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High-throughput single-cell cultivation reveals the underexplored rare biosphere in deep-sea sediments along the Southwest Indian Ridge†

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Microorganisms in the deep sea play vital roles in marine ecosystems. However, despite great advances brought by high throughput sequencing and metagenomics, only a small portion of microorganisms living in the environment can be cultivated in the laboratory and systematically studied. In this study, an improved high-throughput microfluidic streak plate (MSP) platform was developed to speed up the isolation of microorganisms from deep-sea sediments and evaluated with deep-sea sediments collected from the Southwest Indian Ridge (SWIR). Based on our previously reported MSP method, we improved its isolation efficiency with a semi-automated droplet picker and improved humidity control to enable long-term cultivation with a low-nutrient medium for up to five months according to the slow-growing nature of most deep-sea species. The improved MSP method allows the isolation of microbes by selection and investigation of microbial diversity by high throughput sequencing of the pooled sample cultures. By picking individual droplets and scale-up cultivation, a total of 772 strains that were taxonomically assigned to 70 species were isolated from the deep-sea sediments in the SWIR, including 15 potential novel species. On the other hand, based on 16S rRNA gene amplicon sequencing analysis, the microbial diversity of the SWIR was studied and documented with culture-dependent and independent methods in this study. The superiority of the MSP platform in revealing the rare biosphere was also evaluated based on amplicon sequencing. The results show that droplet-based single-cell cultivation of the MSP has a much higher ability than traditional agar plate cultivation in obtaining microbial species and more than 90% of operational taxonomic units (OTUs) detected in the MSP pool belong to the rare biosphere. Our results indicate the high robustness and efficiency of the improved MSP platform in revealing the environmentally rare biosphere, especially for slow-growing species. Overall, the MSP platform has a superior ability to recover microbial diversity than conventional agar plates and it was found to hold great potential for recovering rare microbial resources from various environments.

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Introduction

Microbes have versatile roles in every aspect of biogeochemical cycles in the environment. Isolation of novel microorganisms has been ongoing since the first microbial strain was recovered, and thereby, the study of their biochemical and metabolic characteristics became possible.[1] However, only a small proportion of microorganisms has been cultivated to date[2,3] because of the limited isolation methods. Significant development of sequencing techniques in recent years, such as the Illumina, PacBio, and Nanopore sequencing platforms, has resulted in the identification of many new species of microorganisms. However, we still know little about the physiological properties of these organisms despite the large amount of information we have; thus, the need for robust isolation methods is becoming increasingly urgent.

Many new approaches have been developed to improve the recovery of uncultivated microbes from various environments, such as simulations of *in situ* cultivation,[4,5] the dilution-to-extinction technique,[2] microencapsulation cultivation,[6]

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prolonged incubation times,[7] design/modification of culture media based on the 16S rRNA gene sequences,[8] and microfluidic droplet-based cultivation.[9,10] Among these, droplet-based cultivation encapsulates single cells in nanoliter or picoliter droplets and provides an ultra-high throughput for the isolation of novel functional microbes from samples.[11,12] We recently reported the microfluidic streak plate (MSP) platform for isolating microorganisms. This platform can manipulate tens of thousands of nanoliter droplets on a standard Petri dish for single-cell cultivation.[13] Cultivated droplets can be selected to be scaled up for downstream analysis. We applied the MSP to isolate polycyclic aromatic hydrocarbon (PAH)-degrading bacteria from a soil enrichment experiment,[13] the imidazolinone degrader in a contaminated environmental sample,[14] and the termite gut microbiome.[15] Taken together, the results of these studies indicate that the MSP is a powerful tool for isolating rare species from environments. However, there are still two drawbacks to the MSP platform. First, the selection of the cells using a toothpick or pipette is not very convenient or efficient, and the microbial isolation efficiency is relatively low because many randomly selected droplets remain empty. Additionally, morphological identities can be used in conventional agar plate cultivation to increase the diversity of isolation, while the process of randomly picking droplets is unable to selectively harvest microbes with specific morphology.

Oceans, which account for 71% of the Earth's surface and are the largest ecological realm on the planet, are inhabited by a large number of microorganisms and contain a tremendous amount of microbial diversity. These microorganisms play important roles in the entire Earth's biogeochemical cycle. Accordingly, it is important to study the microbial diversity in these habitats to better understand the entire Earth's ecosystem.[16] The Southwest Indian Ridge (SWIR), which is a major plate boundary of the world's oceans and one of the world's slowest spreading ocean ridges,[17] displays diverse geographic environments and widespread hydrothermal plumes and vents.[18] Because of the remote location of the SWIR, few studies have been conducted to date. In previous studies, many methods have been applied to the microbial diversity investigation such as culture-based methods,[19] non-culture based clone libraries,[20] microbial amplicon sequencing and metagenome sequencing.[21–25] Still, we know very little about the microbes of the SWIR and more studies and approaches are required for better depiction of the deep-sea microbial community of the SWIR.

