Vol. 64: 15–25, 2011
doi: 10.3354/ame01504

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online June 21

Bacterial diversity in the bottom boundary layer of the inner continental shelf of Oregon, USA

A. D. Bertagnolli[1], A. H. Treusch[1], O. U. Mason[2], U. Stingl[4], K. L. Vergin[1], F. Chan[3], B. Beszteri[1], S. J. Giovannoni[1,*]

[1]Department of Microbiology, [2]College of Oceanic and Atmospheric Sciences, and
[3]Partnership for Interdisciplinary Studies of Coastal Oceans, Oregon State University, Corvallis, Oregon 97331, USA
[4]Red Sea Research Center, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia 23955

ABSTRACT: There have been few studies of the bacterial community within the bottom boundary layer (BBL)—the turbulent region of the water column above the benthos—in shallow seas. Typically, the BBL has large amounts of particulate organic matter suspended by turbulence, and it is often the first region of the water column to become hypoxic when oxygen declines. Communities at the surface (5 m) and in the BBL (1 to 10 m above the sea floor) were compared by terminal restriction fragment length polymorphism (T-RFLP) analysis and sequencing of the 16S rRNA gene. Multivariate statistical methods (hierarchical clustering, non-metric multidimensional scaling, and analysis of similarity (ANOSIM)) indicated that the microbial community of the BBL is distinct from the surface community. ANOSIM supported the distinction between surface and BBLs (R values 0.427 and 0.463, based on analysis with restriction enzymes BsuR1 and Hin6I, respectively, $p < 0.1\%$). Six terminal restriction fragments showed an increase in abundance with depth. Cloning, screening and sequencing identified these as a novel environmental clade (Eastern North Pacific *Chromatiales* (ENPC) clade), the ARTIC96BD-19 clade of *Gammaproteobacteria*, the 6N14 and Agg8 clades of the phylum *Planctomycetes*, the OM60/NOR5 clade of *Gammaproteobacteria*, and uncultivated members of the *Roseobacter* clade in the MB11C09 and ULA23 subgroups. To the best of our knowledge, this analysis is the first to focus on the unique composition of microbial communities of the BBL in shallow, inner-shelf regions off the coast of Oregon, USA, and the first to report that an uncharacterized clade of *Chromatiales* is indigenous in this habitat.

KEY WORDS: 16S rRNA gene · T-RFLP · Continental shelf · Northeast Pacific Ocean

INTRODUCTION

The northeast Pacific Ocean along the Oregon continental shelf is a productive marine ecosystem that experiences seasonal upwelling of nutrient-rich mesopelagic waters into the mixed layer. The delivery of nutrient-rich waters to the euphotic zone spurs high primary production by marine phytoplankton, which, in turn, supports higher trophic levels (Pauly & Christensen 1995). Thus, while coastal environments occupy relatively small volumes of the global oceans, they support a substantial proportion (90%) of the world's fisheries yield, making these areas an invaluable natural resource for economic and recreational purposes (Pauly et al. 2002). Coastal Oregon is a particularly interesting ecosystem in light of recent reports of summer anoxic events in shallow, inner-shelf areas having detrimental impacts on pelagic biodiversity (Grantham et al. 2004, Chan et al. 2008).

Bacterial communities are important drivers of biogeochemical cycles in marine ecosystems (Arrigo 2005). Heterotrophic bacteria have a massive impact on the global carbon cycle by transforming organic carbon and remineralizing a large fraction of it to CO_2

*Corresponding author.
Email: steve.giovannoni@oregonstate.edu

(Fuhrman & Azam 1980). Studies of plankton communities in deep waters near the edges of continental shelves, and in ocean gyres, have revealed that bacterial plankton populations frequently are stratified. Members of the SAR202 clade, related to *Chloroflexi* (Giovannoni et al. 1996), as well as the SAR406 clade related to *Fibrobacter* (Gordon & Giovannoni 1996), the SAR324 clade of *Deltaproteobacteria* (Wright et al. 1997) and marine group I *Archaea* (Massana et al. 1997, Karner et al. 2001), display vertical distribution patterns which are often coupled to other physical and biological parameters.

Surveys of microbial diversity in coastal surface waters have revealed that these environments harbor some organisms that are also found in open-ocean surface waters, such as *Alphaproteobacteria* in the SAR11 and SAR116 clades. Other taxa are more frequently detected in coastal environments. These include the OM43 clade of *Betaproteobacteria* related to type I methylotrophs (Rappe et al. 1997, 2000), *Gammaproteobacteria* in the OM60/NOR5 and SAR92 clades (Cho et al. 2007, Stingl et al. 2007a), and *Alphaproteobacteria* affiliated with the *Roseobacter* clade (Buchan et al. 2005). Analyses have been carried out of the bacterial community structure in response to upwelling-induced primary production off the coast of Oregon. 16S rRNA gene clone sequencing, in conjunction with fluorescence *in situ* hybridization, showed that bacterioplankton communities during diatom bloom events differ from non-bloom communities, with members of the genus *Pirellula* and the OM43 clade increasing during these periods (Morris et al. 2006). These studies have painted a detailed picture of the organisms residing in the surface layer off the coast of Oregon. However, knowledge pertaining to microbial diversity below the surface layer, yet above the benthos—i.e. in the bottom boundary layer (BBL)—is lacking for shallow coastal environments.

In shallow seas, the BBL is coupled to physical processes at the surface, such as wind and waves (Grant & Madsen 1986). In deep ocean BBLs, bacteria experience low concentrations of labile organic matter and low temperatures (Turley 2000), whereas in shallow seas, particulate organic matter, including cells sinking from the surface, can transit the water column quickly and enter the BBL. From the perspective of ocean stratification, BBLs in shallow oceans are interesting because decomposition processes—remineralization of dissolved organic matter (DOM)—are compressed vertically, making them susceptible to hypoxia and anoxia. Because microbial communities are important drivers of global nutrient cycles, investigating the phylogeny of the organisms residing in shallow coastal BBLs is a necessary first step in elucidating their putative biogeochemical functions.

The goal of the present study was to examine bacterial diversity in the BBL in near-shore regions (<100 m) of the Oregon shelf, as part of a long-term study of seasonal hypoxia in this region. Analyses of samples taken over a 2 yr period, collected during the spring and summer, showed that the microbial community of the BBL is distinctly different from the microbial community at the surface, and that it harbors previously undescribed microbial taxa specific to this environment.

MATERIALS AND METHODS

Site description. Samples for this study were collected along the Strawberry Hill (SH) line (44° 15′ N) at Stns SH50 (124° 10′ W) and SH100 (124° 27′ W) on the RV 'Elakha'. Sample depth at Stn SH50 is 50 m, and 100 m at Stn SH100. Samples were collected at the surface (5 m) and in the BBLs (1 to 10 m above the sediment). Movement of the ship, due to wave action, can influence the exact placement of water collection equipment and other types of data analysis tools. We maintained a distance of at least 1 m above the benthic water interface for all samples collected; however, during rougher periods, we were more conservative in our collection approach, allowing up to 10 m in order to ensure that the benthos not be disturbed. For a comprehensive list of samples used in this study see Table 1.

