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ORIGINAL ARTICLE

Variability and abundance of the epiphytic bacterial community associated with a green marine *Ulvacean* alga

Niina A Tujula, Gregory R Crocetti, Catherine Burke, Torsten Thomas, Carola Holmström and Staffan Kjelleberg

Centre for Marine Bio-Innovation, School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, New South Wales, Australia

Marine *Ulvacean* algae are colonized by dense microbial communities predicted to have an important role in the development, defense and metabolic activities of the plant. Here we assess the diversity and seasonal dynamics of the bacterial community of the model alga *Ulva australis* to identify key groups within this epiphytic community. A total of 48 algal samples of *U. australis* that were collected as 12 individuals at 3 monthly intervals, were processed by applying denaturing gradient gel electrophoresis (DGGE), and three samples from each season were subjected to catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). CARD-FISH revealed that the epiphytic microbial community was comprised mainly of bacterial cells (90%) and was dominated by the groups *Alphaproteobacteria* (70%) and *Bacteroidetes* (13%). A large portion (47%) of sequences from the *Alphaproteobacteria* fall within the *Roseobacter* clade throughout the different seasons, and an average relative proportion of 19% was observed using CARD-FISH. DGGE based spatial (between tidal pools) and temporal (between season) comparisons of bacterial community composition demonstrated that variation occurs. Between individuals from both the same and different tidal pools, the variation was highest during winter (30%) and between seasons a 40% variation was observed. The community also includes a sub-population of bacteria that is consistently present. Sequences from excised DGGE bands indicate that members of the *Alphaproteobacteria* and the *Bacteroidetes* are part of this stable sub-population, and are likely to have an important role in the function of this marine epiphytic microbial community.

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Introduction

Sessile marine eukaryotes often harbour microbial biofilm communities on their surfaces. These microorganisms have primarily been of interest as potential sources of novel bioactive compounds (El-Gendy *et al.*, 2008; Romanenko *et al.*, 2008; Zhang *et al.*, 2009). However, understanding the complex interactions of host–microbe associations has also become the focus of many microbial ecology studies. Such studies typically focus on the interactions of the microorganisms at one of three different levels, either: (i) within the microbial community; (ii) between the microbial community and the host; or (iii) between the microbial community and colonizing or grazing eukaryotes. There are several recent examples in the literature that address macro-algal bacterial interactions in this context. For example, microbial community interactions have been investigated using bacterial isolates from the green alga *Ulva australis* in experiments addressing competition in dual species biofilms and biofilm development (Mai-Prochnow *et al.*, 2004; Rao *et al.*, 2005). At the microbe–host level, it has been shown that for normal morphological growth of many green foliaceous macroalgae the presence of certain bacterial strains is essential (Nakanishi *et al.*, 1999; Matsuo *et al.*, 2005; Marshall *et al.*, 2006). Finally, algal bacterial epiphytes have been shown to serve as settlement cues and deterrents for a range of eukaryotic larvae and spores (Dobretsov and Qian, 2002; Patel *et al.*, 2003; Huggett *et al.*, 2008). Cultured bacterial isolates were used to study these interactions.

In order to fully assess and explore such interactions, an improved understanding of the composition and dynamics of the surface community is

Correspondence: S Kjelleberg, Centre for Marine Bio-Innovation, School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney 2052, New South Wales, Australia.
E-mail: s.kjelleberg@unsw.edu.au
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required. Community fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE) and T-RFLP, allow for comparisons of many different replicates or samples to answer ecological questions concerning both the membership and stability of microbial communities. To understand the physical distribution and abundance of these microbial communities direct observations are required; fluorescence *in situ* hybridization (FISH) has been applied to study the bacterial communities associated with a number of marine hosts including sponges, corals and ascidians (Ainsworth *et al.*, 2006; Hoffmann *et al.*, 2006; Martinez-Garcia *et al.*, 2007; Santiago-Vazquez *et al.*, 2007; Neulinger *et al.*, 2009), as well as to study the bacteria causing gall formation in the marine red algal genus *Prionitis* (Ashen and Goff, 2000). Wherever the application of FISH directly to algae is difficult because of intense background autofluorescence from algal cells, we previously successfully developed a catalyzed reporter deposition or catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) protocol to study the surface bacterial community of several marine macroalgae (Tujula *et al.*, 2006).

Thus far, very limited data on the epiphytic communities on marine seaweeds is available. Here, we report on a comprehensive spatial and temporal analysis of the bacterial community associated with a green *Ulvacean* alga using a combination of DGGE and CARD-FISH. *U. australis* is found along the rocky shoreline of the Australian coast and belongs to the *Ulvacean* family of marine foliaceous macroalgae, which are distributed worldwide in intertidal and subtidal coastal environments (Womersley, 1996; Woolcott and King, 1999). We investigated the stability of the bacterial community by applying 16S rRNA gene DGGE bacterial community fingerprinting on multiple environmental samples of this alga, collected using a stratified design that allowed for spatial and temporal comparisons. This is the first study to employ such sampling strategy on a green alga and our results suggest that the epiphytic bacterial community is variable, yet contains members that are consistently associated with the host. We also identified the phylogenetic affiliation of members of the bacterial community and report on the presence of members of the *Alphaproteobacteria*, *Gammaproteobacteria* and the *Bacteroidetes*. Moreover, we determined the abundance of several bacterial groups on the algal surface, which revealed the dominance of *Alphaproteobacteria*, and in particular members of the *Roseobacter* clade, on the surface of *U. australis*.

