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REVIEW

Strategies for culturing active/dormant marine microbes

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Abstract
Microorganisms are ubiquitous in the ocean environment and they play key roles in marine ecosystem function and service. However, many of their functions and phenotypes remain unknown because indigenous marine bacteria are mostly difficult to culture. Although many novel techniques have brought previously uncultured microbes into laboratory culture, there are still many most-wanted or key players that need to be cultured from marine environments. This review discusses possible reasons for 'unculturable microbes' and categorizes uncultured bacteria into three groups: dominant active bacteria, rare active bacteria, and dormant bacteria. This review also summarizes advances in cultivation techniques for culturing each group of unculturable bacteria. Simulating the natural environment is an effective strategy for isolating dominant active bacteria, whereas culturomics and enrichment culture methods are proposed for isolating rare active bacteria. For dormant bacteria, resuscitation culture is an appropriate strategy. Furthermore, the review provides a list of the most-wanted bacteria and proposes potential strategies for culturing these bacteria in marine environments. The review provides new insight into the development of strategies for the cultivation of specific groups of uncultured bacteria and therefore paves the way for the detection of novel microbes and their functions in marine ecosystems.

Keywords Uncultured marine bacteria · Culture strategies · Dominant active bacteria · Rare active bacteria · Dormant bacteria · Most-wanted bacteria

Introduction

Although the marine microbiome has been extensively studied for decades, many novel species are still being, or are yet to be, discovered (Louca et al. 2016; Moran 2015; Sunagawa et al. 2015). Despite natural high diversity, there are still only few laboratory cultures of marine microbes, which severely limits our understanding of marine microbial ecology (Rappe et al. 2002; Rappe and Giovannoni 2003). More than 40 years ago, it was found that there were many more bacteria present in the ocean than could be cultured by plating a sample onto media (Hobbie et al. 1977). The low culturability of bacteria is well known as the 'great plate count anomaly' (Staley and Konopka 1985) and it is particularly apparent for marine organisms (Lloyd et al. 2018).

The origins of microbial culture and isolation can be tracked back to the nineteenth century at the inception of the science of bacteriology (Wildfuehr 1982). In that period, solid agar media, streak plate technology and Petri dishes were developed in Robert Koch's laboratory, which laid the foundation for current microbial culture. For more than a century, the cultivation of microbes was the foundation of microbiology and scientists manipulated nutritional requirements and adjusted medium formulations to culture novel microbes. Novel technologies were also developed to bring many previously uncultured microbes into laboratory culture. For example, culturomics technology cultured many novel bacteria from the human gut; in situ culture strategy, mimicking the natural environment, was used to isolate uncultured bacteria in the lab (Kaeberlein et al. 2002; Nichols et al. 2010); novel dilution and isolation

SPECIAL TOPIC: Cultivation of uncultured microorganisms.

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technology, such as gel microdroplets, has enabled high-throughput cultivation and selection (Zengler et al. 2002). However, there are still many bacteria to be isolated and so it remains necessary to analyze the barriers to the isolation of the uncultured bacteria, summarize the different groups of uncultured bacteria, and review the current strategies and their applied ranges for new culturing.

There is little doubt that the establishment of pure cultures, representatives of all bacterial divisions, is one of the major challenges of modern microbiology. However, with the rapid development of high-throughput sequencing technology, the status of cultivation has taken a backseat to the powerful tools developed in the last 20 years (Carini 2019). High-throughput sequencing technology allows the assessment of the diversity and genetic information of uncultured organisms, and as a result, the importance of culturing has started to become forgotten. However, this perception is changing because the limitations of high-throughput sequencing has emerged: although sequencing techniques have been able to speculate many bacteria might play key roles in the environment, their exact ecological roles and physiology need to be identified by pure culture. There is now a revised interest in isolating the most-wanted microbes from a variety of environments. Indeed, isolation is the only way to comprehensively examine the physiology of microbes: to establish which organic substrates are utilized and to determine what secondary metabolites might be released or what biotransformations might be possible. A culture is also required for a full taxonomic characterization and for the formal naming of organisms. Therefore, culturing remains an essential requirement, not only for biodiscovery, but also for determining the role of microbes.