In nature, microbial communities widely exhibit a skewed species abundance distribution known as the long tail of the rare biosphere.[26,27] Many rare microbes may play important ecological roles, such as serving as a microbial seed bank and storing ecological potential, and some rare taxa may be disproportionately active in some ecosystems.[28–30] The increasing resolution of high-throughput sequencing helps us better understand the role of rare microbes compared to that of previously used molecular phylogenetic methods, such as clone libraries and gel fingerprinting.[31] However, culture-dependent methods are still indispensable for surveying microbial diversity and function.[31] Under specific high-throughput sequencing conditions, some rare species may be omitted, while they may be captured by culture methods.[31,32] Moreover, several microbial diversity studies have only a minor overlap in microbial taxa captured by culture-dependent and independent techniques.[32–35] These cases indicate that culture-based methods may select some fast-growing and low abundance microbial species and disproportionately recover some rare-biosphere members.[32,36] To better resolve this issue, we made a great effort to improve the MSP platform and applied it to the cultivation of environmental samples from the SWIR. This present study described the improvements that had been made on the MSP technique, through which the platform can be more adaptive to long-term microbial cultivation and selective isolation based on microbial morphology. We also compared the outcomes of MSP cultivation with traditional agar plate cultivation and sequencing identification to give a deep investigation of the biodiversity of the SWIR, which further confirmed that our strategy is effective at covering more comprehensive microbial diversity and rare organisms.

Experimental

Study site description and sample collection

Five deep-sea surface sediment samples (S1–S5) were collected from the Southwest Indian Ridge on the DY125-39 cruise in May 2016 using a television-guided grab sampler (TVG). The details describing the collected samples are provided in Table 1. Each sample was placed in a sterile plastic bag and kept at −20 °C during transportation. After arriving at the laboratory, 1 g of each sample was subjected for cultivation using the MSP or agar plates. 5 g of each sample was subjected to total DNA extraction using a PowerSoil DNA isolation kit (MO BIO Laboratories). The remaining samples were modified with 12% glycerol and frozen at −80 °C for further cultivation.

Construction of droplet picker for the MSP

The droplet picker consisted of six mechanical parts: a *X–Z* linear translation stage, a support arm, a tubing holder, a capillary tip, a Petri dish clip, and a syringe pump. The tubing holder, dish clip, and support arm were designed using the AutoCAD software and printed with a 3D printer. The translation stage was commercially available. The support arm was fixed on the translation stage with screws. The tubing holder was on the other side of the support arm. The clip for the MSP Petri dish was used to ensure the dish could be revolved to change the observation point along the spiral track without changing the position of the Petri dish. When combined with the translation stage, which moves in an *X–Z* axis manner, the tip of the picker is capable of reaching everywhere on the MSP Petri dish. A unique aspect of the droplet picker is the use of Teflon tubing of two diameters (Zeus, Orangeburg, SC; 250 μm inner diameter [i.d.], 700 μm outer diameter [o.d.], and 150 μm i.d. and

Table 1 Sediment samples collected from the SWIR

Sample ID	Sampling sites	Longitude	Latitude	Depth (m)	Sampling technique
S1	39IV-SWIR-S008TVG05	54.36841	34.802	3272.35	TVG
S2	39IV-SWIR-S011TVG07	54.2143	34.96707	3269.63	TVG
S3	39IV-SWIR-S012TVG08	54.2267	34.87032	3492.67	TVG
S4	39IV-SWIR-S018TVG12	54.6153	34.84631	2335.7	TVG
S5	39IV-SWIR-S010TVG06	54.55891	34.94898	1675.93	TVG

200 μm o.d.) that were connected and sealed with wax. The tube with the larger diameter was connected to a glass syringe (Agilent, Palo Alto, CA, USA), while the smaller one was sealed to the tubing holder and used to store the droplet. The Teflon tubing tip could be used repeatedly for picking a series of droplets (see the ESI†). The syringe and tubing were prefilled with mineral oil and the droplet selection was controlled with a programmable pump (Harvard Apparatus, Holliston, MA, USA) to ensure the precise aspiration and infusion of droplets.

MSP droplet cultivation and bacteria isolation

The modified M13 *Verrucomicrobium* broth, DSMZ Medium 607, was used to cultivate bacteria in sediments as previously described.[37] Suitable antibiotics were added. Actidione was added to suppress the contamination of fungi, and nalidixic acid was added to inhibit Gram negative microbes which were commonly seen under laboratorial conditions. Each sediment sample was pretreated by dispersion and differential centrifugation.[38] The samples were serially diluted with the M13 broth for making droplet arrays. The droplet arrays were streaked on Petri dishes as previously described,[13] with 6–8 dishes for each dilution. About 4000 droplets from a cell suspension diluted with M13 medium were written onto the surfaces of a Petri dish with approximately 30 μL of cell suspension. The Petri dishes were then incubated at room temperature and a humidity of 98–100% for up to 5 months to allow the full growth of the cells. During the cultivation, cell growth was visualized and recorded using an inverted microscope with a 10× objective lens every 1–2 weeks. To compare the total microbiome diversity of the MSP droplet culture technique, three of the six MSP dishes were designed as MSP pool samples, which were washed, after which all of the cells from the incubated nanoliter droplets were pooled in each dish and their DNA was extracted (DL111, Biomed Tech. Co.) for subsequent sequencing analysis. The other three MSP dishes were used for strain isolation and droplets with cell growth were individually selected and transferred into a 96-well plate for scale-up. After 1–3 weeks of cultivation in a 96-well plate, 10 μL of culture liquid in each well was used for agar plating to form pure single colony isolates.

