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Minireview

Cultivation of Uncultured Fastidious Microbes

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A huge number of microorganisms are still unknown and uncultivated. SSU rRNA gene-based analyses of complex microbial communities are unveiling their diversity, distribution and abundance in natural environments. How to isolate uncultivated microorganisms is an everlasting preoccupation for microbiologists even in the era of culture-independent functional analyses such as stable isotope labeling, modernized in situ hybridization techniques and community genomics. Conventional isolation procedures are very laborious, and there is significant room for improvement. In this review, we stress several important clues to the systematic isolation and cultivation of uncultured microorganisms.

Key words: uncultured microorganisms, syntrophy, growth factors, slow growers, signal compounds, gellan gum

Introduction

Over the past decade, molecular approaches primarily based on SSU rRNA gene analyses have been commonly used for analyzing the overall structure of microbial communities. These approaches have uncovered a vast variety of unknown microorganisms present in all kinds of environments[13,29]. The number of bacterial phyla (divisions) has been rising since the 1990's and at present, almost 80 phyla are recognized though only 26 phyla contain cultured representatives[6]. Cumulative sequence information has enabled us to know what phylotypes of microorganisms tend to be present and what phylotypes dominate the communities in particular environments. Such phylogenetic information combined with metagenomics (direct amplification, cloning and sequencing of community genomes) and biochemical analyses (such as microautoradiography, tyramide signal amplification, or RING-FISH (*r*ecognition of *in*dividual *g*enes *f*luorescence *i*n *s*itu *h*ybridization), and stable isotope probing) could provide further insight into the functions and roles of predominating microorganisms in the environment[1,26,32,42,49]. However, such approaches clearly have limitations in terms of understanding what the organisms are really doing and how they make a living in situ. In this context, the isolation of tangible microorganisms is still the most convincing way to know exactly what they are doing and potentially can do. This strategy is obviously the opposite approach to community genomics accomplished by taking advantage of cloning techniques, well-developed DNA sequencers and elaborate gene sequence analyses. However, both strategies can coexist and together may shed light on environmental microorganisms hidden from conventional microbiology.

The isolation of microorganisms is undoubtedly time-consuming and laborious. In addition, the underlying techniques, which have been used for over a century, seem to have limitations for "fastidious" or "elusive" microorganisms. Nevertheless, there is still room for improvement to isolate yet-to-be cultured microbes. This review describes several examples of how we and other investigators have obtained microorganisms in pure cultures that had seemed

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to be very difficult to deal with.

Anaerobic syntrophic microorganisms

From the data collected to date, it is strongly suggested that most microorganisms on the earth thrive in anaerobic environments[46]. Apparently, oxygen is freely available at the surface in environments where O_2-respiring microorganisms dominate. However, a huge number of anaerobic microorganisms are hidden below the surface. Not only terrestrial and oceanic subsurfaces, but places such as animal intestines, rice paddies, wetlands and methane-fermenting processes are well-known habitats for anaerobes.

Under strictly anaerobic conditions, one microorganism shares energy with another for substrate oxidation. Under oxic conditions, simple substrates such as fatty acids can be completely oxidized by one microorganism. In anaerobic environments, however, substrates cannot be completely oxidized by a single species. For instance, an anaerobic fatty acid-oxidizing microorganism that is referred to as a syntroph can, to some extent, oxidize fatty acids to produce H_2 and smaller intermediates, but the H_2 generated in this process inhibits further oxidation for thermodynamic reasons. The organism, therefore, requires another organism that can consume (scavenge) H_2 to keep the H_2 partial pressure extremely low to make the entire oxidation reaction energetically feasible. In general, H_2-consuming methanogens and sulfate-reducing bacteria are in charge of this process.

