

Cultivating the uncultured: limits, advances and future challenges

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Abstract Since the invention of the Petri dish, there have been continuous efforts to improve efficiency in microbial cultivation. These efforts were devoted to the attainment for diverse growth conditions, simulation of in situ conditions and achievement of high-throughput rates. As a result, prokaryotes catalysing novel redox reactions as well as representatives of abundant, but not-yet cultured taxa, were isolated. Significant insights into microbial physiology have been made by studying the small number of prokaryotes already cultured. However, despite these numerous breakthroughs, microbial cultivation is still a low-throughput process. The main hindrance to cultivation is likely due to the prevailing lack of knowledge on targeted species. In this review, we focus on the limiting factors surrounding cultivation. We discuss several cultivation obstacles, including the loss of microbial cell–cell communication following species isolation. Future research directions, including the refinement of culture media, strategies based on cell–cell communication and high-throughput innovations, are reviewed. We further propose that a combination of these approaches is urgently required to promote cultivation of uncultured species, thereby dawning a new era in the field.

Keywords Culture · Isolation · Metabolism · Microbial community · Cell–cell communication · High-throughput cultivation

Introduction

Today, 1.8 million eukaryotic species are recognised, while estimates indicate that our planet hosts five- to ten-fold more species. Until now, only 7031 prokaryotic species have been described (and validated by the International Committee on Systematics of Prokaryotes) in the 120 years since the invention of the Petri dish (Achtman and Wagner 2008), even though we now realise that prokaryotes represent “the unseen majority” (Whitman et al. 1998). The lack of an extensive and accurate picture of the microbial diversity is partly due to a lack in technical advances in the microbiology cultivation field. Over the past three decades, molecular biology was an enormous driving force in microbiology in uncovering the microbial diversity. Many new candidate divisions are now recognised due to 16S rRNA sequence-based approaches and environmental metagenomics (Curtis et al. 2002). These findings exposed a gap between the known phyla and those possessing cultured representatives (Fig. 1). In 1987, when much of our knowledge derived from pure culture techniques, all known phyla possessed cultured representatives. Twenty years later, of the 100 bacterial phyla identified only 30 possess a cultivated representative (Achtman and Wagner 2008). The number of phylum-level divisions possessing cultured representatives has increased at a linear and constant rate over the past two decades. However, this increase was greatly augmented with the emergence of molecular-based approaches. This augmentation is seen when candidate divisions are included in these calculations (Fig. 1).

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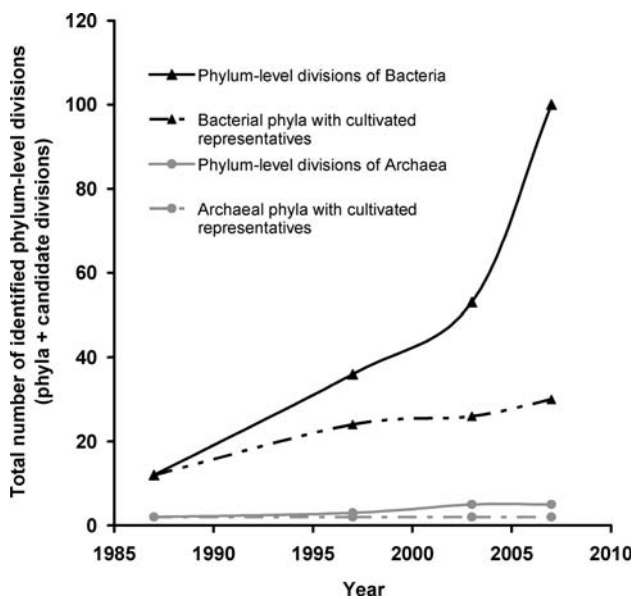


Fig. 1 Numbers of phylum-level divisions (phyla with cultivated members + candidate divisions with no cultivated representative) identified since 1987 among Bacteria (black line) and Archaea (grey line), and numbers of phyla with cultivated representatives (dotted lines). Adapted from Achtman and Wagner (2008)

From a quantitative point of view, the advances made by culture-dependent approaches may appear trivial, especially since only 0.1% of the existing prokaryotes have been cultured so far. Yet, this quantitative approach is reductive since the past 20 years of microbial cultivation have led to unprecedented advances in our knowledge of the microbial world.

Molecular ecology and metagenomics have increased significantly our knowledge of the genetic diversity and have led to interesting hypotheses (Hugenholtz and Tyson 2008). The advanced techniques have also revealed how far we are from measuring the full extent of genetic diversity encoded by microbial life (Hugenholtz and Tyson 2008; Pignatelli et al. 2008). Considering that many of the genes stored in the databases have unknown functions or are incorrectly annotated, it is probable that metagenomes alone will not offer sufficient knowledge to cultivate all organisms. There is evidence that many of the candidate divisions revealed by molecular approaches, and known only from molecular signatures, represent a significant fraction of the microbial diversity. Some members of these ‘not-yet-cultured’ taxa are probably key ecological players. Today, one of the main challenges for microbiologists is to develop strategies to cultivate this uncultured majority. A comprehensive understanding of biology and ecology of prokaryotes will require cultivation. Therefore, it is not surprising to observe an increasing interest for the field of microbial cultivation (Leadbetter 2003).

