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Accessing previously uncultured marine microbial resources by a combination of alternative cultivation methods

Dawoon Jung,[1] Biyin Liu,[1] Xiaoping He,[1] Jeffrey S. Owen,[2] Liwei Liu,[1] Ye Yuan,[1] Weiyan Zhang[1] and Shan He[1]

[1]*Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Research Center, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo, China.*

[2]*Department of Environmental Science, Hankuk University of Foreign Studies, Yongin, Korea.*

Summary

Few microbes can grow under laboratory conditions, highlighting the fact that the majority of microbes in environment are still uncultured and untapped resources. This study used alternative cultivation methods, diffusion chambers (DC), dilution-to-extinction culture (DTE) and modified agar preparation step (PS media) to cultivate previously uncultured marine bacterial species. These methods were applied to samples from a coastal intertidal zone, and the results were compared with those from standard direct plating (SDP) cultivation. Among the strains isolated with DC, DTE and PS media methods, 28%, 48% and 33% were novel species, respectively, while the SDP method resulted in the isolation of only 9% of novel species. Most isolates were unique to the method used for their cultivation. This implies that each method is selective in its own way, which is different from SDP, thus able to access species that are difficult to obtain using conventional approaches. Comparing the diversity showed that 75 genera were recovered by the alternative methods, 2.7 times higher than that of the SDP cultivation, which constituted 45% of total diversity from culture-independent sequencing. We conclude that combining alternative cultivation methods represents a highly promising key for accessing 'microbial dark matter'.

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For correspondence. E-mail heshan@nbu.edu.cn; Tel. +86-574-87604382; Fax +86-574-87604382.
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Introduction

Marine environments are rich, untapped reservoirs of novel natural products and enzymes with tremendous potential in biotechnology and pharmaceutical industries (Xiong *et al*., 2013; Blunt *et al*., 2018). Many studies have suggested that the diverse microbial communities in marine habitats can produce an impressive array of novel bioactive compounds, including antimicrobial, anti-inflammatory, neuroprotective and anti-tumour candidates (Xiong *et al*., 2012; Srilekha *et al*., 2017; Liu *et al*., 2019).

Cultivation and isolation of microbial species are essential steps to enable the discovery of natural products with biotechnological potential (Sekurova *et al*., 2019). However, most of the microbes from natural habitats cannot be cultivated on standard media (Amann *et al*., 1995; Rinke *et al*., 2013), and marine microbes are no exception. Hence, solving the microbial uncultivability challenge is a prerequisite for bioprospecting of marine microbial resources.

To overcome microbial uncultivability, much effort has been devoted to developing alternative cultivation approaches. Modifying growth conditions by adding specific organic or inorganic compounds to media (Nguyen *et al*., 2018), extending incubation times (Davis *et al*., 2005), lowering nutrient concentrations (Janssen *et al*., 2002) and the use of alternative gelling agents (Tamaki *et al*., 2005) have allowed the cultivation of some previously uncultured microbial species. Yet, most of the microbes remain uncultivated. Therefore, the accumulated experience suggests that microbial uncultivability cannot simply be explained by the unfitness of specific strains for certain culture conditions such as medium composition, temperature, pH and other variables (Stewart, 2012).

Other experiments have been conducted to investigate the mechanisms of microbial uncultivability, such as the lack of unknown *in situ* growth factors in the laboratory (Epstein *et al*., 2010), negative effects posed by fast growers (Connon and Giovannoni 2002; Rappé *et al*., 2002) and phosphate-catalysed hydrogen peroxide formation in agar that may be toxic to microbes (Kawasaki and Kamagata, 2017), leading to the development of

more innovative cultivation methods, each utilizing a distinctive approach.

The diffusion chamber (DC) is an *in situ* cultivation device in which environmental microbial cells are inoculated in an agar matrix sandwiched between porous membranes. Incubation of these chambers in the natural environment provides the microbes inside with the growth factors that are present in nature, while maintaining physical separation from other microbes in the environment (Epstein *et al*., 2010). The use of this device has significantly increased the proportion of bacterial species that are culturable from marine sediments (Kaeberlein *et al*., 2002) and other environments (Bollmann *et al*., 2010; Jung *et al*., 2016).

Dilution-to-extinction (DTE) cultivation is one of the most effective approaches for the isolation of marine microbes. In this method, 1 to 10 cells are inoculated into a physically separated well, which isolates cells from mixed populations thus minimizing or eliminating competition with other bacteria (Connon and Giovannoni 2002). This technique led to the cultivation of the first member of the widespread but elusive marine SAR11 clade (Rappé *et al*., 2002) and other uncultivated bacterial groups in marine and aquatic habitats (Yang *et al*., 2016; Castro *et al*., 2017).

Another approach is a simple modification of agar media preparation that reduces phosphate-catalysed hydrogen peroxide formation. The interaction between agar and phosphate during media preparation (autoclaving the phosphate and agar together) can produce hydrogen peroxide, which adversely affects the cultivability of bacteria on media (Tanaka *et al*., 2014). Therefore, hydrogen peroxide scavenging agents such as catalase or pyruvate have been used for the preparation of agar media to improve the efficiency of colony formation and isolation (Kato *et al*., 2018). Recent research has demonstrated that modifying the preparation of agar media, i.e. autoclaving the phosphate and agar separately (PS media), can improve the cultivability of microbes, probably by mitigating oxidative stress (Kawasaki and Kamagata, 2017).

