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PROTOCOL

High-throughput cultivation based on dilution-to-extinction with catalase supplementation and a case study of cultivating acI bacteria from Lake Soyang

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Multi-omics approaches, including metagenomics and single-cell amplified genomics, have revolutionized our understanding of the hidden diversity and function of microbes in nature. Even in the omics age, cultivation is an essential discipline in microbial ecology since microbial cultures are necessary to assess the validity of an *in silico* prediction about the microbial metabolism and to isolate viruses infecting bacteria and archaea. However, the ecophysiological characteristics of predominant freshwater bacterial lineages remain largely unknown due to the scarcity of cultured representatives. In an ongoing effort to cultivate the uncultured majority of freshwater bacteria, the most abundant freshwater *Actinobacteria* acI clade has recently been cultivated from Lake Soyang through catalase-supplemented high-throughput cultivation based on dilution-to-extinction. This method involves physical isolation of target microbes from mixed populations, culture media simulating natural habitats, and removal of toxic compounds. In this protocol, we describe detailed procedures for isolating freshwater oligotrophic microbes, as well as the essence of the dilution-to-extinction culturing. As a case study employing the catalase-supplemented dilution-to-extinction protocol, we also report a cultivation trial using a water sample collected from Lake Soyang. Of the 480 cultivation wells inoculated with a single lake-water sample, 75 new acI strains belonging to 8 acI tribes (acI-A1, A2, A4, A5, A6, A7, B1, B4, C1, and C2) were cultivated, and each representative strain per subclade could be revived from glycerol stocks. These cultivation results demonstrate that the protocol described in this study is efficient in isolating freshwater bacterioplankton harboring streamlined genomes.

Keywords: dilution-to-extinction, cultivation, high-throughput culturing, freshwater, catalase, acI bacteria, *Candidatus* Planktophila

Overview

The notion "the great plate count anomaly" (Staley and Konopka, 1985) was first proposed to describe the vast discrepancy between the number of microbial cells in a natural sample quantitated by epifluorescence microscopy and that of the colony-forming units counted using the standard agar plating method. This concept has been expanded from the simple cell counting to molecular microbial diversity through culture-independent analyses based on 16S rRNA gene and specific metabolism-related genes, such as cloning and sequencing, denaturing gradient gel electrophoresis, fluorescence *in situ* hybridization (FISH), microautoradiography-FISH, stable-isotope probing, and DNA microarray (*see* Fig. 1 for culture-dependent and culture-independent methods). Consequently, it has been confirmed that the majority of prokaryotic taxa have not yet been cultured. More recently, owing to the revolution of sequencing technologies, multi-omics approaches (Zhang *et al.*, 2010) encompassing metagenomics, metatranscriptomics, and metaproteomics have been widely applied to the field of microbial ecology and expanded our knowledge about the hidden diversities and functions of natural microbial assemblages. Countless single-cell amplified genomes and metagenome-assembled genomes retrieved from myriads of multi-omics data have highly contributed to the interpretation of "microbial dark matter" (Rinke *et al.*, 2013; Williams and Embley, 2014) and generated data-driven hypotheses on the microbial metabolism and ecology.

Even in the multi-omics era, where a vast amount of genomics data on natural microorganisms are generated, cultivation is still an irreplaceable approach in microbial ecology. As Giovannoni and Stingl (2007) stated more than a decade ago, "cultures are important because they provide complete genomes and the means to test the hypotheses that emerge from genomic data." Microbial cultures are also important in the evaluation of metagenomics data, functional characterization of genes related to novel physiology, and isolation of bacterial or archaeal viruses. In this context, it has recently been re-emphasized that investments on risky but rewarding culture-based and targeted studies on microbes "most-wanted"

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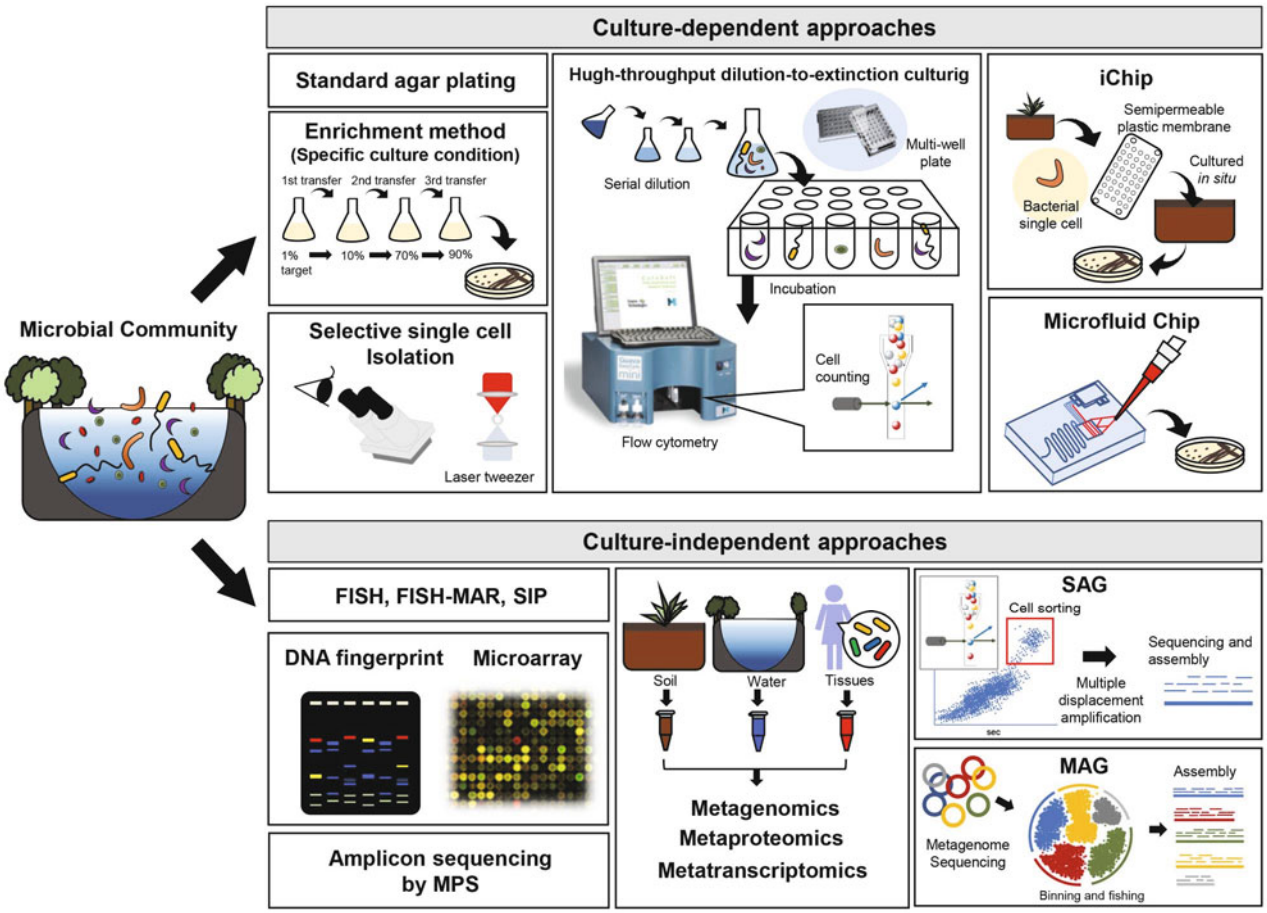

Fig. 1. Culture-dependent and -independent methods to investigate aquatic microbial communities. Abbreviations: FISH, fluorescence *in situ* hybridization; MAR, microautoradiography; SIP, stable-isotope probing; MPS, massively parallel sequencing; SAG, single-cell amplified genome; MAG, metagenome-assembled genome.

in culture are required to maximize the benefits of culturing the uncultured (Carini, 2019; Thrash, 2019).