Types of uncultured bacteria

The current methods of isolating and culturing bacteria are based on Robert Koch's streak plate technology, which is performed by diluting a comparatively high concentration of bacteria to a lower concentration. The dilution of the bacteria should be low enough that colonies are sufficiently spread apart to separate the different types of microbes. Because this method involves dilution, the initial abundance of microorganisms will inevitably, to some extent, affect their culturability. Rare or low-abundance bacteria have a greater natural diversity (Jousset et al. 2017; Lynch and Neufeld 2015). Many uncultured marine bacteria are also in low abundance and even difficult to detect by 16S rRNA gene amplicon analysis. Therefore, when the uncultured bacteria are being isolated from environments, their abundance should be reported. Based on the framework of dilution to pure culture, the uncultured bacteria can be categorized into dominant bacteria and rare bacteria.

In addition, classical microbiology describes viability as being able to multiply (i.e., viability = culturability); however, the concept of dormancy or viable but nonculturable (VBNC) is an oxymoron (Kell and Young 2000). Therefore, bacterial cell physiological status was divided into active, dormant, and dead (Kell and Young 2000). The available data suggests that microbes in a state of dormancy remain metabolically active; most dormant microbes are unculturable (Barer and Harwood 1999). To evaluate whether a microbe can be cultured, its ability to undergo cell division and form a growth curve or a colony is generally assessed. Their physiological status affects their likelihood of culturing; therefore, dormant bacteria can be considered as one group of uncultured bacteria. In total, based on the bacterial abundance and physiological status in the environment, uncultured bacteria can be categorized into three groups: dominant active bacteria, rare active bacteria, and dormant bacteria. There may be several different reasons for the unculturability of the unculturable cells. Different strategies can be applied to culture each category.

Barriers to isolate uncultured marine bacteria

Most abundant marine bacteria are unculturable under laboratory conditions (Mussmann et al. 2017; Rappe et al. 2002). For example, the SAR11 clade is ubiquitous and dominates marine bacterioplankton communities; however, it has taken more than 10 years (based on ribosomal RNA gene analysis) to culture the first type strain since it was first discovered (Giovannoni et al. 1990; Rappe et al. 2002). However, many SAR11 strains remain uncultured. In coastal, abyssal, and bathyal surface sediments, *Woeseiaceae*/JTB255 strains are ubiquitous and core members of microbial communities (Bienhold et al. 2016; Mussmann et al. 2017). However, the first type strain of this group was not isolated until 2015 (Du et al. 2016). The late isolation of the SAR11 clade and *Woeseiaceae*/JTB255 represents a pervasive problem in microbiology: in that it takes a huge effort to even culture an abundant marine species.

Why is it so difficult to culture these most abundant bacteria? One explanation is that scientists were not able to adequately simulate essential components of their natural environment. To date, many reports have shown that different physiological states occur in bacteria, ranging from unstressed living cells to dead cells (Bergkessel et al. 2016). When naturally occuring bacteria are cultured in the laboratory, they need to adjust to their new environments, forming a new growth curve (Fig. 1). If the lag phase on the Petri dish is too long to allow the formation of a colony, these cells cannot be isolated. Minor changes in the environment will possibly prolong the lag phase and this effect might be magnified for oligotrophs. New evidence suggests that numerically abundant microbes in nonhost systems are

