



# Isolation of SAR11 Marine Bacteria from Cryopreserved Seawater

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**ABSTRACT** While marine microorganisms are frequently studied in their natural environment, isolated strains are invaluable resources that can be used in controlled experiments to expand upon direct observations from natural systems. Here, we sought a means to enhance culture collections of SAR11 marine bacteria by testing the use of seawater cryopreserved with glycerol as an inoculum. Using a raw seawater sample collected from the tropical Pacific Ocean, a subsample was diluted in seawater growth medium to create 576 2-ml dilution cultures containing 5 cells each and incubated for a high-throughput culturing (HTC) experiment, while another portion was cryopreserved in 10% glycerol. After 10 months, a cryopreserved aliquot was thawed and used to create a second cultivation experiment of 480 2-ml cultures containing 5 cells each and 470 cultures containing 105 cells each. The raw seawater cultivation experiment resulted in the successful isolation of 54 monocultures and 29 mixed cultures, while cryopreserved seawater resulted in 59 monocultures and 29 mixed cultures. Combined, the cultures included 51 SAR11 isolates spanning 11 unique 16S rRNA gene amplicon sequence variants (ASVs) from the raw seawater inoculum and 74 SAR11 isolates spanning 13 unique ASVs from cryopreserved seawater. A vast majority (92%) of SAR11 isolates from the two HTC experiments were members of SAR11 subclade Ia, though subclades IIIa and Va were also recovered from cryopreserved seawater and subclade Ib was recovered from both. The four most abundant SAR11 subclade Ia ASVs found in the initial seawater environmental sample were isolated by both approaches.

**IMPORTANCE** High-throughput dilution culture has proved to be a successful approach to bring some difficult-to-isolate planktonic microorganisms into culture, including the highly abundant SAR11 lineage of marine bacteria. While the long-term preservation of bacterial isolates by freezing them in the presence of cryoprotectants, such as glycerol, has been shown to be an effective method of storing viable cells over long time periods (i.e., years), to our knowledge it had not previously been tested for its efficacy in preserving raw seawater for later use as an inoculum for high-throughput cultivation experiments. We found that SAR11 and other abundant marine bacteria could be isolated from seawater that was previously cryopreserved for nearly 10 months at a rate of culturability similar to that of the same seawater used fresh, immediately after collection. Our findings (i) expand the potential of high-throughput cultivation experiments to include testing when immediate isolation experiments are impractical, (ii) allow for targeted isolation experiments from specific samples based on analyses such as microbial community structure, and (iii) enable cultivation experiments across a wide range of other conditions that would benefit from having source inocula available over extended periods of time.

**KEYWORDS** HTC, Pelagibacter, SAR11, cryopreserved, cultivation, seawater


The rapid advancement of molecular tools to investigate marine microorganisms in their natural environment has led to unprecedented access to the genomic repertoire and transcription- and protein-based assessments of activity within natural

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 Isolation of marine bacteria, including SAR11, from a cryopreserved seawater sample.

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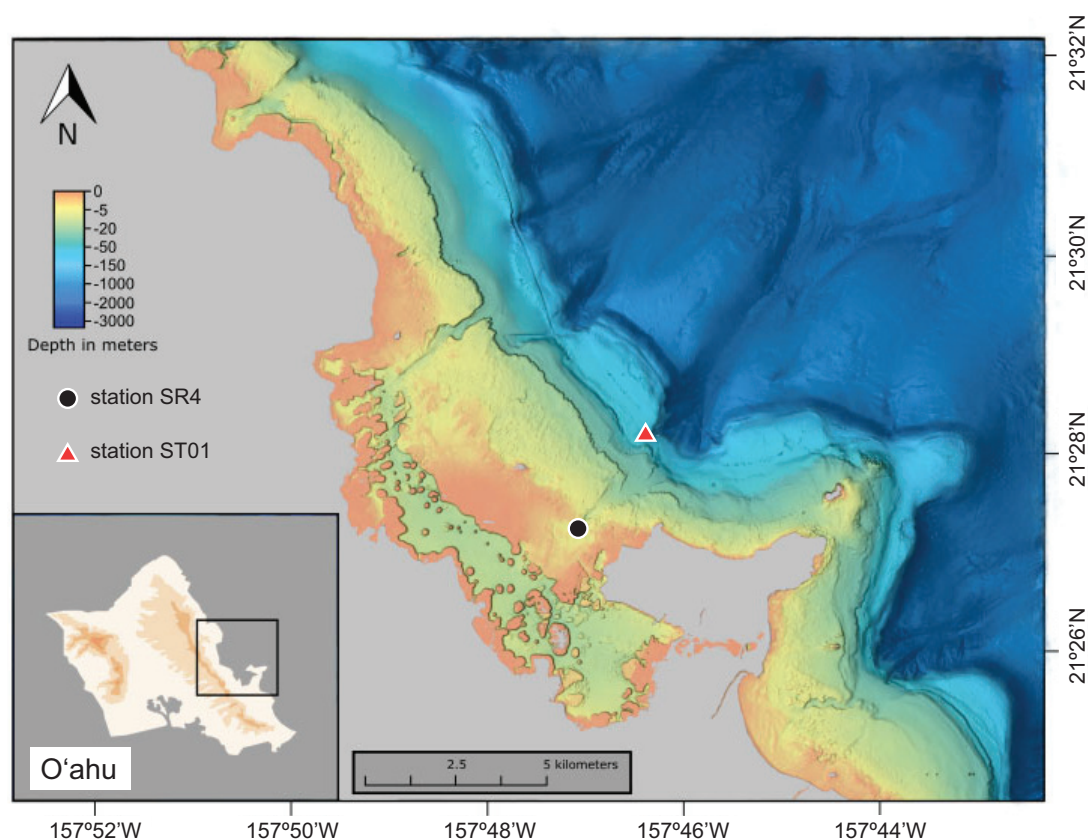
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microbial cells, populations, and communities (1, 2). It is currently feasible for a few liters of seawater to provide sequence data that reveal microbial population structure, the identities of microbial community members, and the presence and activities of potential metabolic functions that they harbor (see, e.g., references 3 and 4). In recent years, however, the value of having cultivated representatives of numerically abundant and environmentally relevant microbial lineages has received renewed recognition (5–8). Access to isolated strains or low-diversity enrichments of marine microorganisms that are commonly found in the natural environment has provided a means to definitively test many hypotheses generated from environmental observations and experiments, as well as whole-genome sequences useful for informing and guiding environmental genomics, transcriptomics, and proteomics research (4, 9, 10). The importance of cultivating environmentally relevant microorganisms from pelagic marine ecosystems for laboratory-based experimentation is now generally appreciated. However, evidence provided by the sequencing of environmental DNA continues to support the conclusion that many of the microorganisms that appear to dominate pelagic marine ecosystems have not yet been cultivated from seawater (11).

Several different isolation methods and strategies have been developed in order to coax recalcitrant environmental microorganisms into laboratory culture (see, e.g., references 12 to 15). Among these novel approaches is an isolation technique based on the dilution-to-extinction culturing methodology first developed by Button and colleagues (16). Although early dilution-to-extinction culturing studies resulted in cultures of novel oligotrophs (16–18), the dilution culture strategy was not without limitations. For instance, the technique yielded only a small number of isolates, while requiring a significant amount of time and effort per experiment. The high-throughput culturing (HTC) approach is a variation of the dilution-to-extinction culturing methodology tailored to facilitate rapid, high-throughput experiments with high rates of replication and greater opportunities to investigate physical, chemical, and biological variables (19, 20).

