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Cultivation of the ubiquitous SAR11 marine bacterioplankton clade

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The α-proteobacterial lineage that contains SAR11 and related ribosomal RNA gene clones was among the first groups of organisms to be identified when cultivation-independent approaches based on rRNA gene cloning and sequencing were applied to survey microbial diversity in natural ecosystems[1]. This group accounts for 26% of all ribosomal RNA genes that have been identified in sea water and has been found in nearly every pelagic marine bacterioplankton community studied by these methods[2]. The SAR11 clade represents a pervasive problem in microbiology: despite its ubiquity, it has defied cultivation efforts. Genetic evidence suggests that diverse uncultivated microbial taxa dominate most natural ecosystems[3–5], which has prompted widespread efforts to elucidate the geochemical activities of these organisms without the benefit of cultures for study[6,7]. Here we report the isolation of representatives of the SAR11 clade. Eighteen cultures were initially obtained by means of high-throughput procedures for isolating cell cultures through the dilution of natural microbial communities into very low nutrient media. Eleven of these cultures have been successfully passaged and cryopreserved for future study. The volume of these cells, about 0.01 μm^3, places them among the smallest free-living cells in culture.

In an effort to isolate some of the ubiquitous uncultivated Bacteria and Archaea that dominate marine bacterioplankton communities[2], we inoculated fresh Oregon coast seawater samples into microtitre dish wells by dilution, such that on average each well received 22 microbial cells. Media consisted of sterile Oregon coast sea water supplemented with either phosphate (as KH_2PO_4) and ammonium (as NH_4Cl), or phosphate, ammonium and a defined mixture of organic carbon compounds. The technique of isolating cells by dilution into sterilized natural waters or other dilute media has been used previously[8,9]; it takes advantage of the fact that

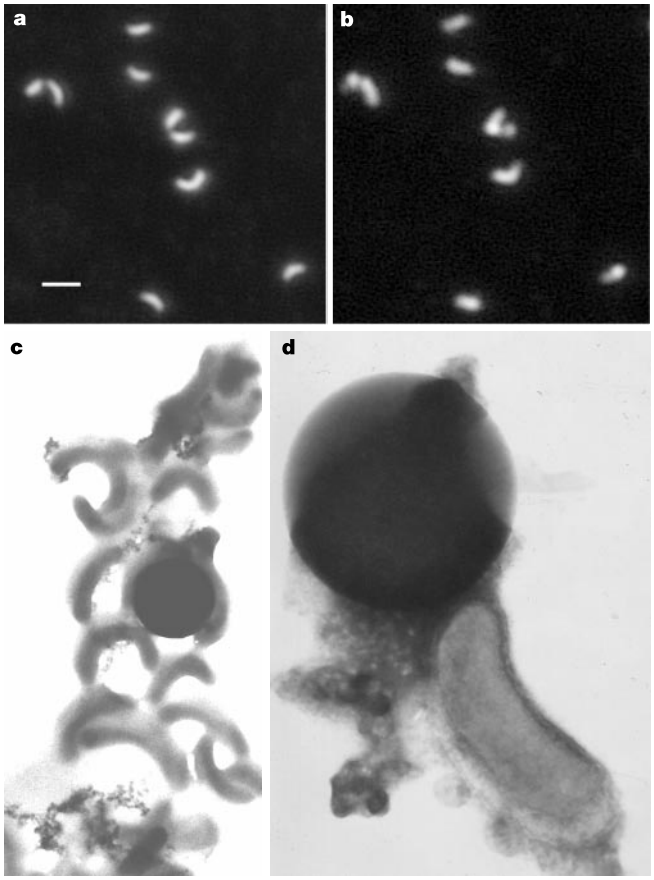

Figure 1 Photomicrographs of a culture of SAR11 clade isolate HTCC1062. **a**, **b**, Fluorescence images of cells in an identical field of view, stained with the DNA-specific dye DAPI (**a**) and after hybridization with four Cy3-labelled oligonucleotide probes targeting SAR11 cells (**b**). Scale bar (**a**, **b**), 1 μm. **c**, **d**, Transmission electron micrographs of strain HTCC1062. **c**, Shadowed cells with the typical SAR11 clade morphology. **d**, Negatively stained cell. The latex beads in **c** and **d** have a diameter of 0.514 μm.