The aim of this review is to provide an overview of the new cultivation-based approaches while documenting their limitations and outcomes. Further, we highlight how cultivation has led to valuable advances in our understanding of microbial physiology and identifying the future challenges for microbiologists in the microbial cultivation field. Last, a discussion is provided on the technical developments that may drive innovative research in the near future.

Review on some cultivation successes

Prokaryotic growth necessitates an energy source (light or chemical compounds), nutrients and proper physicochemical conditions. Challenges for the microbiologist are to identify required nutrients, to provide them in the growth medium in the appropriate concentrations to sustain the microbial growth, and to avoid the co-precipitation of the introduced chemicals. Since different organisms require a different set of nutrients in varying concentrations and forms, the design of growth media remains a difficult task. Conversely, the intrinsic selectivity of any growth medium imposes limitations on the type, diversity and number of prokaryotes recovered from the natural environment. This phenomenon is known as the “great plate count anomaly” (Staley and Konopka 1985). Indeed, there is a difference of several orders of magnitude between colony counts on laboratory medium and total numbers of prokaryotic cells present in natural environments. Only a minor fraction of the naturally occurring microbial community is recovered by conventional selective media (Skinner et al. 1952; Amann et al. 1995). Depending on the nature of the samples, the cultivation efficiency of active cells by standard plating techniques is estimated between 0.001 and 1% (Kogure et al. 1979; Staley and Konopka 1985; Amann et al. 1995). Thus, cultured microorganisms do not reflect the functional and phylogenetic diversity present within any natural habitat.

Two main strategies are used for the isolation of pure cultures in microbiology. In both strategies, enrichment culture is performed as a first step. The first strategy aims to isolate colonies by repetitive streaking on solid medium (or alternatively by performing pour plates or agar shake tubes), while the second strategy aims to isolate cells following repeated series of dilutions in liquid medium. These classical approaches have led to the isolation of a large number of strains belonging to taxa with few or no representatives in pure culture (Janssen et al. 1997; Joseph et al. 2003). Nevertheless, the enrichment and pure culture isolation strategies often select for opportunistic fast-growing organisms also called lab weeds. In nutrient-rich artificial media, the community members with ‘r’-strategy, or fast-growers, often overgrow and out-compete the

naturally abundant ‘K’-strategists (Watve et al. 2000). Consequently, these conventional culture-dependent approaches do not reflect the actual microbial communities (Amann et al. 1995). Certain taxa are still severely under-represented in pure cultures (Hugenholtz et al. 1998). As direct consequence, most of our current knowledge of the nutrition, physiology and biochemistry of prokaryotes is based on easily cultivable organisms. In recent years, novel cultivation strategies were developed to overcome these limitations. Schematically, they are classified into four categories (Fig. 2).

Refinement of standard cultivation strategies

Different studies have demonstrated that a fraction of the “not-yet-cultured” groups of prokaryotes can be grown by the refinement of classical approaches. Changes in the media formulations, including the use of non-traditional electron donors, electron acceptors and carbon sources have proven efficient in recovery of uncultured taxa (Köpke et al. 2005). Diversification of the media and multiplication of culture conditions are simple methods to by-pass approaches that are selective by nature. For instance, the cultivable fraction from coastal sub-surface sediments was shown to yield a higher number and

diversity of isolates when culture collections were performed with diverse electron acceptors and carbon sources (Köpke et al. 2005). The qualitative composition of carbon sources is also a determinant factor for cultivation efficiency. As shown with seawater samples from the North Sea, media prepared using several different carbon sources and complex compounds yield higher number and more diverse isolates than similar media with only one carbon source. These isolates obtained with a single substrate belonged almost exclusively to the *Gammaproteobacteria* while representatives of four other classes grew on complex media (Uphoff et al. 2001). In recent years, it was shown that novel redox reactions, catalysed by specific ecological communities of prokaryotes, can be identified within enrichment cultures onto non-conventional media. Many novel phenotypes can be isolated using this method. Significant advances were made in the field of the anaerobic degradation of hydrocarbons. For example, different microbial consortia of Archaea and Bacteria, which couple the anaerobic oxidation of methane to sulphate or nitrate reduction, were enriched from anoxic marine or freshwater sediments. These findings enhanced our understanding of the global biological cycles (Nauhaus et al. 2002; Raghoebarsing et al. 2006). Moreover, hydrocarbon-degrading bacteria were identified using refined media