To date, there have been very few attempts to grow fastidious microorganisms under laboratory settings by using multi-omics-derived information (Cross *et al.*, 2019). However, with the development of innovative cultivation techniques, many cultivation efforts have been made to domesticate as-yet-uncultured microorganisms. These methodologies modifying the traditional cultivation techniques or adopting recently developed advanced technologies (*see* also Fig. 1) include modified agar plating methods (Janssen *et al.*, 2002; Tamaki *et al.*, 2009; Kato *et al.*, 2020), long-term enrichment cultivation (Daims *et al.*, 2015; Yu *et al.*, 2018), high-throughput culturing based on dilution-to-extinction (HTC) (Connon and Giovannoni, 2002; Cho and Giovannoni, 2004; Henson *et al.*, 2016; Kim *et al.*, 2019), cultivation by the detoxification of reactive oxygen species (Morris *et al.*, 2008; Kim *et al.*, 2016, 2019), single cell isolation using a laser tweezer (Keloth *et al.*, 2018), "culturomics" under hundreds of different types of culture conditions (Oberhardt *et al.*, 2015; Lagier *et al.*, 2016), cultivation with a diffusion growth chamber (Kaeberlein *et al.*, 2002; Jung *et al.*, 2014; Chaudhary *et al.*, 2019), encapsulation of cells in gel microdroplets (Zengler *et al.*, 2002; Dichosa *et al.*, 2014), microfluidic chips (Boitard *et al.*, 2015; Hansen *et al.*, 2019), and isolation chips (Nichols *et al.*, 2010; Gao *et al.*, 2013; Berdy *et al.*, 2017).

These successful cultivation stories mainly rely on the following strategies: physical isolation of target microbes from community members, specific enrichment of target microbes under desired culture conditions, preparation of media composition by simulating natural habitats, and removal of toxic compounds. Of the cultivation methods listed above, HTC fits well with the successful cultivation strategies, because ca. 1–5 cells are inoculated into a physically separated well (Connon and Giovannoni, 2002), seawater- and freshwater-based medium or defined synthetic medium are used to mimic natural environments (Cho and Giovannoni, 2004; Henson *et al.*, 2016; Kim *et al.*, 2017), and chemicals removing toxic substances, such as H_2O_2-detoxifying catalase, are added to the culture medium (Kim *et al.*, 2016, 2019). For this reason, HTC has been initially applied to marine environments and later utilized to cultivate groundwater (Connon *et al.*, 2005) and lake water (Salcher *et al.*, 2015) bacterioplankton. Through HTC, previously uncultured marine oligotrophic bacterioplankton, including the predominant SAR11 clade (*Candidatus* Pelagibacter) (Rappé *et al.*, 2002; Song *et al.*, 2009), SAR-

116 clade (*Candidatus* Puniceispirillum) (Henson *et al.*, 2016; Lee *et al.*, 2019), OM43 clade (Giovannoni *et al.*, 2008), oligotrophic marine *Gammaproteobacteria* group (Cho and Giovannoni, 2004), OM60 clade (Cho *et al.*, 2007), and SUP05 clade (Shah *et al.*, 2017; Spietz *et al.*, 2019) have been successfully domesticated and further studied based on their physiologies and genomes.

Whereas HTC has been successfully applied to cultivate marine bacterioplankton, thereby significantly contributing to marine microbiology, relatively less effort has been made for the cultivation of microorganisms living in freshwater. Many cultivation trials have focused on colony-forming bacteria that easily grow on copiotrophic agar plates (Allen *et al.*, 1983; Hahn and Höfle, 1998). Following the isolation of the SAR11 clade by researchers led by Steve Giovannoni (Rappé *et al.*, 2002), the HTC method was used to isolate freshwater bacterioplankton from Crater Lake (Page *et al.*, 2004) by the same laboratory, resulting in the cultivation of several important isolates belonging to the GKS16 and GKS2-216 clades and the genus *Polynucleobacter*. However, no other HTC study from freshwater environments has been performed until Salcher *et al.* (2015) reported the isolation of freshwater methylotrophs from Lake Zurich through the dilution-to-extinction culturing. As another attempt to isolate small-sized bacterioplankton, the filtration-acclimatization method, which employed 0.2 μm filtered freshwater samples as the inocula, and the serial adaption to high concentrations of organic nutrients as an enrichment method yielded the isolation of *Polynucleobacter* and the *Actinobacteria* Luna clade (Hahn, 2003, 2009; Hahn *et al.*, 2003). The genus *Limnohabitans*, one of the major freshwater bacterioplankton groups, was also isolated using a modified filtration-acclimatization method in predator-free dilution cultures (Kasalický *et al.*, 2010). Since *Polynucleobacter* and *Limnohabitans* are ubiquitous in freshwater ecosystems worldwide and easier to grow than other prevailing microbial groups, studies on their ecological roles, biogeography, and evolution have flourished (Boscaro *et al.*, 2013; Hahn *et al.*, 2016; Sangwan *et al.*, 2016; Horňák *et al.*, 2017; Jezberová *et al.*, 2017). The very first successful report on the cultivation of abundant freshwater bacterioplankton by using the HTC method was about the isolation of freshwater methylotroph LD28 clade ("*Candidatus* Methylopumilus") (Salcher *et al.*, 2015), which is the sister group of marine methylotroph OM43 clade (Giovannoni *et al.*, 2008). Subsequent phenotypic and genomic analyses showed that the members of the LD28 clade follow an oligotrophic lifestyle, with the function of methyl utilization encoded in the streamlined genomes, and might experience a habit transition from freshwater to marine environments through the adaptive gene losses and gains (Salcher *et al.*, 2019).

