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ORIGINAL ARTICLE

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

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The introduction of high-throughput dilution-to-extinction culturing (HTC) of marine bacterioplankton using sterilized natural sea water as media yielded isolates of many abundant but previously uncultured marine bacterial clades. In early experiments, bacteria from the SAR11 cluster (class Alphaproteobacteria), which are presumed to be the most abundant prokaryotes on earth, were cultured. Although many additional attempts were made, no further strains of the SAR11 clade were obtained. Here, we describe improvements to the HTC technique, which led to the isolation of 17 new SAR11 strains from the Oregon coast and the Sargasso Sea, accounting for 28% and 31% of all isolates in these experiments. Phylogenetic analysis of the internal transcribed spacer (ITS) region showed that the isolates from the Oregon coast represent three different subclusters of SAR11, while isolates from the Sargasso Sea were more uniform and represented a single ITS cluster. A PCR assay proved the presence of proteorhodopsin (PR) in nearly all SAR11 isolates. Analysis of PR amino-acid sequences indicated that isolates from the Oregon coast were tuned to either green or blue light, while PRs from strains obtained from the Sargasso Sea were exclusively tuned to maximum absorbance in the blue. Interestingly, phylogenies based on PR and ITS did not correlate, suggesting lateral gene transfer. In addition to the new SAR11 strains, many novel strains belonging to clusters of previously uncultured or undescribed species of different bacterial phyla, including the first strain of the highly abundant alphaproteobacterial SAR116 clade, were isolated using the modified methods.

Introduction

Marine bacterioplankton play an important role in marine food chains and global nutrient cycling (Arrigo, 2005). Phylogenetic analysis based on 16S rRNA genes indicates that bacterioplankton consist mainly of about 10–15 abundant clades, which usually make up about 90% of all prokaryotic gene clones in libraries prepared from ocean surface waters (Rappé and Giovannoni, 2003). Progress has been made in recent years at cultivating members of the numerically abundant clades; for example,

the SAR11 clade (Rappé *et al.*, 2002), the marine group I Crenarchaeota (Konneke *et al.*, 2005) and the OM43 clade (Connon and Giovannoni, 2002) were obtained in pure culture. Much of this success can be attributed to using high-throughput culturing ('HTC'; Connon and Giovannoni, 2002), a method using dilution-to-extinction culturing with pristine sea water as media, mainly based on the ideas and findings reported by Button *et al.* (1993).

With the recent increase in genomic and metagenomic data, bacterial speciation and the microbial species concept are intensively discussed in the recent literature (see Konstantinidis *et al.*, 2006; Staley, 2006). The SAR11 clade is one of the most abundant microorganisms on earth (Morris *et al.*, 2002) and contains well-defined subclusters. The SAR11 clade consists of at least four different subclusters that can be discriminated by their 16S rRNA sequence (Morris *et al.*, 2005). Abundances

of these subclusters change spatially with depth (Field et al., 1997; Morris et al., 2005) and temporally with season (Morris et al., 2005), thus implying different adaptations to the environment for the different subgroups. These attributes make the SAR11 clade a good model for studying bacterial speciation.

The 16S rRNA gene is a slowly evolving evolutionary marker that may not resolve recent speciation events. Sequencing and phylogenetic analysis of the internal transcribed spacer (ITS) region, the non-coding region between the 16S rRNA and the 23S rRNA, has indicated a fine-scale microdiversity within the SAR11 clade (Garcia-Martinez and Rodriguez-Valera, 2000; Brown and Fuhrman, 2005). A key question in the debate on bacterial speciation is whether the microdiversity in clusters of environmental 16S rRNA sequences observed in many environments, including marine habitats (Thompson et al., 2004; Venter et al., 2004; Sogin et al., 2005, 2006; Pommier et al., 2007), coincides with functional divergence. This question has not been answered adequately for most of the major marine bacterial clades (Giovannoni, 2005; Martiny et al., 2006). Answering this question will require culturing and comparative genomic and metabolic studies of the representatives of the divergent subclades. Thus far, cultivation of SAR11 strains by HTC has only been successful in a single experiment, which resulted in the isolation of strains with identical 16S rRNA gene sequences (Rappé et al., 2002). No further cultured strains of this clade have been reported yet.

Apart from a genome analysis (Giovannoni et al., 2005b), physiological data on the SAR11 cluster are scarce, but recent data showed that members of the SAR11 clade play an important role in the marine sulfur cycle and can take up and degrade dimethylsulfoniopropionate (DMSP; Malmstrom et al., 2004; Howard et al., 2006). To improve culturing efficiencies of SAR11, we modified the HTC method by adding DMSP to the media as well as by using ultra-clean Teflon plates as culture vessels. For easy, fast and reliable screening of the cultures, we established a flow cytometry method using a commercial cell counter designed primarily for eukaryotic cells.

The goals of this study were to improve existing methods for culturing marine bacteria to (1) isolate new SAR11 strains from different environments and (2) isolate abundant yet uncultured marine bacteria for further physiological studies.

Two HTC experiments with inoculum from the ultra-oligotrophic subtropical gyre at the Bermuda Atlantic Time Series study site (BATS) and from the nutrient-rich upwelling area at the Oregon coast were performed. The cultures obtained were screened by sequencing of 16S rRNA genes, and, for SAR11 cultures, ITS and proteorhodopsin (PR) genes were sequenced and analyzed.