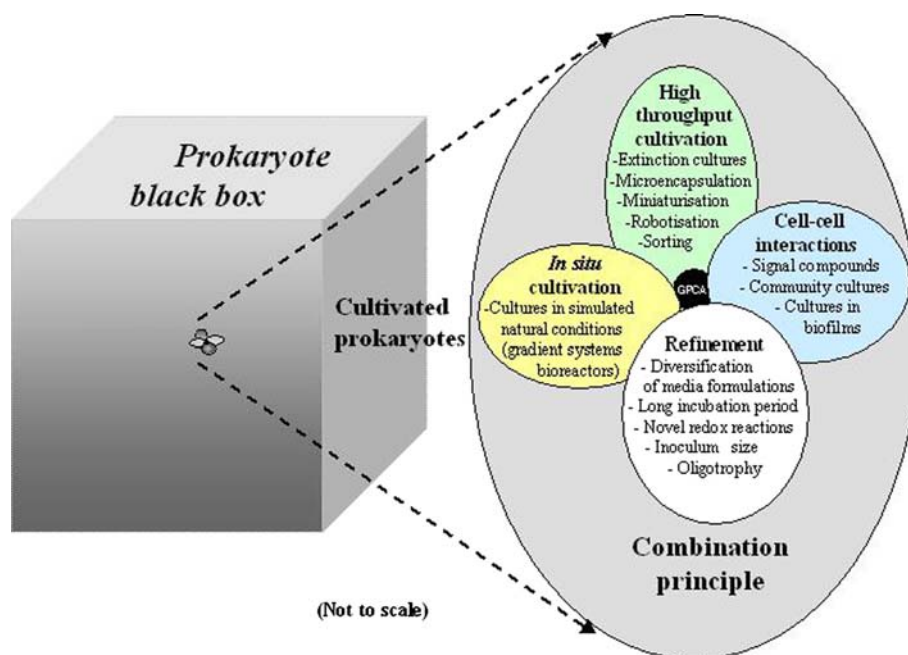


Fig. 2 Schematic representation of the four main categories of cultivation strategies. The small *black circle* schematizes the minor fraction of a naturally occurring microbial community that are recovered onto conventional selective media, the so-called “Great Plate Count Anomaly”. Improved cultivation strategies developed so far aim to enhance one or few aspects of growth conditions among four main categories (represented by four *bullets*) and each allowed

individually recovering more numerous and/or more diverse isolates than traditional approaches. None of these improved approaches is universal. We postulate that the combination (“the combination principle”) of already existing methods belonging to these four categories will help to come close to conditions where a significant fraction of microbes is amenable to culture

containing only hydrocarbon/carbon energy sources and nitrate, iron or sulphate as electron acceptors. These concurrent studies led to a better understanding of the biochemistry and energetics of anaerobes. Several biochemical mechanisms involved in the activation of some of these chemically non-reactive compounds have been identified (Lovley and Lonergan 1990; Rabus et al. 1993; Galushko et al. 1999; Coates et al. 2001; Widdel et al. 2007). However, hydrocarbon-degrading capacities, activation mechanisms and species or ecological guilds, which are involved in hydrocarbon degradation, remain to be discovered. For example, a microbiological anaerobic oxidation of methane with iron (III) or manganese (IV) as a terminal electron acceptor is unknown, yet this reaction is thermodynamically possible (Raghoebarsing et al. 2006). The enrichment culture with anoxic ditch sediment, discovered in 1999 by Zengler and co-workers, was another growth-supporting reaction of relevance. This team demonstrated that the conversion of long-chain alkanes to methane, under strictly anoxic conditions, is biologically performed by an ecological guild assumed to be acetogenic syntrophic bacteria associated with acetoclastic and hydrogenotrophic methanogenic archaea (Zengler et al. 1999). The discovery of this process in nature might help to understand the terminal degradation of organic matter in areas of deep and old marine sediments where sulphate is depleted. Another novel physiotype recently identified due to advances in cultivation and isolation was an autotrophic anaerobe, which couples the oxidation of phosphite (III) to sulphate reduction (Schink and Friedrich 2000). These novel physiotypes along with the newly recognised biological redox reactions are only a few of the several examples of the significant advances made in uncovering the microbial diversity through the enhancement of cultivating techniques.

Some recent successes in improving traditional cultivation methods include the following: the use of relatively low concentration of nutrients to increase the cultivability and to improve the recovery of prokaryotes from different types of natural samples (Button et al. 1993; Janssen et al. 1997; Watve et al. 2000; Connon and Giovannoni 2002; Rappé et al. 2002; Sangwan et al. 2005), the use of increased incubation periods to allow for the development of strains from rarely isolated taxa (Sait et al. 2002; Stevenson et al. 2004; Davis et al. 2005; Sangwan et al. 2005; Stott et al. 2008) and the addition of signalling compounds known to mediate communication between bacteria (Bruns et al. 2002, 2003). Moreover, other less-documented approaches also yielded new isolates. These included the following: the use of gellan gum (phytagel) as a gelling reagent instead of agar (Tamaki et al. 2005; Stott et al. 2008); the decrease in inoculum size (Davis et al. 2005); the addition of electron transporters to the culture media (Stevenson et al. 2004); the

addition of enzymes to cope with reactive oxygen species (Stevenson et al. 2004); the addition of inhibitors of undesired organisms (Leadbetter et al. 1999); and, the combination of an unusual energy source with antibiotics to exclude Bacteria (Könneke et al. 2005). Finally, sophisticated single-cell isolation tools allowing for the manipulation of a targeted cell from a mixed community (with a micro-capillary tube or with 'optical tweezers') have been developed (Huber et al. 1995, 2000; Fröhlich and König 2000). The principal limitations of these single-cell isolation strategies include their labour-intensive requirements and the determination of the suitable growth conditions to cultivate prokaryotic cells of unknown metabolism and systematic affiliation. The identification of a cell of interest among a complex community in the absence of clear distinctive morphological features is also challenging. Altogether, these limitations account for the lack in isolating numerous undiscovered strains.