Environmental parameters. Conductivity, temperature, salinity, and oxygen were measured using a vertical profiling conductivity, temperature and depth system with a mounted oxygen sensor (Sea-Bird Electronics). Dissolved oxygen sensor values were standardized using Winkler titrations.

Sampling and DNA extractions. Seawater was collected using a 30 l Niskin bottle (General Oceanics), and distributed into 20 l sterile polyethylene carboys. Water samples were filtered onto 0.2 µm polysulfone filters (Pall-Gellman). Immediately after collection, filters were frozen in sucrose lysis buffer (50 mM Tris-HCl, 40 mM EDTA, 0.75 M sucrose) and stored at −80°C until processing. Community DNA was isolated as described previously (Giovannoni et al. 1996); in brief, DNA was isolated using a phenol-chloroform-isoamyl alcohol extraction, followed by density centrifugation using cesium trifluoroacetic acid to separate high-molecular-weight community ribosomal RNA from DNA.

Terminal restriction fragment length polymorphism (T-RFLP) analysis. For analysis of bacterial small-subunit (SSU) 16S rRNA genes by T-RFLP using the restriction enzyme BsuR1, 20 ng of environmental DNA was amplified with the bacteria-specific primer 8F and the universal primer 519R (Morris et al. 2005). The

Table 1. Sample collection dates, locations, and associated environmental parameters. BBL = bottom boundary layer; Temp. = temperature; Sal. = salinity

Date	Lat. (N)	Long. (W)	Stn	Depth	Temp. (°C)	Sal. (ppt)	Density (σ)	Oxygen (ml l^{-1})
2 April 2007	44° 15′	124° 10′	SH50	Surface	9.87	31.70	24.40	8.71
2 April 2007	44° 15′	124° 10′	SH50	BBL	8.91	33.65	26.07	2.55
7 May 2007	44° 15′	124° 10′	SH50	Surface	11.08	31.66	24.16	7.73
7 June 2007	44° 15′	124° 10′	SH50	Surface	11.27	33.10	25.25	10.68
7 June 2007	44° 15′	124° 10′	SH50	BBL	7.58	33.92	26.48	2.04
11 July 2007	44° 15′	124° 10′	SH50	Surface	10.10	33.35	25.65	5.24
11 July 2007	44° 15′	124° 10′	SH50	BBL	7.28	33.94	26.54	0.70
8 April 2008	44° 15′	124° 10′	SH50	Surface	9.03	31.77	24.59	7.68
14 May 2008	44° 15′	124° 10′	SH50	Surface	9.64	32.63	25.16	8.42
18 June 2008	44° 15′	124° 10′	SH50	Surface	9.35	33.48	25.87	4.61
18 June 2008	44° 15′	124° 10′	SH50	BBL	7.20	33.94	26.56	1.21
26 June 2008	44° 15′	124° 10′	SH50	BBL	7.13	33.94	26.56	1.21
21 July 2008	44° 15′	124° 10′	SH50	Surface	9.19	33.40	25.83	5.24
21 July 2008	44° 15′	124° 10′	SH50	BBL	7.12	33.92	26.55	1.19
21 July 2008	44° 15′	124° 70′	SH100	Surface	9.46	33.20	25.64	6.29
21 July 2008	44° 15′	124° 70′	SH100	BBL	6.88	33.98	26.63	0.61
30 July 2008	44° 15′	124° 10′	SH50	Surface	10.21	33.76	25.94	8.33
30 July 2008	44° 15′	124° 10′	SH50	BBL	7.51	33.93	26.50	1.88
30 July 2008	44° 15′	124° 70′	SH100	Surface	11.64	33.32	25.35	8.69
30 July 2008[a]	44° 15′	124° 70′	SH100	BBL	7.10	33.96	26.58	0.65

[a]16S rRNA gene clone library constructed from sample

8F primer was labeled at the 5′ end with the phosphoramidite fluorochrome 5-carboxyfluorescein (FAM). Each polymerase chain reaction (PCR) contained final concentrations consisting of 0.25 U Taq polymerase (Fermentas), 2.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotides, and 0.2 µM of each primer. PCR amplifications were performed in a PTC-0200 Thermocycler (MJM Technologies) using the following conditions: 28 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min. FAM-labeled products were visualized on a 1% agarose gel, and cleaned using the QIAquick PCR clean-up kit (Qiagen). Reaction products were then digested at 37°C for 6 h with BsuR1; the digests contained 10 units of restriction enzyme per reaction in 1× Buffer R (Fermentas). Digested FAM-labeled products were cleaned using Sephadex G-50 (Applied Biosystems). Applied Biosystems Genotyper software was used to assess fragment lengths based on the size of the internal standard Map-Marker (Applied Biosystems), which contained 23 discrete size fragments ranging from 50 to 1000 bp. Samples for analysis with the restriction enzyme Hin6I were treated in a way similar to that used with BsuR1, with the following exceptions: PCR products were amplified for 26 cycles, rather than 28, and PCR reactions contained 1 unit of the protein bovine serum albumin. Enzyme digests were precipitated by ethanol using 2 volumes of ethanol and 0.1 volume of sodium acetate. Digests were vacuum dried, resuspended in sterile water, and then analyzed.

Statistical analyses of community structure. Prior to the analyses, T-RFLPs were noise-filtered by eliminating fragments that did not contribute to at least 1% of the relative fluorescence in each sample. Noise-filtered relative fluorescence values were then used for further statistical analysis. Fragments that did not contribute to at least 2 samples were removed. Hierarchical clustering and multidimensional scaling were performed in version 6 of the software package Primer (Plymouth Marine Laboratories). In brief, a distance matrix was calculated using the S17 Bray-Curtis measure. Hierarchical cluster analysis was performed on the resulting matrix. Multidimensional scaling analysis was performed using 25 re-starts and a minimum stress of 0.01. Analysis of similarity (ANOSIM) was used to test the null hypothesis of no difference between surface and BBL samples. The software package R was used for regression analysis of relative T-RFLPs against environmental parameters. The significance of each fragment was examined by testing the correlation between each fragment's logit transformed relative abundance and depth with the cor.test function of the program R, using the Benjamini-Hochberg procedure to correct p-values for multiple testing.

16S rRNA gene cloning. A 16S SSU rRNA gene clone library was constructed from 30 July 2008 at Stn SH100 (Table 1). 16S rRNA genes were amplified using primers 8F and 926R (Lane et al. 1985). PCR

products were amplified with 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min. Cleaned PCR products were ligated into the pGEM T-easy vector (Promega) overnight at 4°C, and the resulting ligation was cloned in *Escherichia coli*-DHα chemically competent cells (Invitrogen), as described previously (Giovannoni et al. 1996). Clones containing the correct size insert were end-sequenced using the primer M13F (5′-ATT AAC CCT CAC TAA AGG GA-3′) to produce a nearly full-length single-strand sequence. Clones from this study were labeled with the library prefix 'OR-SH100-100m'. A total of 106 clones from the library corresponding to 30 July OR-SH100-100m were end-sequenced; of these, 90 were identified as non-chimeric and were further analyzed. To identify T-RFLP fragments we constructed a clone library and analyzed 90 clones. These clones were digested *in silico* with BsuR1 and Hin6I to determine the predicted fragment lengths. Based on these predicted restriction sites, candidate clones were then selected for analysis by T-RFLP to compare predicted and observed restriction site lengths (which often differ slightly), and to match the phylogenetic identification with the environmental T-RFLP peak (Table 2). Sequence information has been deposited in Genbank: accession numbers HQ149693, and HQ173717 to HQ173805.