Materials and methods

Sample collection

Ulva australis was harvested at low tide from the intertidal rock platform at Shark Point, Clovelly, NSW, Australia (33°91′S, 151°26′E). Sampling was conducted at 3 monthly intervals in 2004 to obtain representatives from different time points that corresponded to the four seasons. The water temperature for each sampling occasion was 23.3 °C in summer, 22.5 °C in autumn, 18.0 °C in winter and 19.0 °C in spring. On each occasion, four individual plants were collected from within each of three different tidal pools, located <20 m apart from one another (A–C) ($n=12$). Individual alga were collected into separate sterile plastic bags with approximately 500 ml of seawater from the site and placed on ice for transportation to the laboratory (approximately 0.5 h). Individuals were rinsed thrice with sterile seawater (filtered through a 0.22 μm filter and autoclaved) to remove debris and non-attached bacteria from the sample surface before further processing. Previous microscopic analysis has indicated that *U. australis* does not harbour endophytic bacteria (data not shown), and as such only surface associated bacteria should be analyzed here.

DNA extraction, 16S rRNA gene PCR, DGGE and sequencing

Nucleic acids were extracted from *U. australis* using the FastDNA spin kit for soil (QBiogene, Montreal, QC, Canada) using a standard protocol (Skovhus *et al.*, 2004). For each algal sample, a sterile cork borer was used to cut five circular discs (1.5 cm in diameter, approximately 30% of the thallus length) from the fronds to ensure an equivalent amount (that is, surface area) was extracted. The remainder of the alga was fixed as described below for CARD-FISH analysis. On the first sampling occasion DNA extractions and subsequent PCR and DGGE analysis were carried out in triplicate to address variability on the level of the individual sample. A standard quantity of environmental DNA (approximately 20 ng) was used as template for PCR. PCR amplification and subsequent DGGE analysis of the bacterial 16S rRNA gene amplified with the primers 341F-GC and 907RC (Schafer *et al.*, 2001), were carried out as described previously (Taylor *et al.*, 2004), with two minor modifications. A denaturing gradient of 35–55% was used for DGGE. For data analysis the PRIMER program (v5.2.3) was used to generate Bray-Curtis similarity matrices from the presence–absence matrices of DGGE bands, and similarity dendrograms based on the group average. For the analysis of spatial variation between rock pools, all 12 individuals sampled from each time point were used. For the analysis of temporal variation over seasons, three individual samples were randomly selected from each time point. Selected DGGE bands were excised and sequenced using a standard protocol (Taylor *et al.*, 2005). The TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) was used for DGGE bands for which initial sequencing indicated the presence of multiple sequences, and up to 10 clones per band were sequenced. Wherever bands

were detected across all samples, several bands from the corresponding vertical positions on the gel, but in different lanes, were also sequenced to confirm that they contained the same sequence (data not shown).

Nucleotide sequence accession numbers
The partial sequences of 16S rRNA genes obtained in this study are available from GenBank under accession numbers DQ229319–DQ229347 (see also Figure 3).

Phylogenetic analysis
The ARB software package (Ludwig *et al.*, 2004) was used to carry out phylogenetic analysis. DGGE sequences and their nearest neighbours, determined by using the using seqmatch function with default parameters in the Ribosomal Database Project release 10 (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp), were aligned using the SINA web aligner (http://www.arb-silva.de/aligner/) then imported into the ARB database. The phylogenetic trees were calculated using the maximum likelihood algorithm with near full-length 16S rRNA gene sequences of nearest neighbours (>1300 nt). To exclude the highly variable positions in the 16S rRNA gene a positional variability by parsimony mask was applied that resulted in the comparison of 1077 nucleotides in the tree calculations. Sequences of DGGE bands and other shorter sequences were added to the trees according to maximum parsimony criterion. An uncultured *Crenarchaeote* sp. (AJ535134) was used as an outgroup for the analysis. The robustness of the tree topology was tested by parsimony bootstrap analysis using 1000 resamplings.

CARD-FISH
All algal samples were fixed in paraformaldehyde (2%) and stored in 1 × PBS:ethanol (4:6) at −20 °C using a standard protocol (Amann *et al.*, 2001). The three samples from each season assessed in the above seasonal DGGE comparison were analyzed using an adapted CARD-FISH protocol optimized for algae (Tujula *et al.*, 2006). Probes were chosen to target bacterial groups on *U. australis* based on the DGGE sequences obtained, which although not an exhaustive exploration of the diversity present, should represent the most abundant groups within the community. After matching against the SILVA database in ARB (Pruesse *et al.*, 2007) to assess the likelihood of a match to sequences with high similarity to DGGE sequences obtained in this study, we applied the following probes labeled with horseradish peroxidase (Thermo Electron Corporation, Bremen, Germany): EUBmix (EUB338-i, ii and iii) (Daims *et al.*, 1999) to target the bacterial kingdom, ALF968 (Glockner *et al.*, 1999) which targets the *Alphaproteobacteria*, GAM42a (Manz *et al.*, 1992) for the *Gammaproteobacteria* with unlabeled competitor probes GAM42a_C1033 (Yeates *et al.*, 2003) and BET42a (Manz *et al.*, 1992), CF319a (Manz *et al.*, 1996) for the *Bacteroidetes*, ROS537 (Eilers *et al.*, 2001) to target the *Roseobacter* clade and PSU730 (Huggett *et al.*, 2008) for the *Pseudoalteromonas* genus. The three summer samples had all six probes applied in order to determine the relative abundance of each group. The probes EUBmix, ALF968 and ROS537 were additionally applied to three autumn, winter and spring representatives to assess the variability of these groups between seasons. Hybridization and wash buffers were prepared according to Pernthaler and Pernthaler (2007). Tyramide-labeled $Alexa_{546}$ (Invitrogen) was used as the reporter molecule for signal amplification. Samples were counterstained with SYBR Green II (2 × concentration, Invitrogen). For each sample probed, 20 random images were collected by confocal laser scanning microscopy and quantified using the Image J software program (http://rsb.info.nih.gov/ij) as outlined previously (Tujula *et al.*, 2006).

