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Cultivation of microbes from the deep-sea environments

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ABSTRACT

The deep-sea environment has rich microbial resources, and these resources have been an important subject in the efforts to culture microflora. Special devices that maintain *in situ* pressures have been developed and applied in culturing piezophilic and hyperpiezophilic microbes. However, culturable microorganisms comprise the minority of deep-sea microbes (archaea and bacteria), which reflects the disadvantages of traditional cultivation methods, the ignorance of microbial habitats, and the fastidiousness of microbial growth requirements. This mini-review introduces the diversity of microbes in the deep sea and discusses the deep-sea species that have been identified in the past two years. In addition, this review summarizes almost all of the recognized piezophilic microbes and describes the isolation methods that have been employed. Additionally, we recommend that some of the methods that have been developed to obtain microbes from surface water, freshwater, sediments, soils and organisms should be modified to enable the isolation of the deep-sea microbes. It is anticipated that this mini-review will provide novel insights into exploration of “uncultured” deep-sea microbial resources.

1. Introduction

Oceans cover ~71% of our planet and have an average depth of 3800 m with an average pressure of 38 MPa (380 atm). The deep sea, which generally refers to the sea with a depth of > 1000 m, accounts for ~75% of the total ocean volume (Fang et al., 2010). The salinity of deep seawater is ~35, and the deep-sea temperature is ~1–3 °C (Skropeta and Wei, 2014), except at hydrothermal vents, where the temperature can reach 460 °C at the heart of the chimney though the surrounding water may be only 2 °C (Jebbar et al., 2015). The pH is ~7.8–7.9 in most deep-sea locations that have been examined (Park, 1966; Tan et al., 2012). The deep ocean lacks sunlight because light can only reach to a water depth of 250 m. Current speeds at the sea bottom are ~10 cm s⁻¹ at bathyal depths, and ~4 cm s⁻¹ at abyssal depths (Skropeta and Wei, 2014). Pressure increases by 1 atm for every 10-m depth increase. The oxygen concentration of bottom water in the deep northeast Pacific Ocean is 200–300 μmol/kg (Cai and Reimers, 1995), and D'Hondt et al. (2015) found that oxygen penetrates the entire sediment column underlying the South Pacific Gyre, where sediment slowly accumulates in a shallow layer. The concentration of dissolved organic carbon in the deep ocean is ~35–48 μmol/kg (DeLong et al., 2006; Bercovici and Hansell, 2016). A variety of topographical features exist in the deep-sea environments, such as hydrothermal vents, cold

seeps, oceanic trenches, seamounts, and bathyal plains, which possess different physical and chemical properties. The extreme environment supports a particular microbial community, which, in turn, affects the surrounding habitat.

All the above mentioned factors may have considerable influences on microbial life. However, most critical factors (in order of importance) are nutrient concentration, temperature, and hydrostatic pressure (Jannasch et al., 1982). Moreover, pressure effects on microbes are influenced by temperature. High hydrostatic pressure is a most significant physical characteristic of the deep sea, and distinguishes it from shallow seas. Thus, deep-sea bacteria may be considered as piezotolerant (growth from 0.1 to 10 MPa), piezophilic (growth from 10 to 70 MPa), and hyper-piezophilic (no growth at pressure lower than 50 MPa) based on their optimal growth pressures (Fang and Bazylinski, 2008). As piezotolerant bacteria do not have strict requirement of high pressure, they have been regularly cultivated and the diversity of piezotolerant bacteria is relatively high (Table 1 and Table 2). Piezophilic microbes have also been cultured, but their diversity is very low and mainly includes representatives of five bacterial genera belonging to γ-proteobacteria, i.e., *Photobacterium*, *Shewanella*, *Colwellia*, *Psychromonas* and *Moritella*, whereas piezophilic archaea are mostly (hyper-) thermophiles from *Thermococcales* (Oger and Cario, 2013). Obligate piezophilic microbes, whose activities are restricted by unique

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Table 1
Novel bacterial species isolated from deep-sea environments during 2014-2015 and their isolation methods.