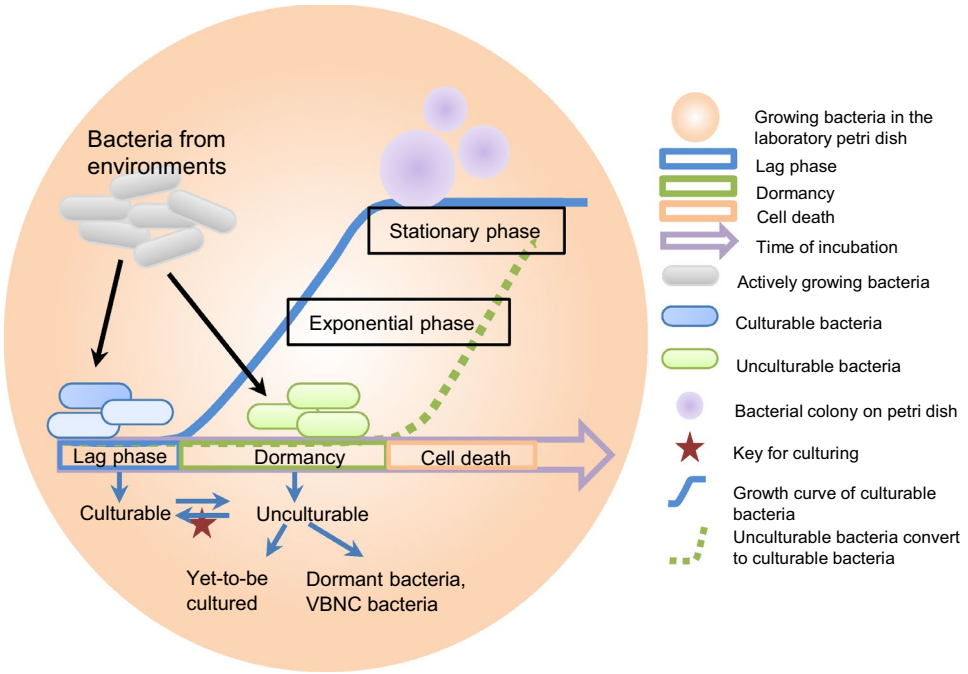

Fig. 1 Schematic diagram of the transition between culturable bacteria and unculturable bacteria under laboratory conditions. When the bacteria are cultured on the Petri dish from natural environments, culturable bacteria may adapt to laboratory conditions and form a growth curve with a short lag phase. The lag phase of uncultured bacteria is too long to produce a complete growth curve. Some uncultured bacteria may change to dormant bacteria or VNBC bacteria. When dormant bacteria cannot be revived and become culturable, they will most likely die with prolonged culturing times

oligotrophs (Gao and Wu 2018). In general, oligotrophs are hard to culture because they are small, slow-growing cells and high yields on low-nutrient media are not achieved (Carini 2019). Therefore, simulating local environments in the laboratory to reduce the bacterial lag phase on the Petri dish is the key to culture dominant bacteria.

To better mimic the natural environment to culture dominant bacteria, the types of environmental factors the bacteria require need to be identified. Unfortunately, even complete genome sequencing of bacteria cannot reveal what types of and how many environmental factors an uncultured bacterium requires (Carini 2019). Furthermore, even if the substrates requirement of a particular species is known, this does not mean that all members of that species utilize the same substrates (Joint et al. 2010). Therefore, the potential key environmental factors based on a specific natural environment in which a strain lives still need to be speculated. Commonly, both biotic factors and abiotic environmental factors affect a microorganism's growth: Abiotic factors are essential for microorganism growth and are an important selective force for microbial community structure and dynamics (Thompson et al. 2017). Abiotic factors have been well studied and are commonly recognized. When attempting to tailor synthetic media, the first focus is usually on nutrients, pH, osmotic conditions, temperature, and oxygen. However, biotic factors are complicated and may involve biological interactions or signaling; the biotic factors for culturing are largely unknown. For this reason, Rappe et al. (2002), used filtered seawater as media to simulate the original abiotic environment but was able to culture only a limited number of the dominant marine bacteria. In natural environments, interspecies symbiosis based on nutrient exchange (syntrophy) are well known (McInerney et al. 2008). As the Black Queen Hypothesis describes, microorganisms can sometimes lose the ability to perform a function that appears to be necessary for their survival; thus, they manage to survive by depending on "helper" microbes. In these "helper" microbe situations, necessary functions are performed by other bacteria, an adaptation that can encourage microorganisms to live in cooperative communities (Morris et al. 2012). As a result, simulating biotic exchange is one of the key factors in simulating an environment to culture bacteria.

One of the key barriers to culturing rare or low-abundance bacteria is their rarity. Dilution separation might miss most of them, resulting in failure of isolation. Low cell abundance can be the result of many factors, such as biotic/abiotic interactions and inherent trade-offs in life-history strategies (Jousset et al. 2017). External abiotic and biotic factors can play pivotal roles in species rarity. For instance, a highly specialized species showing a very narrow environmental niche may be abundant in a few habitats but remain rare in most others. Low abundance may also be the result of fitness trade-offs. Slow-growing species might not reach a high density but may survive well under stressful conditions (Gudelj et al. 2010). These microbes are called "k-strategists", and rapid growth will not occur in most environmental conditions (Janssen 2009). Some rare species might be slow-growing in any environment.

Many dormant microbes can remain generally inactive and at low density most of the time, only becoming dominant when more favorable conditions arise. This state

is also referred to as viable but nonculturable (VBNC) (Xu et al. 1982). Dormant bacteria might have a specific adaption in local environments; even if essential aspects of their local environments can be mimicked, they may still not grow well and are difficult to isolate (Mu et al. 2018). As a result, the key barrier to isolating dormant bacteria is an understanding of how to prompt cell division.