For over a decade since its initial discovery in 1990, the marine planktonic bacterial lineage known as SAR11 served as a notorious example of an abundant and widespread microorganism in nature that was recalcitrant to cultivation as an isolated strain in a controlled laboratory setting (21, 22). The value of the HTC strategy was solidified when early trials yielded the first cultured representatives of many marine microbial groups that were previously known only from environmental small-subunit (SSU) rRNA genes (19), including the first cultivated strains of SAR11 (20). In general, the HTC approach employs growth media created from natural or artificial seawater in order to dilute the cells within a fluid sample, which is then arrayed in high-density replicate cultures, propagated, and monitored under controlled conditions. In addition to diverse SAR11 strains (20, 23–28), numerous important lineages of marine bacteria, including OM43 (19, 29), SAR116 (23, 30), SAR92 (23, 31), and SUP05 (32), among others, have resulted from the application of this method. While efforts have succeeded in isolating many abundant planktonic marine bacteria, the genetic diversity harbored by these lineages in nature still greatly surpasses what has been isolated in the laboratory. For example, the SAR11 lineage currently encompasses a bacterial order-level divergence (the *Pelagibacterales*) within the class *Alphaproteobacteria* and includes nine subclades: Ia, Ib, Ic, IIa, IIb, IIIa, IV, Va, and Vb (33). While five of these subclades contain cultivated representatives (Ia, Ib, IIa, IIIa, and Va), the vast majority of strains are concentrated within subclade Ia and thus leave much of the genetic diversity of SAR11 lacking cultivated models.

A current limitation of the HTC approach is that, thus far, it has been used only with freshly collected inoculum. This presents a potential constraint on HTC experiments using fluid samples collected in the field, as it requires that all of the resources necessary for setting up an HTC experiment (appropriate laboratory space, biosafety cabinet, etc.) be available at or near the time and location of sampling. The preservation of cultivated bacterial strains by freezing in the presence of cryoprotectants such as glycerol

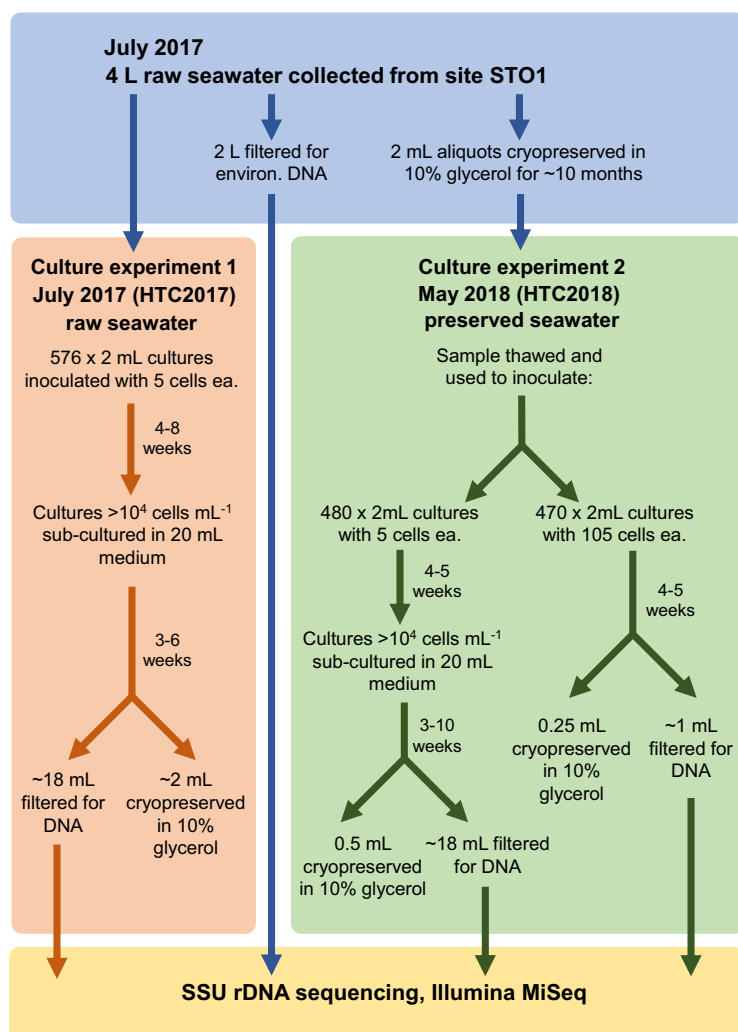


**FIG 1** Map indicating the locations of stations ST01 (red triangle) and SR4 (black circle) in the vicinity of Kāneʻohe Bay on the island of Oʻahu, Hawaiʻi, where seawater used as inocula (ST01) and medium preparation (SR4) were collected.

or dimethyl sulfoxide (DMSO) has proved an effective method for preserving viable cells, including cultivated strains of SAR11, over long time periods (i.e., years) (20, 34, 35). However, to our knowledge, cryopreservation has not previously been tested for its efficacy in preserving raw seawater for subsequent use as inocula for high-throughput cultivation. In this study, we conducted HTC experiments to compare the use of a raw seawater sample collected from the coast of Oʻahu, Hawaiʻi, in the tropical Pacific Ocean with a subsample of the same seawater that was cryogenically preserved for nearly 10 months. In particular, we sought to determine if members of the SAR11 lineage of marine bacteria could be isolated from cryopreserved seawater and thus open the possibility of expanding existing culture collections of SAR11 to potentially include any locations where seawater samples can be collected and preserved.

## RESULTS

**Overview of HTC experiments.** Using seawater sampled outside Kāneʻohe Bay on the island of Oʻahu, Hawaiʻi (Fig. 1), two high-throughput cultivation experiments were conducted: one that used fresh seawater as an inoculum, labeled HTC2017, and one that used a cryopreserved sample of the same seawater ~10 months later (HTC2018) (Fig. 2). Of 576 initial 2-ml cultures inoculated with raw seawater for the HTC2017 experiment, 150 exhibited positive growth after 56 days of incubation. Of these, 123 contained a sufficient volume of culture to be subcultured into 20 ml of fresh medium. Following DNA extraction, sequencing, and the assignment of amplicon sequence variants (ASVs), 54 monocultures and 29 mixed cultures were recovered (Table 1). The remainder either did not yield an amplification product or did not contain an ASV that was  $\geq 50\%$  of the culture and thus were not considered further. Fifty-four isolates were



**FIG 2** Outline of experiments performed in this study.

identified in the 29 mixed cultures (see Table S1 in the supplemental material); the 108 unique isolates identified in the HTC2017 experiment (monocultures plus isolates contained in mixed cultures) were distributed among 28 ASVs in total (Table 2; Table S1). The HTC2017 experiment yielded a culturability of 3.1% (2.5% to 3.9%) when both

**TABLE 1** Culturability statistics from fresh (HTC2017) and cryopreserved (HTC2018) seawater cultivation experiments

Inoculum source	Inoculum size (no. of cells) <sup>a</sup>	No. of inoculated cultures	Mono- and mixed cultures		Monocultures only	
			No. of positive cultures <sup>b</sup>	% culturability <sup>c</sup>	No. of positive cultures <sup>b</sup>	% culturability <sup>c</sup>
HTC2017 fresh seawater	5	576	83	3.1 (2.5, 3.9)	54	2.0 (1.5, 2.6)
HTC2018 cryopreserved seawater	5	480	50	2.2 (1.6, 2.9)	39	1.7 (1.2, 2.3)
	105	94	38	0.5 (0.3, 0.7)	20	0.2 (0.1, 0.4)

<sup>a</sup>In a culture volume of 2 ml.

<sup>b</sup>Calculated as the number of inoculated cultures that resulted in the growth of either a monoculture or mixed cultures (see Materials and Methods for definitions). The constituent members of all mixed cultures are listed in Table S1 in the supplemental material.

<sup>c</sup>Ninety-five percent confidence intervals are shown in parentheses.