In this study, we used the alternative cultivation methods, DC, DTE and PS media to cultivate microbes from a coastal intertidal zone. This habitat presents its inhabitants with multiple challenges, owing to the daily tidal cycles (Ortega-Morales *et al*., 2010). Microbes living in intertidal sediments must cope with wide variations in temperature and moisture, exposure to ultraviolet radiation and intense biological competition for nutrients and space (Decho, 2000; Zhu, 2018). These conditions have likely led to unique adaptations and thus unique cell chemistry in microbial populations in this environment. We expected that the unique habitats in the intertidal zone are ideal environments for isolating microbial species as rich sources of valuable natural compounds.

Our strategy for isolating new microbial resources in the intertidal zone is the use of multiple cultivation methods that utilize different key principles to access uncultured microbes. We hypothesized that each of the cultivation methods would result in a method-specific culture collection because each method addresses a different limitation of conventional cultivation and would lead to cultures that are otherwise difficult or impossible to isolate. In addition, combining these approaches should provide a better understanding of microbial cultivability and new insights to the advantages of these alternative cultivation methods. To evaluate the hypothesis, we used three alternative cultivation methods and the conventional agar plating method to compare the resulting culture collection.

Results

Identifying isolates based on the 16S rRNA gene

We performed culture-dependent and independent methods for three samples (sediment without plants, S1; sediment with plants, S2; and seawater, W) from the intertidal zone (see Experimental procedures section for details). A total of 1,276 bacterial isolates from the culture-dependent methods were identified. These cultures included 255 isolates obtained from DC, 430 from DTE, 299 from PS media and 292 from SDP (Fig. 1). The DC, DTE and PS cultivation enabled isolation of 423 species (defined as operational taxonomic units [OTUs] composed of 16S rRNA gene sequences sharing over 97% identity) from 11 taxonomic groups (Table S1–S3). SDP cultivation enabled isolation of 124 species from seven taxonomic groups (Table S4). The alternative cultivation methods yielded 384 species that were absent from the SDP collection, with an overlap of 39 species (Fig. S1). The collections at the species level differed depending on the sampling site and cultivation methods (Fig. S2).

The proportion of novel species, defined as a strain with $\leq$ 97% 16S rRNA similarity to the closest known relative among the isolates in EzBioCloud, differed among the alternative methods and the SDP. Among the strains obtained by DC, DTE and PS, 28%, 48% and 33% were novel species, respectively, while only 9% of SDP isolates were novel (Fig. 2). In particular, isolates obtained by DTE from the sediment with plants (S2) were overwhelmingly (60%) hitherto-uncultivated species. We carried out partial sequencing of all isolated strains for practical reasons and considered the limitations of assigning novelty based on such partial sequences. To verify the level of novelty, analysis of full sequences was performed for one quarter of the novel isolates, and we confirmed that most of tested novel species maintained their similarity level compared to the results based on partial sequences.

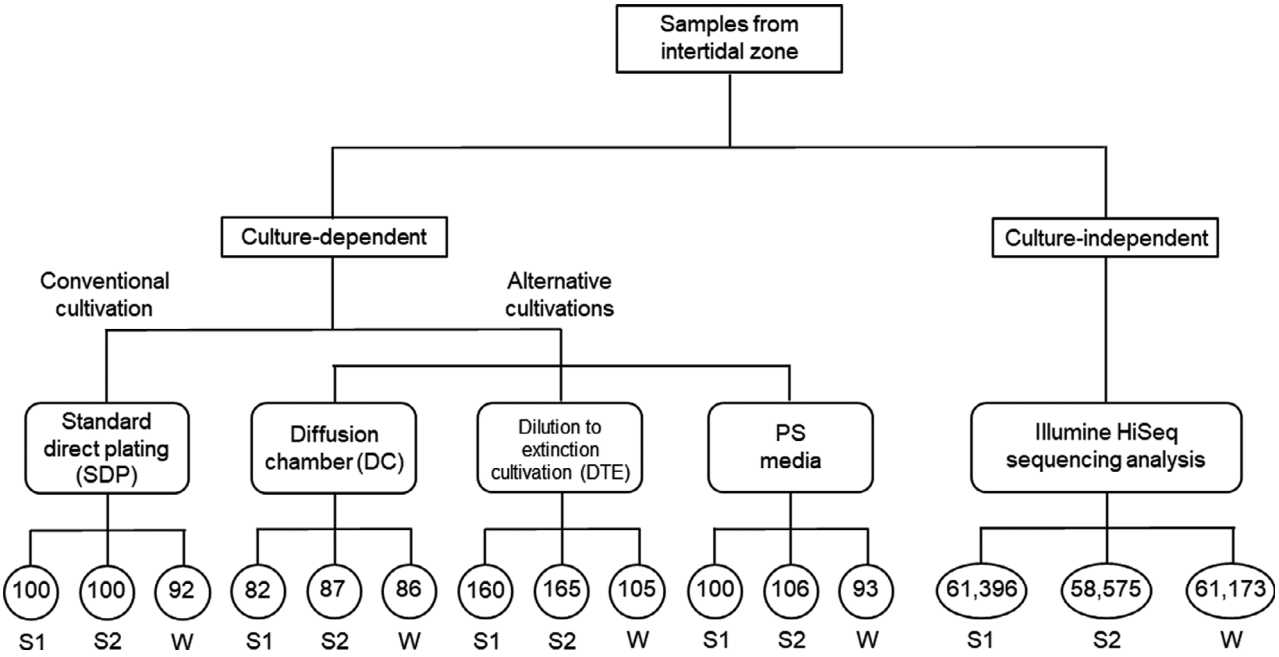

Fig. 1. Flow chart of culture-dependent and culture-independent experiments. The numbers in each circle represent the number of isolates obtained from each culture-dependent method and site and reads numbers from Illumina HiSeq sequencing analysis of each sample. The sample names, S1, S2 and W represent sediment without plants, sediment with plants and seawater, respectively.