Strategies to solve the challenge of cultivation

For dominant active bacteria

The dominant or abundant bacteria in environments commonly exhibit high environmental adaption and cell activity (Rivett and Bell 2018). If a natural environmental sample, such seawater, is used as a growth medium, combined with diluting a sample "to extinction" (Rappe et al. 2002), abundant bacteria would theoretically be able to be isolated and cultivated (Fig. 2). In fact, approaches based on simulated environments have resulted in many abundant bacteria being isolated from culturable marine environments (Table 1) (Stewart 2012). One of the best-known cases is the isolation and culturing of the SAR11 clade (*Pelagibacterales*) (Rappe et al. 2002). Based on this strategy, members of the microbial majority, including SAR116, OM60/NOR5, SAR92, and *Roseobacter*, were isolated and cultured (Henson et al. 2016). Nonetheless, it is difficult to replicate a natural environment at an arbitrarily high level of fidelity if parameters for the growth of a given bacterial taxon are unknown. Therefore, one alternative is to take the bacteria back to the environment to grow them, often by moving a portion of the environment sample into the laboratory (Stewart 2012). Based on this theory, some in situ culture methods have been developed and have isolated and cultured many previously uncultured bacteria (Table 1) (Kaeberlein et al. 2002; Nichols et al. 2010). Another method for isolating dominant bacteria in their environment has been developed based on gel microdroplets. Indeed, encapsulated gel microdroplets can be incubated in natural environments as a growth medium (Zengler et al. 2002). By using this method, some uncultured bacteria have been isolated as microcolonies in gel microdroplets (Table 1), including lineages of *Planctomycetales* that were previously uncultured (Zengler et al. 2002).

Co-culture, a strategy that focuses on unknown biotic factors that may be effective in isolating uncultured organisms, has been proposed as another approach to simulating an environment. In fact, co-culture dependence has been recognized since the 1900s, the classic example that is used in microbiology classes being the dependence of *Haemophilus influenzae* on *Staphylococcus aureus* (Davis 1921). *H. influenzae* was found to need an exogenous source of both heme and NAD for aerobic growth. In 1919, Fleming found that the dependence of *H. influenzae* on *S. aureus* can

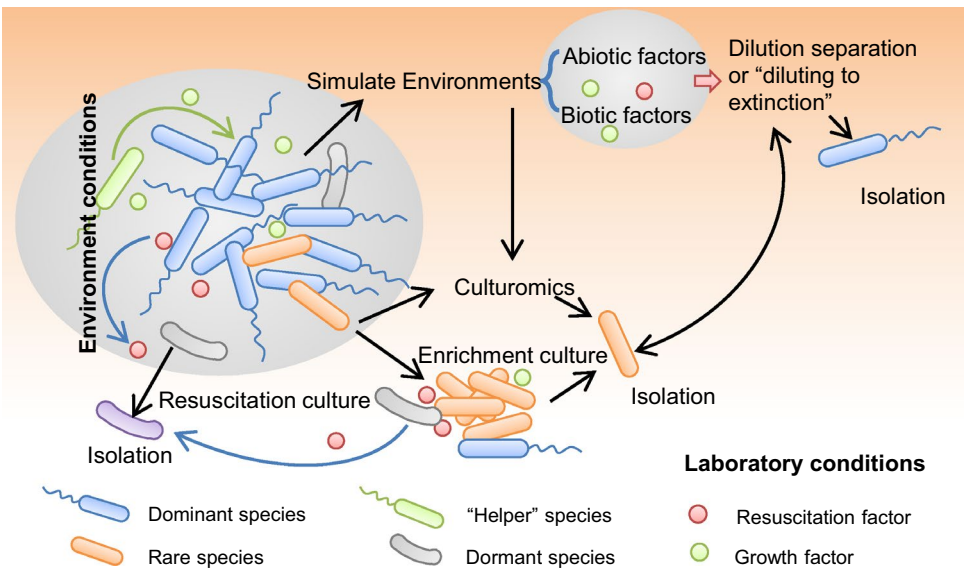

Fig. 2 Potential strategies for culturing different types of uncultured bacteria. Dominant bacteria and rare bacteria refer to active taxa that live well and undergo cell division in natural environments. Dormant bacteria refer to bacteria that have poor adaption to local environments with low metabolic activity. For culturing active bacteria, the core strategy is simulating natural environments. The dilution method is suitable for further isolation of dominant bacteria, and culturomics and enrichment culture are two approaches for further isolating rare active bacteria. For culturing dormant bacteria, the core strategy is "wake up", and resuscitation culture is assumed to be a feasible approach. The key to growing rare or dormant bacteria is "change": culture conditions should be changed, so as to make the "rare" be "dominant", and to convert "dormancy" to "resuscitation". Various approaches can be invoked, with important innovation

