RESEARCH LETTER

Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean

Jaeho Song, Hyun-Myung Oh & Jang-Cheon Cho

Division of Biology and Ocean Sciences, Inha University, Incheon, Korea

Correspondence: Jang-Cheon Cho, Division of Biology and Ocean Sciences, Inha University, Incheon 402-751, Korea. Tel.: +82 32 860 7711; fax: +82 32 876 5541; e-mail: chojc@inha.ac.kr

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Abstract

Although the SAR11 clade of the *Alphaproteobacteria* represents the most abundant and ubiquitous bacterioplankton in the ocean, very few laboratories have successfully cultured SAR11 cells. All of the SAR11 strains isolated thus far have been retrieved from the Oregon coast and the Sargasso Sea. In this study, a modified dilution-to-extinction culturing with prolonged incubation at low temperature was applied in an effort to cultivate major bacterioplankton lineages in the East Sea, Western Pacific Ocean. Five to 10 cells were inoculated into each well of 48-well plates, followed by the incubation of the plates at 10 °C for 4, 8, 20, and 24 weeks. Among a total of 35 isolated strains, 18 strains assigned to the SAR11 clade were isolated after 8, 20, and 24 weeks of incubation, whereas no SAR11 cells were detected in the samples after 4 weeks of incubation. The SAR11 isolates, noticeably, comprised 64–82% of the total isolates from the plates incubated for 20 and 24 weeks. Extinction cultures belonging to the *Roseobacter*, OM43, and SAR92 clades were also cultivated. The results of this study suggest that long-term incubation at low temperatures might prove an alternative for the efficient cultivation of new variants of the members of the SAR11 clade.

Introduction

The SAR11 clade of the class *Alphaproteobacteria*, the most abundant and ubiquitous clade of heterotrophic bacteria in the ocean, accounts for *c.* 25–50% of the total microbial community in surface waters (Morris *et al.*, 2002). On the basis of the 16S rRNA gene sequence analyses, the clade consists of at least four subgroups, the clonal abundances of which change differently with depth and season (Field *et al.*, 1997; Morris *et al.*, 2005). Despite the significant abundance and ubiquitous distribution of the SAR11 clade, none of the members of the clade had been cultured until several coastal strains represented by strain HTCC1062 (*Candidatus* Pelagibacter ubique) were brought in culture from the Oregon Coast (Rappé *et al.*, 2002). Cultivation of members of the SAR11 clade has provided important clues to the physiological and ecological roles of the cosmopolitan marine bacteria. The representative SAR11 isolate, *Can.* Pelagibacter ubique HTCC1062, harbors the second smallest genome (1 308 759 bp) among known free-living bacteria (Giovannoni *et al.*, 2005b, 2008) and it adapts to oligotrophic marine environments with proteorhodopsin-based photoheterotrophy (Béjà *et al.*, 2000; Giovannoni *et al.*, 2005a). Later, it was determined that strain HTCC1062 assimilates the 3-dimethylsulfoniopropionate (DMSP) associated with global climate (Tripp *et al.*, 2008).

The successful culturing of SAR11 cells was credited to the use of a high-throughput culturing (HTC) method based on dilution-to-extinction culturing (Connon & Giovannoni, 2002), using pristine seawater as an incubation medium. The HTC method has resulted in the successful cultivation of marine bacterioplankton not only in members of the SAR11 clade, but also in a battery of marine isolates, including the OM43 clade (Giovannoni *et al.*, 2008), the OM60 clade (Cho *et al.*, 2007), the phylum *Lentisphaerae* (Cho *et al.*, 2004), and the OMG group (Cho & Giovannoni, 2004). One of the reasons for this success is considered to be the application of culture conditions that mimic closely the chemical composition of aquatic environments. In this regard, the HTC method using a freshwater medium was also successful in delivering previously uncultured Antarctic lake bacterioplankton (Stingl *et al.*, 2008).

Although numerous reports have been filed regarding the presence of members of the SAR11 clade in different seas,

only a few laboratories have thus far succeeded in culturing SAR11 cells, and these were retrieved only from the Oregon coast and Sargasso Sea. In a recent study, several SAR11 strains have been successfully isolated using a modified dilution-to-extinction culturing method, characterized by custom-made 24-well Teflon plates (Stingl *et al.*, 2007b). They claimed that the application of Teflon plates and the addition of DMSP in the culture medium were effective in the acquisition of other SAR11 isolates. In the current study, we applied the dilution-to-extinction culture method in the East Sea of Korea, using a conventional polystyrene plate. The inoculated multi-well plates were incubated at 10 °C for a prolonged period of up to 24 weeks. Consequently, 18 axenic cultures belonging to the SAR11 clade were isolated among 37 isolated strains. We suggest that long-term incubation at low temperatures may prove useful for the cultivation of new variants of the SAR11 clade.

