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Innovations to culturing the uncultured microbial majority

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Abstract | Despite the surge of microbial genome data, experimental testing is important to confirm inferences about the cell biology, ecological roles and evolution of microorganisms. As the majority of archaeal and bacterial diversity remains uncultured and poorly characterized, culturing is a priority. The growing interest in and need for efficient cultivation strategies has led to many rapid methodological and technological advances. In this Review, we discuss common barriers that can hamper the isolation and culturing of novel microorganisms and review emerging, innovative methods for targeted or high-throughput cultivation. We also highlight recent examples of successful cultivation of novel archaea and bacteria, and suggest key microorganisms for future cultivation attempts.

Enrichments
Assemblages of several strains that evolve from a taxonomically diverse inoculum in response to controlled environmental selection pressures (such as substrates or temperature).

Pure cultures
Cultures containing cells belonging to the same strain, ideally originating from a single cell or colony, that have minimal genetic variation between them. Also often called axenic cultures.

Co-cultures
Defined assemblages of two or more strains, often artificially introduced and grown together in the laboratory, which may establish interspecies metabolic relationships with one another.

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Advances in genome-sequencing technologies and sophisticated metagenomics and phylogenetic methods have contributed to drastically change our views on the diversity of microbial life, including the very shape of the tree of life[1,2]. Yet the marked expansion of genomic data, which has led to an improved understanding of archaeal and bacterial diversity, is contrasted by our inability to culture representatives for many of the novel lineages. Consequently, most of what we currently know about archaea and bacteria is either derived from a minority of well-studied cultured lineages or from reconstructed genomes belonging to uncultured lineages. Although this period of rapid, genome-driven discovery has provided numerous important new insights into microbial life on our planet, it is essential to isolate and culture species from these uncultured lineages to test genome-based predictions about their cell biology and physiology and to properly understand their ecological roles. Such a need is emphasized by examples of completely novel enzymatic reactions and pathways that have been discovered through experimental testing of microbial enrichments or cultures[3–5], and some of these pathways were undetectable by genomic methods alone[4].

Microbial cultivation can be used to generate pure cultures, which provide a continuous supply of cells from the same species or strain. Such cultures can be used to investigate microbial traits in experiments performed in replicates, which improves reproducibility and statistical confidence. Without pure cultures (or, in some cases, highly enriched co-cultures that contain a small number of species that depend on each other to grow) it is difficult to accurately determine microbial features such as growth characteristics, metabolism, physiology and cell biology for a single organism. These features are also difficult to infer from genome sequences alone because genomic data provide no indication of which genes are functionally expressed, and therefore no indication of how the active proteome adapts under certain conditions. Although metatranscriptomics and metaproteomics can provide some insights, the data that these approaches generate are still difficult to interpret without fundamental knowledge of the underlying physiology. Thus, without cultivation, many questions about the role of organisms in their natural settings remain unanswered.

To improve our understanding of the uncultured archaeal and bacterial diversity, it is essential to increase our capacity for bringing microorganisms from the environment into culture[6–9]. To achieve this task in a more efficient manner, prioritizing cultivation efforts for microorganisms that are thought to be the most interesting (for example, because they represent a poorly characterized group) or are most likely to improve our understanding of a particular process (TABLE 1) is ultimately required[10].

Traditional microbiological methods (BOX 1) are hugely important and indispensable assets for cultivation, and are continually used to successfully isolate numerous microorganisms of interest. However, these approaches often require substantial amounts of time and patience to succeed, as well as painstaking and meticulous testing of media combinations and of different physicochemical conditions. To circumvent or at least minimize these potential limitations, innovative new technologies (some of which incorporate and expand on classical methods) have broadened the

Table 1 | **Key targets for cultivation**

Target microorganism or group[a]	Common environments	Superphylum or phylum	Reasons they are of interest for cultivation
Archaea			
Anaerobic methanotroph clades 1, 2 and 3	Sediments	Euryarchaeota	They function as an important sink for the greenhouse gas methane, which they metabolize as it seeps out from methane reservoirs beneath marine sediments. They therefore help to limit the amount of methane that is released into the atmosphere and are the only known organisms capable of oxidizing methane under anaerobic conditions[127].
Bathyarchaeota	Sediments	TACK archaea	They are a group of globally widespread metabolic generalists that are abundant in anoxic sediments. They contain some of the few known putative methanogen lineages from outside the Euryarchaeota[41].
Verstraetearchaeota	Sediments	TACK archaea	Some of the few known putative methanogens from outside the Euryarchaeota belong to this phylum[128].
Candidate phyla Heimdallarchaeota, Helarchaeota, Lokiarchaeota, Odinaracheota and Thorarchaeota	Marine sediments and hydrothermal vents	Candidate superphylum Asgard archaea	These archaea belonging to the Asgard superphylum are important for understanding the origin of eukaryotes. The Heimdallarchaeota are currently the best-supported sister linage of eukaryotes, and are therefore the most important target for cultivation. Some lineages are also abundant in some marine sediments[124].
DPANN archaea	Assorted	DPANN archaea	They are a major archaeal group, currently thought to consist of at least 12 different phyla, with 6 cultured representatives across the entire group. They typically have small cell and genome sizes, limited metabolic capabilities and are likely to be symbionts or parasites of other microorganisms[129].
Marine Group II, III and IV archaea	Marine	Euryarchaeota	Marine Group II are abundant in some marine environments and are thought to be important for the degradation of organic carbon[130]. Marine Groups III and IV are abundant and widespread in some marine environments, and there are currently no cultured representatives for any of these clades[131].
Water column B Thaumarchaeota	Marine	Thaumarchaeota	They have a key role in biogeochemistry by participating in carbon and nitrogen cycling in the deeper layers of oceans[132].
Bacteria			
Acidobacteria	Soil	Acidobacteria	They are a widespread and abundant phylum of versatile heterotrophs, thought to have a major impact on the ecology of some terrestrial environments[133].
Candidate phylum Rokubacteria	Soil	Candidate phylum Rokubacteria	They are a novel phylum with unusually small cell sizes but large genomes and are widespread in terrestrial ecosystems[134].
Candidatus Actinomarinidae	Marine	Actinobacteria (OM1)	A class with no cultured representatives in the Actinobacteria (which otherwise have numerous cultured representatives (FIG. 1)). They have streamlined genomes, ultra-small cell sizes and are putative photoheterotrophs as their genomes encode genes for rhodopsins[135].
Candidatus Atribacteria	Sediments	Candidate phylum Atribacteria (OP9/JS1)	They are globally distributed, and in some environments are abundant, and contain species that are thought to be anaerobic hydrocarbon degraders[136] as well as some that are thought to be syntrophic propionate oxidizers[137].
Candidatus Dormibacteraeota and Candidatus Eremiobacteraeota	Soil	Candidate phylum AD3 and Candidate phylum WPS-2, respectively	These novel phyla contain species that are thought to survive on the consumption of trace atmospheric gases. Their cultivation could provide wider insight into the growth strategies used by bacteria that are abundant in oligotrophic soils[138].
Candidatus Marinimicrobia	Marine	Candidate phylum marine group A	They are an abundant and highly diverse group, participating in sulfur and nitrogen cycles, driving the biogeochemistry of oceans, and might also function as a potential sink for the greenhouse gas nitrous oxide[139].
Candidatus Poribacteria	Marine	Candidate phylum Poribacteria	They are often dominant and widespread members of microbial communities associated with marine sponges[139].
'*Candidatus* Udaeobacter copiosus'	Soil	Verrucomicrobia	They are metabolically efficient heterotrophs with unusually small genomes, which are widespread and abundant in many soils[140].
Dehalogenating bacteria	Assorted	Chloroflexi, Firmicutes and others	Some of these bacteria have been shown to respire anthropogenic chemicals that are common environmental contaminants, suggesting they could be useful for bioremediation[141].
CL500-11	Aquatic	Chloroflexi	Members of this clade are abundant globally in the low-temperature layers of deep freshwater lakes[142].
SAR202	Marine	Chloroflexi	They are abundant in mesopelagic and bathypelagic marine layers, where they are thought to have major roles in sulfur cycles[143].