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substrate concentrations and cell numbers in natural waters are typically about three orders of magnitude less than in common laboratory media. The approach that we used to isolate members of the SAR11 clade was similar but involved modifications to a microtitre dish format and a newly developed procedure for making arrays of cell cultures on microscope slides coupled with fluorescence *in situ* hybridization (FISH)[10,11]. These procedures were designed to increase the rate at which cultures could be obtained and identified. In this experiment, 288 extinction cultures of 1 ml were inoculated and screened.

After incubation at 15 °C for 23 d either in the dark or under a 14 h/10 h light/dark cycle (irradiance ≈ 400 μmol m^{-2} s^{-1}), culture wells were tested for replicating cells by arraying small culture volumes on polycarbonate membranes. The limit of detection with this method is roughly 2×10^3 cells per ml, and it requires only 100 μl of culture (~200 cells). Arrays were stained with the DNA-binding dye 4′,6-diamidino-2-phenylindole (DAPI)[12] and positive wells were screened further by FISH with a set of four fluorescently labelled oligonucleotide probes specific for SAR11 clade 16S rRNA (Fig. 1). Ten cultures consisting of 100% SAR11 clade cells and eight mixed cultures containing about 10–90% of SAR11 clade cells were identified preliminarily. Cultures were obtained in microtitre plates incubated in the dark or under a light/dark cycle, and in both the medium containing only natural organic carbon and the medium supplemented with a defined mixture of carbon compounds. Eleven isolates were propagated and cryopreserved for future study.

Phylogenetic relationships of the isolates were investigated by a combination of 16S rRNA gene and 16S–23S rDNA intergenic spacer sequence analysis. Ribosomal RNA nucleotide sequences of 414–608 bases, obtained from the 3′ end of the 16S rRNA gene of all 11 isolates, were identical. Intergenic spacer sequences (415–417 bases) indicated, however, that there were three genetically distinct lineages among the strains. Strains HTCC1002, 1016, 1025, 1056, 1057 and 1061 differed from HTCC1004, 1013, 1040 and 1062 at ten sequence positions and by an indel of two nucleotides. An eleventh isolate (HTCC1051) differed from the latter group at two nucleotide positions. The two principal sequence groups contained strains that were originally isolated from dark and light/dark incubations, as well as from both media types. Complete 16S rRNA gene sequences obtained from representatives of the two main intergenic spacer groups (HTCC1002 and HTCC1062) were found to differ by a single base and were more than 99% similar to over 30 published nucleotide sequences from rRNA-gene-containing clones recovered from sea water. These include nearly full-length rDNA clones obtained from the Arctic Ocean[13,14], from the coast of Plymouth, UK, in the Atlantic Ocean[15] (Fig. 2) and from the Santa Barbara Channel[16] (data not shown).

Comparative sequence analysis of 16S rRNA genes showed that SAR11 and its relatives are a deeply branching cluster of the α-subclass of Proteobacteria. Members of this group show less than 82% sequence similarity to cultivated members of the α-Proteobacteria. Since their original discovery in the Sargasso Sea, members of the SAR11 clade have been recovered in every 16S rRNA gene clone library that has been constructed with universal or bacterial polymerase chain reaction (PCR) primers from marine prokaryotic plankton samples, including coastal and near-shore waters[16,17] and seawater samples from depths up to 3,000 m (ref. 18). Relatives of the SAR11 clade have even been detected in freshwater lakes[19].