**TABLE 2** Summary of isolates from fresh (HTC2017) and cryopreserved (HTC2018) seawater cultivation experiments, including the relative abundance of each taxonomic group in the environmental sample based on SSU rRNA gene sequencing

Taxonomy <sup>a</sup>	HTC2017		HTC2018		Seawater	
	No. of ASVs	No. of Strains	No. of ASVs	No. of Strains	No. of ASVs	Abundance (relative %) <sup>b</sup>
<i>Alphaproteobacteria</i> , SAR11 subclade Ia	9	47	9	68	10	10.23
<i>Alphaproteobacteria</i> , SAR11 subclade Ib	2	4	2	3	14	7.74
<i>Alphaproteobacteria</i> , SAR11 subclade IIIa	0	0	1	2	4	0.42
<i>Alphaproteobacteria</i> , SAR11 subclade Va	0	0	1	1	2	2.13
<i>Alphaproteobacteria</i> , <i>Rhodobacteraceae</i>	4	14	5	5	12	5.81
<i>Alphaproteobacteria</i> , SAR116 clade	1	1	0	0	23	5.41
<i>Alphaproteobacteria</i> , PS1 clade	1	3	3	4	3	0.42
<i>Gammaproteobacteria</i> , <i>Haliaceae</i> , OM60(NOR5) clade	4	24	5	21	5	1.91
<i>Gammaproteobacteria</i> , K189A clade	1	1	0	0	4	0.60
<i>Betaproteobacteriales</i> , <i>Burkholderia-Caballeronia-Paraburkholderia</i>	1	4	1	1	2	0.11
<i>Betaproteobacteriales</i> , <i>Methylophilaceae</i> , OM43 clade	2	5	0	0	1	0.04
<i>Gammaproteobacteria</i> , <i>Pseudomonadaceae</i>	1	2	0	0	0	0
<i>Gammaproteobacteria</i> , <i>Rhodanobacteraceae</i>	1	2	0	0	0	0
<i>Actinobacteria</i> , <i>Corynebacteriaceae</i>	0	0	1	1	0	0
<i>Actinobacteria</i> , <i>Geodermatophilaceae</i>	0	0	1	1	0	0
<i>Bacteroidetes</i> , <i>Chitinophagaceae</i>	0	0	1	1	0	0
Fungi	1	1	1	2	0	0

<sup>a</sup>Abbreviated taxonomy adapted from Silva release 132; the full taxonomy of each ASV is available in Table S1.

<sup>b</sup>Environmental relative abundance was calculated using read counts of all environmental ASVs detected in the seawater sample from within each taxonomic grouping, after curation to remove ASVs originating from chloroplasts.

monocultures and mixed cultures were considered and a culturability of 2.0% (1.5% to 2.6%) when only monocultures were considered (Table 1).

For the cryopreserved seawater experiment (HTC2018), wells were inoculated with either 5 or 105 cells. Of the 480 initial 2-ml cultures inoculated with 5 cells well<sup>-1</sup> of cryopreserved seawater (HTC2018), 142 exhibited positive growth after 30 days of incubation. The 142 positive wells were subcultured into 20 ml seawater medium, and after 72 days, 95 subcultures ultimately exhibited growth. Following DNA extraction, sequencing, and the assignment of ASVs, 39 monocultures and 11 mixed cultures were identified (Table 1). The remainder either did not yield an amplification product or did not contain an ASV that was  $\geq 50\%$  of the culture and thus were not considered further. Sixteen isolates were identified in the 11 mixed cultures (Table S1). Combined, the 55 unique isolates identified in the HTC2018 5-cells-well<sup>-1</sup> experiment were distributed among 17 ASVs (Table 2; Table S1). The HTC2018 5-cells-well<sup>-1</sup> experiment yielded culturabilities of 2.2% (1.7% to 2.9%) when both monocultures and mixed cultures were considered and 1.7% (1.2% to 2.3%) when only monocultures were considered (Table 1).

Of the 470 initial 2-ml cultures inoculated with 105 cells well<sup>-1</sup> of cryopreserved seawater (HTC2018), 343 exhibited positive growth after 31 days of incubation. A single 96-well cultivation plate containing 64 positive wells and 2 uninoculated control wells were selected for further processing. Following DNA extraction, sequencing, and the assignment of ASVs, 20 monocultures and 18 mixed cultures were identified (Table 1). The remainder either did not yield an amplification product or did not contain an ASV that was  $\geq 50\%$  of the culture and thus were not considered further. Thirty-five isolates were identified in the 18 mixed cultures (Table S1); combined, the 55 unique isolates identified in the 105-cells-well<sup>-1</sup> HTC2018 experiment were distributed among 21 ASVs (Table 2; Table S1). The HTC2018 105-cells-well<sup>-1</sup> experiment yielded culturabilities of 0.5% (0.3% to 0.7%) when both monocultures and mixed cultures were considered and 0.2% (0.1% to 0.4%) when only monocultures were considered (Table 1).



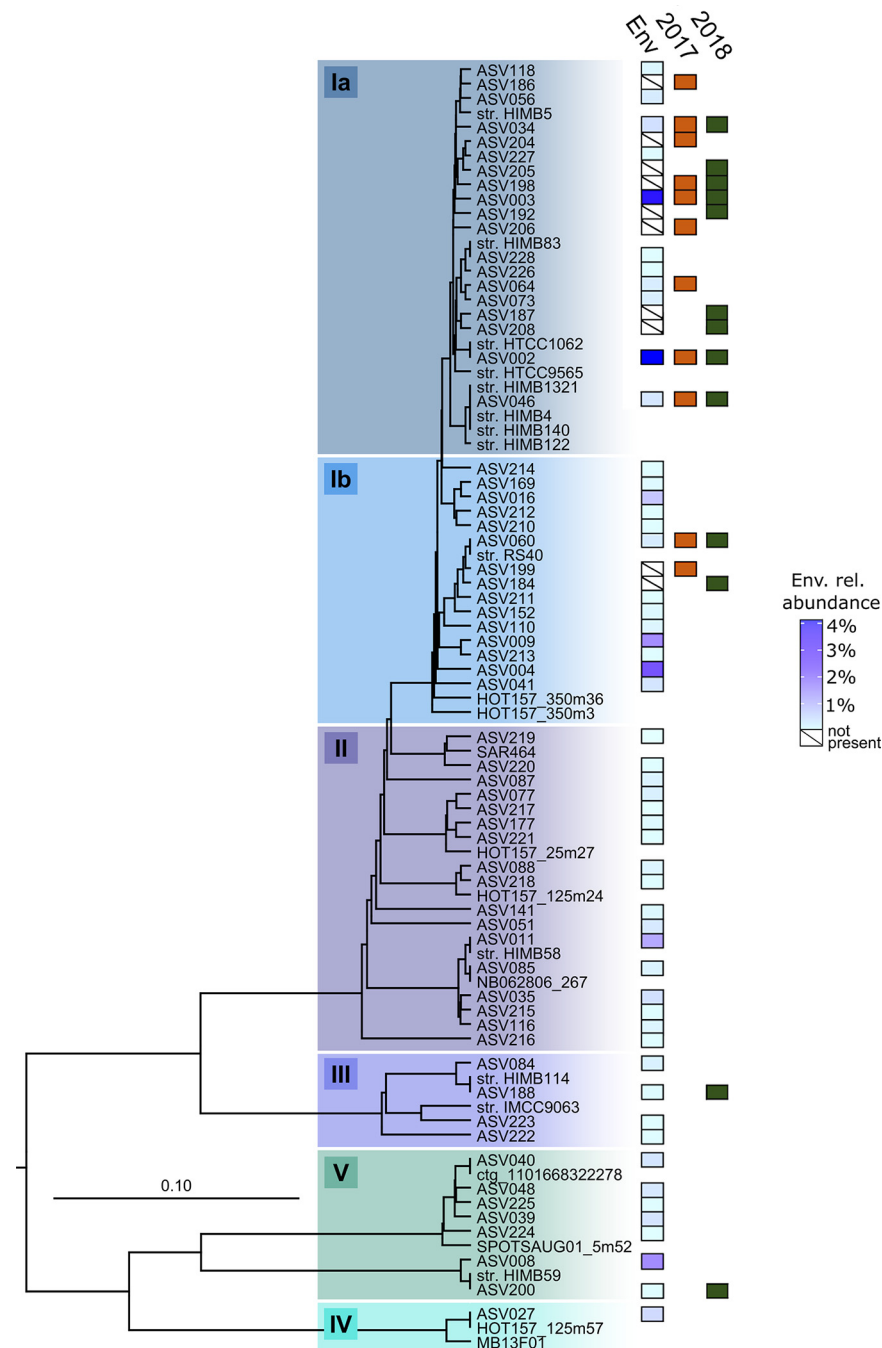
**Identities of isolates.** Each culture was sequenced to an average depth of  $14,047 \pm 8,014$  (SD), and a range of 679 to 57,557 quality-controlled reads. Regardless of whether they originated from raw or cryopreserved seawater, the broad, bacterial family-level taxonomic identities of isolates revealed substantial overlap between culture experiments (Table 2; Table S1). Members of alphaproteobacterial SAR11 subclade I, the marine gammaproteobacterial family *Haliaceae*, and the alphaproteobacterial family *Rhodobacteraceae* were the first-, second-, and third-most-abundant families isolated from both the fresh seawater (HTC2017) and cryopreserved seawater (HTC2018) cultivation experiments (Table 2; Table S1). Combined, these three groups made up 82% (89 of 108) and 88% (97 of 110) of isolates recovered from HTC2017 and HTC2018, respectively. Other bacterial families with isolates shared between HTC2017 and HTC2018 include the PS1 clade of *Alphaproteobacteria* and *Burkholderiaceae* within the *Betaproteobacteria* (Table 2; Table S1). When the five bacterial families shared between HTC2017 and HTC2018 are considered, the fresh seawater and cryopreserved seawater cultivation experiments shared 89% (96 of 108) and 93% (102 of 110) of isolated strains, respectively.