Table 1 The approaches and strategies for isolating different types of uncultured

Types of uncultured	Strategy	Cultured case	Approach	References
Dominant bacteria	Simulate environments	SAR11	Using seawater as a growth medium and dilution-to-extinction	Rappe et al. (2002)
		SAR11, SAR116, OM60/NOR5, SAR92, *Roseobacter*	Using artificial seawater media and dilution-to-extinction	Henson et al. (2016)
		Cytophaga-Flexibacter-Bacterioides (CFB) group *Arcobacter* spp. *Deltaproteobacteria* *Epsilonbacteria*	In situ culture (take the bacteria back to the environment)	Kaeberlein et al. (2002); Nichols et al. (2010)
		SAR11, *Cytophaga–Flavobacterium–Bacteroides* group, SAR116 clade, and *Planctomycetales*	Gel micro-droplets	Zengler et al. (2002)
		Psychrobacter	Co-culture	Nichols et al. (2008)
		Faecalibacterium, *Bacteroides*, *Bilophila*, *Gordonibacter*, and *Sutterella*	Co-culture	Fenn et al. (2017)
		Firmicutes KLE1738 In the Health's "Most Wanted" list	Co-culture	Strandwitz et al. (2019)
Rare bacteria	Culturomics (directly isolate)	247 new prokaryote species in the gut	Based on 212 different culture conditions and dilution	Lagier et al. (2016)
	Enrichment culture (increase the abundance of rare bacteria)	*Bradymonadales*, *Marinilabiliales*	Enrichment culture with Low-nutrient medium containing 10 mM sodium pyruvate	Mu et al. (2018)
		79 strains of *Planctomycetes*	Enrichment culture based on *N*-acetylglucosamine and antibiotics selection	Wiegand et al. (2020)
		Kiritimatiellaeota	Enrichment culture	Spring et al. (2016); van Vliet et al. (2019)
		ammonia-oxidizing bacteria	Continuous enrichment culture	Bollmann and Laanbroek (2001)
Dormant bacteria	Resuscitation culture	Cellulose-degrading bacterial community	Rpf protein	Su et al. (2018)
		Vibrio cholerae	Autoinducers	Bari et al. (2013)
		Marinilabiaceae, *Tangfeifania*, *Draconibacterium*, *Saccharicrinis*	Enrichment culture	Mu et al. (2018)

be overcome by preheating the blood (creating the oddly named "chocolate" agar); this heat treatment both releases heme and inactivates the enzyme that breaks down NAD (Fleming 1919). Recently, the groups of Epstein and Lewis noticed that some organisms forming colonies in a diffusion chamber can grow on a Petri dish but only in the presence of other species from the same environment (Kaeberlein et al. 2002; Nichols et al. 2008). In a following study, they found that short peptides (Nichols et al. 2008), siderophores (D'Onofrio et al. 2010), quinones (Fenn et al. 2017), and γ-aminobutyric acid (Strandwitz et al. 2019) were key biotic factors for culturing "uncultivable" microorganisms (Table 1). In addition to growth factors, "helper" microbes can support other forms of biotic factors to enhance the growth of an "uncultivable" microorganism. Some "helper" microbes may help other microorganisms to remove toxic

compounds, such as by secreting catalase to scavenge reactive oxygen species. For example, the ubiquitous actinobacterial acI lineage was cultured by supplying the biochemical "helper" catalase (Kim et al. 2019).

For rare active bacteria

To culture these low-abundance bacteria, one strategy is to tailor different synthetic media for culturing bacteria that adapt to specific environmental niches (Fig. 2). Baas Becking's dictum that "everything is everywhere, but the environment selects" (de Wit and Bouvier 2006) also suggests that different environments can be designed to select different bacteria. Based on this strategy, culturomics was developed and it has achieved great success in culturing previously uncultured members of the human gut microbiota (Table 1) (Lagier et al. 2016). In fact, the original environment was inadvertently altered when microbes were isolated. For example, marine broth 2216 was used to simulate marine environments, however, there are large differences between sea water and marine broth 2216, such as differences in concentrations of sulfur, carbon, and nitrogen (Henson et al. 2016). This may be one reason why when using marine broth 2216, most cultured bacteria have been rare species (Mu et al. 2018).