Growth rates for the 11 SAR11 clade isolates replicating at 15 °C in sterile Oregon coast seawater supplemented with 0.1 μM phosphate, 1.0 μM ammonium and a defined mixture of organic carbon compounds ranged from 0.40 to 0.58 d^{-1}. Although this rate of cell division is low in comparison to values typical of cultivated bacteria, it is not dissimilar to the measured growth rates of marine bacterioplankton communities in nature, which vary from 0.05 to 0.3 d^{-1} (ref. 20). All of the isolates produced a logistic growth curve (Fig. 3). In subsequent experiments with strain HTCC1062, which was originally isolated in seawater media supplemented with the defined organic carbon compound and vitamin mixtures, removal of these amendments did not negatively affect growth rate or maximum cell abundance (Fig. 3). However, the addition of dilute proteose peptone (0.001%) inhibited growth (Fig. 3).

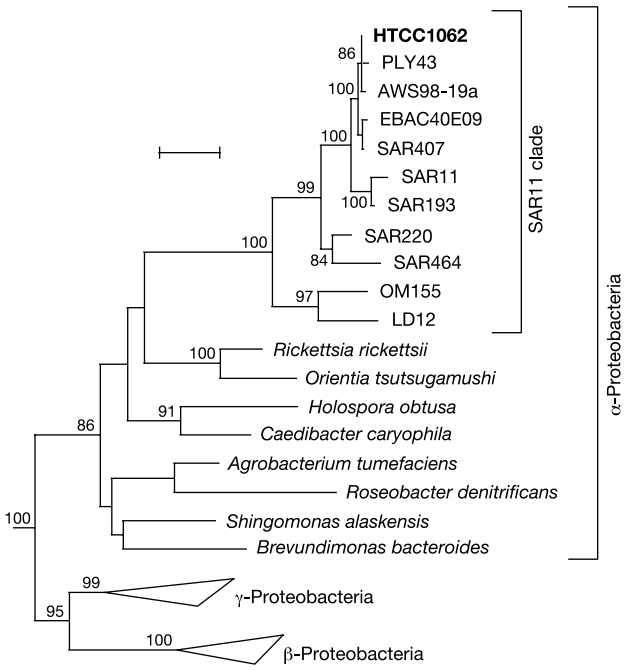

Figure 2 Phylogenetic relationships between strain HTCC1062 and representatives of the SAR11 clade and α-Proteobacteria inferred from 16S rRNA gene sequence comparisons. The Gram-positive bacteria *Bacillus subtilis* and *Marinococcus halophilus* were used as outgroups. Bootstrap proportions over 70% that supported the branching order are shown. Scale bar corresponds to 0.05 substitutions per nucleotide position. Also included in the analysis were the γ-Proteobacteria *Alteromonas macleodii* and *Marinobacter hydrocarbonoclasticus*, and the β-Proteobacteria *Methylophilus methylotrophus* and *Polynucleobacter necessarius*.

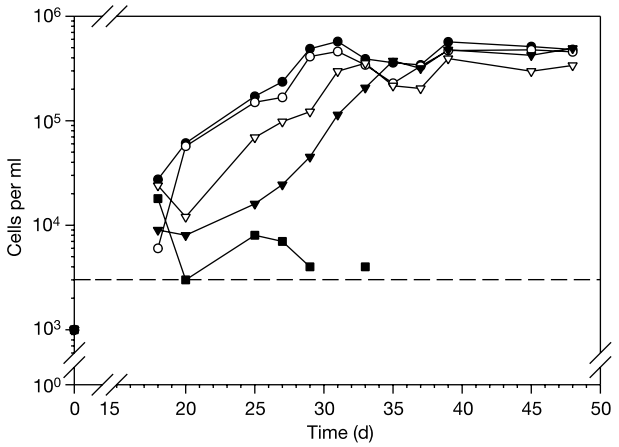

Figure 3 Growth of strain HTCC1062 in Oregon coast seawater media. Media consisted of sterile sea water supplemented with 1.0 μM NH_4Cl and 0.1 μM KH_2PO_4 (filled circles), 1.0 μM NH_4Cl, 0.1 μM KH_2PO_4, and mixed carbon (open circles), 1.0 μM NH_4Cl, 0.1 μM KH_2PO_4, and Va vitamins (filled triangles), 1.0 μM NH_4Cl, 0.1 μM KH_2PO_4, mixed carbon and Va vitamins (open triangles), 1.0 μM NH_4Cl, 0.1 μM KH_2PO_4, mixed carbon, Va vitamins and 0.001% (w/v) proteose peptone (filled squares). For all cultures, cell counts attempted on days 7 and 12 were below the limit of detection (dotted line, 3,000 cells per ml), as were counts on day 31 and after day 33 for the culture containing proteose peptone. The point at day 0 is the inoculum density.