Phylogenetic analysis and taxonomic assignment. Cloned sequences were edited to remove vector contamination with Vec-Screen, an online tool for sequence analysis based on the BLAST algorithm (Altschul et al. 1990). Edited sequences were aligned with the NAST-alignment tool in Green-Genes and then analyzed with Bellerophon to identify putative chimeras (DeSantis et al. 2006). Sequences were imported into the software package ARB and aligned to the SILVA alignment version 92 (Ludwig et al. 2004). Sequence alignments were then manually edited, and phylogenetic trees were constructed. Tree topologies were analyzed using neighbor-joining, maximum-likelihood, and parsimony phylogenetic analyses; boot-strap values were based on 1000 re-samplings.

RESULTS

Bacterioplankton community structure

Bacterial community structure was assessed by T-RFLP targeting of the 16S rRNA gene of bacteria with 2 independent restriction enzymes (BsuR1 and Hin6I). Hierarchical clustering and multidimensional scaling analyses of relative fragment abundances supported a clear distinction between samples collected in the surface area of the water column and those collected in the BBL, with BBL samples forming a distinct cluster at the 40% similarity level under both analyses (Fig. 1). ANOSIM also supported a significant difference in community structure between surface and BBL communities ($R = 0.427$ and 0.426 with BsuR1 and Hin6I, respectively, $p < 0.1$).

To identify bacterial taxa associated with the BBL, we examined the contribution of individual terminal restriction fragments to the BBL cluster in each ordination, and evaluated the depth dependency of each fragment by regression analyses. Overall, we identified 6 terminal restriction fragments that significantly contributed to the BBL; 4 were identified with enzyme BsuR1 (254, 287, 323 and 253 bp; Fig. 2A–D) and 2 were identified with Hin6I (340 and 354 bp; Fig. 2E,F) (see also Table 2). To determine the taxonomic identities of these fragments, a 16S rRNA gene clone library was phylogenetically characterized and analyzed for the aforementioned fragments.

Table 2. R values for depth computed with the restriction enzymes BsuR1 and Hin6I. Only fragments that showed correlation values of 0.7 or greater with Benjamini-Hochberg corrected p-values of less than 0.05 are reported. Phylogenetic associations for observed fragments are listed as well as an index describing the initial phylogenetic characterization of each organism from the marine library. Observed length shows observed T-RFLP fragment length of selected clone on ABI 3700; Predicted shows predicted T-RFLP fragment length based on 16S rRNA gene clone sequence. ENPC: Eastern North Pacific *Chromatiales*

Clone no.	Observed length	Predicted	R value depth	Phylogenetic identification	Clade designation
Enzyme BsuR1					
6 (Fig. 3)	254	257	0.84	*Gammaproteobacteria* ENPC clade	Present study
3 (Fig. 4)	287	287	0.83	*Planctomycetes* 6N14	Present study
50 (Fig. 3)	323	322	0.78	*Gammaproteobacteria* ARTCIC96BD-19	Suzuki et al. (2004)
11 (Fig. 4)	253	252	0.71	*Planctomycetes* agg8	DeLong et al. (1993)
Enzyme Hin6I					
48, 9, 53 (Fig. 5)	340	339	0.91	*Roseobacter* (MBC1109 & ULA23)	Suzuki et al. (2004) & Present study
63 (Fig. 3)	354	353	0.80	*Gammaproteobacteria* OM60/NOR5	Rappe et al. (1997)

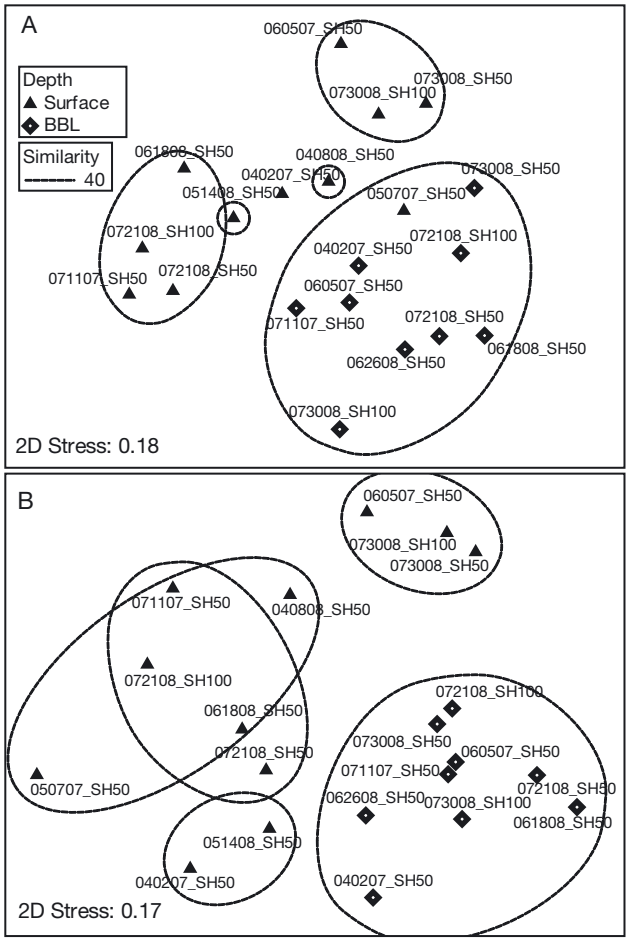

Fig. 1. Non-metric multi-dimensional scaling (NMDS) analysis of relative terminal restriction fragments produced from samples collected in this study with the enzymes (A) BsuR1 and (B) Hin6I. Hierarchical clustering analysis was performed as well, and similarity levels based on these analyses are represented at the 40% similarity level. Samples are labeled with the month, day and year followed by the site designation; example: 040207_SH50 is interpreted as 2 April 2007, Stn SH50. BBL: bottom boundary layer

