Environmental Microbiology (2006) **8**(8), 1361–1370 doi:10.1111/j.1462-2920.2006.01029.x

Pirellula and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom

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Summary

Although bacterioplankton and phytoplankton are generally perceived as closely linked in marine systems, specific interactions between discrete bacterioplankton and phytoplankton populations are largely unknown. However, measurements of bacterioplankton distributions during phytoplankton blooms may indicate specific microbial lineages that are responding to phytoplankton populations, and potentially controlling them by producing allelopathic compounds. Here we use a comprehensive molecular approach to identify, characterize and quantify bacterioplankton community responses to an Oregon coast diatom bloom. Total DAPI counts increased by nearly sevenfold in bloom samples, reaching 5.7×10^9 cells l^{-1}, and lineage-specific cell counts using fluorescence *in situ* hybridization (FISH) indicated that Bacteria accounted for approximately 89% of observed increases. Several dominant members of the bacterial community present outside the bloom (SAR11 and SAR86) did not contribute significantly to observed increases in bloom samples. Clone library and FISH data indicated that uncultured planctomycetes most closely related to *Pirellula*, and members of the OM43 clade of beta proteobacteria, reached 0.5×10^8 and 1.2×10^8 cells l^{-1}, respectively, and were among the dominant lineages in bloom samples.

Introduction

In the 1980s and 1990s activity measurements and cell counting procedures showed that bacterioplankton consume 30–60% of the organic carbon being produced by phytoplankton (Williams, 1981; Ducklow, 1983; Cole and Pace, 1988). These observations led to a revision of thinking about food chains and the 'microbial loop' hypothesis was formulated to explain the much larger role assumed by bacterioplankton (Pomeroy, 1974; Azam *et al*., 1983). However, despite a rich literature on the microbial loop, the mechanisms by which phytoplankton release organic carbon for consumption by bacterioplankton are not well understood. Grazing and viral predation on phytoplankton certainly account for some of the loss, but it is not known why bacterioplankton assimilate as much as 60% of production in some systems, whereas in other systems they recover as little as 30% (del Giorgio *et al*., 1997; Carlson *et al*., 1998).

It is generally assumed that the linkage between bacterioplankton production and algal production is passive, with algae releasing a varying proportion of production depending on circumstances, and bacterioplankton consuming this carbon, subject to the availability of other nutrients (N, P and trace elements). The surprisingly high amounts of carbon sometimes released by phytoplankton could be explained by the hypothesis that bacterioplankton are not just passive consumers but instead actively interfere with phytoplankton metabolism, acting either as pathogens, or parasites. In 1996, Fukami and colleagues reported on the relationship between bacterial assemblages and algal succession in a study that included *Skelotonema costatum* and the toxic algal species *Heterosigma akashiwo* (Fukami *et al*., 1996). Yoshinaga and colleagues (Yoshinaga *et al*., 1997) and Lovejoy and colleagues (Lovejoy *et al*., 1998) showed that specific cultures of marine bacteria could inhibit or kill algal cultures by producing a heat-labile substance. These first reports laid important groundwork, but they did not establish whether the production of these compounds by marine bacteria could control algal populations in nature (Long and Azam, 2001). Here we use a comprehensive molecular approach to identify, characterize and quantify novel bacterioplankton responses during an inshore Oregon coast diatom bloom (bloom) in contrast to increases in *Synechococcus* and picoeukaryotes observed offshore (non-bloom). Specifically, we used non-metric multidimensional scaling (NMS), terminal-restriction fragment length polymorphism (T-RFLP), 16S rDNA cloning and fluorescence *in situ* hybridization (FISH) to track microbial lineages in bloom and non-bloom samples collected off the Oregon coast in May 2002.

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Results

Bacterioplankton distributions and abundances

We monitored relative T-RFLP fragment abundances to identify shifts in bacterioplankton community structure associated with surface bloom and surface non-bloom seawater samples (15 m) and samples collected from the upper mesopelagic zone (250–500 m). NMS ordination analyses supported predefined groupings, which indicated that bacterioplankton T-RFLP profiles varied along horizontal (axis 1) and vertical (axis 2) gradients (Fig. 1). The lengths of lines shown in the joint plot (Fig. 1A) correspond to nutrient measurements and microbial abundance estimates correlated with ordination axes. A line length along an axis indicates the strength of its correlation with that axis. Chlorophyll concentrations had a strong positive correlation with near-shore surface samples along axis 1. Maximum chlorophyll concentrations were 1.81 and 2.44 μg l^{-1} at NH10 and NH15 respectively. Diatom and total heterotrophic cell counts showed similar trends, indicating that both diatom and bacterioplankton abundances contributed to an Oregon coast bloom at NH10 and NH15. While the taxonomic composition of the diatom bloom was not determined during this experiment, previous research off the Oregon coast has revealed diatoms that include *S. costatum*, *Chaetoceros* spp., *Leptocylindrus minimus*, *Pseudo-nitzschia* spp., *Actinopytchus* sp., *Asterionellopsis glacialis* and *Thalassiosira* spp. (Moses and Wheeler, 2004; Wetz *et al*., 2004). Conversely, the abundances of small phytoplankton, picoeukaryotes and *Synechococcus*, were negatively correlated with axis 1, indicative of higher concentrations at sampling stations further from shore. Nitrate, phosphate and silicate concentrations increased with depth and were strongly correlated with upper mesopelagic samples along ordination axis 2.

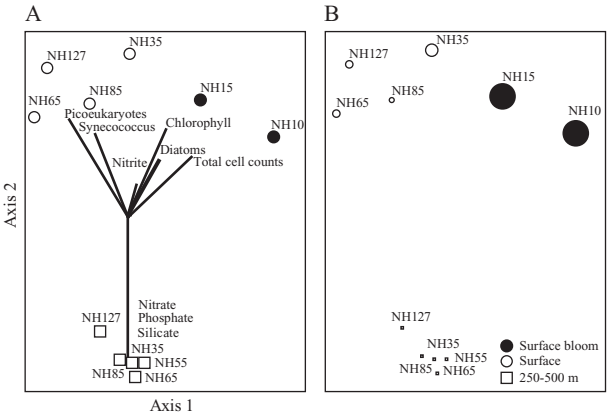

Fig. 1. Non-metric multidimensional scaling (NMS) of bacterial 16S rDNA terminal restriction fragments collected along the Newport Hydroline in May 2002.
A. Nutrient measurements and microbial abundance estimates (flow cytometric) correlated with ordination axes are indicated by line lengths along axes in the joint plot. A priori sample groupings indicate bacterioplankton surface bloom and non-bloom (15 m) and upper mesopelagic (250–500 m) samples.
B. *Pirellula* (fragment 207) contributions to samples in the ordination. Larger symbol sizes correspond to increases in the relative abundance of *Pirellula* fragments. Integrated fragment areas were divided by the total for their corresponding sample to transform the data into relative units. Correlation coefficients in Table 1 indicate the strength (magnitude) and directionality (sign) of a fragment's contribution to ordination axes. Positive and negative signs in Table 1 indicate an increase or decrease along an ordination axis in Figure 1 respectively. The NH10 surface sample exhibited strong increases in fragments contributing to the bloom grouping and was selected for clone library analysis.