Methods	Taxon	References
Plating methods using regular media	Marine agar 2216	<i>Nocardioideis pacificus</i> Fan et al. (2014a)
		<i>Luteimonas abyssi</i> Fan et al. (2014b)
		<i>Luteococcus sediminum</i> Fan et al. (2014c)
		<i>Oceanobacillus pacificus</i> Yu et al. (2014)
		<i>Achromobacter sediminum</i> Zhang et al. (2014)
		<i>Roseivivax marinus</i> Dai et al. (2014)
		<i>Salipiger nanhaiensis</i> Dai et al. (2015)
	R2A agar	<i>Desmospora profundinema</i> Zhang et al. (2015)
		<i>Lentisphaera profunda</i> Choi et al. (2015)
		<i>Marinithermophilum abyssi</i> Zhang et al. (2015)
Plating methods using modified media	Marine agar 2216 containing MnCl ₂	<i>Altererythrobacter atlanticus</i> Wu et al. (2014)
Plating methods using special media	R2A agar containing cycloheximide	<i>Caviceella subterranea</i> França et al. (2015)
	Marine agar 216L	<i>Mameliella atlantica</i> Xu et al. (2015)
Others methods	FRPFO medium	<i>Marinibacterium profundimaris</i> Li et al. (2015)
		<i>Zunongwangia atlantica</i> Shao et al. (2014)
		<i>Anoxybacter fermentans</i> Zeng et al. (2015b)
		<i>Caloranaerobacter ferrireducens</i> Zeng et al. (2015a)
	PAH medium	<i>Celeribacter indicus</i> Lai et al. (2014)
Others methods	Continuous-flow bioreactor	<i>Spirochaeta psychrophila</i> Miyazaki et al. (2014)
	Dilution to extinction technique	<i>Sulfurovum aggregans</i> Mino et al. (2014)
		<i>Thermococcus cleftensis</i> Hensley et al. (2014)
		<i>Thermococcus paralvinellae</i> Hensley et al. (2014)
	Potential controlled electrode	<i>Brevundimonas denitrificans</i> Tsubouchi et al. (2014)
	Hungate tubes	<i>Thermococcus nautili</i> Gorlas et al. (2014)
		<i>Crassaminicella profunda</i> Lakhal et al. (2015)
		<i>Methanoculleus taiwanensis</i> Weng et al. (2015)

temperature and pressure requirements, may be indigenous deep-sea residents, such as *Psychromonas hadalis/kaikoe*, *Colwellia hadaliensis/piezophila*, *Shewanella benthica*, and *Moritella yayanosii* (Table 2), whereas some piezotolerant bacteria may originate from other environments, such as spore-forming bacteria like *Clostridium* strains (Lauro et al., 2004) and *Bacillus stearothermophilus* (Bartholomew and Rittenberg, 1949).

Only a minor fraction of the microorganisms in the deep sea has been obtained in culture (Deming and Baross, 2000). On one hand, cells may be active *in situ*, but they can be resistant to culturing or can remain inactive (dormant) *in situ* due to starvation or other stresses for long periods and require recovery using particular incubation conditions. On the other hand, there are common problems inherent to culture as follows: (1) Medium: concentration and composition of organic/inorganic constituents may be inappropriate; growth of some cells may be activated or suppressed by certain chemicals; some cells may not grow on a solidified agent. (2) Cultivation condition: physicochemical factors may be unsuitable, such as pressure, temperature and the concentration of O₂; the communication among cells may be limited or destroyed. (3) Method of detection or separation: growth of some cells is very slow and/or growth may reach saturation at low cell concentrations, so that some simple, rapid and highly sensitive methods of detection or separation are required.

In this mini-review, we summarize the current knowledge of recognized piezophilic microbes (including their isolation methods), and recommend effective cultivation strategies with the aim of presenting novel insights into culturing microbes from deep-sea environments.

2. Diverse deep-sea microbes

The deep sea is the largest biome, and it contains more than half of the ocean's microbes (Salazar et al., 2016). By culture-independent methods, highly diverse bacteria have been found in deep-sea sediments (Schauer et al., 2010) and deep water. For example, using a massively parallel tag sequencing strategy, Sogin et al. (2006) indicated that most of the diversity was due to numerous, low-abundance populations of microbes (or so called "rare biosphere") in deep-sea water. Using high-throughput sequencing of the 16S rRNA gene, Salazar et al.

(2016) found that most abundant pelagic prokaryotes present in the deep ocean at a global scale were *Gammaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria*, *Thaumarchaeota* and *Deltaproteobacteria*, and ~50% of the operational taxonomic units (OTUs) belong to previously unknown prokaryotic taxa, most of which are rare and appear in just a few samples. In addition, the study of He and Zhang (2016) revealed that *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the predominant phyla in deep-sea hydrothermal vents.