**(i) SAR11.** A total of 51 strains representing 11 unique ASVs of SAR11 marine bacteria (alphaproteobacterial order *Pelagibacterales*) were cultivated in HTC2017, while 74 strains representing 13 ASVs were cultivated in HTC2018 (Fig. 3; Table 2; Table S1). They make up 47% and 67% of the isolates recovered in the two experiments, respectively. The vast majority of these isolates were members of SAR11 subclade Ia, including 47 strains from HTC2017 and 68 strains from HTC2018. Each experiment resulted in the isolation of nine subclade Ia ASVs, including five that were common between the two experiments (Fig. 3 and 4; Table S1). The two most-often-isolated SAR11 subclade Ia ASVs were shared between the two experiments: ASV003 (25 and 37 isolates) and ASV002 (11 and 13 isolates) from HTC2017 and HTC2018, respectively (Fig. 3; Table S1). Two other subclade Ia ASVs (ASV034 and ASV046) consisted of multiple strains from both experiments, while 7 subclade Ia ASVs consisted of a single isolate from one experiment (Fig. 3; Table S1).

Strains affiliated with SAR11 subclade Ib were also isolated from both fresh and cryopreserved seawater, including 4 isolates across 2 ASVs from HTC2017 and 3 isolates across 2 ASVs from HTC2018 (Fig. 4; Table S1). SAR11 subclade Ib ASV060 consisted of 3 and 2 isolates from HTC2017 and HTC2018, respectively, while each experiment also yielded an isolate with a unique subclade Ib ASV (Fig. 3; Table S1). Two SAR11 subclades were isolated only from the cryopreserved seawater sample, including two isolates from subclade IIIa (ASV188) and one isolate from subclade Va (ASV200) (Fig. 3; Table 2; Table S1).

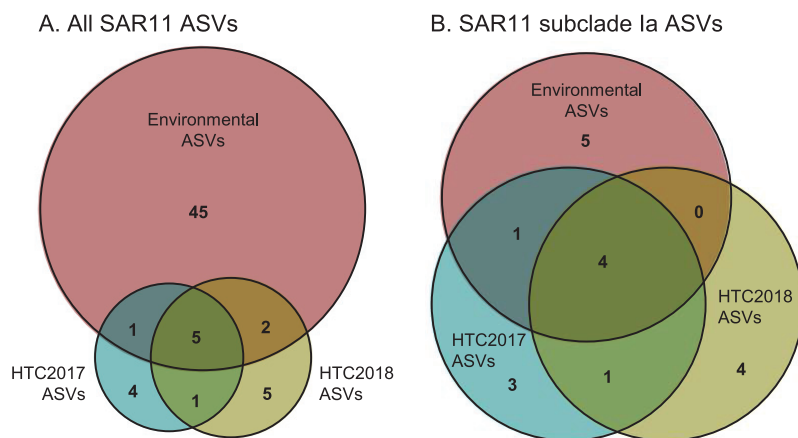
**(ii) OM60(NOR5).** Within the gammaproteobacterial family *Haliaceae*, the marine OM60(NOR5) clade made up 24 and 21 isolates, or 22% and 19% of HTC2017 and HTC2018, respectively (Table 2). The 4 ASVs that accounted for the 24 isolates from HTC2017 were shared with HTC2018, where they accounted for 19 of the 21 isolates recovered from that experiment (Fig. 5; Table S1). One additional OM60(NOR5) ASV (ASV201) consisting of 2 isolates was recovered from cryopreserved seawater (Fig. 5; Table S1). Two closely related ASVs (ASV032 and ASV018) accounted for most of the OM60(NOR5) strains isolated from both experiments of this study (Fig. 5; Table S1). ASV32 was identical to strain HIMB55, a genome-sequenced member of the OM60 (NOR5) clade previously isolated from Kāne'ohe Bay, Hawai'i (36).

**(iii) Rhodobacteraceae.** The marine alphaproteobacterial family *Rhodobacteraceae* made up 14 and 5 isolates, or 13% and 5% of HTC2017 and HTC2018, respectively (Table 2). The strains were distributed among 4 (HTC2017) and 5 (HTC2018) ASVs, including three (ASV12, ASV71, ASV124) that were shared between the two experiments (Fig. 5; Table S1). Eight of 14 *Rhodobacteraceae* isolates recovered from HTC2017 belonged to a single ASV (ASV012). ASV71 was identical to strain HIMB11, a genome-sequenced member of the *Rhodobacteraceae* family previously isolated from Kāne'ohe Bay, Hawai'i (Fig. 5) (37).



**FIG 3** Phylogenetic analysis of the SAR11 clade illustrating relationships among 16S rRNA gene ASVs recovered from isolates and the source seawater used as the inoculum in this study. The scale bar corresponds to 0.1 substitution per nucleotide position. A variety of *Alphaproteobacteria* were used as an outgroup. Previously cultured isolates (“str.”) and select environmental gene clones were included as references. Boxes labeled “Env. rel. abundance” indicate the relative environmental abundance of each ASV (blue gradient), while orange (“2017”) and green (“2018”) boxes indicate the presence of an ASV in the fresh (HTC2017) and cryopreserved (HTC2018) seawater cultivation experiments, respectively. Boxes containing a slash in the “Env” column indicate that ASVs were found in a culture but were not detected in the environmental sample.

**(iv) Other isolates.** In addition to the SAR11, OM60(NOR5), and *Rhodobacteraceae* ASVs described above, one ASV was isolated in both experiments; ASV190 within the betaproteobacterial family *Burkholderiaceae* was represented by 4 strains in HTC2017 and 1 strain in HTC2018 (Table S1). While identical ASVs were not isolated, members of



**FIG 4** (A) Venn diagrams comparing SAR11 ASVs identified within the environmental seawater sample used as the inoculum, isolates from the fresh seawater cultivation experiment (HTC2017), and isolates from the cryopreserved seawater cultivation experiment (HTC2018). (B) Same as for panel A, except the values are limited to SAR11 subclade Ia ASVs.