Microbial cultivation with traditional agar plates

The sample pretreatment for the cultivation on agar plates was the same as for the MSP cultivation and the M13 broth was modified with 1.8% agar (A8190, Solarbio) as a solid support. The treated samples were diluted 10-fold, 100-fold, and 1000-fold with artificial seawater and 100 μL of each dilution was plated on the surface of the M13 agar media. The agar plates were then incubated at room temperature and the plates that were cultivated for 14 days, 30 days, and 60 days were used as agar pool samples. Cells on those agar plates were harvested and pooled together and DNA was extracted for amplicon sequencing analysis.

Identification of isolated strains

Genomic DNA of single MSP colonies was extracted, after which the bacterial 16S rRNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The products were purified and sequenced using an ABI 3730XL automated DNA sequencer, after which the 16S rRNA gene sequences were assembled with the DNASTAR software and used for the BLAST searches of the EzBioCloud database (http://www.ezbiocloud.net/).[39]

16S rRNA gene amplicon DNA sequencing

Total genomic DNA from marine sediments, the MSP pool and agar pools were extracted as described above. Amplicon high-throughput sequencing was performed by sequencing PCR-amplicon products produced using the 341F (5′-CCTACG GGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAAT CC-3′) primers with barcodes, which targeted the V3–V4 region of the 16S rRNA gene.[40] Amplicon products of all of the samples were sent to Novogene (Beijing, China) using an Illumina HiSeq2500 platform for sequencing. Library construction and quality control were all processed by the Novogene Company.

The 16S rRNA gene amplicon sequencing data process and analysis

The raw sequences were split into individual samples according to the barcode sequences. After removing the primers and barcodes, the raw tags were obtained by merging PE reads with FLASH (version V1.2.7).[41] Next, clean tags were generated by filtering the raw tags with the QIIME software package (version V1.9.1).[42] The final effective tags were subsequently produced after checking for chimeras in clean tags with vsearch[43] and the UCHIME algorithm.[44] The effective tag sequences were then clustered into OTUs based on the relatedness of the sequences (97% identity) using the

UPARSE software.[45] The most abundant sequence in each OTU was selected by UPARSE as the representative sequence. The representative sequences were then annotated with the SSUrRNA of the SILVA dataset from Mothur.[46] Sample data were normalized according to the sample that has the lowest data size and the subsequent analysis of alpha and beta diversity was based on the normalized data. Several commonly used diversity indices that indicate the microbial communities' complexity such as Observed-OTUs, Chao1, Shannon index, and Simpson index were estimated by the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) (version V1.9.1). An analysis based on these indices was taken for comparing microbial communities from different methods.

Data deposit

Raw amplicon sequence data of all the samples were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the BioProject accession numbers PRJNA523706 and PRJNA524769. The 16S rRNA gene sequences of the isolated strains were deposited in the GenBank database under the accession numbers MK318567–MK318637 and KY800369–KY800371.

Results & discussion

The procedural framework of high-throughput single-cell cultivation

To explore the microbial diversity of deep-sea sediments, recovery of rare microbial resources with our MSP platform, and comparison of its efficiency with currently available methods, we designed a comparative study (Fig. 1). The overall culture efficiency of the improved MSP platform was evaluated based on microbial diversities revealed by the 16S rRNA gene amplicon sequencing of the pooled droplets after cultivation (MSP pool), then compared with that of the original samples (sample) and pooled cultured samples from the agar plates (agar pool). In addition to amplicon sequencing, nanoliter droplets were recovered with a newly developed droplet picker and transferred into 96 microwell plates for scaled-up cultivation, then further identified using 16 rRNA gene sequencing.

Technical advances of the MSP platform

To enable long-term cultivation and strain recovery of marine species with the MSP, we improved the MSP technique in three aspects. First, we dramatically improved the MSP for the long-term cultivation of slow-growing environmental