On the other hand, many groups have made efforts to explore and culture special and diverse deep-sea microbes from various environments. Thermophile archaea and bacteria (Zeng et al., 2015b; Zhao et al., 2015) were isolated and identified from deep-sea hydrothermal vent samples (i.e. sediments, black smoker chimney, or sulfide). Gram-positive piezophilic bacteria were often isolated from deep subsurface sediments (Runke et al., 2014). Polycyclic aromatic hydrocarbonoclastic bacteria were obtained from deep-sea sediments, such as the high-latitude Arctic Ocean (Dong et al., 2014) and the Middle Atlantic Ridge (Cui et al., 2008). Recently, Ciobanu et al. (2014) endeavored to cultivate microbes from 1922 m below the seafloor of the Canterbury Basin using methods that mimicked *in situ* conditions, and showed that both bacteria (belonged to *Alpha*-, *Beta*-, *Gamma*-*proteobacteria*, *Actinobacteria* and *Armatimonadetes*) and fungi (e.g., *Cadophora*) could be recovered from deep sediment layers. In addition, bacteria from deep-sea water have been regularly cultured and identified (Cao et al., 2014; Huo et al., 2015; Li et al., 2015). Deep-sea eukaryotes, namely, amphipods (Lauro et al., 2007), deep-sea fishes (Nakayama et al., 1994), holothurians (Deming et al., 1984) and sponges (Xin et al., 2011), also provide habitats for microbial life. Sinking particles in the benthic environment were revealed to carry abundant heterotrophic bacteria that could be cultured (Deming, 1985). Overall, microbes with various characteristics are widely distributed in almost all areas of the deep sea.

Using traditional culturing methods for surface seawater, freshwater and soil microorganisms, many researchers have obtained deep-sea microbes. However, better success has followed from the use of modified approaches or special strategies for isolating deep-sea microbes. Recently, using modified media, dilution-to-extinction techniques, or special devices, more than 40 novel deep-sea bacteria and archaea have

Table 2
List of known cultured facultative and obligate piezophiles.

Isolation methods	Physiology	Strains	Taxon	Origin	Depth (m)	Sample location	References
Silica gel pour tubes (Dietz and Yayanos, 1978)	Facultative piezophiles	CNPT3	<i>Psychromonas marina</i>	decaying amphipods; amphipod trap water	5800	North Pacific	Yayanos et al. (1979); Yayanos (1986); Delong and Yayanos (1986)
		PE31/PE35	-	seawater	3584	Eastern Pacific	Yayanos et al. (1982)
		BBS2/3	-	sediment	4120	North Atlantic	Deming (1985)
		BBDT1/2	-	fecal pellet	3920	North Atlantic	Deming (1985)
		BBP5/6	-	fecal pellet	4695	North Atlantic	Deming (1985)
		BBG3/4	-	holothurian	4120	North Atlantic	Deming (1985)
		PE36	<i>Moritella</i> sp.	amphipod trap water	3584	East Pacific	Yayanos (1986); Delong and Yayanos (1986)
		SC2A	<i>Shewanella</i> sp.	seawater	1957	East Pacific	Delong and Yayanos (1986)
		AT7	<i>Carnobacterium</i> sp.	seawater	2500	Aleutian Trench	Delong and Yayanos (1986); Lauro et al. (2007)
		AT12	<i>Carnobacterium</i> sp.	seawater	2500	Aleutian Trench	Lauro et al. (2007)
	Obligate piezophiles	MT41	<i>Codwellia</i> sp.	decaying amphipods	10476	Mariana Trench	Yayanos et al. (1981); Delong and Yayanos (1986)