Although the most abundant marine bacterial groups *Candidatus* Pelagibacter (Rappé *et al.*, 2002), *Prochlorococcus* (Chisholm *et al.*, 1992), and *Nitrosopumilus* (Könneke *et al.*, 2005) were cultivated during the 1990s and 2000s, the most abundant freshwater bacterial groups (the acI clade of *Actinobacteria* and the LD12 clade of *Alphaproteobacteria*) could not be domesticated for a long time. However, a representative of the LD12 clade (*Candidatus* Fonsibacter), which is a freshwater sister group of the marine SAR11 clade, has recently been isolated from coastal brackish water through a modified HTC method employing defined synthetic media (Henson *et al.*, 2018). The acI clade is the predominant freshwater *Actinobacteria* group, often representing > 50% of the microbial cells in a lake ecosystem. This group was first cultivated from Lake Soyang, Korea, through the HTC method, whereby four complete acI bacterial genome sequences were obtained (Kang *et al.*, 2017). One year later, 16 acI cultures were retrieved from Lake Zurich by using almost the same dilution-to-extinction culturing method (Neuenschwander *et al.*, 2018). However, all efforts to maintain stably-growing acI cultures had failed until two acI strains belonging to the acI-A1 ("*Candidatus* Planktophila rubra") and acI-A4 ("*Candidatus* Planktophila aquatilis") subclades originally cultured from Lake Soyang could be stably grown in a freshwater-based medium supplemented with catalase (Kim *et al.*, 2019). The simple catalase-supplemented HTC method, developed by our research group, lowers the hydrogen peroxide concentration in the culture medium. Since this method was effective in maintaining acI cultures, it has been suggested that the same method can be widely used for the cultivation of genome-streamlined bacterioplankton.

The protocols described in this study are based on this successful cultivation story of acI bacteria. Here, we present the detailed procedures for isolating aquatic oligotrophic bacteria through the catalase-supplemented HTC technique, including medium preparation, inoculation, screening, and preservation. We also report the result of a cultivation case study that applied the catalase-supplemented HTC method to a single lake-water sample collected from Lake Soyang in 2016. From 480 dilution-to-extinction wells inoculated with this single sample, 75 new acI strains belonging to 8 acI tribes were successfully cultivated using the catalase-supplemented HTC method, demonstrating the efficacy of the technique in isolating bacterioplankton with streamlined genomes.

Applications

As Thrash *et al.* (2017) indicated in their methodology paper, HTC can theoretically be applied to any type of water ecosystems, such as ocean, lake, river, stream, groundwater, and soil pore water, as long as a sufficient amount of sample can be obtained to prepare the culture media. Being a laboratory using HTC, with a focus on cultivating marine and freshwater bacterioplankton, our laboratory began cultivating microorganisms that inhabit aquifer systems. The preliminary results of using the HTC method to cultivate aquifer microorganisms are remarkable; many previously uncultured representatives from diverse phyla, noticeably from a novel phylum, could be cultured for the first time (unpublished data), which serves as a proof-of-concept of this method. A modified HTC method has also recently been applied to oligotrophic soil environments (Bartelme *et al.*, 2020). In that study, 5 cells/ml extracted using a buoyant density gradient centrifugation from the forest soil were inoculated into a defined synthetic medium, whereby some of the previously uncultured *Actinobacteria* and *Alphaproteobacteria* have been shown to be readily cultivable (Bartelme *et al.*, 2020). Through a modified HTC method employing a seawater-based medium supplemented with sulfate as an electron acceptor in an anaerobic growth

chamber, we have also recently isolated new groups of sulfate-reducing bacteria inhabiting anoxic tidal flat sediments (unpublished data). Therefore, we believe that the key concept of the HTC method, inoculation of physically separated cells into culture media mimicking natural environments, works for any environment harboring active microorganisms.

Despite the success in cultivating the previously uncultured majority, such as "*Candidatus* Pelagibacter"and "*Candidatus* Planktophila", through the HTC method, it should be noted that the initial cultivation of these bacteria was a "serendipity" since HTC cannot target specific groups of microorganisms. Only after the genomes of the isolates were sequenced, stable culture conditions hinted by the genomic information could be determined to maintain the isolates, such as optimization of carbon sources, vitamins, amino acids, and catalase supplements (Tripp *et al.*, 2008; Carini *et al.*, 2013, 2014; Kim *et al.*, 2019). Due to this limitation of the HTC method, the "most-wanted" microbes, such as the clades SAR202, SAR86, OM1, marine group A, and several "microbial dark matter" (Carini, 2019), remain uncultivated despite the tremendous effort given to domesticate them. Innovative and creative cultivation trials, such as the recently reported method for targeted microbial isolation through reverse genomics (Cross *et al.*, 2019), are needed to enrich the cultured representatives. In this regard, our case study, which will be discussed in detail later, is noteworthy since it reports the cultivation of 75 novel strains belonging to 8 subclades (acI-A1, A2, A4, A5, A6, A7, B1, B4, C1, and C2) of the acI clade through the catalase-supplemented HTC method developed using the information deduced from acI genome sequences. Since the growth of marine photoautotroph *Prochlorococcus* and ammonia-oxidizing *Nitrosopumilus* was enhanced at low concentrations of hydrogen peroxide (Morris *et al.*, 2011, 2012; Kim *et al.*, 2016), the employment of the catalase-supplemented HTC method in relevant culture conditions (under the light environment or high concentration of ammonium) may help expand the culture collection of such microorganisms.

Methods

Preparation of culture medium

Almost all successful stories in cultivating as-yet-uncultured microorganisms from aquatic ecosystems relied on the use

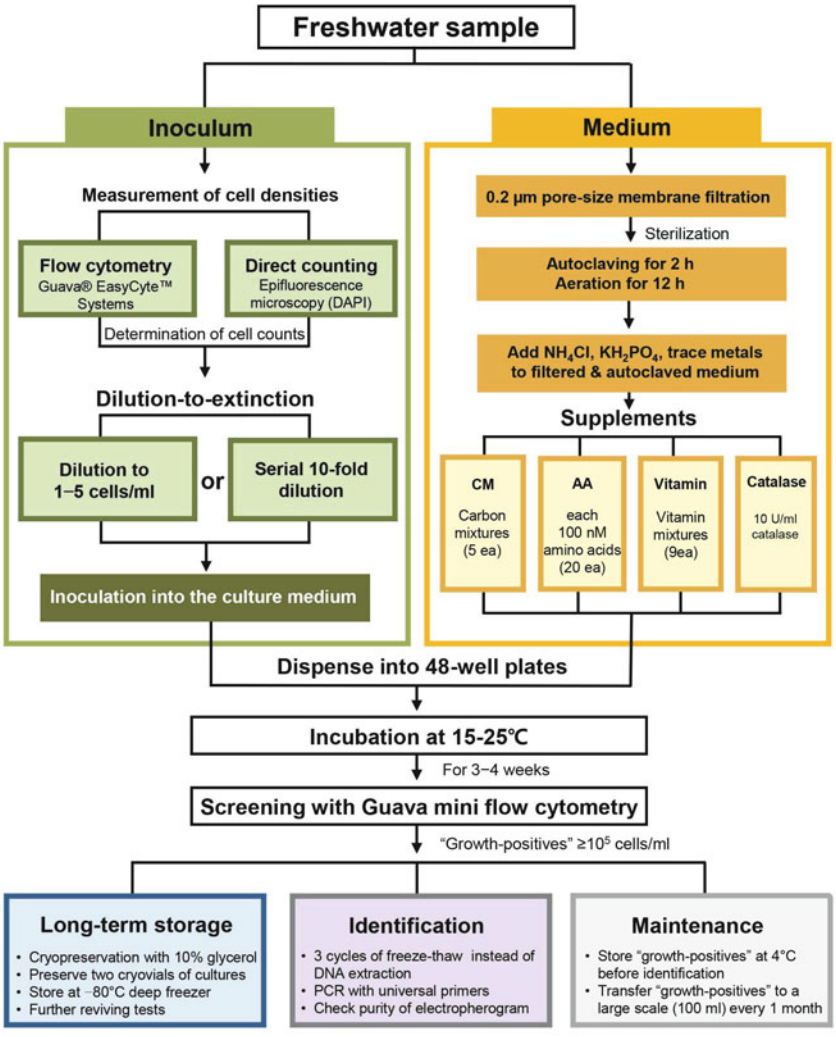

Fig. 2. **Experimental scheme of the dilution-to-extinction culturing applied to the cultivation of the freshwater acI clade.**

