MINIREVIEW

Strategies for culture of 'unculturable' bacteria

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Abstract

Molecular ecology methods are now well established for the culture-independent characterization of complex bacterial communities associated with various environmental and animal habitats and are revealing the extent of their diversity. By comparison, it has become clear that only a small minority of microorganisms are readily cultivated *in vitro*, with the majority of all bacteria remaining 'unculturable' using standard methods. Yet, it is only through the isolation of bacterial species in pure culture that they may be fully characterized, both for their physiological and pathological properties. Hence, the endeavour to devise novel cultivation methods for microorganisms that appear to be inherently resistant to artificial culture is a most important one. This minireview discusses the possible reasons for 'unculturability' and evaluates advances in the cultivation of previously unculturable bacteria from complex bacterial communities. Methods include the use of dilute nutrient media particularly suited for the growth of bacteria adapted to oligotrophic conditions, and the provision of simulated natural environmental conditions for bacterial culture. This has led to the recovery of 'unculturables' from soil and aquatic environments, likely to be due to the inclusion of essential nutrients and/or signalling molecules from the native environment.

Introduction

For the purpose of this minireview, the terms 'unculturable' and 'as yet uncultivated' are used to describe organisms that have yet to be grown on artificial media *in vitro*.

It is well established that only approximately 1% of bacteria on Earth can be readily cultivated *in vitro* – the so-called 'great plate count anomaly', based on the observation that microscopic counts are considerably larger than the equivalent total viable counts (Staley & Konopka, 1985; Amann *et al.*, 1995; Hugenholtz *et al.*, 1998). There are currently estimated to be 61 distinct bacterial phyla, of which 31 have no cultivable representatives (Hugenholtz *et al.*, 2009). The topology of the archaeal phylogenetic tree remains uncertain, but it is clear that the 54 species of *Archaea* cultured to date represent only a fraction of the total diversity, with 49 lineages mostly uncultured (Auguet *et al.*, 2010).

Because the majority of bacteria and archaea remain unculturable, the diversity of complex bacterial communities is inevitably underestimated using standard cultivation methods. Furthermore, organisms of key importance to the community and the entire ecosystem in the environment or pathogens of plants and animals may be overlooked if they are unculturable. Consequently, with the development of molecular culture-independent techniques, there has been a move towards the characterization of mixed bacterial populations within biomass from the environment and in samples from animals (including humans) using PCR amplification of housekeeping genes particularly that encoding 16S rRNA gene, cloning for purification and sequencing for identification (Giovannoni *et al.*, 1990; Pace, 1997). As a result, numerous novel phylotypes have been identified among bacterial communities from a wide range of habitats: from seawater and soil to the health- and disease-associated microbiota of humans (Munson *et al.*, 2002; Rappe & Giovannoni, 2003; Zhou *et al.*, 2004; Aas *et al.*, 2005).

Despite the availability of varied molecular methods for the evaluation of bacterial communities, cultural analyses are far from redundant. It is only through the isolation of individual bacterial species in pure culture that a comprehensive characterization of physiological properties and a full assessment of virulence potential may be undertaken. Hence, there has been a significant focus in recent years on

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developing methods for the *in vitro* culture of those species hitherto refractory to cultivation.

Reasons for 'unculturability'

The finding that certain bacterial species have never been identified by culture may be a simple matter of coincidence: an organism that has a low prevalence or is particularly slow-growing may have been overlooked in cultural analyses. Additionally, many genetically distinct phylotypes are phenotypically indistinguishable and are lumped together if conventional biochemical methods for identification are used.

Conversely, some bacteria are genuinely resistant to culture in isolation on conventional media. Certain bacteria have fastidious growth requirements including the need for specific nutrients, pH conditions, incubation temperatures or levels of oxygen in the atmosphere. Kopke *et al.* (2005) investigated the effect of different substrates and culture conditions on the growth of bacteria from comparable samples of coastal sediments, and found that the various cultivation approaches resulted in the isolation of different groups of bacteria specific to each method, confirming the impact of cultivation conditions on the yield of culture. Thus, if the specific requirements for the growth of a bacterium are not met by the artificial medium and incubation conditions, or if there is competition for nutrients among mixtures of organisms cultured together, some bacteria may not grow. Growth may also be inhibited by bacteriocins released from other bacteria in a mixed culture or by antibacterial substances present within the medium (Tamaki *et al.*, 2005). In order to make the best estimate of the true diversity of the community present, multiple methods of cultivation should be used.