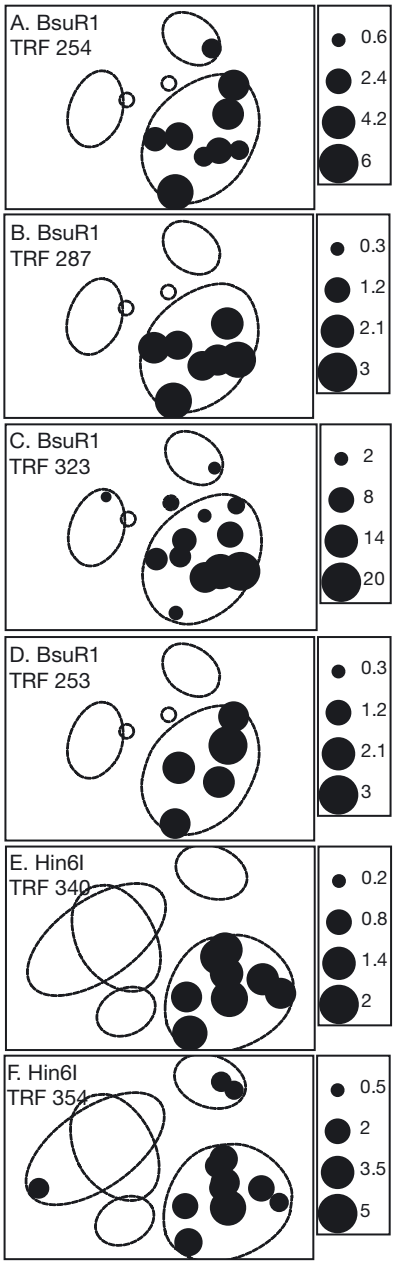

Fig. 2. Individual terminal restriction fragment (TRF) contributions to the non-metric multi-dimensional scaling (NMDS) ordinations based on analysis with (A–D) BsuR1 and (E,F) Hin6I. The inset of each plot indicates the relative abundance of a given fragment

Lineage identification

BsuR1

Clone library analyses identified several clones with a 323 bp signature. These clones corresponded to a group of *Gammaproteobacteria* in the ARTIC96BD-19 clade (Figs. 2C & 3). This clade comprises clones from several marine 16S rRNA gene surveys, in addition to an uncharacterized bacterial strain (HTCC8012) which was isolated from coastal Oregon seawater by dilution to extinction culturing (Stingl et al. 2007b). These clones were 99% similar to strain HTCC8012 and 93% similar to the putative sulfur-oxidizing symbiont of the hydrothermal vent clam *Calyptogena magnifica*.

Clones attributed to a 254 bp restriction fragment formed a novel clade in the order *Chromatiales* of the *Gammaproteobacteria* (Figs. 2A & 3). This clade has no cultured representatives. The nearest relatives in the NCBI-nr database were retrieved from the Saanich Inlet, British Columbia (clone SHBC668) (Walsh et. al. 2009). The physiologically described organisms closest to this

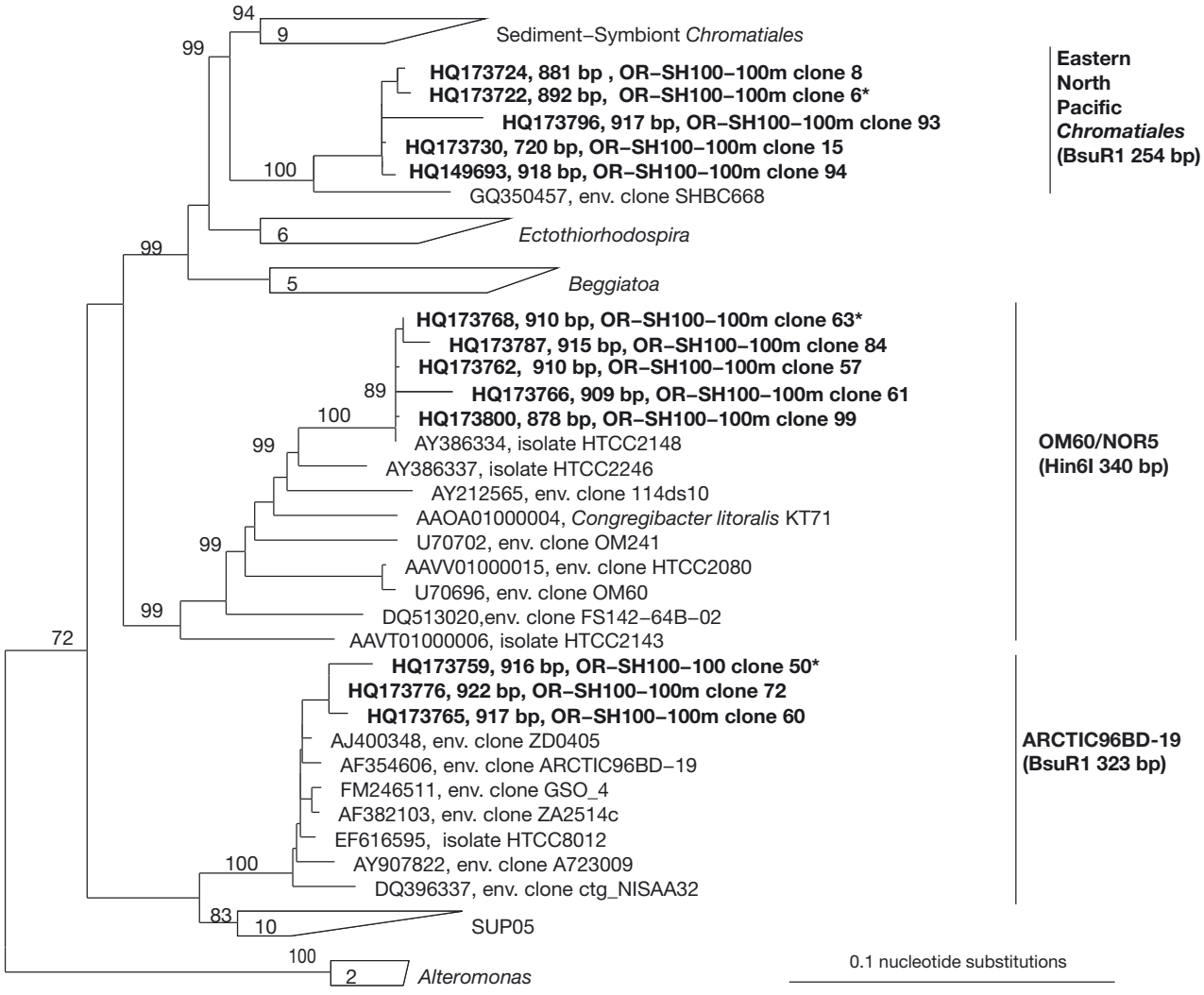

Fig. 3. Neighbor-joining tree describing the phylogeny of 16S rRNA genes affiliated with the *Gammaproteobacteria* from this study (bold) that could be assigned to environmental fragments showing a positive correlation with depth. The observed fragment size for selected 16S rRNA gene clones, indicated by an asterisk (*), is in parentheses under each clade designation. Sequences affiliated with the SILVA92 reference database included with the ARB software package are listed as well. NCBI accession numbers, followed by clone indicators or organism names, are listed. Bootstrap support values of 70% or greater based on 1000 re-samplings are displayed. *Polaribacter dokdonesis* MED152 was used as the outgroup (not shown)

clade are purple sulfur bacteria in the family *Ectothiorhodospiraceae* (87 to 89%). Bootstrap support for this clade based on neighbor-joining analysis was greater than 70%, and tree topologies based on parsimony and maximum likelihood were congruent, suggesting that this is a novel marine group, referred to as the Eastern North Pacific *Chromatiales* clade (ENPC clade).