Table 1 (cont.) | **Key targets for cultivation**

Target microorganism or group[a]	Common environments	Superphylum or phylum	Reasons they are of interest for cultivation
Bacteria (cont.)			
Most wanted taxa from the Human Microbiome Project[80]	Human	Assorted	These are bacteria recognized based on 119 OTUs that have been prioritized owing to their evolutionary distance from already characterized strains and their frequency among healthy human-derived samples. Cultivation of such microorganisms is thought to be essential for providing a better understanding of human health and diseases[80], and for example include bacteria belonging to the phyla Bacteroidetes, Firmicutes and TM7.
SAR324	Marine	Deltaproteobacteria	They are metabolically diverse and globally distributed throughout the deeper layers of the oceans[144].
SAR86	Marine	Gammaproteobacteria	They are abundant in the surface layers of oceans and widespread globally[145].
Most wanted taxa in soil[146]	Soil	Assorted	These bacteria are thought to be crucial for accurately forecasting the ecological consequences of ongoing global environmental change, and are important for better understanding soil bacterial communities[146]. The most ubiquitous and globally abundant of these include bacteria belonging to the phyla Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Acidobacteria and Planctomycetes.
Candidate Phyla Radiation	Assorted	Candidate Phyla Radiation	This is a major group in Bacteria, currently thought to consist of at least 74 different phyla, for which there are an extremely limited number of cultured representatives.
Most wanted taxa in wastewater treatment plants[147]	Wastewater treatments	Assorted	They are essential for municipal and industrial wastewater purification, by removal of pollutants, to protect public and environmental health and have importance for improving the performance of wastewater treatment plants[147]. The most globally abundant and ubiquitous of these include bacteria belonging to the phyla Betaproteobacteria, Gammaproteobacteria and Bacteroidetes.
Others			
Most-wanted chemolithoautotrophic 'spookmicrobes'[148]	Assorted	Assorted	These microorganisms from various taxonomic groups are thought to have important roles in global methane, sulfur and nitrogen cycles. They also participate in recently discovered processes, including complete ammonia oxidation (comammox) and as yet undiscovered processes, such as iron and manganese-dependent methane and ammonium oxidation[148].
'*Candidatus* Parakaryon myojinensis'[149]	Hydrothermal vent	Unknown	This microorganism is represented by an unusual microscopically investigated cell without molecular data, which is interesting as it has structural similarities to both prokaryotes and eukaryotes yet is seemingly distinct from both[149].

[a]A subjective overview of microorganisms that could be considered as key targets for cultivation. Although this summary is far from exhaustive, given that every researcher has different interests, the organisms presented here were selected owing to wide general interest in them or because they bear significant relevance to particular scientific questions. The table is updated and expanded from previous work[10].

toolkit for microbial isolation and the efficient determination of suitable culture conditions. Although many of these technologies remain in their infancy, with wide-ranging applicability having not yet been demonstrated for diverse species and environments, other technologies are already used widely by researchers and are starting to have a positive impact on microbiological research. In this Review, we revisit the capabilities and limitations of traditional isolation and cultivation methods, and provide an overview and discussion of more recent innovative technologies that have potential to improve our ability to isolate, culture and characterize microorganisms from poorly studied groups. We also highlight recent successes in culturing elusive microorganisms and present a list of some examples of microorganisms to prioritize in future cultivation attempts.

Recent successes in cultivation

In recent years there have been a number of important cultivation successes, some of which have garnered considerable far-reaching interest from different fields. In most cases, the interest lies in the novelty of the microorganisms that were cultured, or because the cultured microorganisms provided insights and an improved understanding of certain natural processes.

Among archaea, a notable example is the first representative of the Asgard archaea superphylum, '*Candidatus* Prometheoarchaeum syntrophicum', representing the closest archaeal relative of eukaryotes cultured to date, which was highly enriched in a co-culture containing two species. This feat was achieved using an innovative bioreactor system and traditional enrichment methods over the course of 12 years, partially owing to this organism having extremely slow growth rates[11]. The first representative of the Nanohaloarchaeota phylum, '*Candidatus* Nanohaloarchaeum antarcticus', was recently co-cultured with a Euryarchaeota host, *Halorubrum lacusprofundi*, by combining classical enrichment methods with single-cell sorting selecting for cells of appropriate sizes, as inferred from fluorescence in situ hybridization (FISH) experiments[12]. Two archaea belonging to closely related genera, '*Candidatus* Argoarchaeum ethanivorans'[13] and '*Candidatus* Ethanoperedens thermophilum'[14], are the first organisms shown to oxidize ethane in

Isolation
The physical separation of a single cell, strain or species from others found in the same sample or habitat.

Fluorescence in situ hybridization
(FISH). A method of labelling cells with a fluorescent signal by binding fluorophore-coupled oligonucleotide probes to complementary target molecules (usually 16S rRNA) in biological samples. Probes can be designed to be highly taxon-specific, making it possible to taxonomically identify microorganisms on the single-cell level.

Box 1 | **Classical cultivation strategies and methods**

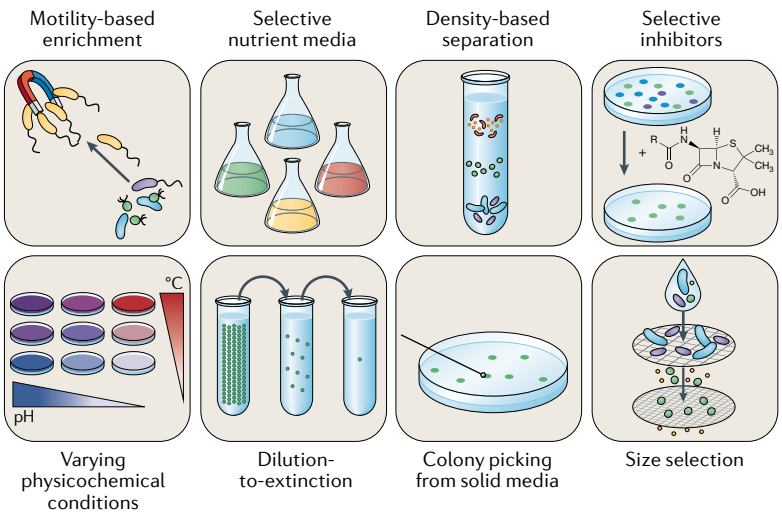
The origins of microbial cultivation can be traced back to the middle of the nineteenth century, and many modern-day cultivation efforts rely on some of the same early principles introduced more than a century ago[154]. Several strategies can be applied to enrich and later isolate specific microorganisms, many of which rely on direct observation of the physiological behaviour of the culture and the phenotypic and genotypic characteristics of the microorganisms it contains (see the figure). Experience of the researcher with microbial isolation is also important when it comes to the selection of the most appropriate measure for isolation.

Examples of techniques to enrich specific taxa include the design of selective nutrient media (for example, with specific substrates), application of selective physicochemical conditions (for example, temperature, pH, salinity and gas-phase composition), addition of selective inhibitors (for example, antibiotics, toxic compounds and metabolic inhibitors) and the addition or omission of specific growth factors (for example, amino acids, vitamins and metals). The effect that each of these strategies has on the growth and number of a specific population of microorganisms can be monitored and used to define further isolation methods.

Observing cultures under the microscope can be a useful way to define strategies for isolation. For example, when the target microorganism has a substantial difference in size or shape from others in the culture, size fractionation by filters with various pore sizes and mass-based separation by gradient centrifugation can be used to separate them. Microscopic observation over time sometimes enables the detection of different growth rates for microorganisms, which can then be used to inform subculture periods to select for faster-growing microorganisms (by transferring cultures at an earlier incubation stage).

Growing cultures on a surface of solid media, commonly agar, and colony picking is a common way to isolate organisms, and using alternative solidifying agents such as gellan gum and agarose can target different microorganisms[38,155]. It is also possible to isolate microorganisms in liquid media by dilution-to-extinction and design experiments for the selection of motility phenotypes (such as phototaxis, aerotaxis, chemotaxis, galvanotaxis or magnetotaxis)[156–158].

Another consideration is the method used to sterilize growth medium, the most common being autoclaving. However, besides the risk of degradation of certain components, the presence of certain components during autoclaving can lead to the formation of toxic by-products, such as hydrogen peroxide[159], that can inhibit growth. Autoclaving media components separately or, instead, using filter sterilization has been shown to avoid these problems[159].



Dilution-to-extinction
A method of serially diluting a mixed community culture with the aim of isolating single cells that will grow and divide to establish monoclonal and axenic cultures. Can also be called limited dilution.

syntrophic interactions with sulfate-reducing bacteria, and were cultured using traditional selective enrichment methods.

Among bacteria, 79 different isolates belonging to diverse lineages of Planctomycetes were recently cultured using several traditional methods, including selective enrichment, antibiotic treatment and solid media streaking combined with colony picking[15]. The first freshwater representative of the widely abundant SAR11 Alphaproteobacteria clade, '*Candidatus* Fonsibacter ubiquis', was cultured by high-throughput dilution-to-extinction in an oligotrophic medium[16]. Three species belonging to the phylum Saccharibacteria (TM7) of the Candidate Phyla Radiation (CPR), a broad clade that has few cultured representatives, were isolated together with their host Actinobacteria from human saliva samples in the first demonstration of the reverse genomics[17] method. '*Candidatus* Manganitrophus noduliformans' is the first organism shown to be capable of manganese oxidation in syntrophic interaction with a betaproteobacterium, and was cultured using selective substrate enrichment and dilution-to-extinction[18]. *Casimicrobium huifangae*, the first isolate of a novel family in the Betaproteobacteria with the potential to support improved understanding of and processing in wastewater treatment plants, was cultured using traditional methods[19].

Although the examples mentioned above are far from an exhaustive list of all microorganisms that were successfully cultured in recent years, the total combined strains that are currently maintained in culture in various laboratories or culture collections around the world represent only a miniscule fraction of the total microbial diversity that exists.