Another strategy for the isolation of low-abundance bacteria is attempting to make them dominant (Fig. 2), whereby the above approaches for culturing dominant bacteria can be applied. Enrichment is a long-used and common practice that effectively increases the populations of target organisms. This is generally achieved by introducing nutrients or environmental conditions that only allow the growth of the organism of interest. At the end of the nineteenth century, Sergei Winogradsky manipulated the concentration of ammonium salts, pH and determined the effect of the added organic material; they obtained a medium in which nitrification proceeded rapidly, and eventually isolated nitrifying microorganisms (Dworkin 2012). This was, in effect, the invention of enrichment, a technique that has proven to be a powerful tool for the isolation of specific nutritional or physiological types of microorganisms. By employing this approach, many rare, uncultured microorganisms have been isolated or highly enriched (Table 1). Sandra Wiegand et al. (2020) isolated 79 strains of the poorly represented and slow-growing phylum *Planctomycetes* by employing a developed enrichment strategy based on their preference for *N*-acetylglucosamine and resistance to certain antibiotics. Strains of the novel phylum *Kiritimatiellaeota*, which is a member of the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum, were isolated from saline lakes and marine sediments by an anaerobic enrichment method (Spring et al. 2016; van Vliet et al. 2019). Also, sublineage I *Nitrospira* were isolated by a combined method: selective enrichment using a continuous feeding bioreactor to increase the cell abundance, then purification followed by sub-cultivation via a cell sorting system by focusing on the unique characteristics of the target bacteria (Fujitani et al. 2014). Additionally, our own examinations isolated one strain within phylum *Kiritimatiellaeota* (16S rRNA gene similarity of less than 85% to the type strain accession number: MN909984) by anaerobic enrichment culture. The isolation of some bacteria displays a dependence on enrichment culture. For instance, *Marinilabiliales* [an order within the phylum *Bacteroidetes* (Wu et al. 2016)] contains approximately 50 species; more than 70% of these have been isolated by enrichment treatment (Mu et al. 2018). Enrichment is also a process of selection, as anaerobic enrichment can significantly decrease the abundance of obligate aerobes and enrich facultative anaerobes and obligate anaerobes. If enriched microbes are cultured on plates under aerobic conditions, enriched obligate anaerobes cannot grow, while the enriched facultative anaerobes can be isolated. This approach is efficient for isolating facultative microbes in marine sediments or intertidal zone sediments, such as some marine bacteria in the phylum *Bacteroidetes* (i.e., *Bacteroidetes*). Most members of *Marinilabiliales* are facultative microbes and can be enriched and isolated by employing this approach (Table 1) (Ben Hania et al. 2017; Mu et al. 2018). In addition, continuous enrichment culture using oligotrophic medium can selectively enrich slow-growing microorganisms (Table 1), for example, the isolation of ammonia-oxidizing bacteria (Bollmann and Laanbroek 2001). The approach of long-term continuous enrichment culture of archaea may also be employed in culturing slow-growing bacteria (Imachi et al. 2020).

For dormant bacteria

Although the simulated environment method has been a successful approach for isolating bacteria, especially for dominant species in an environment, it has some limitations for isolating dormant species, as natural environments do not commonly have the optimum conditions for the growth of most bacteria. In fact, many bacteria are dormant in natural environments and some strains are VBNC bacteria (Mu et al. 2018). In addition, a scout model of the microbial life cycle in dormant cells and activity cells was proposed, which postulated that dormant cells were able to stochastically wake into activity in situ (Epstein 2013). It is possible that scout cells can accrue and then induce the remaining dormant cells to activity if they detect suitable environmental factors. Otherwise, the scout may not accrue and form a population in situ. As a result, the molecules in the environment that serve as a 'wake-up call' and

stimulate the growth of the scout and dormant cells should be determined.