A 16S rDNA clone library was constructed from water collected at NH10 to identify bacterioplankton lineages with T-RFLP fragments that matched environmental fragments collected from surface bloom samples (Table 1). Correlation coefficients with ordination axes shown in Table 1 indicate the strength (magnitude) and directionality (sign) of environmental fragments and fragment designations. Positive and negative numbers indicate an increase or decrease along either axis in Fig. 1 respectively. Four *Pirellula* clones were identified with fragments similar to a 207 bp environmental fragment strongly correlated with ordination axis 1 (0.71) (Fig. 1B, Table 1). Larger symbols at NH10 and NH15 in Fig. 1B indicate an increase in the relative abundance of 207 bp fragments in surface bloom T-RFLP profiles. Other cloned fragments matching environmental fragments strongly correlated with ordination axis 1 included a single OM43 clone (0.81) and four *Cytophaga* clones (0.66–0.77). Three of the four *Cytophaga* clones identified in the NH10 library lacked the internal restriction site (GGCC) and had predicted amplicons that were either 522 or 225 bp in length. The fourth *Cytophaga* clone had a predicted restriction fragment that was 409 bp long. Although lineages with predicted fragment sizes less than 50 bp (39 bp) were common, they were below the limit of detection used for these analyses and were discarded.

Initial FISH profiles using probe suites targeting Bacteria and representatives from abundant bacterioplankton lineages (Table 2) supported similar shifts in community structure indicated by NMS (Fig. 2A). On average, bacteria accounted for 77.4% (± 10.3) of all 4,6-diamidino-2-phenylindole (DAPI)-stained cells in the upper 30 m of the water column and decreased to 53.7% (±13.8) below 30 m (Fig. 2B). Members of the SAR11 ('*Pelagibacter ubique*') clade exhibited similar patterns of abundance and distribution and averaged 25.9% (±9.5) and 14.8% (±7.5) of total upper surface and > 30 m cell counts respectively. SAR86 and SAR202 cell counts were similar to previously published results, which indicated that members of these lineages are stratified (Eilers *et al*., 2000; Morris *et al*., 2004). Members of the SAR11 clade and SAR86 cluster were most abundant in upper surface waters (1–30 m) and SAR202 cells were detected only at

Table 1. Cloned 16S rDNA terminal restriction fragment lengths in nucleotides (A) observed on an ABI 3100 Fragment Analyzer and (B) predicted from gene sequences, and corresponding correlations with ordination axes (Fig. 1).

Clone ID	Accession no.	T-RFLP fragment designation	Fragment length		Total	Correlation coefficient	
			A	B		Axis 1	Axis 2
NH10_24	DQ372848	*Roseobacter*	ND	39	9	ND	ND
NH10_29	DQ372849	*Roseobacter*	ND	39	9	ND	ND
NH10_44	DQ372852	OM60 clade	ND	39	9	ND	ND
NH10_46	DQ372853	*Roseobacter*	191	193	1	ND	ND
NH10_02	DQ372839	*Pirellula*	207	207	13	0.71	0.58
NH10_04	DQ372841	*Pirellula*	206	206	13	0.71	0.58
NH10_19	DQ372846	*Pirellula*	207	208	13	0.71	0.58
NH10_22	DQ372847	*Pirellula*	206	206	13	0.71	0.58
NH10_03	DQ372840	OM43 clade	223	223	13	0.81	0.49
NH10_01	DQ372838	*Actinobacteria*	230	232	1	–0.26	–0.48
NH10_09	DQ372844	*Actinobacteria*	280	284	1	–0.70	0.58
NH10_05	DQ372842	*Roseobacter*	292	293	1	0.02	–0.86
NH10_07	DQ372843	*Cytophaga*	408	409	1	0.68	0.48
NH10_11	DQ372845	*Cytophaga*	523	522[a]	2	0.77	0.30
NH10_37	DQ372850	*Cytophaga*	523	522[a]	2	0.77	0.30
NH10_40	DQ372851	*Cytophaga*	526	525[a]	1	0.66	0.21

a. Predicted amplicon lengths shown for sequences lacking an internal HaeIII (GGCC) restriction site.
No data (ND) indicates gene clones with observed fragment lengths smaller than the minimum standard used for T-RFLP analyses (50 bp), which were therefore not present in the ordination analysis. Fragment designations are based on similarities with cloned 16S rDNA sequences identified by T-RFLP, sequencing and phylogenetic analyses.

depths ≥100 m. The bacterial fraction of total cell counts in bloom samples at NH10 and NH15 increased by approximately 14% (Fig. 2C), which supports the occurrence of a surface bacterioplankton bloom in these samples. Interestingly, the SAR11 fraction of total cell counts dropped by approximately 18% at NH10 and NH15. No significant differences were observed in the relative abundance of SAR86 cells in bloom compared with non-bloom samples (Fig. 2C).

Fragment identification and lineage contributions to bloom communities

Phylogenetic affiliations were assigned to terminal restriction fragments by direct comparison of observed and predicted fragment lengths obtained from 16S rDNA clone library data (Table 1). Terminal restriction fragment analyses of full-length inserts from the NH10 library ($n = 32$) identified 16 clones containing unique or single fragments,

Table 2. Oligonucleotide probes and stringency conditions targeting bacterioplankton lineages identified by T-RFLP, and 16S rDNA cloning and sequencing analyses.