The uncultured majority

The tree of life, arguably one of the most important concepts in biology, has been vastly expanded with several archaeal and bacterial groups of high taxonomic rank over the past decades[1,2,20]. Contemporary best-supported ideas for the structure of the tree of life divide prokaryotes into two primary domains, Archaea and Bacteria, which together are estimated to comprise anywhere from hundreds to even thousands of phyla[1,21–26] — a figure that has increased as genome data have accumulated, but can differ substantially depending on the estimation method. Based on 16S rRNA gene sequence data, the total number of archaeal and bacterial species has been calculated to be around 400,000, comprising around 60,000 genera[22], although estimates of the actual number of archaeal and bacterial species on Earth potentially exceeds this by several orders of magnitude[27–30]. However, only ~14,000 archaeal and bacterial species — distributed over 3,500 genera and 38 phyla — have been cultivated and validly described[31–33]. Of these species, ~97% belong to just four bacterial phyla (Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria)[31] (FIG. 1). Conversely, all other bacterial phyla, and Archaea as a whole, are poorly represented by comparatively few cultivated species (FIGS 1,2).

Nevertheless, uncultivated or under-represented phyla are known to dominate various environments, where they are likely to have pivotal ecological roles[34]. Therefore, cultivation of representative members of these groups is important to uncover their physiological and metabolic properties. Given the huge breadth in diversity of microbial life, cultivating every microbial species inhabiting our planet is practically impossible. Therefore, in order to maximize effectiveness, attempts to grow archaea and bacteria should prioritize representatives of the most interesting or useful groups, or those

Growth factors
Any substance that can be used by an organism to facilitate growth.

Symbiosis
The association, usually a physical or metabolic interaction, of two or more organisms, which typically has an influence on the fitness of one or more of the partners involved.

Syntrophy
An interspecies relationship in which metabolites produced by one species are used as growth substrates by another species.

without any cultured representatives, and take advantage of the most recent and innovative technologies to do so.

Factors influencing culturability

Identification of substrates and growth conditions. The difficulties associated with the isolation and cultivation of archaea and bacteria have long been recognized. In the mid 1980s, 16S rRNA gene sequencing of environmental samples revealed large numbers of uncultured taxa[35]. From then on, the discrepancy between microorganisms present in a given environment and those that could be cultured in the laboratory was referred to as the 'great plate count anomaly'[36].

Since then, our understanding of microbial physiology has improved substantially. The necessary substrates, electron donors and acceptors, or other media components for growing particular microorganisms can, in some cases, be used to enrich and/or isolate specific strains. However, cultivation of many microorganisms strictly depends on specific environmental conditions and on the presence of various growth factors (such as vitamins, amino acids, nucleotides, inorganic compounds, humic acids or other external electron shuttles), which are often difficult to identify and therefore challenging to mimic in the laboratory[37,38]. Additionally, several inorganic compounds (metals, sulfur and nitrogen compounds) are involved in cryptic cycles and can be present at concentrations below the detection limit in the environment, despite their importance in biogeochemical cycles[39].

Although for some microorganisms the broad category of substrates that they use can be inferred from genome sequences, it is possible that each uses a highly specific subset of substrates from those categories, which can be difficult to determine without functional data. For example, genomic data suggest that Bathyarchaeota and Asgard archaea commonly have heterotrophic pathways for energy conservation and the ability to degrade various organic substrates, including complex carbohydrates, peptides, amino acids, alcohols, fatty acids and hydrocarbons[40,41]. This variability could perhaps partially explain why there is currently only one cultured representative[11] from these two diverse groups of archaea.

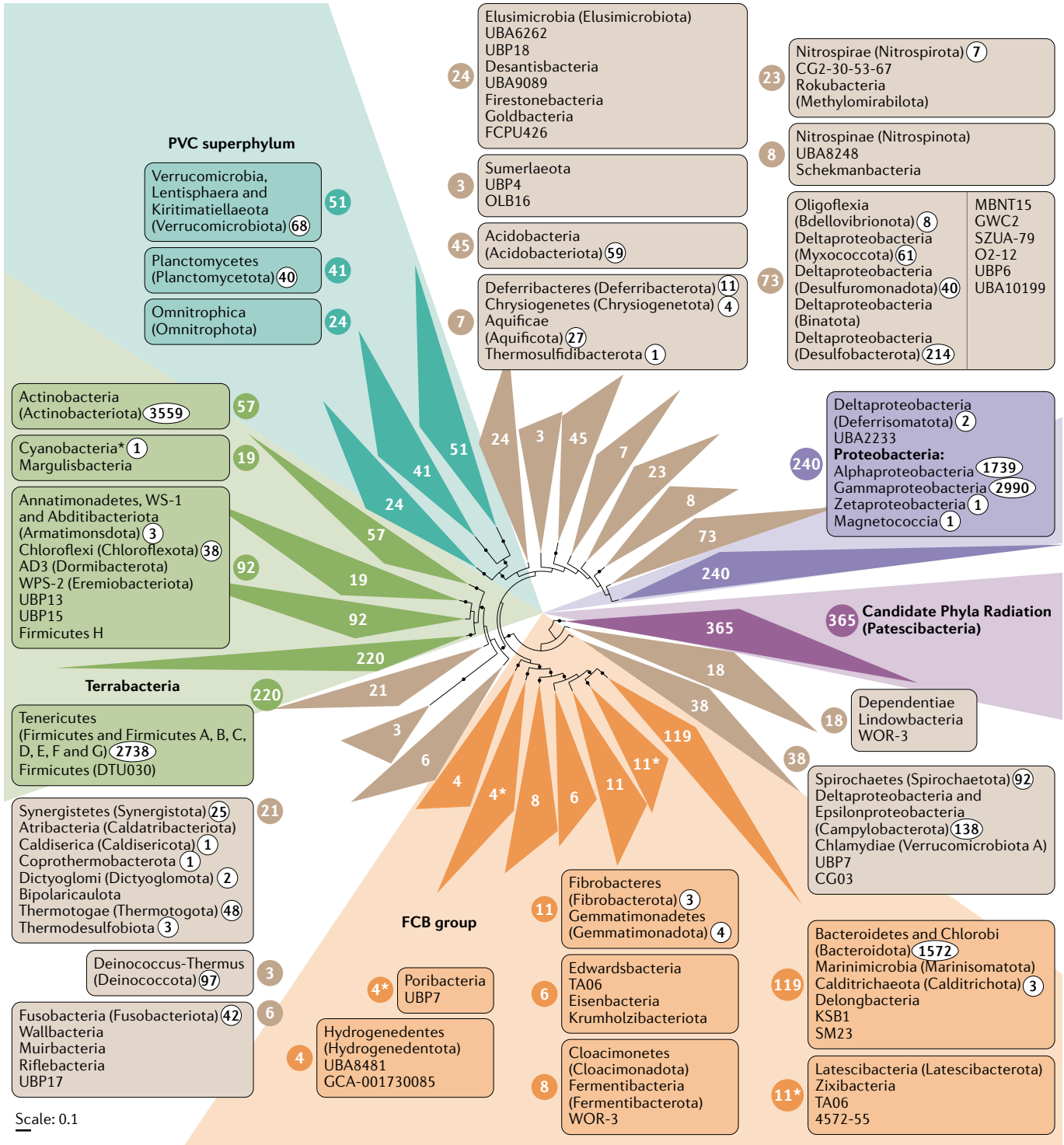
Resuscitation of dormancy. Microbial populations can include persisters, which are phenotypic variants of the wild-type cells whose function is survival[42]. Persisting microorganisms are dormant, non-dividing cells, and in conditions of low nutrient and energy availability, such as in the deep biosphere, dormancy might represent the default state of prokaryotic life[43,44]. As a large proportion of microorganisms that currently remain uncultured reside in environments that are potentially dominated by persisters, resuscitation of dormancy represents an essential hurdle in microbial cultivation efforts. Although a substantial body of literature exists on microbial dormancy, relatively little is known about the potential mechanisms that underpin how microorganisms transition between dormant and active states. Resuscitation of dormancy has been proposed to be a stochastic process[45], which might be influenced by certain signalling compounds[46,47]. Still, microorganisms have probably evolved different mechanisms to regulate dormancy, which deems it unlikely that a unified solution exists to resuscitate them from dormant states. Hence, this potential variability might represent a further complication for cultivation studies.

Symbiotic interdependencies. In some cases, essential molecules or electrons (including microbially produced electron shuttles, such as H_2 and formate) are directly exchanged between members of a microbial community[11,48–51], in an interspecies dependency commonly known as symbiosis (or 'syntrophy' if the two organisms depend on each other for the degradation of a substrate to overcome thermodynamic limitations). In the case of mutualistic or syntrophic microorganisms, using methods that can co-isolate both microbial partners, such as cell sorting in a combinatorial fashion, could be advantageous for establishing a stable co-culture.

Given their (sometimes obligate) interdependence, separating symbiotic or syntrophic partners and growing them in monocultures can be challenging. However, attempts have been made to demonstrate that one syntrophic partner can be abiotically replaced, by investigating co-cultures of H_2-producing bacteria and H_2-consuming methanogens[52,53]. In one study, a H_2-stripping bioreactor system was used to enrich ethanol-oxidizing bacteria from a methanogenic enrichment[53]. However, methanogenic activity was not inhibited completely, suggesting that the H_2 consumption by the methanogen was not entirely replaced[53]. Similar results were obtained in another study that used a bioelectrochemical system to mimic H_2 consumption by methanogens in a co-culture, thereby greatly enriching an 'obligately' syntrophic bacterium (*Syntrophomonas zehnderi*)[52]. However, the bacterium was not separated from the methanogens completely or maintained in a monoculture.