The formation of biofilms appears to be an inevitable result of bacterial colonization of surfaces and has been identified in the earliest fossil records (Hall-Stoodley *et al.*, 2004). Bacterial biofilms have many of the features of multicellular organisms and individual species within biofilms cooperate to resist external stresses (Stoodley *et al.*, 2002). Such interactions enable the biofilm to function as a complex unit (Stoodley *et al.*, 2002; Marsh, 2005; ten Cate, 2006). There may be cross-feeding or metabolic cooperation between species for the provision of nutrients (Belenguer *et al.*, 2006), such as the production of lactic acid (through fermentation of carbohydrates) by *Streptococcus mutans*, which is utilized as a source of carbon by *Veillonella* spp. (Mikx & Van der Hoeven, 1975). Another key feature of biofilm communities is bacterial communication through networks of signals (Davey, 2008). These include quorum-sensing mechanisms that are involved in the regulation of the bacterial community structure, properties and survival (De Kievit *et al.*, 2001; Konaklieva & Plotkin, 2006; ten Cate, 2006).

Some bacterial growth factors have been considered analagous to mammalian cytokines – circulating regulatory molecules that mediate cellular communication. The term 'bacterial cytokine' was coined by Mukamolova *et al.* (1998) for the resuscitation-promoting factor (Rpf), a protein that revived dormant *Micrococcus luteus* cells and increased the growth rate of vegetative cells. Rpf also stimulated the growth of other members of the *Actinobacteria* including *Mycobacterium tuberculosis*, and a family of related growth factors was identified (Kell & Young, 2000). A family of proteins with a similar function in the *Firmicutes* was subsequently discovered (Ravagnani *et al.*, 2005). Rpf was later demonstrated to have a lysozyme-like structure and muralytic activity (Cohen-Gonsaud *et al.*, 2005). How Rpf stimulates the growth of dormant cells remains to be determined, but it is possible that remodelling of the peptidoglycan in the cell walls of dormant cells is required before growth can resume. Interestingly, it has been demonstrated recently that peptidoglycan fragments bind to PrkC, a serine/threonine protein kinase, in *Bacillus subtilis* to stimulate spore germination (Shah *et al.*, 2008). Muropeptides generated by Rpf degradation of peptidoglycan may interact with PknB, a homologue of PrkC in *M. tuberculosis*, and thereby initiate resuscitation and stimulate growth (Kana & Mizrahi, 2009).

Signalling molecules present only within the natural habitat are thought to be essential for the growth of many bacteria (Lewis, 2007; Nichols *et al.*, 2008).

In the absence of these beneficial interactions and signals, some bacteria may struggle to grow in monoculture. Furthermore, faced with an unfamiliar environment devoid of essential factors, bacteria may, as a survival strategy, enter into a temporary state of low metabolic activity accompanied by the inability to proliferate or to form colonies on culture media (Barcina *et al.*, 1990; Colwell, 2000; Lewis, 2007; Nichols *et al.*, 2008), which may be mistaken for a constitutional resistance to culture.

Techniques used to culture the 'unculturables'

Significant efforts have been made in recent years to devise culturing methods for as-yet-uncultivated species. Developments in the last decade, particularly in the field of environmental microbiology, have led to the recovery of unculturables from diversely populated habitats including soil and aquatic (marine and freshwater) environments.

The majority of culture media used to date have been nutrient-rich. It is now thought that these conditions may favour the growth of faster-growing bacteria at the expense of slow-growing species, some of which thrive in nutrient-poor environments (Koch, 1997; Connon & Giovannoni, 2002), and may be inhibited by substrate-rich conventional

media. Consequently, the use of dilute nutrient media has led to the successful cultivation of previously unculturable bacteria from various aquatic and terrestrial habitats (Watve *et al.*, 2000; Connon & Giovannoni, 2002; Rappe *et al.*, 2002; Zengler *et al.*, 2002).

Various methods have been used to physically reduce the number and diversity of bacteria within mixed samples before cultivation. These include filtration methods (Hahn *et al.*, 2004), density-gradient centrifugation or elutriation and extinction-dilution whereby samples are diluted, ideally down to single cells, before their culture in isolation (Watve *et al.*, 2000; Connon & Giovannoni, 2002; Ben-Dov *et al.*, 2009; Song *et al.*, 2009; Wang *et al.*, 2009).

Many bacteria, particularly those that are oligotrophic in the environment, are very slow-growing. Extended incubation times are a prerequisite for the cultivation of such bacteria, with the added benefit that faster-growing members within the mixed populations progressively die off over time, reducing the bacterial competition. The culture of soil bacteria for up to 12 weeks has revealed increasing colony counts and an increased recovery of rarely isolated strains with time (Davis *et al.*, 2005). Similarly, long-term incubation for up to 24 weeks has been successful for the isolation of strains from the SAR11 clade (Song *et al.*, 2009). Even members of the TM7 Division, which have yet to be cultivated in isolation, were able to form colonies visible to the naked eye when incubation times of 50 days were used [unpublished observation reported in a review by Hugenholtz (2002)].