Methods

Sampling and media
Inoculum was sampled with Niskin bottles in May 2006 at station NH-5, 5 miles off the coast along the Newport, Oregon hydroline, from a depth of 10 m, and in July 2006 at the BATS from 10 and 200 m depth. Samples from BATS were collected in a rinsed 1-liter Teflon bottle and shipped overnight on ice to Oregon State University. Samples from the Oregon coast were collected in the same bottles and transported on ice to Oregon State University. Preparation of the media followed the protocol of low-nutrient heterotrophic media ('LNHM'; Connon and Giovannoni, 2002), with sea water collected at NH-5 in May 2006. The media was amended with DMSP (100 nM), a mix of vitamins (Rappé et al., 2002), NH_4Cl (10 μM), K_2HPO_4 (1 μM) and a mixture of carbon compounds ('MC'; 0.001% (w/v); Rappé et al., 2002).

Preparation of Teflon plates
Custom-made 24-well Teflon plates with a well volume of 6 ml were obtained from Cowie-Tech (Wilmington, DE, USA). To prevent metal contaminations from the autoclave steam, plates were sterilized using a combination of microwaving and UV irradiation. The plates were rinsed five times with nanopure water and soaked overnight in 10% omnipure HCl. After soaking, plates were rinsed five times with nanopure, filled up with nanopure, and put into a seal-a-meal bag and microwaved at maximum power for 10 min. The boiling water was discarded, the bags were sealed and UV-irradiated for at least 60 min on each side.

Inoculation
The cell densities of the inocula were determined by 4′,6-diamidino-2-phenylindole (DAPI) counting as described by Connon and Giovannoni (2002). Dilutions of the inoculum were performed in media. Wells were filled with 5 ml media including inoculum. Final inoculation densities were calculated based on the dilution factor of the original inoculum (Table 1). Incubation of the plates was performed at 16°C in the dark.

Screening of the cultures
Plates were screened after 4, 8 and 12 weeks with a Guava EasyCyte cell counter (Guava Technologies, Hayward, CA, USA). Samples of 200 μl were transferred to a 96-well plate and stained with SYBR Green1 (final dilution 1:2000, Invitrogen, Eugene, OR, USA) for 60 min. The samples were run for 5 s each with the green photomultiplier set at 700 V; the two other photomutlipliers were set to a low value (400 V). The limit of detection of this method was tested with a pure culture of Candidatus Pelagibacter ubique and determined to be

Table 1 Setup and results of dilution-to-extinction experiments

Location and depth	Inoculation density (cells/ml)	Number of inoculated wells	Positive wells	Pure cultures	SAR11 isolates
Oregon, NH-5, 10 m	1	96	52	43	12
Oregon, NH-5, 10 m	3	96	96	ND	ND
BATS, 10 m	1	168	4	4	2
BATS, 10 m	3	168	11	9	3
BATS, 200 m	1	168	1	1	0
BATS, 200 m	5	168	3	2	0

Abbreviations: BATS, Bermuda Atlantic Time Series study site; ND, not determined, microscopic analysis showed many mixed cultures.

2×10^3 cells/ml. For the HTC experiments, wells with cell densities of 10^4 cells/ml and higher were considered positive. Samples (200 μl) of positive cultures were fixed with formalin, stained with DAPI and transferred to a polycarbonate membrane according to Connon and Giovannoni (2002). Micrographs of cultures were taken with a digital camera at $\times 1000$ magnification. For all wells that were called positive based on the results of the cell counter, cells were visible under the microscope.

Culture storage, DNA extraction, PCR and RFLP analysis
For long-term storage of the cultures, two 200-μl samples were stored in 10% glycerol and frozen in liquid nitrogen. Usually about 90% of the cultures can be easily revived using this method. DNA extractions, PCR of 16S rRNA genes and restriction fragment length polymorphism (RFLP) analysis were performed as described previously (Connon and Giovannoni, 2002). Briefly, positive PCR products were digested with *Hae*III for 2 h at 37°C. Digests were electrophoretically separated on 3% agarose gels, stained with SYBR safe (Invitrogen) and visualized under UV light. If the sum of the length of the bands was equal to or less than one 16S rRNA gene ($\sim$1500 bp), then the culture was considered pure. RFLPs with the same pattern from the same environment were grouped together.

Phylogenetic analysis
16S rRNA gene sequences of at least two randomly chosen strains from each RFLP group (if applicable) were sequenced with primer 27F on an ABI 111 sequencer (Oregon State University, CSL facility, Corvallis, OR, USA). ABI traces were manually checked using DNAStar (GATC, Konstanz, Germany) and yielded high-quality sequences of 520–1050 bp. Sequence data were analyzed with the ARB software package (Ludwig et al., 2004). The new sequences were added to the ARB database and aligned with the Fast Aligner tool. Alignments were checked and corrected manually where necessary. 16S rRNA gene sequences from the isolates were compared to sequences in public databases with BLASTn (Altschul et al., 1997); 16S rRNA gene

sequences with high similarities to those determined in this study were retrieved and added to the alignment. Highly variable regions of the 16S rRNA gene and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences. Framework trees were calculated with fastDNAmL, a maximum-likelihood method implemented in ARB, using only almost-full-length sequences ($>$1400 bp). Shorter sequences ($<$1400 bp) were added to these trees with the ARB parsimony tool, which allows the addition of short sequences to existing phylogenetic trees without changing global tree topologies (Ludwig et al., 1998). The stability of the branching pattern was tested with the neighbor-joining and maximum-parsimony (DNAPARS) methods included in the PHYLIP package as implemented in ARB.