Lineage (position)	Probe sequence (5′-3′)	Reference	mM NaCl	% Formamide	Wash (°C)
EUB (8R[a])	CTGAGCCAKGATCRAACTCT	Morris and colleagues (2002)	150	15	50
EUB (338Rpl)	GCWGCCWCCCGTAGGWGT				
EUB (700R)	CTAHGCATTTCACYGCTACAC				
EUB (700Ral)	CTACGAATTTCACCTCTACAC				
EUB (1522R)	AAGGAGGTGATCCANCCVCA				
SAR11 (152R)	ATTAGCACAAGTTTCCYCGTGT	Morris and colleagues (2002)	150	15	55
SAR11 (441R)	TACAGTCATTTTCTTCCCCGAC				
SAR11 (542R)	TCCGAACTACGCTAGGTC				
SAR11 (732R)	GTCAGTAATGATCCAGAAAGYTG				
SAR86 (61R)	GATACTTTCTCGCACGAC	This study	150	15	55
SAR86 (736R)	TCAGTACAGATCCAGGAG				
SAR86 (1093R)	TGCGCTCGTTATCCGACT				
SAR86 (1247R)	GCTTAGCGTCCGTCTGTA				
SAR202 (104R)	GTTACTCAGCCGTCTGCC	Morris and colleagues (2004)	70	35	60
SAR202 (312R)	TGTCTCAGTCCCCCTCTG				
*Pirellula*1 (1223R)	CATTGTAGGACGTGTGCAG	Gade and colleagues (2004)	70	35	50
OM43 (62R)	CTCTCTATGCTGCCGTTCGA	This study	70	35	50
Roseobacter (536R)	CAACGCTAACCCCCTCCG	Brinkmeyer and colleagues (2000)	70	35	52
*Cytophaga*318 (319R)	TGGTCCGTGTCTCAGTAC	Manz and colleagues (1996)	70	35	50
NEG (338F)	TGAGGATGCCCTCCGTCG	Morris and colleagues (2002)	70	35	50

a. EUB-8R is synonymous with the previously published designation EUB-27R.

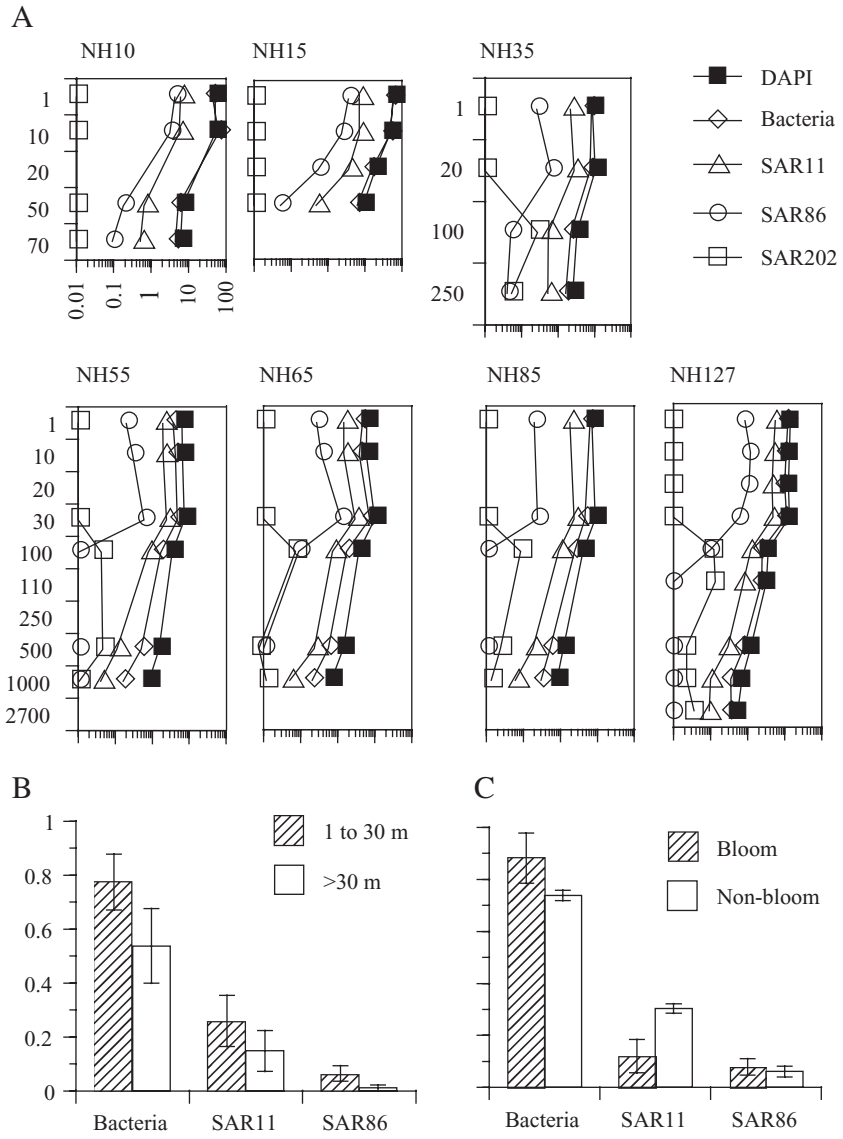

Fig. 2. Group-specific fluorescence *in situ* hybridization and prokaryotic cell counts (DAPI staining particles) collected along the Newport Hydroline in May 2002.
A. Depth profiles of near-shore diatom bloom (NH10 and NH15) and non-bloom (NH35, NH55, NH65, NH85 and NH127) samples from the North Pacific show vertical and horizontal abundance estimates for abundant bacterioplankton lineages.
B. Average Bacteria, SAR11 and SAR86 relative abundances in surface and > 30 m depths.
C. Average Bacteria, SAR11 and SAR86 relative abundances in surface bloom and non-bloom samples.

which were sequenced for phylogenetic analyses. Four sequences representing 13 clones with predicted fragment sizes that ranged from 206 to 208 bp in length were most closely related to *Pirellula* and one clone with a predicted fragment of 223 bp in length was most closely related to OM43 (Fig. 3A). Differences in T-RFLP profiles and fragment contributions from bloom (NH10) and non-bloom (NH35) samples suggest that *Pirellula*, OM43, *Roseobacter* and *Cytophaga* were among the dominant lineages identified in bloom samples (Fig. 3B). Clones representing other bacterioplankton lineages were most closely related to the OM60 cluster and *Actinobacteria* (Table 1). No SAR11 or SAR86 clones were identified in the NH10 library.

