 °C. A formamide concentration of 10% in the HB was sufficient to achieve target specificity for probe AMAC137R. A formamide concentration of 18% was optimal for probe ROSEO536R, whereas concentrations exceeding 20% dramatically reduced the fluorescent signal.

As a test of potential probe application, the clade probes ROSE536R and AMAC137R were hybridized with samples from cultures of the toxic marine dinoflagellates *Alexandrium tamarense* (NEPCC407) and *A. lusitanicum* (NEPCC253). Figs 21, 22 and 23, 24 are hybridizations of fluorescein-labelled ROSEO536R with *A. tamarense* and *A. lusitanicum* cells, respectively. Figs. 25, 26 and 27, 28 are hybridizations of fluorescein-labelled AMAC137R with *A. tamarense* and *A. lusitanicum* cells, respectively. The green probe signal is easily distinguishable from autofluorescing bacteria and dinoflagellate cells (Figs 22, 24, 26 and 28, large arrowheads). It is evident from these experiments that bacteria belonging to these clades are maintained in these cultures and appear to be loosely associated with dinoflagellate cells.

Discussion

The clade- and species/cluster-specific probes developed in this study were designed to be used as a set, in either *in situ* or dot-blot hybridizations, to detect bacteria in toxic algal cultures and to examine bacterial/toxic algal interactions. T_d values for the probes ranged from 55 to 65 °C, which aids simultaneous probe application to samples. Testing of probes against non-target strains containing one to five mismatches (all available mismatch combinations) in their 16S rRNA sequences provided a high level of confidence in probe specificity. Chemiluminescent and fluorescent signal of the clade probes, ROSEO536R and AMAC137R, was strong in both dot-blot and whole-cell *in situ* hybridizations, indicating no probe self-complementation and that the target nucleic acids were easily accessible and not blocked by possible backfolding. The fluorescein label of the probes for *in situ* hybridization proved to be a good choice as the green probe-conferred signal was readily visualized and distinguishable from autofluorescing bacteria and dinoflagellate cells. Similarly, chemiluminescent and fluorescent signals of taxon-specific probes 407-20/1446R, 4αVS3/210R and 253-19/175R were strong in both dot-blot and whole-cell *in situ* hybridizations. Cluster-specific probe 667-12/191R proved to be suitable primarily for *in situ* hybridizations. Although 253-11/1423R and 407-2/209R were not functional as probes for whole-cell *in situ* hybridization in the conditions used here, they have been successfully used in field trials by changing the length of the hybridization to overnight and by changing the label to CY3 (Töbe *et al.*, in press). The weak signal of probe 667-12/994R has been intensified by using a fluorescent label with a higher quantum yield, such as CY3, making this probe functional for *in situ* hybridization (data not shown).

The clade probe AMAC137R matched 100% to taxa inferred to be in the *Alteromonas* clade and the clade probe ROSEO536R matched 100% to most (95%) of the taxa inferred to belong to the *Roseobacter* clade and one species outside the clade. Three of seven of the *Roseobacter* clade SSU rRNA genes that did not contain the target site for the *Roseobacter* clade-specific probe had substitutions of a single base that did not preserve the secondary structure of the SSU rRNA molecule, raising the possibility that these may be sequencing or PCR errors. In addition, the SSU rRNA gene for the gall symbiont of *Prionitis lanceolata* (Ashen & Goff, 1996; U37762) possesses an unresolved base (N/G mismatch) in the region targeted by the ROSEO536R clade probe. This sequence has

a 'C' in the nucleotide position across the helix of this region of the SSU rRNA secondary structure, which provides a measure of evidence that this sequence does in fact contain the probe target site. The SSU rRNA for *Sulfitobacter pontiacus* (Sorokin, 1995; Y13155) contains a single base pair substitution that results in a non-canonical base pair but does not possess a reciprocal substitution across the helix of this region of the SSU rRNA secondary structure. All other available *Sulfitobacter* spp. SSU rRNA sequences do not have this substitution but instead possess the probe target site exactly. The ROSEO536R probe also showed 100% similarity to the 16S rRNA sequence of *Rhodovulum iodosum* (Straub *et al*., 1999), which is not associated with the *Roseobacter* clade. Ideally, hybridization of these microorganisms with the probe would confirm whether or not the target sequence is present. Although the clade probe ROSEO536R could not be tested on all members of its clade target, our analyses using the *Roseobacter* clade isolates from *Alexandrium* spp. (S. Gallacher, E. Mass, E. Moore & G. Hold, unpublished data), cultures obtained from the DSMZ and several isolated strains of *Octadecabacter articus* and *Octadecabacter antarcticus* (R. Brinkmeyer, K. Ravenschalg, R. I. Amann & E. Helmke, unpublished data) indicated that our probe is highly specific. Hybridization testing of AMAC137R with *Alteromonas* spp. isolates from Gallacher *et al*. (unpublished data) and *A. macleodii* also indicated high target specificity of this probe.