Results

Spatial and seasonal variability in 16S rRNA gene DGGE bacterial community fingerprints
A total of 48 environmental samples of *U. australis* collected as 12 individuals at 3 monthly intervals were processed. Cluster analysis of DGGE profiles showed that the variability between the communities of individual hosts was generally high (Figure 1). The range of variability between individuals was lowest for the autumn time point (22%) and was highest in summer (40%), followed by winter (35%) and spring (33%). In terms of spatial variation, cluster analysis indicated that generally the differences in banding patterns between individuals collected from different tidal pools were no greater than the differences between individuals from within a tidal pool (see Figure 1). Thus, we randomly selected three individuals from each seasonal sampling point to assess temporal variation. With the exception of one individual from winter, the community banding profiles of samples from a given time point were more similar to each other, than to samples from another season (Figure 2b). This cluster analysis also revealed that the bacterial communities of samples collected during different seasons still share approximately 60% of bands.

Phylogenetic analysis of excised 16S rRNA gene DGGE bands
Selected bands from the DGGE gels were excised and sequenced to identify the phylogenetic affiliations of representative members of the surface community. A total of 34 sequences were obtained, from which 28 distinct sequence types were subjected

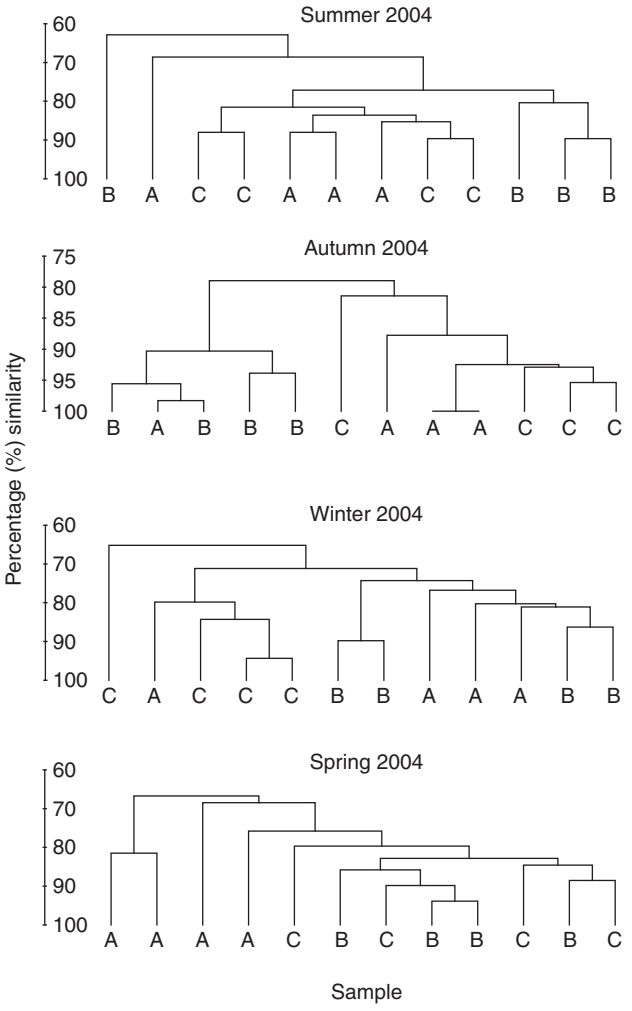

Figure 1 16S rRNA gene-based similarity dendrogram showing the relationships among the microbial communities of different individuals of *Ulva australis* collected from three different tidal pools (A, B, C) over four seasonal time points. This dendrogram was generated using the PRIMER program.

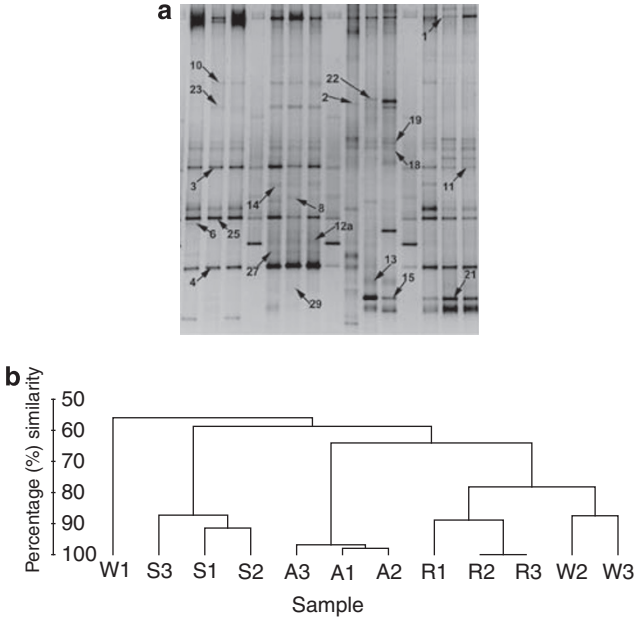

Figure 2 Seasonal comparison of microbial communities of *Ulva australis*. (**a**) 16S rRNA gene DGGE gel representing individuals (1–3) from summer (S), autumn (A), winter (W) and spring (R). M=marker. Numbers indicate which bands were sequenced for phylogenetic analysis, and correspond to band numbers in Figure 3. (**b**) 16S rRNA gene-based cluster diagram representing the data from (**a**). This dendrogram was generated using the PRIMER program.