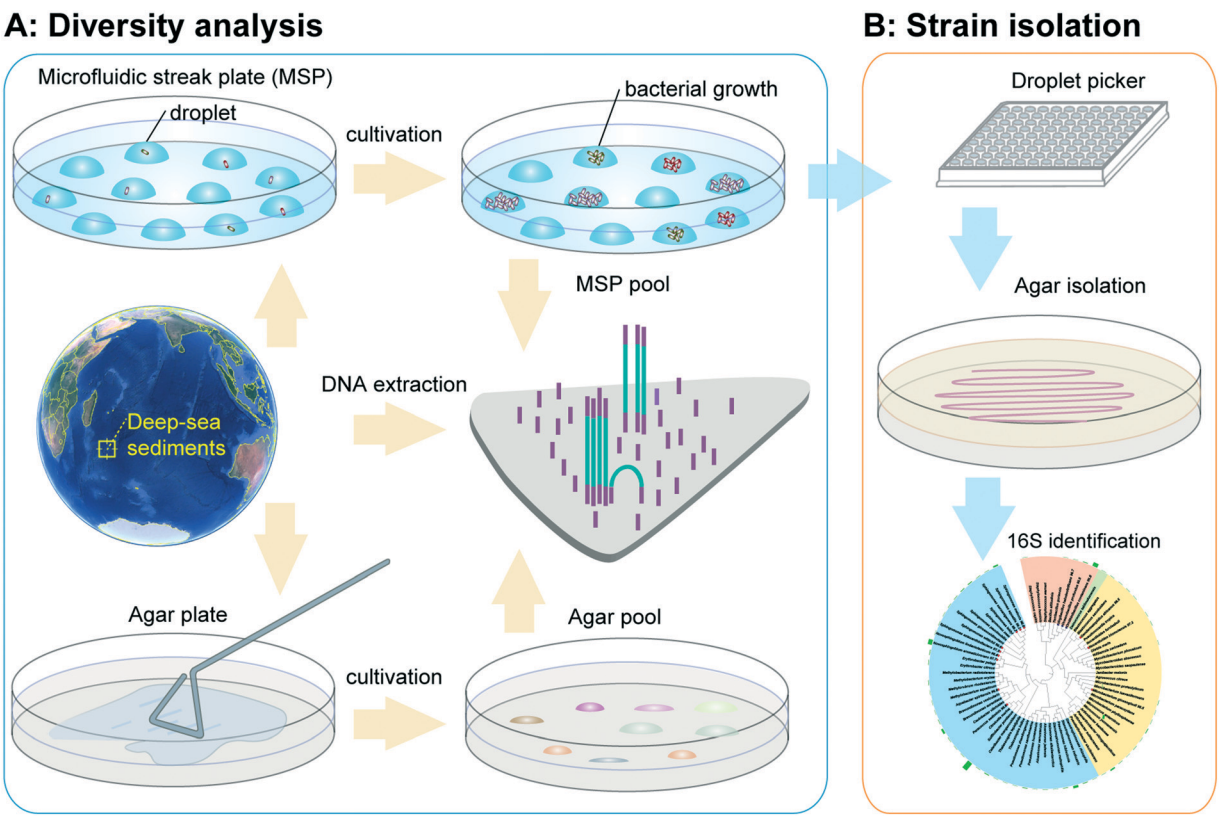

Fig. 1 A schematic workflow of the microbiome recovery from the SWIR sediments in this study. The two columns include (A) the microbiome investigation using the culture-dependent amplicon method. Samples include the direct amplicon sequences of the original sediments (sample), the MSP droplet enriched pool sample (MSP pool), and the agar plate enriched pool samples (agar pool). (B) Strain isolation procedure. The improved MSP droplet picker was used here, after which the droplets were transferred to well-plates and then cultivated on agar plates, and their phylogenetic positions were identified.

microbes by controlling the moisture stability using a sealed chamber. The droplet evaporation was effectively constrained to allow the extension of cultivation time. The chamber can store tens of droplet containing dishes, which can be kept in the dark for up to five months at room temperature with limited evaporation, compared with the previous method (Fig. S1 and S2A†). During incubation, the moisture of the sealed chamber was monitored using a remote humidity meter and kept at around 98–100%. To the best of our knowledge, this is the longest cultivation of microbes within nanoliter droplets. Overall, the results indicate that our MSP method can be an alternative for isolating most slow-growing microbes from the environment.

We next introduced a rapid and contamination-free droplet picker that can precisely locate and pick up a single droplet by coordinated operation of a syringe pump and a manual translation stage (Fig. 2A). The droplet picker was fixed on the translation stage of an inverted microscope and the droplet dish was fixed with the dish clip, allowing the facile tracking of droplets along the spiral track by rotating the dish. Importantly, we introduced a disinfection procedure by rinsing the Teflon tubing of the droplet picker; thus, we did not need to replace the tubing in between the collection of adjacent droplets (Fig. S2B†). The operation of the droplet picker is shown in Fig. 2B and Movie S1.† In addition to picking sessile droplets, which are not stabilized with surfactants, as shown in Movie S2,† the picker could also be used for picking surfactant-stabilized droplets, which are not fixed on the surface. This greatly reduced the time required for droplet collection and allowed efficient and contamination-free selection of hundreds of droplets in one experiment.