The *Roseobacter* clade, inferred by our phylogenetic analyses and recently referred to as the '*Roseobacter* group' by González *et al*. (1999), encompasses a physiologically and geographically diverse group of bacteria within the α-subclass of the Proteobacteria. These heterotrophic bacteria are proving to be important in biogeochemical processes such as a DMSP degradation (Ledyard *et al*., 1993; González *et al*., 1999), lignin transformation (González *et al*., 1997) and methyl and inorganic sulphur compound utilization (Sorokin *et al*., 1995; Holmes *et al*., 1997; González *et al*., 1999; Pukall *et al*., 1999*a*). Bacteria of this clade have been discovered in a wide range of marine habitats including coasts (González & Moran, 1997; Rappé *et al*., 1997; Giuliano *et al*., 1999), sediments (Sobecky *et al*., 1998), snow aggregates (Rath *et al*., 1998) and as endosymbionts or in association with macroalgae (Ashen & Goff, 1996; Shiba, 1991) and invertebrates (Barbieri *et al*., 1996; Ruiz-Ponte *et al*., 1998). *Octadecabacter antarcticus* and *O. arcticus* (Gosink *et al*., 1997) isolated from polar sea ice, *Antarctobacter heliothermus* (Labrenz *et al*., 1998) and *Roseovarius tolerans* (Labrenz *et al*., 1999) isolated from a hypersaline lake in Antarctica and *Silicibacter lacuscaerulensis* (Petursdottir & Kristjansson, 1997) isolated from a geothermal lake in Iceland represent members of this clade found in extreme environments.

The significance of the *Roseobacter* clade in marine biogeochemical processes was first recognized by González & Moran (1997) and González *et al*. (1999). They used oligonucleotide probes specific for a portion of the α-subclass of the Proteobacteria to identify bacteria in the *Roseobacter* clade that could metabolize dimethyl sulphoniopropionate (DMSP) and other sulphur compounds. However, these probes only partially targeted the *Roseobacter* clade and also did not target a DMSP-degrading *Roseobacter* clade bacterium that had been earlier described by Ledyard *et al*. (1993). Our ROSEO536R clade probe targets the entire clade and would be a more useful probe to identify *Roseobacter* clade bacteria in environmental samples and in studies of endosymbiosis. *Roseobacter* clade bacteria have also been isolated from cultures of toxic dinoflagellates (Gallacher *et al*., unpubl.; Hold *et al*., submitted; Lafay *et al*., 1995). Provic *et al*. (1998) found a predominance of *Roseobacter* spp. bacteria in cultures of the toxic dinoflagellate, *Prorocentrum lima*. Autonomous toxigenesis has been observed in *Roseobacter* clade bacteria that were isolated by Gallacher *et al*. (unpublished data) from cultures of toxic *Alexandrium* spp. These include bacterial strains 253-11 and 253-13. The taxon-specific probe 253-11/1423R was designed to target these toxigenic bacteria. Other probes targeting non-toxigenic isolates of the *Roseobacter* clade, together with the ROSEO536R clade probe, can also provide much-needed answers to questions of bacterial/toxic algal dynamics.

The *Alteromonas* clade of the γ-subclass Proteobacteria includes *Alteromonas macleodii*, for which the clade was named, bacteria isolated from *Alexandrium* spp. (S. Gallacher, E. Mass, E. Moore, & G. Hold, unpublished data), unidentified γ-Proteobacteria isolated from deep-sea sites off the southern coast of Japan (Takami *et al*., 1998) and uncultured γ-Proteobacteria sampled in the Mediterranean Sea (Acinas *et al*., 1999; Pukall *et al*., 1999*b*). The *Alteromonas-Pseudomonas-Vibrio* group is often found in association with toxic dinoflagellates in culture and dinoflagellate blooms and many strains have been documented as toxigenic (Buck & Pierce, 1989; Tosteson *et al*., 1989; Doucette & Trick, 1995; Gallacher & Birkbeck, 1993, 1995; Onji *et al*., 1995; Doucette & Powell, 1998). Probes 253-19/175R, 4αVS3/210R and 407-2/209R are specific for toxigenic bacteria within the *Alteromonas* clade. These bacteria have been observed to excrete 1×10^{-4} fmol of toxin (saxitoxin or STX equivalents per cell) (Gallacher *et al*., 1997). Localization of these bacteria within or on algal cells of the *Alexandrium* spp. cultures from which they were isolated may explain their function in this interaction. Whether these bacteria are associated with *Alexandrium* spp. and other toxic dinoflagellates during bloom events is an interesting question. Our *Alteromonas* clade probe, AMAC137R, and the taxon-specific probes targeting members of this clade should reveal more information regarding the distribution of these bacteria and their possible role in toxigenesis.

Preliminary testing of the probes ROSEO536R and AMAC137R on samples from *Alexandrium* spp. cultures indicated that bacteria affiliated with the *Roseobacter* and *Alteromonas* clades were present and easily observable. The clade- and species/cluster-specific probes designed in

this study can be applied to other cultures of toxic algae to determine whether *Roseobacter* spp. and *Alteromonas* spp. are persistent in the bacterial flora. In addition, these probes could be used to further examine succession of bacterial community structure during the course of HABs. The application of our taxon/cluster-specific probes could further define community structure by detecting the presence or absence of potential toxigenic and non-toxigenic bacteria. However, we must emphasize that detection of bacteria with our taxon-specific probes designed for toxigenic bacteria is not reliable proof of toxigenesis. The genes responsible for toxin production are probably not associated with the 16S rRNA and the ecology of toxin production is relatively unexplored. Additionally, the specificity of oligonucleotide probes is based upon 16S rRNA sequences available during the design process. New 16S rRNA sequences are introduced into the GenBank database on a regular basis and the specificity of our taxon-specific probes must be continually reaffirmed. The probes ROSEO536R and AMAC137R, first designed in the spring of 1997, remain specific for the *Roseobacter* and *Alteromonas* clades, respectively. The application of our probes to screen samples taken during the 1999 PSP monitoring season in the Orkney Islands is presented in Töbe *et al*. (in press). Localization of toxigenic and non-toxigenic bacteria on or within cells of *Alexandrium* spp. will be the next step in utilizing the clade- and taxon/cluster-specific probes to provide insights into bacterial/toxic algal interactions.

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