		PT99	<i>Shewanella benthica</i>	decaying amphipod	8600	Philippine Trench	Yayanos (1986)
		PT48	<i>Shewanella benthica</i>	amphipod trap water	6163	Philippine Trench	Delong and Yayanos (1986); Delong et al. (1997)
		MT199	-	amphipod trap water	3584	North Pacific	Yayanos (1986)
		YC-1	<i>Profundimonas piezophila</i>	seawater	6000	Puerto Rico Trench	Cao et al. (2014)
The DEEP-BATH system (Kyo et al., 1991)	Facultative piezophiles	HS11	<i>Psychromonas</i> sp.	sediments	5800	Hawaii coast	Delong and Yayanos (1986)
		PT64	-	decaying amphipods	3584	North Pacific	Yayanos (1986); Delong and Yayanos (1986)
		#302	<i>Shewanella benthica</i>	sediments	3064	Japan Sea	Arakawa et al. (2006)
		#304	<i>Moritella yayanosii</i>	sediments	3064	Japan Sea	Arakawa et al. (2006)
		CT01	<i>Shewanella</i> sp.	sediments	5000	Kurile Trench	Sekiguchi et al. (2010)
	^a Piezophiles	CT03	<i>Shewanella</i> sp.	sediments	5000	Kurile Trench	Sekiguchi et al. (2010)
		CT08	<i>Moritella</i> sp.	sediments	5000	Kurile Trench	Sekiguchi et al. (2010)
		CT12	<i>Moritella</i> sp.	sediments	5000	Kurile Trench	Sekiguchi et al. (2010)
		JT01	<i>Moritella</i> sp.	sediments	5000/7000	Japan Trench	Sekiguchi et al. (2010)
		36B1/36C1	-	deep-sea fish	4700	Northwestern Pacific	Yano et al. (1997)
	Facultative piezophiles	16C1	-	deep-sea fish	3100	Northwestern Pacific	Yano et al. (1997)
		47A1	-	deep-sea fish	3200	Northwestern Pacific	Yano et al. (1997)
		33E1	-	deep-sea fish	5300	Northwestern Pacific	Yano et al. (1997)
		2D2	-	deep-sea fish	6100	Northwestern Pacific	Yano et al. (1997)
		56D1/56E1	-	deep-sea fish	5600	Northwestern Pacific	Yano et al. (1997)
The dorayaki method (Nakayama et al., 1994)	Facultative piezophiles	38D1	-	deep-sea fish	5900	Northwestern Pacific	Yano et al. (1997)
		DB5501	<i>Shewanella benthica</i>	sediment	2485	Suruga Bay	Kato et al. (1995)
		DB6101	<i>Shewanella benthica</i>	sediment	5110	Ryukyu Trench	Kato et al. (1995)
		DB-172F/R	<i>Shewanella benthica</i>	sediment	6499	Izu-Bonin Trench	Kato et al. (1996); Qureshi et al. (1998)
		DSS12	<i>Shewanella violacea</i>	sediment	5110	Ryukyu Trench	Nogi et al. (1998b)
	Obligate piezophiles	DSK 1	<i>Moritella japonica</i>	sediment	6356	Japan Trench	Nogi et al. (1998a)
		DSJ4	<i>Photobacterium profundum</i>	sediment	5110	Ryukyu Trench	Nogi et al. (1998c)
		DB21MT-2	<i>Shewanella benthica</i>	sediment	10898	Mariana Trench	Nogi and Kato (1999); Kato et al. (1998)
		DB21MT-5	<i>Moritella yayanosii</i>	sediment	10898	Mariana Trench	Nogi and Kato (1999); Kato et al. (1998)
		Y223G	<i>Codwellia piezophila</i>	sediment	6278	Japan Trench	Nogi et al. (2004)
Growing the cells with low-melting agarose under the high pressure (Kato et al., 1995)	Facultative piezophiles	Y251E	<i>Codwellia piezophila</i>	sediment	6278	Japan Trench	Nogi et al. (2004)
		DB6705	<i>Shewanella benthica</i>	sediment	6356	Japan Trench	Kato et al. (1995)
		DB6906	<i>Shewanella benthica</i>	sediment	6269	Japan Trench	Kato et al. (1995)
		JT403	-	seawater	4000	Japan Trench	Sakiyama and Ohwada (1997)
							(continued on next page)
	^a Piezophiles						
	Facultative piezophile						