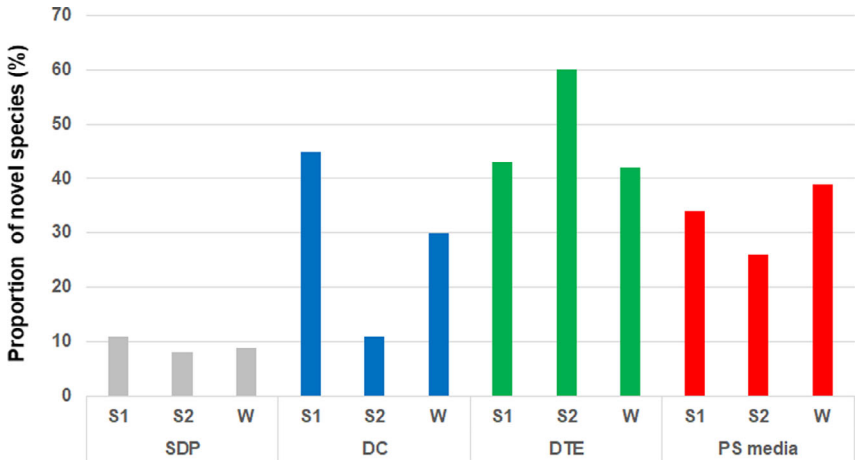

Fig. 2. Comparison of the proportion of new species from SDP and alternative cultivation methods for the three samples. Novel species were defined as a strain with ≤ 97% 16S rRNA similarity to the closest known relative in GenBank databases. The sample names S1, S2 and W represent sediment without plants, sediment with plants and seawater, respectively.

The diversity at the family level of isolates is shown in Fig. 3. A total of 74 families were isolated by culture-dependent approaches. Among these, 42 families were obtained exclusively by the alternative cultivations and 29 were isolated by both SDP and the alternative cultivations. Only three families were isolated exclusively with the SDP method.

The bacterial community of isolated species obtained using the alternative cultivation methods is shown in Fig. 4. The phylogenetic tree shows that 48% of bacterial species are candidates for novel species (Fig. 4a). These methods also allowed for isolation of several novel strains belonging to *Actinobacteria* (Fig. 4b). Among 26 actinobacterial OTUs, 10 show novelty at or above the species level.

Bacterial community composition determined by the culture-independent method

The microbial community composition in the three sample types was analysed by Illumina HiSeq sequencing based on 16S rRNA gene. A total of 61 396, 58 575 and

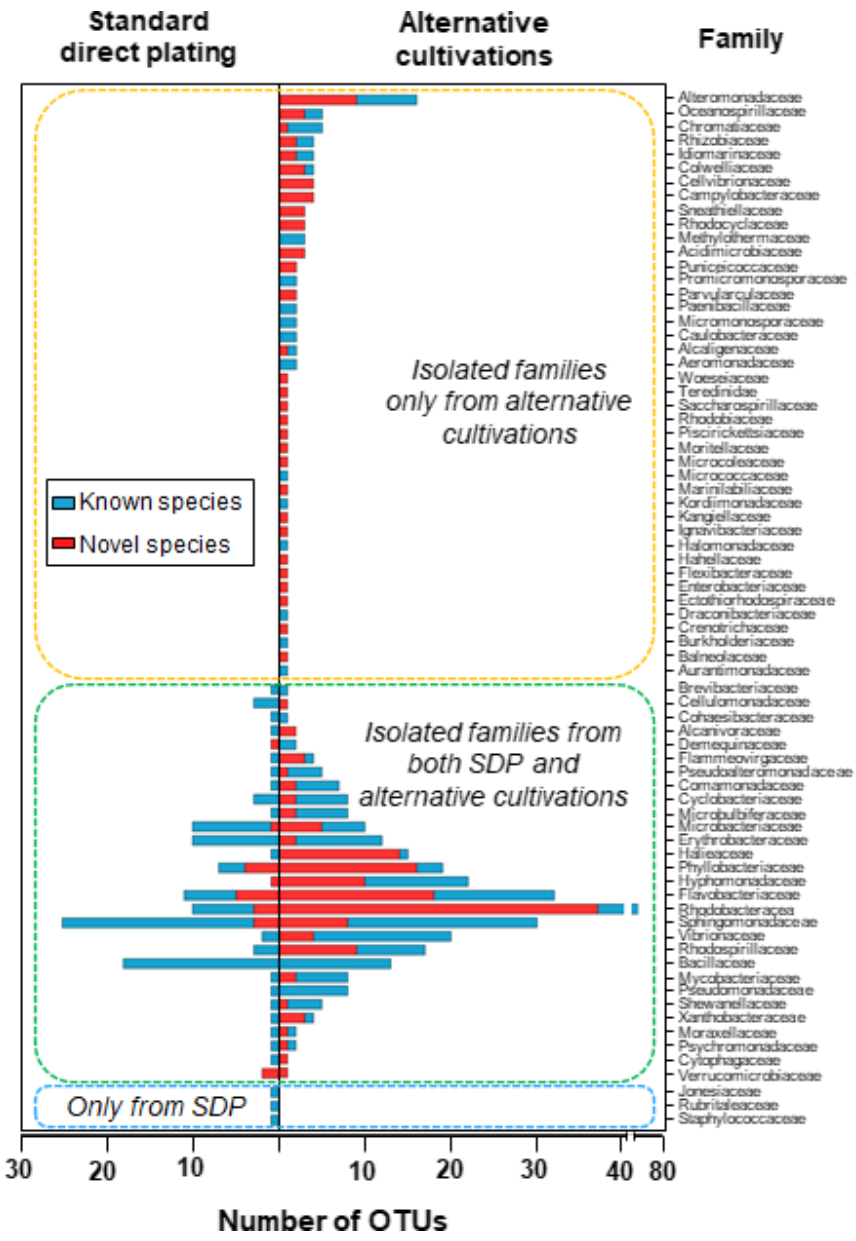

Fig. 3. Diversity of isolates obtained from SDP and alternative cultivation methods at the family level. The bars represent the number of OTUs belonging to each family.