Clones with fragment sizes of 287 bp were identified as the 6N14 clade of uncultured microorganisms related to the genus *Pirellula* (Figs. 2B & 4). This clade was named after fosmid clone 6N14 (98 to 99% similarity to sequences retrieved here) (Vergin et al. 1998), constructed from nucleic acids collected near the edge of the continental shelf off the coast of Oregon (Stein et al. 1996). The cultured, and well characterized, representative nearest to these clones is *Rhodopirellula baltica* SH1, at 93 to 94% sequence similarity.

A clone corresponding to a fragment size of 253 bp was affiliated with the uncharacterized Agg8 clade of the phylum *Planctomycetes* (Figs. 2D & 4). This clade also contained representatives from 16S rRNA gene surveys of marine bacterial diversity studies, including those of phytoplankton aggregates (DeLong et al. 1993). The clone retrieved in this study was 99% similar to the 16S rRNA gene located on the fosmid clone 6FN (Woebken et al. 2007). The cultured, most well characterized representative closest to the clone retrieved in this study is *Planctomyces maris* (84% similar).

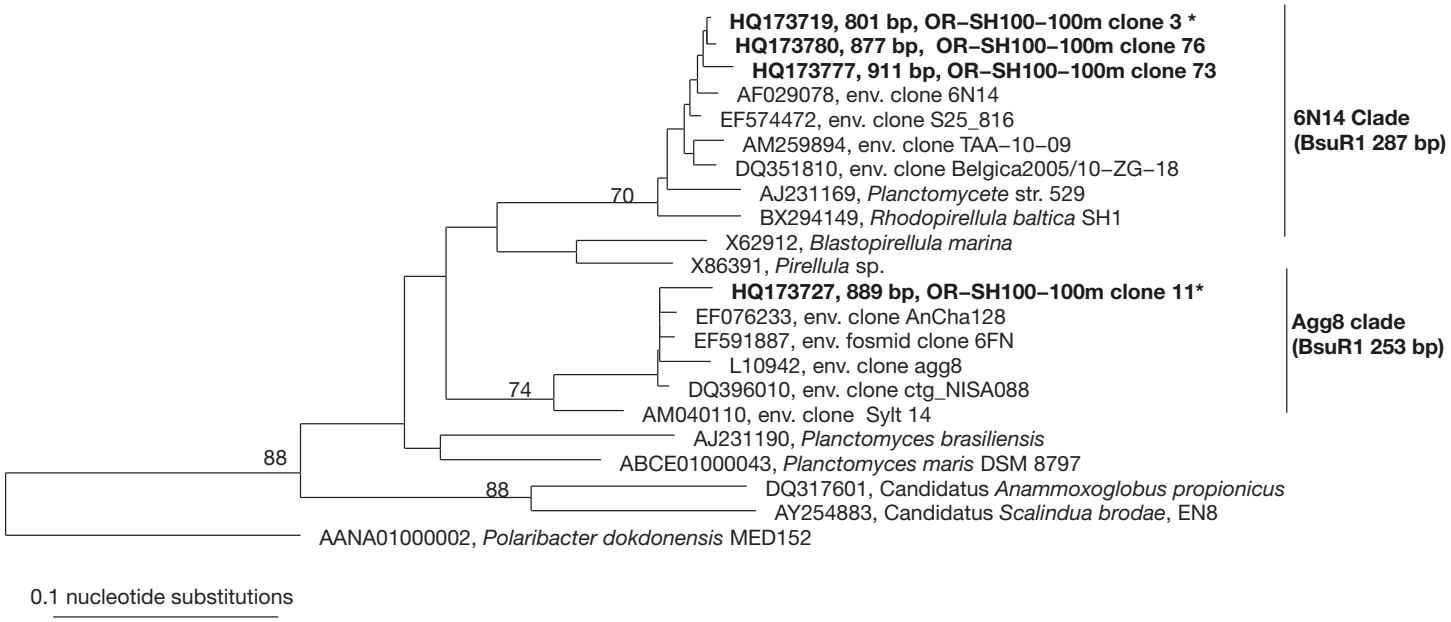

Fig. 4. Neighbor-joining tree describing the phylogeny of 16S rRNA genes affiliated with the *Planctomycetes* from this study (bold) that could be assigned to environmental fragments showing a positive correlation with depth. The observed fragment size for selected 16S rRNA gene clones is shown as in Fig. 3. NCBI accession numbers, clone indicators or organism names, and bootstrap support values of 70% or greater are indicated as in Fig. 3. *Polaribacter dokdonesis* MED152 was used as the outgroup

Hin6I

The additional information provided by digestion with enzyme Hin6I enabled us to identify 2 organisms that were associated with the BBL community (Fig. 2E,F). Clones producing terminal restriction fragments of 354 bp were identified as members of the OM60/NOR5 clade of marine *Gammaproteobacteria*. Several microorganisms from this clade have been retrieved in culture; Candidatus *Congregibacter litoralis* strain KT71 represents the physiologically best described organism (92 to 93% similar to clones reported here), and HTCC2148 is a cultured organism most closely related to clones reported here (99 to 100% similar, Fig. 3).

Sequences producing terminal restriction fragments of 340 bp were attributed to 2 sub-clades of the marine *Roseobacter* group, referred to here as the MB11C09 and ULA23 clades (Fig. 5). These environmental clades contain no cultured representatives; they are closely related to physiologically described organisms. The MB11C09 clade is closely related to *Marinosulfi-*

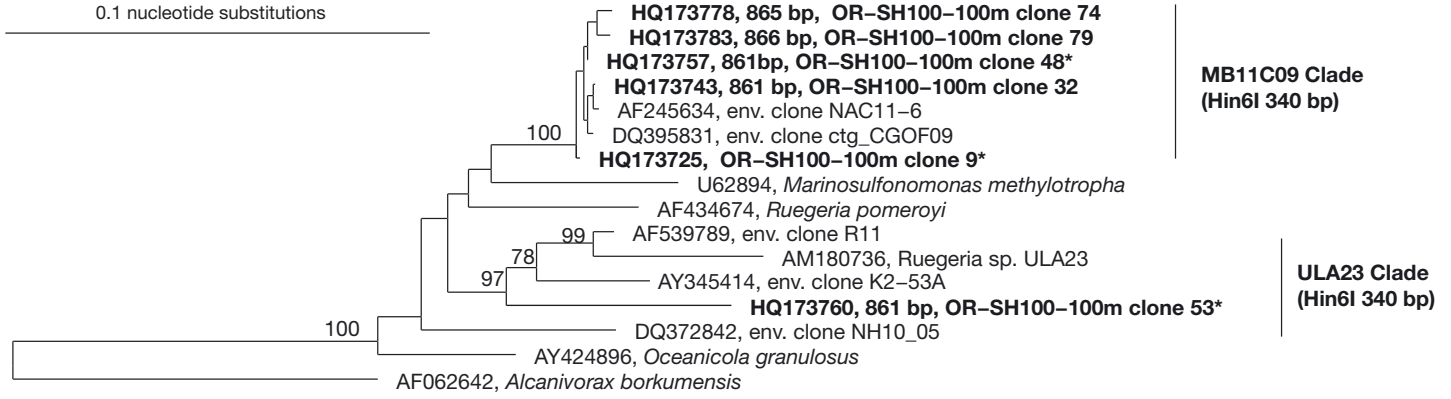

Fig. 5. Neighbor-joining tree describing the phylogeny of 16S rRNA genes affiliated with the *Roseobacter* group from this study (bold) that could be assigned to environmental fragments showing a positive correlation with depth. The observed fragment size for selected 16S rRNA gene clones is shown as in Fig. 3. NCBI accession numbers, clone indicators or organism names, and bootstrap support values of 70% or greater are indicated as in Fig. 3. *Alcanivorax borkumensis* was used as the outgroup