to phylogenetic analysis (Figure 3). The majority of sequences (15) obtained were affiliated with the *Alphaproteobacteria* and belonged to the families *Rhodobacteraceae*, *Phyllobacteriaceae* and *Sphingomondaceae*. It is to be noted that a number (7) of related sequences (89–97%) obtained clustered within the *Roseobacter* clade. The second most represented phylogenetic group of sequences belonged to the *Bacteroidetes* phylum (7). Of these, the majority belonged to the *Sphingobacteriales* (6), however, one sequence fell within the *Flavobacteriales*. We also obtained a sequence belonging to the *Gammaproteobacteria* (1), an unclassified *Protoebacteria* sequence (1) and several unaffiliated sequences (4) that were related (87–93% identity) to clone sequences from a carbonate chimney in the Lost City hydrothermal field and clones within the candidate bacterial phylum OP11.

It is interesting to note that sequences from five DGGE bands were detected in all samples analyzed, although one represented three different sequences (band 19). The sequences from three bands were affiliated with the *Alphaproteobacteria* and one within the *Bacteroidetes*. Specifically, DGGE bands 25 and 4 were closely related (>99%) to *Alphaproteobacteria* clone sequences earlier obtained from two seaweed surfaces, as well as uncultured clone sequences (>97%) from healthy coral tissue. *Hellea balneolensis* was the nearest cultured relative of DGGE band 18, which was also related to an *Alphaproteobacteria* clone (>94%) derived from the microbial community of *U. australis* (Longford *et al*., 2007). The DGGE band within the *Bacteroidetes* (DGGE band 1) was related (97%) to clones derived from *U. australis* (Longford *et al*., 2007) and was almost identical (99.6%) to the ocean sediment isolate *Krokinobacter diaphorus* (AB198089) (Figure 3).

In situ *abundance of epiphytic bacterial groups on the surface of* U. australis

In order to gain an understanding of the physical representation and abundance of bacterial groups identified by DGGE on the host, CARD-FISH was applied. A hierarchical approach was used when selecting and applying probes to these samples. Initially, the domain *Bacteria* was targeted and found to account for an average of 90% (±1.6) of the cells in the microbial community on the seaweed surface