61 173 reads with a median length of 250 base pairs (bp) (V3-V4 ~ 433–682 bp) assigned to 1891, 2297 and 603 OTUs, respectively, were obtained from each sample: sediment without plants (S1), sediment with plants (S2) and seawater (W). In total, 8 major taxonomic groups (cut-off < 0.5%), *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, *Firmicutes* and *Proteobacteria* were detected from the samples (Fig. S3). The isolates from the culture-dependent methods belonged to five groups, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Proteobacteria*, but *Acidobacteria*, *Gemmatimonadetes* and *Nitrospirae* did not appear in the culture collection. The most abundant phylum was *Proteobacteria* in all samples, which is similar to the results obtained by the culture-dependent methods (Fig. 4). We also analysed the 16S rRNA sequences from Illumina HiSeq sequencing at the genus level and compared the results with those of culture-dependent approaches (Fig. 5). A total of 168 major genera were identified (with a cut-off of < 0.02%) in the environmental samples. Among those, the isolates from multiple cultivation methods belonged

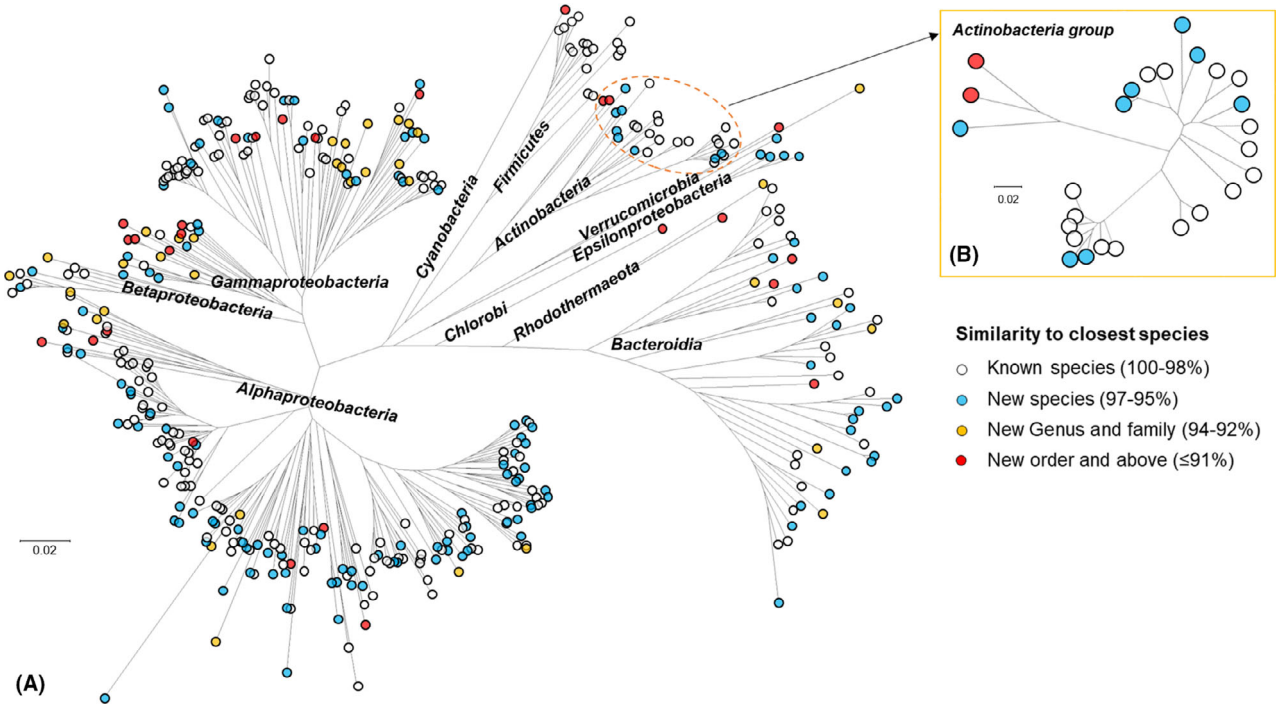

Fig. 4. Phylogenetic tree showing the microbial diversity and novelty of isolated OTUs obtained by the alternative cultivation methods for all bacterial species (A) and species belonging to *actinobacteria* (B). The trees are maximum-likelihood trees (fast bootstrap, 1000 replicates) based on the 16S rRNA gene of isolates. The colours of the circles indicate the degree of 16S rRNA similarity to the closest known species in GenBank.

to 75 genera (26, 59 and 46 in DC, DTE, and PS, respectively), while the SDP isolates belonged to 28 genera.

Discussion

The majority (> 99%) of microbes from natural habitats do not form colonies on the standard agar media traditionally used for microbial isolation. This low culturability might reflect the conditions inherent in conventional methods, e.g. the lack of specific key factors required for microbial growth. Hence, applying more advanced alternative cultivation methods will be prerequisite for improving bioprospecting of marine microbial resources.