nomonas methylotropha (94 to 95%), while the ULA23 clade is closely related to *Ruegeria pomeroyi* (90%).

DISCUSSION

The present study provides evidence in support of the hypothesis that surface and BBL communities located in shallow regions of coastal Oregon are different, and it identified several taxa that are associated with the BBL. Multivariate statistical analysis (hierarchical clustering, multidimensional scaling, and ANOSIM), using 2 separate T-RFLP analyses of environmental samples, revealed evidence in support of the hypothesis that the surface and BBL are distinctly different. The partitioning of community structure by depth has emerged as a common theme in marine environments (Giovannoni et al. 1996), but, to the best of our knowledge, this is the first report to focus on the distinctive composition of the microbial community of the BBL in the shallow inner-shelf region off the coast of Oregon. Due to its close proximity with the benthos, and lower ventilation in comparison to the surface waters, especially during summer months, this community is often the first to experience hypoxic and anoxic conditions (Grantham et al. 2004).

We observed 3 distinct *Gammaproteobacteria* clades specifically associated with the BBL: ARTIC96BD-19, the novel ENPC clade, and OM60/NOR5. The ARTIC96BD-19 clade has been recovered in 16S rRNA gene surveys from various marine environments, including open-ocean and coastal samples from various depths (Suzuki et al. 2001, Bano & Hollibaugh 2002, Zubkov et al. 2002). This clade accounted for a substantial proportion of the 16S rRNA genes recovered from the permanent oxygen-minimum zone of northern Chile (Stevens & Ulloa 2008), and is closely related to putative thiotrophic symbionts of metazoans in the SUP05 environmental clade (Walsh et al. 2009).

A second BBL clade was in the order *Chromatiales.* These clones were not significantly related to any physiologically described organisms (16S rRNA gene similarity <97%), and are referred to as the ENPC clade (Fig. 3). The 16S rRNA gene sequences nearest to clones reported here were recovered from the Saanich Inlet of British Columbia, an area that experiences seasonal anoxia (Walsh et al. 2009). Several members of the order *Chromatiales* are capable of anaerobic anoxygenic photosynthesis; commonly referred to as purple sulfur bacteria, these organisms are often found in low-oxygen aquatic environments where they utilize reduced sulfur compounds as electron donors to fuel photosynthesis (Peduzzi et al. 2003).

Physiologically described organisms closest to the ENPC clade are members of the family *Ectothiorhodospiraceae*. Isolates from this family are known to inhabit lake systems, colonizing phytoplankton biomass under anoxic conditions while oxidizing reduced sulfur compounds (Bryantseva et al. 1999). In the clones described here, the 16S rRNA genes were not sufficiently similar to those of cultured representatives to permit any assumptions to be made regarding the physiology of these organisms. However, the ecological distribution of members of both the order *Chromatiales* and the ARTIC96BD-19 clade in other low-oxygen aquatic environments, in conjunction with their evolutionary relationships to other characterized or putative sulfur-oxidizing bacteria, may be taken as evidence in support of the hypothesis that microbial metabolism involving reduced sulfur compounds could be occurring in BBLs off the coast of Oregon.

Previous investigations on the production of volatile sulfur compounds during the respiration of algal biomass under anaerobic conditions have shown that hydrogen sulfide and other reduced sulfur compounds can be produced in large quantities (Zinder et al. 1977). Hydrogen sulfide fluxes from the benthos have been observed in other productive coastal environments, such as the south Atlantic off the coast of Namibia (Emeis et al. 2004). A recent molecular diversity survey has provided evidence to suggest that these fluxes are consumed by *Gammaproteobacteria* closely related to putative sulfur-oxidizing symbionts (Lavik et al. 2009). Whether similar biogeochemical processes are occurring off the coast of Oregon remains to be determined. Dissolved oxygen (DO) was present in all of the samples described in this study (2.55 to 0.65 ml l^{-1}), and nitrate, although not measured, is typically found in the BBL at micromolar concentrations (10 to 30 µM). Both oxygen and nitrate are potential electron acceptors for the oxidation of hydrogen sulfide. While transient hypoxia DO < 1.4 ml l^{-1}), severe hypoxia (DO < 0.5 ml l^{-1}), and anoxia (undetectable oxygen) have been reported off the coast of Oregon in recent years (2002 to 2006, Chan et al. 2008), only hypoxic and non-hypoxic samples were detected in the BBL environment during this study (2007 to 2008). Surprisingly perhaps, DO concentrations were not significantly correlated with microbial community composition in the BBL.

The third *Gammaproteobacteria* clade associated with the BBL was OM60/NOR5. These organisms are commonly retrieved during ribosomal gene surveys of coastal surface waters, where they reach as much as 11% of the total bacterial numbers (Yan et al. 2009). In a previous study, direct quantification of the OM60/NOR5 clade along a longitudinal gradient off the coast of Oregon revealed a clear increase in cell density in

the coastal zone, with a vertical distribution coupled to the deep-chlorophyll maximum (Cho et al. 2007). Candidatus *Congregibacter litoralis* KT71, the most well characterized member of the OM60/NOR5 clade, is an aerobic anoxygenic photosynthetic (AAnP) organism that grows optimally at oxygen partial pressures of 12% and whose photosynthetic antennae complex is regulated in response to oxygen and light (Spring et al. 2009). Clones described in this study were very similar to HTCC2148 (99 to 100%), an uncharacterized yet genome-sequenced isolate in the OM60/NOR5 clade, but they diverged too much from KT71 (92 to 93%) to support physiological assumptions by phylogenetic inference (Thrash et al. 2010). The presence of members of the OM60/NOR5 clade in a coastal BBL sheds new light on the distribution of OM60/NOR5 representatives.