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The maximum cell densities reached by the SAR11 clade isolates differed between seawater samples collected on different dates and locations in the Pacific Ocean off of the Oregon coast, regardless of the addition of organic carbon. Overall, the maximum carrying capacity of the different seawater batches varied from 2.5×10^5 cells per ml to 3.5×10^6 cells per ml. These results suggest that natural factors present in seawater control the cultured SAR11 populations. Without nutrient amendments, some sterile sea water batches supported the growth of these isolates up to cell densities of 2.5×10^5 cells per ml. Other Oregon coast seawater would only promote the growth of these isolates when at least 1.0 μM ammonium and 0.1 μM phosphate were added. The growth of these cells did not seem to be affected by light. Overall, these observations have important implications for oceanographic research because they suggest that cultured SAR11 clade cells growing in sea water can be used to identify chemical factors that control their natural abundance in the environment.

SAR11 clade cells are crescent-shaped (vibrioid), 0.37–0.89 μm in length, and have an average cell diameter of 0.12–0.20 μm (Fig. 1). The size and morphology of the isolates was identical to cells of the SAR11 clade that have been observed in natural populations by fluorescence *in situ* hybridization. Size estimates made by transmission electron microscopy on a culture of HTCC1062 cells indicate that it is one of the smallest free-living and replicating pure cultures of cells known (Fig. 1). The size differences of these estimates arise from the natural variation in cell length that is associated with cell division, and uncertainty about whether a lightly staining structure observed on the outwardly curved lateral surface of cells is enclosed by membrane (Fig. 1). In addition, the cells were measured after fixation in glutaraldehyde, which may have caused shrinkage. For a cell of $0.4 \times 0.2\ \mu m^2$, which is a conservative estimate of the size of an HTCC1062 cell emerging from division, the cell volume is about $0.01\ \mu m^3$. The small size of the SAR11 clade isolates is noteworthy, particularly considering that this organism is well adapted to a life of autonomous replication. The genome size of strain HTCC1062 was estimated to be 1.54 Mb by pulsed-field gel electrophoresis.

The success of this cultivation approach with members of the SAR11 clade may be attributed to several factors, including the use of pristine sea water as a medium, the relatively large number of cultures screened, and the low detection threshold of the procedure. Access to members of the SAR11 clade in culture will provide an unusual opportunity for genome sequence analysis of an organism that has global biogeochemical significance and can be manipulated in culture. It may also provide insight into the adaptations of cells to very low nutrient systems. Because of their extraordinarily small size, the study of these isolates should also refine our understanding of the minimal macromolecular machinery required for autonomous cellular replication. On the basis of the preliminary characteristics presented here, we propose the status '*Candidatus* Pelagibacter ubique' gen. nov., sp. nov., for this taxon. □

Methods

Sample collection and culturing

A water sample was collected in a 2-l Niskin bottle fitted with a Teflon-coated spring from a depth of 10 m at a station 27.6 km off of the coast of Oregon on the Newport Hydrographic line (44° 39.1′ N, 124° 24.7′ W). The bottle was immediately stored in a cooler with cold packs to maintain a temperature similar to surface water at this station (12.0 °C) until further laboratory processing (3 h from sample collection). The primary cultivation experiments used two types of media: filtered (0.2 μm), autoclaved, CO_2- and air-sparged coastal Pacific Ocean sea water amended with 1.0 μm NH_4Cl and 0.1 μM KH_2PO_4; and an ammonium- and phosphate-enriched medium amended with 0.001% (w/v) D-glucose, D-ribose, succinic acid, pyruvic acid, glycerol, *N*-acetyl glucosamine, 0.002% (v/v) ethanol and Va vitamin mix at a 10^{-4} dilution of stock[21]. Growth curve experiments used the same nutrient concentrations.