Cultures in situ or cultures in simulated natural conditions

Often, the laboratory conditions poorly mimic the natural environmental conditions. Therefore, strategies aimed at simulating natural conditions or culturing in situ have been proven efficient. Schematically, two types of "in situ colonisation devices" have been developed: the diffusion chambers and the carriers (of organic or inorganic nature) (Kaeberlein et al. 2002; Ferrari et al. 2005; Yasumoto-Hirose et al. 2006; Bollmann et al. 2007). Diffusion chambers are apparatuses equipped with filter membranes, which restrict the movement of cells in the chamber. They allow for the removal of low-molecular weight inhibitory end-products, as well as the exchange of chemicals between the chamber and the environment, thereby making high-density cultivation possible (Pörtner and Märkl 1998; Kaeberlein et al. 2002). Different types of membrane-based systems have been developed to grow microbial communities directly in the natural habitats (Kaeberlein et al. 2002; Plugge and Stams 2002; Ferrari et al. 2005, 2008; Bollmann et al. 2007). Uncultured bacteria from soil, marine or activated sludge were grown in diffusion chambers. This led to the hypothesis that in situ cultivation of environmental prokaryotes in diffusion chambers either enriches sufficiently the strains for their subsequent isolation onto classical solid media, or conditions them for growth under otherwise prohibitive in vitro conditions (Bollmann et al. 2007). Interestingly, slow-growing organisms were cultivated using this method.

In natural ecosystems, many prokaryotes live attached to surfaces. This is well known for microbes living in sediments, soils, or in association with eukaryotes. It is less

recognised for microbes living in aquatic habitats, where free-living forms were supposed to be dominant, but other associations, with various interfaces, exist. The attached existence provides several advantages for the prokaryotes. Attached cells escape grazing better than their free-living neighbours. Attachment also allows cells to develop metabolic inter-relations, resistance to different stresses and better access to adsorbed substrates (Schink 1999). In situ colonisation carriers are useful tools to overcome cultivation limits induced by attachment of prokaryotes to solid surfaces. Several publications describe the deposition in natural ecosystems of different carriers such as glass, ceramic, titanium devices, porous inorganic substrates or polyurethane foams (Araki et al. 1999; Alain et al. 2004; Yasumoto-Hirose et al. 2006). In situ collectors coated with selective substrates are effective for the selective enrichment of targeted prokaryotes (Yasumoto-Hirose et al. 2006). In addition, specialised techniques simulating one or several important spatial or physical parameters allow the cultivation of novel phenotypes. For instance, gradient systems (Nelson and Jannasch 1983; Emerson and Moyer 1997), high-pressure reactors (Marteinsson et al. 1999; Alain et al. 2002), flow-through devices (Houghton et al. 2007) and gas-lift reactors (Postec et al. 2005, 2007) were successfully used. Pressure is also an essential parameter given that high-pressure environments occupy the largest fraction of the known biosphere. This parameter was often neglected in microbial cultivation. However, its effects on microbial life are as important as those of temperature or salinity. Pressure acts upon physiology and upon biochemical reactions. Thus, high-pressure of reactors are effective to grow microbial strains from high-pressure habitats. However, only few piezophilic prokaryotes have been enriched or isolated under elevated pressures (i.e. Yayanos et al. 1979, 1981; Marteinson et al. 1999; Alain et al. 2002; Houghton et al. 2007). Recently, the first obligate piezophilic and hyperthermophilic archaeon (*Pyrococcus* sp. strain CH1) was isolated from the deepest hydrothermal vent field explored so far, using a high-pressure reactor (Zeng et al. 2009). Finally, different types of bio-reactors are used to reproduce diverse environmental conditions. The physical and chemical conditions of seafloor hydrothermal systems are among the most difficult to reproduce in laboratories, considering the multiplicity of in situ physical and chemical gradients. Gas-lift reactors and flow-through devices are powerful tools to simulate hydrothermal vent in situ conditions (Postec et al. 2005, 2007; Houghton et al. 2007). Flow-through devices allow for continuous cultures under in situ temperature, pressure and fluid flow. They make possible the simulation of the deep-sea vent changes of fluid chemistry (Houghton et al. 2007). Both systems allow for

continuous enrichment culturing under controlled conditions. Significant fractions of microbial communities were grown using these systems (Postec et al. 2007).

Cultures of microbial communities

The cultivation methods allowing for the growth of mixed populations offer great potential to cultivate not-yet cultivated organisms. Indeed, in natural environments, most organisms live as a part of a community in which distinct cells work in concert and communicate either by trading metabolites, by exchanging dedicated signalling molecules, or by competition for limited resources (West et al. 2007; Nadell et al. 2009). These relationships, in addition to complex cell–cell communications (CCCs), are hardly reproducible in monocultures. This “in group” lifestyle, in biofilm or multi-cellular assemblage, is thought to generate robustness for nutritional, biotic and abiotic changes by providing a capacity of adaptation to environmental fluctuations. It is critical for microbial ecology and evolution. A striking example is biofilm, in which CCC determines biofilm structure, maturation and microbial niche construction thereby optimising microbial survival and reproduction. Mixed microbial assemblages have also the capacity to perform multiple-step functions that are often not possible for individual strains or species. Examples are the degradation of cellulose or the methanogenic conversion of complex organic matter (Brenner et al. 2008). In some cases, the cooperation within the microbial community is based on sharing metabolic intermediates, micronutrients (e.g. vitamins) or chelating agents that either assist or compromise the growth of other community members. When identified, such facultative dependencies can be reproduced experimentally by supplying the medium with these micronutrients or co-substrates. Besides facultative associations, syntrophic associations are often compulsory and this interdependence cannot easily be by-passed or suppressed by the addition of factors to the media (Schink 1999). Many syntrophic associations are explained by unfavourable energetic conditions. Both facultative and syntrophic associations are widespread in natural habitats.