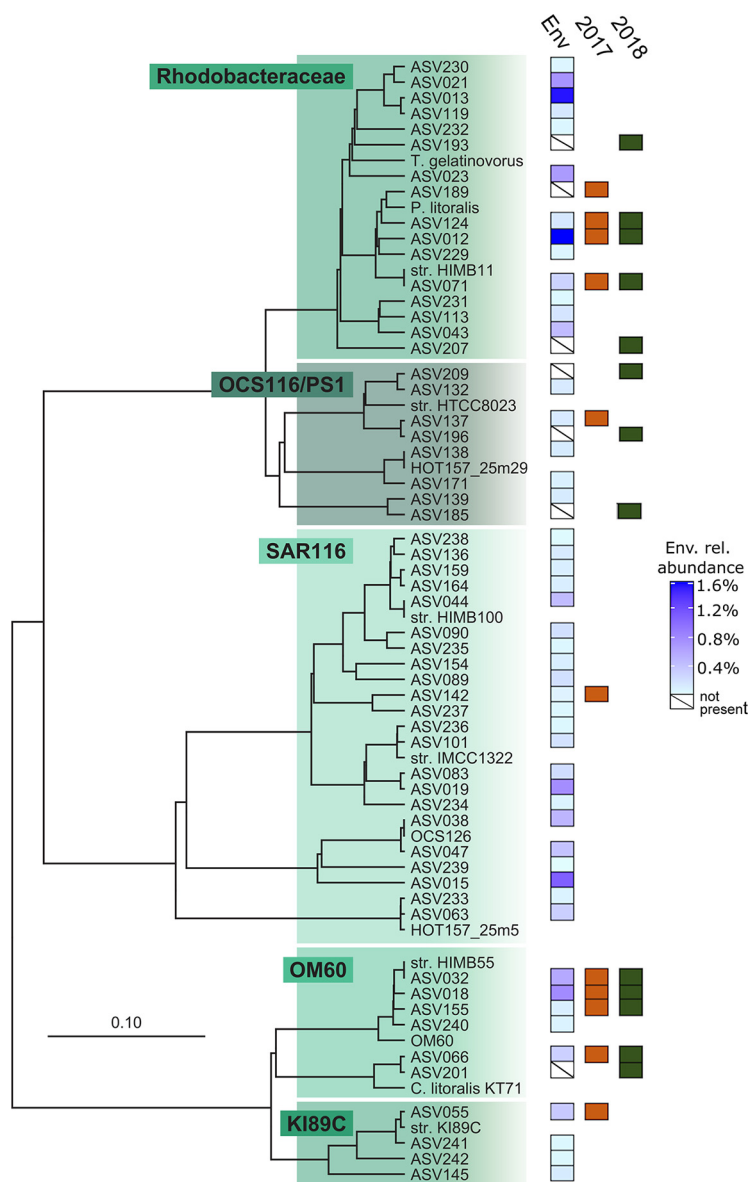
the marine alphaproteobacterial PS1 clade within the order *Parvibaculales* were recovered from both cultivation experiments, including 3 isolates from a single ASV (ASV137) in HTC2017 and 4 isolates from 3 ASVs in HTC2018 (Fig. 5; Table S1). The remaining ASVs were recovered as either singletons or pairs of strains, except for the OM43 clade of the *Betaproteobacteriales*, which consisted of five strains across 2 ASVs from the fresh seawater inoculum (HTC2017) only (Table S1).

**Comparisons with inoculum microbial community.** The inoculum seawater sample was sequenced to a depth of 65,924 quality-controlled reads. This sample harbored a microbial community dominated by typical marine bacteria, including the marine picocyanobacteria *Prochlorococcus* and *Synechococcus*, multiple subclades of the SAR11 lineage, the family *Flavobacteriaceae* of the bacterial phylum *Bacteroidetes*, diverse members of the gammaproteobacterial SAR86 and OM60(NOR5) lineages and alphaproteobacterial SAR116 and *Rhodobacteraceae* lineages, and *Actinomarinaceae* of the bacterial phylum *Actinobacteria*, among others (Table S1).

Within the inoculum sample, 53 SAR11 ASVs totaling 26% of the microbial community were identified. These spanned a diverse array of subclades that included Ia, Ib, IIa, IIIa, IV, Va, and Vb (Fig. 3; Table S1). Eight of the 53 SAR11 ASVs were isolated from at least one cultivation experiment, including ASVs within subclades Ia, Ib, IIIa, and Va, while 5 of the 8 were isolated from both HTC2017 and HTC2018 (Fig. 3 and 4; Table S1). Of 10 SAR11 subclade Ia ASVs present in the inoculum, the four that were most abundant were cultivated from both experiments (ASV002, ASV003, ASV034, and ASV046), including the second- and third-most-abundant individual ASVs in the inoculum seawater community (Table S1). A fifth was cultivated in HTC2017 only (Fig. 3; Table S1). Eight SAR11 subclade Ia ASVs that did not appear in the inoculum seawater community were isolated (Fig. 3 and 4; Table S1).

In contrast to SAR11 subclade Ia, other SAR11 subclades present in the inoculum seawater microbial community were cultivated rarely or not at all. For example, only 1 of 14 subclade Ib ASVs that appeared in the inoculum was isolated (ASV060) (Fig. 3; Table S1), although it was cultivated in both the HTC2017 and HTC2018 experiments. From the HTC2018 experiment, one of four subclade IIIa ASVs (ASV188) was isolated, as well as one of two subclade Va ASVs (ASV200) (Fig. 3; Table S1). Thirteen *Rhodobacteraceae* ASVs were identified in the environmental sample, of which the same three (ASV012, ASV071, ASV124) were cultivated from both fresh and cryopreserved seawater (Fig. 5; Table S1). Four of the five total most environmentally abundant OM60(NOR5) clade ASVs were also cultivated in both experiments (Table S1). Despite





**FIG 5** Phylogenetic analysis of select lineages of *Alpha*- and *Gammaproteobacteria* illustrating relationships among 16S rRNA gene ASVs recovered from isolates and the source seawater used as the inoculum in this study. The scale bar corresponds to 0.1 substitution per nucleotide position. A variety of *Betaproteobacteria* were used as an outgroup. Previously cultured isolates (“str.”) and select environmental gene clones were included as references. Boxes labeled “Env. rel. abundance” indicate the relative environmental abundance of each ASV (blue gradient), while orange (“2017”) and green (“2018”) boxes indicate the presence of an ASV in the fresh (HTC2017) and cryopreserved (HTC2018) seawater cultivation experiments, respectively. Boxes containing a slash in the “Env” column indicate that ASVs were found in a culture but were not detected in the environmental sample.

19 ASVs appearing in the inoculum, only one SAR116 ASV (ASV142) (Fig. 5; Table S1) was cultivated in the HTC2017 experiment.

**Mixed cultures.** Twenty-nine mixed cultures were identified within each of the HTC2017 and HTC2018 experiments, yielding a total of 58 mixed cultures (Table S1). A majority of the mixed cultures contained ASVs from either SAR11 subclade Ia or the OM60(NOR5) clade, which is logical given the high recovery of monocultures from these two groups in both cultivation experiments (Fig. S1). Of nine mixed cultures containing OM60(NOR5) ASV018, eight also contained SAR11 subclade Ia ASV002, ASV003, or ASV034 (Fig. S1; Table S1). Eight of the 11 mixed cultures containing OM60(NOR5)

ASV032 also contained a SAR11 subclade Ia ASV as well (Fig. S1; Table S1). All cultivated OM43 clade ASVs were in mixed cultures; both of the OM43 clade ASV202 isolates appeared in coculture with the OM43 clade ASV195 (Fig. S1; Table S1).