Direct cell counts were determined for bloom-associated lineages to quantify trends identified by NMS ordination analyses, which are subject to biases associated with sample collection, 16S rDNA amplification and lineage designations. Lineage-specific FISH cell counts indicated that representatives of the *Pirellula*, OM43, *Roseobacter* and *Cytophaga* lineages exhibited similar, approximately 6.1-fold (±0.7) increases in surface bloom waters (Fig. 4A). *Pirellula* and OM43 cell counts were below the limit of detection at stations NH35 and NH55, but on average increased to 0.4×10^8 and 0.8×10^8 cells l^{-1} in surface bloom waters, respectively (Fig. 4A). *Pirellula* cells were typically cocci, nearly 1 μm in diameter, and usually found in clusters or attached to diatoms (Fig. 4B). In contrast, OM43 cells were more evenly distributed comma-shaped rods that were typically 1–2 μm long (Fig. 4B). *Roseobacter* and *Cytophaga* contributions to non-bloom surface cell counts at NH35 and NH55 were

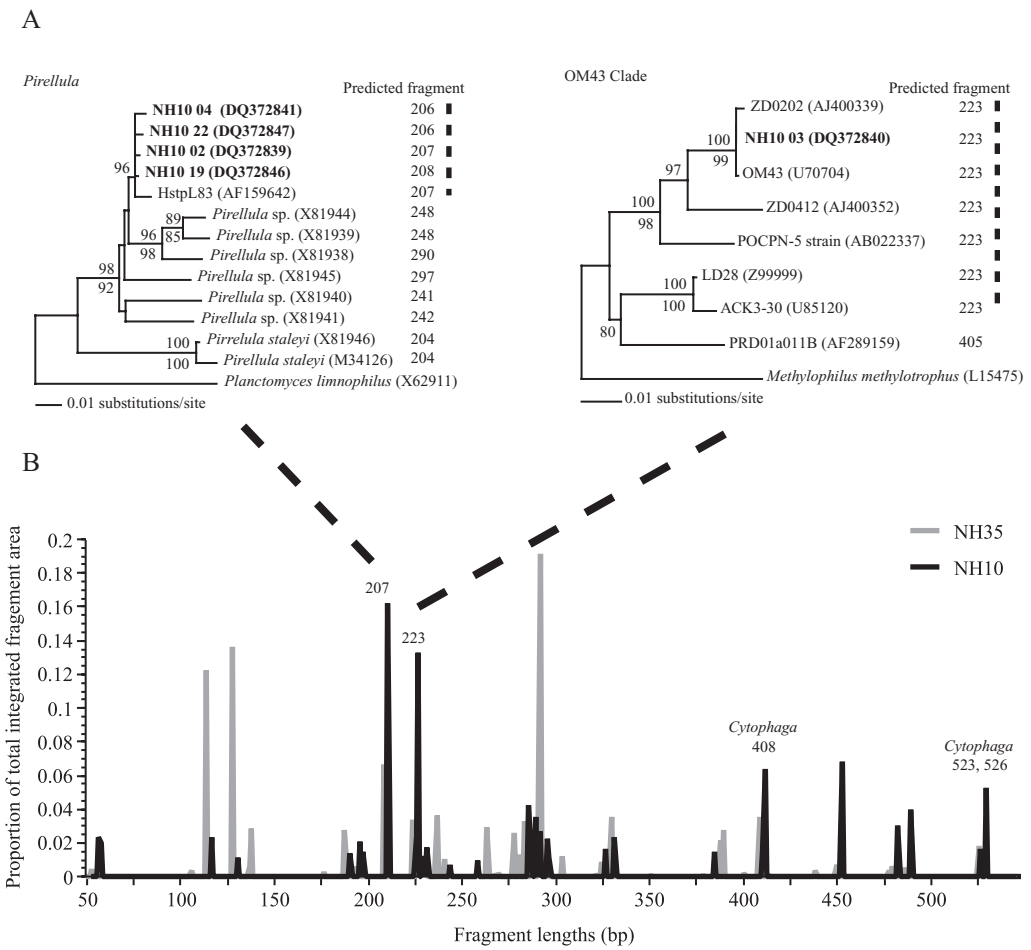

Fig. 3. (A) Neighbour-joining 16S rRNA phylogenetic trees showing terminal restriction fragment sizes and relationships among representative members of the *Pirellula* and OM43 lineages and (B) corresponding fragments from surface bloom and non-bloom T-RFLP profiles. Clones obtained in this study are shown in bold type and representative sequences derived from public databases are labelled with clone names and accession numbers. Bootstrap proportions over 70% from both neighbour-joining (above nodes) and maximum parsimony (below nodes) are indicated for 1000 re-samplings. *Planctomyces limnophilus* and *Methylophilus*/*Methylobacillus* were used as outgroups for *Pirellula* and OM43 trees respectively. Terminal restriction fragment sizes expected from the 16S rRNA gene sequence data are shown to the right.

high and averaged 2.9×10^8 and 2.3×10^8 cells l^{-1} respectively. Of the lineages enumerated using FISH, only *Pirellula* cells were observed attached to diatoms.

Discussion

Members of the *Roseobacter*, *Cytophaga*, *Pirellula* and OM43 lineages were strongly associated with an Oregon coast diatom bloom. While previous studies have shown that representatives of abundant lineages such as *Roseobacter* and *Cytophaga* are associated with phytoplankton (González *et al*., 2000; Riemann *et al*., 2000), significant *Pirellula* and OM43 contributions to bacterioplankton increases in bloom communities have not been reported. The roles of the bloom-associated lineages identified in this study are currently unknown. However, the physical association of the *Pirellula* lineage with diatom cells suggests the possibility that these organisms may interact directly by unknown mechanisms.