Consequently, approaches based on community cultures are effective methods to grow facultative associations and syntrophic organisms. Using community culture approaches in addition to dialysis membrane reactors, thermophilic syntrophic anaerobic glutamate-degrading consortia from anaerobic sludge have been successfully enriched (Plugge and Stams 2002). Similarly, batch reactors operating under anaerobic–aerobic cycling conditions have allowed the enrichment of mixed microbial sludge communities (Crocetti et al. 2002).

High-throughput automatable microbial culture formats

The past decade was marked by the emergence of high-throughput cultivation methods (Connon and Giovannoni 2002; Zengler et al. 2002, 2005; Ingham et al. 2007). Several advances in high-throughput culture formats have originated from industrial endeavours. Indeed, the myriad of organisms inhabiting our planet represents a tremendous reservoir of bio-molecules for pharmaceutical, agricultural, industrial and chemical applications. While culture-independent recombinant approaches are used to screen novel molecules and enzymes from natural samples, cultivation of organisms greatly simplifies such studies and allows the use of a strategy biased for the desired phenotype.

A very efficient high-throughput cultivation method resulted from modifying the pioneering work, called extinction cultures, of Button et al. 1993. Briefly, extinction culturing requires dilutions of natural communities to a low number of cells (1–5 cells/ml) in natural environmental water followed by their incubation in defined conditions. Following incubation, growth is measured. The initial procedure was refined by Giovannoni and colleagues to increase high-throughput rates by using microtiter plates and fluorescence microscopy screening (Connon and Giovannoni 2002; Stingl et al. 2007). This improved technique resulted in better sensitivity and cultivation efficiency. This technique allows notably for the growth of slow-growers. It also allowed for the isolation of the first representatives of two bacterial clades: the SAR11 clade, a ubiquitous alpha-proteobacterial lineage found abundantly in marine pelagic environment (Rappé et al. 2002) and the OM43 clade (Connon and Giovannoni 2002).

Another technology for massive cultivation of prokaryotes was developed recently. This high-throughput approach consists in the encapsulation of cells in gel microdroplets (GMDs) incubated in a single column for long intervals of time under low nutrient flux conditions. Microcapsules are subsequently sorted by flow cytometry. This technique is applied to samples from different habitats and provides more than 10000 bacterial and fungal isolates per natural sample (Zengler et al. 2005). To our knowledge, this technology, which allows the development and detection of micro-colonies (20–100 cells), has never been used for anaerobes. It is advantageous as cell-containing micro-droplets are grown together in capsules allowing for the exchange of signal compounds and metabolites between cells from different micro-colonies, but originating from the same natural community. Using the micro-encapsulation approach, members of numerically abundant clades were isolated. Although very promising, this technique is not yet easily implemented in microbiology laboratories (Zengler et al. 2002, 2005).

Finally, a multiwell microbial culture-chip was recently developed by a team of microbiologists, nanotechnologists and micro-engineering experts (Ingham et al. 2007). This micro-Petri dish is composed of a unique porous ceramic subdivided into millions of compartments in which cultures can be separately grown. The growth of microorganisms is sustained by the nutrients diffused through a porous membrane. The innovation lies in the conception of the chip that is composed of micron-scale wells, is readily manufactured, affordable and easy-to-use in microbiology laboratories not equipped with micromanipulator. This system combines automation and miniaturisation, prerequisites for modern microbiology.

All approaches described in this chapter aimed to optimise prokaryote cultivation efficiency (Fig. 2). Overall, these studies allow for the isolation of numerous novel species and permit major breakthroughs in cultivation. Despite continuous efforts, culture-dependent approaches undergo changes characterised by gradual improvements rather than by a radical paradigm shift. It is noteworthy that the so-called “Moore Law” in microbiology (Gefen and Balaban 2008) applies to the volume used in culture and not to the output in number of novel species described.

Why do so many microbes resist cultivation?

Despite extensive studies on ecosystems where sampling is conducted, the conditions used in classical cultivating conditions are often far from endogenous abiotic and biotic conditions required for microbial growth. Thus, it is not surprising that only a small number of the microbial repertoire has been cultured so far.

The lack of efficient cultivation techniques stems from many factors that largely remain unknown. These factors include limited knowledge of (1) the diverse organisms, (2) the chemistry of the natural habitats, (3) the natural biotic and abiotic interactions and (4) the global functioning of the ecosystems at microbial level. Cultivation aims to create an artificial system mimicking in situ conditions. However, we do not have sufficient knowledge to reproduce the natural conditions in the laboratory or to create viable synthetic conditions for all organisms. This lack of knowledge has led to regrettable mistakes. For example, traces of tungsten element, which inactivates nitrogenase, have been included for a long time in the culture media for *Spirochaetes* (Leadbetter 2003). It was recently discovered that *Spirochaetes* are able to grow via nitrogen fixation (Lilburn et al. 2001), a property that can be expressed only in tungsten-depleted media. Another remarkable example is the unexpected discovery of nanoarchaea in enrichment cultures (Huber et al. 2002). This discovery was unattainable by current PCR-based approaches since the universal

primers commonly used for diversity studies could not target this group.