Table 2 (continued)

Isolation methods	Physiology	Strains	Taxon	Origin	Depth (m)	Sample location	References
medium under the high pressure (Sakiyama and Ohwada, 1997)	Obligate piezophiles	JT761 JTW-863 MTW-1 MTW-13 23/27/72 27A/AB/AS/ 51A	- <i>Moritella yayanosii</i> <i>Shewanella benthica</i> - - -	seawater seawater seawater seawater amphipods	7481 6000 10500 10500 4900 5100	Japan Trench Japan Trench Japan Trench Japan Trench Northwest Atlantic Northwest Atlantic	Sakiyama and Ohwada (1997) Radjasa (2013) Radjasa (2013) Radjasa (2013) Jannasch and Wirsén (1984) Jannasch and Wirsén (1984)
Isolation procedure on certain medium under the high pressure	Facultative piezophiles						
Enrichment cultures in certain condition; pure culture on agar plate	Facultative piezophiles	UM40 W145 2674	<i>Vibrio</i> sp. <i>Vibrio</i> sp. <i>Moritella profunda</i>	amphipod holothurian sediments	5920 4575 2815	Puerto Rico Trench South Atlantic Atlantic Ocean	Deming et al. (1984) Deming et al. (1984) Rüger and Tan (1992); Xu et al. (2003a)
		2693	<i>Moritella abyssi</i>	sediments	2815	Atlantic Ocean	Rüger and Tan (1992); Xu et al. (2003a)
		2825	<i>Psychromonas profunda</i> .	sediments	2770	Atlantic Ocean	Rüger and Tan (1992); Xu et al. (2003b)
		LT13a JAL-1	<i>Shewanella profunda</i> <i>Methanocaldococcus jannaschii</i>	sediments hydrothermal vent sample	4795 2600	Nankai Trough East Pacific Rise	Toffin et al. (2004) Jones et al. (1983); Jebbar et al. (2015)
Enrichments using certain medium, purified with an agarose medium	Obligate piezophile	IHB1 WHB46	<i>Thermosiphon japonicus</i> <i>Shewanella benthica</i>	black smoker chimney seawater	972 4995	Iheya Basin Weddell Sea	Takai and Horikoshi (2000) Liesack et al. (1991); Fang et al. (2010)
Enrichment using certain medium, purified by agar shake dilution series	Facultative piezophiles	500-1 80-55	<i>Desulfovibrio profundus</i> <i>Desulfovibrio profundus</i>	sediment sediment	1400 980	Japan Sea Japan Sea	Bale et al. (1997) Bale et al. (1997)
Enrichments using certain medium, purified by serial dilutions	Facultative piezophilic archaeon	GB5 TVS	<i>Pyrococcus abyssi</i> <i>Thermococcus guaymasensis</i>	a fluid sample sediment	2000 2000	North Fiji basin Guaymas Basin	Erraou et al. (1993) Canganella and Jones (1994); Canganella et al. (1998); Canganella et al. (2000)
		TY	<i>Thermococcus aggregans</i>	sediment	2000	Guaymas Basin	Canganella and Jones (1994); Canganella et al. (1998); Canganella et al. (2000)
		Archaeon MP KT27	<i>Thermococcus barophilus</i> <i>Cotwellia</i> sp.	chimney samples amphipods	3550 9856	Mid-Atlantic Ridge Kermadec Trench	Marteinsson et al. (1999) Lauro et al. (2007); Lauro et al. (2013);
		KT99	<i>Shewanella benthica</i>	amphipods	9856	Kermadec Trench	Lauro et al. (2007); Lauro et al. (2013);
Enrichment cultures in certain condition.	Facultative piezophiles	116 106 108	<i>Methanopyrus kandleri</i> <i>Thiopropindum lithotrophica</i> <i>Piezobacter thermophilus</i>	black smoker fluid black smoker chimney black smoker chimney	2420 3626 3626	Central Indian Ridge Mid-Atlantic Ridge Mid-Atlantic Ridge	Takai et al. (2008) Takai et al. (2009) Takai et al. (2009)
Purified by the dilution-to-extinction method		DY22613 Archaeon DMJ Archaeon DY20341 Archaeon A501	<i>Anoxybacter fermentans</i> <i>Palaeococcus ferrophilus</i> <i>Palaeococcus pacificus</i> <i>Thermococcus eurythermalis</i>	hydrothermal sulfides black smoker chimney sediment the spire of chimney of a hydrothermal vent	2891 1338 2737 2006.9	East Pacific Rise Ogasawara Trough East Pacific Ocean Guaymas Basin	Zeng et al. (2015) Takai et al. (2000) Zeng et al. (2013) Zhao et al. (2015)
		BNL-1 KA3 Archaeon CH1	<i>Cotwellia hadaliensis</i> <i>Marinoga piezophila</i> <i>Pyrococcus yanosii</i>	particle-rich seawater chimney samples black smoker sulphide	7410 2630 4100	Puerto Rico Trench East-Pacific Rise Mid-Atlantic Ridge	Deming et al. (1988) Alain et al. (2002) Zeng et al. (2009); Birrien et al. (2011)
Dilution-to-extinction cultivation methods	Obligate piezophile	PRT1	<i>Roseobacter</i> sp.	seawater	8350	Puerto Rico Trench	Eloe et al. (2011)
Enrichment using SRB growth medium. Purified by repeated use of the roll-tube method	Facultative piezophiles	AM13 CITLV30	<i>Desulfovibrio hydrothermalis</i> <i>Desulfovibrio piezophilus</i>	hydrothermal chimney sample wood falls placed on deep-sea sediments	2600 -	East Pacific Rise Eastern Mediterranean	Alazard et al. (2003) Khelaifa et al. (2011)
Filter sampler with attached transfer unit	Facultative piezophile	FIA	<i>Shewanella benthica</i>	seawater	4900	North Atlantic	Jannasch and Wirsén (1977); Wirsén et al. (1986); Delong et al. (1997)
Seawater samples were filtered and filters were	Facultative piezophile	ANT-2200	<i>Photobacterium phosphoreum</i>	seawater	2200	Northwestern	Al Ali et al. (2010); Martini et al. (continued on next page)

Table 2 (continued)

Isolation methods	Physiology	Strains	Taxon	Origin	Depth (m)	Sample location	References
incubated on agar medium							
Successively growing end-point dilutions	Facultative piezophile	Archaeon OG1	<i>Thermococcus peptonophilus</i>	hydrothermal vent sample	1380	Mediterranean Sea Western Pacific	(2013) González et al. (1995); Canganella et al. (1997)
-	Facultative piezophiles	SS9 MT1.1 WP2 WP3	<i>Photobacterium profundum</i> <i>Denacoccus abyss</i> <i>Shewanella psychrophila</i>	amphipod sediment sediment	2551 10898 1914	Sulu Sea Mariana Trench West Pacific	Delong et al. (1997) Pathom-aree et al. (2006) Xiao et al. (2007)
	Obligate piezophiles	JT7301 JT7304 K41G	<i>Shewanella piezotolerans</i> <i>Psychromonas kaikōae</i> <i>Psychromonas halidii</i>	sediment sediment sediment	1914 7434 7434 7542	West Pacific Japan Trench Japan Trench Japan Trench	Xiao et al. (2007) Nogi et al. (2002) Nogi et al. (2002) Nogi et al. (2007)