of growth conditions (e.g., media components and physical parameters) that mimic the physicochemical conditions of the corresponding natural environments. Because marine environments, especially pelagic zone, are nutrient-limited, oligotrophic microorganisms adapted to low-nutrition environments predominate. Nutritional conditions of surface freshwater environments vary according to anthropogenic effects, but many large and deep lakes are also known to be oligotrophic. For this reason, marine agar 2216, nutrient agar, trypticase soy agar, R2A agar, and their non-agar broths, which have been traditionally used to isolate aquatic microorganisms, have failed to cultivate predominant oligotrophic microorganisms. In the 1990s, the dilution-to-extinction culturing method pioneered by Button *et al.* (1993) showed for the first time that unamended environmental seawater could be successfully used as a culture medium to cultivate oligotrophic and heterotrophic marine bacteria. Our protocol, outlined in Fig. 2, is based on the ideas of Button *et al.* (1993, 1998). It is also founded on a procedure that was developed and modified by Steve Giovannoni and colleagues, including ourselves (Connon and Giovannoni, 2002; Rappé *et al.*, 2002; Cho and Giovannoni, 2004; Stingl *et al.*, 2007; Song *et al.*, 2009; Henson *et al.*, 2016; Shah *et al.*, 2017; Kim *et al.*, 2019).

There are two different ways to make a culture medium that mimics the natural aquatic environment, through the use of i) natural water, such as seawater and freshwater, collected on-site for a cultivation trial and ii) defined synthetic media based on the chemical composition of the aquatic system. Each of these methods has distinct advantages and disadvantages. The culture medium based on natural water acts as a sterilized habitat for microorganisms living in the aquatic ecosystem and provides an environment in which fastidious microorganisms can successfully proliferate. However, standardization of such a medium for use as a generalized culture medium is difficult because the chemical composition of a water sample depends on the sampling time and location. The formulas of defined synthetic media are designed artificially based on the chemical composition of the habitat of microorganisms to be cultured, in consideration of concentration of basic salts, carbon, nitrogen, phosphorous, trace metals, amino acids, vitamins, and other nutritional or chemical requirements (Carini *et al.*, 2013, 2014; Henson *et al.*, 2016; Thrash *et al.*, 2017). The defined synthetic medium is, of course, easy to be standardized so that the medium can be used to proliferate microbial isolates in any microbiological laboratory, thereby circumventing the need to collect and transport natural water. However, a disadvantage of a defined synthetic medium over a natural water-based medium is the difficulty of making the chemical composition identical to that of the natural environment. Thus, the cultivation rate for predominate oligotrophic microorganisms in a defined medium is likely to be lower than that in a natural water-based medium. Microbial isolates that initially proliferated in a natural water-based medium may be further adapted to a defined synthetic medium. Therefore, this protocol, which was also used for the case study, focuses on the use of a culture medium based on natural lake water.

The first step for using natural water as a culture medium is to remove microorganisms present in the water sample, and this step can be achieved through filtration. In the HTC method, in order not to interfere with the cell count determined through flow cytometry, the water sample should be filtered through a 0.2 μm filter. After the first filtration of natural water with 0.2 μm, autoclaving is preferred to make the medium sterile. Although autoclaving can sterilize the fluid completely, the sterilization process causes loss of oxygen and carbonate buffering capacity and decomposition of several essential nutrients such as heat-labile vitamins. Therefore, after autoclaving, aeration is required. For seawater-based medium, CO_2 sparging is also needed to restore bicarbonate buffering capacity before aeration. However, for freshwater-based medium, since the amount of bicarbonate lost during autoclaving is negligible, CO_2 sparging is not necessary. To finalize the preparation of the freshwater-based medium, any elements lost during autoclaving should be added alongside the additives that enhance the growth. In this stage, to support heterotrophic growth, diverse carbon sources, amino acids, and vitamins are usually added. Especially, because many oligotrophic bacteria that have streamlined genomes, such as SAR11 and acI bacteria, exhibit auxotrophy for several vitamin B compounds, reduced sulfur, and several amino acids (Tripp *et al.*, 2008; Carini *et al.*, 2014; Kang *et al.*, 2017), all vitamin and amino acid families should be better supplemented. The last requirement for cultivating acI bacteria is to supplement the culture medium with catalase since the simple catalase-supplemented HTC method can maintain stably growing acI cultures (Kim *et al.*, 2019).

Dilution and inoculation

The cultivation approach described in this protocol uses the dilution-to-extinction method. The essence of the dilution-to-extinction has been practically applied to the most probable number (MPN) method since the 1930s (Halvorson and Ziegler, 1933; Cochran, 1950). The MPN method, which is historically called the method of ultimate or extinction dilution, is a statistical method to estimate viable cell numbers in a sample by inoculating a broth medium in serial 10-fold dilutions and has been applied to environmental samples where measurement of colony-forming units is unsuitable. Since the MPN method dilutes the original population to almost extinction to grow, the method is called an extinction dilution method. The HTC method uses the same theory of dilution culturing. However, the MPN method targets to predict the number of cells in a pre-diluted sample according to the positive or negative growth rate in the broth tubes. In contrast, the HTC method relies on the actual cell number of "growth-positives" to obtain actively growing isolates.

Once the natural water-based medium is prepared, the next step for HTC is to dilute the original microbial populations to near extinction in the culture medium. Total cell counts in the water sample are better to be determined by using the following methods: flow cytometry and epifluorescence microscopy. As long as microbial cells in the water sample are stained well with SYBR Green I and diluted suitable to be detected with the flow cytometry, the cell counts determined by the flow cytometry would be preferred because epifluorescence microscopic counts might cause systematic error according to the user's proficiency. Microbial cells can be diluted into 48-well microtiter plates through either of the following two methods: dilution to a specific number of cells

(usually 1–5 cells/ml) or serial 10-fold dilution to near extinction. If the numbers of cells estimated by flow cytometry and epifluorescence microscopy are comparable, and the water sample does not contain much solid material interrupting the cell counting, a specific desired number of cells are preferred to be inoculated into the medium. For sediment, soil slurry, and a water sample containing high amounts of suspended particles, serial 10-fold dilution until the extinction of the microbial population is recommended. For example, assuming that a surface lake water has 10^6 cells/ml, 10^{-5}, 10^{-6}, and 10^{-7} dilution would inoculate 10, 1, and 0.1 cells/ml into the medium. Since too much dilution would yield low culturability, and too little dilution would cultivate mixed populations rather than pure cultures, screening multi-well plates that have a reasonable number of "growth-positives" would minimize failure in the cultivation trials. By diluting natural microbial populations in this way, slowly replicating oligotrophic bacteria are physically separated and can be prevented from being outcompeted by rapidly growing "weed" microbes.