ITS and proteorhodopsin sequences of SAR11 strains
PCR for ITS regions of SAR11 strains was performed as described previously (Rappé et al., 2002). Phylogenetic analysis of ITS sequences followed the protocol of Brown and Fuhrman (2005) with newly obtained ITS sequences (390–410 bp) added to the existing alignment that was kindly provided by Dr Mark Brown, University of Hawaii. Phylogenetic tree was calculated in ARB using the neighbor-joining algorithm according to Brown and Fuhrman (2005).

PCR for PR was performed with a mixture of previously described and newly developed primers deduced from SAR11-like PR sequences from the Sargasso Sea dataset (Venter et al., 2004). As forward primer, we used either (5′-MGNTAYATHGAYTGG YT-3′; Sabehi et al., 2005) or PR_SAR11_F1 (5′-ATG AAAAACTTAAACTGTTT-3′), reverse primers were (5′-GGRTADATNGCCCANCC-3′; Sabehi et al., 2005) or PR_SAR11_R1 (5′-TGCAGCAGCCCAGATTACT-3′). Annealing temperature was determined empirically. Reactions (100 μl) were performed using 1 μl extracted DNA as template, 500 nM of each primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, 2 units of Taq polymerase (MBI Fermentas, Hanover, MD, USA) and $\times$1 of the respective buffer provided by the manufacturer. Routinely, reactions were amplified in a thermocycler (MJ Research PTC 200, MJ, Ramsey, MI, USA)

with the following protocol: initial denaturing step of 3 min at 94°C, 50 cycles of 30 s at 94°C, 45 s at 44°C, 1 min at 72°C, which were followed by a final extension step for 5 min at 72°C.

The obtained PR nucleotide sequences were between 370 and 570 bp. Sequences were aligned with ClustalW as integrated in ARB. The closest related sequences were retrieved by BLASTn from Genbank and added to the alignment. Nucleotide sequences were translated and a phylogenetic tree of the amino-acid sequences was calculated using the neighbor-joining algorithm as implemented in ARB.

Accession numbers

The sequences reported in this study are deposited with GenBank under accession numbers EF616578–EF616643.

Results

Initial experiments with a pure culture of *Candidatus* Pelagibacter ubique HTCC1002 showed that cells grow better in Teflon plates than in polystyrene plates (data not shown). The increased volume per well of the Teflon plates (5 ml instead of 2 ml) also allows for more sampling of the cultures, which is valuable because of highly variable lag phases and growth rates of marine bacteria.

Screening of the plates using the Guava EasyCyte, a 96-well flow cytometer primarily designed for counting eukaryotic cells, is both fast and reliable. Counting of four 24-well plates takes approximately 45 min. Limit of detection was determined with a pure culture of *Candidatus* Pelagibacter ubique HTCC1062 and is in the range of 2×10^3 cells/ml.

Overview on isolated strains

HTCC experiments were performed with inoculum from the Sargasso Sea (10 and 200 m depths, 14 plates each) in July 2006 and from the Oregon coast (10 m depth, eight plates) in May 2006 (Table 1). Culturing efficiencies were highest for the Oregon inoculum and yielded 52 positive wells. PCR of 16S rRNA genes and following RFLP analysis resulted in 43 potential pure cultures and 9 mixed cultures (Table 1). The pure cultures could be clustered into 12 different RFLP patterns each containing 1 to 12 strains (Table 2). Sequence analysis of the representatives of the different RFLPs showed that most of the strains were from the SAR11 cluster (12 strains, representing 28% of the isolates). Other strains of abundant environmental groups that previously had no closely related cultured representatives include the SAR116 group (HTCC8037, one strain), the OCS14 clade (HTCC8023, one strain), two groups of Verrucomicrobia (HTCC8039 and HTCC8042), one strain of Bacteroidetes (HTCC8036), and two strains of a group of Gammaproteobacteria that are closely related to uncultivated sulfur-oxidizing

symbionts (HTCC8012 and HTCC8014). The other strains mainly clustered among Alphaproteobacteria and Gammaproteobacteria (Table 2), and were closely related to groups that have previously isolated and systematically described, or were present in prior HTCC experiments.

Dilution-to-extinction culturing using inoculum from the Sargasso Sea yielded 19 positive wells, 4 from 200 m samples and 15 from surface samples. RFLP analysis indicated that 16 were pure cultures that could be grouped into 7 RFLP groups with 1 to 5 representatives. Phylogenetic analysis showed that five SAR11 strains were among the cultures (31% of isolates). Among the BATS isolates, one strain of a so far unnamed group of Alphaproteobacteria clustered together with many environmental sequences (HTCC7212) and had no closely related described species (Table 2).

SAR11 cultures

There were no obvious morphological differences between novel isolates from the Sargasso Sea and from the Oregon coast. Morphology of the newly isolated SAR11 strains is virtually identical to *Candidatus* Pelagibacter ubique 1062 (Rappé et al., 2002). A phylogenetic analysis of the 16S rRNA genes of the isolated SAR11 strains showed that they were closely related with sequence identities of 97.6%–99.6%. Sequence similarities to *Candidatus* Pelagibacter ubique were between 97.7% and 99.7%. Strains obtained from the Oregon coast were more diverse (97.6%–99.6% identical) than strains from the Sargasso Sea (99.9%–100% identical). Nearly identical sequences were obtained from the Sargasso strains and some Oregon strains. All of the obtained strains clustered among 16S rDNA sub-cluster 1A of the SAR11 clade (Figure 1).