This study used multiple cultivation methods that have been demonstrated to significantly enhance microbial cultivability. These alternative approaches yielded increased novelty of isolates from the intertidal habitats sampled in this study, as 28% 48% and 33% of isolates obtained by DC, DTE and PS media were novel species, respectively, while only 9% of SDP isolates were novel (Fig. 2). Isolates obtained by the alternative cultivation methods were more novel than those from the SDP cultivation (chi-square test, $x^2 = 89.4$, $P < 0.01$).

In addition, the alternative approaches led to the isolation of diverse bacterial groups that were not obtained by the SDP cultivation method. Multiple cultivation methods allowed for isolation of additional 42 families that could not be isolated by SDP cultivation (Fig. 3), and many species belonging to these families were novel species (58/96, 60%). Perhaps many strains belonging to these families are fastidious for cultivation with standard cultivation methods, thus other growth factors in the alternative methods are required to cultivate such bacteria. This suggests that a combination of more advanced cultivation approaches can show the hidden bacterial diversity in the intertidal microbial community, which is not accessible by conventional approaches.

Comparing the bacterial composition of the 16S rRNA gene from the culture-independent method and that of the culture collections indicates that many previously uncultured bacterial groups can be isolated with alternative cultivation approaches. Fig. 5 shows that the alternative cultivation methods led to the isolation of several bacterial groups at the genus level that were present in the environmental samples observed by Illumina HiSeq sequencing analysis, but were not cultivated in the SDP method. A total of 28 and 75 genera appeared in the culture collection for SDP and alternative cultivations, respectively, these were about 17% and 45% of the genera in the environmental samples. Therefore, it seems that isolates obtained from alternative cultivation

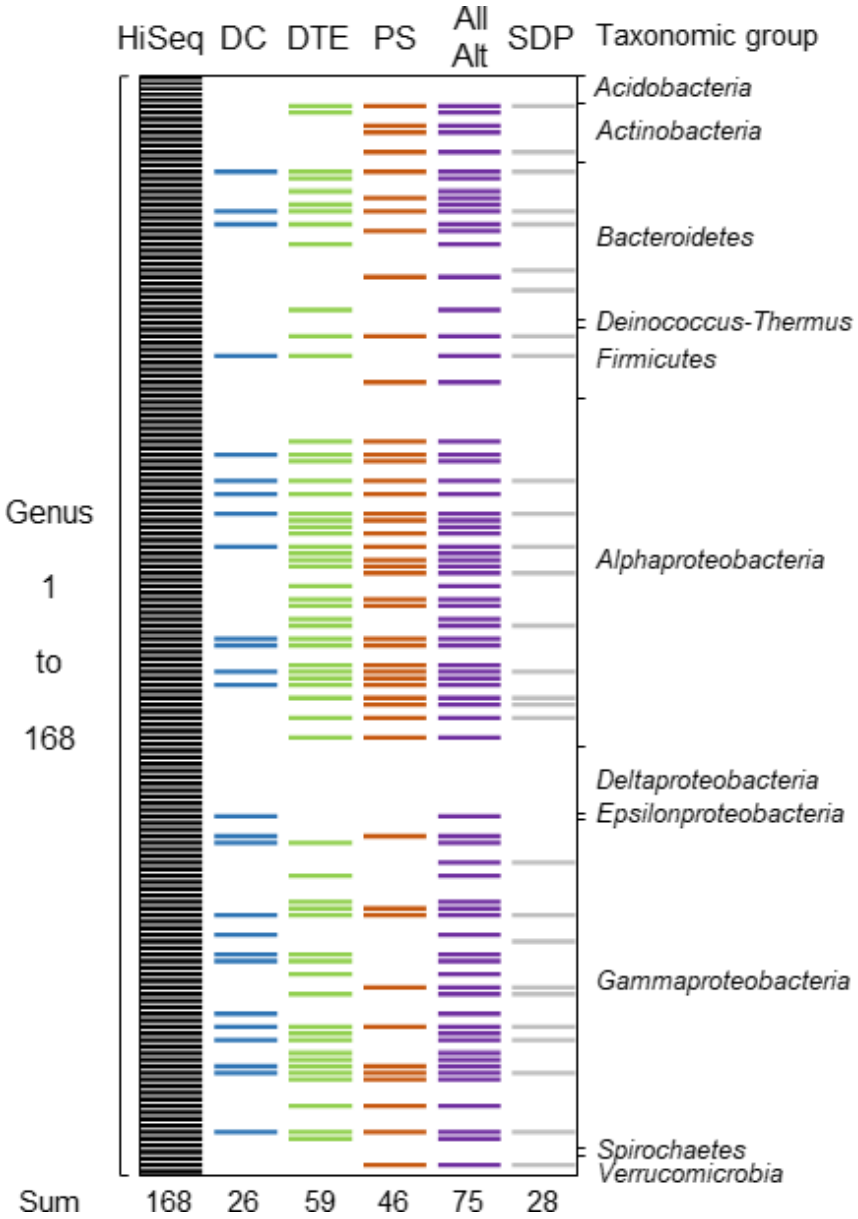

Fig. 5. Band chart showing the presence of genera for the environmental samples by HiSeq sequencing analysis and for the isolates obtained by the alternative cultivations and SDP methods. A total of 168 major genera were detected (with a cut-off of < 0.02%) in environmental samples (black bands). Blue, green, red, and grey bands show the number of isolates cultured from DC, DTE, PS and SDP cultivation in those genera. All Alt indicates the sum of all the results of the alternative cultivation methods (purple bands).

methods reproduced a much more complete diversity in the samples compared to SDP cultivation. This suggests that numerous species discovered via culture-independent methods, which have been widely known as 'uncultured', may be isolated simply, if the alternative cultivation approaches in this study are used for the field samples. On the contrary, we noted an absence of *Acidobacteria* and *Deltaproteobacteria* in our culture collection, even though a high proportion of these groups were found in the intertidal zone (Fig. 5). These microbes may require specific growth conditions not met in our study.