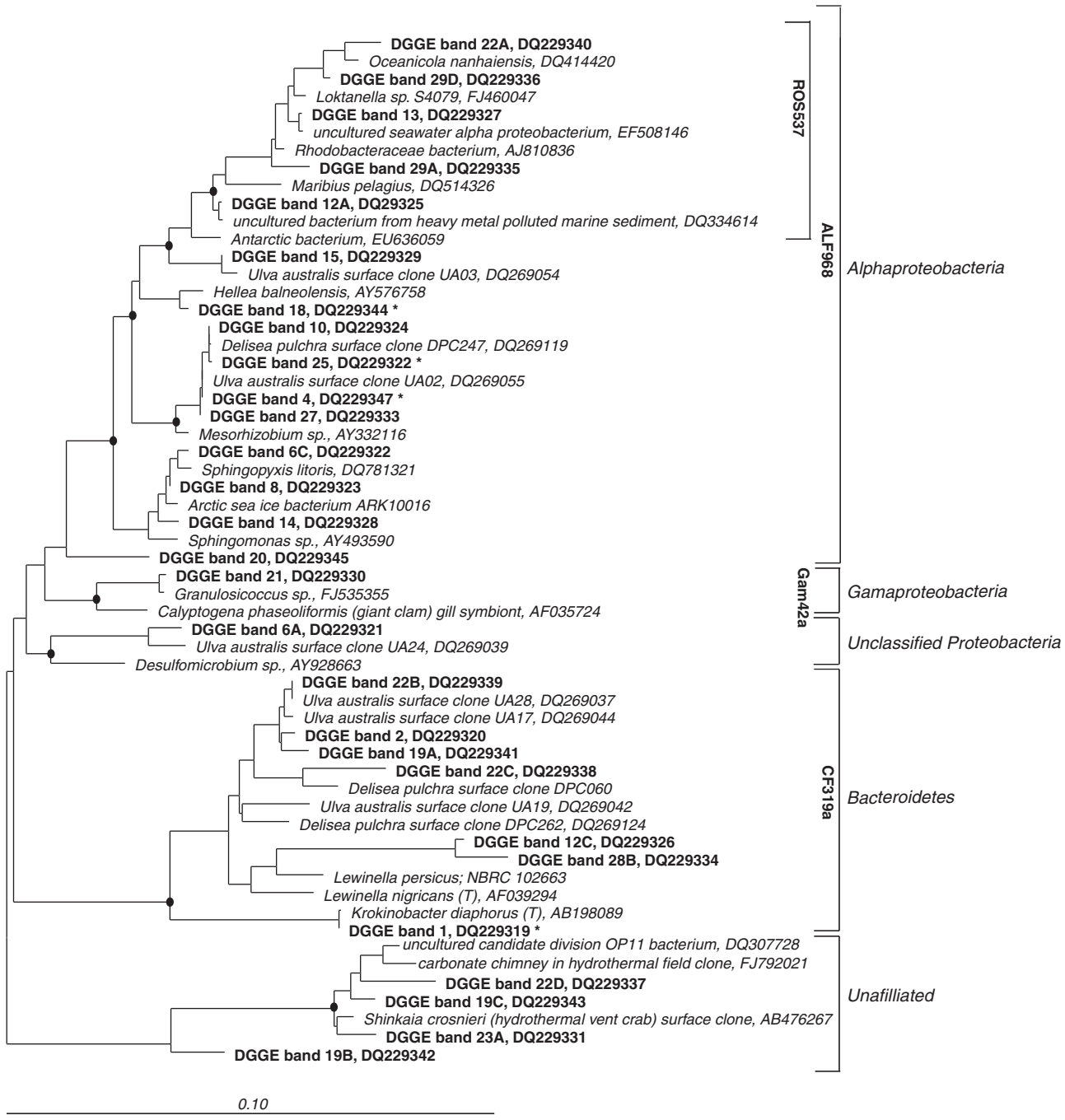

Figure 3 Maximum likelihood tree showing the phylogenetic position of *Ulva australis* surface bacterial 16S rRNA gene bands excised from DGGE gels (shown in bold). The tree was generated in ARB by comparing 1077 nucleotides of the 16S rRNA gene, with shorter DGGE sequences added afterwards using the Parsimony insertion tool. Closed circles at nodes indicate >90% bootstrap support by parsimony analysis, using 1000 resamplings. An uncultured *Crenarchaeote* sp. (AJ535134) was used as an outgroup for the analysis but is not shown in the tree. The scale bar indicates 0.1 nucleotide changes (10%) per nucleotide position. * denotes a sequence from a DGGE band that was detected in all samples; letters after a number indicate where multiple clones were sequenced from one band.

(Figure 4a), visually comprising a diverse range of cell morphologies (see Figure 5). Many bacteria were observed in the form of microcolonies (such as in Figure 5f) often along the algal intercellular cell wall depressions, and were comprised of a variety of cell morphologies (data not shown).

Quantification of the *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* showed that the *Alphaproteobacteria* comprised on average 70% (± 1.0) of the cells in the microbial community (Figure 4a). Four distinct alphaproteobacterial cell morphotypes were noted (Figures 5b–e): (i) thin rods

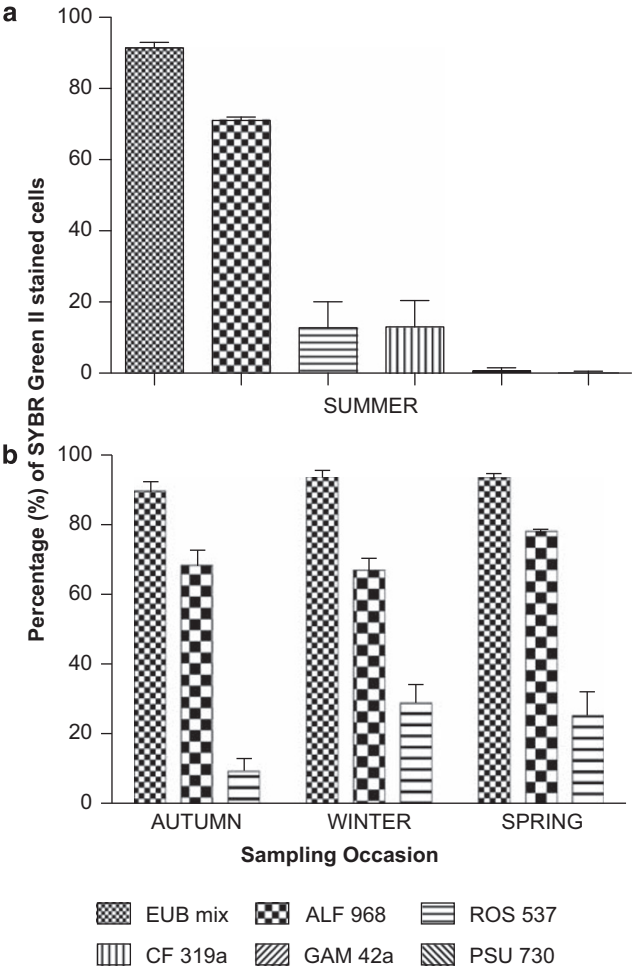

Figure 4 Percentage of specific bacterial groups enumerated by CARD-FISH of the total number of SYBR Green II-stained cells on the surface of *Ulva australis*. Error bars indicate standard deviation. (**a**) Samples collected in summer 2004 ($n=3$) were hybridized with the six probes listed in the key above, targeting *Bacteria* (EUBi-iii), *Alphaproteobacteria* (ALF968), *Roseobacter* (ROS537), *Bacteroidetes* (CF319a), *Gammaproteobacteria* (GAM42a) and *Pseudoalteromonas* (PSU730). (**b**) Samples collected in autumn ($n=3$), winter ($n=3$) and spring ($n=3$) 2004 were further hybridized with probes targeting *Bacteria, Alphaproteobacteria* and *Roseobacter*.

(0.5 μm in diameter and 3 μm in length); (ii) filamentous cell chains (1.5 μm diameter, 10–15 μm in length); (iii) diploid large rods (1.5 μm diameter, 3 μm long, combined length 6 μm); and (iv) coccibacilli (approximately 1.5 μm in diameter).