Materials and methods

Sample collection and dilution-to-extinction culturing

A seawater sample was collected from a depth of 10 m at the GS1 station (38°12′72″N, 128°39′86″E) in the East Sea, Korea, in November 2007. The water temperature and salinity at the sampling site were 8.4 °C and 33.4 psu, respectively. The sample was immediately maintained in darkness at 4 °C until further processing. A low-nutrient heterotrophic media (LNHM) for the HTC method was prepared in accordance with the previously described protocol (Connon & Giovannoni, 2002). In brief, the seawater collected from the GS1 station was filtered with a 0.2-μm pore-sized filter and autoclaved for 4 h, followed by 16 h of CO_2 sparging and 24 h of aeration. Finally, the culture medium was prepared via the addition of the following chemicals: 1.0 μM NH_4Cl, 0.1 μM KH_2PO_4, 0.001% (w/v) of D-glucose, D-ribose, glycerol, *N*-acetyl-D-glucosamine, methylamine, pyruvic acid, ethanol, and a 10^{-4} dilution of a vitamin mixture (Davis & Guillard, 1958). The inoculum was diluted to 5–10 cells mL^{-1} in the culture medium and dispensed into a 48-well-polystyrene microtiter plate. Each of the wells was filled with 1 mL of media containing five or 10 cells. After incubation at 10 °C for 4, 8, 20, and 24 weeks in darkness, 180 μL of each well was fixed with formalin, stained with 4′, 6-diamidino-2-phenylindole, and filtered onto 0.2-μm pore-sized black polycarbonate membranes (48 × 60 mm, Osmonics) in a custom-made 48-well cell arrayer. Cellular growth was assessed via epifluorescence microscopy (Nikon 80i, Nikon, Japan). Wells with *c.* 2.0×10^5 cells mL^{-1} cell densities and higher were considered positive, and they were stored as 10% (v/v) glycerol suspensions at − 80 °C for further analyses.

DNA extraction, PCR, and 16S rRNA gene sequencing

Genomic DNA was extracted from 200-μL aliquots of the positive wells using a DNeasy tissue kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The 16S rRNA genes were amplified via PCR using the 27F-B and 1492R primers as described previously (Cho & Giovannoni, 2004). Thermal cycling conditions modified from the touch-down PCR method (Don *et al.*, 1991) were as follows: 94 °C for 5 min followed by 20 cycles of 94 °C for 1 min, annealing at temperatures lowered from 60 °C in decrements of 1 °C per cycle, and 72 °C for 1 min. After 20 cycles of touch-down PCR, 25 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min were conducted. The PCR products were grouped initially by restriction fragment length polymorphism (RFLP), using HaeIII restriction patterns. After purification using a SolGent PCR Purification Kit (Solgent, Korea), the 16S rRNA genes were sequenced using an ABI 3730XL capillary DNA Sequencer (Applied Biosystems, Foster City, CA) with the following primers: 800R (5′-TACCAGGGTAT CTAATCC-3′), 519F (5′-CAGCMGCCGCGGTAATWC-3′), 27F-B, and 1492R.

Phylogenetic analysis

The 16S rRNA gene sequences of the isolates were imported into the ARB software package (Ludwig *et al.*, 2004) and automatically aligned using the FAST ALIGNER tool, followed by manual correction of the alignments. The sequences were then compared with the sequences deposited in the GenBank using BLASTN (Altschul *et al.*, 1997) and with validly published species in the EzTaxon server (Chun *et al.*, 2007). Only unambiguously aligned nucleotide positions were used for phylogenetic analyses with the ARB database and PAUP* 4.0 beta 10 (Swofford, 2002). The 16S rRNA gene sequence similarity values between sequences were determined using ARB software. Phylogenetic trees were inferred the neighbor-joining method (Saitou & Nei, 1987) using the Jukes-Cantor model, and the resultant trees were evaluated by bootstrap analyses based on 1000 resamplings. Shorter sequences (< 1000 bp) were added to the trees using the parsimony insertion tool implemented in the ARB software.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study are available in GenBank database under the accession numbers FJ532479–FJ532499.