To date, immense efforts to isolate syntrophic microorganisms have been made by many investigators. The key to successfully isolating such microorganisms is either isolating them in cocultures with H_2-consuming organisms or isolating them using different substrates that allow them to grow in pure cultures. The former approach requires H_2-consuming organisms that can support the growth of syntrophs. Typically, H_2-consuming organisms are pregrown and mixed with inoculum so that the syntrophs grow and form colonies on a "lawn" of H_2 consumers in solidified medium. The latter approach is on a trial and errors basis to find appropriate substrates for growth in pure culture. However, one important clue is that most syntrophs are capable of fermenting certain substrates using intermolecular disproportionation, or they may have a respiratory system that uses fumarate, sulfate or Fe(III) as a terminal electron acceptor. We have accumulated much knowledge and have succeeded in isolating several very fastidious syntrophic microorganisms in pure culture[11,14,15,27,28,33–35] (Fig. 1).

Thermacetogenium phaeum is an obligately anaerobic organism that oxidizes acetate slowly with the concomitant formation of H_2. It grows on acetate only in coculture with an H_2-scavenging methanogen[10,11]. The organism was isolated from an enrichment culture which oxidized 80 mM acetate to produce methane in one month. Initially, we attempted to isolate a "pure coculture" of the acetate-oxidizing organism and methanogens by diluting the enrichment culture in the presence of pregrown H_2-scavenging methanogen cells. The isolation of two-membered cocultures was attempted using a methanogen-containing agar medium (lawn culture) as described above. However, no colony was obtained in tubes containing high dilutions. We then attempted to isolate the target organism in pure culture using different substrates. Of the substrates tested, pyruvate was found to allow an organism that predominated in the enrichment culture to grow. The organism was eventually purified after repeated passage to pyruvate agar medium. To confirm whether it was the target organism, we reconstructed a coculture with H_2-scavenging methanogens to verify if acetate is oxidized together with growth. However, there was a pitfall in this step. It took years to determine that the reconstruction (restart of growth in coculture) needs several months. We first thought that the organism isolated was *NOT* the target, since neither growth nor acetate oxidation occurred even after a month. Moreover, we found that the species of "partner methanogen" is crucial, as it turned out later that one species of the genus *Methanothermobacter* (formerly *Methanobacterium*) used in the earlier experiments was not suitable for the reconstruction but the species we used later was. It eventually took almost twelve years to isolate the organism, establish a coculture and characterize the organism in detail. Nevertheless, this strategy is commonly used for the isolation of anaerobic syntrophs in our laboratories, since it is facilitated by the full-cycle 16S rRNA approach. Currently, we would clone 16S rRNA genes from a community or enrichment of interest, sequence them and predict the sequence of the target organism. Once the sequence is obtained, we design a probe for in situ hybridization and apply it to various enrichment cultures under different selective pressures to find the best substrate(s) that allows the target organism to grow.

Organisms that require growth factors produced by other organisms

Syntrophic association in anaerobic environments is, as mentioned above, underlain, to a large extent, by the interspecies transfer of H_2 between H_2-producing microbes and H_2-scavenging microbes. This is a common way of life for anaerobes. By contrast, how aerobic microorganisms inter-

***Thermacetogenium phaeum* gen. nov. sp. nov. (*Firmicutes*)**

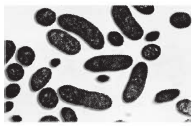
(Hattori *et al.*, 2000)

A thermophilic acetate-oxidizing H_2-producing syntroph that requires methanogens for growth. Grows very slowly (requiring approx. 30 days for complete mineralization of 40 mM acetate). In axenic culture, it utilizes H_2/CO_2, pyruvate, methanol, some methoxybenzoates, and some alcohols. Once it grows in pure culture, it is hard to return to cocultivation with methanogens.

***Pelotomaculum thermopropionicum* gen. nov. sp. nov. (*Firmicutes*)**

(Imachi *et al.*, 2002)

A thermophilic propionate-oxidizing H_2-producing syntroph that requires methanogens for growth. Grows very slowly (requiring nearly 80 days for complete mineralization of 20 mM propionate). Growth is sometimes unstable. Growth with methanogens is faster on ethanol than on propionate. In pure culture, it reduces fumarate with several organic substances as electron donors.