Another reason that can explain the failure to cultivate many prokaryotes is our lack of patience and sensitive detection methods for low cell yields. As previously discussed, in some cases, cultivation efficiency was significantly increased by long periods of incubation. This is true for organisms originating from oligotrophic habitats and potentially in a non-growing or dormancy state. It is important to note that transition from a non-growing to a growing state in a synthetic medium is a critical and stressful event. Thus, adaptations of cells to laboratory growth conditions may require increased cultivation times. For instance, the duration of growth log phase can depend on the cell status (i.e. healthy, stressed or sub-lethally damaged cells) and the gap between in situ and in vitro conditions (de novo synthesis of an enzymatic set to grow in the synthetic medium, etc.). Without permitting adequate time intervals for growth, many organisms and many redox reactions would never have been discovered. For example, without lengthy incubation periods, the observation that methane is formed from long-chain alkanes under anaerobic conditions would not have been documented. Under tested conditions, gas formation in the presence of hexadecane started only after 4-month incubation of the culture (Zengler et al. 1999). While bioenergetics calculations can predict thermodynamical feasibility of a reaction, our current knowledge cannot predict kinetics for ‘resuscitation’ and for the growth of de novo enrichment cultures via a given reaction under a defined experimental conditions. Indeed, the characteristics of natural uncultured organism(s) (physico-chemical optima for growth, transport systems, etc.) and of their enzymes (catalytic rates, substrate affinities, regulation mechanisms) are unknown. At best, we can suppose that enrichment cultures performed with highly stable substrates, weak oxidants and calculated to have low net free energy gain will require long-term incubations and patience. Otherwise, extremely long growth kinetics or low cell densities are sometimes observed in enrichment or pure culture. Inappropriate or non-optimal growth conditions might be responsible for these low yields. Thus, once parameters that inhibit growth are identified, “normal” growth kinetics and high yields can be restored (i.e. Flagan et al. 2003). For the organisms characterised by meagre yields, highly sensitive detection technologies such as tangential flow filtration and concentration are critical (Giovannoni and Stingl 2007).

Another rational put forward for prokaryotic resistance to cultivation is inherent to the in vitro cultivation techniques widely used. More specifically, the in vitro cultivation techniques used paradoxically aim to isolate strains in pure culture, while most organisms in nature live in community and establish complex relationships (see

previous chapter). The main consequence of this general practice is a disruption of inter- and intra-species communication during the very first stage of isolation. The impact of this perturbation on cultivability is unknown for most prokaryotic species and might vary depending of the species. CCC has gained considerable attention in recent years, in particular density-dependent cell-signalling mechanisms known as quorum sensing (QS). Cell–cell interactions have been investigated mainly in Bacteria due to their tremendous importance in health, environmental and industrial applications. Bacteria respond to a wide range of signalling molecules at intra-species level (species-specific compounds) and/or at inter-species level (Camilli and Bassler 2006). Inter-species interactions appear to be ubiquitous among prokaryotes and are not limited to signalling molecules such as autoinducer-2 (AI-2) and *N*-acyl-homoserine lactones (*N*-AHLs) but extend to antibiotics at sub-inhibitory concentrations (Ryan and Dow 2008). Globally, prokaryotes and eukaryotes have co-existed for millions of years and frequently have co-evolved in the same environments. Therefore, it is not surprising that interactions between them range from mutually beneficial to virulent. QS compounds like AHLs found initially in Bacteria are involved in virulence, biofilm formation, motility and antibiotic production and are recognised by eukaryotes with effects on immuno-modulation, intracellular calcium signalling and apoptosis (Hughes and Sperandio 2008). QS mechanisms have been extensively investigated during the past decade. Many Bacteria have one QS circuit while some display two or three circuits to coordinate their population density. Yet, these circuits are themselves under the control of a master QS regulator (Hooshanghi and Bentley 2008). Recently, Kolodkin-Gal et al. (2007, 2008) showed that the mode of action of antibiotics in *E. coli* is determined by the ability to communicate through the extra-cellular death factor (EDF) as a function of cell density. CCC was initially considered as an exception limited to a few specialised bacteria, but has recently emerged as the norm in the bacterial world. Several indications strengthen this trend: (1) the high frequency of QS among genome-sequenced bacteria (40% of the 800 sequenced bacterial genomes contain the *luxS* gene suggesting that the AI-2 precursor functions as a universal signal (Pereira et al. 2008)) and (2) the discovery of signalling molecules in microbial metagenomic data. In addition, there is growing evidence that QS signals provide to bacteria more information than cell density. Surprisingly, the abundance of results on QS, biofilms and cell–cell communication, at intra-species or inter-species levels have been analysed in the perspective of pathology (virulence, biofilm formation and control), biotechnology, synthetic biology (Hooshanghi and Bentley 2008), evolution (Keller and Surette 2006; West et al.

2007), ecology (Nadell et al. 2008), but seldom for cultivation purposes.

To account for CCC mechanisms in cultivation procedures remains a challenge due to our lack of knowledge on the cell–cell interaction requirements of targeted species. Considering the small fraction of prokaryotes cultivated, it seems likely that most CCC mechanisms are unknown. CCC mechanisms described up to now, like those classically observed with *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Escherichia coli*, operate at high cell densities. We cannot exclude that some CCC mechanisms could also act at low cell densities (10^2 – 10^4 cells/ml) to regulate cell growth of some species. In that case, it may be necessary to reconsider isolation procedures accordingly.