Results and discussion

Dilution-to-extinction culturing experiments were conducted with inocula obtained from a depth of 10 m in the East Sea, in November 2007 (Table 1). In this study, 192

Table 1. Summary of dilution-to-extinction culturing results

Incubation period (week)	Inoculum size (cells mL^{-1})	Number of inoculated wells	Number of positive wells*	Number of PCR negatives	Culturing efficiency (%)†	SAR11 isolates
4	5	48	0	0	0	0
4	10	48	2	0	4.2	0
8	10	144	11	6	7.6	2
20	5	96	11	2	11.5	9
24	5	48	11	3	22.9	7

*Above 2.0×10^5 cells mL^{-1}.

†Number of positive wells/number of inoculated wells.

Table 2. Taxonomic affiliations of extinction cultures based on RFLP and 16S rRNA gene sequencing

Incubation period (week)	Phylum/class (arbitrary phylogenetic group)	Strains with identical RFLP	Closest described species (16S rRNA gene similarity)	16S rRNA gene-sequenced isolates
4	*Alphaproteobacteria* (genus *Paracoccus*)	1	*Paracoccus marcusii* (96%)	IMCC10440
	Betaproteobacteria (OM43 clade)	1	*Methylophilus methylotrophus* (91%)	IMCC10439
8	*Alphaproteobacteria* (SAR11 clade)	2	*Candidatus* Pelagibacter ubique (100%)	IMCC10436, IMCC10437
	Alphaproteobacteria (Roseobacter clade)	1	*Thalassobius gelatinovorus* (94%)	IMCC10434
	Betaproteobacteria (OM43 clade)	1	*Methylophilus methylotrophus* (92%)	IMCC10438
	Gammaproteobacteria (SAR92 clade)	1	*Microbulbifer salipaludis* (91%)	IMCC10433
20	*Alphaproteobacteria* (SAR11 clade)	9	*Candidatus* Pelagibacter ubique (98–100%)	IMCC10400, IMCC10402 IMCC10404, IMCC10405 IMCC10406-10410
24	*Alphaproteobacteria* (SAR11 clade)	7	*Candidatus* Pelagibacter ubique (98–99%)	IMCC10411, IMCC10417, IMCC10422, IMCC10425, IMCC10428
	Firmicutes (genus *Paenibacillus*)	1	*Paenibacillus ehimensis* (92%)	IMCC10419

wells each containing five or 10 cells were incubated at 10 °C, because the ambient seawater temperature at the sampling site was 8.4 °C. Another reason for selecting an incubation temperature of 10 °C was that 16 or 20 °C, previously reported as optimal temperatures for SAR11 cells (Rappé *et al.*, 2002), may allow the faster-growing bacteria to flourish in the wells before the expansion of more slowly growing bacteria, such as SAR11 and OM43 cultures.

In the screening of 384 inoculated wells after 4, 8, 20, and 24 weeks of incubation, a total of 35 dilution cultures were isolated (Table 1). Wells with *c.* 2.0×10^5 cells mL^{-1} and higher were considered positive, as 2.0×10^5 cells mL^{-1} of cell density was empirically regarded as the threshold for PCR amplification in the initial experiment (data not shown). Initial screening for 96 wells after 4 weeks of incubation yielded only two positive cultures. Because of the low culturability in the plates incubated for 4 weeks, the remainders of the inoculated 48-well plates were incubated for longer periods. Culturing efficiencies increased with increasing incubation periods; the highest culturability (22.9%) was noted in the plates incubated for 24 weeks. Among 35 extinction cultures, 18 isolates (51%) were identified as members of the SAR11 clade on the basis of the 16S rRNA gene sequences. The majority of the SAR11 isolates were obtained from the plates after 20 and 24 weeks of incubation.