- The data were unavailable.
^a piezophiles; unclear whether obligate or facultative piezophiles.

been isolated, identified and recognized; these organisms were recovered from various habitats (data for statistics were from published papers in the International Journal of Systematic and Evolutionary Microbiology). Most of these bacteria belong to phyla *Actinobacteria*, *Proteobacteria* (α -, β -, γ - and ϵ -), *Bacteroidetes*, and *Firmicutes*. For archaea, most of the novel species belong to *Euryarchaeota*. A greater proportion of piezotolerant bacteria may be found than piezophilic bacteria (Table 1).

3. Cultivation of piezophilic microbes

Since the first obligate piezophilic bacterium (*Colwellia* sp. MT41) was isolated from a deep-sea decompressing amphipod in the Mariana trench (Yayanos et al., 1981), numerous deep-sea piezophilic microorganisms have been isolated from seawater, sediments and hydrothermal vents (Table 2). In general, the procedure to isolate piezophilic microbes is a combination of traditional methods and high pressure techniques; the first and most essential step is to place the cells at an *in situ* pressure as soon as possible after collection. In addition, elevated pressure, as a necessary condition for piezophilic growth, should be available throughout part or all of the isolation processes. Several high-pressure laboratory techniques have been developed, including pressure vessels for maintaining *in situ* pressure, which permit the recovery and examination of colonies at high pressure. Yayanos and his group were the first to successfully isolate a piezophilic bacterium (*Psychromonas marina* CNPT3) using silica gel pour tubes (Dietz and Yayanos, 1978). Subsequently, many piezophilic bacteria have been cultured using this method. Later, JAMSTEC (Japan Marine Science and Technology Center) designed and developed a DEEP-BATH system (deep-sea baro-piezophile and thermophile isolation and cultivation system) for collecting, isolating and cultivating deep-sea microbes (Kyo et al., 1991), with which many valuable piezophilic bacteria have been obtained. Different approaches have also been attempted. For example, Imachi et al. (2011) cultivated the methanogenic community from subseafloor sediments using a continuous-flow bioreactor.

Overall, it seems that there is a limited diversity of cultured piezophilic bacteria (Table 2), possibly reflecting artificial selection due to the use of rich, heterotrophic growth media. Therefore, it is likely that one strategy for increasing the diversity of piezophilic bacterial isolates is to develop special growth media. A second strategy is to obtain good samples, which should be kept in the dark, at a temperature and pressure commensurating with the *in situ* environmental conditions.

4. Cultivation of piezotolerant microbes

It has been reported that piezotolerant aerobic strains were often dominant whenever attempts were made to isolate microbes from some deep-sea environments. Early research on the deep sea reported that the number of bacteria that could grow in a nutrient medium at 1 atm was almost as many as could grow at 700 atm; however, only a few bacteria from Indian Ocean Deeps could grow at 1000 atm (ZoBell and Morita, 1959). More recently, Takami et al. (1997) found various non-extremophilic bacteria from the Mariana Trench. Later, Parkes et al. (2009) developed the DeepIsoBUG system for enriching and isolating deep-sea microbes without depressurization, and the results showed that almost all the enriched bacteria could grow at atmospheric pressure. Similar results were also shown by Jannasch et al. (1982) with 4000 m deep seawater samples. These studies increased our attention to piezotolerant microbes. Recently, many deep-sea bacteria and archaea have been isolated and identified, most of which were piezotolerant (Table 1). In brief, the study of piezotolerant bacteria is as important as the study of piezophiles.

Laurent and Karine (2014) suggested that a main challenge for microbiologists studying the deep biosphere was the development of culturing strategies for currently uncultured microorganisms. Many techniques with high culture efficiency that have been developed for

isolating bacteria from surface water, freshwater, sediments, soils and organisms will be described here, which can be developed to explore the deep-sea biosphere, especially for isolating piezotolerant microbes.