Another option for the enrichment or isolation of H_2-producing syntrophs could be the catalytic removal of H_2. Previous studies have demonstrated the hydrogenation of fatty acids using a palladium catalyst (both fatty acids and H_2 were produced through fermentation of cellulose)[54]. However, hydrogenation rates in the liquid phase of these experiments were low, and the effect of the catalytically formed compounds on the growth of H_2-producing bacteria are not known, as this approach was never tested for the purpose of microbial isolation.

Some microorganisms, such as the DPANN archaea and the CPR bacteria, commonly have small genomes and small cell sizes, which combined with an understanding of the lifestyles of the few cultured representatives of these clades suggests that DPANN archaea and CPR bacteria are predominantly dependent on other host organisms to some degree, either in the form of symbiosis or parasitism[2]. As such, this requirement provides additional complications for cultivation as appropriate conditions must be identified that satisfy both microbial partners, which will likely require an understanding of the basis for the relationship between the partners.



Physical contact or spatial proximity. Physical contact or spatial proximity between symbiotic or syntrophic partners seems to be an important element for microbial growth, as indicated by lower growth rates of microbial partners grown in compartments separated by dialysis membranes than for those grown together[55], which limits the application of such systems for microbial isolation. Some researchers have applied Percoll gradient techniques to separate syntrophic partners[56,57], but trials to further grow the segregated microorganisms often fail, likely owing to the lack of growth factors typically provided by other microorganisms. However, such segregated fractions could still be used, for example, for enzyme assays, giving further insights into the activity of the separate microorganisms.

Physicochemical environmental conditions. Physicochemical environmental conditions, such as temperature, pH, salinity and redox conditions, are also important determinants for microbial cultivation, and

◀ Fig. 1 | **Cultured bacteria are biased towards Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria.** A phylogenetic species tree for bacteria, inferred from concatenated alignments of a minimum of 5 out of a total 15 ribosomal proteins per species, encoded by 1,541 bacterial genomes that were obtained from the Genome Taxonomy Database[21]. Numbers in white font in coloured circles are the number of individual taxa in each collapsed clade, and are also used to connect corresponding taxa names to clades. Numbers in black font in white ellipses next to taxa names indicate the total number of species-level cultured isolates described for those taxa, based on the number of species type strains assigned to each clade that are present in the BacDive database[31] (last accessed 6 April 2020). Taxa without numbers have no cultured isolates recorded in BacDive[31]. Numerous cultured representatives have been reported in the scientific literature that are not represented in the numbers in this figure, because cultures have not been officially described and/or deposited in culture collections, and are therefore not included in BacDive[31] (a comprehensive database recording all cultured bacteria including those not officially described or deposited in culture collections is currently lacking). The tree was generated from datasets containing homologous proteins from the different species included, which were aligned separately using MAFFT (L-INS-i)[150] and the alignments for each protein then concatenated, such that those proteins belonging to the same species were combined to form a single sequence. Poorly conserved sites in the concatenated alignment were removed using trimAl[151] with the option -gt 0.5. A phylogeny was generated from this trimmed alignment using the model LG + C60 + F + R10 in IQ-TREE[152] with 1,000 ultrafast bootstrap replicates[153]. Branches labelled with black dots have support values ≥95%. Given the limited protein data set used to infer this phylogeny, in some cases the deeper relationships between some species or groups may not reflect more widely accepted relationships based on more in-depth and better supported analyses. Particularly, Deinococcus-Thermus (Deinococcota) and Chlamydiae (Verrucomicrobiota A) do not group with other lineages of Terrabacteria and the PVC superphylum, respectively. *Although numerous cultured representatives for numerous cyanobacterial lineages exist, they are particularly under-represented in BacDive[31]. Unlike most bacteria, and owing to historical reasons, Cyanobacteria are mostly classified using the Botanical code (that is, International Code of Nomenclature for algae, fungi and plants). As a result, Cyanobacteria lack defined type strains and are therefore not extensively listed in BacDive[31], and a comprehensive database of existing Cyanobacteria cultures is lacking.

all of these factors can vary sharply in natural environments across microscale distances. In a microbial community, some microorganisms contribute to making the environmental conditions amenable for others, which complicates the isolation of the microorganisms that depend on these effects. Similarly, the cultivation of strictly anaerobic microorganisms is also technically demanding, in particular with modern high-throughput techniques involving cell sorting and growth in microtitre plates (see below). Working in anaerobic tents or glove boxes is often the most convenient solution for enrichment and cultivation of anaerobic microorganisms (for example, for plating and sorting of cells and transferring enrichment cultures). However, aseptic conditions can be difficult to maintain in these settings, although the possibility of contamination can be reduced by incorporating an air filtration unit.

Low abundance and competition. Many prokaryotes exist in nature at low abundance in complex microbial communities, yet may still exert substantial influences on certain processes[58]. A possible reason for this is that metabolic rates of substrate degradation and growth rates are not necessarily linked. This means that, in some cases, a low-abundance microorganism might metabolize a substrate at a faster rate than another, more abundant, microorganism with higher growth rates found in the same environment.

To have the best chance of isolating such rare, yet ecologically relevant microorganisms, identifying environments in which these cells are naturally present at the highest relative abundance would benefit further enrichment efforts. One way to identify such environments is by analysing publicly available 16S rRNA gene data, or generating such data de novo for uncharacterized sites, to select the best locations to sample. However, even if a microorganism is obtained at a high relative abundance, cultivation attempts can still fail if faster-growing microorganisms are also present. Faster-growing microorganisms have the potential to quickly outcompete slow-growing target microorganisms, meaning that even if a microorganism is initially enriched in a sample, its relative abundance can soon be diminished when both of these types of microorganisms are co-inoculated. Such competition often happens when cultivation media are supplemented with rich substrates (for example, yeast extract or peptone) or when easily fermentable substrates are used as carbon sources. Likewise, some microorganisms can have a high affinity for a particular substrate, consuming it efficiently when the substrate is present in limited concentrations. Thereby, these microorganisms can prevent the growth of other microorganisms that are able to use the same substrate but have a lower affinity for it[59]. To tackle these problems, several recent methods[17,60–63] focus on isolating single cells from environmental samples and using these as inocula, rather than gradually enriching microorganisms from mixed communities. Additionally, for oligotrophs that are poorly adapted to a nutrient-rich environment, the use of low-nutrient media has proven successful for their cultivation[64,65].

An additional difficulty for the targeted cultivation of slow-growing microorganisms involves the long timescales of research, which has both practical and economic[6] implications for researchers. Microbial growth rates can be affected by suboptimal conditions provided in the laboratory, and substantially differ from the 'natural' growth rates in the environment. Although growth rates might be improved by attempting to optimize growth conditions, slow-growing microorganisms might represent less appealing targets for many researchers given the extended timescales and increased associated research costs.

Innovative techniques

Most current methods that aim to increase the rate at which microorganisms of interest are isolated broadly follow at least one of two main strategies (FIG. 3). They either rely on scaling-up the number of cell isolations to increase the chance of isolating a species that is interesting (high-throughput isolation and cultivation), or aim to selectively isolate organisms with specific functional characteristics or that belong to a specific taxonomic group (targeted isolation). Methods that fall into these two categories (FIG. 4) are described in the sections below.

Membrane diffusion-based cultivation. Our inability to produce culture media that sufficiently replicate all of the necessary growth factors present in natural habitats remains a limitation for many cultivation experiments[7,66]. With this limitation in mind, several cultivation technologies centre on the principle of physically separating

Anaerobic
An organism that grows in the absence of molecular O_2.

Inocula
Samples of microorganisms introduced to fresh medium for initiating the growth of a new culture.

