RESEARCH ARTICLE

Bacterioplankton composition of the coastal upwelling system of 'Ría de Vigo', NW Spain

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Abstract

Catalysed reported deposition-FISH and clone libraries indicated that *Roseobacter*, followed by *Bacteroidetes*, and some gammaproteobacterial groups such as SAR86, dominated the composition of bacterioplankton in Ría de Vigo, NW Spain, in detriment to SAR11 (almost absent in this upwelling ecosystem). Since we sampled four times during the year, we observed pronounced changes in the structure of each bacterioplankton component, particularly for the *Roseobacter* lineage. We suggest that such variations in the coastal upwelling ecosystem of Ría de Vigo were associated with the characteristic phytoplankton communities of the four different hydrographical situations: winter mixing, spring bloom, summer stratification, and autumn upwelling. We retrieved new sequences among the major marine bacterial lineages, particularly among *Roseobacter*, SAR11, and especially SAR86. The spring community was dominated by two *Roseobacter* clades that had previously been related to phytoplankton blooms. In the other seasons, communities with higher diversity than the spring one were detected.

Introduction

Coastal upwelling systems are some of the most productive areas in the world's oceans, contributing significantly to the export of primary production to the ocean interior. The Ría de Vigo (Galicia, NW Spain) is located in the northern boundary of the northwest African upwelling system. Therefore, it is in an area where the along-shore winds interact with the coastal topography to generate upwelling–downwelling dynamics (cross-shore Ekman transport) on the continental shelf (Arístegui *et al.*, 2006). The upwelling season in this ecosystem occurs on average from March to September, while downwelling conditions predominate during the rest of the year (Figueiras *et al.*, 2002).

The coastal upwelling induces the inflow of subsurface oceanic Eastern North Atlantic Central Water into the estuaries of Galicia, generating a circulation pattern in which a part of the biomass produced inside the estuary is transported offshore to be remineralized before coming back into the Rías. This fertilizing process is responsible for the high productivity of the Ría de Vigo, reflected in the high shellfish production of this ecosystem (Figueiras *et al.*, 2002).

Marine microbial communities play critical roles in carbon and nitrogen cycling through their influence on the formation and fate of dissolved organic matter (Azam *et al.*, 1994) and many other processes. Despite their importance, few studies are available about bacterioplankton diversity in coastal upwelling ecosystems. In particular, little is known about the bacterioplankton of Ría de Vigo, where previous studies had been centred in the number of heterotrophic culturable bacterioplankton (CFU), and had probably little to do with the dominant bacterioplankton. These numbers were strongly affected by the upwelling events (Zdanowski & Figueiras, 1999).

In the world's oceans, six bacterial clades account for *c.* 84% of the bacterioplankton 16S rRNA genes recovered

(Fuhrman & Hagström, 2008). Among them, the SAR11 and *Roseobacter* clades from *Alphaproteobacteria*, and the *Gammaproteobacteria* SAR86, have been found to be abundant in coastal waters (e.g. González *et al.*, 2000; Morris *et al.*, 2002; Alonso-Sáez *et al.*, 2007). Some of these groups are comprised almost completely of clone sequences of uncultured bacteria. In fact, SAR86 isolates have not yet been obtained, in spite of their abundance (Eilers *et al.*, 2000). In contrast, *Roseobacter* is considered to be the most readily cultivated of these major marine lineages and recent studies have also revealed a high diversity of clusters within this and in the SAR11 lineages (Field *et al.*, 1997; Selje *et al.*, 2004; Buchan *et al.*, 2005).

Few traits are representative of the entire *Roseobacter* clade, thus being a heterogeneous lineage formed by at least 17 genera, 36 described species, and literally hundreds of uncharacterized isolates and clone sequences. *Roseobacter* are often more abundant in bacterial communities associated with marine algae, including natural phytoplankton blooms (González *et al.*, 2000; Zubkov *et al.*, 2002; Wagner-Dobler & Biebl, 2006). Following the thorough revision of Buchan *et al.* (2005), 13 major clusters were established and linked to characteristic habitats or ecological niches. For instance, the DC5-80-3 cluster is characteristic of oceanic waters, the OCT cluster is related to cold waters, the NAC11-7 cluster is commonly associated with algal blooms, and so forth. Although 16S rRNA gene sequence data alone are not reliable predictors of an ecological niche, a good classification of clone sequences can give us clues about the functional activities of at least some of the clusters.

Here, we provide an insight into the bacterioplankton diversity in the Ría de Vigo using culture-independent molecular techniques. Samples were taken under four different oceanographic conditions characteristic of this ecosystem (Table 1): spring bloom, summer stratification, autumn upwelling, and winter mixing. Relatively extensive clone libraries, supported with catalysed reported deposition (CARD)-FISH with common probes, were used to describe the diversity and seasonal trends of the abundance of the dominant marine bacterial groups. Since the Ría de Vigo is subject to continuous pollution from industrial and urban sewage, we also specifically looked for specialized hydrocarbonoclastic bacteria in the clone libraries and used a specific FISH probe to detect the genus *Cycloclasticus* (Maruyama *et al.*, 2003), which was previously identified as a major player in the degradation of low-molecular-weight soluble aromatic hydrocarbons (Chung & King, 2001).

Materials and methods

Sampling

The Ría de Vigo is a coastal embayment that is driven completely by marine water mass circulation, and has minor freshwater influence. We sampled a position in the middle of the Ría (42°13.9′N; 8°51.0′W) at the four most relevant periods of the seasonal plankton cycle in the coastal northeast Iberian Atlantic waters: the spring bloom (March 2005), summer stratification (July 2005), autumn upwelling (September 2005), and winter mixing (January 2006). The water was collected with large (1.5 m in diameter and 2 m deep) bags, which were filled from their bottom through a 200-mm mesh, in order to exclude mesozooplankton. Once filled, the bags were closed with a stopper and transported to the shore to be used as the initial water for a series of mesocosm experiments (which are reported elsewhere: Teira *et al.*, 2007, 2008). The clone libraries were constructed with the initial water, before the addition of any supplement, but after a few hours of mesocosm fill-up. We lost the initial sample from March 2005, and instead used the control (with no additions) sample from Day 1. Since little changes were observed in bacterial abundance between times 0 and 1 (Lekunberri, 2008), and since the denaturing gradient gel electrophoresis analyses of the other three amplified samples revealed no visible changes between times zero and Day 1 (details not