To get a better resolution on the phylogeny of the isolated SAR11 strains, phylogenetic analysis of the ITS region was performed (Figure 2). All of the Sargasso Sea strains and two of the Oregon strains clustered together with environmental sequences from the Sargasso Sea, the Mediterranean Sea and the San Pedro channel in the eastern Pacific in ITS surface 2 group. The rest of the Oregon strains clustered together either with previously described strains of *Candidatus* Pelagibacter ubique and clones from surface waters off Antarctica and Greenland in ITS surface group 1, or with sequences obtained from the Mediterranean Sea and the San Pedro channel in ITS surface group 1(a) (Figure 2).

PRs are bacterial light-driven proton pumps that are homologous to bacteriorhodopsins of halophilic archaea (Beja et al., 2000). PRs were shown to be actively expressed in oceanic surface waters (Beja et al., 2001) and are assumed to play a pivotal role in the metabolism of marine bacteria (Eiler, 2006; Gomez-Consarnau et al., 2007). The first reported isolate of the SAR11 clade, *Candidatus* Pelagibacter ubique (Rappé et al., 2002), was shown to possess an

Table 2 Detailed summary of RFLP analysis results and 16S rRNA gene sequencing of isolates

Location	Depth (m)	Strains with identical RFLP	Phylogenetic group	Closest described species (% 16S rDNA identity)	Representatives with sequenced 16S rRNA gene	Accession numbers
BATS	10	5	Alphaproteobacteria	*Candidatus* Pelagibacter ubique (98%)	HTCC7211, 7214, 7215, 7216, 7217	EF616578, EF616587–90
BATS	10	4	Bacteroidetes	*Aureimonas pelagi* (98%)	HTCC7202, 7213	EF616579, EF616586
BATS	10	2	Gammaproteobacteria	*Marinobacter lipolyticus* (96%)	HTCC7208	EF616583
BATS	10	1	Alphaproteobacteria	*Hyphomonas jannaschiana* (95%)	HTCC7206	EF616582
BATS	10	1	Alphaproteobacteria	*Woodsholea maritima* (85%)	HTCC7212	EF616585
BATS	200	1	Alphaproteobacteria	*Phycococcus omphalius* (96%)	HTCC7201	EF616578
BATS	200	1	Alphaproteobacteria	*Hyphomonas jannaschiana* (95%)	HTCC7204	EF616580
BATS	200	1	Gammaproteobacteria	*Oleiphilus messinensis* (96%)	HTCC7205	EF616581
Oregon coast	10	12	Alphaproteobacteria	*Candidatus* Pelagibacter ubique (98%)	HTCC8010, 8022, 8038, 8041, 8043, 8045–8049, 8051, 8052	EF616599, EF616605, EF616608, EF616610, EF616612–18
Oregon coast	10	9	Alphaproteobacteria	*Loktanella tamlensis* (97%)	HTCC8002	EF616592
Oregon coast	10	8	Gammaproteobacteria	OMG[a]	HTCC8009, 8011, 8019, 8030, 8032, 8044	EF616593, EF616594, EF6166597, EF616601, EF616602, EF616612
Oregon coast	10	4	Gammaproteobacteria	*Pseudomonas putida* (91%)	HTCC8020	EF616598
Oregon coast	10	2	Gammaproteobacteria	*Methylobacter luteus* (91%)	HTCC8012, HTCC8014	EF616595, EF616596
Oregon coast	10	1	Alphaproteobacteria	*Telmatospirillum siberiense* (89%)	HTCC8037	EF616604
Oregon coast	10	1	Verrucomicrobia	*Rubritalea marina* (96%)	HTCC8042	EF616609
Oregon coast	10	1	Bacteroidetes	*Flavosolibacter ginsengiterrae* (91%)	HTCC8036	EF616603
Oregon coast	10	1	Alphaproteobacteria	*Ruegeria atlantica* (97%)	HTCC8001	EF616591
Oregon coast	10	1	Alphaproteobacteria	*Pedomicrobium fusiforme* (90%)	HTCC8023	EF616600
Oregon coast	10	1	Betaproteobacteria	*Methylophilus methylotrophus* (94%)	HTCC8040	EF616607
Oregon coast	10	1	Verrucomicrobia	*Fucophilus fucoidanolyticus* (94%)	HTCC8039	EF616606