Fluorescent microscopy

For the identification of SAR11 clade-containing cultures by FISH, we fixed 100–200 μl from each culture well with 0.2-μm-filtered paraformaldehyde (2% final concentration) and filtered it onto 0.2-μm polycarbonate membrane filters. FISH was done essentially as described[22], with the following modifications (R. Morris, unpublished data). Hybridization reactions were carried out at a temperature of 35 °C for 3–12 h in hybridization buffer consisting of 900 mM NaCl, 20 mM Tris (pH 7.4), 0.01% (w/v) SDS, 15% formamide and four Cy3-labelled oligonucleotide probes targeting SAR11 clade ribosomal RNA at a final concentration of 2 ng μl^{-1} each (SAR11-152R, 5′-ATTAGCAC AAGTTTCCYCGTGT-3′; SAR11-441R, 5′-TACAGTCATTTTCTTCCCCGAC-3′; SAR11-542R, 5′-TCCGAACTACGCTAGGTC-3′; SAR11-732R, 5′-GTCAGTAATGATCC AGAAAGYTG-3′). We achieved optimal hybridization stringency by washing the membranes for two 15-min intervals at 55 °C in 150 mM NaCl, 20 mM Tris (pH 7.4), 6 mM EDTA and 0.01% SDS. After mounting filters in Citifluor (Ted Pella), Cy3-positive and DAPI-positive cells were counted for each field of view[23] using a Leica DMRB epifluorescence microscope equipped with a Hamamatsu ORCA-ER CCD digital camera, filter sets appropriate for Cy3 and DAPI, and Scanalytics IPLab v3.5.5 scientific imaging software.

Electron microscopy

Cells were initially concentrated by centrifugation or Vivascience Vivaspin 500 ultrafiltration concentrators and resuspended in sterile sea water containing 0.5% glutaraldehyde. After centrifugation of concentrated cell suspensions onto Formvar-coated copper grids, cells were prepared for imaging by either negative staining with 2% uranyl acetate (pH 4.0), or shadowing with gold/platinum. We included fluoresbrite beads (diameter, 0.514 ± 0.015 μm) in the preparations as an internal size standard.

Genome size

Concentrated cells of strain HTCC1062 were embedded in agarose, and individual cell plugs were digested with the homing endonuclease I-*Ceu*I or the restriction endonucleases *Not*I or *Sfi*I. We carried out electrophoresis with a CHEF DRII unit in 1% agarose for 18 h at 6 V cm^{-1}, with an initial switch time of 50 s and a final switch time of 90 s. Genome size was measured by comparison to a yeast chromosome PFG marker. The I-*Ceu*I endonuclease apparently linearized the chromosome, resulting in a single band. The restriction endonucleases *Not*I and *Sfi*I did not cut the genomic DNA of strain HTCC1062.

Phylogenetic analysis

For small-volume (~1 ml) cultures, genomic DNA was isolated from 200 μl of culture using a DNeasy Tissue kit (Qiagen) after two freeze–thaw cycles. For large volume cultures (~100 ml), cells were filtered onto 0.1-μm pore size Supor-100 polysulfone filters and the DNA was extracted as described[24]. Crude nucleic acid extracts were purified further with a DNeasy Tissue kit. Owing to the small amount of starting material, a semi-nested PCR reaction was sometimes required to obtain sufficient product for further characterization. We characterized the 3′ end of the 16S rRNA gene and the intergenic transcribed spacer in a single reaction by PCR amplification with the 16S rRNA gene primer 926F and the 23S rRNA gene primer 23S-117R (ref. 25), or in semi-nested reactions using the forward primer corresponding to the SAR11-542R oligonucleotide followed by PCR amplification with the 926F primer (both paired with 23S-117R). Some semi-nested reactions also used 926F followed by 1100F as forward primers (both paired with 23S-117R). For nearly complete 16S rRNA gene sequencing, genes were initially amplified in a PCR reaction using the primers 27F and 1492R, followed by a second reaction using 27F and 1406R. PCR products were cleaned with a QiaQuick PCR purification column (Qiagen) and sequenced on an ABI 377 or ABI 3100 automated sequencer.