Two environmental clades of *Planctomycetes* (6N14 and Agg8) were associated with the BBL. Clones affiliated with 6N14 were similar to the 16S rRNA gene located on the large insert clone 6N14 (97 to 99%) (Vergin et al. 1998), which was recovered previously from samples collected off the coast of Oregon at a depth of 200 m (Stein et. al. 1996). Recent sequence analysis of this 42 kbp fosmid sequence revealed gene suites involved in the degradation of phytoplankton detritus, such as glycosyl hydrolase, in addition to genes involved in phosphate acquisition, such as 3-dehydroquinate synthase (Woebken et al. 2007). Interestingly, the cultured representative closest to the 6N14 cluster is *Rhodopirellula baltica* SH1 (93 to 94% sequence similarity), which possesses suites of genes that are active under microaerobic and anaerobic conditions—such as genes encoding cytochrome *d* oxidase and a putative fermentative pathway, respectively (Glöckner et al. 2003). Additionally, the genome of *R. baltica* contains 110 sulfatases. Glöckner et al. (2003) hypothesized that these genes would be advantageous for accessing sulfated carbon compounds in a nutrient-depleted environment. 16S rRNA gene similarities were not significant enough to make a direct link between clones recovered in this study and *R. baltica*.

The second *Planctomycetes* clade associated with the BBL was the agg8 clade, described previously (DeLong et al. 1993). The clone recovered here was 97% similar to sequences from the agg8 fosmid metagenome clone 6FN, recovered previously from the continental shelf off the coast of Namibia, a region that experiences pronounced deficits in DO. Sequence analysis of this 40 kbp fosmid library revealed gene suites involved in methylotrophy, such as a formylmethonfuran dehydrogenase, and a methonal dehyrogenase regulator (Woebken et al. 2007). The closest cultured representative to this clade is *Planctomyces maris* at 84% similarity to clones described here, far too diverged for any meaningful 16S rRNA gene comparisons.

The *Planctomycetes* are a physiologically, ecologically, and genetically distinctive phylum. These organisms utilize protein rather than peptidoglycan in their cell wall, are capable of intracellular compartmentalization, divide by budding, and, in some cases, are capable of gliding motility (Ward et al. 2006). *Planctomycetes* are frequently recovered in freshwater and marine ecosystems and are often attached to particulate matter, including algal cells, by means of specific holdfast structures. They typically produce flagellated swarmer cells which apparently enable them to colonize new surfaces. We postulate that the propensity of *Planctomycetes* for particle colonization makes them well adapted to occupy the BBL, where organic matter accumulates and is re-circulated by turbulence during decomposition.

Two uncharacterized lineages in the marine *Roseobacter* group were affiliated with the BBL. The *Roseobacter* group is commonly recovered during 16S rRNA gene surveys of marine environments (Rappe et al. 1997, Gonzalez et al. 1999, 2000), with higher frequency in coastal environments (Buchan et al. 2005). Cultivated members of the *Roseobacter* clade are metabolically diverse, capable of utilizing a wide range of carbon and sulfur compounds (Buchan et al. 2005). Specifically, certain *Roseobacter* clade members utilize the reduced sulfur compound dimethylsulfoniopropionate, which has a tremendous impact on the global sulfur cycle (Gonzalez et al. 1999, 2000, Howard et al. 2008), and in addition, several cultivated representatives contain gene suites involved in the oxidation of reduced sulfur compounds, specifically the *sox* gene cluster. Like the *Planctomycetes*, members of this clade are often found in physical association with algal biomass, suggesting a link between this group and primary productivity (Morris et al. 2006, Slightom & Buchan 2009).

CONCLUSIONS

The goal of the present study was to examine the bacterial community associated with the BBL off the coast of Oregon. The analysis provided strong statistical support for taxa specifically associated with this region of the coastal ocean. Two of these clades, the *Gammaproteobacteria* OM60/NOR5 clade and *Alphaproteobacteria Roseobacter* clade, harbor both taxonomically described and genome-sequenced organisms. However, the 16S rRNA gene similarities of clones recovered in this study were too diverged from characterized isolates to draw meaningful comparisons

between these organisms. Two of the environmental (undescribed) clades—ARTIC96BD-19 and the ENPC clade—are distantly related to either organisms that can oxidize hydrogen sulfide or those that can harbor the necessary genetic components to do so. Two BBL clades were affiliated with uncultured *Planctomycetes* clades 6N14 and agg8. Both *Planctomycetes* and *Roseobacter* organisms are often found associated with particles (Slightom & Buchan 2009).

The BBL off the coast of Oregon resides in close proximity to the benthic surface. Re-suspended matter from this environment is a likely source of bacterial diversity in the BBL, especially during turbulent mixing events. Subsurface benthic areas often contain layers where very little or no oxygen exists, and hydrogen sulfide from sulfate-reducing bacteria can accumulate. Given the presence of transient hypoxic and anoxic conditions in the BBL, the benthic surface and subsurface environments provide a suitable niche for organisms in the BBL environment to inhabit. A study regarding the genetic diversity of organisms in free-living, aggregate-associated, and sediment-surface-associated communities in the shallow intertidal zone of the Wadden Sea provided evidence that considerable overlap exists between the 3 types of community, suggesting that exchange processes between these communities may be occurring (Stevens et al. 2005). Whether similar processes occur between the benthos and BBL community off the coast of Oregon remains to be seen.

We contrasted the BBL community with the surface community to identify taxa that might be specifically associated with this region of the water column where DOM decomposition is elevated, photosynthesis is minimal, and oxygen demand is high. Future studies aimed at understanding the geochemistry of marine DOM oxidation and coastal hypoxic zones may find the unusual microflora of this habitat fruitful topics for investigation.

Acknowledgements. We thank the Center for Genome Research and Biocomputing at Oregon State University, Cameron Thrash for help in manuscript preparation, and the captain and crew of the RV 'Elakha'.

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Arrigo KR (2005) Marine microorganisms and global nutrient cycles. Nature 437:349–355

Bano N, Hollibaugh JT (2002) Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Appl Environ Microbiol 68:505–518

Bryantseva I, Gorlenko VM, Kompantseva EI, Imhoff JF, Suling J, Mityushina L (1999) *Thiorhodospira sibirica* gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium from a Siberian soda lake. Int J Syst Bacteriol 49:697–703

Buchan A, Gonzalez JM, Moran MA (2005) Overview of the marine roseobacter lineage. Appl Environ Microbiol 71: 5665–5677

Chan F, Barth JA, Lubchenco J, Kirincich A, Weeks H, Peterson WT, Menge BA (2008) Emergence of anoxia in the California current large marine ecosystem. Science 319: 920

Cho JC, Stapels MD, Morris RM, Vergin KL and others (2007) Polyphyletic photosynthetic reaction centre genes in oligotrophic marine *Gammaproteobacteria.* Environ Microbiol 9:1456–1463

DeLong EF, Franks DG, Alldredge AL (1993) Phylogenetic diversity of aggregate-attached vs free-living marine bacterial assemblages. Limnol Oceanogr 38:924–934

DeSantis TZ, Hugenholtz P, Keller K, Brodie EL and others (2006) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 34:W394–W399

Emeis KC, Bruchert V, Currie B, Endler R and others (2004) Shallow gas in shelf sediments of the Namibian coastal upwelling ecosystem. Cont Shelf Res 24:627–642

Fuhrman JA, Azam F (1980) Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl Environ Microbiol 39: 1085–1095