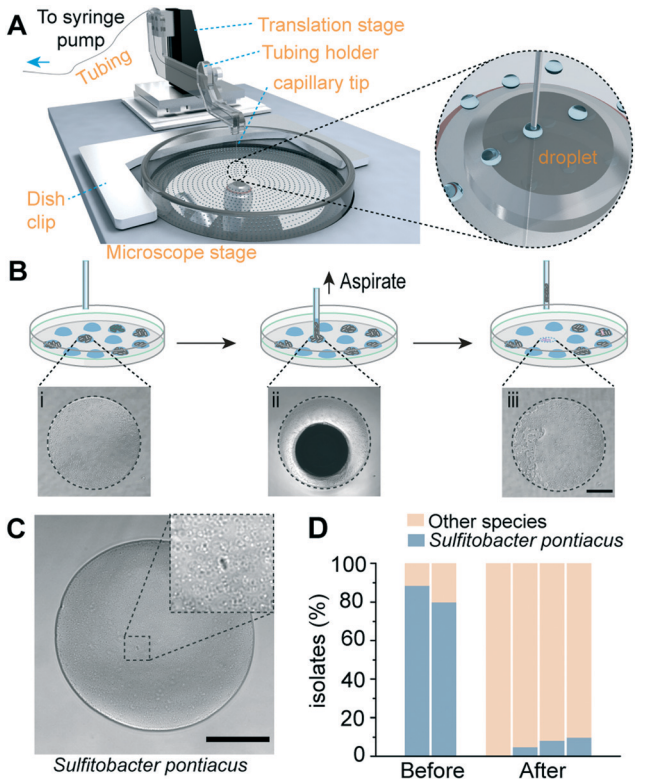

Fig. 2 Improvements that have been made on the MSP platform to improve efficiency and overcome contamination. (A) Schematic diagram of the semi-automatic droplet picker device, which contains a translation stage, a tubing holder, a capillary tip, a dish clip, and a syringe pump. (B) Diagram showing the droplet picking process observed under a microscope. Once the droplet is located (Bi), the tip of the droplet picker will move to the correct position (Bii) and the droplet is then aspirated into the tubing. The position where the droplet settles becomes empty and only a few microbial cells remain (Biii). (C) Microscopic image of a sessile droplet containing motile and high density *Sulfitobacter pontiacus* cells. (D) The ratio of *Sulfitobacter pontiacus* decreased significantly after removing duplicates according to their morphology. The scale bar is 100 μm.

Third, to improve the microbial diversity of MSP isolation, we not only check the growth of cells in droplets before we pick up the droplet, but also use microscopic morphological analysis to exclude duplicated isolations of the dominant strains. For the sediment samples we studied, when we check the growth of microbes every 1–2 weeks, we found that a large portion of droplets contains a motile microbe with a high cell density. Indiscriminate picking of droplets resulted in high frequency of isolation of *Sulfitobacter pontiacus* as confirmed by the PCR and sequencing (Fig. 2C), and led to the reduced diversity of isolation. By excluding suspected droplets based on microscopic morphological analysis, reduction of highly duplicated species was achieved and the diversity of the isolation improved significantly (Fig. 2D). The above technical advances comprehensively improved the efficiency of the MSP platform in the isolation of microbial species from environmental samples.

Microbial isolation with the improved MSP platform

To evaluate the efficiency of bacterial isolation using the improved MSP platform, we cultivated five deep-sea sediment samples from the SWIR (S1–S5) for 5 months and collected a total of 1344 droplets with visible bacterial growth under the microscope during 5 months. We then scaled up the droplets to microliters in microwell plates and translated them to agar plates. In total, 772 bacterial strains were successfully isolated from these droplets. After removing the duplicated strains based on the colony morphology, 627 bacterial strains were biologically classified (EZBioCloud BLAST) to 4 phyla, 39 genera and 70 species (Fig. 3). *Proteobacteria* was the most abundant phylum (50.7%), which was similar to the amplicon sequencing results, followed by *Actinobacteria* (39.2%), *Firmicutes* (9.88%), and *Deinococcus–Thermus* (0.015%). *Williamsia serinedens* and *Sulfitobacter pontiacus* were the two most abundant species, accounting for 20.7% and 18.0% of the total isolates, respectively (Table S1†). Among these isolates, 15 were found to be potentially novel species according to their 16S rRNA similarity (<98.7%), and three were identified as novel species.[37]

Effectiveness of the MSP platform in the recovery of rare species

To demonstrate the superiority of the MSP platform in revealing the rare biosphere of deep-sea sediments along the