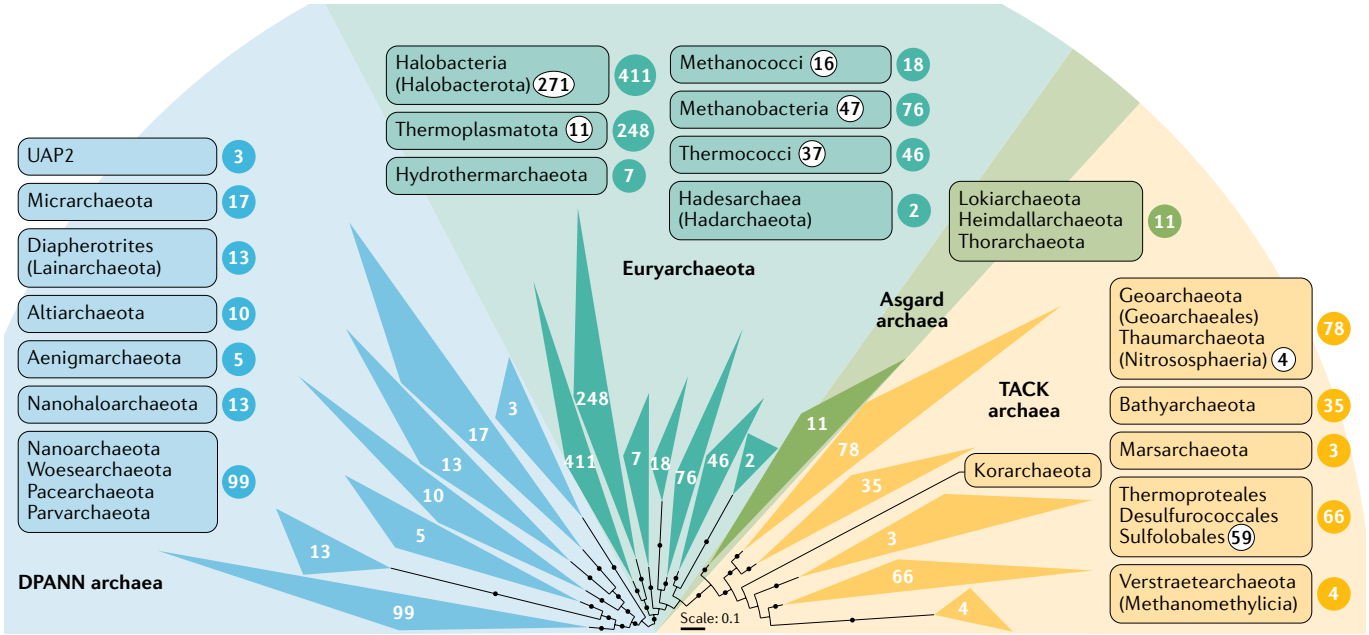


Fig. 2 | **Archaeal diversity is dominated by uncultured groups.** A phylogenetic species tree for archaea, inferred from concatenated alignments of a minimum of 5 out of a total 15 ribosomal proteins per species, encoded by 1,166 archaeal genomes that were obtained from the Genome Taxonomy Database[21]. Numbers in white font in coloured circles are the number of individual taxa in each collapsed clade, and are also used to connect corresponding taxa names to clades. Numbers in black font in white ellipses next to taxa names indicate the total number of species-level cultured isolates described for those taxa, based on the number of species type strains assigned to each clade that are present in the BacDive database[31] (last accessed 6 April 2020). Taxa without numbers have no cultured isolates recorded in BacDive[31]. Numerous cultured representatives have been reported in the scientific literature that are not represented in the numbers in this figure, because cultures have not been officially described and/or deposited in culture collections, and are therefore not included in BacDive[31]. A comprehensive database recording all cultured archaea including those not officially described or deposited in culture collections is currently lacking. The tree was generated from datasets containing homologous proteins from the different species included, which were aligned separately using MAFFT (L-INS-i)[150] and the alignments for each protein then concatenated, such that those proteins belonging to the same species were combined to form a single sequence. Poorly conserved sites in the concatenated alignment were removed using trimAl[151] with the option -gt 0.5. A phylogeny was generated from this trimmed alignment using the model LG + C60 + F + R10 in IQ-TREE[152] with 1,000 ultrafast bootstrap replicates[153]. Branches labelled with black dots have support values ≥95%. Given the limited protein dataset used to infer this phylogeny, in some cases the deeper relationships between some species or groups may not reflect more widely accepted relationships based on more in-depth and better supported analyses.

cells, while allowing them limited contact with their natural habitats. Typically, separation is achieved by a filter or membrane, with a pore size small enough to enable the diffusion of growth factors but not cells. In these set-ups, cells maintain access to essential growth factors from their natural environments or syntrophic partners, while replicating in isolation, forming ideally axenic cultures or colonies[66]. Furthermore, potential growth-suppressing metabolites produced by the isolated microorganisms can freely diffuse away rather than accumulate locally[61,67]. Mimicking environmental in situ growth conditions in this way avoids the meticulous development of suitable artificial conditions, in particular avoiding excessive quantities of nutrients typically provided by classical media, which can be detrimental to the growth of some species[65].

One such method is the hollow-fibre membrane chamber device[61], which consists of numerous hollow fibres (porous tubes) connected to syringes that function as cell isolation chambers. The fibres are each inoculated with a single cell, by serially diluting environmental cell suspensions, and are then submerged in an environmental water sample that provides the cells with growth factors. The hollow fibres are inoculated using the syringes, which can also be used to subsample the chambers while still incubating the remaining sample in situ.

Another membrane diffusion-based technology with the capacity to perform high-throughput cultivation experiments is the i(isolation)Chip[60] (and derivatives[68,69]), which consists of a plate accommodating an array of small holes that function as micro-chambers to capture and isolate single cells from environmental samples. These holes are sealed with a membrane, and the whole device is then incubated in the environment from which the cells were originally sampled, providing in situ conditions for isolated cells to grow. One notable success of this technology was the discovery of a novel antibiotic from bacteria inhabiting soil[70].

The soil substrate membrane system[71,72] (and related methods[73,74]) is another membrane diffusion method that specifically targets archaea and bacteria inhabiting soils. This system involves dispensing cells from an environmental (soil) extract on the upper side of a membrane. The membrane is then placed on top of a sample of the soil, which the cells can then access and use as a growth substrate. The system is then incubated, enabling clonal colonies to form, which can be screened for

species of interest or to inoculate media for continued cultivation. Other, similar techniques have isolated bacteria by growing them on the surface of filters floating on liquid media, which enabled colonies to form for species that did not grow on more conventional solid media[75,76].

For larger-volume cultivations, a previous study[77] described the diffusion bioreactor, which provides cells access to their natural growth factors. This device consists of an inner chamber containing inoculated growth medium and an outer chamber containing a substrate, such as soil or sediment, which are connected by several holes sealed by membranes. This set-up enables cells to grow and proliferate in conditions resembling their natural environment before isolating single strains using classical methods, such as dilution-to-extinction and spread-plate colony picking (BOX 1).

Many of these diffusion cultivation devices have been used to facilitate the growth of phylogenetically novel species, beyond those that were recovered from the same environment using traditional cultivation methods alone[60,61,71,76,77].

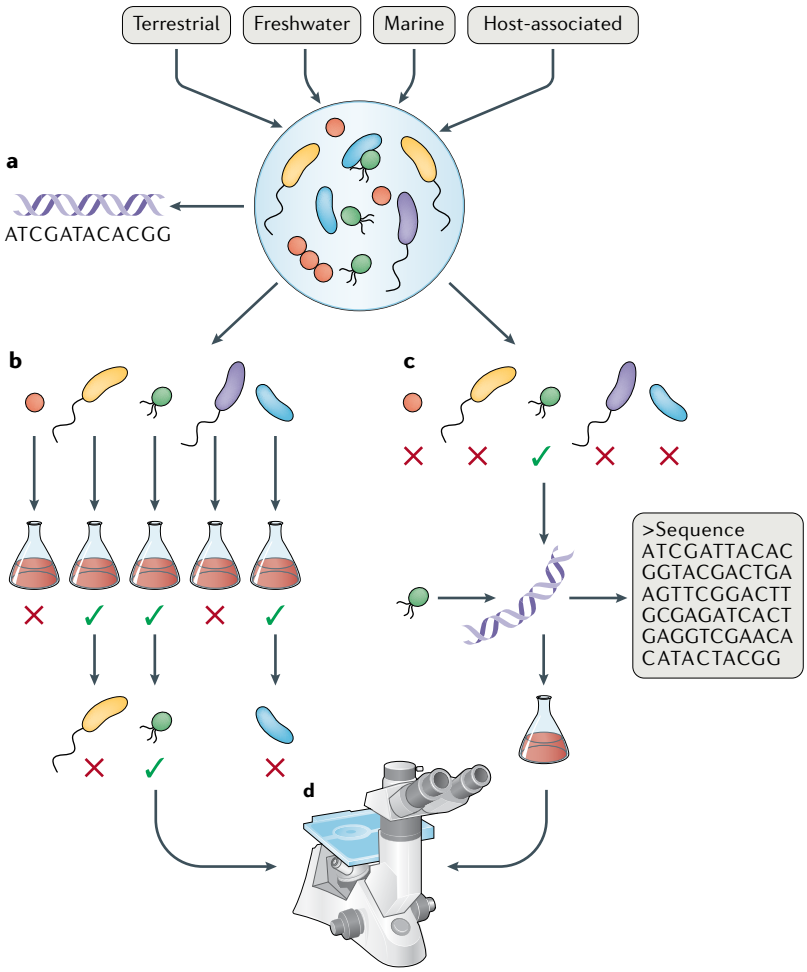


Fig. 3 | **Workflows for isolating novel microorganisms for cultivation using high-throughput or targeted approaches. a** | Sequencing-based screening of habitats can be used to identify locations with high relative abundance of target organisms, followed by collection of cell samples from these sites. **b** | High-throughput approaches can be achieved by inoculating media with single cells to establish large numbers of monocultures, incubating cultures and then screening for growth, followed by screening of viable cultures for those containing species of interest. **c** | Targeted approaches rely on isolation of cells belonging to specific taxonomic or functional groups. **d** | Cultured isolates can be used for downstream characterization and experimentation to investigate their biology.

Microfluidic systems for cultivation. Microfluidic systems are widely used for various biological research applications, including cultivation. Generally, the benefits of these systems include increased scalability, and therefore throughput, by miniaturizing overall experimental set-ups[78], as well as the ability to manipulate large numbers of single cells from environmental samples in parallel and in the presence of a range of substrates or under different physicochemical conditions[78]. In some cases, these benefits can also be extended to the cultivation of anaerobic microorganisms, as some systems can maintain low levels or an absence of O_2 (REF.[62]).