The average proportion of the *Bacteroidetes* cells was 13% (±7.4) (Figure 4a). The majority of cells were thin and rod shaped in appearance, and can be seen in association with microcolonies of other bacteria (Figure 5f). Very few *Gammaproteobacteria* cells (<1%) were detected in this study (Figure 4a).

At the final level in our probe hierarchical analysis, a group from each of the *Alphaproteobacteria* and *Gammaproteobacteria* was quantified. The marine-alphaproteobacterial lineage, which includes *Roseobacter*, was selected as it was represented by many of the DGGE band sequences. The *Roseobacter* population was found to have a relative abundance of 12% (±7.3) (Figure 4a) and a sole coccobacilli morphotype was observed with cells often noted together in aggregates or as microcolonies (Figure 5f). The gammaproteobacterial genus *Pseudoalteromonas* was chosen, even though no such DGGE sequences were obtained, because several studies have reported a high proportion of culturable isolates of this genus from the surface of *Ulvacean* algae (Egan *et al*., 2000, 2001; Dobretsov and Qian, 2002; Patel *et al*., 2003). However, *Pseudoalteromonas* cells were not readily detected.

Following the observed seasonal variation in the bacterial community composition (see Figure 2), the three individuals from each of the seasonal sampling points were further investigated to assess the variability of the *Alphaproteobacteria* and the *Roseobacter* clade. Across all four seasons, the abundance of *Bacteria* within the epiphytic community was 91.9% (±1.9). *Alphaproteobacteria* and *Roseobacter* accounted for 71.0% (±4.9) and 19.1% (±9.4), respectively, although the abundance of *Roseobacter* exhibited highest variability and was most abundant in the winter samples (Figure 4b).

Discussion

This is the first study to address the spatial and temporal variability in the bacterial community associated with a cosmopolitan *Ulvacean* algae, employing both 16S rRNA gene DGGE and CARD-FISH analysis. DGGE data available on other marine macroalgae derived from *rpoB* based DGGE show differences in banding patterns among individuals of up to 16% for *Amphiroa anceps*, 30% for *Corallina officinalis* and 30% for *Delisea pulchra* (Huggett *et al*., 2006). Studies of sponge-associated bacteria based on DGGE analysis of the 16S rRNA gene reported differences in DGGE banding patterns of <30%, even for samples of the same species collected over a 500 km range (Taylor *et al*., 2005). Here, we report differences of up to 40% when comparing individuals of *U. australis* from within the same, and between three different, tidal pools separated by <20 m, which indicates that the phylogenetic composition of this alga is perhaps more variable than other host-associated communities from similar habitats.

In our temporal comparison, community variability correlated with the season of sample collection. Seasonal differences have been observed in non-DGGE based investigations of epiphytic microbial communities of algae and have been attributed to the growth state of the plant (Laycock, 1974; Sieburth and Tootle, 1981). Leaf age is considered to be a significant inherent source of variation in spatial and temporal assessments (Baker, 1998), and this has been demonstrated recently in a study of the brown alga *Laminaria saccharina*, wherein