Table 1. Site characteristics for each sampling

	Sample characteristics			
	Spring	Summer	Autumn	Winter
Sampling date (mm/dd/yy)	03/02/05	07/02/05	09/20/05	01/27/06
Water temperature (°C)	10.5 ± 0.0	20.8 ± 0.0	15.4 ± 0.0	12.4 ± 0.0
Salinity	35.48 ± 0.02	35.02 ± 0.01	35.73 ± 0.00	35.60 ± 0.00
Dissolved inorganic nitrogen (DIN) (μM)	4.40 ± 0.08	0.58 ± 0.11	5.66 ± 0.73	7.70 ± 0.44
Dissolved inorganic phosphorus (DIP) (μM)	0.52 ± 0.00	0.15 ± 0.01	0.51 ± 0.08	0.48 ± 0.02
Silicate (SiO_4) (μM)	3.17 ± 0.04	0.59 ± 0.05	0.41 ± 0.02	3.72 ± 0.15
Particulate organic carbon (POC) (μM)	16.1 ± 1.1	23.4 ± 0.3	33.9 ± 1.6	9.2 ± 0.6
Particulate organic nitrogen (PON) in μM	2.7 ± 0.2	3.4 ± 0.2	6.2 ± 0.4	1.3 ± 0.1
Prokaryotic abundance (PA) ($\times 10^5$ cell mL^{-1})	7.2 ± 0.3	11.7 ± 0.5	17.4 ± 0.2	5.8 ± 0.3
Chlorophyll *a* concentration (mg m^{-3})	3.2 ± 0.2	1.9 ± 0.1	10.6 ± 0.7	0.5 ± 0.0

shown), we consider this sample to also be representative of the *in situ* community at this time of the year.

Collection of community DNA

Microbial biomass was collected by sequentially filtering around 8 L of seawater through a 3-μm pore size polycarbonate filter (Poretics) and a 0.2-μm Sterivex filter (Durapore, Millipore) using a peristaltic pump. The Sterivex units were filled with 1.8 mL of lysis buffer (50 mM Tris-HCl, pH 8.3, 40 mM EDTA, pH 8.0, 0.75 M sucrose) and kept at −80 °C. Microbial biomass was treated with lysozyme, proteinase K, and sodium dodecyl sulphate, and the nucleic acids were extracted with phenol and concentrated using a Centricon-100 (Millipore), as described previously (Massana *et al.*, 1997). Here we report the results of the 0.2-μm filter.

Construction of the 16S rRNA gene clone library

An almost complete 16S rRNA gene was PCR amplified using primers F27 and R1492 (Edwards *et al.*, 1989; Lane, 1991). The PCR reaction (25 μL) included 10 mM Tris-HCl (pH 8.3); 50 mM KCl (pH 8.3); 2.5 mM $MgCl_2$; 200 μM of each deoxynucleoside triphosphate; 1.25 U of AmpliTaq-Gold DNA polymerase (PE Applied Biosystems, Foster City, CA); 0.4 μM of each primer; and 100 ng of the metagenomic DNA extracted from seawater samples. The reaction mixtures were subjected to a hot start (5 min at 95 °C) and, then, to the following thermal cycling: (1) 5 min at 95 °C; (2) 40 cycles, with one cycle consisting of 30 s at 96 °C for denaturation, 30 s at 54 °C for annealing, and 1.5 min at 72 °C for elongation; and (3) a final extension step of 10 min at 72 °C. This cycle was performed using a Perkin-Elmer GeneAmp 2700 Thermocycler (Applied Biosystems).

PCR products of approximately 1500 bp were cloned into a pCR®2.1-TOPO® vector and transformed into competent *Escherichia coli* TOP 10F′ cells, following the manufacturer's protocol of the TOPO T/A Cloning Kit (Invitrogen).

PCR products of each clone using primers M13F and M13R were separated by electrophoresis in a 1% (w/v) agarose gel in 1 × TAE buffer stained with ethidium bromide and photographed immediately under UV light using Gel Doc XR system and QUANTITY ONE software (Bio-Rad, Hercules, CA). A 100-bp ladder was used as a size marker (Promega).

One hundred different clones with the correct size insert were sequenced in both directions using overlapping primers F341 and R907 (Edwards *et al.*, 1989).

Clone library coverage estimations

Operational taxonomic units (OTUs) were defined for the sequences under study to facilitate their classification. Sequences with > 97% identity were considered as belonging to the same OTU (Table 2). Two estimates were used to calculate the coverage of our clone libraries: the Good (1953) and Chao *et al.* (1993) coverages. The number of ribotypes in a clone library of infinite size that would be represented in a smaller library can be estimated from a nonparametric calculation using the clone coverage defined by Good. Chao and colleagues' coverage estimator is nonparametric and based on the abundance of the dominant OTUs in the community. The sample coverage, C_{ACE}, is estimated as the number of sequences in relatively rare OTUs that occur more than once in a library. The S_{Chao1} nonparametric estimator yields an unbiased estimate of the probable total number of OTUs present in the source assemblage (Lee & Chao, 1994). We performed these calculations to evaluate the coverage of our clone libraries (Table 2). Furthermore, pair alignments and the DNA-distance matrix between all possible combinations of the four libraries were analysed using the LIBSHUFF software (Singleton *et al.*, 2001) to test for the null hypothesis that any two 16S rRNA gene libraries are samples of the same prokaryotic community.