Abbreviations: BATS, Bermuda Atlantic Time Series study site; OMG, oligotrophic marine Gammaproteobacteria; RFLP, restriction fragment length polymorphism.
[a]Multiple RFLP patterns related to different groups of the OMG (Cho and Giovannoni, 2004).
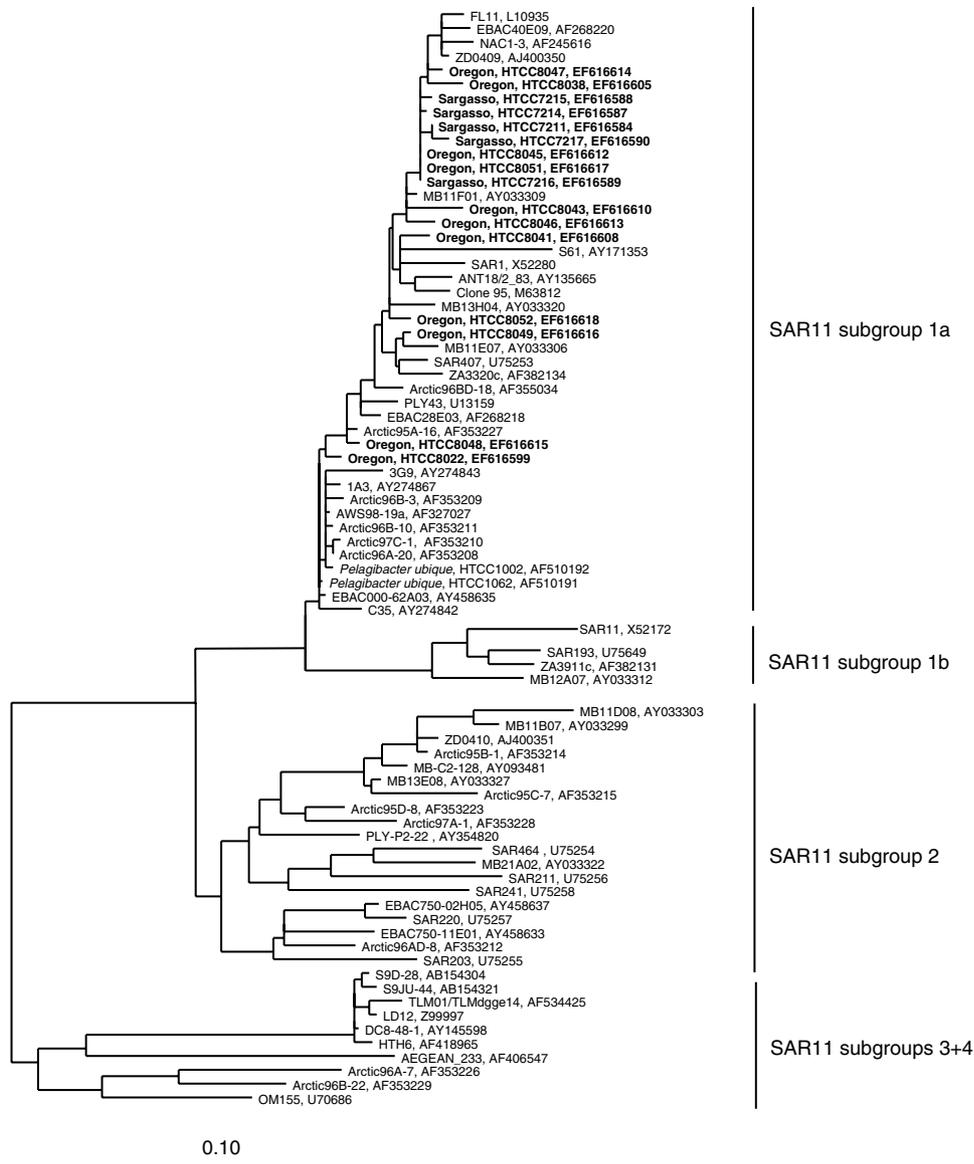
Figure 1 Dendrogram depicting relationships of 16S rRNA gene sequences of newly isolated strains from the Oregon coast and the Sargasso Sea (bold) among members of different subgroups of the SAR11 clade. Bar represents 0.10 substitutions per site.

actively expressed PR (Giovannoni *et al.*, 2005a). PR genes were amplified from 11 newly isolated SAR11 strains (Figure 3). A single amino acid ('position 105') was shown to be responsible for wavelength tuning of PR (Man *et al.*, 2003). Six out of seven strains isolated from the Oregon coast had a leucine at position 105, indicating wavelength tuning towards green light, one strain had a glycine at this position, indicating a wavelength tuning towards blue. In contrast, all isolates from the Sargasso Sea were tuned towards blue light (Table 3 and Figure 3). A phylogenetic analysis of the sequences showed that they clustered together with previously published PR sequences from *Candidatus* Pelagibacter ubique (Giovannoni *et al.*, 2005a, b), bacterial artificial chromosome (BAC) clones (Sabehi *et al.*, 2005; McCarren and DeLong, 2007) and sequences from

PCR surveys (Sabehi *et al.*, 2003). PR sequences from cultured SAR11 strains and closely related sequences clustered together according to their spectral tuning, independent of their origin (Figure 3). Interestingly, phylogenies of ITS sequences and PR sequences of the new SAR11 isolates were not congruent. A summary of the isolated SAR11 strains is presented in Table 3.