Incubation, screening, and identification

Incubation conditions such as temperature, period, aerobic or anaerobic, and light or dark affect the cultivation efficiency. The rule of thumb for cultivation experiments is to imitate the natural environment. In the case study using the catalase-supplement HTC method presented below, the incubation temperature was set at 25°C, because the water temperature in Lake Soyang in July was 27°C. Since incubation temperature 2–5°C lower than ambient water temperature empirically worked well for the proliferation of aquatic microbes in our laboratory, we usually set the incubation temperature for the HTC in Lake Soyang to 15–25°C.

Considering the dilution rates leading to near extinction, long-term incubation is a prerequisite for the HTC method. Doubling times of marine bacterioplankton range from 1 day to 1 week (Button *et al.*, 1993), i.e., nearly 2 days for "*Candidatus* Pelagibacter ubique" in the laboratory culture condition (Carini *et al.*, 2013). Doubling times of freshwater bacterial isolates, including *Actinobacteria* Luna-I and *Sphingobacterium* sp. were reported to be 14–37 h (Hörtnagl *et al.*, 2011). If the doubling time of target freshwater bacteria for cultivation is 1 day and 1 cell is inoculated per ml of cultivation well, cell numbers exceeding the detection limit (10^4 cells/ml) of the Guava flow cytometry and 10^6 cells/ml are achieved after 14 and 20 days of incubation, respectively. In this regard, we usually screen the inoculated multi-well plates after 3–4 weeks of incubation. As Song *et al.* (2009) showed, an extended incubation may be required to obtain more "growth-positive" cultures if the number of positive wells is too low. Screening for "growth-positives" has been performed using a 48-well manifold for enumeration by epifluorescence microscopy (Connon and Giovannoni, 2002). However, a flow cytometric method is widely preferred over epifluorescence microscopy, owing to facileness and effectiveness (Tripp, 2008).

After "growth-positive" wells are screened from the multi-well plates, the initial liquid cultures can be efficiently identified based on the routine 16S rRNA gene-based sequencing method. For the 16S rRNA gene PCR of marine microbial isolates, a DNA extraction step is required to prevent PCR inhibition by salts. However, it is noteworthy that the DNA extraction step for PCR is not necessary for freshwater bacterial cultures. We found the 16S rRNA genes of most freshwater bacterial cultures isolated by the HTC were successfully amplified without any interference after three cycles of freeze-thaw.

Materials

Reagents

- Bovine catalase (C9322, Sigma-Aldrich)
- 4′,6-diamidino-2-phenylinole (DAPI, Sigma-Aldrich)
- SYBR Green I (10,000× solution, Invitrogen)
- Formalin (37% formaldehyde solution, Merck)
- Hydrochloric acid (35–37%, Samchun Chemicals)
- Ethanol (100%, Merck)
- Glycerol (Sigma-Aldrich)
- R2A Agar (BD Difco)
- TE buffer: 10 mM Tris, 1 mM EDTA (pH 8.0)
- *Taq* polymerase (Ex Taq, Takara Bio)
- PCR and sequencing primers: 27F-B (5′-AGRGTTYGA TYMTGGCTCAG-3′), 1492R (5′-GGYTACCTTGTTA CGACTT-3′), 800R (5′-TACCAGGGTATCTAATCC-3′), 518F (5′-CCAGCAGCCGCGGTAATACG-3′)
- Media components: ***See* Tables 1 and 2**

Equipment

- Niskin water sampler (Model 1010, General Oceanics)
- Polycarbonate carboys (1 L, 2 L, 4 L, and 20 L; Nalgene)
- Sterile polyethylene water-collection bottles (2 L and 4 L, Daihan)
- Peristaltic pump (Masterflex, Cole-Parmer)
- 142 mm filter unit (Geotech)
- 47 mm polyethersulfone membrane filter with 0.2 μm pore size (Pall)
- 47 mm cellulose acetate membrane filter with 0.2 μm pore size (Advantec)
- HEPA-VENT (0.3 μm glass microfiber), PolyVENT (0.2 μm PTFE membrane), and Polydisc TF (0.1 μm PTFE membrane) in-line filter devices (Whatman)
- Heat-resistant silicone tubing (5 × 9 mm, Korea Ace Scientific)
- Aquarium air pump (Daekwang)

Table 1. Inorganic components added to the filtered and autoclaved freshwater medium

Component(s)	Compound	Final concentration	Stock concentration
Nitrogen	NH_4Cl	10 μM	100 mM
Phosphorous	KH_2PO_4	10 μM	100 mM
Trace metals	$FeCl_3{\cdot}6H_2O$	117 nM	11.7 mM
	$MnCl_2{\cdot}4H_2O$	9 nM	900 μM
	$ZnSO_4{\cdot}7H_2O$	800 pM	80 μM
	$CoCl_2{\cdot}6H_2O$	500 pM	50 μM
	$Na_2MoO_4{\cdot}2H_2O$	300 pM	30 μM
	Na_2SeO_3	1 nM	100 μM
	$NiCl_2{\cdot}6H_2O$	1 nM	100 μM

Table 2. Organic components added to the filtered and autoclaved freshwater medium

Components	Compound	Final concentration	Stock concentration
Vitamin mixture	Thiamine·HCl (B1)	59 nM	590 μM
	Riboflavin (B2)	1 nM	500 μM
	Niacin (B3)	81 nM	810 μM
	Ca-pantothenate (B5)	84 nM	840 μM
	Pyridoxine (B6)	59 nM	590 μM
	Biotin (B7)	409 pM	4.09 μM
	Folic acid (B9)	453 pM	4.53 μM
	Cobalamin (B12)	70 pM	700 nM
	myo-inositol	555 nM	5.55 mM
	p-Aminobenzoic Acid	7 nM	70 μM
Carbon mixture	Pyruvate	50 μM	500 mM
	D-Glucose	5 μM	50 mM
	N-Acetyl-d-glucosamine	5 μM	50 mM
	D-Ribose	5 μM	50 mM
	Methyl alcohol	5 μM	50 mM
Amino acid mixture	L-Alanine, L-Arginine Hydrochloride, L-Asparagine, L-Aspartic Acid, L-Cysteine hydrochloride, L-Glutamic Acid, L-Glutamine, Glycine, L-Histidine hydrochloride, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine	100 nM each	100 μM each[a]

[a] Amino acid stock solutions (all 20 mM, except that L-tyrosine was prepared at 2 mM) are prepared first, followed by the preparation of the amino acid mixtures with each amino acid at 100 μM.