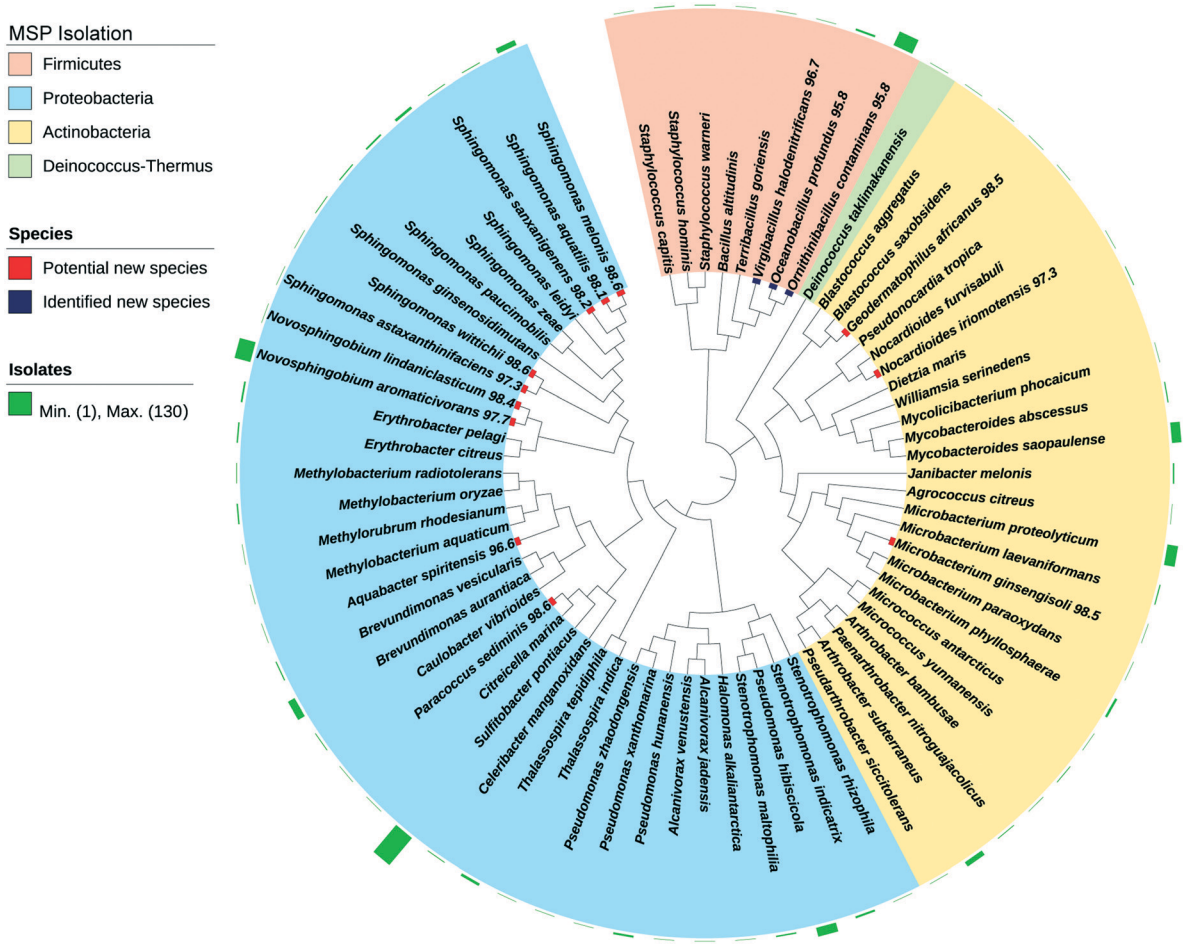

Fig. 3 Phylogenetic tree of the MSP isolated strains. The tree was built with the 16S rRNA gene sequences (amplified with 27F/1492R). Most isolates belonged to the phyla *Proteobacteria* (blue) and *Actinobacteria* (yellow), while only a few isolates belonged to the phyla *Deinococcus–Thermus* (green) and *Firmicutes* (orange). Red annotations at the node ends indicate potential new species (similarity of <98.7%) and blue annotations indicate strains that have already been identified and published as novel stains. Green bars indicate the number of strains isolated with the MSP platform.

SWIR, we should first glimpse at the whole microbial community in the MSP and non-MSP cultured samples. We compared the 16S rRNA gene amplicon sequencing of the original samples from the deep-sea samples (S1–S5), the MSP pooled cultures (M1–M5) and the agar plate pooled cultures (A1–A5) and further compared their microbial diversities. We failed to obtain the sequences from samples S5, M5, and A5; therefore, these samples were omitted upon analysis. Overall, a total of 12 samples were sent for sequencing (S1–S4; M1–M4; A1–A4). After paired-end joining and quality control, approximately 780 000 qualified reads were recovered with an average length of 400 bp. An operational taxonomic unit (OTU) table that contains the distribution information of each OTU in all the samples based on the 97% sequence similarity was generated, and the taxonomy of each OTU was classified using the Ribosomal Database Project (RDP) classifier. The rarefaction curves of each sample indicated that the sequencing depth was adequate to reflect the bacterial diversity in these samples (Fig. S3A†). A total of 3415 OTUs assigned to 57 known phyla were obtained in these samples. The detailed descriptions of the microbial diversity along the SWIR revealed by the amplicon high-throughput sequencing of the original samples, MSP pooled samples and agar pooled samples are shown in Fig. S3B and C† and are well documented in the ESI.†

After data processing, the major differences between the MSP and agar pool samples are shown in the statistical graphs (Fig. 4). Based on the amplicon sequencing results of the original, agar-pooled and MSP-pooled samples, the difference between MSP cultivation and agar plate cultivation was compared. Firstly, principal coordinates analysis (PCoA) was used to evaluate the diversity difference of the three datasets of the original samples, MSP pool and agar pool (Fig. 4A), which indicates that the samples using the same experimental approach are more similar to each other than samples using different experimental approaches. Then, the Shannon index was calculated to show the diversity of all the samples (Fig. 4B), and again, the MSP pool sample showed a much higher Shannon index than the agar pool, suggesting a higher ability in recovering microbial species of the MSP

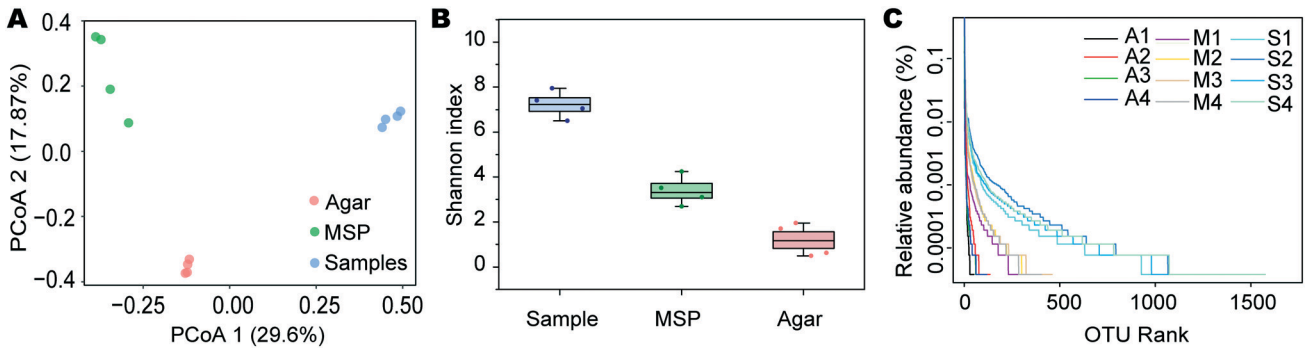

Fig. 4 Comparison of diversity indices based on amplicon sequencing results in this study. (A) Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity representing the differences among the sample, MSP pool and agar pool results. (B) Shannon index showing the alpha diversity of the three methods, indicating a higher ability of the MSP in capturing microbial species than that of traditional agar plate cultivation. (C) Rank–abundance curves showing the microbial abundance and evenness of the three methods.