SAR116 culture
The SAR116 clade of Alphaproteobacteria is one of the most abundant groups of marine bacterioplankton (Rappé *et al.*, 2000; Suzuki *et al.*, 2001; Rappé and Giovannoni, 2003; Giovannoni and Stingl, 2005). The closest described species are among the *Rickettsiales*, but none of these species shares more
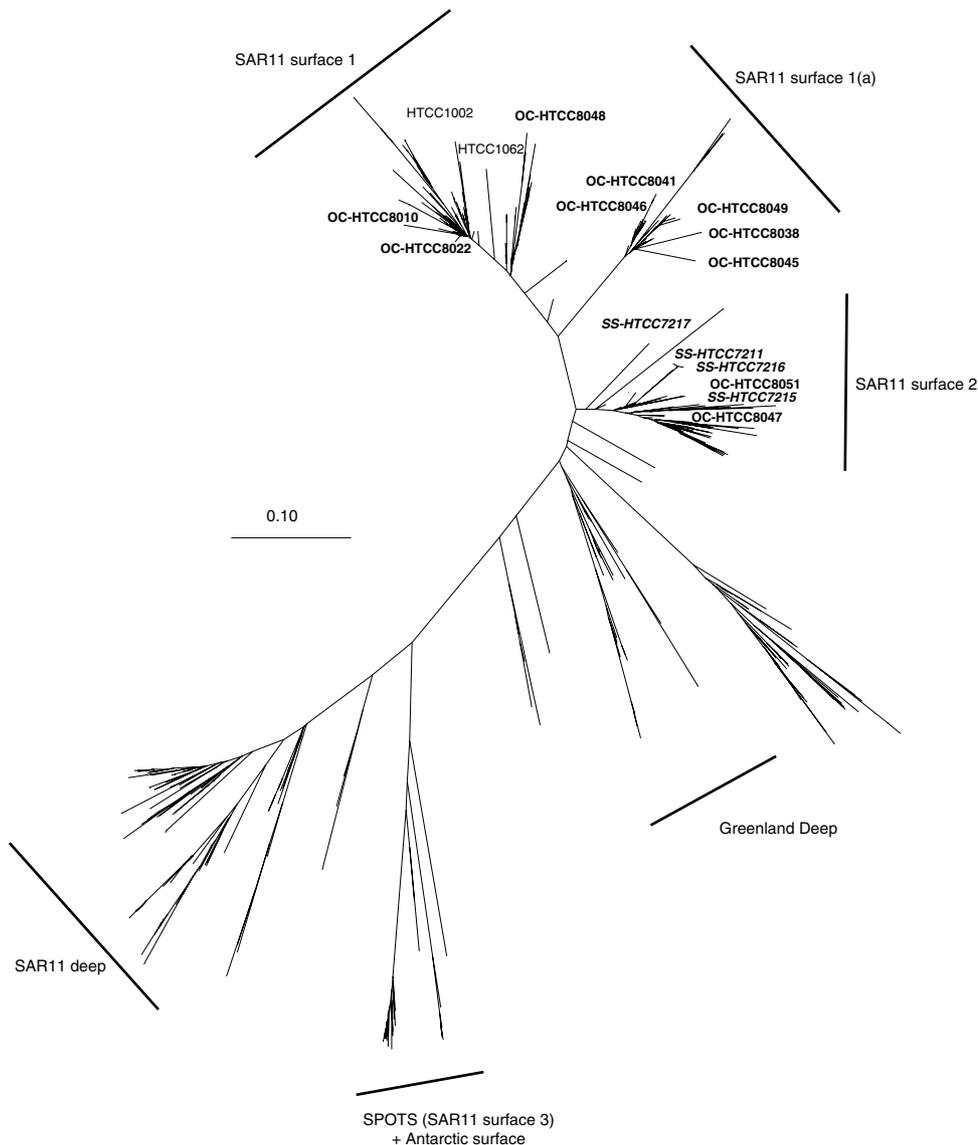
Figure 2 Phylogenetic diversity of ITS sequences of cultivated and uncultivated members of the SAR11 clade based on the alignment of Brown and Fuhrman (2005). Newly isolated strains from the Oregon coast ('OC-', bold) and the Sargasso Sea ('SS-', bold italics) were added to the alignment and a new phylogeny was calculated using neighbor-joining. Names of environmental sequences were omitted for clarity. Accession numbers for new ITS sequences are EF616619–EF616632. Bar represents 0.10 substitutions per site. ITS, internal transcribed spacer.

than 90% sequence similarity in the 16S rRNA gene. HTCC8037, isolated from the Oregon coast, is the first cultured representative of this clade (Figure 4).

Discussion and conclusion

Thus far, cultivation of SAR11 strains has only been reported in a single experiment (Rappé *et al.*, 2002), and, although recently many representatives of hitherto uncultivated groups have been isolated with the HTC method (see Cho and Giovannoni, 2004; Cho *et al.*, 2004), some abundant groups of marine bacteria, like the SAR86 clade of Gamma-