Interestingly, most isolated species were unique to their respective isolation approach (Fig. S2). This indicates that each alternative cultivation method has its own selectivity, which is different from that of the SDP method (Fig. S1); this is not surprising considering that each of the methods used is based on a different principle described below.

Previous work on *in situ* cultivation methods using similar reasoning has led to the isolation of uncultivated and phylogenetically new and industrially important isolates (Sizova *et al*., 2011; Ling *et al*., 2015; Berdy *et al*.,

2017). This is likely because the first step of the method, *in situ* cultivation, mimics natural conditions and allows for the growth of only those species that are active in the environment (Kaeberlein *et al*., 2002; Epstein *et al*., 2010). Recently, we argued that one principle mechanism behind the success of *in situ* cultivation is that certain unknown growth factors present in nature are behind the induction of otherwise non-growing strains (Jung *et al*., unpublished). In a previous study, some chemical substances in the environment (sponge extract in that study) improved cell recovery on agar plates (colony formation efficiency); these signalling molecules selectively work on the isolates from *in situ* cultivation. We also confirmed that such signalling-like molecules do not promote the growth (neither growth rate nor maximum growth) of the isolates from *in situ* cultivation but initiate their growth. During the *in situ* cultivation, these signalling-like compounds, defined as 'growth initiation factors', are provided by the natural environment. These factors may enable the resumption of microbial growth, leading to their enrichment inside the DCs. Following that, some microbes, those which initiate growth and thrive during *in situ* incubation, continue their growth even during the subcultivation step *ex situ*. Therefore, microbial resuscitation by the growth triggering factor might be a key mechanism supporting the cultivation of some uncultivated microbes and the reason why DC cultivation in this study resulted in isolating these bacterial types.

The exact nature of microbial adaptations that fosters colony formation on solid media vis-a-vis liquid is still unknown (Schut *et al*., 1993). Liquid media may provide better conditions for some marine bacteria even with similar nutrient content (Jung *et al*., unpublished). In addition, slower growing microbes can proliferate with dilution to extinction given the absence of fast growers (Connon and Giovannoni 2002; Rappé *et al*., 2002). The combined characteristics of cultivation in liquid and dilution in the DTE method might explain the success of the method in cultivating a high proportion of novel species.

Cultivation using PS media is the simplest, straightforward and most economical method among the techniques used in this study. The only difference between PS and conventional media is whether phosphates and other nutrient components were autoclaved separately from agar or not. Autoclaving these components separately reduces the amount of reactive oxygen species and their unfavourable effect (Tanaka *et al*., 2014). This technique appears to be highly effective in improving culturability of recalcitrant microbes from diverse habitats (Kawasaki and Kamagata, 2017). Our results also support the idea that separate autoclaving leads to an increase in microbial cultivability (Fig. 2).

In this study, we also simplified procedures in the alternative cultivation methods to increase efficiency for bioprospecting. Our main focus was on efficiency and simplicity in the cultivation of novel species. This could lead to scaling up the isolation of new microbial species from marine habitats. Therefore, we followed modified procedures from recent studies or simplified procedures to achieve higher throughput for microbial recourse mining. For example, in the original DC method (Kaeberlein *et al*., 2002), grown microbes were subcultured for purification by picking single colonies under the microscope. In this study, we plated DC-grown material directly on agar plates after thoroughly mixing instead. This led to a much simpler and faster process to obtain diverse bacterial isolates. In DTE cultivation, total cell numbers in the samples were often counted to determine the optimal dilution in a liquid medium, so that aliquoting the dilution into the multi-well plate would result in one to a few cells per well (Connon and Giovannoni 2002). Here, we applied a simpler approach that has been used for the ichip, an *in situ* cultivation technique (Berdy *et al*., 2017). We bypassed counting by preparing multiple well plates in a serial dilution manner and then selecting the optimal dilution for further subculturing. The simplified procedure resulted in some empty chambers but is more practical because the importance of fresh samples was paramount. In addition, in the original method, grown microbes in liquid media were screened with flow cytometry analysis and transferred to larger scale cultivation (e.g. 48-well plates). In this study, we inoculated the grown microbes (1 μl) directly on agar plate and then only isolates forming pure colony were used for further cultivation and identification. This is a much simpler and faster process compared to original procedure, but its efficiency of isolating of novel species has been shown to be adequate (48% of isolates from DTE cultivation were novel species). Therefore, the alternative cultivation methods produced a large number of previously uncultured microbes from the intertidal zone and did so more efficiently than in any previous studies (approximately 48% of isolated bacterial species from the alternative cultivation methods were novel species; Fig. 4a). Newly discovered microbes are now being explored as sources of valuable secondary metabolites. Particularly interesting is that we isolated numerous novel Actinobacteria (Fig. 4b) known to be prolific producers of bioactive compounds (Karuppiah *et al*., 2016).