***Syntrophothermus lipocalidus* gen. nov. sp. nov. (*Firmicutes*)**

(Sekiguchi *et al.*, 2000)

A thermophilic butyrate-oxidizing H_2-producing syntroph that requires methanogens for growth. Grows slowly (requiring nearly 14 days for complete mineralization of 20 mM butyrate). Crotonate is the only substrate that allows the organism to grow in pure culture.

***Sporotomaculum syntrophicum* sp. nov. (*Firmicutes*)**

(Qiu *et al.*, 2003)

A mesophilic benzoate-oxidizing H_2-producing syntroph that requires methanogens for growth. Grows very slowly (requiring nearly 24 days for complete mineralization of 5 mM benzoate). Crotonate is the only substrate that allows the organism to grow in pure culture. Benzoate also allows growth in pure culture but only in the presence of crotonate.

***"Pelotomaculum terephthalicum"* (*Firmicutes*)**

(Qiu *et al.*, unpublished)

A mesophilic terephthalate-oxidizing H_2-producing syntroph that requires methanogens for growth. Grows extremely slowly (the slowest isolate as far as the authors know) requiring up to 3 months to completely degrade 1 mM terephthalate. Growth is sometimes unstable. Crotonate is the only substrate that allows the organism to grow in pure culture. A very tiny colony (0.1-0.15 mm in diameter) is formed in crotonate agar medium after 1 month of incubation.

***"Pelotomaculum isophthalicum"* (*Firmicutes*)**

(Qiu *et al.*, unpublished)

A mesophilic terephthalate-oxidizing H_2-producing syntroph that requires methanogens for growth. Grows very slowly requiring up to 1 month to completely degrade 1 mM isophthalate. A very tiny colony (0.1-0.2 mm in diameter) is formed in the presence of methanogens in agar medium after 3 months of incubation. No substrate has been found to support axenic growth (unpublished).

***Anaerolinea thermophila* gen. nov. sp. nov. (*Chloroflexi*)**

(Sekiguchi *et al.*, 2003)

A thermophilic carbohydrate-fermenting organism. Grows very slowly (requiring more than two weeks for fermentation of several mM of glucose). Coculturing with H_2-scavanging methanogens leads to faster growth. Widely distributed in anaerobic methanogenic processes. The first cultured organism in *Chloroflexi* subphylum I.

Fig. 1. Anaerobic syntrophic organisms that were recently isolated in our studies and their traits as fastidious organisms. All of the traits described above are taken from references 11, 14, 15, 27, 28, 33, 34, and 35.

act remains unclear, although most microbial ecologists suppose that cell-cell communication (material transfer in other words) routinely occurs between different species of microorganisms.

One of the prominent studies is the isolation of *Symbiobacterium thermophilum. S. thermophilum*, which is phylogenetically placed in the high G+C Gram positive group (the phylum *Actinobacteria*), was first isolated in a coculture with a *Bacillus* strain[37] and later an axenic culture of the organism was established using dialyzable compounds produced by the *Bacillus* strain[24,25,44]. Moreover, it was found that the ability to support the growth of *S. thermophilum* is not limited to the *Bacillus* strain but is distributed among other species of the family *Bacillaceae* and even among a wider variety of bacterial species. Molecular ecology has revealed that the organism and its relatives are widely distributed in natural environments[43], suggesting that this form of commensalism may be ubiquitous in microbial communities. The genome sequencing of *S. thermophilum* is now complete[45] and much more molecular information will be accumulated.

We also attempted to isolate microorganisms whose growth is stimulated by other microorganisms. A bacterium isolated from activated sludge and later designated *Catellibacterium nectariphilum* did not show significant growth on nutrient broth. However, the growth was significantly stimulated by the addition of supernatants from other bacterial cultures[40,41]. The culture filtrate of a strain related to the genus *Sphingomonas*, in particular, increased the cell yield and growth rate. The supernatant could not be replaced by known cofactors or amino acids. The growth factor remains uncharacterized but based on its chemical traits, it is not cAMP, *N*-acyl homoserinelactones, or peptides.