## DISCUSSION

For a variety of reasons, SAR11 marine bacteria remain a target for culturing experiments, despite being first isolated nearly 20 years ago (20). In large part, this is driven by the enormous genomic diversity harbored by this lineage and the probability of ecotypic differentiation across the SAR11 phylogenetic tree (3, 26, 38–40). Living cultures offer a direct means of characterizing and quantifying the cellular and physiological features that underlie differences in abundance or activity observed via direct environmental sampling (4, 9, 41). The primary goal of this study was to test the hypothesis that SAR11 marine bacteria can be isolated from cryogenically preserved seawater. We reasoned that, since existing SAR11 isolates could be cryopreserved in the presence of 10% glycerol (see, e.g., references 20, 33, and 34), then there was no *a priori* reason to believe that natural populations of SAR11 cells could not similarly be preserved. One of many unknown variables, however, was whether or not the process of cryopreservation would result in significant cell loss and thus affect cultivation efficiency. With equivalently sized inocula of five cells, we found that not only were SAR11 strains able to be cultivated from cryopreserved seawater but also the overall levels of culturability were similar between the fresh and cryopreserved seawater samples. Both experiments resulted in the isolation of representatives from the four most abundant SAR11 subclade Ia ASVs in the original inoculum seawater sample, as well as strains from subclade Ib. The cryopreserved seawater sample also proved capable of serving as an inoculum to isolate other SAR11 subclades, as evidenced by the recovery of isolates from within subclades IIIa (two strains) and Va (one strain) from the cryopreserved sample only.

In addition to yielding numerous isolates from SAR11 subclade Ia that appear to represent abundant ASVs in the seawater sample used as the inoculum for these experiments, this study yielded seven strains of SAR11 subclade Ib in either mono- or mixed cultures. Despite being a widespread and frequently abundant lineage of SAR11 in the global surface ocean (42–44), only one cultivated representative of subclade Ib had been previously reported, from the Red Sea (28). In addition to the novel isolates of subclade Ib, two strains of subclade IIIa and one of subclade Va were isolated from cryopreserved seawater, indicating that a broad range of SAR11 diversity covering at least four major sublineages can be cultivated by this approach, with no apparent negative affect from the cryopreservation treatment itself.

As demonstrated by their recovery here, a range of other oligotrophic marine bacteria can be isolated from cryopreserved seawater coupled with an HTC approach. This includes representatives from the OM60(NOR5) clade, a ubiquitous lineage of oligotrophic marine *Gammaproteobacteria* (OMG) that has consistently been isolated via HTC approaches (see, e.g., references 19, 27, and 31), including from coastal Hawai'i (36). The OM60(NOR5) lineage was the second-most-commonly isolated group of marine bacteria, behind only SAR11 subclade Ia, whether fresh or cryopreserved seawater was used as the inoculum. Of five OM60(NOR5) ASVs present in the seawater used as the inoculum, the four most abundant were isolated in both cultivation experiments, indicating no apparent effect of using cryopreserved seawater as an inoculum for isolating members of the OM60(NOR5) lineage. A similar pattern emerged for the *Rhodobacteraceae* lineage of marine *Alphaproteobacteria*, where isolates from the same 3 ASVs were recovered in each of the two cultivation experiments, out of 13 total *Rhodobacteraceae* ASVs identified in the environmental sample. This included the most abundant *Rhodobacteraceae* ASV from the seawater inoculum, as well as an ASV identical to the previously isolated and genome-sequenced strain HIMB11 from the same sampling location (37). We found only one abundant (>3) set of strains that was isolated by using raw seawater as the inoculum without corresponding strains also being isolated from cryopreserved seawater; five strains

belonging to two ASVs within the OM43 clade of *Betaproteobacteria* were isolated in mixed and monocultures. While this may indicate that the cryopreservation process had a negative impact on the viability of OM43 clade cells, we note that previously isolated members of this lineage have successfully been cryopreserved in a fashion identical to the method employed in the current study (29, 45). Thus, there is also the potential that this difference stems from stochasticity related to diluting the two inocula nearly 1,000,000-fold.

Combining a barcoded next-generation 16S rRNA gene amplicon sequencing approach with a high-throughput dilution culture strategy proved to be an efficient and sensitive means with which to identify strains and assess the constituent taxa within mixed cultures. By barcoding and sequencing each individual culture in the same manner as if it were a mixed microbial community, we obtained taxonomic and proportional data on the microorganisms growing within 58 mixed cultures of up to four constituent ASVs. Recent studies have highlighted the intricacies that interweave the metabolisms of microorganisms inhabiting seawater (see, e.g., references 4 and 46); in natural systems, it is probable that a portion of marine microorganisms require as-yet-unknown growth factors from coexisting cells (47, 48). These dependencies can be identified and investigated by combining a miniaturized, high-throughput approach to cultivate and screen 100s to 1,000s of dilution cultures with an inoculum size aimed at growing mixed consortia and a rapid sequence-based screening method that is appropriate for mixed communities, like the one used here.

While it is common for HTC experiments to use an inoculum size of one to a few cells per initial culture, a set of cultures inoculated with 105 cells each were included here in order to guard against the potential for significant cell loss through cryopreservation of the raw seawater. However, the 5-cell-per-culture inoculum resulted in ample growing cultures to test the utility of this approach. For this reason, the 105-cell inoculum was ultimately not needed for this experiment and subsequently not included in the labor-intensive subculturing step. However, to provide an initial glimpse of the microorganisms growing within these dilution cultures, they were identified directly from ~1 ml of the initial culture volume from one of five 96-well growth plates. Thus, comparisons of cultivability between the two different inoculum sizes are complicated because the cultures were processed differently. What is clear, however, is that a 105-cell-per-well inoculum would have had to exhibit growth in nearly every inoculated culture vessel in order to approach the same culturability statistic as found for the 5-cell-per-well inoculum. The reasons why it did not are not readily apparent from this experiment.

Consistently with recent observations (27), this set of experiments resulted in the isolation of several bacterioplankton lineages that have been isolated numerous times via HTC and thus appear readily amenable to cultivation via this approach, including members of SAR11 subclade Ia, the OM60(NOR5) lineage, and the *Rhodobacteraceae*. However, a large portion of the diversity of marine microbes is still being missed in contemporary cultivation efforts. For example, when considering only the putatively heterotrophic, noncyanobacterial fraction of the microbial community targeted in this study, major lineages, including the *Flavobacteriaceae*, SAR86 clade, *Marinimicrobia* (SAR406 clade), and marine actinobacteria ("*Candidatus Actinomarina*") were missed completely. At the single-nucleotide resolution of ASVs, abundant lineages of SAR116 and SAR11 subclades Ib, IIa, and Vb were also conspicuously missed. While this study does not offer a panacea for isolating any of these well-known but as-yet-uncultivated (or undercultivated) lineages in laboratory culture, it presents a method by which high-throughput isolation experiments can be repeatedly performed on identical sets of cryopreserved seawater samples such that requirements for growth can be systematically tested in a cumulative fashion.

In summary, we have demonstrated that a broad range of marine bacterioplankton taxa can be isolated from glycerol-cryopreserved seawater via an HTC approach and that the cryopreservation process itself did not negatively affect culturability or

influence the taxonomic identities of the resulting isolates. Strains of SAR11 subclades Ia, Ib, IIIa, and Va, as well as other abundant lineages of marine bacteria, such as OM60 (NOR5), oligotrophic *Rhodobacteraceae*, and the PS1 clade, are amenable to isolation from cryopreserved seawater. This study demonstrates that cryopreserved seawater can be used as a means to expand the breadth of HTC studies to anywhere cryopreserved stocks can be made and opens new opportunities for repeatedly interrogating individual water samples or selectively targeting specific samples for cultivation once ancillary data are in hand.