- Acrodisc syringe filters with Supor membrane (0.2 μm, 0.1 μm, and 25 mm; Pall)
- Slide (76 × 26 mm) and cover (24 × 24 mm) glasses (Paul Marienfeld)
- Guava EasyCyte Plus Flow Cytometry (Millipore)
- Epifluorescence microscope (80i, Nikon)
- Flat-bottom 48-well Clear Cell Culture Plate (BD Falcon)
- Round-bottom 96-well Assay plate (BD Falcon)
- Multichannel pipettes (Eppendorf)
- Cryovials (Nunc)
- Incubator
- Deep freezer
- Thermal cycler

Protocols

A. Preparation of media ingredients

1. Prepare the media ingredients listed in Tables 1 and 2.
2. Prepare 100mM stock solutions of NH_4Cl and KH_2PO_4 in Milli-Q water, sterilize by using 0.2 μm syringe filters, and aliquot into 2 ml cryovials. Store at -20°C.
3. Prepare mixture stock solutions of trace metals, carbons, vitamins, and amino acids separately. To prepare four separate mixture solutions, dissolve each component (e.g., each 20 amino acids for the amino acid mixture) in an appropriate volume of autoclaved Milli-Q water separately, mix them together, adjust the final concentration, filter-sterilize through 0.2 μm syringe filters, and aliquot into 2 ml cryovials (suitable for 2–3 times use). Store the trace-metal, carbon, vitamin, and amino acid mixtures separately at -20°C. Keep the vitamin mixture in dark or foil-covered vials. For immediate use, keep the mixtures at 4°C.
   ➢ **NOTE**: For easy combination, it is recommended to store media components in separate cryovials. Thereby, the components can be easily mixed whenever necessary.
4. Dissolve an appropriate amount of lyophilized bovine catalase powder in 10 mM PBS (pH 7.4) to make a 10^5 U/ml stock solution; store at 4°C.
   ➢ **NOTE:** The concentration of the bovine liver catalase product on the label depends on the lot number. Therefore, to calculate the amount of catalase powder needed, it is necessary to check the exact concentration of the product.
   ➢ **NOTE:** The lyophilized catalase powder is not completely soluble in 10 mM PBS buffer. Although, it dissolves better in a PBS buffer of higher concentration, high concentrations of PBS increase the salinity. High salinity has little effect on marine microorganisms but affects freshwater microbes. Therefore, this protocol uses a saturated solution of catalase in 10 mM PBS buffer, successively filtered through 0.2 μm and 0.1 μm syringe filters.

B. Preparation of freshwater-based culture medium

1. Thoroughly wash all the culture equipment directly contacting with the water samples and media, such as carboys, bottles, and flasks. Then acid-wash the reusable culture hardware by immersing them for at least 24 h in a water bath containing 10% (v/v) HCl. Rinse them five times with Milli-Q water, dry completely, and autoclave. After autoclaving, dry the culture hardware to remove residual moisture.
   ➢ **NOTE**: The autoclaved hardware can be stored with dust-caps or wrapped in foil at room temperature for a long time, but we recommend that they are autoclaved prior to use.
2. Collect the freshwater samples at the desired sampling depth by using a pre-washed Niskin water sampler. Transfer the samples to sterile 20 L or 4 L carboys in a cold-storage box.

Immediately transport them to the laboratory to prevent bacterial overgrowth.
➢ **NOTE**: Carboys of 4 L may be used for bacterial inocula, and 20 L carboys for culture media. Physicochemical parameters, such as temperature, dissolved oxygen, pH, and conductivity should be measured on-site.
3. After the transport to the laboratory, filter the water samples through 0.2 μm filters (142 mm) by using a peristaltic pump, and collect the filtrates in 4 L carboys.
4. Autoclave the filtered freshwater sample for 2 h, and cool it in a laminar flow at room temperature.
5. Set up the air sparging apparatus by sequentially attaching the 0.3, 0.2, and 0.1 μm in-line filter devices through silicone tubing; autoclave and then dry.
6. Attach the air sparging apparatus into a 4 L carboy, sparge gently with air for 12 h by using an air pump.
➢ **NOTE**: If seawater is used as the culture medium, a successive CO_2 and air sparging for 6 h and 12 h, respectively, is required to restore the carbonate buffering capacity.
7. Transfer 1 L of the filtered, autoclaved, and aerated lake water into a new acid-washed polycarbonate carboy or flask with a screw top. Use 25 ml or 50 ml sterile serological pipettes to transfer the medium.
8. To 1 L of the above medium, add 100 μl of NH_4Cl, 100 μl of KH_2PO_4, and 10 μl of the trace metal mixture stocks listed in Table 1, and designate the resulting mixture as "filtered and autoclaved freshwater medium" (FAM).
➢ **NOTE**: FAM can be stored at 4°C or room temperature. However, vitamins, carbons, amino acids, and catalase solution should be added to FAM immediately before use.
9. To 1 L of FAM, add 100 μl of vitamin mixture, 100 μl of carbon components, and 1 ml of amino acid mixture listed in Table 2, and 100 μl of catalase stock (final concentration, 10 U/ml).

C. Enumeration of total microbial cells for inoculum

1. To prevent the bottle effect, the water samples used as the inocula should be filled in 4 L or 20 L carboys.
➢ **NOTE**: Be sure to keep the water sample for inoculum in a separate cooler to prevent any chemical or biological contamination.
2. To determine the total cell numbers of the sample for the inoculum using the Guava flow cytometry, transfer 198 μl of the water sample into a round-bottom 96-well plate in triplicate.
3. Add 2 μl of 500× SYBR Green I solution to 198 μl of the sample (5× final concentration SYBR Green I) and incubate the plate for 1 h in the dark.
4. Count the total cell numbers by using Guava flow cytometry at 730 V green fluorescence for 10 sec or until the number of cells reaches 5,000. The samples with ≥ 500 cells/μl should be diluted with a filter-sterilized medium.
➢ **NOTE**: For the detailed protocols of using the Guava flow cytometry to count total cells or small cells such as *Pelagibacter*, refer to Tripp (2008) and Thrash *et al.* (2017).
5. To compare and confirm the total cell counts determined by the flow cytometry, DAPI-stained epifluorescence microscopy is recommended to be additionally used. For total microbial cell counts, stain 5 ml of the water sample with 15 μg/ml DAPI solution for 15 min in the dark.
6. Filter the stained sample through a 0.2 μm Nucleopore polycarbonate filter, dry in the dark, and mount the dried membrane on a glass slide by using immersion oil and a cover glass.
7. Count the number of cells from at least 30 randomly selected fields under an epifluorescence microscope, and determine the cell counts in consideration of the dilution factor.