The extinction cultures were grouped according to RFLP patterns to assess their purity, and their 16S rRNA gene sequences were determined (Table 2). Comparative 16S rRNA gene sequence analyses using BLASTN searches and phylogenetic analyses (Figs 1 and 2) revealed that the extinction cultures belonged to the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Firmicutes*. Only two isolates, IMCC10439 and IMCC10440, were cultured from the 4-week-incubated plates, and were most closely related to the uncultured bacterium ZD0412 (GenBank Accession, AJ400352; 98% similarity) in the OM43 clade of the *Betaproteobacteria* and *Paracoccus* bacterium JL1148 (DQ985067, 99%) in the *Rhodobacterales*, respectively. In the 48-well plates incubated for 8 weeks, 11 wells were considered positive cultures. However, only five strains (IMCC10433, 10434, 10436, 10437, and 10438) were amplified under the given PCR conditions (Table 1). The amplification failures of six cultures might be attributable to problems with DNA extractions and/or overestimations of cell titers. The strains were grouped into four categories based on RFLP and sequence information, and they were affiliated with the SAR11 clade (IMCC10436, IMCC10437)

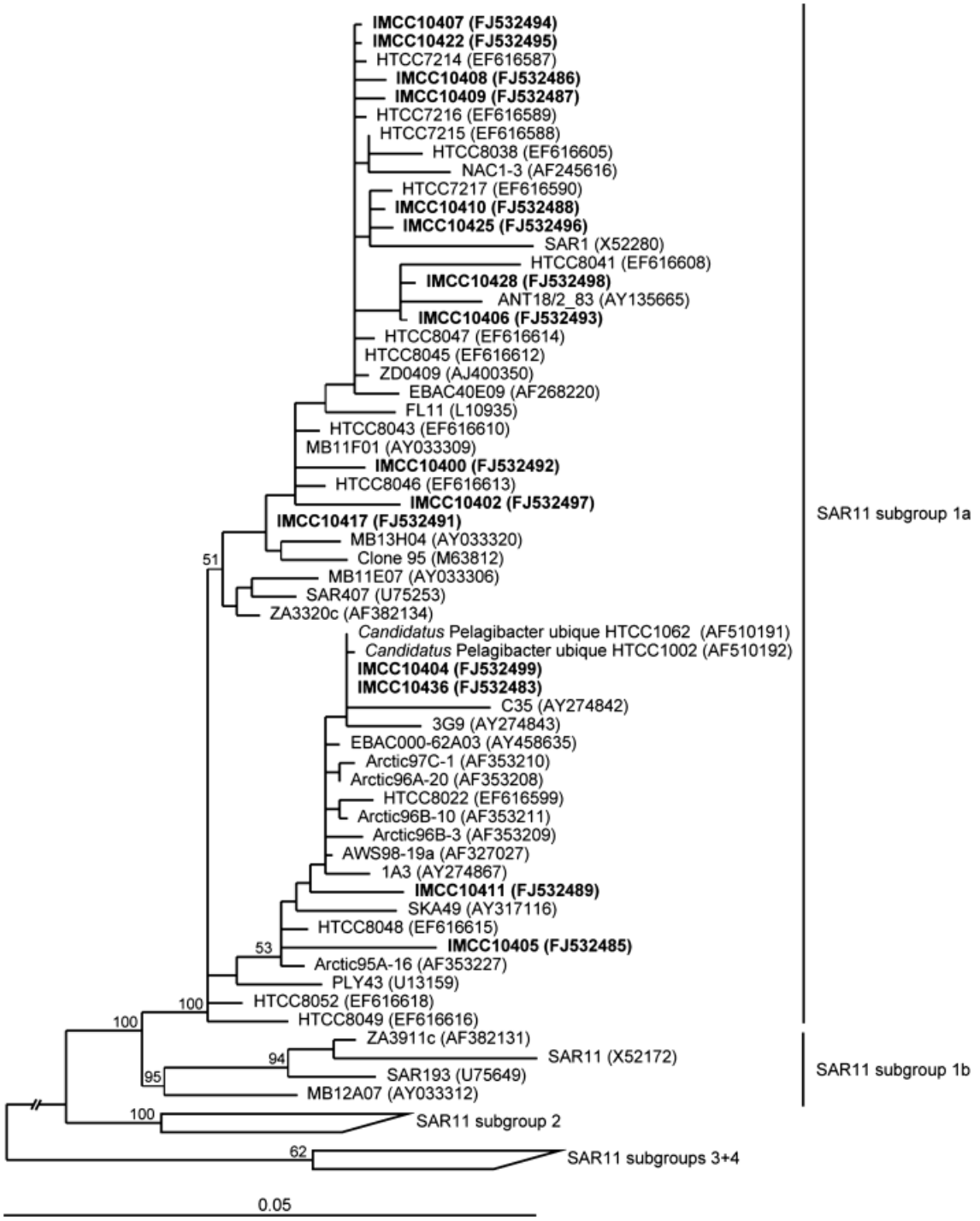

Fig. 1. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between isolates cultured from the East Sea, the Western Pacific Ocean, and representatives of the SAR11 clade of the *Alphaproteobacteria*. Boldfaced texts represent SAR11 strains cultured in this study. Bootstrap values in excess of 50% are shown. Bar, 0.05 substitutions per nucleotide position.