In summary, we used three different alternative, more advanced cultivation methods to access the biodiversity of the marine microbial communities and to successfully cultivate a diverse group of bacterial species, including numerous novel species (a total of 201) that could not be achieved by a conventional cultivation method. These methods appear efficacious as well, as their application

leads to a much higher proportion of novel species per collection, including those from biotechnologically interesting groups *Actinobacteria* and *Verrucomicrobia*. We also verified the hypothesis that each of the alternative methods would produce different culture collections of bacterial species. Therefore, our result shows that integrating different cultivation methods that utilize different principles is important to maximize the microbial resources obtained from nature environment.

We suggest that the alternative methods used in this study are valuable tools for bioprospecting not only in coastal marine microbial communities but in a variety of other environmental habitats. The combination of multiple cultivation methods used here represents another step forward in addressing the challenges of accessing previously uncultured microbes and the discovery of valuable natural compounds derived from these microbial communities.

Experimental procedures

Sample collection

Three types of samples from the intertidal zone were collected on the northwest coast of Meishan Island (Ningbo, China) between June and September of 2019. The sampling site is approximately 1 kilometre from our laboratory in Ningbo University. One sediment sample was collected in an area without macrophytes (hereafter referred to as sediment without plants, S1). The second and third types were a sediment sample and seawater sample collected in an area with macrophytes (hereafter referred to as sediment with plants, S2 and seawater, W respectively). Details of sampling locations and site characteristics are listed in Table S5. All samples were transported immediately to the laboratory for further culture-dependent and culture-independent experiments.

Media

For the microbial cultivation experiments, we used the following media: (i) 1:2 diluted marine broth 2216 (50% of manufacturer's suggested concentration, Difco, Franklin Lakes, NJ, USA); (ii) 1:10 diluted R2A liquid medium (10% of the manufacturer's suggested concentration; Hopebio, Qingdao, China); (iii) 1:100 diluted nutrient broth (1% of the manufacturer's suggested concentration; Hopebio). All media were supplemented with additional artificial sea salt (Instant Ocean, Blacksburg, VA, USA) to a final concentration of 2%.

Diffusion chamber in situ *cultivation*

The overall experimental design of the cultivation experiments is shown in Fig. 1. Diffusion chambers (DC) were prepared as described previously (Bollmann *et al*., 2010; Jung *et al*., 2016). Chambers were constructed using 0.03 μm pore size polycarbonate membranes (GVS, Sanford, ME, USA) glued to an iron washer with a waterproof adhesive (Binyuan, Yiwu, China). To prepare inoculum for sediment samples, 1 g of each sample was placed into a 15 ml conical tube with 10 ml of sterile artificial seawater (2%) and homogenized using a vortex machine for 10 mins. The seawater sample was used directly as a source of inoculum. Each sample was diluted 10^{-3} to 10^{-5} with sterilized artificial seawater and mixed with warm (45°C) agar containing media. We used three kinds of media, 1:2 diluted marine broth, 1:10 diluted R2A and 1:100 diluted nutrient broth with 1.5% of agar for DC cultivation. The mixture was placed on the chamber membrane glued to the bottom of the washer, then after inoculation, the second membrane was glued on top, sealing the agar containing bacteria inside the chamber. The size and structure of the DC in this study is nearly the same as the original DC in previous studies (Kaeberlein *et al*., 2002; Bollmann *et al*., 2010).

The DCs were incubated for a week on top of each sediment sample or suspended in the water sample. After incubation in conditions mimicking *in situ* conditions, the devices were retrieved and opened. The agar with the grown material was homogenized by passing it through a syringe equipped with a 25-gauge needle, diluted with sterile artificial seawater and plated on agar media. The same type of media used in DC cultivation was used for subculture. We randomly selected and attempted to subculture 100 colonies per sample for microbial purification and identification.

Dilution-to-extinction cultivation in liquid media

The same inoculum prepared for DC cultivation in each 15 ml centrifuge tube was centrifuged for 5 min at 500 rpm. The supernatant was diluted 10^{-5} to 10^{-8} with three types of liquid media, 1:2 diluted marine broth, 1:10 diluted R2A broth and 1:100 diluted nutrient broth and 100 μl of each dilution was then dispensed into a 96-well clear flat-bottom polystyrene microplate (BIOFIL, Guangzhou, China). We prepared multiple plates as a dilution series, with each dilution made in triplicate. After incubation at room temperature for 3 weeks, cellular growth in each well was scored visually (roughly OD_{660} nm > 0.05). After incubation, multi-well plates showing 10–70% of positive wells per plate were selected and used for further subcultivation. Next, 1 μl of the microbial cell suspension from each positive well was inoculated on agar plates supplemented with same media used in liquid cultivation. After 3 weeks of incubation, all pure colonies observed under a microscope (CNOPTEC, Chongqing, China) were used for taxonomic identification and subculturing.

Agar plating method using PS medium

The media were prepared by autoclaving nutrient components including phosphate and agar separately. For this, each type of media (1:2 diluted marine broth, 1:10 diluted R2A broth and 1:100 diluted nutrient broth) and agar was autoclaved separately and then mixed just before solidification. The same inocula mentioned above were serially diluted and 50 μl aliquots from different dilutions were plated. The plates were incubated for 3 weeks, and about 100 colonies per sample were selected randomly and pure cultured for microbial identification.

Standard direct plating cultivation

Standard direct plating (SDP) cultivation employed the same inocula used for the alternative cultivation methods. Samples were serially diluted and 50 μl aliquots from different dilutions were plated on agar (1.5%) plates using the media, 1:2 diluted marine broth, 1:10 diluted R2A broth and 1:100 diluted nutrient broth. To better follow more conventional cultivation methods, each type of media and agar were autoclaved together. The plates were incubated for 3 weeks, and 100 colonies were selected randomly and further purified for microbial identification.