Resuscitation-promoting factor (Rpf) was the first-reported protein that revived dormant gram-positive cells and increased the growth rate of vegetative cells (Mukamolova et al. 1998); a family of related growth factors was identified in further studies (Kell and Young 2000). Rpf was later demonstrated to have a lysozyme-like structure and muralytic activity (Cohen-Gonsaud et al. 2005). One potential resuscitation mechanism of Rpf is remodeling of the peptidoglycan in the cell wall of dormant cells, generating muropeptides that may serve as a 'wake-up call', stimulating the growth of dormant cells (Kana and Mizrahi 2010). Su et al. (2018) enhanced the cellulose-degrading capability of the bacterial community in composting by using the Rpf protein to resuscitate viable but nonculturable bacteria (Table 1). In gram-negative bacteria, gene expression regulator autoinducers (AIs), with the ability to stimulate growth rates, have also been discovered (Freestone et al. 1999). Unculturable *Vibrio cholerae* cells were found to be resuscitated by AI-2 in aquatic reservoirs (Bari et al. 2013). In addition to purified autoinducers, the supernatant of growing cells in the late logarithmic phase has a positive effect on bacterial resuscitation (Pinto et al. 2011). Other biotic molecules, such as siderophores (Lankford et al. 1966) and pyruvate (Morishige et al. 2013; Mu et al. 2018) also promote exit from dormancy. It has been suggested that uncultured bacteria only commit to division in a familiar environment, which they recognize by the presence of growth factors released by their neighbors (D'Onofrio et al. 2010). As a result, co-culture or mixed culture is one way to 'wake up' dormant bacteria, though the actual resuscitation factors have yet to be determined (Table 1). Compared with pure cultures, enrichment culture is a mixed culture system that involves competition, cooperation, or coordination among bacterial communities (Imachi et al. 2020; Mu et al. 2018). In addition, this approach might culture "uncultured" bacteria not only by enriching the abundance of "uncultured" strains but also through the resuscitation mechanism (Mu et al. 2018).

Targeted culture of key bacterial players in marine environments

It is impossible to culture all microbes from marine environments. As a result, it is necessary to set specific, achievable, and relevant cultivation goals. A greater effort has been made to isolate the most-wanted or key players from the marine environment. As stated by Paul Carini (Carini 2019), key bacterial players may include those that (1) have a high relative abundance, (2) play key role in biogeochemistry or bioremediation, (3) have the potential to produce natural products, and (4) substantially diverge from cultured taxa. Following Paul Carini's suggestion, Table 2 lists some marine bacteria that might be defined as key, but uncultured players in marine environments. Some key players were found to have various functions, such as *Pelagibacterales* (initially, this taxon was known solely by metagenomic data and known as the SAR11 clade), a group of small, carbon-oxidizing bacteria that reach a global estimated population size of 2.4×10^{28} cells. In both the euphotic zone and the deeper ocean, *Pelagibacterales* cells oxidize many labile organic compounds and produce CO_2 as well as other volatile organic compounds (i.e., dimethyl sulfide and methanethiol) that can enter the atmosphere, playing a key role in global biogeochemistry cycling (Giovannoni 2017).

Many more key players remain uncultured and their ecological roles have only been predicted by metagenomics analysis. However, phenotypic properties cannot always be predicted from sequence information and their exact ecological functions need to be further identified by isolation. The *Woeseiaceae*/JTB255 group are among the most abundant bacteria at the surface of coastal, abyssal, and bathyal sediments. The global estimated population of these bacteria might reach of 5×10^{26} cells (Hoffmann et al. 2020). *Woeseiaceae*/JTB255 cells appear to have a higher biovolume (on average 0.13 μm^3) compared with the water column clade SAR11 [0.01 μm^3; (Rappe et al. 2002)] and thus may account for a considerable fraction of microbial biomass in the oceans. Metagenomics analysis has shown that some representatives of *Woeseiaceae*/JTB255 might be involved in sulfur oxidation, carbon fixation (Mussmann et al. 2017) and cycling of a major class of refractory sediment organic matter (Hoffmann et al. 2020). However, the solo cultured strain *Woeseia oceani* XK5^T in this group did not exhibit sulfur oxidation or carbon fixation abilities. Simulating sediment environments might be useful for isolation of *Woeseiaceae*/JTB255 bacteria. Nevertheless, more strains need to be isolated to determine the key factors required for culturing this group. The unicellular cyanobacteria group A (UCYN-A) is another uncultured group that is abundant and potentially significantly contributes to N_2 fixation in the surface waters of oceans (Martinez-Perez et al. 2016). Unlike the uncultured *Woeseiaceae*/JTB255 group, these bacteria display unprecedented genome reduction and a symbiotic association with a unicellular prymnesiophyte, which might be one reason for their unculturability. In addition, as with the hosts that live in marine environments, an increasing focus is being placed on bacteria from the human microbiome, together, the potential role of host-associated unculturable bacteria is being investigated. "*Candidatus Entotheonella*" is a typical host (sponge)-associated marine unculturable bacteria. These bacteria are filamentous symbionts that produce almost all known bioactive