technique over that of traditional agar plate cultivation. Besides the two diversity indices used above, the advantages of droplet-based single-cell cultivation of the MSP were further confirmed by the OTU rank–abundance curves (Fig. 4C), with higher species richness and better evenness than those of the conventional agar pool samples (Fig. 4C). Together, these results demonstrated that compared to the conventional agar plate, the MSP method is a superior tool in recovering microbial species with higher diversity from environmental samples.

The robustness of the MSP platform in rare microbe recovery was further confirmed by looking deeply into the OTU table. Briefly, an OTU table gives a statistic overview of the taxonomic composition and abundance of the microbial community. Rare species are those microbes whose relative abundances are $<0.01\%$.[31] According to the Venn diagram based on the OTU table, a total of 3415 OTUs were detected with the three methods in this present study, which is 486 OTUs higher than the 2929 OTUs that were only detected in the original samples (Fig. 5A). Although the original samples constituted most of the OTUs detected in this study, the MSP culture expanded the OTU number with 384 OTUs, which did not appear in the original samples. Those OTUs belong to rare microbe species below the detection limit of the current sequencing method. Besides, as shown in Fig. 5B, more than 90% of the OTUs in the MSP pool samples belong to the rare biosphere, which abundance is lower than 0.01% in the original samples. This shows that the MSP has great potential for uncovering the microbial rare biosphere in deep-sea sediments. The heatmap shown in Fig. 5C also indicated that the rare microbes were highly enriched after MSP cultivation. Agar plate incubation also obtained a number of OTUs that were not detected in the original samples, but with a much less frequency (133, about 1/3 to that of the MSP method). These results again proved that the MSP method is superior to the conventional agar plate, with a higher ability in revealing the rare biosphere, and the two methods can be combined with high-throughput sequencing to give a more comprehensive understanding of microbial diversity in the environment, and can also be a powerful tool in isolating novel strains from extreme environments, especially for those that need long-term incubation.

High-throughput sequencing revealed a skewed distribution throughout the three datasets of the original samples, agar pool and MSP pool in Fig. 5A or Fig. 5C, which is commonly observed in microbial communities.[31] Importantly, a large part of OTUs was only observed by one method, indicating the necessity of combined approaches. First of all, chances of contamination were largely excluded with rigorous aseptic procedures including the washing step and operation in a clean bench. Moreover, our marine-specific cultivation medium with high concentration of sea salt is not suitable for laboratorial environmental microbial growth. The possible mechanism responsible for divergence is that molecular techniques can only reveal partial microbial diversity, and culture-based methods have a "culture clash" in recovering microbial species.[47] Similar comparisons between sequencing and cultivation were also reported with traditional agar plate cultivation,[32,34,48–50] soil slurry membrane system (SSMS),[35] and culturomics.[51] Besides, it was estimated that about 10^{11} reads in sequencing depth might be needed if one wants to capture all of the microbial species.[52] At the given sequencing depth, some rare species may be omitted by high-throughput sequencing.[31] Moreover, culture-based methods, including the MSP and agar plates, might disproportionately enrich or select some copiotrophs or opportunistic taxa, which have low abundance in the environment and can flourish at favorable nutrient conditions.[31] Therefore, the combination of culture-dependent and culture-independent methods can reveal more comprehensive microbial diversity.

It is anticipated that after MSP cultivation or traditional agar plate cultivation, many taxa in the original samples were decreased or missed (Fig. 5C). This phenomenon occurs because most environmental microbes cannot be cultivated using artificial medium, and only some microbes can be enriched and isolated currently.[3] However, the results showed that the number of taxa enriched by the MSP was significantly higher than that of the traditional agar plate cultivation. The possible mechanism responsible for the ability of recovering more microbes from samples might be