The first cultured representative within the genus *Pirellula* was initially described as a stalkless aggregate-forming bacterium similar in morphology to *Pasteuria ramosa* (Staley, 1973). Isolation of a similar spore-forming parasite of daphniae eventually resulted in its reclassification as *Pirellula staleyi* (Starr *et al*., 1983; Schlesner and Hirsch, 1987; Ward *et al*., 1995). Related sequences have since been recovered from diverse marine environments, including tissue cultures from the giant tiger prawn (Fuerst *et al*., 1991) and microaggregates associated with marine snow (DeLong *et al*., 1993). Although far less is known about the OM43 gene clade, a representative genome is currently being sequenced (M. Rappé, pers. comm.). Our results indicate that bacterioplankton community structure shifted in response to a diatom bloom, representatives

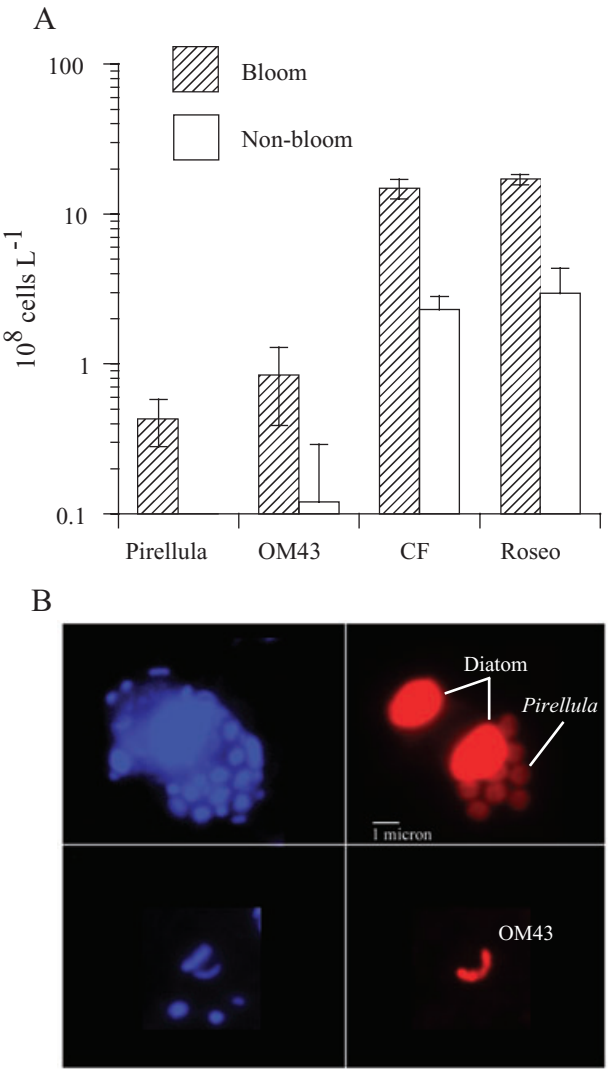

Fig. 4. A. Average lineage-specific *Pirellula*, OM43, *Cytophaga* and *Roseobacter* fluorescence *in situ* hybridization cell counts from surface (1–10 m) bloom (NH10 and NH15) and non-bloom (NH35 and NH55) samples collected along the Newport Hydroline in May 2002. B. Representative images of cells stained with DAPI (left) and hybridized to *Pirellula* and OM43 probes (right).

from *Pirellula* and OM43 were among the dominant lineages observed in bloom samples, and that cells hybridizing to the *Pirellula* probe suite were frequently attached to diatoms.

An important goal for future studies is to identify functionally relevant categories within bacterioplankton lineages by correlating measurements of distribution and abundance with oceanographic processes. Functionally relevant phylogenetic categories for most bacterioplankton lineages have not been well defined. This is due largely to extensive genetic diversity in natural microbial communities and the lack of cultivated representatives available for detailed physiological characterization. Acinas and colleagues (2004) identified fine-scale structure in a coastal bacterioplankton community and Thompson and colleagues (Thompson *et al.*, 2005) reported the occurrence of more than 1000 genotypes in a coastal population of *Vibrio splendidus*. These and other studies highlight the extensive genetic variability and unknown phenotypic diversity that exists within bacterioplankton populations.

In cases where molecular data are used to indicate species abundance, operational taxonomic units can be assigned to different phylogenetic groups and used to evaluate lineage-specific contributions to observed trends in community structure. Community structural analyses of molecular data can extract predominant trends from large heterogeneous community data sets and correlate those trends with measured environmental variables. Potential biases can be overcome by direct quantification using FISH probes or quantitative polymerase chain reaction (PCR) primers designed to target specific lineages.

In this study of marine bacterioplankton, shifts in 16S rDNA fragments corresponding to *Pirellula*, OM43, *Cytophaga* and *Roseobacter* clones were verified by direct quantification using FISH cell counts. While the exact roles of *Pirellula* and other bloom-associated lineages are currently unknown, the complete genome sequence of the marine isolate *Pirellula* sp. strain 1 (*Rodopirellula baltica*) indicates that this organism derives its energy from cleavage of sulfated polymers produced by algae (Glöckner *et al.*, 2003; Schlesner *et al.*, 2004). To date there is no direct evidence that *Pirellula* strains possess adaptations that allow them to interfere directly with the metabolism of eukaryotic cells. However, specific lineage increases in an Oregon coast diatom bloom suggest that representatives identified in this study may play a role in phytoplankton ecology.

Experimental procedures

Sample collection and nucleic acid extraction

Water from a Eastern Pacific Ocean coastal transect was collected from various depths along the Newport Hydroline (44°65′N) at stations NH10 (124°18′W), NH15 (124°25′W), NH35 (124°53′W), NH55 (125°13′W), NH65 (125°36′W), NH85 (126°3′W) and NH127 (127°3′W) as previously described (Morris *et al.*, 2004). Surface (15 m) and upper mesopelagic (250–500 m) high-molecular-weight (HMW) RNA and DNA samples were filtered through 0.2 μm polysulfone filters (Supor-200; PALL, Ann Arbor, MI) housed in an *in situ* water transfer system (McLane, East Falmouth, MA). HMW RNA and DNA was extracted from the filters and processed as described (Giovannoni *et al.*, 1996).

Environmental parameters

Chlorophyll concentration was used as a proxy for phy-

toplankton biomass. Discrete water samples were filtered through GF/F filters and kept frozen at −80°C for up to 1 month before processing. In this study, 90% HPLC grade acetone was added to the filters and allowed to extract overnight at −20°C. Chlorophyll *a* and pheopigment concentrations were determined using a Turner Designs 10-AU fluorometer (Strickland and Parsons, 1972). Samples for nutrient analysis were stored frozen (−20°C) in 60 ml high-density polyethylene bottles. The analyses for phosphate, nitrate plus nitrite (N + N), nitrite and silicic acid (silicate) were performed using a hybrid Technicon AutoAnalyzerII™ and Alpkem RFA300™ system following protocols modified from Gordon and colleagues (1994). Nitrate concentrations were determined by subtracting nitrite values from N + N values.