Finally, one last reason for which prokaryotes remain uncultured is enrichment-isolation process whereby the abiotic interactions are broken down. This disruption of the bio-geochemical factors, that collectively represent the environment, is a source of stress for the organisms from natural habitats. The depreciation of these bio-geochemical factors from the native extra-cellular habitats leads the experimentalists to design synthetic conditions, which might introduce a stressful parameter or a main change in resource type or concentration. For example, a phenomenon similar to substrate-accelerated death might occur. Growth inhibition is observed when cells are suddenly grown in the presence of concentrations greater than 1–10 μ M of a given substrate (i.e. ammonia, phosphate or glucose), whereas they were previously starved of this substrate (Overmann 2006).

Cultivating the uncultured: future directions

There is current growing consensus among microbiologists that improvements are needed in microbial cultivation. A comprehensive understanding of the microbial world will undoubtedly require cultivation. It is clear that the keys to cultivation will not come from a single technological breakthrough but will depend on our knowledge of the natural microbial systems. The numerous examples of successes in cultivation that we have mentioned in this review demonstrate that through patience and advances in technology, cultivation is a surmountable obstacle for many organisms. We still have a lot to learn from pure culture microbiology, even if these isolates might display unnatural behaviours in the synthetic laboratory. Pure culture microbiology still represents the best method to study microbial physiology including detailed investigations on the role of genes, proteins and metabolic pathways. This is true since several unexpected physiological discoveries were made after isolation of novel species belonging to taxa presumed to be already well documented.

In turn, these data provide important guidance for the optimization of cultivation media.

While several difficulties remain in cultivating microorganisms, future directions can be summarised as follows: refining culture medium, mimicking nature through in situ cultivation systems or designing devices supporting CCC and developing automated procedures through robotics. Undoubtedly, combinations of these diverse approaches will yield successes in cultivation.

Refinement of culture media

Culture medium optimization will require the use of various complementary tools including the following: (1) the ability to define the range of electron donors, acceptors and key elements sources based on a better knowledge of the environment, (2) molecular probes for screening novel species and (3) high-sensitive methods with low detection thresholds to uncover rare and slow-growing species in culture. In the future, results from single-cell genomics and metagenomics analyses will contribute to better isolation strategies for prokaryotes.

Design of isolation strategies based on cell–cell interactions

As seen previously, microbial landscapes are dominated by biofilms or aggregates. Being isolated is not the preferred state of most Bacteria and Archaea. Despite the growing knowledge on cell–cell-signalling molecules, the use of antibiotics and auto-inducers in culture is limited. Indeed, the vast majority of these compounds were characterised from microorganisms in pure culture, some of which were subsequently retrieved in metagenomic analyses. Metagenomics will yield numerous genes coding for novel auto-inducers or their precursors, but these will be classified as unknown genes until discovered in culture. Therefore, it is not surprising that our previous analytical knowledge is hardly applicable to isolation procedures of novel species. For isolation of a strain, one way to preserve the endogenous CCC mechanisms is to grow it in microbial community conditions, using bioreactors, either under planktonic stage, or incorporated in gel micro-droplets (GMDs) (Zengler et al. 2002), or attached to a surface. So far, the community culture step is more often followed by an isolation step through various means: streaking, dilution-to-extinction, sorting (mechanically or by FACS). For the last method, single-cells are distributed in wells or micro-wells filled with appropriate medium and incubated for further culture. The major drawback is that the appropriate medium used to grow the cells after the isolation step is different from the medium that supported growth in the community culture, and that all chemical CCC are

suddenly disrupted. What is the impact on the cultivability of some strains? In order to address this question it seems necessary to design novel culture equipments. The easiest method would be to substitute culture micro-plates by micro-bioreactors in the Zengler's method (Fig. 3). In such a system, the flow from the community culture would nourish each well of the micro-bioreactor with all the metabolites produced by the community culture. This system would combine community culture and isolation. Further, it would allow for the circulation of chemical compounds in the micro-bioreactor, partly preserving CCC, even though physical cell–cell contacts would be disrupted. Since the community culture would operate as a black box for signalling compounds, we cannot exclude that some metabolites could have inhibitory effects. The benefits of this combination of enrichment culture and isolation remain to be assessed. By dissociating and, in a following step, combining community culture and isolation, this system could address the culture of consortia components and symbionts, which are almost impossible to solve with current practices.

High-throughput issues

A quiet revolution occurred during the past decade in liquid handling applied to life sciences with the development of pipetting workstations. The main line of action was the reproduction at high-throughput of manual procedures by pipetting robots. Culture medium distribution in micro-plate wells and inoculation of a single-cell or GMD per well is now performed routinely by robots. The combination of GMD encapsulation and flow cytometry sorting is effective for aerobes (Akselband et al. 2006). Further, these studies show that some cells remain viable after staining and sorting; however, these findings await confirmation. This remains a challenge for obligate anaerobes, especially due to the sorting step with flow cytometers. FACS equipment could be adapted to operate in anaerobic chambers, but might prove inconvenient and costly. The development of direct sorting of GMDs on liquid handling workstations would be an excellent alternative to avoid FACS analysis, to limit the cost of equipments and to allow dissemination of these approaches in microbiology laboratories. What seems also to be certain is that the widespread use of high-throughput cultivation procedures implies a simultaneous development of affordable high-throughput identification procedures.