Table 2. Clone library characteristics

	Values for OTU at > 97%/ > 99% similarity threshold			
	Spring	Summer	Autumn	Winter
Number of clones sequenced	96	96	96	96
Number of clones discarded as chimeras	0	2	1	2
Number of clones discarded as plastids	2	1	2	5
Number of clones analysed	94	93	93	89
Observed OTU richness	27/40	36/54	39/54	38/49
S_{Chao1} estimates	49/81	54/125	47/92	55/82
Good's coverage estimate (%)	81/72	77/57	83/65	79/66
C_{ACE} coverage estimate (%)	45/54	66/46	80/65	74/58

Sequencing and phylogenetic analyses

Partial 16S rRNA gene sequences using primers F341, R907 (Edwards *et al.*, 1989) were obtained, edited, and analysed as described previously (Alonso-Gutiérrez *et al.*, 2008). The Check Chimera program of the Ribosomal Database Project (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) was used to detect heteroduplexes that were eliminated from the analyses. Then, the sequences were examined using the BLAST search alignment tool comparison software (BLASTN) to detect the closest bacterial sequences within the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Alignments with reference sequences obtained from GenBank were performed using CLUSTALW (http://align.genome.jp/), edited with MACCLADE 4.06, and directly transferred to version 4.0b10 of PAUP* software (Sinauer Associates Inc., Sunderland, MA). MODELTEST software version 3.6 (Posada & Crandall, 1998) was run together with PAUP*, as a guide to determine the best-fit maximum likelihood (ML) model for the edited alignment. MODELTEST examines ML models, ranging from simple to complex. The best-fit models of nucleotide evolution, calculated by MODELTEST and PAUP*, were incorporated into software PHYML (Guidon & Gascuel, 2003), which uses a single, fast, and accurate algorithm to estimate large phylogenies by ML. The same alignments were also used to generate phylogenies by maximum parsimony and neighbour-joining (NJ) using PAUP*. Finally, the trees created by PHYML were edited using the FIGTREE v1.1.2 software (http://tree.bio.ed.ac.uk/software/figtree/).

To describe such complex communities properly, several phylogenetic analyses were performed in a hierarchical way. First, a general phylogenetic tree (using reference sequences from all *Eubacteria*, details not shown) was performed to obtain a better view of the phylogenetic adscription of each clone. Alignments for the preliminary classification of sequences containing numerous and too divergent taxa were modified using GBLOCKS software (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). This computer program eliminates poorly aligned positions and divergent regions of the DNA alignment, which are convenient to delete before phylogenetic analysis. Second, those clones that clustered together in the general tree were separately analysed with the closest reference sequences described in previous studies, such as those of the *Roseobacter* lineage (Buchan *et al.*, 2005), SAR11 (Field *et al.*, 1997), or SAR86 clades (Suzuki *et al.*, 2001; Sabehi *et al.*, 2004). These phylogenies allowed us to classify all clones in the already-described major groups, detect possible new clusters, and suggest seasonal trends for each group detected in the Ría de Vigo.

CARD-FISH

Five-millilitre samples were fixed with 0.2-mm-filtered paraformaldehyde (2% final concentration) and subsequently stored at 4 °C in the dark for 12–18 h. Thereafter, each sample was filtered through a 0.2-μm polycarbonate filter (Millipore, GTTP, 25 mm filter diameter) supported by a cellulose nitrate filter (Millipore, HAWP, 0.45 μm), washed twice with Milli-Q water, dried, and stored in a microfuge vial at −20 °C until further processing in the laboratory.

The *in situ* abundance of different bacterial populations was determined using CARD-FISH with oligonucleotide probes specific for the domain *Eubacteria* (EUB338) (Amann *et al.*, 1990), *Alpha-* (ALF968) (Glockner *et al.*, 1999), *Beta-* (BET42a) (Manz *et al.*, 1992), and *Gammaproteobacteria* (GAM42a) (Manz *et al.*, 1992) subclasses, the *Bacteroidetes* group (CF319a) (Manz *et al.*, 1996), the *Roseobacter* lineage (ROS537) (Eilers *et al.*, 2001), the SAR11 cluster (SAR11-A1 and SAR11-441R) (Field *et al.*, 1997; Morris *et al.*, 2002), the SAR86 cluster (SAR86-1249) (Eilers *et al.*, 2000), and the hydrocarbon-degrading *Cycloclasticus* bacteria (CYPU829) (Maruyama *et al.*, 2003). The filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme (Pernthaler *et al.*, 2002; Teira *et al.*, 2004). They were then cut into sections and hybridized at 35 °C with horseradish peroxidase (HRP)-labelled oligonucleotide probes for 2–4 h. Tyramide-Alexa488 was used for signal amplification (for 30–40 min) as described previously (Teira *et al.*, 2004). We used 55% of the formamide for all of the probes, except ALF968, SAR11A1, and SAR11-441R (45% formamide). The hybridization of these three probes was performed overnight (Alonso-Sáez *et al.*, 2007). Cells were counter-stained with a 4′-6-diamidino-2-phenylindole (DAPI)-mix [5.5 parts of Citifluor (Citifluor Ltd), 1 part of Vectashield (Vector Laboratories Inc.), and 0.5 parts of phosphate-buffered saline with DAPI (final concentration of 1 μg mL^{-1})].

The slides were examined using a Leica DMBL microscope equipped with a 100 W Hg lamp and appropriate filter sets for DAPI and Alexa488. More than 800 DAPI-stained cells were counted per sample. For each microscope field, two different categories were enumerated: (1) total DAPI-stained cells and (2) cells stained with the specific probe. Negative control counts (hybridization with HRP-Non338) averaged 0.5% and were always below 1.5% of DAPI-stained cells. The counting error, expressed as the percentage of SE between replicates, was $< 2\%$ for DAPI counts and $< 9\%$ for FISH counts. The EUB338 probe detected $81 \pm 5\%$ of the total DAPI-stained cells. The average number of membrane nonpermeable cells determined with physiological probes in the samples was 82% (author's unpublished data), thus indicating a probable low presence of *Archaea* in the Ría.

Nucleotide sequence accession numbers

The nucleotide sequences identified in this study have been deposited in the GenBank database under accession numbers EU600492–EU600587 for spring library clones (locus 1_XX), EU600588–EU600681 for summer library clones (locus 2_XX), EU600397–EU600491 for autumn library clones (locus 3_XX), and EU600682–EU600775 for winter library clones (locus 4_XX). Same locus codes, shaded in grey, were used in phylogenetic trees (Figs 2 and 3) to classify sequences derived from each season in the Ría.

Results

Clone library coverage estimations

At least 95 clones were analysed in each of the libraries corresponding to the four different hydrographical situations. Sequences of different length, ranging from 550 to > 800 bp, were obtained after eliminating low-quality peaks and joining the sequences obtained with each primer. Some of them included 16S rRNA genes from phytoplankton plastids, which were excluded from further work (Table 2). We used a minimum of 97% and 99% similarity threshold to group sequences in the same OTU (Table 2). The Good and Lee-Chao (C_{ACE}) estimators indicated that our clone libraries covered on average 80% and 66%, respectively, of the total bacterioplankton richness present in Ría de Vigo for a 97% threshold (Table 2). S_{Chao1}, as expected, presented higher values for a similarity threshold of 99%. LIBSHUFF analysis confirmed good coverages of the libraries and showed a marked difference between the libraries of each season (all *P*-values were lower than the critical *P*-value of 0.0043 for comparison between four libraries).