## MATERIALS AND METHODS

**Processing of seawater for growth experiments.** On 26 July 2017, a 4-liter seawater sample was collected in an acid-washed polycarbonate (PC) bottle from a depth of 2 m at station STO1 (N 21° 28.974', W 157° 45.978') outside Kāne'ohe Bay, O'ahu, Hawai'i (Fig. 1). Within 1 h of collection, subsamples of the raw seawater were used to enumerate planktonic microorganisms, cryopreserve subsamples, collect microbial biomass for environmental DNA, and serve as inocula for a high-throughput cultivation experiment (Fig. 2). Microbial cells were enumerated by staining them with SYBR Green I nucleic acid stain (Invitrogen, Carlsbad, CA, USA) and counted on a Guava easyCyte 5HT flow cytometer equipped with a high-powered 150-mW blue (488-nm) laser (Millipore, Burlington, MA, USA) according to a previously published protocol (49). To cryopreserve the raw seawater, individual 1.5-ml subsamples were added to 375  $\mu$ l of a 50% (vol/vol) glycerol solution (in sterile Kāne'ohe Bay seawater; 10% final concentration) in 2-ml cryovials (Nalgene, Rochester, NY, USA) at room temperature (24°C), mixed by inverting the vials, and cooled at a rate of  $-1^{\circ}\text{C min}^{-1}$  with a Cryo 1°C freezing container (Nalgene) inside a  $-80^{\circ}\text{C}$  ultracold freezer. Approximately 1.3 liters of the raw seawater sample was collected on a 25-mm-diameter, 0.1- $\mu$ m-pore-sized polyethersulfone membrane (Supor-100; Pall Gelman Inc., Ann Arbor, MI). The filter was submerged in 500  $\mu$ l DNA lysis buffer (50, 51) and stored at  $-80^{\circ}\text{C}$  until DNA extraction.

**High-throughput cultivation experiment with raw seawater.** Growth medium was made by following previously published methods (52). Briefly, 20-liter seawater samples were collected on 8 July 2017 and subsequently again on 20 September 2017 from a depth of 2 m at station SR4 (N 21° 27.699', W 157° 47.010') near Kāne'ohe Bay, O'ahu, Hawai'i (Fig. 1), in acid-washed 4-liter PC bottles. Within 1 h of collection, the seawater was sequentially filtered through preirradiated (10 liters of sterile water followed by 10 liters of seawater) 0.8-, 0.2-, and 0.1- $\mu$ m-pore-sized polyethersulfone (PES) membranes (AcroPak 20 and Supor 100; Pall Corp., Port Washington, NY, USA) into clean 4-liter PC bottles. Bottles were then autoclaved for 3 h at  $121^{\circ}\text{C}$  and allowed to cool. The sterile seawater was sparged with  $\text{CO}_2$  to restore the inorganic carbon chemistry, and then with air, through three in-line HEPA vent filters (0.3- $\mu$ m glass fiber to 0.2- $\mu$ m polytetrafluoroethylene [PTFE] to 0.1- $\mu$ m PTFE; Whatman, GE Healthcare Life Sciences, Chicago, IL, USA) and stored at  $4^{\circ}\text{C}$  until use. The pH of the seawater was checked prior to being autoclaved and after being sparged to ensure continuity of the inorganic carbon chemistry.

Subsamples of raw seawater were diluted in the sterile seawater to  $2.5 \text{ cells ml}^{-1}$  and arrayed in 2-ml volumes (5-cell inoculum) into 576 wells of custom-fabricated 96-well Teflon microtiter plates. Plates were sealed with breathable polypropylene microplate adhesive film (VWR, Radnor, PA, USA) and incubated at  $27^{\circ}\text{C}$  in the dark. The presence of cellular growth was monitored at 3.5 and 8 weeks via a Guava easyCyte 5HT flow cytometer equipped with a high-powered 150-mW blue (488-nm) laser (50). Instrument settings included gain controls of 11.81 for forward scatter, 16.0 for side scatter, and 2.71 for green fluorescence. Custom gates were determined from an isolated SAR11 culture. This experiment is here referred to as HTC2017.

Wells that exhibited positive growth of  $>10^4 \text{ cells ml}^{-1}$  were subcultured by transferring 1 ml into 20 ml of sterile seawater medium amended with  $400 \mu\text{M}$   $(\text{NH}_4)_2\text{SO}_4$ ,  $400 \mu\text{M}$   $\text{NH}_4\text{Cl}$ ,  $50 \mu\text{M}$   $\text{NaH}_2\text{PO}_4$ ,  $1 \mu\text{M}$  glycine,  $1 \mu\text{M}$  methionine,  $50 \mu\text{M}$  pyruvate,  $800 \text{ nM}$  niacin (B3),  $425 \text{ nM}$  pantothenic acid (B5),  $500 \text{ nM}$  pyridoxine (B6),  $4 \text{ nM}$  biotin (B7),  $4 \text{ nM}$  folic acid (B9),  $6 \mu\text{M}$  myo-inositol,  $60 \text{ nM}$  4-aminobenzoic acid, and  $6 \mu\text{M}$  thiamine hydrochloride (B1). Subcultures were subsequently incubated at  $27^{\circ}\text{C}$  in the dark and monitored for growth after 4.5 weeks. Those that again reached  $>10^4 \text{ cells ml}^{-1}$  were cryopreserved (500  $\mu$ l of culture with 125  $\mu$ l of 50% (vol/vol) glycerol solution in Kāne'ohe Bay seawater, 10% final concentration) in 2-ml cryovials (Nalgene) at room temperature ( $24^{\circ}\text{C}$ ), mixed by inverting the tubes, and cooled at a rate of  $-1^{\circ}\text{C min}^{-1}$  with a Cryo 1°C freezing container (Nalgene) inside a  $-80^{\circ}\text{C}$  ultracold freezer. Cells in the remaining volume of culture ( $\sim 18 \text{ ml}$ ) were collected by filtration through 13-mm-diameter, 0.03- $\mu$ m-pore-sized PES membrane filters (Sterlitech, Kent, WA, USA), submerged in 250  $\mu$ l DNA lysis buffer, and stored at  $-80^{\circ}\text{C}$  until DNA extraction.

**High-throughput cultivation experiment with cryopreserved seawater.** After 42 weeks of storage at  $-80^{\circ}\text{C}$ , one cryopreserved stock of raw seawater from station STO1 was hand-thawed to room temperature ( $\sim 24^{\circ}\text{C}$ ) and diluted 10-fold in sterile seawater growth medium, and organisms were enumerated via staining with SYBR Green I and flow cytometry. The cryopreserved sample was subsequently diluted with nutrient-amended sterile seawater growth medium to two different concentrations: 2.5 and 52.5  $\text{cells ml}^{-1}$ . The 2.5- $\text{cells ml}^{-1}$  dilution was used to create 480 2-ml dilution cultures (5-cell inoculum) in custom-fabricated 96-well Teflon microtiter plates, while the 52.5- $\text{cells ml}^{-1}$  dilution was used to create 470 2-ml dilution cultures (105-cell inoculum). Ten control wells containing uninoculated sterile seawater growth medium were also included. Teflon plates were sealed with breathable polypropylene microplate adhesive film (VWR) and incubated at  $27^{\circ}\text{C}$  in the dark. Growth was monitored at 2, 3, and

5 weeks post-inoculation as described above. Dilution cultures from the 5-cell inoculum showing positive growth ( $>10^4$  cells  $\text{ml}^{-1}$ ) after 5 weeks of incubation were subcultured by distributing 1 ml of initial culture into 20 ml of sterile seawater growth medium and monitored for growth during incubation for up to 10 weeks at 27°C in the dark. This experiment is here referred to as HTC2018.

Subcultures from the 5-cell cryopreserved seawater inoculum that reached  $>10^4$  cells  $\text{ml}^{-1}$  were cryopreserved (500  $\mu\text{l}$  of culture with 10% [vol/vol] glycerol, final concentration) as described for cultures obtained with a raw seawater inoculum. Cells in the remaining volume ( $\sim 18$  ml) were collected by filtration through 0.03- $\mu\text{m}$ -pore-sized PES membrane filters (Sterlitech), submerged in 250  $\mu\text{l}$  DNA lysis buffer, and subsequently stored at  $-80^\circ\text{C}$  until DNA extraction.