One example of these microfluidic systems is the SlipChip[79], which was originally designed for use in chemistry but was later repurposed for high-throughput cultivation of bacteria[62]. The repurposed version works by incubating single cells separately in thousands of microcompartments, which can contain various media and substrates. The microcompartments are formed by co-aligned wells present in the interfacing surfaces of two adjoining plates (that together form the chip). Once inoculated, the chip is incubated, giving the cells time to multiply and form micro-sized cultures. The plates are then 'slipped' apart, dividing each microcompartment in two, thereby forming two identical replicate microcultures for each compartment. The replicates in the wells of one plate can then be individually screened for growth and/or taxonomic identification (which is typically destructive) and the corresponding wells of the other plate preserve live cells for continued cultivation.

Additionally, multiple SlipChips can also be used to screen a range of different growth conditions in parallel. To do this, the contents of all cell-containing wells from one plate are pooled. Pooling is done separately for multiple chips, which have all been incubated in separate conditions. These pools are then genetically assayed (for example, by PCR) to screen for the presence of species of interest, indicating growth of that species under a particular condition. The data this generates can be used to narrow down the number of potential culture conditions, thereby efficiently tailoring a suitable subset for growing a target microorganism[78]. A previous study[78] demonstrated the effectiveness of this approach for culturing the first representative of a *Ruminococcae* genus corresponding to one of the 'most wanted' taxa in the Human Microbiome Project[80].

As discussed in the sections above, many microorganisms rely on products of syntrophic partners for growth. In such cases, the use of microfluidic chips that grow single microorganisms in isolation, in fully sealed chambers, likely prohibit successful cultivation. To overcome this limitation, a previous study designed the nanoporous microscale microbial incubator system[63], which incorporates both microfluidic and membrane diffusion-based technologies. Nanoporous microscale microbial incubator chips comprise an array of thousands of micro-scale diffusion chambers organized on a microfluidic slide[63]. Once sealed, the chambers physically isolate individual cells, but facilitate the transfer of

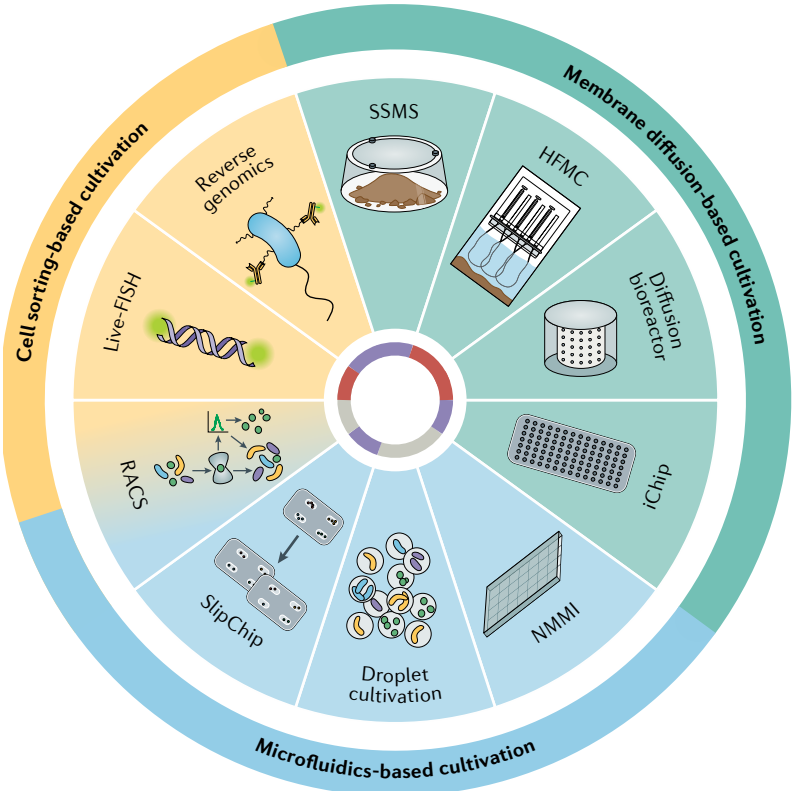


Fig. 4 | **Innovative methods for the isolation and cultivation of novel microorganisms.** Membrane diffusion-based cultivation methods (green), such as the i(isolation)Chip[60], hollow-fibre membrane chambers (HFMC)[61], diffusion bioreactors[77] or the soil substrate membrane system (SSMS)[71,72], use permeable membranes that enable nutrients and metabolites to diffuse into the cultivation medium and thereby mimic more natural conditions during cultivation. Microfluidics-based cultivation methods (blue), such as nanoporous microscale microbial incubators (NMMI)[63] or the SlipChip[78,79], are able to manipulate cells in small volumes and large numbers of replicates, and can also be combined with various droplet cultivation methods[87]. Cell sorting-based techniques (yellow), such as Raman-activated cell sorting (RACS)[98,100], fluorescence in situ hybridization of live cells (live-FISH)[97] or reverse genomics[17], provide a way to target a functional or taxonomic subset of cells for isolation.

growth factors and signalling compounds between all cells in the slide by passive diffusion through the permeable chamber walls. Although this system has strong potential for the isolation and cultivation of interesting syntrophic organisms, there are currently no published examples.

Finally, systems that encapsulate single cells or small populations of cells, in either liquid[81–84] or gel[85,86] droplets, are also commonly used for cultivation[87,88]. The cell-containing droplets are typically manipulated and incubated in a microfluidic device. These encapsulation methods decrease competition between species, because cells are grown in isolation from those in other droplets, and have also been demonstrated in some cases to recover more phylogenetically diverse microorganisms than were recovered using traditional cultivation methods for the same samples[84]. Furthermore, as the number of simultaneous cultivation experiments can be vastly increased by manipulating millions of individual droplets in parallel, these techniques offer extremely high rates of experimental throughput[89].

Isolation of cells by sorting and selection for taxonomy or function. Cell-sorting technologies are a mainstay for many areas of biological research, and can be used to isolate single cells from cell suspensions of mixed communities. Cell sorting can be performed with many different technologies, some of which perform at high speed, such as droplet-based[90] and microfluidic-based[91] sorters, that are available commercially. Other technologies, such as microscopically guided optical tweezers[92], can precisely manoeuvre and isolate single cells but with a lower rate of throughput. Such an approach was used for the isolation and co-cultivation of the nanosized hyperthermophilic archaeon *Nanoarchaeum equitans* and its host *Ignicoccus hospitalis*[93]. However, unlike droplet sorters, which can expose cells to considerable pressure, optical tweezers typically exert less pressure, so are less detrimental (although, in some cases, they can cause photodamage[94]). Many cell sorters can sort cells stochastically, which is useful for high-throughput cultivation experiments, as large numbers of single cells can be distributed into separate wells containing growth media. However, the likelihood of isolating particular target cells can be increased by selectively sorting cells based on detectable distinguishing phenotypes.

Fluorescence-activated cell sorting (FACS) is a common method for sorting cells based on fluorescence signals. Whereas some organisms have intrinsic fluorescence properties (autofluorescence), some low-toxicity or non-toxic fluorescent dyes can also be used to stain different cellular targets, such as DNA and phospholipid membranes, making cells more distinguishable from background levels of fluorescence. In addition, if only a subset of species autofluoresce when excited at a certain wavelength of light or are better stained than other species by a particular dye, FACS could be used to separate cells based on these properties, and enrich a fraction of the community. However, more sophisticated labelling methods can be applied to increase the taxonomic or functional specificity of isolated microorganisms.

FISH is a widely used fluorescent labelling method, which can be used to identify and quantify cells belonging to specific taxonomic groups in a given sample[95]. FISH-labelled cell samples can also be sorted with FACS to enrich cells belonging to selected taxonomic groups for sequencing-based studies[96]. In the vast majority of FISH protocols, cell viability is not maintained, because cells are chemically fixed and their membranes permeabilized to give molecular probes access to their intracellular target, while also maintaining the structure of the cells. However, a recent study[97] has demonstrated a 'live-FISH' method, which avoids cell fixation and permeabilization, and instead incorporates probes into living cells by chemical transformation. Using this technique, living Alphaproteobacteria from natural

Optical tweezers
A method for isolating single cells from cellular suspensions by microscopy and laser capture. Many optical tweezer set-ups are now automated and operate in microfluidic chips. Cells are passed through these chips in a suspension, and those with a detectable phenotype are captured, relocated from the main flow to a sterile outlet and collected.

Phenotypes
The observable or detectable traits of an organism influenced by its genes (genotype) and factors of its environment.

Fluorescence-activated cell sorting
(FACS). The dispersion of cells into separate containers, such as test tubes or wells, based on either natural or artificially induced fluorescent properties (for example, by fluorescent stains or labelling techniques).

Genome-resolved metagenomics
The reconstruction of genome sequences from metagenomic data, typically obtained through bioinformatics approaches in which contigs from a single microorganism are grouped ('binned') together.

seawater were first labelled with FISH probes, sorted by FACS and, subsequently, cultivated. Despite optimizing the protocol to maximize cellular viability during the live-FISH procedure, the best survival rates of cells in this study were relatively low (1.24–2.82%, depending on the strain tested), and therefore, in its current form, live-FISH would most likely be unsuitable for isolating microorganisms found in low abundance.