Bacterioplankton composition

Bacterioplankton community was mainly composed of *Alphaproteobacteria* throughout the year. In this group, FISH results showed that *Roseobacter* was the dominant group, while SAR11 existed in low numbers, at the four sampling times (Table 3). The highest abundance of *Roseobacter* occurred in the spring and then reached a minimum in the autumn and winter (Fig. 1a). The FISH abundances were similar to clone frequencies in the libraries, where most clones were related to *Roseobacter* (30–70%, Table 3).

Phylogenetic analysis of the *Roseobacter*-related sequences suggested the presence of the already-described main clusters (Fig. 2, black characters), and also new clusters composed exclusively of sequences derived from this study (Fig. 2, grey). The VIGO-ROS-A cluster contained two subclades with bootstrap values high enough to be proposed as a potential new cluster inside *Roseobacter* (Fig. 2, grey).

Phylogenetic analysis also suggested some seasonal tendencies for the different observed clusters. For example, two different 16S rRNA gene sequences close to *Roseobacter*, accounting for the highest frequencies among all clones, were mainly detected at two specific seasons. One of them, closely (99–100%) related to the uncultured *Roseobacter* strain DC5-80-3 (AY145589), was detected with 21 and 18 clones in the spring and summer libraries, respectively (Fig. 1c). The other sequence, 99–100% identical to NAC11-7 (AF245635), was represented by 22 clones in the spring library and 11 in the winter one (Fig. 1c). The DG1128 clade appeared in summer and autumn, but was not detected during the rest of the year. Although present in all libraries, CHAB-I-5 was mainly detected in the summer. Other *Roseobacter* clades similar to *Loktanella*, *Jannaschia*, or AS-21 were also detected in specific seasons while clades such as *Ruegeria–Silicibacter*, TM1040, *Roseivivax–Salipiger*, ANT9093, *Sulfitobacter–Staleya–Oceanibulbus*, *Citricella–Leisingera*, *Roseovarius*, and *Rhodobacter* did not show up in our libraries.

Both FISH and clone libraries revealed a low detection of SAR11 in the Ría during the whole year. FISH results obtained with two different FISH probes showed a

Table 3. Relative abundance (CARD-FISH, *F*% ± SE) and clone library characteristics (frequency, *C*%, and number of OTUs) observed for each major group of marine bacteria found during each season

	Spring			Summer			Autumn			Winter		
	Fish	Clone library		Fish	Clone library		Fish	Clone library		Fish	Clone library	
Major groups	*F*%	*C*%	OTUs	*F*%	*C*%	OTUs	*F*%	*C*%	OTUs	*F*%	*C*%	OTUs
Alphaproteobacteria	28 ± 1	86	17	25 ± 1	73	19	18 ± 2	53	12	15 ± 3	52	17
Roseobacter	52 ± 7	70	11	43 ± 5	52	14	28 ± 5	30	8	28 ± 8	29	10
SAR11	5 ± 0.5	2	3	0.5 ± 0	0	2	2 ± 0.5	12	2	1 ± 0.3	11	4
Gammaproteobacteria	7 ± 2	6	4	11 ± 1	13	9	6 ± 1	15	5	16 ± 5	27	9
SAR86	5 ± 0.5	0	0	15 ± 3	9	2	20 ± 2	11	2	5 ± 1	1	1
Cycloclasticus	0 ± 0	0	0	0 ± 0	0	0	0.1 ± 0	0	0	1 ± 0.5	3	1
Bacteroidetes	14 ± 2	5	4	27 ± 3	11	5	23 ± 3	23	16	29 ± 6	6	4
Betaproteobacteria	3 ± 1	2	1	0.2 ± 0	0	0	3 ± 1	0	0	1 ± 0.2	2	1
Verrucomicrobia	NA	0	0	NA	3	3	NA	2	1	NA	0	0
Actinobacteria	NA	0	0	NA	0	0	NA	1	1	NA	0	0
Spirochaetes	NA	0	0	NA	0	0	NA	0	0	NA	1	1
Unclassified bacteria	NA	1	1	NA	0	0	NA	6	4	NA	12	6

F% data for SAR11 is the average of the results obtained with the two different probes used (A1 and 441R; Fig. 1).
Diversity indices were measured only when the percentages of clones were ≥5%.
NA, not measured or not applicable.