and the *Roseobacter* clade (IMCC10434) in the *Alphaproteobacteria*, the OM43 clade of the *Betaproteobacteria* (IMCC10438), and the SAR92 clade of the *Gammaproteobacteria* (IMCC10433). In 20- and 24-week-incubated plates, 16 extinction cultures were most closely related to *Can*. Pelagibacter ubique HTCC1062 with a 16S rRNA gene sequence similarity of 98–100%. Another isolate, IMCC10419, which is affiliated with the *Firmicutes*, evidenced the highest 16S rRNA

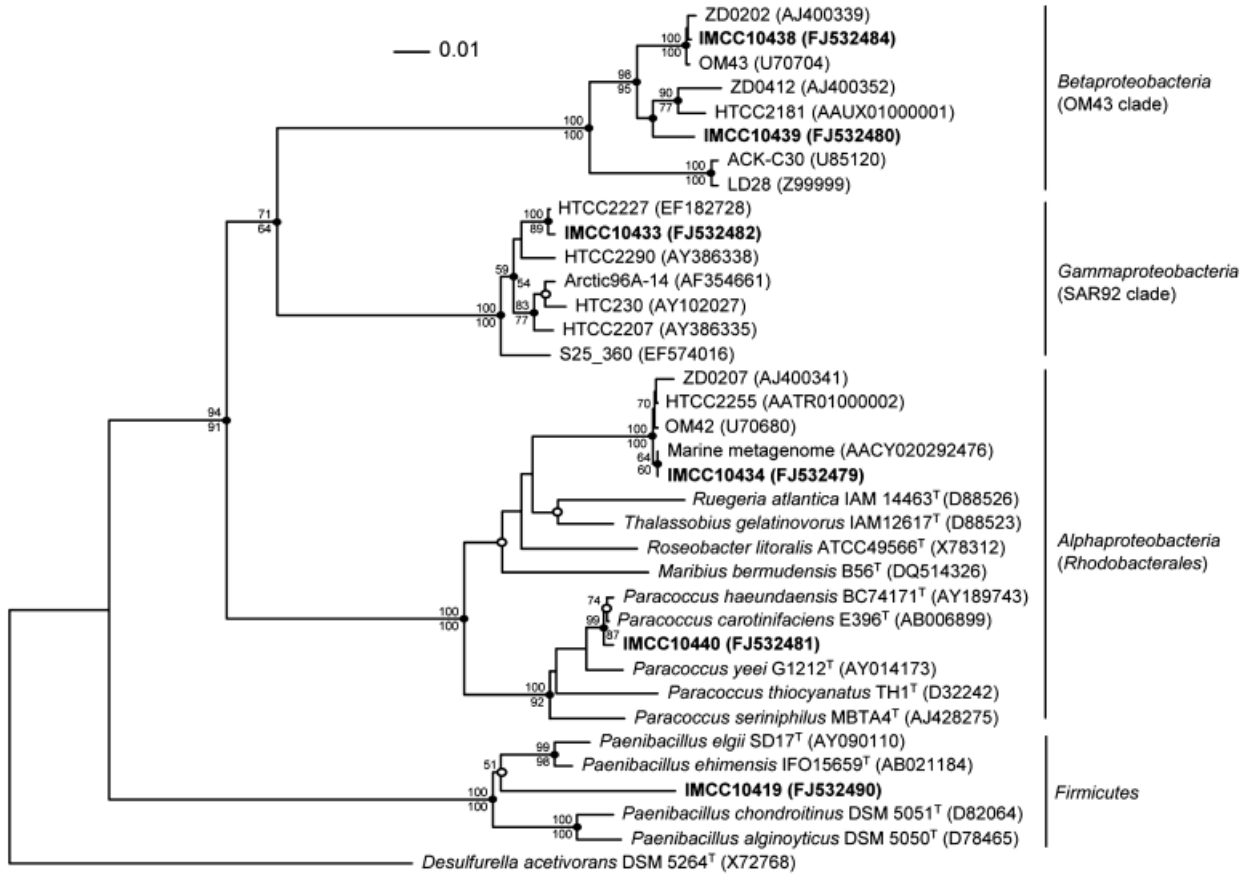

Fig. 2. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between the isolates cultured from the East Sea, Western Pacific Ocean and representatives of the *Alpha-*, *Beta-*, *Gammaproteobacteria*, and *Firmicutes*. Boldfaced texts represent non-SAR11 strains cultured in this study. Bootstrap proportions (> 50%) determined from both the neighbor-joining approach (above the nodes) and the maximum-parsimony approach (below the nodes) are shown. Bar, 0.01 substitutions per nucleotide position.

gene sequence similarity with *Paenibacillus* sp. SJH06 (EF114680, 94%).