We aligned sequences against those in a database of over 11,500 SSU rDNA sequences maintained with the ARB software package[26]. We carried out phylogenetic analyses with the program PAUP* 4.0 beta 8 (ref. 27), and included 951 unambiguously aligned nucleotide positions. The tree topology was inferred by maximum likelihood with a heuristic search and tree bisection-reconnection (TBR) branch swapping, a transition/transversion ratio that was estimated from the data (1.426) and nucleotide frequencies that were estimated from the data. We determined bootstrap proportions from 1,000 resamplings using evolutionary distances calculated with the Kimura 2-parameter model for nucleotide change, a transition/transversion ratio that was estimated from the data, and neighbour joining.

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Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to S.J.G. (e-mail: steve.giovannoni@orst.edu). The sequences have been deposited in GenBank under accession numbers AF510191 to AF510193.

An unexpected specialization for horizontal disparity in primate primary visual cortex

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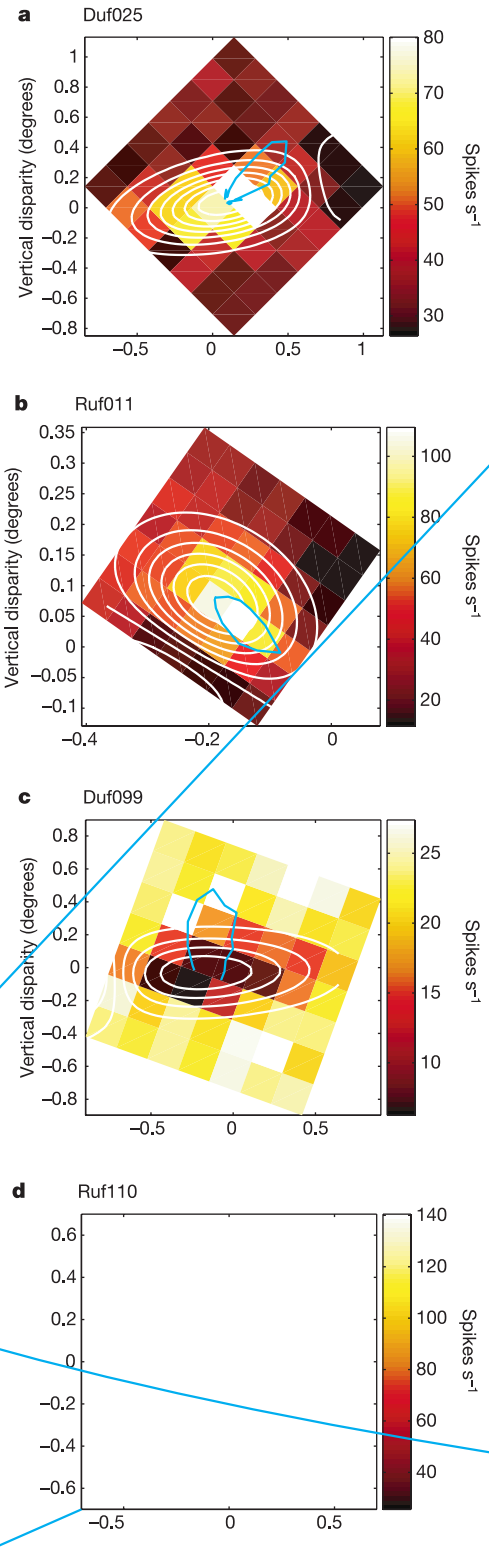
The horizontal separation of the eyes means that objects nearer or farther than the fixation point project to different locations on the two retinae, differing principally in their horizontal coordinates (horizontal binocular disparity). Disparity-selective neurons have generally been studied with disparities applied in only one direction[1] (often horizontal), which cannot determine whether the encoding is specialized for processing disparities along the horizontal axis. It is therefore unclear if disparity selectivity represents a specialization for naturally occurring disparities. I used random dot stereograms to study disparity-



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