Table 2 Examples of marine bacteria that are most-wanted in culture

Candidate organism or group	Biotopes	Phylum	Reason for cultivation
"*Candidatus Atelocyanobacterium thalassa*" (Martinez-Perez et al. 2016; Thompson et al. 2012)	Surface seawater	Unicellular cyanobacteria group A (UCYN-A)	The most abundant of the unicellular diazotrophs in oceans and key role in biogeochemistry
Methylphosphonic acid aynthesis bacteria (Metcalf et al. 2012)	Seawater	Assorted	Key roles in methane generation in the aerobic ocean
Dimethylsulfoniopropionate (DMSP) synthesis bacteria (Curson et al. 2017)	Seawater	Assorted	Key roles in DMSP generation in the aerobic ocean
SAR202 (Mehrshad et al. 2018)	Seawater	*Chloroflexi*	Abundant in mesopelagic waters and dark ocean
SAR86 (Dupont et al. 2012)	Seawater	*Gammaproteobacteria*	Abundant in surface waters and key role in biogeochemistry
SAR324 or Marine Group B (Sheik et al. 2014)	Seawater	*Deltaproteobacteria*	Ubiquitous in the dark ocean
"*Candidatus Actinomarinidae*" (Ghai et al. 2013)	Seawater and sediment	*Actinobacteria* (OM1)	Streamlined genome and key role in biogeochemistry
"*Candidatus Marinimicrobia*" (Bertagnolli et al. 2017)	Seawater, oxygen minimum zones (OMZs)	Candidate phylum marine group A	Abundant and key role in biogeochemistry
"*Candidatus Atribacteria*" (Nobu et al. 2016)	Sediment	Candidate phylum *Atribacteria* (OP9/JS1)	Key role in biogeochemistry
Woeseiaceae/JTB255 (Mussmann et al. 2017)	Surface Sediment	*Gammaproteobacteria*	Abundant in surface sediments and key role in biogeochemistry
"*Candidatus* Electrothrix" and "*Candidatus* Electronema" (Kjeldsen et al. 2019; Muller et al. 2020)	sediment	*Deltaproteobacteria*	Cable bacteria and key role in biogeochemistry
Assorted bacteria (Boetius et al. 2000)	sediment	*Proteobacteria*	Anaerobic oxidation of methane by sulfate
'*Candidatus* Methylomirabilis oxyfera' (Ettwig et al. 2010)	sediment	Candidate division NC10	Anaerobic oxidation of methane by nitrite
Assorted bacteria (Beal et al. 2009)	sediment	Assorted	Anaerobic oxidation of methane by iron and manganese
"*Candidatus Entotheonella*" (Wilson et al. 2014)	sponge	"*Candidatus Entotheonella*"	With large and distinct metabolic repertoire for ecological studies and drug discovery
Any representative of the candidate phylum radiation	Assorted	Assorted	Divergent from all cultured bacteria

compounds derived from the Lithistida sponge *Theonella swinhoei* (Lackner et al. 2017; Wilson et al. 2014). Genome analysis has shown that these bacteria are auxotrophic for multiple amino acids, suggesting that providing free amino acids would be crucial for cultivating them (Liu et al. 2016).

Importantly, the 'reverse genomics' approach should be considered for targeted culturing of key players (Cross et al. 2019). With this method, antibodies against predicted membrane proteins can be used to target and culture microbial cells from a specific taxonomic group (Cross et al. 2019). However, the efficiency of methods for isolating uncultured microbes from environments that harbor more complicated microbiota should be further explored. Moreover, follow-up culturing of the targeted strains is still associated with the above difficulties (Lewis and Ettema 2019).

Concluding remarks

Although most natural bacteria cannot be isolated in the laboratory, microbiologists have made great efforts to determine the reasons for the 'great plate count anomaly' and have developed several effective approaches for growing uncultured bacteria. In retrospect, culturing in situ or bringing the environment into the laboratory has allowed the cultivation of many novel isolates, and co-culture with other

bacteria has identified how biotic environmental factors affect isolation. Enrichment culture enables the cultivation of low-abundance or dormant bacteria. In addition, novel technologies, such as encapsulation into gel microdroplets and the 'reverse genomics' approach, are increasing the rate of isolation of unculturable bacteria and with this pool of isolates, it will be possible to identify more of the mechanisms underlying the inability of these bacteria to grow in the laboratory.

Regardless of how the technology develops, culturing and isolating uncultured bacteria remains a time-consuming and laborious task. Thus, the most-wanted microbial groups in marine environments should be cultured first. A deeper understanding of the biogeochemical cycles mediated by microorganisms in marine environments requires better knowledge about the unculturable majority of bacteria, their relationships with coexisting members of the microbial community, and how they can be integrated as key players in biogeochemical processes.

"Revolution has not yet succeeded, and comrades still need to work hard." Sun Yat-sen (The Revival of China, chapter 1.7).

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Compliance with ethical standards

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