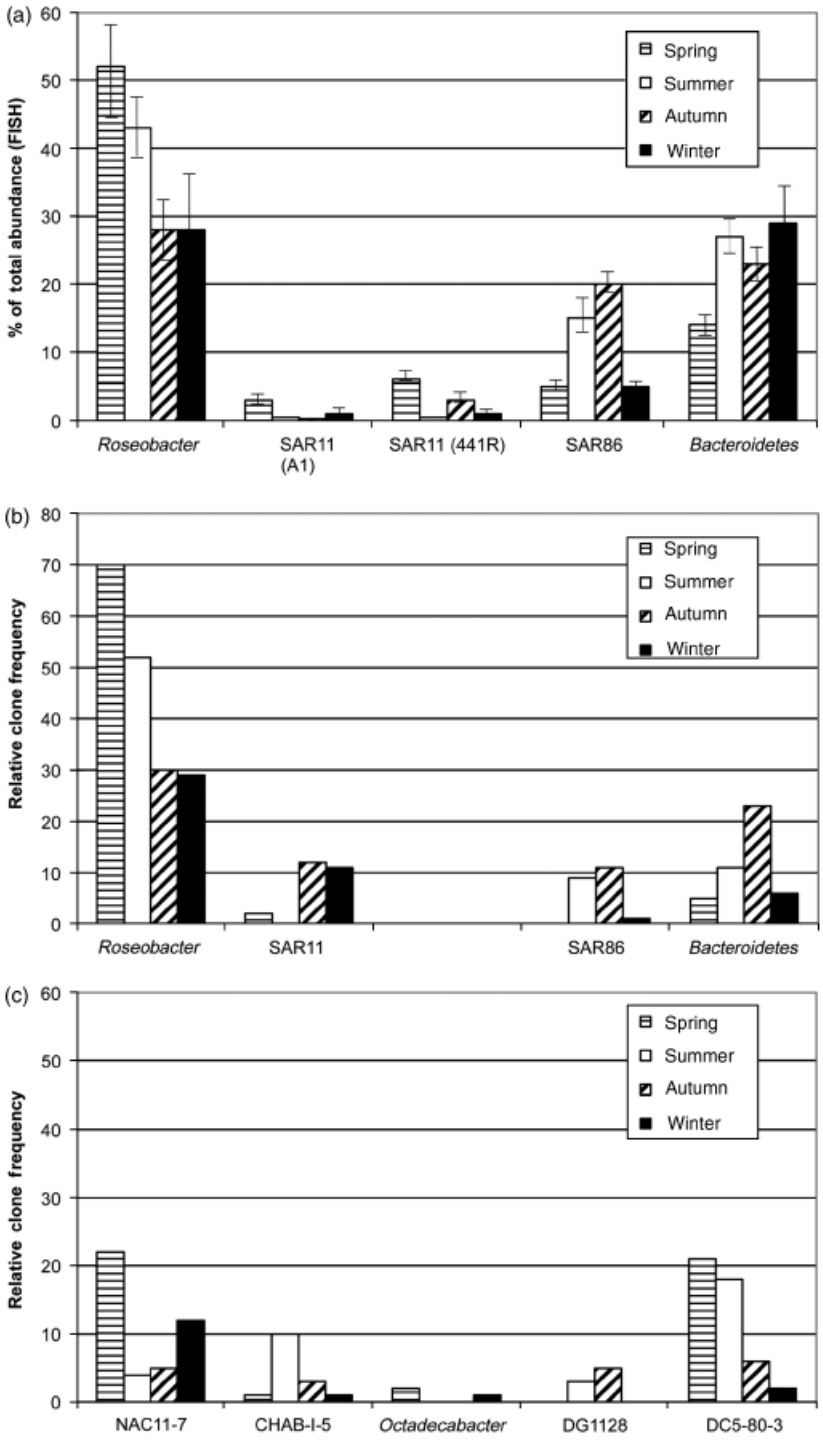

Fig. 1. Relative abundances (using CARD-FISH) (a) and relative clone frequency (b) of the most relevant groups of bacteria detected in the Ría de Vigo at different times of the year. The oligonucleotide probes used for CARD-FISH cover part of the *Bacteroidetes* group (CF319a) (Manz *et al.*, 1996), the *Roseobacter* lineage (ROS537) (Eilers *et al.*, 2001), the SAR11 cluster (SAR11-A1 and SAR11-441R) (Field *et al.*, 1997; Morris *et al.*, 2002), and the SAR86 cluster (SAR86-1249) (Eilers *et al.*, 2000). In the case of the SAR11 clade of *Alphaproteobacteria*, two different probes were used [A1 (Field *et al.*, 1997) and 441R (Morris *et al.*, 2002)]. The results for other groups of bacteria with a lower representation in the community [e.g. *Betaproteobacteria* (BET42a) (Manz *et al.*, 1992) and *Cycloclasticus* (CYPU829) (Maruyama *et al.*, 2003)] are presented in Table 3. Percentages from FISH for each group are referred to in the total counts of *Eubacteria*-positive cells (with probe EUB338; Amann *et al.*, 1990). Relative clone frequencies belonging to different clades of *Roseobacter* (Fig. 2) are also illustrated (c).

constantly low population (around 3% on average, Fig. 1). SAR11 clone sequences were mainly detected in the autumn and winter (about 10–12% of the library, Table 3), with low and no representation in the spring and summer libraries, which was exactly opposite to what was found for the most abundant *Roseobacter* clones (NAC11-7 and DC5-80-3) (Fig. 2). A phylogenetic tree was constructed to classify our SAR11 clone sequences using the reference clusters already described by Field *et al.*, 1997 (not shown). Approximately half of these autumn and winter clones were represented by SAR1 (X52280)-like sequences. The rest of the autumn clones possibly formed new subclusters within this cluster