proteobacteria or the SAR202 clade, have not yet been cultured (Rappé and Giovannoni, 2003; Giovannoni and Stingl, 2005). Why these groups remain recalcitrant to cultivation is unknown, but several hypotheses exist. The most crucial point in cultivation is meeting the media requirements of an organism. Use of pristine sea water as the basis for the media, according to the ideas of Button (1993), significantly increased culturing efficiencies. To isolate bacteria with pristine sea water as media, living cells are first removed or inactivated, usually by autoclaving, resulting in changes of the chemical composition and the necessity of amending the media with nutrients that other cells would provide in the natural environment. Metal impurities in
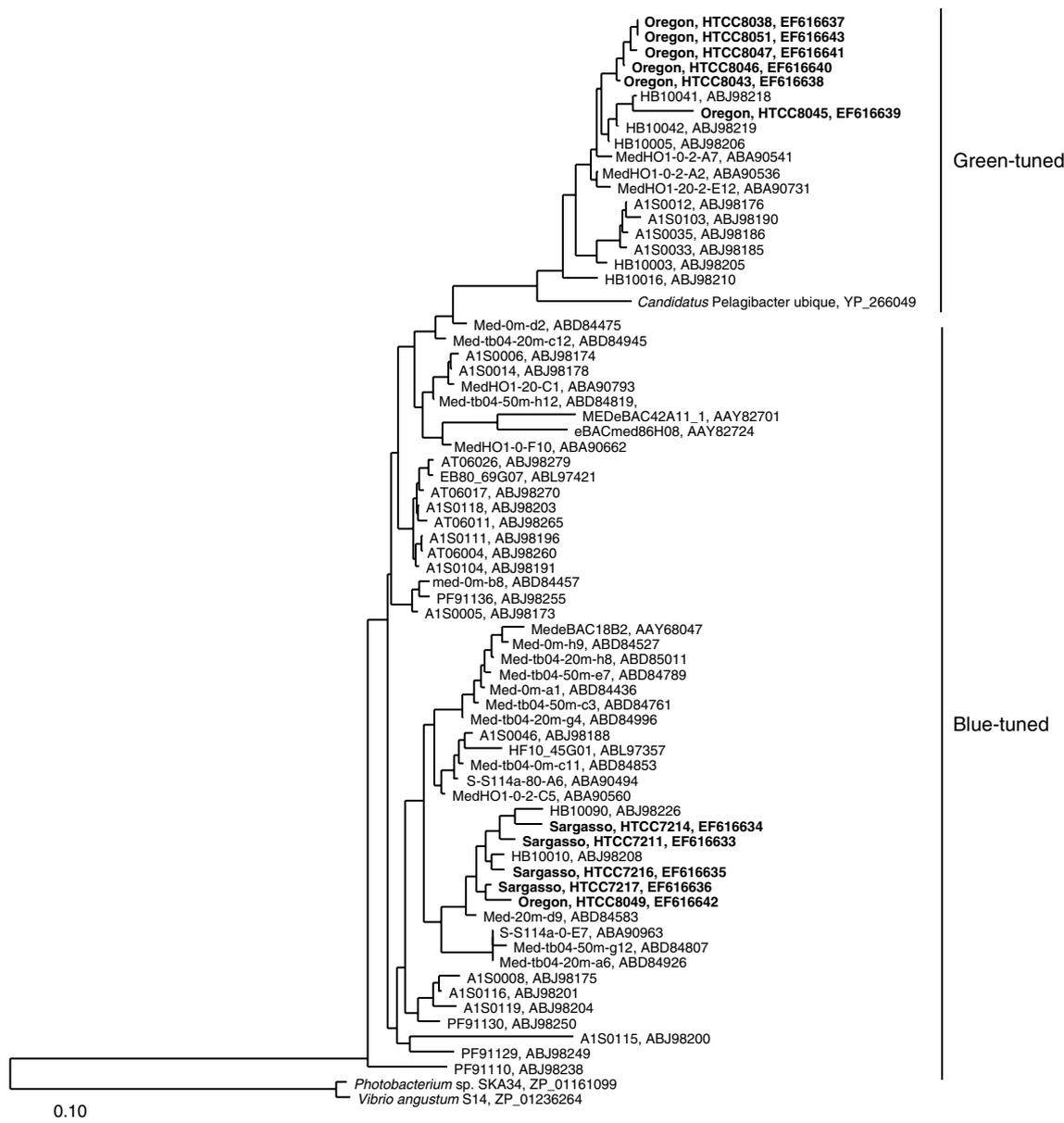
Figure 3 Neighbor-joining tree showing the relationships of proteorhodopsin (PR) amino-acid sequences of newly isolated SAR11 strains (bold) to environmental sequences retrieved from PCR assays and BAC clones. Bar represents 0.10 substitutions per site. BAC, bacterial artificial chromosome.

either media amendments or culturing vessels, as well as attachment of cells to the walls of the culture vessel may reflect additional reasons for cells not to divide. To circumvent some of these problems, and mainly to get more SAR11 strains into culture, we used custom-made Teflon plates that were cleaned with trace metal-free HCl, and avoided autoclaving during sterilization of the plates. Additionally, we added DMSP to the medium. DMSP is excreted by certain algae and may provide a source of carbon and sulfur that was shown to be taken up and degraded by members of the SAR11 clade (Malmstrom et al., 2004; Howard et al., 2006). These modifications not only led to the isolation of 17

new strains of SAR11, but also to the isolation of several, previously uncultured abundant groups of marine bacteria. These isolates will be important for a better understanding of carbon fluxes and ecology of marine environments as well as for studies on evolution and speciation in the highly diverse SAR11 clade.

The noncohesive naming of SAR11 16S rRNA subclusters (Field et al., 1997; Morris et al., 2002, 2005) and ITS subgroups (Garcia-Martinez and Rodriguez-Valera, 2000; Brown and Fuhrman, 2005) led to some confusion. Here, we resolve some of the existing confusion by showing that three of the ITS clusters (surfaces 1, 1(a) and 2) represent

Table 3 Summary on SAR11 strains isolated in this study

Location	HTCC number	16S rDNA cluster[a]	ITS cluster[b]	Spectral tuning of PR[c]
Oregon coast	8010	ND	Surface 1	ND
Oregon coast	8022	1A	Surface 1	ND
Oregon coast	8038	1A	Surface 1(a)	Green
Oregon coast	8041	1A	Surface 1(a)	ND
Oregon coast	8043	1A	ND	Green
Oregon coast	8045	1A	Surface 1(a)	Green
Oregon coast	8046	1A	Surface 1(a)	Green
Oregon coast	8047	1A	Surface 2	Green
Oregon coast	8048	1A	Surface 1	ND
Oregon coast	8049	1A	Surface 1(a)	Blue
Oregon coast	8051	1A	Surface 2	Green
Oregon coast	8052	1A	ND	ND
Sargasso Sea	7211	1A	Surface 2	Blue
Sargasso Sea	7214	1A	ND	Blue
Sargasso Sea	7215	1A	Surface 2	ND
Sargasso Sea	7216	1A	Surface 2	Blue
Sargasso Sea	7217	1A	Surface 2	Blue