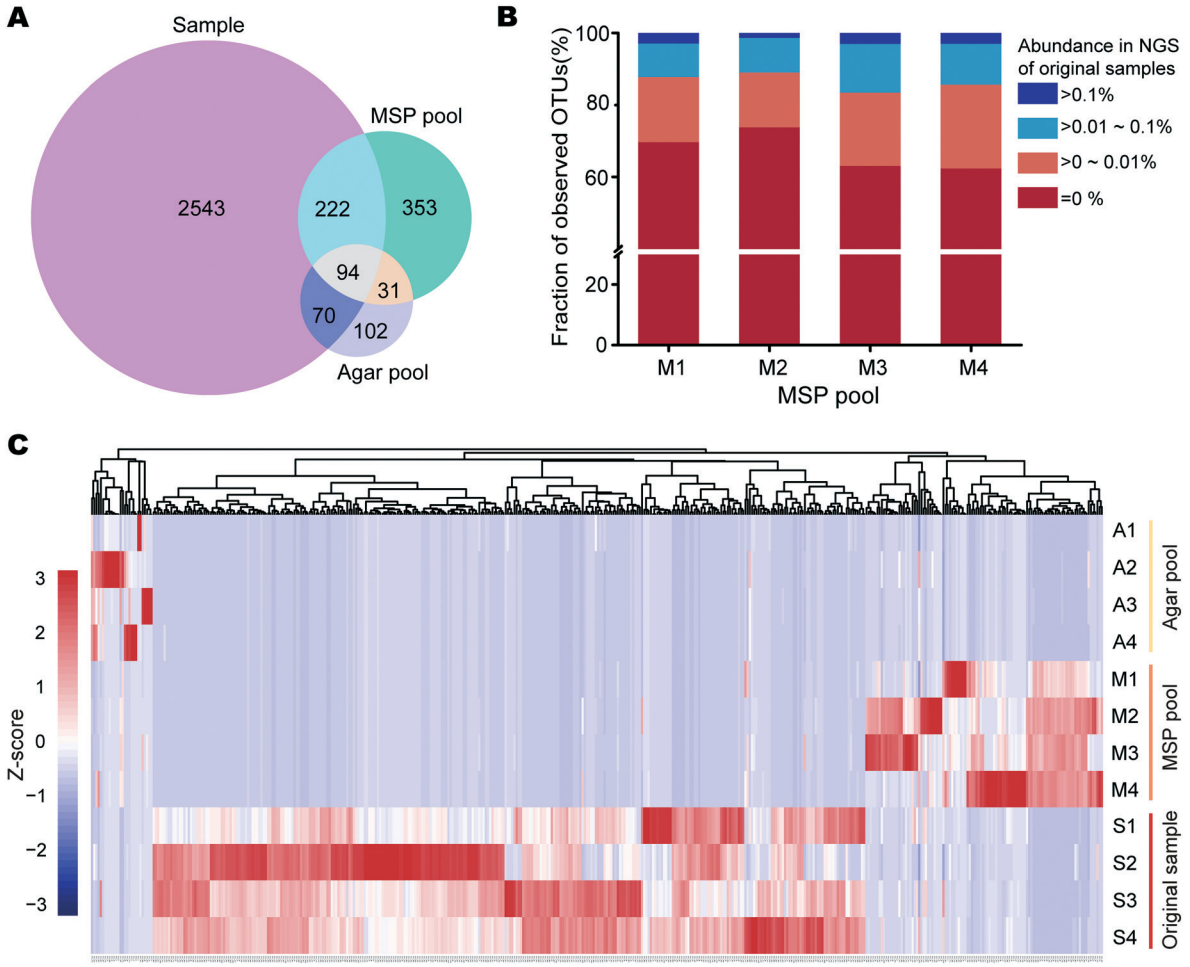

Fig. 5 Comparison of MSP cultivation and traditional agar plate cultivation using the amplicon sequencing results of the original sediment samples, the MSP pool samples, and the agar pool samples. (A) Venn diagram indicating the communal and unique operational taxonomic units (OTUs) of different sample types. (B) Bar chart constructed with all OTUs of the four MSP pooled samples indicates the ability of the MSP in revealing the rare biosphere. >0.1%, >0.01–0.1%, >0–0.01% and 0% indicate the abundance of OTUs in original samples; 0% indicates that these OTUs are detected in the MSP pool but absent in the original samples. (C) A heatmap was constructed with these OTUs that have an abundance of higher than 0.01% in the original, MSP pool and agar pool samples. The abundance of the OTUs was normalized and the scale bar is the *Z*-score value of the OTUs.

the isolation-efficiency introduced by the MSP nanoliter droplets. Specifically, the encapsulation of single-cells in the nanoliter droplets provides an isolated space for each cell, thereby fully occupying the space and media without competition with other cells for nutrients. Moreover, the liquid culture in droplets provides a physically and chemically different environment than conventional agar-based solid media. It can avoid the growth-inhibitory effect of reactive oxygen species (ROS) such as hydrogen peroxide generated during the autoclave treatment of agar media.[53,54] Furthermore, cells on agar plates or in the environment often face severe competition and are, therefore, always suppressed by fast-growing species, preventing the growth and recovery of slow-growing rare species.

Conclusions

This study explains the retrieval of bacteria from environmental samples using our improved MSP platform based on single-cell cultivation in nanoliter sessile droplets. We significantly improved the MSP method to enable more efficient, long-term, and selective cultivation of environmental microbiomes. We also developed a facile droplet picker that greatly improves the microbial isolation efficiency. For long-term cultivation of deep-sea sediments, the new platform allows the cultivation of nanoliter droplets for more than 5 months with limited evaporation and contamination. The high-throughput sequencing of pooled droplets from the MSP allows the microbial diversity assessment of culturable species in the sample, which was compared with the microbial diversity of the original sediments and the pooled sequences from conventional agar plate cultures.

In summary, the new MSP platform provides an unprecedented tool in discovering the rare species in the environmental communities, especially for those slow-growing rare species that cannot be recovered with conventional agar plate or under the detection limit of the

in-fashion NGS technology. Pure cultures of novel strains were also isolated with the platform, indicating its great potential for excavating novel microbial resources. We believe that the MSP platform will be a powerful tool in harvesting valuable microbial resources, and the combination of the MSP with the conventional culture methods and NGS will enable more comprehensive depiction of microbial diversity and rare biosphere in various environments.

Conflicts of interest

There are no conflicts to declare.

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