Giovannoni SJ, Rappe MS, Vergin KL, Adair NL (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 FO, Kube M, Bauer M, Teeling H and others (2003) Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. Proc Natl Acad Sci USA 100: 8298–8303

Gonzalez JM, Kiene RP, Moran MA (1999) Transformation of sulfur compounds by an abundant lineage of marine bacteria in the alpha-subclass of the class Proteobacteria. Appl Environ Microbiol 65:3810–3819

Gonzalez JM, Simo R, Massana R, Covert JS, Casamayor EO, Pedros-Alio C, Moran MA (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. Appl Environ Microbiol 66:4237–4246

Gordon DA, Giovannoni SJ (1996) Detection of stratified microbial populations related to *Chlorobium* and *Fibrobacter* species in the Atlantic and Pacific Oceans. Appl Environ Microbiol 62:1171–1177

Grant WD, Madsen OS (1986) The continental-shelf bottom boundary-layer. Annu Rev Fluid Mech 18:265–305

Grantham BA, Chan F, Nielsen KJ, Fox DS and others (2004) Upwelling-driven nearshore hypoxia signals ecosystem and oceanographic changes in the northeast Pacific. Nature 429:749–754

Howard EC, Sun S, Biers EJ, Moran MA (2008) Abundant and diverse bacteria involved in DMSP degradation in marine surface waters. Environ Microbiol 10:2397–2410

Karner MB, DeLong EF, Karl DM (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 409: 507–510

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid-determination of 16S ribosomal-RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955–6959

Lavik G, Stuhrmann T, Bruchert V, Van der Plas A and others (2009) Detoxification of sulphidic African shelf waters by blooming chemolithotrophs. Nature 457:581–586

Ludwig W, Strunk O, Westram R, Richter L and others (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Massana R, Murray AE, Preston CM, DeLong EF (1997) Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. Appl Environ Microbiol 63:50–56

Morris RM, Vergin KL, Cho JC, Rappe MS, Carlson CA, Giovannoni SJ (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

Morris RM, Longnecker K, Giovannoni SJ (2006) *Pirellula* and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom. Environ Microbiol 8: 1361–1370

Pauly D, Christensen V (1995) Primary production required to sustain global fisheries. Nature 374:255–257

Pauly D, Christensen V, Guenette S, Pitcher TJ and others (2002) Towards sustainability in world fisheries. Nature 418:689–695

Peduzzi S, Tonolla M, Hahn D (2003) Isolation and characterization of aggregate-forming sulfate-reducing and purple sulfur bacteria from the chemocline of meromictic Lake Cadagno, Switzerland. FEMS Microbiol Ecol 45:29–37

Rappe MS, Kemp PF, Giovannoni SJ (1997) Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. Limnol Oceanogr 42:811–826

Rappe MS, Vergin K, Giovannoni SJ (2000) Phylogenetic comparisons of a coastal bacterioplankton community with its counterparts in open ocean and freshwater systems. FEMS Microbiol Ecol 33:219–232

Slightom RN, Buchan A (2009) Surface colonization by marine roseobacters: integrating genotype and phenotype. Appl Environ Microbiol 75:6027–6037

Spring S, Lunsdorf H, Fuchs BM, Tindall BJ (2009) The photosynthetic apparatus and its regulation in the aerobic Gammaproteobacterium *Congregibacter litoralis* gen. nov., sp nov. PLoS ONE 4:e4866

Stein JL, Marsh TL, Wu KY, Shizuya H, DeLong EF (1996) Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. J Bacteriol 178:591–599

Stevens H, Ulloa O (2008) Bacterial diversity in the oxygen minimum zone of the eastern tropical South Pacific. Environ Microbiol 10:1244–1259

Stevens H, Brinkhoff T, Simon M (2005) Composition of free-living, aggregate-associated and sediment surface-associated bacterial communities in the German Wadden Sea. Aquat Microb Ecol 38:15–30

Stingl U, Desiderio RA, Cho JC, Vergin KL, Giovannoni SJ (2007a) The SAR92 clade: an abundant coastal clade of culturable marine bacteria possessing proteorhodopsin. Appl Environ Microbiol 73:2290–2296

Stingl U, Tripp HJ, Giovannoni SJ (2007b) Improvements of high-throughput culturing yielded novel SAR11 strains and other abundant marine bacteria from the Oregon coast and the Bermuda Atlantic Time Series study site. ISME J 1:361–371

Suzuki MT, Beja O, Taylor LT, DeLong EF (2001) Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. Environ Microbiol 3:323–331

Thrash JC, Cho JC, Ferriera S, Johnson J, Vergin KL, Giovannoni SJ (2010) Genome sequences of strains HTCC2148 and HTCC2080, belonging to the OM60/NOR5 clade of the *Gammaproteobacteria*. J Bacteriol 192:3842–3843

Turley C (2000) Bacteria in the cold deep-sea benthic boundary layer and sediment-water interface of the NE Atlantic. FEMS Microbiol Ecol 33:89–99

Vergin KL, Urbach E, Stein JL, DeLong EF, Lanoil BD, Giovannoni SJ (1998) Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order Planctomycetales. Appl Environ Microbiol 64:3075–3078

Walsh DA, Zaikova E, Howes CG, Song YE and others (2009) Metagenome of a versatile chemolithoautotroph from expanding oceanic dead zones. Science 326:578–582

Ward N, Staley JT, Fuerst JA, Giovannoni S, Schlesner H, Stackebrandt E (2006) The order Planctomycetales, including the genera *Planctomyces*, *Pirellula*, *Gemmata*, and *Isosphaera*, and the *Candidatus* genera Brocadia, Kuenenia, and Scalindua. In: Dworkin M (ed) The prokaryotes, 3rd edn. Springer-Verlag, New York, NY, p 757–793

Woebken D, Teeling H, Wecker P, Dumitriu A and others (2007) Fosmids of novel marine *Planctomycetes* from the Namibian and Oregon coast upwelling systems and their cross-comparison with planctomycete genomes. ISME J 1: 419–435

Wright TD, Vergin KL, Boyd PW, Giovannoni SJ (1997) A novel delta-subdivision proteobacterial lineage from the lower ocean surface layer. Appl Environ Microbiol 63: 1441–1448

Yan S, Fuchs BM, Lenk S, Harder J, Wulf J, Jiao NZ, Amann R (2009) Biogeography and phylogeny of the NOR5/OM60 clade of *Gammaproteobacteria.* Syst Appl Microbiol 32: 124–139

Zinder SH, Doemel WN, Brock TD (1977) Production of volatile sulfur-compounds during decomposition of algal mats. Appl Environ Microbiol 34:859–860

Zubkov MV, Fuchs BM, Archer SD, Kiene RP, Amann R, Burkill PH (2002) Rapid turnover of dissolved DMS and DMSP by defined bacterioplankton communities in the stratified euphotic zone of the North Sea. Deep-Sea Res II 49:3017–3038

Editorial responsibility: Gerhard Herndl, Vienna, Austria

Submitted: August 30, 2010; Accepted: March 7, 2011
Proofs received from author(s): June 1, 2011