D. Inoculation and incubation

1. Calculate a dilution factor to reach the target cell density based on the enumeration obtained above.
➢ **NOTE**: The target cell density for the HTC is typically set in the range of 1–5 cells/ml. Considering the Poisson distribution of the cells inoculated into each cultivation well in a multi-well plate, using 1 cell/ml as the target cell density often produces too low "growth-positive" wells.
2. Pre-dilute the inoculum to a cell density of 10^3 cells/ml in 40 ml of the culture medium (FAM supplemented with carbon, amino acids, and vitamins).
3. Dilute the pre-diluted inoculum to the target cell density (1–5 cells/ml) in 1 L of the culture medium.
4. Dispense 1 ml of the medium containing 1–5 cells into each well of a flat-bottom 48-well clear cell culture plate by using a multichannel pipette.
5. Seal the plates with Parafilm and incubate at the desired temperature and condition for 3–4 weeks.

E. Screening and preservation of growth-positives

1. To detect cellular growth through flow cytometry, transfer 99 μl of the cultures from each well of the 48-well plates into each well of round-bottom 96-well assay plates.
2. Add 1 μl of 500× SYBR Green I to 99 μl of the sample and incubate for 1 h in the dark.
3. Count the cell numbers using the Guava flow cytometry at 730 V green fluorescence for 10 sec or until the counts reach 5,000.
➢ **NOTE**: Since the purpose of this step is to determine

Table 3. Summary of the catalase-supplemented dilution-to extinction culturing of a single water sample collected from Lake Soyang

Inoculum size (cells/ml)	Number of inoculated wells	Number of "growth-positive" wells[a]	Number of pure cultures[b]	Culturing efficiency (%)[c]	Culturability (V)[d]
1	240	68	44	28.3	0.333
3	240	155	116	64.6	0.346
Total	480	223	160	46.5	ND

[a] Cell counts ≥ 1×10^5 cells/ml after incubation
[b] Based on the clear electropherogram peaks of the 16S rRNA gene sequences
[c] Number of "growth-positive" wells/number of inoculated wells
[d] $V = -\ln(1 - p)/X$, where p is the proportion of "growth-positive" wells and X is the number of inoculated cells

positive or negative cellular growth, dilution of the cultures to enumerate the precise cell numbers is not necessary.
4. Cell counts exceeding 10^4 cells/ml are generally considered as "growth-positive" in the Guava flow cytometry settings. Our laboratory empirically sets 5×10^4 cells/ml or 10^5 cells/ml as the thresholds for "growth-positive", depending on the cultivation results.
5. Transfer 100 µl of "growth-positive" cultures to Eppendorf tubes for 16S rRNA gene sequencing.
6. Transfer 200 µl of the "growth-positive" cultures to 2 ml cryovials in duplicate. Add 50 µl of 50% (v/v) glycerol (final concentration, 10%), and gently mix. Cryopreserve the cryovials in a -80°C deep-freezer or liquid N_2 tank.
7. Drop 10 µl of "growth-positive" cultures onto R2A, 1/10 R2A, or desired agar plates, and wait until the drops dry completely. Seal the agar plates with Parafilm and incubate at the desired temperature (usually 15–20°C).
8. Keep the 48-well plates holding the remaining cultures at 4°C until the "growth-positives" are identified by 16S rRNA gene sequencing.

F. 16S rRNA gene PCR and classification

1. Freeze and thaw the "growth-positive" cultures three times.
   ➢ **NOTE**: The freshwater cultures freeze-thawed three times are used directly as templates for PCR. In most cases, PCR works well for freshwater cultures. However, for seawater cultures, DNA extraction is required for PCR since sea salts inhibit PCR.
2. Perform PCR by using 27F and 1492R primers for the identification of *Bacteria*. To identify *Bacteria* and *Archaea* simultaneously, 515F and 926R primers can be used.
3. Follow the standard methods for 16S rRNA gene sequencing and identification.
   ➢ **NOTE**: The microbial community structure analyses without cultivation are often necessary to compare culture-dependent results. For the community structure analyses, refer to recently published protocols (Jo *et al.*, 2020; Kim *et al.*, 2020)

Results from the case study using the catalase-supplemented HTC method

The catalase-supplemented HTC experiment was performed using a single surface lake water sample collected from a depth of 1 m at the Soyang Dam in Lake Soyang (37°57′11″ N, 127°49′02″ E) in July 2016. The culture medium was prepared following the protocols mentioned above. In brief, filtered and autoclaved lake water was supplemented with bovine catalase, nitrogen, phosphate, and mixtures of metals, carbons, amino acids, and vitamins. A total of 480 wells from 10 multi-well plates (BD Falcon) were inoculated with 1 or 3 cells/ml (1 ml/well) and incubated at 25°C in the dark. After 4 weeks of incubation, microbial growth (≥ 10^5 cells/ml) was detected in 223 "growth-positive" wells using the Guava flow cytometry, resulting in 46.5% cultivation efficacy (Table 3). The culturability of 1 cell- and 3 cells-inoculated culture sets was 0.333 and 0.346, respectively, as determined by the equation $V = -\ln(1 - p)/X$, where V is culturability, p is the proportion of "growth-positive" wells, and X is the number of inoculated cells (Button *et al.*, 1993), showing the increased culturability compared to 0.122 of the HTC experiment for marine bacteria in the East Sea (Yang *et al.*, 2016). Based on the above protocols, 16S rRNA gene sequences were obtained after PCR by using 27F 1492R universal primers and 100 µl of "freeze-thawed" cultures as templates. Of 223 "growth-positive" cultures, 160 isolates (71.7%) were considered as putative pure cultures, according to clear electropherogram peaks generated using 518F and 800R sequencing primers. The remaining 63 wells were considered co-cultures and excluded from further analysis.

Taxonomic assignment of 16S rRNA gene sequences obtained from the 160 putative pure cultures was performed using the classification algorithm implemented in Mothur (Schloss *et al.*, 2009) with Silva database version 132 (Quast *et al.*, 2013). The HTC isolates were assigned to four phyla/classes (classes for *Proteobacteria*): *Actinobacteria* (76 strains), *Bacteroidetes* (9 strains), *Alphaproteobacteria* (27 strains), and *Gammaproteobacteria* (48 strains) (Table 4). At the family-level, three bacterial families, *Sporichthyaceae* (75 strains)

Table 4. Taxonomic assignment of the HTC isolates according to the SILVA taxonomy by using the 16S rRNA gene sequences

Phylum	Class	Order	Family	No. of Isolates
Actinobacteria	*Actinobacteria*	*Frankiales*	*Geodermatophilaceae*	1
			Sporichthyacea [a]	75
Bacteroidetes	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	1
			Saprospiraceae	2
		Flavobacteriales	*Crocinitomicaceae*	2
			Flavobacteriaceae	4
Proteobacteria	*Alphaproteobacteria*	*Acetobacterales*	*Acetobacteraceae*	2
		Caulobacterales	*Hyphomonadaceae*	2
		Rhizobiales	A0839	1
		Rhodobacterales	*Rhodobacteraceae*	5
		Sphingomonadales	*Sphingomonadaceae*	17
	Gammaproteobacteria	*Betaproteobacteriales*	*Burkholderiaceae*	42
			Methylophilaceae	4
		Enterobacteriales	*Enterobacteriaceae*	2
Total				160