Regarding well-known signal compounds such as cAMP, *N*-acyl homoserinelactones, siderophore, and some peptides, there are several important reports on the isolation of microorganisms yet to be cultured or significant increases in cultivability using these compounds[2–4,7]. The effectiveness of these compounds varies depending upon the concentrations used and culture conditions, but these studies strongly suggest that it is possible to recover novel lineages of organisms that would otherwise escape detection.

Slow growers, the slower the better

Considering the concentrations of available substrates in the natural environment, it would not be surprising if a number of prokaryotic populations prefer low concentrations of the nutrients routinely used to grow "lab-tamed microorganisms" such as *Escherichia coli*. Based on preferable nutrient concentrations together with growth rates, we could classify microorganisms into four groups. Group 1: microorganisms that do not grow at high concentrations of nutrients, but grow reasonably quickly. Group 2: microorganisms that do not grow at high concentrations of nutrients, and grow slowly. Group 3: microorganisms that prefer high concentrations of nutrients, and grow quickly. Group 4: microorganisms that prefer high concentrations of nutrients, but grow slowly. Readily cultivable *E. coli*-type microorganisms represent Group 3. In Groups 1 and 2, microorganisms that grow in conditions of extreme nutritional deficiency, can be defined as oligotrophs. Organisms classified into Groups 1 and 3 are relatively easy to isolate. By contrast, Groups 2 and 4 microorganisms are very difficult to isolate from a complex community, simply because fast growers (Groups 1 and 3) outgrow them and dominate the culture medium even if they are not numerically significant.

The key to isolating these microorganisms from a complex community is eliminating the fast-growing microorganisms. There are no general solutions, but we have succeeded in isolating a variety of slow-growing microorganisms by limiting nutrient concentrations to suppress the growth of fast growers or by using appropriate inocula in which slow growers are already predominant[8,19,21,22,34,36,38,48]. One organism that we previously isolated was *Gemmatimonas aurantiaca. G. aurantiaca* is the only cultivated representative of the phylum *Gemmatimonadetes*, which was formerly called candidate phylum BD (or KS-B). Environmental sequence data suggest that this phylum is widespread in nature and has phylogenetic breadth. A strategy was adopted to isolate slowly growing bacteria that may be important but uncharacterized members of activated sludge operated under enhanced biological phosphorus removal conditions. We focused on heavier cells within the aggregates, plated the ultrasonically dispersed cells on a low-nutrient medium and incubated them for up to 12 weeks. By doing so, this organism was isolated in pure culture. The organism forms a tiny colony 1–2 mm in diameter after two weeks of incubation. Interestingly, it was found to utilize a limited range of substrates, and to have unusual cell envelope constituents. Very recently, Davis *et al.*[5] isolated one strain within this phylum as a slow grower using a diluted nutrient broth solidified with gellan gum, though its physiological traits remain unknown. The genomic sequencing of *G. aurantiaca* is now under way and so we will soon be able to confirm the evolutionary and functional novelty of this strain.

Gellan gum as an alternative gelling reagent for the isolation of novel lineages of microbes

Since the era of Robert Koch, most microbiologists have been using agar plates for the cultivation and isolation of microorganisms without question. Besides agar, gelling reagents such as gellan gum and silica gel are also being used for the cultivation of hyperthermophiles, acidiphiles, or organisms that are extremely sensitive to organic materials. However, only a few studies have focused on gelling reagents for the cultivation of previously uncultured microbes.