A recent technique that bridges the gap between non-taxa-specific cellular stains that can label cells while maintaining their viability and highly taxa-specific but usually destructive FISH is reverse genomics[17] (FIG. 5). This technique takes advantage of the ease with which near-complete genome sequences for uncultured microorganisms can be reconstructed from environmental samples using genome-resolved metagenomics. From these genomes, membrane proteins with extracellularly exposed domains that are conserved only among target microorganisms are then predicted in silico, and used as epitopes for the production of antibodies, which are then tagged fluorescently. When used in complex environmental cell suspensions, the raised antibodies should bind to the matching protein epitopes of the target cells, thereby marking the cells with fluorescent labels. Provided the selected epitopes have low sequence conservation with other microorganisms, labelling should be taxa-specific, enabling detection of target cells and separation from the remaining sample by FACS. Single cells labelled using this method were shown to retain viability after being sorted and were successfully used as inocula to establish new cultures[17].

Raman-activated cell sorting[98] offers an alternative to fluorescence-based labelling and a way of isolating viable cells, while selecting for those that are most active under certain conditions, thereby corresponding to particular ecological functions. For Raman-activated cell sorting, cells are incubated in the presence of deuterium (D_2O) under growth conditions that are likely to favour the activity of target microorganisms[99]. Deuterium is proportionally incorporated into the synthesized lipids of more active cells, thereby conferring those cells with a chemical label[99]. The deuterium labels can then be detected using Raman microspectroscopy in a microfluidic device, with the corresponding cells then captured and immediately sorted with optical tweezers[100]. The isolated cells can then be used as inocula for downstream cultivation.

Limitations. Although many of the techniques discussed above have potential for increasing rates of species isolation, their success might not be consistent across the existing diversity of microbial life. Theoretically these methods could be applied to target many taxa, but in some cases their practical application could be much more challenging. For example, microorganisms that form biofilms could be more challenging to separate for cell sorting[101]. Likewise, many microorganisms are sampled from environments, such as sands, soils, sediments and faecal material, that contain non-biological particles that can interfere with molecular labelling and flow-based methods. In these cases, cells need to be separated from the particles before isolation, and procedures to achieve separation are often not trivial[101,102].

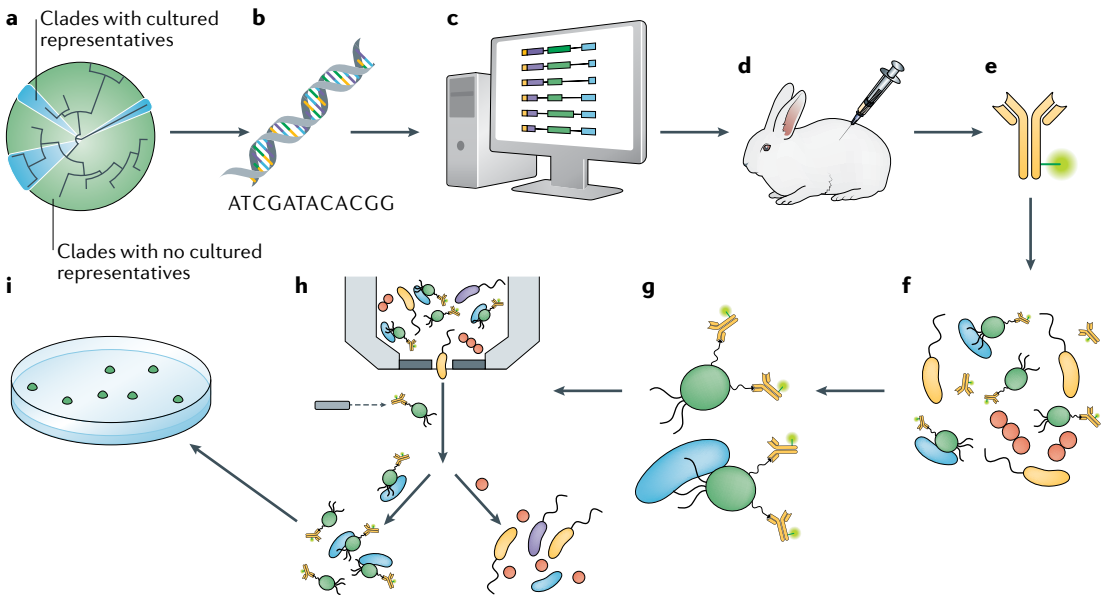


Fig. 5 | **Reverse genomics for targeted isolation and cultivation of novel microorganisms.** Reverse genomics[17] can be used for targeted cultivation of novel lineages. **a** | First, the target microorganism belonging to novel or important clades is identified. **b** | The genome of the target microorganisms can be reconstructed from metagenomic data. **c** | Based on these data, proteins can be predicted and highly expressed membrane proteins with extracellular domains can be identified. **d** | This is followed by the synthesis of a target-protein domain antigen and inoculation into a suitable animal for antibody production. **e** | The raised antibodies are then purified and coupled to a fluorescent dye. **f** | Antibodies are added to environmental cell samples. **g** | The antibodies label the target cells. **h** | Cells can then be sorted by fluorescence-activated cell sorting based on the antibody-conferred signal. **i** | Cells are sorted onto liquid or solid growth media. If the targets are symbionts that physically associate with each other and if one cell is labelled, both microorganisms could be co-sorted together and used to inoculate a syntrophic co-culture.

Anoxic
A state of complete absence of molecular O_2, for example, in an environment or a culture.

Optical density
A common spectrophotometric method for assessing the cell density of a liquid suspension, typically by measuring the extent at which light at a 600 nm wavelength is scattered by cells as it passes through a sample.

Another limitation of many of these methods, particularly if working with anaerobic microorganisms, is that isolation experiments must be performed in anoxic conditions. Although anaerobic chambers have been used for traditional microbial culturing methods for many decades, newer techniques such as those involving cell sorters typically require much larger equipment, which is difficult to fit and operate in a typical anaerobic chamber. The use of larger and more accessible chambers could be a solution; another option is to adapt the cell isolation equipment in such a way that cells can be manipulated under anoxic conditions. Indeed, several companies have developed fluorescence-based cell sorters that are either small enough to fit into typical anaerobic chambers or, otherwise, perform cell sorting in an enclosed flow cell, which can be loaded into an anaerobic chamber before being removed and loaded into the sorter.

Another difficulty for high-throughput cultivation methods is the supply of gaseous substrates, such as H_2, CO_2, CO and CH_4. Currently, devices to supply gas to microtitre plates are unavailable, with the main challenge being the complete isolation of the headspace of the individual wells. Furthermore, cultivation at high temperatures has its own intrinsic challenge owing to liquid evaporation, which is particularly problematic when cultivating cells in a small volumes as evaporation can lead to cultures drying out. In addition, evaporation can also cause condensation build-up inside well lids or seals, which can prevent the monitoring of growth by automatic optical density measurement.

It must also be recognized that the isolation of cells is typically just the first part of a two-part puzzle. To then culture a microorganism that has been isolated, maintaining its growth continuously, a suitable medium and physicochemical conditions must be found. One way of finding optimal conditions is by inferring phenotypic features from metagenome-assembled genomes (complemented by proteomic and transcriptomic data) from uncultured target microorganisms, and selecting the medium and conditions based on this information[103]. Numerous published strategies and tools are available for estimating physiological and ecological traits, such as the optimum growth temperature[104] and antibiotic susceptibility[105], from genome sequences. Such strategies could help provide clues for ways in which a microorganism can be successfully grown or enriched in cultures. However, a genome sequence alone often provides insufficient data for accurately determining all necessary culture conditions to grow a particular microorganism successfully.

As an alternative, or in addition to genome-based methods, sophisticated 'next-generation' physiology approaches[106] can be used to more accurately determine metabolic and physiological properties for target microorganisms in enrichments or environmental samples. These approaches include techniques such as bioorthogonal non-canonical amino acid tagging, stable isotope probing and the detection of substrates incorporated at the single-cell level by technologies such as NanoSIMS, Raman microspectroscopy and BrdU staining[106]. Combining the insights that these methods provide with careful observation of microorganisms in their natural environments, while being particularly attentive to their physical and metabolic traits, can inspire ingenuity and help researchers to find a successful way of isolating and culturing a particular microorganism. Likewise, complementing modern innovative methods with traditional methods will also help researchers to achieve cultivation.

Screening methods

For experiments that generate several enrichments, cultures or colonies, one must determine which of these enrichments contain cells that are actively growing and which contain organisms of interest. If done efficiently, such screening will help researchers determine cultures to prioritize for further study, because maintaining vast numbers of cultures, including those in which cells are not viable or contain microorganisms of limited interest, is costly and will decrease the overall effectiveness of research. Screening is particularly important for high-throughput cultivation experiments, which therefore require equally high-throughput screening methods. The following sections outline various such methods, but ultimately researchers must determine which methods are most appropriate for their particular experimental setting.

Direct visualization. The observable formation of a colony on solid media indicates the presence of active cells and the use of commercial colony-picking robots can increase the rate at which colonies are taxonomically screened and reinoculated[107]. However, many microbial strains grow very slowly on solid media[108] or stop growing after their colonies reach a small size[109]. These 'microcolonies' can be invisible to the human eye without magnification. For cases such as these, it is unclear whether current commercially available colony-picking robots are sufficiently sophisticated and precise. Therefore, without improvements to this technology, manually picking microcolonies, while observing them under a microscope, might be a more efficient method. Alternatively, one technological solution avoids these difficulties by growing single cells in individual liquid droplets arranged on the surface of solid media, which are easier to manipulate in an automated fashion[81].