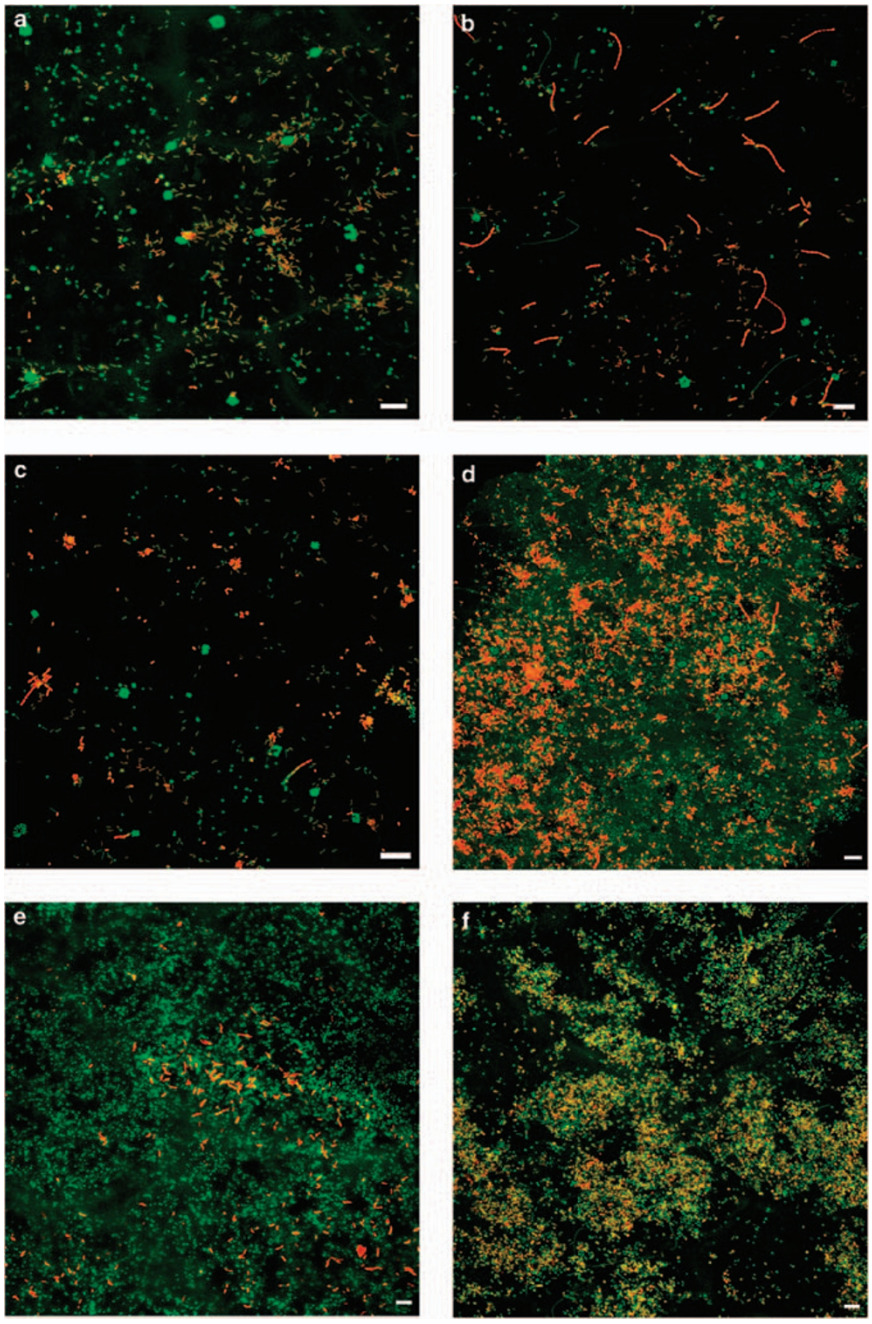

Figure 5 Confocal laser scanning micrographs of bacterial cells on *U. australis* visualized by CARD-FISH using horseradish peroxidase probes targeting the *Alphaproteobacteria* (**a–d**), *Bacteroidetes* (**e**) and *Roseobacter* (**f**). $Alexa_{546}$ was used as the reporter signal and SYBR Green II as a counter stain, the probe-conferred signal is presented in red and SYBR Green II counterstaining is presented in green. The scale bar on all panels is 10 μm.

DGGE profiles of the young meristem and cauloid sections from different plants were more similar to each other than the ageing phyloid section of the same plant (Staufenberger *et al.*, 2008). It is possible that leaf age also contributes to spatial and temporal variability observed in this study, as attempts to collect algal samples of similar size, a crude estimator of age, proved difficult in the field. However, the data reported here indicates that despite levels of variability, the epiphytic community includes a sub-population of bacteria that are consistently associated with *U. australis*, as reflected by the DGGE bands present in all samples. The consistent detection of these sequences indicates that this sub-population may have an important role in the function of the bacterial community on *U. australis*. Similar observations of specificity have recently been reported for bacterial assemblages associated with marine diatoms (Grossart *et al.*, 2005), sponges (Taylor *et al.*, 2005; Lee *et al.*,

2009) and corals (Webster and Bourne, 2007), suggesting that studies of such microbial communities will increasingly add to our understanding of marine bacterial sessile host interactions.

The majority of DGGE sequences obtained in this investigation were affiliated with the *Alphaproteobacteria* and the *Bacteroidetes*, including bands detected in all samples, indicating that representatives of these broad phylogenetic groups constitute the key members of the epiphytic community. A prevalence of sequences from these two bacterial groups has been reported in a DGGE based study of phytoplankton (Jasti *et al.*, 2005), and on brackish submerged macrophytes (Hempel *et al.*, 2008). In addition to these two groups, *Gammaproteobacteria* and unaffiliated sequences similar to the candidate bacterial phylum OP11, were identified in this study. A 16S rRNA gene clone library of the epiphytic bacterial community associated with *U. australis* and the red alga *Deslisea pulchra* (Longford *et al.*, 2007) also contained sequences belonging to these bacterial groups and several of our DGGE bands were expectedly related to these clones. These broad phylogenetic groups, with the exception of the OP11-like sequences, have also been detected in molecular-based community analyses of bacterial assemblages associated with the tropical green macro-alga *Caulerpa taxifolia* (Meusnier *et al.*, 2001), filamentous green algae of sphagnum bogs (Fisher *et al.*, 1998), marine diatoms (Grossart *et al.*, 2005) and phytoplankton (Jasti *et al.*, 2005), and hence appear to emerge as important associates of algae in general.

The confocal laser scanning micrographs of the bacterial community of *U. australis* (Figure 5) complement the early scanning electron micrographs of *U. lactuca* thalli (Sieburth, 1975) that have aptly been described as luxuriant microbial gardens. By using CARD-FISH analysis we found that bacteria accounted for more than 90% of the cells in the microbial community over all four seasons. This is relatively high in comparison to the average of 56% determined in a review of FISH studies in aquatic environments (Bouvier and del Giorgio, 2003) and could be a reflection of the importance of bacteria for the normal morphological growth of *Ulva* (Provasoli and Pintner, 1980; Nakanishi *et al.*, 1996; Matsuo *et al.*, 2003). The larger non-hybridized cells observed frequently on the surface of *U. australis* are possibly diatoms and microalgae, which is in agreement with scanning electron micrographs-based observations of microfouling of small diatoms and blue-green algae on seaweed surfaces (Sieburth, 1975; Provasoli and Pintner, 1980; Dobretsov and Qian, 2002).