Many bacteria have specific nutrient or chemical requirements for growth (Graber & Breznak, 2005; Tripp *et al.*, 2008). For example, members of the genera *Abiotrophia* and *Granulicatella*, previously known as the nutritionally variant streptococci, require pyridoxal or L-cysteine for growth (Ruoff, 1991), while *Tannerella forsythia* requires an exogenous source of *N*-acetyl muramic acid (Wyss, 1989). The characterization of phylogenetically related species may provide clues to the metabolic requirements of organisms that are so far resistant to culture. Cultivation media may be modified or enriched with this in mind, resulting in the isolation of previously 'unculturable' organisms (Sait *et al.*, 2002; Davis *et al.*, 2005). However, simply adding the required substrate to cultivation media may not, in all cases, enable culture of the target organism. For example, slow-growing acetotrophs of the genus *Methanosaeta* are often outcompeted by faster-growing *Methanosarcina* spp. in mixed culture. On the other hand, Janssen (2003) found that the incorporation of acetone and isopropanol as enrichments led to the production (by species that ferment these substrates) of a slow and steady source of acetate that allowed *Methanosaeta* spp. to flourish.

Because of a reliance on beneficial bacterial interactions within the source environment, attempts to cultivate certain bacteria under laboratory conditions have sometimes been met with success only when these bacteria are cocultivated with helper strains (Ohno *et al.*, 1999, 2000; Nichols *et al.*, 2008).

Factors released from helper bacteria into the environment are often growth-stimulatory for otherwise unculturable bacteria even in the absence of the actual helper strain. Thus, the conditioning of media with spent culture supernatants or cell-free extracts derived from helper strains has been used for the growth stimulation of species such as *Catellibacterium* spp., *Psychrobacter* spp., *Sphingomonas* spp. and *Symbiobacterium* spp. (Tanaka *et al.*, 2004; Bae *et al.*, 2005; Kim *et al.*, 2008a, b; Nichols *et al.*, 2008).

Signalling molecules may be responsible for such growth promotion. Empirical testing of known signal molecules, cyclic AMP (cAMP) and acyl homoserine lactones was shown to significantly increase the cultivation efficiency of marine bacteria (Bruns *et al.*, 2002) – the addition to liquid media of 10 μM cAMP led to cultivation efficiencies of up to 100%. This remarkable result has not, however, been corroborated by other studies investigating the effect of cAMP on the growth of individual species. Coppola *et al.* (1976) observed a growth inhibition of *Escherichia coli* in media supplemented with 5 mM cAMP, and in a study by Chen & Brown (1985), the addition of cAMP at levels ranging from 0.01 to 100 μM showed no consistent influence on the growth rates of *Legionella pneumophila*. A cAMP concentration-dependent effect on growth may explain the differences in the results of the various studies. It is also possible that use of the most-probable-number method in the study by Bruns *et al.* (2002) led to an overestimation of cell numbers. Another study (Nichols *et al.*, 2008), in this case investigating the growth stimulation of a *Psychrobacter* strain, successfully characterized the growth-promoting factor responsible and identified this as a 5-amino-acid peptide.

An alternative approach for the culture of as-yet-uncultivated organisms is to simulate their natural environment *in vitro*. Kaeberlein *et al.* (2002) constructed a diffusion chamber that allowed the passage of substances from the natural environment (intertidal marine sediment) across a membrane and successfully grew bacteria from marine sediment that were previously uncultivated. These bacteria were subsequently cultured on solid media, but grew only in the presence of other bacteria, implying codependency. Similar diffusion chambers have been constructed since, to culture 'uncultivable' or rarely cultivated bacteria from marine (Nichols *et al.*, 2008) and freshwater environments (Bollmann *et al.*, 2007). The latter study reported a significantly greater diversity of recovered isolates using the diffusion chamber than on conventional agar plates.

Also mimicking the natural environment, sterile fresh- (Stingl *et al.*, 2008; Wang *et al.*, 2009) and marine- (Rappe *et al.*, 2002; Song *et al.*, 2009) waters have been used to

culture previously uncultivated bacteria. Ben-Dov *et al.* (2009) encapsulated individual bacteria (following dilution of samples) in agar spheres encased in a polysulphonic polymeric membrane before incubation in a simulated or a natural environment, and was successful in growing several novel organisms.

Another innovative technique mimicking natural conditions, this time used for the microcolony cultivation of uncultivated soil bacteria, is the soil substrate membrane system (Ferrari *et al.*, 2005, 2008), which includes a polycarbonate membrane support and soil extract as a substrate. Although this system allowed the microcultivation of novel bacterial strains, the bacteria remained part of a mixed community on the membrane. A recent development of the method has enabled the detection of live microcolonies on the membrane using viability staining, and the subsequent micromanipulation of such colonies for their isolation (Ferrari & Gillings, 2009).