Flow cytometric analysis of bacteria

A Becton–Dickinson FACSCalibur flow cytometer was used for cell enumeration. Water samples in 3 ml aliquots were fixed with 0.2% w/v paraformaldehyde (final concentration), stored in the dark for at least 10 min at room temperature to harden cells, and quick-frozen in liquid nitrogen. Samples were then stored at −80°C until sample processing on shore. After thawing, heterotrophic cells were enumerated after staining with a 1× working stock of SYBR Green I (Molecular Probes, Eugene, OR) and 25 mM potassium citrate for 15 min following a protocol modified from Marie and colleagues (1997). Photoautotrophic cells were enumerated using unstained aliquots of the water sample. Cytograms of FL3 (red) fluorescence versus FL2 (orange) fluorescence were used to count *Synechococcus* and picoeukaryotes, while cytograms of side scatter versus FL3 fluorescence were used to count diatoms.

PCR, restriction digest and T-RFLP analysis of 16S rDNA

Ribosomal RNA genes from mixed communities were amplified for T-RFLP analyses as previously described (Morris *et al*., 2005). In short, 16S rRNA genes were amplified by PCR with *Taq* polymerase (Fermentas, Hanover, MD) and variations of commonly used bacterial primers: 8F-FAM (Morris *et al*., 2004) and 519R (Morris *et al*., 2005). The 8F-FAM primer was 5′ end-labelled with the phosphoramidite fluorochrome 5-carboxy-fluorescein (6-FAM). Amplifications were performed in a PTC-0200 thermocycler (MJ Research, Cambridge, MA) using the following conditions: 28 cycles, annealing at 55°C for 1 min, elongation at 72°C for 2 min and denaturation at 94°C for 30 s. PCR products were restricted with the BsuR1 (HaeIII) restriction enzyme (Fermentas, Hanover, MD) and cleaned using a MultiScreen 96-well filtration plate (Millipore, Billerica, MA) containing Amersham Sephadex G-50 superfine (Amersham, Piscataway, NJ). Terminal restriction fragments were resolved on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), and ABI Genotyper software was used to size fragments based on an internal lane standard with 23 discrete fragments ranging from 50 to 1000 base pairs in length (Bioventures MapMarker 1000, Cambio, UK).

Cloning

A bacterial 16S rRNA gene clone library was constructed from bacterioplankton collected from NH10 surface waters as described previously (Giovannoni *et al*., 1996). Briefly, ribosomal RNA genes were amplified from environmental DNA for cloning by PCR with *Taq* polymerase (Fermentas, Hanover, MD) and variations of commonly used bacterial primers, 8F and 1492R (Morris *et al*., 2004). Amplifications were performed in a PTC-0200 thermocycler (MJ Research, Cambridge, MA) using the following conditions: 35 cycles, annealing at 55°C for 1 min, elongation at 72°C for 2 min and denaturation at 94°C for 30 s. A single band of the predicted length was observed by agarose gel electrophoresis. A clone library was constructed using the resulting mixed-template amplicon, and the pGEM-T-Easy (Promega, Madison, WI) vector following the manufacturer's instructions. The clones were assigned the prefix 'NH10' and numbered from 01 to 40. All clones were stored at −20°C in Luria–Bertani (LB) broth containing 10% glycerol (w/v). The clone library was screened using a streamlined protocol (Vergin *et al*., 2001) modified for T-RFLP analyses. Cloned 16S rDNA fragments were amplified, restricted, cleaned and resolved as described above for environmental samples. In short, plasmid DNA was extracted and 16S rDNA inserts were amplified using the 8F-FAM labelled and 519R primers used for environmental T-RFLP analyses. Clones exhibiting similar terminal restriction fragments were grouped together and group representatives were selected for sequencing (Table 1).

Sequencing and phylogenetic analysis

Cloned 16S rRNA genes were grouped according to terminal restriction fragment length and representatives were sequenced and used for phylogenetic analysis. Only clones that contained full-length inserts (~1500 bp), and for which there was a single or dominant observed terminal restriction fragment, were selected for sequencing (Table 1). 16S rRNA gene sequences were compared with sequences available in the GenBank database by BLAST (Altschul *et al*., 1997), and aligned with the ARB software package and database (Ludwig *et al*., 2004). Only unambiguously aligned nucleotide positions were used for phylogenetic analyses. In total, 834 nucleotide positions (*Escherichia coli* numbering, 35–1065) were used for *Pirellula* and 1098 nucleotide positions (*E. coli* numbering, 53–1189) were used for OM43 phylogenetic analyses with PAUP* 4.0 beta 10 (Swofford, 2002). Phylogenetic trees were inferred by neighbour-joining analyses (Saitou and Nei, 1987) using Juke–Cantor evolutionary distance correction. The tree topology was also compared with the phylogenetic trees inferred by both maximum parsimony with a heuristic search and maximum likelihood with a heuristic search, a transition/transversion ratio of 1.7326, and nucleotide frequencies estimated from the data. The neighbour-joining and parsimony trees were evaluated by bootstrap analyses based on 1000 re-samplings.

Statistical analysis of community structure

Non-metric multidimensional scaling searches for the best positions of *n* entities (samples) on *k* dimensions (axes) that

minimize stress of the final configuration. Calculations are based on a distance matrix generated from the species matrix, and stress is measured as the departure from monotonicity between distance in the original space and distance in ordination space (McCune and Grace, 2002). Sorensen distance and autopilot slow and thorough options were selected for NMS analyses of T-RFLP data using a random starting configuration and the software package PC-ORD (McCune and Grace, 2002). Normal analyses were performed on a data matrix consisting of 10 samples in rows and 139 fragments in columns. Fragments occurring in fewer than two samples were excluded. Remaining fragments were transformed into relative units by dividing integrated fragment areas by the total for their corresponding sample. Categorical variables were assigned to surface bloom, surface non-bloom and upper mesopelagic samples based on suspected patterns associated with surface bloom samples and known patterns in community variability with depth. A stable two-dimensional solution was identified with final stress of 3.5 and instability near zero. The probability of a similar stress obtained by chance (0.0196) was calculated by comparing 50 randomized Monte Carlo runs, with 40 runs of real data. Axes were rotated to maximize orthogonality (> 99%).

SAR86 and OM43 probe analyses

In this study, 84 SAR86 and 12 906 total 16S rRNA sequences were analysed using the ARB sequence analysis package (Ludwig *et al*., 2004) to determine probe specificity. In total, 78 SAR86 sequences contained sequence spanning at least one target site that was an exact match to at least one probe sequence (Table 2). A minimum of three of the four probes matched exactly all rRNA genes with sequence spanning all target sites ($n = 25$), and the majority of these sequences (72%) matched all four probes identically. None of the probes used in the SAR86 probe suite matched sequences outside of the SAR86 clade. Of 52 OM43 sequences analysed 40 contained target sites with an exact match to probe OM43-82R, including clone NH10-03. Two SSU rRNA sequences outside the OM43 clade, including a gamma proteobacteria symbiont of *Thyasira flexuosa* and a plastid sequence, also had the probe target sequence. Both sequences are unlikely to contribute to cell counts reported in this study of pelagic bacterioplankton.