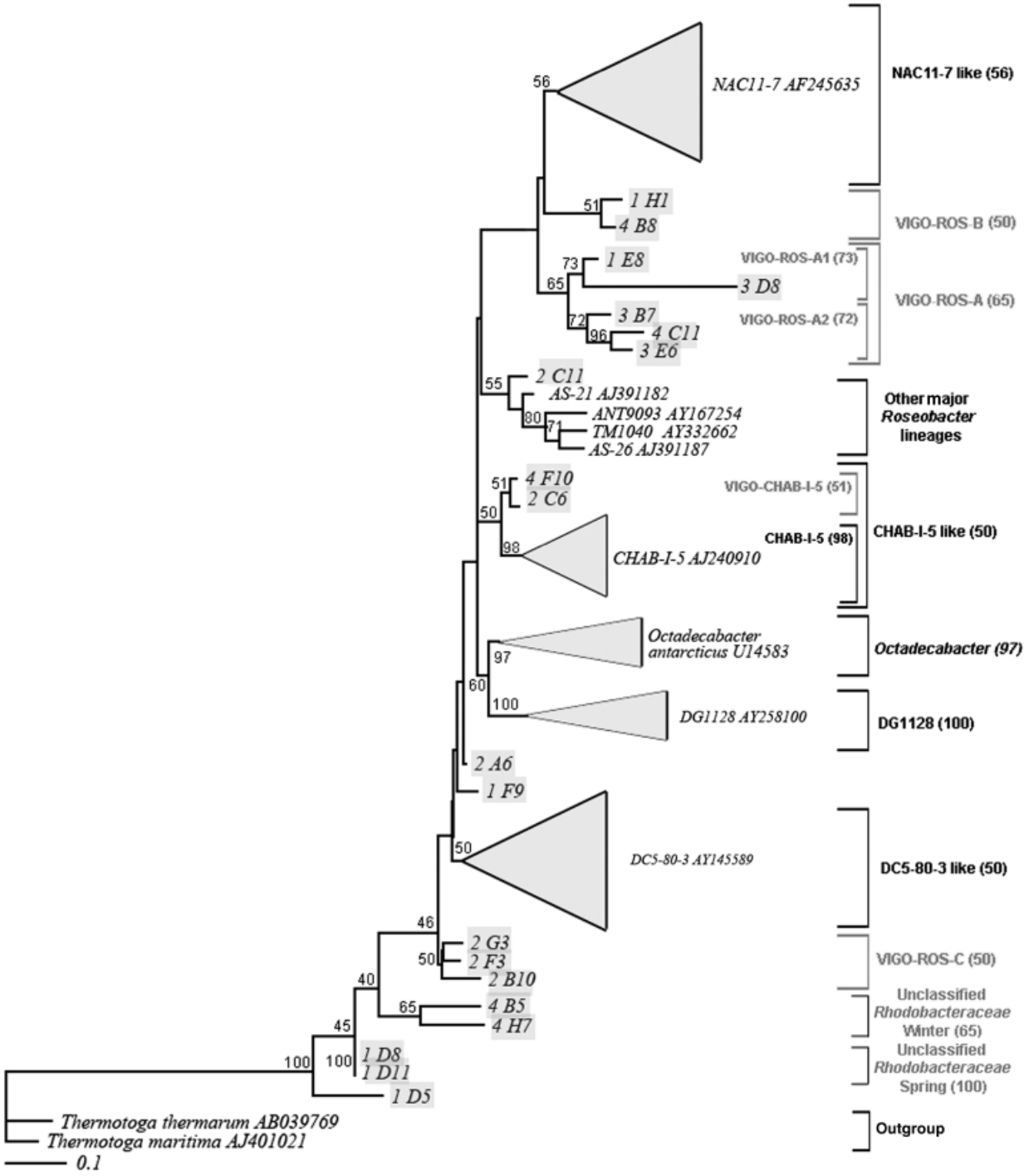

Fig. 2. ML phylogenetic tree of the clones related to major *Roseobacter* clades as described by Buchan *et al*. (2005). Clone sequences derived from this study are shaded in grey, while type sequences (and its accession numbers) for each clade defined by Buchan and colleagues are in black characters. Triangles in grey colour represent sequences derived from this study that grouped with previously described clades. Already-described clades are given in black characters, while clades composed exclusively by clone sequences derived from this study are in grey characters. Phylogenies are based on alignments of 650 bp. Parameters for searching trees in Phyml were selected by MODELTEST and PAUP* using the Akaike information criterion (AIC) (model of nucleotide evolution: GTR+I+G; Pinvar: 0.2630). Bootstrap values of ≥50% are shown at branch nodes (1000 iterations). *Thermotoga* spp. was used as the outgroup.

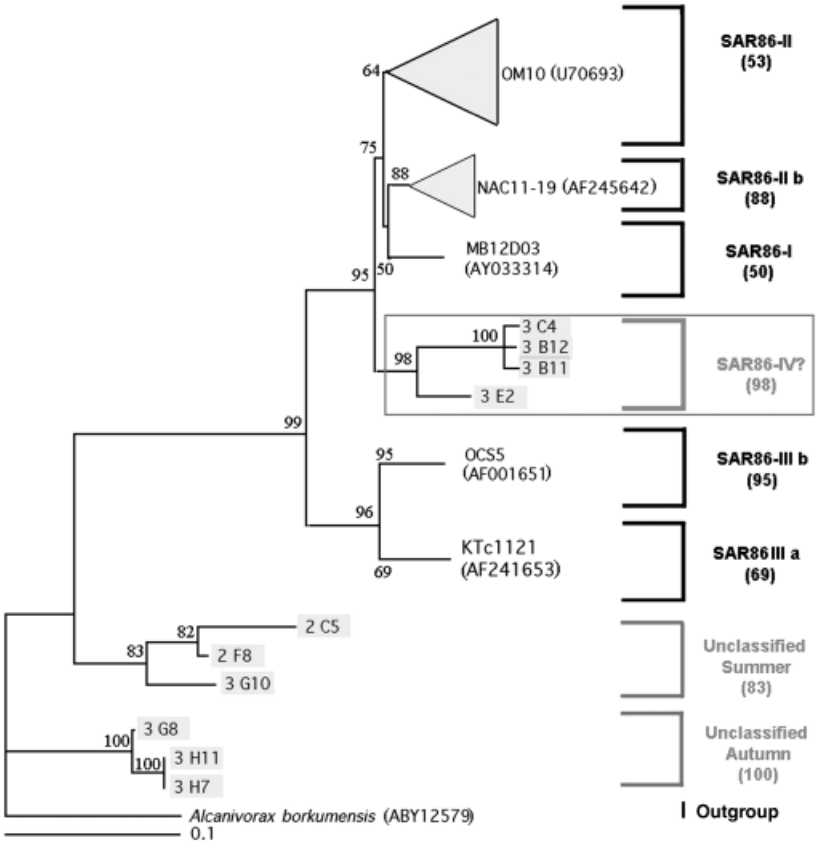

Fig. 3. ML phylogenetic tree of the clones related to major SAR86 clades as proposed previously (Suzuki *et al.*, 2001; Sabehi *et al.*, 2004). Phylogenies are based on alignments of 600 bp. Parameters for searching trees in Phyml were selected by MODELTEST and PAUP using the Akaike information criterion (AIC) (model of nucleotide evolution: GTR+I+G; Pinvar: 0.4683). Bootstrap values of $\geq 50\%$ are shown at branch nodes (1000 iterations). The *Gammaproteobacteria Alcanivorax borkumensis* (Y12579) was used as an outgroup. The same criteria as in Fig. 2 have been used to distinguish between already-described sequences and clades as those derived from this study.

A, with a high bootstrap support. The other large SAR11 clusters (-D/-B/-G) were only represented by two winter clones that did not cluster with any of the reference sequences used. Other sequences of difficult adscription were left as unclassified *Alphaproteobacteria*, because they appeared between the SAR11 cluster and the *Rhodobacteraceae* family.

Gammaproteobacteria was composed of diverse groups (SAR86, *Alteromonas* spp., *Glaciecola* spp., *Saccharophagus* spp., *Oleispira* spp., *Cycloclasticus* spp., etc.) (Table 3). Both FISH and clone libraries showed the same seasonal trend for SAR86. Relative abundances of approximately 20% were detected by FISH in the summer and autumn, while the group was almost absent from the winter and spring communities. The highest abundance and diversity in this group was found in the autumn library, where at least one novel clade of SAR86 seemed to exist (Fig. 3). A high bootstrap value of 98, and its position between clades SAR86-I, -II, and -III, support the description of a new cluster inside SAR86 named SAR86_IV (Fig. 3, boxed, grey).