4.1. Improving traditional cultivation methods

Compared with common microbes, deep-sea bacteria have different growth requirements regarding nutrients and environmental factors. The information from cultured microbes provides essential knowledge about necessary growth elements for the isolation of currently uncultured bacteria, such as carbon sources, whose composition is of great importance (Uphoff et al., 2001; Alain and Querellou, 2009), but low concentration of nutrients contributes more to obtaining several conventionally-uncultured microbes. For instance, *Sphingomonas alaskensis* RB2256^T could only grow in a liquid seawater medium containing less than 1 mg l⁻¹ of dissolved organic carbon when it was first cultivated (Vancanneyt et al., 2001). Sulfur sources are also very important. For instance, Tripp et al. (2008) indicated that ‘*Candidatus* Pelagibacter ubique’ relies exclusively on reduced sulfur compounds that originate from other plankton. In addition, cultivation temperature, pH and oxygen concentration should be adjusted to simulate the *in situ* environment of the deep-sea microbes to be isolated. Moreover, bacterial physiology and function should be considered as a guide for culturing, i.e. increasing incubation time and supplying growth-requirement molecules (signal molecules, electron acceptors or cell-free extracts) (Table 3). Some disadvantages of the plating method include the production of H₂O₂ after autoclaving; this may be controlled by supplying catalase or sodium pyruvate to degrade the H₂O₂. The enrichment method may be appropriate for the recovery of some bacteria (Fichtel et al., 2012). The end result is that many deep-sea microbes have been isolated by using improved versions of traditional cultivation methods (Table 1).

4.2. Single cell isolation techniques

Spreading inocula over the surface of agar plates may have drawbacks. For example, plates have limited space and nutrients, which make cells compete with one another. Fast-growing bacteria could inhibit the growth of slow growers, contributing to the “great plate count anomaly” (Staley and Konopka, 1985). Single cell sorting approaches have been developed, and show great advantages, such as overcoming the shortcomings of spread plating. Dilution-to-extinction cultivation technique is one of the most popular methods, and it can recover many novel strains. The most abundant bacterium (SAR11) in the ocean was difficult to culture, but it was isolated using this method (Rappé et al., 2002; Stingl et al., 2007; Song et al., 2009). Another example involves

the recovery and cultivation of sulfate-reducing bacteria (Colin et al., 2013). Micromanipulation is another important technology that was used in detecting and separating single microbial cells (Fröhlich and König, 2000; Ashida et al., 2010). More recently, flow cytometry has been used, and it has enabled several thousands of single cells to be cultured. Moreover, Slava Epstein (a microbiologist at Northeastern University in Boston, USA) and his group developed and used Ichip (or minitrap) for the high-throughput *in situ* cultivation of “uncultivable” microbial species (Nichols et al., 2010; Sizova et al., 2012). Additionally, Ma et al. (2014) designed a microfluidics-based workflow for genetically targeted isolation and cultivation of microorganisms from complex clinical samples. Many more single cell isolation techniques have been reported, some of which have been used to explore the deep sea, as testified by the isolation of strain PRT1 using dilution-to-extinction method (Eloe et al., 2011).

4.3. Coculture

Many researchers are focused on communication among bacteria. Traditional methods have the disadvantage of selectivity, and single cell sorting approaches limit the inter-communication of bacteria. It is known that some bacteria need the existence of other bacteria to grow. A simple, modified process was developed to culture previously uncultured bacteria in the presence of cultured organisms from the same environment. Using this approach, D’Onofrio et al. (2010) recovered previously uncultured isolates from marine sediment biofilm grown on a Petri dish. There is a classic case in which autotrophic ammonia-oxidizing archaea from marine sediments were successfully enriched and cultivated in coculture with sulfur-oxidizing bacteria (Park et al., 2010). Culturing all bacteria together in an *in situ* environment is another coculture method. For example, a diffusion growth chamber (DGC) was developed to provide a settled place where all the strains could be kept. The chamber can be placed in a simulated *in situ* environment (Kaeberlein et al., 2002; Bollmann et al., 2007, 2010). A hollow-fiber membrane chamber (HFMC), which is a modified DGC chamber, was developed as an *in situ* cultivation device for environmental microorganisms (Aoi et al., 2009). Moreover, DGC can be successfully applied in living marine organisms, such as sponges (Steinert et al., 2014).

4.4. Combination of different techniques

It is well known that any one method cannot culture all types of microorganisms. Success can be improved if different methods are combined. Zengler et al. (2002) developed the combination of gel microdrop (GMD) technique and flow cytometry method (FCM) to

Table 3
Improved traditional cultivation methods.