Fluorescence in situ *hybridization*

Hybridization reactions were performed as previously described for SAR11 and SAR202 lineages (Morris *et al*., 2002; 2004). In short, reactions were performed on one quarter membrane sections at 37°C for 12–16 h in hybridization buffer [900 mM NaCl, 20 mM Tris (pH 7.4), 0.01% (w/v) sodium dodecyl sulfate (SDS) and 15% or 35% formamide] and Cy3-labelled oligonucleotide probe suites specific for Bacteria, SAR11, SAR86, SAR202, *Pirellula*, OM43, *Roseobacter* or *Cytophaga* (Table 2). A control hybridization reaction was performed with a low-stringency buffer containing 35% formamide and a Cy3-labelled nonsense oligonucleotide (338F). All probes had a final concentration of 2 ng μl^{-1} each. Optimal hybridization stringency was achieved by washing the membranes in hybridization wash [20 mM Tris (pH 7.4), 6 mM ethylenediaminetetraacetic acid (EDTA), 0.01% SDS and 70 or 150 mM NaCl] for two 10 min intervals. Experimentally determined or previously published temperatures of dissociation (Td) specific for each probe or probe suite were used to control stringency in the hybridization washes (Table 2). Nucleic acid staining occurred by transferring the membrane to a chilled (4°C) hybridization wash containing DAPI at a final concentration of 5 $\mu g\ ml^{-1}$ for 10 min. The DAPI was rinsed for 2 min in a final hybridization wash chilled to 4°C. All reagents were 0.2 μm filtered.

Fluorescent microscopy

After mounting the filters in Citifluor (Ted Pella, Redding, CA), Cy3-positive and DAPI-positive cells were counted for each field of view using a Leica DMRB epifluorescence microscope equipped with a Hamamatsu ORCA-ER CCD digital camera, filter sets appropriate for Cy3 and DAPI, and Scanalytics IPLab v3.5.6 scientific imaging software. Consistent exposure times of 1 and 5 s were used for DAPI and Cy3 images respectively. Cy3 images were manually segmented in IP Lab and automatically made to overlay corresponding DAPI image segmentations in order to identify positive probe signals coincident with DAPI signals. Negative control counts were determined from the 338F hybridization using the same technique and subtracted from positive probe counts to account for objects detected using the Cy3 and DAPI filter sets in the absence of the positive probe set, such as autofluorescent cells. Partial images (0.25) were counted from bloom-surface waters (1–10 m) at NH10 and NH15 to adjust for significant increase observed in bacterioplankton abundances.

Nucleotide sequence accession numbers

Gene sequences were deposited in GenBank and given accession numbers DQ372838–DQ372853.

Acknowledgements

We thank Mike Rappé and Stephanie Connon for designing OM43 and SAR86 oligonucleotide probes. We also thank Barry and Evelyn Sherr for their support, the officers and crew of the *Wecoma* for their valuable assistance, and three anonymous reviewers and the editor for their many constructive comments. This study was supported through a Cornell University Biogeochemistry and Biocomplexity Postdoctoral Research Fellowship for R.M.M., an Oregon Sea Grant (NA16RG1039, Project No. R/HAB-01), the National Science Foundation (MCB-0237713 and OCE-0002236 to B.F. Sherr and E.B. Sherr) and in part by the Gordon and Betty Moore Foundation Award No. 607.

References

Acinas, S.G., Klepac-Ceraj, V., Hunt, D.E., Pharino, C., Ceraj, I., Distel, D.L., and Polz, M.F. (2004) Fine-scale

phylogenetic architecture of a complex bacterial community. *Nature* **430:** 551–554.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A., and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. *Mar Ecol Progr Ser* **10:** 257–263.

Brinkmeyer, R., Rappé, M.S., Gallacher, S., and Medlin, L. (2000) Development of clade (*Roseobacter* and *Alteromonas*) and taxon-specific oligonucleotide probes to study interactions between toxic dinoflagellates and their associated bacteria. *Eur J Phycol* **35:** 315–331.

Carlson, C.A., Ducklow, H.W., Hansell, D.A., and Smith, W.O. (1998) Organic carbon partitioning during spring phytoplankton blooms in the Ross Sea polynya and the Sargasso Sea. *Limnol Oceanogr* **43:** 375–386.

Cole, J.J., and Pace, F.L. (1988) Bacterial production in fresh and saltwater ecosystems. *Mar Ecol Progr Ser* **43:** 1–10.

DeLong, E.F., Franks, D.G., and Alldredge, A.L. (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol Oceanogr* **38:** 924–934.

Ducklow, H.W. (1983) Production and fate of bacteria in the oceans. *Bioscience* **33:** 494–501.

Eilers, H., Pernthaler, J., Glockner, F.O., and Amann, R. (2000) Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66:** 3044–3051.

Fuerst, J.A., Sambhi, S.K., Paynter, J.L., Hawkins, J.A., and Atherton, J.G. (1991) Isolation of a bacterium resembling *Pirellula* species from primary tissue culture of the giant tiger prawn (*Penaeus monodon*). *Appl Environ Microbiol* **57:** 3127–3134.

Fukami, K., Yuzawa, A., Nishijma, T., and Hata, Y. (1996) Effect of bacterial assemblages on the succession of blooming phytoplankton from *Sketetonema costatum* to *Heterosigma skashiwo*. In *Harmful and Toxic Algal Blooms.* Yasumota, T., Oshima, Y., and Fukuyo, Y. (eds). Paris, France: Intergovernmental Oceanographic Commission of UNESCO, pp. 335–338.

Gade, D., Schlesner, H., Glockner, F.O., Amann, R., Pfeiffer, S., and Thomm, M. (2004) Identification of planctomycetes with order-, genus-, and strain-specific 16S rRNA-targeted probes. *Microb Ecol* **47:** 243–251.

del Giorgio, P.A., Cole, J.J., and Cimbleris, A. (1997) Respiration rates in bacteria exceed phytoplankton production in unproductive aquatic systems. *Nature* **385:** 148–151.