A phylogenetic analysis using 16S rRNA gene sequences revealed that all of the SAR11 strains isolated in the East Sea of Korea belonged to the SAR11 subgroup 1a (Morris *et al.*, 2005), which is the most abundant subclade among the SAR11 subclusters (Fig. 1). Sequence similarities among the East Sea SAR11 strains ranged between 97.5% and 100%. The East Sea SAR11 isolates were clustered with *Can.* Pelagibacter ubique HTCC1062 and other SAR11 strains cultured from the Oregon Coast and Sargasso Sea (Fig. 1). They were also as diverse as the strains retrieved from the Oregon Coast and Sargasso Sea. This finding coincides with the finding that nutrient-rich coastal environments harbor a variety of SAR11 strains (Stingl *et al.*, 2008). As the SAR11 strains cultured in the present study were the first SAR11 variants obtained from the West Pacific Ocean rather than the Oregon Coast or Sargasso Sea, the results of this cultivation study will provide insight into SAR11 ecotypes and global distribution (Field *et al.*, 1997; Wilhelm *et al.*, 2007).

Other isolates, other than the SAR11 clade, evidenced a distribution similar to that of previous studies using the HTC method (Connon & Giovannoni, 2002; Stingl *et al.*, 2008) (Fig. 2). Strains IMCC10438 and 10439 were assigned to the OM43 clade, members of which are xanthorhodopsin-containing methylotrophic bacteria (Giovannoni *et al.*, 2008) and constitute one of the most prevalent bacterial clusters observed during a diatom bloom (Morris *et al.*, 2006). Strain IMCC10433 was a member of the SAR92 clade, which is known to harbor proteorhodopsin facilitating light-mediated proton translocation (Stingl *et al.*, 2007a). Strain IMCC10434 evidenced a 100% 16S rRNA gene similarity with strain HTCC2255 (AATR01000002), which is a genome-sequenced and proteorhodopsin-containing member of the *Roseobacter* clade, the second largest clade in the marine bacterial community (Rappé *et al.*, 2000). These strains, including IMCC10419 and 10440, were most closely associated with the validly published species *Paenibacillus ehimensis* IFO 15659^T (92%), *Microbulbifer salipaludis* SM-1^T (91%), *Thalassobius gelatinovorus* IAM12617^T (94%),

Methylophilus methylotrophus NCIMB 10515^T (92%), *M. methylotrophus* NCIMB 10515^T (91%), and *Paracoccus marcusii* DSM 11574^T (96%) (Table 2). This result indicates that these East Sea isolates might constitute novel genera or species.

A recent cultivation study (Stingl *et al.*, 2007b) with Teflon plates cleaned with metal-free HCl, microwave sterilization instead of autoclaving, and the addition of DMSP yielded many new SAR11 isolates from the Oregon Coast and the Sargasso Sea. In our study, we used γ-irradiated conventional polystyrene plates as cultivation vessels, and did not use DMSP as a growth-enhancing chemical for the enrichment of SAR11 cells. From our results showing that various SAR11 isolates were cultivated via conventional HTC approaches, the application of new culture vessels may not have been essential for the acquisition of more SAR11 isolates. Rather, we have incubated the inoculated plates at 10 °C for up to 24 weeks. In the plates incubated for 20 and 24 weeks, SAR11 isolates comprised 64–82% of the total isolates. Therefore, the successful cultivation of diverse SAR11 isolates might be attributable to long-term incubation at a low temperature. Growth characteristics of SAR11 cells that showed a long lag period (Rappé *et al.*, 2002; Stingl *et al.*, 2008) may allow them to divide slowly in oligotrophic liquid culture medium after prolonged incubation. The SAR11 isolates obtained after 20 and 24 weeks of incubation did not lose their reviving activity from glycerol stocks; thus, these new isolates will require further investigations via polyphasic taxonomy and ecological studies.

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Authors'contribution

J.S. and H.-M.O. contributed equally to this study.

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