Recently, we have demonstrated that gellan gum was effective for the cultivation and isolation of hitherto-uncultured microbes in freshwater sediment of a shallow eutrophic lake[39] (Fig. 2). CFU counts from gellan gum-based medium were about 10 times higher than those from agar-based medium. Furthermore, approximately 60% of the microbes grown on the gellan gum medium were considered novel, at least at the species level, and the percentage was twice as high as that on the agar medium. Some of these novel isolates showed significantly low similarity (<90%) in the 16S rRNA gene sequence with known species. Interestingly, we observed that more than half of these novel isolates were not able to form colonies on agar medium of the same composition under the same culture conditions (unpublished data). Gellan gum-based cultivation would reduce the discrepancy in the composition of bacterial communities between culture-dependent and -independent analyses.

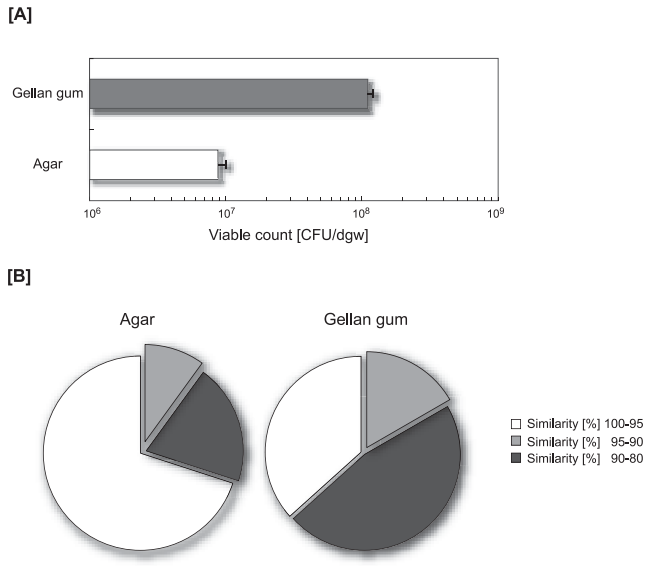


Fig. 2. Effect of gelling reagents in PE03 medium[39] on [A] viable counts and [B] cultivation of novel microbes in freshwater sediment. The similarity values shown are the 16S rRNA gene sequence similarities between our isolates and their closest relatives in the GenBank database.

Janssen and coworkers[5,16,17,31] have been extensively investigating the effectiveness of gellan gum-based media in their studies on the cultivation of soil microorganisms. They successfully cultivated and isolated novel microbes belonging to rarely isolated lineages such as the phyla *Acidobacteria, Chloroflexi, Gemmatimonadetes, Planctomycetes* and *Verrucomicrobia* using gellan gum-solidified media.

Gellan gum is an extracellular polysaccharide produced by *Sphingomonas* spp. (*S. paucimoblis*[30] or *S. elodea*[9]). The molecular structure is one of a linear heteropolysaccharide consisting of the repeating units of beta-D-glucose, beta-D-glucuronic acid, beta-D-glucose, and alpha-L-rhamnose. Gellan gum forms thermostable gels which are also stable over a wide range of pH. The gels have high clarity and require low concentrations (approx. 0.5–1.0%) to provide high gel strength. Compared with agar, the clarity of the gels may be a distinct advantage for the cultivation of microbes since it enables one to detect and select micro-colonies on plates. Gellan gum-based culture media may also stimulate, or may not affect, the growth of microbes whose growth is suppressed by agar.

As a replacement for agar, new gelling reagents such as gellan gum will become important for increasing the cultivability of organisms yet to be cultured.

Other strategies and concluding remarks

There are many other factors that have to be taken into consideration when attempting to isolate uncultured organisms. Physicochemical factors such as temperature, pH, redox potential, O_2 concentration and salinity seem very important to improve or modify isolation strategies. Ultimately, it would be best if we could mimic the natural environment[12]. Indeed, there are some reports on the cultivation of uncultured organisms by simulating the natural environment[18]. In contrast to this approach, several groups are developing modernized and systematic methods of isolation using flow cytometry[20,23,47].

In conclusion, as long as microbiologists believe that no microorganism is uncultivable, the isolation and cultivation of yet-to-be cultured organisms will remain an everlasting challenge.

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