Growing the recalcitrant

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Unculturable microorganisms are those that have been identified by microscopy, by their activity or by detection of phylogenetic markers such as their 16S rRNA genes, and have not been able to be cultured, despite reasonable efforts having been made. Recent successes in the cultivation of so-called unculturable microorganisms have revealed that the key ingredient in the recipe for growing them in the laboratory is patience. Beyond that, there is probably no single secret to success and microbial diversity must be matched by experimental ingenuity.

A recalcitrant 'kicks out its heels' and is obstinately disobedient. The microbiologist wishes to bring the recalcitrant bacterium, archaeon or eukaryote into laboratory culture, but it does not do what is expected. It does not multiply into a visible colony or a turbid culture on a standard medium within a timescale that the microbiologist considers acceptable. However, the problem lies with the microbiologist, not with the microbe, and the recalcitrant might better be termed 'misunderstood'. Widely used isolation methods select for microorganisms that respond rapidly after transfer to high nutrient levels, and standard isolation media favour microorganisms that are able to cope with and react quickly to nutrient flushes. The majority of microbes are probably adapted to grow at low and more or less steady-state nutrient concentrations, and may not respond rapidly to such conditions. The microbiologist's expectations must be matched to the microbe; good microbiology is not possible the other way around.

Try new media and growth conditions

The first step should be to use a new medium or new conditions – the standard ones have probably failed for one or more reasons.

MICRO-FACT

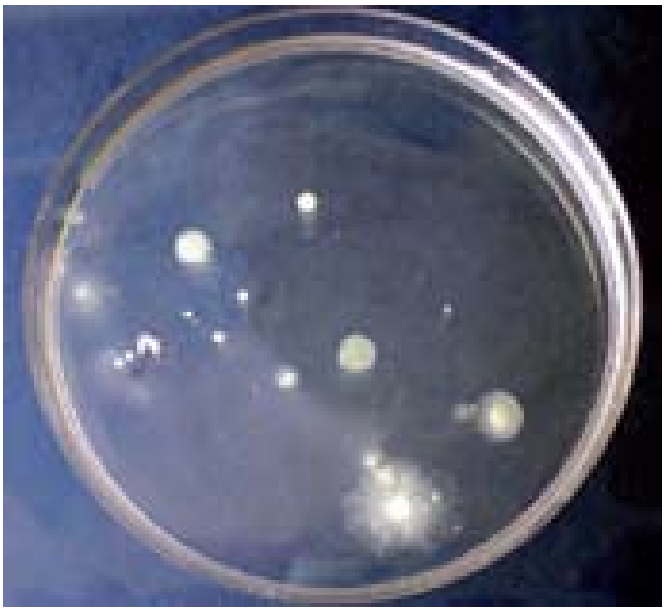
Soils are living because they contain a wide range of microorganisms including bacteria, archaea, and the eukaryotes – fungi, algae, protozoa, nematodes and other fauna including microarthropods, macroarthropods and earthworms.

Gelling agents, buffers and trace elements may be inhibitory, and high substrate concentrations may lead to growth inhibition, so medium design is a significant factor in success. Concentrations of most medium components need to be much lower than those routinely used and breakthroughs in culturing soil bacteria and many environmentally-significant anaerobes have come about from matching the synthetic medium composition to the natural environment [1,2]. However, sometimes samples of the environment are better growth media, at least initially. The square halophile *Haloquadratum walsbyi* and the marine bacterium *Pelagibacter ubique* are abundant in their environments, but appear to initiate growth only rarely. Both were isolated by using natural water sources as the growth media and after screening a large number of cultures for the few that contained the target microorganisms [3,4]. Initial cultures did not produce a visible turbidity and different means of assessing growth were used. Similarly, soil bacteria of the phylum *Verrucomicrobia* were isolated by screening over 1,200 colonies to find 14 cultures; an abundance well below their natural occurrence in the sample material [5].

Accommodate different growth rates

Extending the incubation time is a worthwhile modification to any isolation method. The first recognised isolation of

Fig. 1. Colonies of soil bacteria on a plate of gellan-solidified 1/100 nutrient broth after three months of incubation at 25°C. In addition to a variety of larger colonies, a number of very small colonies can be seen, aided by the extreme clarity of the medium.



Helicobacter pylori was achieved by incubating plates for longer than usual [6]. Similarly, many so-called unculturable soil bacteria are readily culturable on plates of appropriate media (Figure 1) that are incubated for longer than usual periods of weeks and months rather than days [2]. Moving away from enrichments and ensuring good physical separation of cells that allows each to develop into a single culture or colony without overgrowth by other, faster-growing species, is very important. Traditional plate methods with very small inocula, serial dilution of liquid cultures to single cells, and other cell separation methods have been successfully applied to isolate a great range of microbial diversity [2-5,7,8]. In these examples, medium choice, appropriate methods for detecting growth or colonies, longer incubation times, and the understanding that not all cells would initiate growth, played parts in eventual success.

Interactions may be important

The addition of signal molecules may be appropriate where they are known, but chemically different molecules are required by members of different phyla. Acyl-homoserine lactones may increase culturability in systems dominated by *Proteobacteria* [9,10], but cannot be expected to have much effect on members of other bacterial phyla. Using other microorganisms to provide such signals, to remove inhibitory products or to produce substrates at low rates, may overcome some unculturability problems [11-14]. Some physiologies require end product removal to allow detectable growth in culture [15,16] and, at least initially, not all species can be grown as pure cultures. Understanding the relationships between different species has allowed co-cultures of syntrophic bacteria to be isolated [15-17]. Informed isolation strategies using metagenomic data will be useful if enough sequence information can be gathered [18], but attention to detail and a good understanding of microbial physiology will still be required. Consideration of ecological strategies [2] and thermodynamic constraints will guide cultivation methods, especially for anaerobes and lithotrophs [15-17,19].

New and sometimes sophisticated approaches have been developed to isolate microorganisms [19], but simple methods, combined with consideration of how microbes might behave, are still very powerful [2,3,13,17,19]. There is certainly no reason to assume that most microorganisms are unculturable. However, the cultivation of many will be laborious and frustrating. Careful selection of microorganisms worthy of detailed study, combined with powerful new cultivation-independent methodologies to investigate them in situ, will bring rewards for those microbiologists who try to understand the recalcitrants and have the patience to coax them into the laboratory.

MICRO-FACT

The Australian Antarctic Territory represents about 42% of Antarctica, the fifth largest continent on earth.

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