Giovannoni, S.J., Rappé, M.S., Vergin, K.L., and Adair, N.L. (1996) 16S rRNA genes reveal stratified open ocean bacterioplankton populations related to the Green Non-Sulfur bacteria. *Proc Natl Acad Sci USA* **93:** 7979–7984.

Glöckner, F.O., Kube, M., Bauer, M., Teeling, H., Lombardot, T., Ludwig, W., *et al.* (2003) Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. *Proc Natl Acad Sci USA* **100:** 8298–8303.

González, J.M., Simó, R., Massana, R., Covert, J.S., Casamayor, E.O., Pedrós-Alió, C., and Moran, M.A. (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. *Appl Environ Microbiol* **66:** 4237–4246.

Gordon, L.I., Jennings, J.J.C., Ross, A.A., and Krest, J.M. (1994) A suggested protocol for continuous flow automated analysis of seawater nutrients (phosphate, nitrate, nitrite and silicic acid) in the WOCE Hydrographic Program and the Joint Global Ocean Fluxes Study. In *WOCE Operations Manual, WOCE Report No. 18/91. Revision 1, 1994.* Woods Hole, MA: Woods Hole Oceanographic Institute.

Long, R.A., and Azam, F. (2001) Antagonistic interactions among marine pelagic bacteria. *Appl Environ Microbiol* **67:** 4975–4983.

Lovejoy, C., Bowman, J.P., and Hallegraeff, G.M. (1998) Algicidal effects of a novel marine *Pseudoalteromonas* isolate (class *Proteobacteria*, gamma subdivision) on harmful algal bloom species of the genera *Chattonella*, *Gymnodinium*, and *Heterosigma*. *Appl Environ Microbiol* **64:** 2806–2813.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363–1371.

McCune, B., and Grace, J.B. (2002) *Analysis of Ecological Communities.* Gleneden Beach, OR, USA: MJM Software.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., and Schleifer, K.H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* **142:** 1097–1106.

Marie, D., Partensky, F., Jacquet, S., and Vaulot, D. (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Appl Environ Microbiol* **63:** 186–193.

Morris, R.M., Rappé, M.S., Connon, S.A., Vergin, K.L., Siebold, W.A., Carlson, C.A., and Giovannoni, S.J. (2002) SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420:** 806–810.

Morris, R.M., Rappé, M.S., Urbach, E., Connon, S.A., and Giovannoni, S.J. (2004) Prevalence of the Chloroflexi-related SAR202 bacterioplankton cluster throughout the mesopelagic zone and deep ocean. *Appl Environ Microbiol* **70:** 2836–2842.

Morris, R.M., Vergin, K.L., Cho, J.C., Rappé, M.S., Carlson, C.A., and Giovannoni, S.J. (2005) Temporal and spatial response of bacterioplankton lineages to annual convective overturn at the Bermuda Atlantic Time-series Study site. *Limnol Oceanogr* **50:** 1687–1696.

Moses, W.C., and Wheeler, P.A. (2004) *Seasonal and Across-shelf Trends of the Phytoplankton Community of the Oregon Coast Environment.* Data Report no. 194, Northeast Pacific GLOBEC Long-term Observation Program, College of Oceanic and Atmospheric Sciences, Oregon State University.

Pomeroy, L.R. (1974) The ocean's food web, a changing paradigm. *Bioscience* **24:** 499–504.

Riemann, L., Steward, G.F., and Azam, F. (2000) Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* **66:** 578–587.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4:** 406–425.

Schlesner, H., and Hirsch, P. (1987) Rejection of the genus name *Pirella* for pear-shaped budding bacteria and proposal to create the genus *Pirellula* gen. nov. *Int J Syst Bacteriol* **37:** 441.

Schlesner, H., Rensmann, C., Tindall, B.J., Gade, D., Rabus, R., Pfeiffer, S., and Hirsch, P. (2004) Taxonomic heterogeneity within the Planctomycetales as derived by DNA–DNA hybridization, description of *Rhodopirellula baltica* gen. nov., sp. nov., transfer of *Pirellula marina* to the genus *Blastopirellula* gen. nov. as *Blastopirellula marina* comb. nov. and emended description of the genus *Pirellula*. *Int J Syst Evol Microbiol* **54:** 1567–1580.

Staley, J.T. (1973) Budding bacteria of the Pasteuria-Blastobacter group. *Can J Microbiol* **19:** 609–614.

Starr, M.P., Sayre, R.M., and Schmidt, J.M. (1983) Assignment of ATCC27377 to *Planctomyces staleyi* sp. nov. and conservation of *Pasteura ramosa* Metchnikoff 1988 on the basis of type descriptive material. Request for an opinion. *Int J Syst Bacteriol* **33:** 666–671.

Strickland, J.D.H., and Parsons, T.R. (eds) (1972) *A Practical Handbook of Seawater Analysis.* Ottawa, Canada: Fisheries Research Board of Canada.

Swofford, D.L. (2002) *PAUP: Phylogenetic Analysis Using Parsimony (*and Other Methods).* Sunderland, MA, USA: Sinauer Associates.

Thompson, J.R., Pacocha, S., Pharino, C., Klepac-Ceraj, V., Hunt, D.E., Benoit, J., *et al.* (2005) Genotypic diversity within a natural coastal bacterioplankton population. *Science* **307:** 1311–1313.

Vergin, K.L., Rappé, M.S., and Giovannoni, S.J. (2001) Streamlined method to analyze 16S rRNA gene clone libraries. *BioTechniques* **30:** 938–940, 943–944.

Ward, N., Rainey, F.A., Stackebrandt, E., and Schlesner, H. (1995) Unraveling the extent of diversity within the order *Planctomycetales*. *Appl Environ Microbiol* **61:** 2270–2275.

Wetz, M.S., Wheeler, P.A., and Letelier, R.M. (2004) Light-induced growth of phytoplankton collected during the winter from the benthic boundary layer off Oregon, USA. *Mar Ecol Prog Ser* **280:** 95–104.

Williams, P.J.L. (1981) Incorporation of microheterotrophic processes in the classical paradigm of the planktonic food webs. *Kieler Meeresforsch* **5:** 1–28.

Yoshinaga, I., Kawai, T., and Ishida, Y. (1997) Analysis of algicidal ranges of the bacteria killing the marine dinoflagellate *Gymnodinium mikimotoi* isolated from Tanabe Bay (Wakayama Pref., Japan). *Fish Sci* **61:** 780–786.