Specific probes and clone library results showed exactly the same trend in the microbial community for *Cycloclasticus*, a polyaromatic hydrocarbon (PAH)-degrading bacterium (Table 3). It appeared in the winter, while, during the rest of the year, it remained in almost undetectable numbers. All sequences from the winter were identical and similar (99–100%) to the described species *Cycloclasticus spirillensus* (DQ659429).

In terms of *Betaproteobacteria*, both FISH and clone library data indicated low relative populations of around $2 \pm 1\%$ throughout the year. The sequences detected during the winter and spring were related to members of the *Methylophilaceae* family. However, the sequences from each season were quite different from those of the spring season, albeit close to members of this family, and were likely to constitute a novel group inside the *Betaproteobacteria* (tree not shown).

FISH results showed a relatively constant and high contribution of *Bacteroidetes* to the bacterial assemblage throughout the year ($20 \pm 5\%$ of DAPI counts), with the exception of spring, which seemed to be dominated by the presence of *Roseobacter*. The libraries' results showed that this group was the most diverse, with almost all clones being represented only once in each library (Table 3). Sequences close to the genera *Flavobacteria*, *Bacteroidetes*, *Polaribacter*, *Gelidibacter*, *Flexibacter*, *Cytophagales*, and *Winogradskyella* were detected in Ría de Vigo.

Besides *Bacteroidetes* and *Proteobacteria* (*Alpha-*, *Gamma-*, and *Betaproteobacteria*), other phyla of bacteria, such as *Verrucomicrobia* and *Actinobacteria*, were detected.

Verrucomicrobia-related sequences were detected during the summer and autumn ($3 \pm 1\%$ of each clone library), but not during the rest of the year, while *Actinobacteria* were detected only during the autumn (1% of the clone library). Sequences from both groups were similar to uncultured species of an undefined affiliation.

Discussion

Methodological considerations

In general, the results from FISH were considered as quantitative tools in this study, while clone sequences were used to describe diversity. The limited number of studied clones in each situation (around 95) and the well-known methodological biases (e.g. von Wintzingerode *et al.*, 1997; Cottrell & Kirchman, 2000; Eilers *et al.*, 2000; Alonso-Sáez *et al.*, 2007) prevent an exhaustive and quantitative description of the whole bacterioplankton community using libraries. However, the coverage was good enough to generate an image of the bacterial diversity existing in the Ría throughout the year. Being aware of its limitation, we used clone frequencies belonging to specific clades to establish hypotheses about possible seasonal preferences of these groups, which should therefore be interpreted with caution.

The *Roseobacter*, SAR11, and *Cycloclasticus* probe data matched well with the clone sequence data (Table 3). Other probes, such as that for SAR86, showed major discrepancies with sequences of this group detected in the Ría, as we will discuss later. The worst case was found for the *Gamma-* and *Alphaproteobacteria* groups, which were drastically underestimated by FISH counting. In these two cases, there was a considerable inconsistency between counts with the general probes (GAM42a and ALF968) and the more specific probes (SAR86-1249 and ROS537) (Table 3). Amann & Fuchs (2008) found that the group coverage for ALF968 and GAM42a probes was 81% and 76%, respectively. Therefore, if some nondetected members are particularly abundant in a sample, the underestimation using the general probe can be considerable. This seemed to occur in the present study.

Using the current rRNA database (SILVA), we found that 9% of the sequences (mainly from uncultured *Rhodobacteraceae*) detected with the ROS537 probe are not detectable with the ALF968 probe. *In silico* alignments of the ALF968 probe sequence matched perfectly with most of the clones detected. However, 20 out of the 80 different sequences classified as *Rhodobacteraceae* in this study did not match with the ALF968 probe. It is very likely that some of these nondetected members were particularly abundant in the Ría de Vigo. This explains the large discrepancy in FISH counts. In environments where SAR11 dominates over *Roseobacter*, such as Blanes Bay (Mediterranean Sea), FISH counts match fairly well with the library data (Alonso-Sáez *et al.*, 2007). The same occurred when checking *Gammaproteobacteria* probes; the vast majority (98%) of the sequences that were detected with SAR86-1249 were not detected with the GAM42a probe.

The fraction of EUB338 counts accounted for by the sum of general probes (ALF968, BET42a, GAM42a, and CF319a) was only 58%. However, the sum of the ROS537, SAR11-441R, BET42a, GAM42a, SAR86-1249, and CF319a probes accounted for only 86% of the total EUB338 counts. This analysis demonstrates that the old general probes must be used with caution and points to the urgent need to refine phylum- and class-specific probes using the high-quality rRNA databases (Amann & Fuchs, 2008).

Bacterial diversity in the Ría de Vigo

Changes in bacterioplankton community structure and diversity were found throughout the year in Ría de Vigo (Table 3), including the detection of new members in all major groups of bacteria.

Both culture-independent (i.e. 16S rRNA gene-based) and culture-dependent studies have indicated that members of the *Alphaproteobacteria* belonging to the *Roseobacter* lineage are abundant in coastal (González & Morán, 1997; González *et al.*, 1999) and open ocean environments, where they are often found in association with phytoplankton (Prokic *et al.*, 1998; González *et al.*, 2000).

In Ría de Vigo, the hydrographic conditions are highly variable throughout the year. The *Roseobacter* lineage clearly dominated the bacterioplankton in all seasons, having its higher contribution to community structure in the spring, when the phytoplankton community was dominated by actively growing chain-forming diatoms (Teira *et al.*, 2007).

The marked difference between seasons in the detection of the two major clades of *Roseobacter* (NAC11-7 and DC5-80-3) could likely reflect a seasonal trend. Both seemed to overlap in the spring phytoplankton bloom, when intermediate oceanographic characteristics exist (moderate temperatures and high inorganic nutrient inputs) (Table 1, Fig. 1c). The different clades could use different strategies, or have different environmental preferences, including an association with different phytoplankton species (Wagner-Dobler & Biebl, 2006; West *et al.*, 2008). The absence of eight *Roseobacter* clades is not diagnostic, but could provide some circumstantial evidence of the dominance of NAC11-7 and DC5-80-3.