For experiments with inoculated liquid medium cultures, visible turbidity can sometimes be observed, indicating microbial growth. However, some archaea and bacteria cultivated in the laboratory may only reach such low maximum cell densities that they defy visible detection by eye. Attempts to visualize cells by light microscopy could also fail if cells are very small and/or transparent, and can be laborious for a high-throughput set-up. Cells can be made more conspicuous by staining them with fluorescent live stains and visualizing them with a fluorescence microscope; however, it is still difficult to confidently rule out microbial growth in a sample if no cells are visualized.

Optical detection of growth. Photospectrometer plate-readers can be used to perform optical density measurements for liquid samples in separate wells of a multiwell plate, thereby determining increased cell

Flow cytometry
A technique used to detect and count cells based on physical or chemical properties.

MALDI-TOF mass spectrometry
MALDI is an ionization technique used in mass spectrometric analysis based on embedding samples in a special matrix from which they are desorbed by laser light. The technique allows the analysis of biomolecules and organic molecules.

density indicating growth in a scalable, high-throughput manner. However, this method can be unsuitable for species that have a low per-cell density or that only grow to low population densities. Optical density measurements also do not provide an accurate indication of the number of cells present in a liquid sample. An alternative and more sensitive method is flow cytometry, which can be used to efficiently screen for growth and also quantify cell numbers from as little as tens of microlitres of a culture, thereby retaining greater volumes for further experiments. Many flow cytometers are automated so that they can screen and quantify several cultures grown in separate wells of multiwell plates, making them suitable for processing large numbers of liquid samples such as those generated by high-throughput dilution-to-extinction experiments[110].

PCR and sequencing-based screening. Once viable colonies or cultures in a large-scale experiment are identified, they can be screened for species of interest. If a limited number of species are targeted, performing PCR with primers specific for target species is an effective and scalable screening method. In some cases, PCR can be performed using just a small subsample of a culture as the input, with cells being lysed and DNA released for amplification by the initial (typically ~95–98 °C) denaturation step of the PCR. However, more sophisticated lysis methods are required for many robust cell types.

For cultures containing naturally occurring PCR inhibitors (many of which exist[111]), direct PCR often is unsuccessful. Although the effects of some inhibitors can be mitigated[111], carefully designed controls are needed to determine whether a negative result is really caused by the absence of target microorganisms in a sample (although this is inherently difficult to conclusively determine) or whether PCR inhibition or failure has occurred (a false negative). Alternatively, there are vast numbers of protocols and commercial kits available to extract and purify DNA from cells, helping to remove most inhibitors. However, a proportion of the cell material or extracted DNA is typically lost during most of these protocols, meaning they could be unsuitable for small and precious samples. Furthermore, DNA extraction can be time consuming and costly at a large scale.

Another widely used PCR-based identification method is 16S rRNA gene amplicon sequencing, which can also help to determine the relative abundance and species diversity in a sample. 16S rRNA genes are present in all archaeal and bacterial genomes, and contain several variable regions that can be used for taxonomic discrimination, as well as highly conserved regions. Against these conserved regions, 'universal' primers can be designed that capture large swathes of the total known diversity of archaea and bacteria, while also discriminating between different species[112]. Indeed, various primer sets targeting conserved regions of this gene are described in the scientific literature and are widely used in diversity studies[113–115]. The amplified PCR products from several different samples can be sequenced in multiplex using various contemporary high-throughput technologies. The resulting data can be used to infer diversity and the relative abundances from different samples. 16S rRNA amplicon sequencing can therefore be particularly useful to screen or continually monitor enrichment cultures in which a mixed community is present[116].

Amplicon sequencing can suffer from primer biases, however, which in some cases lead to substantial portions of the known microbial diversity being missed[117]. Especially, microorganisms of as yet uncultivated phyla such as the CPR and novel groups of archaea are frequently overlooked by amplicon sequencing approaches because insertions in their 16S rRNA genes[25] or mismatches with commonly used primer sets impede their detection[117]. If a specific taxonomic group is being targeted for cultivation, primers that better capture the total breadth of diversity in that group could be advantageous for screening both sampling sites and enrichments.

With the increased availability of cheap sequencing technologies, 16S rRNA gene amplicon sequencing is now quick and more affordable, which shortens the duration between sampling and data analysis, meaning that enrichment cultures can be monitored on finer timescales. Although amplicon sequencing provides insight into relative abundances, these measurements do not provide absolute abundances or total cell quantities. To achieve absolute measurements, sequencing data can be complemented with cell enumeration data generated (for example, by flow cytometry) for the same sample, thereby providing a more complete understanding of microbial community composition in a culture or enrichment[118,119].

Furthermore, one recently developed platform has used affordable sequencing technologies and microfluidics to automate DNA preparation and whole-genome sequencing for screening large numbers of samples in parallel, such as those generate by high-throughput cultivation experiments[120]. This platform was also shown to obtain high-quality genomic data for low biomass samples, making it suitable for screening isolates that grow to low population densities in cultures or form microcolonies on solid media.

MALDI-TOF mass spectrometry. An alternative method of taxonomic identification, for which media composition and the presence of inhibitors are less of a consideration, is MALDI-TOF mass spectrometry. This method has proven to be both fast and cost-effective for identifying isolates while filtering out conspecifics and non-target taxa. As MALDI-TOF mass spectrometry is highly sensitive, only a relatively low cell mass is needed to record a mass profile for taxonomic identification[121]. Commercially available systems typically provide profile databases that make identification possible at the genus to species level for a query isolate. Currently, however, these systems are mostly used to identify microorganisms from clinical or food-associated environments, rendering their databases unsuited for the identification of taxa from other environments, as well as novel isolates[122]. Therefore, to become a useful taxonomic identification platform for large-scale cultivation experiments, databases would need to be complemented with profiles of a

wide diversity of microorganisms. Broadening of databases could be achieved by including profiles of novel cultured taxa with confirmed identity (for example, by 16S rRNA gene sequencing[121]). Although databases are likely to improve in the future, another limitation is that MALDI-TOF mass spectrometry identification currently cannot be used for samples in which several species are present, as these will not produce a clear profile.

Targets for culturing

What qualifies as an interesting microorganism differs depending on the researcher, according to their own interests and motivations. TABLE 1 lists groups of microorganisms for which a strong case could be made that it would be interesting to culture them, based on, for example, their inferred functions and/or ecology, or because they so far have no cultured representative.

Groups that are highly abundant in an environment likely have an important role in the biogeochemistry of that environment, as exemplified by Bathyarchaeota, various Marine Group archaea, Acidobacteria, SAR202 and SAR86 (TABLE 1). Therefore, such groups are interesting to culture to help better understand their functions. Identifying microorganisms that fit this profile can be relatively straightforward, as abundances can be estimated for particular groups from publicly available 16S rRNA gene amplicon data (for example, from data in the Sequence Read Archive[123]) or can be generated for individual environments de novo.

Microorganisms also could be deemed interesting to culture if they belong to a large group with no, or few, previously cultured representatives, with key examples including the CPR bacteria and DPANN archaea (FIGS 1,2; TABLE 1). Furthermore, microorganisms that shed light on evolutionary processes owing to their proximity in the tree of life to an important evolutionary event are also interesting to culture. One clear example of this is the Asgard archaea, in particular the Heimdallarchaeota, which are thought to be the closest archaeal relatives of eukaryotes[124–126]. Culturing further representatives of this group will facilitate comparisons between their cellular features and those of eukaryotes, thereby potentially helping to establish the major evolutionary changes that occurred during early eukaryotic evolution (eukaryogenesis) (TABLE 1).

Ultimately, in-depth knowledge and topic-specific priorities will help researchers to identify the targets that are likely to be the most rewarding for cultivation efforts.

Conclusions

The wide-scale need for microbial isolation and cultivation has led to the development of numerous innovative methods. Most of these methods adopt either a targeted (for example, reverse genomics[17], Raman-activated cell sorting[98] and live-FISH[97]) or a high-throughput (for example, iChip[60], SlipChip[62] and nanoporous microscale microbial incubators[63]) strategy to isolate cells from communities and environments, although some methods incorporate both strategies to varying degrees. Some of these methods, such as reverse genomics[17], have proven to be viable and accessible options for bringing interesting microorganisms belonging to poorly sampled clades into culture, while circumventing the traditionally long time spans that can be associated with culturing. Other approaches, however, although theoretically appealing, have not yet been successful beyond isolating members of mock or low-complexity communities (FIG. 4). There are two main, generally opposing, possible explanations for why this might be the case. For one, these methods currently fail to overcome poorly understood yet limiting biological processes (such as microbial dormancy), and are therefore only capable of culturing a certain range of microorganisms. Alternatively, the other explanation is that these methods have not yet been applied widely enough, or developed to the point at which they will be most effective.

With current available methods, we are only able to culture microorganisms that represent a small fraction of the existing landscape of microbial diversity. To culture new microorganisms, the further development and maturation of advanced cultivation technologies will be required. The innovative methods discussed in this Review may well represent avenues for a future revolution in successful cultivation efforts.

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