Identification of isolates based on 16S rRNA gene sequencing

Taxonomic identification was performed by sequencing about 750 bp long fragments of the 16S rRNA gene. The colony material was used directly as a template for PCR. The universal primers 27F and 1492R were used to amplify the 16S rRNA gene with a PCR system recommended by the manufacturer (Sangon Biotech, Shanghai, China). The PCR products were sequenced commercially (Sangon Biotech) by fluorescent dye terminator sequencing. The sequences were compared to databases in EzBioCloud (https://www.ezbiocloud.net) to determine their closest relatives. Distance matrices and phylogenetic trees based on 16S rRNA sequences were built, and OTUs sharing 97% 16S rRNA gene sequence identity established, according to the Kimura two-parameter model and neighbour-joining algorithms using the MEGA program (MEGA software, Tempe, AZ, USA).

To obtain full sequences of candidates for novel species, purified PCR products were cloned into vector pMD19-T and then sequenced. The recombinant plasmid transformed into *Escherichia coli* DH5α and then commercially sequenced. The almost-complete 16S rRNA gene sequence (1459–1519 nt) was compared in databases in EzBioCloud (https://www.ezbiocloud.net) to determine their closest relatives.

DNA extraction and amplicon sequencing targeting the 16S rRNA gene

To compare the cultivated bacterial diversity with the microbial molecular signatures in the samples, 16S amplicon sequencing was performed. In the laboratory, sediments (1 g) and water (30 ml) samples were stored frozen at −20°C. The total DNA from sediment samples was extracted using a Magnetic Soil and Stool DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Total DNA from seawater samples was isolated after suspending microorganisms on the surface of 0.2 μm sterile polycarbonate filters (Merck Millipore, Burlington, MA, USA). DNA from the shredded filters was extracted using the CTAB method (Raimundo *et al.*, 2018). DNA concentration and purity were detected in 1% agarose gels, and DNA was diluted to 1 ng μl^{-1}. The 16S rRNA gene in the extract was amplified using the amplicon forward primer 341F (5′-CCTAYGGGRBGCASCAG-3′) and reverse primer 806R (5′-GGACTACNNGGGTATC TAAT-3′) (Yu *et al.*, 2005) with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) and then purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). DNA was sequenced by Novogene (Beijing, China) using an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Each read was assigned taxonomically using Quantitative Insights Into Microbial Ecology (QIIME) software (V1.7.0).

Nucleotide sequence accession numbers

Newly determined sequence data have been deposited in GenBank (www.ncbi.nlm.nih.gov) under accession numbers MT254895 to MT254897, MT260804 to MT260840, MT254913, MT254915, MT254918, MT254929 to MT254931, MT254933, MT254935 to MT254940, MT254942 to MT254945, MT254955 to MT254965, MT254971 to MT254972, MT254983, MT254985 to MT254986, MT254988, MT254990 to MT254993, MT254995 for isolates from DC, MT626377 to MT645477, MT678662, MT740345 to MT740346 for isolates from DTE, MT636561 to MT636650, MT645470 to MT645472, MT678753 to MT678758 for isolates from PS and MT628712 to MT628748, MT678759, MT678761, MT740349 for isolates from SDP cultivation.

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Conflict of interest

The authors declare that they have no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Overlap among culture collections obtained using alternative cultivation and standard direct plating (SDP) methods. Values in the centre of each circle represent the total number of OTUs (defined at 97% 16S rRNA gene sequence identity) by methods; values in the overlapping areas represent the numbers of co-isolated species. The numbers in parentheses show the numbers of novel species. Venn diagrams were made with Venn Diagram Plotter v. 1.5.5228.29250 (Pacific Northwest National Laboratory http://www.pnl.gov/; http://omics.pnl.gov/).

Fig. S2. Overlap between culture collections obtained using three different alternative cultivation approaches (A) and three different sampling sites (B). Values in the centre of each circle represent the total number of OTUs (defined at 97% 16S rRNA gene sequence identity) in each section. Values in the overlapping areas represent the numbers of co-isolated species. Venn diagrams were made with Venn Diagram Plotter v. 1.5.5228.29250 (Pacific Northwest National Laboratory http://www.pnl.gov/; http://omics.pnl.gov/).

Fig. S3. Bar graph showing OTU analysis at the phylum level for the samples by HiSeq sequencing analysis.

Table S1. Phylogenetic affiliations of isolates with the DC method on the basis of 16S rRNA gene sequences. The letters M, R and N refer to used media, 1:2 diluted marine broth, 1:10 diluted R2A broth, and 1:100 diluted nutrient broth media.

Table S2. Phylogenetic affiliations of isolates with the DTE method on the basis of 16S rRNA gene sequences. The letters M, R and N refer to used media, 1:2 diluted marine broth, 1:10 diluted R2A broth, and 1:100 diluted nutrient broth media.

Table S3. Phylogenetic affiliations of isolates with the PS media method on the basis of 16S rRNA gene sequences. The letters M, R and N refer to used media, 1:2 diluted marine broth, 1:10 diluted R2A broth, and 1:100 diluted nutrient broth media.

Table S4. Phylogenetic affiliations of isolates with the SDP method on the basis of 16S rRNA gene sequences. The letters M, R and N refer to used media, 1:2 diluted marine broth, 1:10 diluted R2A broth, and 1:100 diluted nutrient broth media.

Table S5. Location and environmental characteristics of the sampling sites.