In contrast to previous results, which indicated that SAR11 clade members dominate surface bacterioplankton communities (Morris *et al.*, 2002), the communities of Ría de Vigo seem to be dominated by other groups, and SAR11 is present in very low abundances. A low abundance of SAR11 was also reported for the estuarine environment of Ría de Aveiro (Portugal) (Henriques *et al.*, 2004), which is

located on the same coastline as Ría de Vigo (around 200 km from Vigo), and it is also impacted by the northwest African upwelling system. Mary *et al.* (2006) also found a relatively low abundance of SAR11 throughout the year at a coastal station on the western English Channel (13% of DAPI counts). On the other hand, a high variability in the relative abundance of SAR11 (6–37% of DAPI counts) has been found in north-west Mediterranean coastal waters (Alonso-Sáez *et al.*, 2007) and in Oregon coastal surface waters (17–38%) (Morris *et al.*, 2002). Low SAR11 abundance had been previously observed in the Baltic Sea (Riemann *et al.*, 2008) and in the low-salinity part of Eastern North American estuaries (Kirchman *et al.*, 2005). However, the low SAR11 contributions in the case of Ría de Vigo, which is a special coastal upwelling system different from an estuary, cannot be related to the low salinity levels (Table 1).

In oligotrophic coastal waters, *Roseobacter* have been observed to thrive only during chlorophyll *a*-rich periods (upwelling seasons) (Alonso-Sáez *et al.*, 2007). Recent studies have revealed a higher metabolic activity of *Roseobacter* in competing with SAR11 for nutrients under eutrophic conditions (Alonso & Pernthaler, 2006). The Vigo embayment, characterized by a high primary production, provides a rich environment where *Roseobacter* members probably develop in detriment of SAR11, which are more adapted to the oligotrophic conditions characteristic of the open waters (Rappé *et al.*, 2002).

The SAR86 lineage of *Gammaproteobacteria* has also been found to be abundant within previously studied bacterioplankton communities (González *et al.*, 2000; Suzuki *et al.*, 2004). However, and as occurred with the other lineages, SAR86 members contributed little to community structure as compared with *Roseobacter* in Ría de Vigo. SAR86 reached maximum abundances in the autumn, when the relative abundance of *Roseobacter* was the lowest. During its peak in population, the new cluster SAR86-IV was detected (Fig. 3), which could be characteristic of the waters under study.

We detected bacteria phylogenetically related to the species *C. spirillensus* in the Ría de Vigo, considered to be key organisms for PAH degradation in seawater (Chung & King, 2001; Kasai *et al.*, 2002; McKew *et al.*, 2007). We observed that the detection of *Cycloclasticus* in winter water increased proportionally with the initial concentration of the added PAHs (Teira *et al.*, 2007), supporting the theory of the existence of a preadapted hydrocarbon-degrading community in the Ría, as was suggested previously (Alonso-Gutiérrez *et al.*, 2008).

Bacteroidetes were represented by a small number of highly diverse clones with low similarity among them or with any sequence in the GenBank database (Table 3). The *Bacteroidetes* group has been reported to dominate during bloom periods (Pinhassi & Hagstrom, 2000; Eilers *et al.*, 2001; Mary *et al.*, 2006), when high nutrient inputs could favour these organisms, as demonstrated recently (Alonso & Pernthaler, 2006). In our study, a relatively constant and high percentage (23% as a mean from the FISH results) is present throughout the year. In a system that is constantly provided with nutrients, such as the Ría de Vigo system, *Roseobacter* and *Bacteroidetes*, which were better adapted to these situations (Alonso & Pernthaler, 2006), maintained a constantly high population throughout the year, in contrast to typical open ocean organisms, such as SAR11.

Sequences almost identical to *Betaproteobacteria* cluster OM43 were detected in winter (99–100% of identity with U70704). Members of this clade are commonly associated with phytoplankton blooms (Giovannoni *et al.*, 2008). The phylogenetic analysis (tree not shown) of sequences detected in the spring suggested their classification as a novel group inside *Betaproteobacteria*. Low numbers of *Betaproteobacteria* (typically considered 'freshwater' groups; Methé *et al.*, 1998), detected both by FISH and clone libraries, support the low influence of freshwater bacteria compared with those of marine origin in this environment.

Detection of new clusters

Information from review studies of major marine bacteria, such as *Roseobacter* (Buchan *et al.*, 2005), SAR11 (Field *et al.*, 1997), or SAR86 (Suzuki *et al.*, 2001; Sabehi *et al.*, 2004), and from our four clone libraries (retrieved from different oceanographic conditions) enabled us to detect new clades in specific seasons. This information should be further incorporated into the design of new, more specific, FISH probes to describe with higher precision the bacterial communities of upwelling ecosystems.

From the new clusters described in the analyses performed, the most interesting ones belonged to SAR86 clones. The clone sequences from this study that grouped in already described clusters (e.g. SAR86-II, SAR86-I, SAR86-IIIa, b) matched perfectly with the probe used (SAR86-1249). However, three of the novel clusters proposed (Fig. 3) showed significant mismatches with the sequence. For example, the proposed VIGO_SAR86-IV and the two unclassified groups (summer and winter) had 6–8, 5–7, and 4 bp mismatching, respectively, to the probe sequence. The last two clusters are not clearly related to the rest of SAR86. However, cluster SAR86-IV, boxed in grey colour in Fig 3, is clearly between the described clusters of SAR86, from which it is separated by a high bootstrap value. Tree topology and bootstrap support were also obtained by parsimony and NJ methods (not shown). High bootstrap values and the large number of sequences of proposed new clusters in SAR86 (Fig. 3) and SAR11 (tree not shown) prompt the design of more specific probes to detect members of these groups.

Conclusion

Ría de Vigo is an upwelling-affected coastal ecosystem of high productivity in which phytoplankton blooms frequently occur. Nutrients are readily available throughout the year and especially during the spring, and our study shows that this favours the existence and dominance of different *Roseobacter* subclades. High nutrient levels, associated with phytoplankton blooms, are probably the cause of the low presence of the SAR11 lineage throughout the year. We suggest that changes in the phytoplankton community characteristic of each season drive the composition and dominance of the *Roseobacter* clade. These changes could also explain the appearance of new subclades in all major lineages (SAR11, SAR86, *Bacteroidetes*, *Betaproteobacteria*, and even inside *Roseobacter*) that would be particularly adapted to the changing environment of the Ría.

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