Many bacteria were observed in microcolonies (as can be seen in Figure 5f) in association with the algal intercellular cell-wall junctions (data not shown). This observation has also been made for kelp species of the genera *Laminaria* and *Ecklonia* (Linley *et al.*, 1981; Koop *et al.*, 1982; Corre and Prieur, 1990) and is thought to be related to the accumulation of nutrients in the depressions between cells on the algal surface. The intercellular spaces of an unclassified *Ulva* sp. have been found to contain packed layers of polysaccharides containing xylose and glucose (Bobin-Dubigeon *et al.*, 1997), which most likely act as carbon source for bacterial microcolonies observed in these areas. The formation of microcolonies may also protect bacteria from potential stresses within shallow tidal pools such as desiccation, osmotic stress and UV radiation, as has been shown for bacteria on terrestrial leaf surfaces (Monier and Lindow, 2003).

The selected probes used for CARD-FISH analysis were based on the DGGE sequence information obtained in this study. Both methods correlated in identifying *Alphaproteobacteria* as the most abundant group on the surface of *U. australis*. It is difficult to relate the dominance observed in this study to particular functional phenotypes, because the *Alphaproteobacteria* are morphologically and metabolically extremely diverse. However, recent evidence indicates that the *Alphaproteobacteria* contribute significantly to dimethylsulfoniopropionate (DMSP) assimilation in oceans and have an important role in global sulphur cycling (Malmstrom *et al.*, 2004). Furthermore, DMSP is an osmolyte produced by phytoplankton and algae, including *Ulva* sp., which has been demonstrated to provide protection to bacteria during salt stress (Pichereau *et al.*, 1998; Cosquer *et al.*, 1999). Thus, we speculate that the high proportion of *Alphaproteobacteria* may be linked to DMSP utilization.

The *Roseobacter* clade is an important marine alphaproteobacterial lineage (for review see Brinkhoff *et al.* (2008)) that includes many species highly efficient in DMSP degradation and assimilation (Moran *et al.*, 2003; Malmstrom *et al.*, 2004). Bacteria within this clade are known to be ubiquitous and rapid colonizers of surfaces in coastal environments (Dang and Lovell, 2000) and have been enumerated as the most abundant group within the bacterial assemblages associated with some marine algal cultures and phytoplankton blooms in nature (Gonzalez *et al.*, 2000; Riemann *et al.*, 2000; Alavi *et al.*, 2001). Given that many of the DGGE sequences fell within the *Roseobacter* clade, this group was selected for a detailed quantification on the surface of *U. australis*. The seasonal CARD-FISH data support the idea that *Roseobacter* species are an integral part of the epiphytic community of *U. australis*, in agreement with the finding that an epiphytic *Roseobacter* isolate was able to outcompete all other strains trialed in dual species biofilm competition experiments with bacterial isolates from *U. australis* (Rao *et al.*, 2005).

Members of the *Bacteroidetes* were the second most abundant bacteria on the algal surface. The abundance of *Bacteroidetes* on the surface of environmental samples of *U. australis* in the present

study is approximately 13% of surface associated bacterial cells. As *Bacteroidetes* bacteria have been found to contribute 15–30% of the DMSP assimilation in the North Atlantic Ocean and the Gulf of Mexcio (Malmstrom *et al.*, 2004), it is possible that the relatively high abundance of this group may also be linked to DMSP production by the alga. *Bacteroidetes* bacteria have also been linked with the maintenance of normal thallus structure in the green alga *Monostroma oxyspermum* (Matsuo *et al.*, 2003), although bacteria from a range of phyla including the *Proteobacteria* have also been shown to induce normal morphology in the green alga *Ulva linza* (Marshall *et al.*, 2006). Six of the seven *Bacteroidetes* sequences are closely related to the *Saprospiraceae* family, strains of which have been isolated from seawater (Lee, 2007; Oh *et al.*, 2009) and algae (Hosoya *et al.*, 2006). *Saprospiraceae* sequences have also been detected in a previous study of *U. australis* and the red alga *Delisea pulchra* (Longford *et al.*, 2007).

In this investigation we obtained one gammaproteobacterial DGGE sequence and using CARD-FISH quantified the abundance of this group to be <1%. This low *in situ* abundance appeared to be in contrast with numerous reports of a high proportion of gammaproteobacterial species isolated from the surface of *Ulvacean* algae (Egan *et al.*, 2000, 2001; Vairappan and Suzuki, 2000; Dobretsov and Qian, 2002; Patel *et al.*, 2003) and highlights the culturing bias frequently observed in microbial ecological studies (Hugenholtz, 2002). However, recent investigations suggest that organisms having a low *in situ* abundance should not be disregarded in terms of their function within the community. For example, members of the genera *Pseudoalteromonas*, *Vibrio* and *Shewanella* present at an abundance of <1% of the epiphytic community of the algae *C. offencialis* and *A. anceps* significantly contribute to induce the settlement of sea urchin larvae (Huggett *et al.*, 2008).

Concluding remarks

The two community profiling techniques DGGE and CARD-FISH analysis employed in this study identified *Alphaproteobacteria* as the dominant bacterial group, which together with the *Bacteroidetes* constitute the majority of bacterial cells in the epiphytic community on the surface of *U. australis*. The temporal and spatial comparisons carried out have revealed that the bacterial community associated with *U. australis* is variable both between individual plants and across different seasons. However, there remains a sub-population of bacteria that are found consistently across space and time, again affiliated with the *Alphaproteobacteria* and the *Bacteroidetes* suggesting that they are likely to have an important role in the function of this bacterial community. The presence of stable sub-populations within host associated bacterial communities is a feature that is likely to be germane to the ecology of host-associated microbial communities.

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