Abbreviations: ITS, internal transcribed spacer; ND, not determined, no PCR product obtained; PR, proteorhodopsin.
[a]According to Morris *et al.* (2005).
[b]According to Brown and Fuhrman (2005).
[c]According to Man *et al.* (2003).
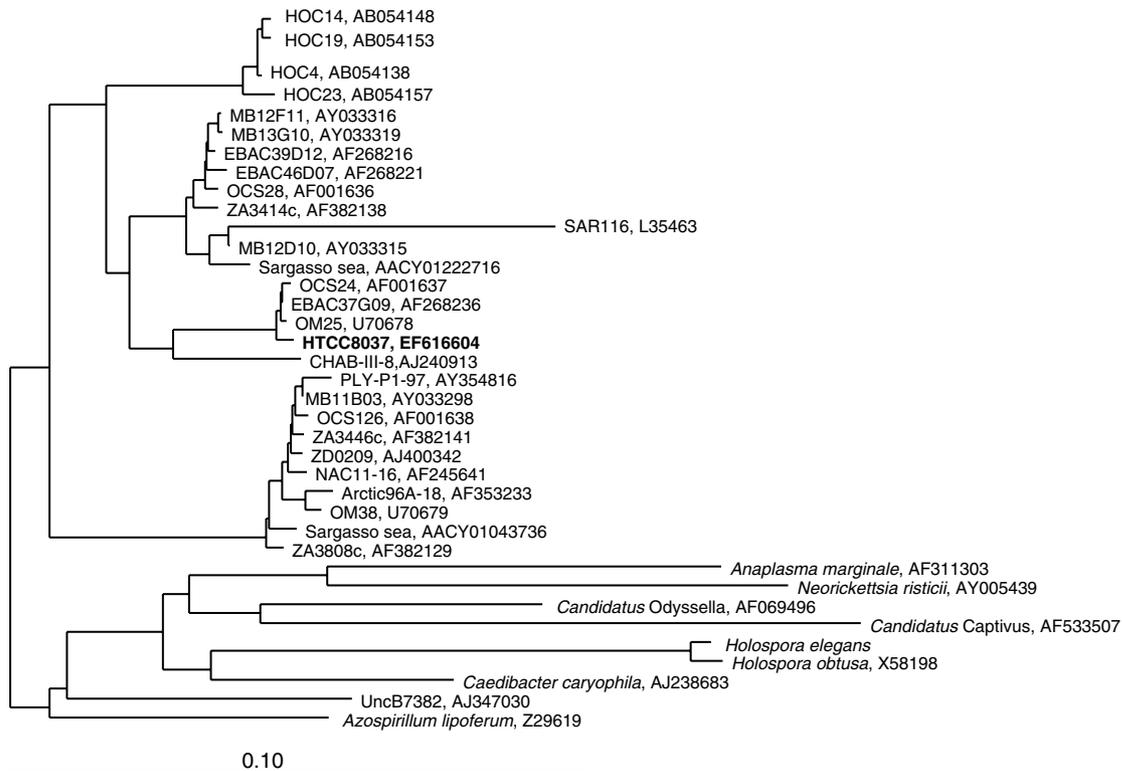
Figure 4 Dendrogram depicting relationships of 16S rRNA gene sequences of HTCC8037, environmental clones in the SAR116 clade and related sequences of validly described species of *Rickettsiales*. Bar represents 0.10 substitutions per site.

SAR11 bacteria from the 16S rRNA cluster 1A, which is the most abundant subgroup in the SAR11 clade. Most of the different ITS clusters do not seem to correlate with geographical location as environmental sequences and, as presented in this study, isolates with very similar sequence (in the SAR11 surface 2 cluster) were retrieved from completely different environments.

Interestingly, our data suggest that SAR11 microdiversity is more pronounced in nutrient-rich coastal environments like the Oregon coast compared to the nutrient-deficient Sargasso Sea. A similar conclusion

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was reached by Brown and Fuhrman (2005), who analyzed all available ITS sequences from the SAR11 clade. We speculate that higher nutrient concentrations in coastal areas may provide additional metabolic niches for members of the SAR11 clade, thereby facilitating ecotype speciation. Analogous to a higher diversity based on ITS analysis, spectral tuning of PR in the SAR11 clade also seems to be more complex in nutrient-rich habitats like the Mediterranean Sea (Sabehi et al., 2007) and the Oregon coast (this study) compared to the oligotrophic Sargasso Sea (Venter et al., 2004; Sabehi et al., 2007). In nutrient-replete environments, SAR11 populations with both green and blue-tuned PRs are present; however, the oligotrophic Sargasso Sea only contains SAR11 populations with blue-tuned PR. Although several hypotheses have been proposed for the exclusive existence of blue-tuned PRs in the Sargasso Sea (Sabehi et al., 2007), the underlying metabolic basis is not yet understood. Physiological experiments on the newly obtained strains as well as a detailed analysis of the SAR11 clade in the GOS data (Rusch et al., 2007) will provide further insights on the importance of PR spectral tuning in the SAR11 clade in relation to their environment.

The phylogenies of PR and ITS sequences of newly isolated SAR11 strains were incongruent, which is most likely due to a high degree of lateral gene transfer among members of the SAR11 clade (Vergin et al., 2007) and among PR (Frigaard et al., 2006; McCarren and DeLong, 2007).

Time series based on sequencing of ribosomal genes and ITS demonstrated that different groups or subclusters of marine bacteria vary in abundance on a temporal scale (Morris et al., 2005; Fuhrman et al., 2006; Johnson et al., 2006). This temporal variation should be taken into account in future culturing experiments. Therefore, as is done with gene-based time series studies, cultivation efforts should be performed in time series to match abundances of the target organisms with composition of important nutrients in the water used for media.

Acknowledgements

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