[a] All the isolates assigned to the family *Sporichthyaceae* belonged to 8 tribes of the acI clade

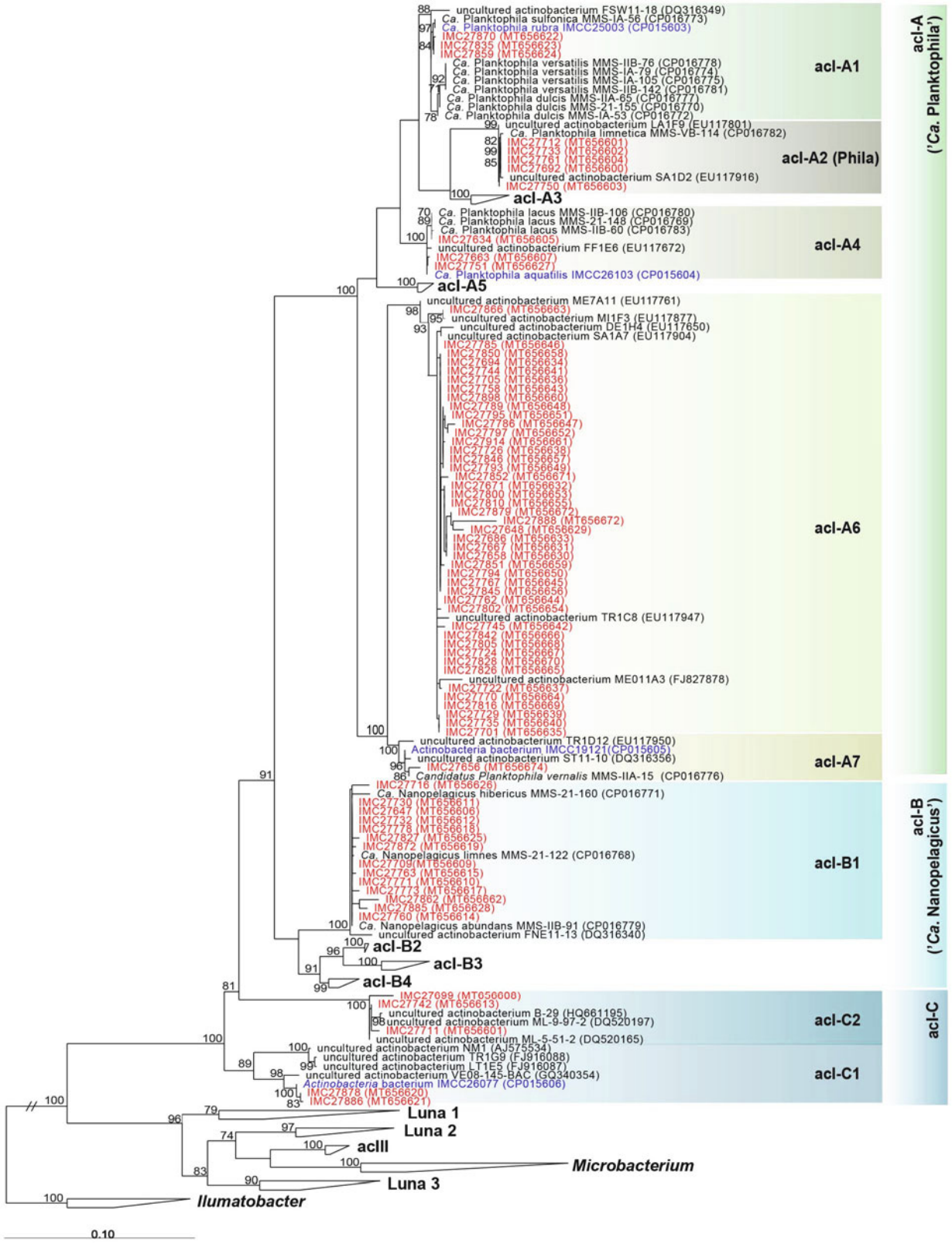

Fig. 3. Maximum-likelihood tree based on 16S rRNA gene sequences, showing the phylogenetic positions of the 75 strains belonging to the acI clade and cultivated in this case study. The tree was generated using the RAxML software (Stamatakis, 2014) and imported into the ARB software (Pruesse *et al.*, 2007) for phylogenetic grouping. All the isolates cultured in this case study are marked in red. *Illumatobacter coccineus* YM16-304 (AP012057), *Illumatobacter nonamiensis* YM16-303 (BAOL01000001), and *Illumatobacter fluminis* YM22-133 (AB360343) were used as outgroups. Bootstrap supporting values (from 500 replicates) > 70% are shown at the nodes. Bar, 0.10 substitutions per nucleotide position.

of *Actinobacteria*, *Burkholderiaceae* (42 strains) of *Gammaproteobacteria,* and *Sphingomonadaceae* (17 strains) of *Alphaproteobacteria*, were identified to be predominant.

Of these isolates, we focused on the ones assigned to *Sporichthyaceae*, the family that the acI clade belongs to, according to the Silva database, since we believed that the catalase-supplement HTC method would increase the chances of culturing the acI bacteria. The sequences assigned to *Sporichthyaceae* were aligned using the SINA web aligner (Ludwig *et al.*, 2004) and imported into the ARB-SILVA database (SSURef NR 99, release 123) (Pruesse *et al.*, 2012). A maximum-likelihood phylogenetic tree was generated in RAxML 8.2.7 (Stamatakis, 2014) using the aligned sequences, and each 16S rRNA gene sequence was phylogenetically assigned to a subclade or a tribe according to the freshwater bacterioplankton taxonomic framework (Newton *et al.*, 2011). All 75 *Sporichthyaceae* sequences were affiliated with the acI clade encompassing 8 diverse tribes; 3 strains of acI-A1, 5 strains of A2 (Phila), 3 strains of A4, 44 strains of A6, 1 strain of A7, 14 strains of B1, 2 strains of C1, and 3 strains of C2 (Fig. 3). Since only 20 strains of the acI clade have been reported to be cultivated from Korea (4 strains) and Europe (16 strains) to date, the new 75 strains of the acI clade are important resources in enriching the diversity of acI bacterial isolates. Particularly members of the acI-A6 and acI-C2 tribes were first cultivated in our present study. To check whether cryopreserved acI strains can resuscitate in a culture medium, a single strain selected from each acI tribe was melted from the cryopreserved stocks and inoculated to the fresh medium. All strains successfully revived from the glycerol stocks, indicating that the new acI strains are stably cultivated and maintained. Conclusively, the catalase-supplement HTC method described in this protocol allowed us to cultivate 75 stably-growing acI strains distributed in diverse acI phylogenetic tribes, suggesting that the method can be widely employed to cultivate previously uncultivated aquatic microbes, especially harboring streamlined genomes.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the acI isolates have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers MT656600-MT656674.

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Conflict of Interest

The authors declare that this study was conducted without any commercial or financial relationship that could be construed as a potential conflict of interest.

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