Combination principle

Microbiologists have long recognised that our understanding of the microbial world critically depends on the technological advances that broaden the knowledge-base for

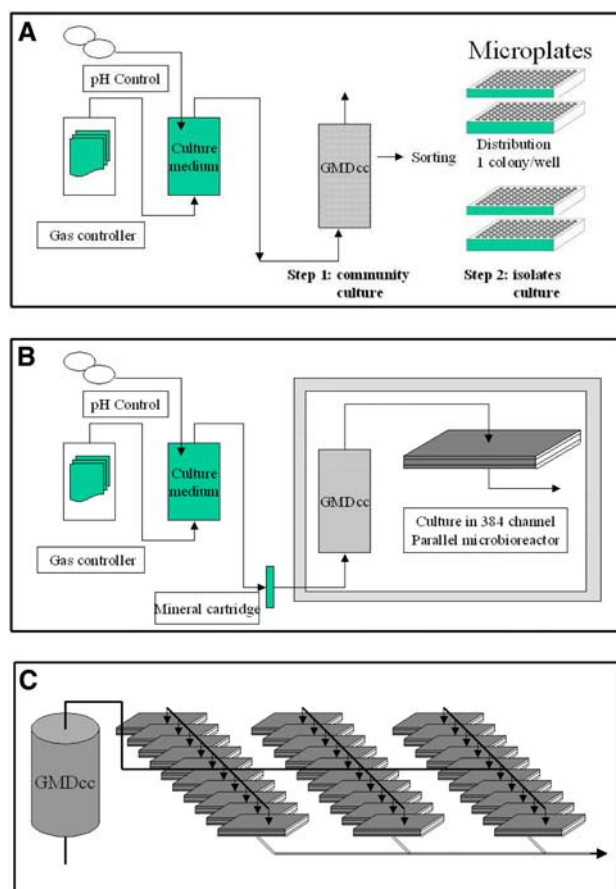


Fig. 3 Schematic diagram of cultures and isolation procedures based on the combination principle. *GMDcc* community culture of microbial cells incorporated in gel micro-droplets. **a** *GMD* community culture followed by sorting and microplate cultivation (From Zengler et al. 2002, modified). **b** Flow-through culture in parallel micro-bioreactors nourished by community culture medium and metabolite products. Micro-plates are replaced by micro-bioreactors directly connected to the *GMD* community culture. This system can be operated in aerobic or anaerobic conditions. Micro-bioreactors (either 96 or 384 parallel channels SBS format) and flow-through equipments await development. **c** *GMD* and micro-bioreactors (384 channels) coupled in a nearly theoretical 10000 parallel channel configuration

chemical, biological and physical processes. Today, it is established that an integration of the knowledge from multiple hierarchical levels of organisation and from the molecule to the ecosystem are also required. Despite these facts, most efforts in cultivation focused only in the optimization of a limited set of parameters. We are convinced that this situation will change in the near future for several reasons. First, a consensus is gradually emerging: culture is not only needed to describe randomly novel species as a function of researcher main field of interest. Culture appears more and more as the sine qua non condition for understanding how the microbial world functions and is a prerequisite to predict changes in the frame of global change. Second, it seems that the complexity of the microbial world emerging from the

growing knowledge of genomic and proteomic advances cannot be analysed only by combining these approaches and relying on systems biology. Finally, the advent of automation in culture and the combination of innovative methods lead to a new era in cultivation, assuming that some specific equipment are developed to handle the cell–cell interaction mechanisms during the clonal culture phase. Combinations theoretically possible are detailed in Fig. 3.

Ecosystems encompass abiotic conditions, living species and all their interactions (resource limitations, competition, predation, parasitism, among others). It is obvious that any cultivation attempt is by nature highly reductive and cannot reproduce the conditions observed in nature. The combination of existing methods and the development of novel approaches will help to come close to conditions where a significant fraction of microbes is amenable to culture (Fig. 2).

Concluding remarks

The extent of microbial diversity is still unknown and prokaryotes have undoubtedly many secrets to reveal. The success of microbial life on Earth stems from its great physiological diversity that collectively allows prokaryotes to derive energy from a wide range of redox reactions and to colonise several types of habitats, including the extreme ones. It is remarkable how much we have learned about microbial life by studying only a small fraction of its diversity. New examples of microbial ingenuity were recently discovered due to cultivation, either independently or in combination with metagenomics. There are still important essentials hidden in the 99.9% of the microbial world that are not yet cultured. They might change our understanding of biochemical processes, redox reactions, physiological adaptations and microbial behaviours. We postulate that in the future major advances in the understanding of microbial life will be achieved by innovative approaches in cultivation, but not cultivation alone. Coordinated efforts of researchers studying microbial systems at different levels should guide us to rethink culture strategies and to design growth conditions as close as possible to the natural interactions and conditions. In conclusion, the different cultivation successes discussed in this review demonstrate that contrary to widely held beliefs, many prokaryotes can be cultivated. In view of the gap between the number of phylotypes with or without representative cultured species, an additional question arises: is there a need to define relative priorities between phylotypes? The division amongst microbiologists at the international realm suggests that a consensus is not possible. The only consensus that could emerge, and successfully funded, is the need to investigate novel cultivation approaches, to develop

new equipments and bring microbial cultivation in the twenty-first-century as a technologically advanced and a data rich discipline.

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