Methods	Results	Ref.
Add organic sources	Yielding higher number and more diverse isolates	Uphoff et al. (2001)
Oligo-culture	Increasing the concentration of fastidious bacteria	Tripp et al. (2008)
Increase incubation times	Obtaining conventionally-uncultured species	Vancanneyt et al. (2001)
	Cultivation and detection of previously uncultured microbe	Stevenson et al. (2004)
	Allowing the development of visible colonies of members of rarely isolated groups	Davis et al. (2005)
Supply signal molecules	Isolating previously uncultured species	Stott et al. (2008)
	High cultivation efficiencies	Bruns et al. (2002)
Use electron acceptors	Inducing an “uncultivable” microorganism to grow in vitro	Nichols et al. (2008)
	Recovering previously uncultured hyperthermophiles	Kashefi et al. (2002)
	High cultivation efficiencies	Kopke et al. (2005)
Add catalase or sodium pyruvate	Resuscitating viable but nonculturable cells	Mizunoe et al. (2000)
	Improving recoverability of microbial colonies	Olson et al. (2000)
Add cell-free extract	Isolating uncultivable anaerobic thermophiles	Kim et al. (2008)
Use of other gelling reagents	Increasing culturability	Tamaki et al. (2005)
	Isolating previously uncultured species	Stott et al. (2008)
Adhesion to solid surfaces	Enriching previously uncultured bacteria	Gich et al. (2012)

Table 4

The applications of GMD and FCM techniques.

Samples	Encapsulation method	Cultivation	Media	Aim	Ref.
Seawater	Agarose; Glass membrane filter	Chromatography columns	Filtered seawater alone	High throughput culture	Ji et al. (2011)
Strain	Agarose; CellSys 100 microdrop maker	Test tubes/ flasks	Artificial seawater-based medium	Enrichment of non-growing bacteria	Akselband et al. (2006)
Seawater	Agarose; CellSys 100 microdrop maker	Chromatography columns	Filtered seawater alone	High throughput culture	Zengler et al. (2002)
Soil	Agarose; CellSys 100 microdrop maker	Chromatography columns	Diluted soil extract	High throughput culture	Zengler et al. (2002)
Strain	Agarose; Glass membrane filter	Test tubes/flasks	Thiamin-free synthetic medium	Enrichment of non-growing bacteria	Manome et al. (2001)
Strain	Agarose; CellSys 100 microdrop maker	Test tubes/ flasks	Synthetic dextrose medium	Enrichment of non-growing bacteria	Gift et al. (1996)

selectively enrich non-growing bacterial cells in an artificial model system. This technique combines the encapsulation of cells in gel microdroplets for massively parallel microbial cultivation under low nutrient flux conditions, followed by flow cytometry to detect microdroplets containing microcolonies. Under these conditions, single encapsulated cells grow and form microcolonies within the microcapsules. Flow cytometry has been used as a sensitive tool to detect growth within the microcapsules (Zengler et al., 2005). Methods have been developed to enrich mixed bacterial populations for slow-growing microorganisms using GMD combined with fluorochrome staining and flow cytometry (Table 4). Our group has used GMD method to culture the “uncultured” microbes from surface seawater (Ji et al., 2011) and has also attempted to obtain diverse bacteria from deep-sea samples (Zhang et al., unpublished). In our results, over 82% of the strains using the GMD method were not obtained by a plating method from a hydrothermal sediment at the Okinawa Trough, and 50% of the genera belonged to the class *α-proteobacteria* which was present at a very low relative abundance (0.93%) in the sample. In addition, over 44% of the strains from the GMD method were not obtained from a plating method for the polymetallic nodule area. In this case, 50% of the genera belonged to the phylum *Actinobacteria*, which was present at a very low relative abundance (1.04%) in the sample.

5. Perspectives

Although considerable progress has been made, due to the complexity of the deep-sea environment, microbes remain mysterious. The journey to explore the deep-sea world still faces enormous challenges. Four suggestions are made for the future. First, advanced deep-sea exploring devices, such as deep-water robots, deep-sea sampling and detecting equipment, should be further designed and produced. Second, special materials (organic or inorganic) that can remain stable in deep-sea conditions should be further developed. For example, new encapsulation materials would improve the application of GMD technique for culturing deep-sea bacteria under *in situ* conditions. Third, highly sensitive observation and detection technologies (such as fluorescence microscopy technology, flow cytometry technology, microbial staining technology, and other advanced methods) should be more widely developed and applied in culturing and separating microbes. Fourth, culture-independent technology (such as metagenomics, metatranscriptomics, and single-cell sequencing) remains essential for obtaining more information on uncultured microbes. It is also highly important to establish a connection between microbial growth requirements and sequence data, though this is difficult.

In the omics age today, cultivation of microbes is still necessary, as cultivation can contribute to comprehensively understanding the detailed physiology of microbes and the complex environmental processes associated with them. In addition, cultures can provide complete genomes and the means to verify potential metabolic ability or ecosystem function of microbes which have been predicted on the basis of

genomics data, for example, which organic substrates are utilized, what compounds are produced, or how metabolic reactions are performed. Moreover, cultivation is the first step for the microbes to be applied on environmental remediation, energy and pharmaceutical industry. In summary, the deep-sea remains a mystery, but cultivation of more deep-sea microbes may be a breakthrough for exploring the deep sea, and any attempt to create and develop cultivation methods would be worthwhile.

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