The 105-cell inoculum was not subcultured. Instead, wells from one 96-well microtiter plate that exhibited growth ( $>10^4$  cells  $\text{ml}^{-1}$ ) were cryopreserved by combining 60  $\mu\text{l}$  of 50% glycerol solution with 250  $\mu\text{l}$  of subculture (10% [vol/vol] final concentration) in 2-ml cryovials (Nalgene) at room temperature (24°C), mixed by inverting the vials, and cooled at a rate of  $-1^\circ\text{C min}^{-1}$  with a Cryo 1°C freezing container (Nalgene) inside a  $-80^\circ\text{C}$  ultracold freezer. Cells in the remaining volume of subculture ( $\sim 1$  ml) were collected by filtration through 0.03- $\mu\text{m}$ -pore-sized PES membrane filters (Sterlitech), submerged in 250  $\mu\text{l}$  DNA lysis buffer, and subsequently stored at  $-80^\circ\text{C}$  until DNA extraction.

**DNA extraction and sequencing.** Genomic DNA was extracted from the environmental sample and all 5-cell subcultures that exhibited growth using the Qiagen DNeasy blood and tissue kit (Qiagen, Germantown, MD, USA), with modifications (53). Genomic DNA was extracted from one 96-well microtiter plate of the 105-cell cryopreserved seawater inoculum cultures that exhibited growth using the DNeasy 96 blood and tissue kit (Qiagen) in accordance with the manufacturer's protocol. Genomic DNA from the environmental sample and all 5-cell subcultures was used as the template for PCR amplification (Bio Rad C1000 Touch; Bio Rad, Hercules, CA, USA) using barcoded 515Y and 926R primers targeting the V45 region of the SSU rRNA gene (54) in a total reaction volume of 25  $\mu\text{l}$  containing 2  $\mu\text{l}$  of the genomic DNA template, 0.5  $\mu\text{l}$  of each forward and reverse primer, 10  $\mu\text{l}$  of 5PRIME HotMasterMix (Quantabio, Beverly, MA, USA), and 12  $\mu\text{l}$  of  $\text{H}_2\text{O}$ . The reaction included an initial denaturing step of 3 min at 94°C, followed by 40 cycles of 45 s at 94°C, 1 min at 50°C, and 1.5 min at 72°C, and a final extension of 10 min at 72°C.

A nested-PCR approach was used to amplify SSU rRNA gene fragments from genomic DNA recovered from the 105-cell inoculum cryopreserved seawater cultures. The first reaction employed bacterial 27FB (55) and 1492R (56) primers in a 25- $\mu\text{l}$  total reaction volume as described above. The reaction included an initial denaturing step of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 1 min at 50°C, and 45 s at 72°C, and a final extension of 18 min at 72°C. PCR products from the first amplification were then used as the template for a second amplification reaction using the barcoded 515Y and 926R primers (54), using the same reaction conditions as described for the 5-cell inoculum samples.

All PCR products were quantified (Qubit 2.0; Invitrogen), pooled at a concentration of 240 ng per sample, and cleaned (QIAquick PCR purification kit; Qiagen). Pooled products were sequenced via three Illumina MiSeq 250-bp paired-end runs using v.2 reagent kits.

**Sequence analysis.** The three Illumina MiSeq runs were each separately imported into QIIME2 v2019.4.0 and demultiplexed, and paired ends were analyzed for sequence quality and merged (57). The DADA2 software package (58) was then used to denoise sequences, which included removal of chimeras. Due to the low quality at the end of the sequences, 10 bases were truncated from the 3' end of the reverse reads. Sequence reads from the three runs were then merged postdenoising. Amplicon sequence variant (ASV) identities were defined by DADA2 for all reads that varied by at least 1 bp. Taxonomy was assigned to each ASV using a Naïve Bayes classifier trained on the Silva rRNA v132 database (59), clustered at 99% similarity, and subsequently modified manually based on phylogenetic analyses and the results of previous work. Denoised sequences, ASVs, and taxonomy classifications were imported into R v3.5 (60) using the phyloseq v1.26.1 package (61) for additional manual curation as outlined below. Visualizations were created in R using ggplot2 (62) and in BioVenn (63).

The identities of ASVs found within the cultures and the environmental sample were assigned by QIIME2. For each culture, ASVs represented by fewer than 20 reads were discarded from the data set in order to account for potential sequencing error. Subsequently, the proportion of each ASV in an individual culture was calculated using the read count for that ASV divided by the total number of reads from the culture postcuration. Cultures were functionally divided into three separate categories: "monocultures," "mixed cultures," and cultures with no discernible, dominant member. All cultures that consisted of  $\geq 90\%$  of reads from a single ASV and contained no other ASVs that were  $\geq 5\%$  of reads were categorized as "monocultures," and that ASV was assigned a unique isolate identifier in the Hawai'i Institute of Marine Biology Culture Collection (prefix "HIMB" followed by a unique number). Cultures were defined as "mixed" if (i) they contained an ASV that accounted for  $< 90\%$  but  $\geq 50\%$  of the total reads for that particular culture (this ASV, as well as any other ASV within the mixed culture that contained  $> 5\%$  of the total reads, was assigned a unique HIMB identification number) and (ii) the culture consisted of  $\geq 90\%$  of reads from a single ASV and an additional ASV that was  $\geq 5\%$  of reads. Each of these were also assigned unique HIMB identification numbers. The final category consisted of culture wells that contained no ASVs accounting for  $\geq 50\%$  of the total reads; these were not considered further in the context of this study.

**Analysis of environmental samples.** All ASVs represented by  $< 20$  reads in the environmental sample were removed in order to account for sequencing error and artifacts. All ASVs that were taxonomically identified as "chloroplast" at the bacterial-order level in the Silva taxonomy were also removed from the data set. The relative abundance of each remaining ASV was calculated as the read count of



the individual ASV divided by the total number of reads in the environmental sample postcuration. Unique identifiers were assigned to all ASVs that remained in the data set postcuration.

**Culturability statistics.** Fundamental culturability statistics were derived as outlined previously (16). Briefly, percent viability (culturability), or  $V$ , is defined as the ratio of the number of viable cells to the total number of cells initially present. It was calculated using the formula  $V = -\ln(1 - p)/X$ , where  $p$  is the proportion of wells that scored positive for growth, and  $X$  is the number of cells used for the initial inoculation. To obtain 95% confidence intervals, the exact upper and lower 95% confidence limits for  $p$  were calculated and inserted back into the original viability equation in place of  $p$ . The result is the exact upper and lower 95% confidence limits for percent culturability. For this experiment,  $p$  is defined as the number of cultures that were determined to be either monocultures or mixed cultures as described above. Two separate culturability statistics were calculated: one including mono- and mixed cultures and one including only monocultures.

**Phylogenetic analyses.** Amplicon sequences corresponding to all ASVs were imported into the ARB software package (64) and aligned using SINA v.1.2.11 (65) to a curated database of marine bacterial 16S rRNA gene sequences based on Silva v.95. Phylogenetic analyses were performed using the RAxML maximum likelihood method with the GTR model of nucleotide substitution under the gamma and invariable models of rate heterogeneity (66). The heat map of ASV relative abundance was constructed in R v.3.5 (60) using the ggplot2 package (62).

**Data availability.** Amplicon sequencing data are available in the Sequencing Read Archive (SRA) under BioProject number [PRJNA673898](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA673898). Strains are archived at the Hawai'i Institute of Marine Biology Culture Collection (HIMBCC) and available upon request.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**FIG S1**, TIF file, 2.4 MB.

**TABLE S1**, XLSX file, 0.1 MB.

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