The study of bacteria with an obligate intracellular lifestyle presents a particular challenge and it can be difficult to determine and reproduce the environmental conditions required for metabolic activity. For example, initial work investigating the metabolism of *Coxiella burnetii* used neutral pH buffers and concluded that there was negligible activity (Ormsbee & Peacock, 1964). When acidic buffers were used, metabolism was markedly enhanced (Hackstadt & Williams, 1981). Further refinements of this approach including the use of a citrate buffer, provision of complex nutrients and high (140 mM) chloride have enabled metabolic activity to be maintained for over 24 h (Omsland *et al.*, 2008), enabling the investigation of the physiology of this important species.

Many of the methods described above use an open-ended approach with the aim of cultivating all bacteria present in a sample. As a result, they have led to the cultivation of numerous fastidious bacteria. However, the phylogenetic targeting of specific bacterial strains of interest requires alternative approaches.

Isolation of targeted 'unculturables'

Advances in molecular biology have enabled the detection and sorting of specific target bacteria with a view to their selective enrichment or physical isolation. Oligonucleotide probes can be designed to target phylotypes with no known cultivable representatives. Using methods such as FISH or catalysed reporter deposition (CARD)-FISH for added sensitivity, target-specific probes can detect cells of previously 'unculturable' taxa among mixed populations (Amann *et al.*, 1995, 2001; Ferrari *et al.*, 2006; Vartoukian *et al.*, 2009), enabling the visualization of their cellular morphology. A limitation of these methods is that the cells detected within a sample are no longer viable after cell permeabilization and fixation procedures, and may not therefore be subsequently cultured in isolation.

The colony hybridization method, on the other hand, is undertaken on membrane transfers from plate cultures that remain viable (Salama *et al.*, 1993). Consequently, hybridization detections on membranes may be used to locate matched microcolonies within mixed cultures, from where they may be isolated. This method has been used in recent work (Vartoukian *et al.*, 2010), leading to the successful isolation from dental plaque of a previously uncultivated member of the *Synergistetes* phylum. Enrichment of the subcultured microcolonies with candidate feeder organisms from the original mixed cultures was found to facilitate the growth of the microcolony-forming bacteria.

Flow cytometry and cell sorting (FACS) is a method with numerous applications in microbiology (Alvarez-Barrientos *et al.*, 2000). In an effort to cultivate as-yet-uncultivated taxa, Zengler *et al.* (2002) used gel microdroplets to encapsulate single bacterial cells (from dilutions of mixed environmental samples), which then formed microcolonies *in situ*. Based on characteristic light-scattering properties, any microdroplets that contained microcolonies (as opposed to single or no cells) were detected and sorted by FACS, and subsequently analysed phylogenetically. When the intention is to detect and sort specific bacterial species, however, target-specific fluorochrome-labelled antibody or oligonucleotide probes are usually required. Whereas antibody-conjugated probes may preserve cellular viability, oligonucleotide probes do not, preventing the subsequent cultivation of sorted cells. Although FACS of 'unculturable' bacterial cells may not therefore directly lead to their cultivation, FACS in conjunction with whole-genome amplification has been used to obtain a partial genome sequence for a member of the TM7 phylum (Podar *et al.*, 2007). Knowledge of the genomes of as-yet-uncultivated organisms will help characterize these species and provide clues that will aid their *in vitro* cultivation in the future. For example, genomic analysis of '*Candidatus Pelagibacter ubique*' has revealed a deficiency of the genes that are necessary for assimilatory sulphate reduction in the production of sulphur, which is essential for biosynthesis in aerobic marine bacteria (Tripp *et al.*, 2008).

The micromanipulation of single bacterial cells for their isolation in pure culture has potential applications for the isolation of 'unculturable' bacteria (Frohlich & Konig, 2000). Optical tweezers, in the form of an infrared laser, are used to trap and isolate single cells within a cell separation unit from where they are ultimately transferred to growth media for cultivation. This method was used successfully by Huber *et al.* (1995) to isolate a previously uncultivated archaeal strain following visual recognition of its cellular morphology from targeted whole-cell hybridization. Raman tweezers, as used by Huang *et al.* (2009), involve a similar

technique of optical trapping differing only in the method of cell recognition, which is based on the characteristic profile of spectral peak shifts within the Raman spectrum of individual cells.

Concluding remarks

It is clear that there are many approaches to the cultivation of as-yet-uncultivated bacteria. Furthermore, the use of combinations of techniques has proven successful on several occasions. For example, Nichols *et al*. (2008) cultured a novel *Psychrobacter* strain by the application of appropriate environmental conditions for growth, together with the use of helper strains and conditioned media. The signalling molecules involved in growth stimulation were identified and domesticated variants emerged that were capable of independent growth after repeated cultivation in coculture with helper strains. It is likely that such combinatorial approaches will be required in the future to further improve the range of bacterial life on Earth that can be cultured in the laboratory.

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