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Review

Cultivation strategies for growth of uncultivated bacteria

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ABSTRACT

Background: The majority of environmental bacteria and around a third of oral bacteria remain uncultivated. Furthermore, several bacterial phyla have no cultivable members and are recognised only by detection of their DNA by molecular methods. Possible explanations for the resistance of certain bacteria to cultivation in purity *in vitro* include: unmet fastidious growth requirements; inhibition by environmental conditions or chemical factors produced by neighbouring bacteria in mixed cultures; or conversely, dependence on interactions with other bacteria in the natural environment, without which they cannot survive in isolation. Auxotrophic bacteria, with small genomes lacking in the necessary genetic material to encode for essential nutrients, frequently rely on close symbiotic relationships with other bacteria for survival, and may therefore be recalcitrant to cultivation in purity.

Highlight: Since in-vitro culture is essential for the comprehensive characterisation of bacteria, particularly with regard to virulence and antimicrobial resistance, the cultivation of uncultivated organisms has been a primary focus of several research laboratories. Many targeted and open-ended strategies have been devised and successfully used. Examples include: the targeted detection of specific bacteria in mixed plate cultures using colony hybridisation; growth in simulated natural environments or in co-culture with 'helper' strains; and modified media preparation techniques or development of customised media eg. supplementation of media with potential growth-stimulatory factors such as siderophores.

Conclusion: Despite significant advances in recent years in methodologies for the cultivation of previously uncultivated bacteria, a substantial proportion remain to be cultured and efforts to devise high-throughput strategies should be a high priority.

Contents

1. Introduction

Evidence started emerging over 50 years ago for the existence of a far greater variety of bacterial species than cultural analyses alone would suggest [1]. A discrepancy was noted between the numbers of bacteria counted under a microscope and viable counts in culture – the so-called Great Plate Count anomaly [2,3]. Furthermore, molecular analyses of 16S rRNA gene sequences, performed in some studies in parallel with cultural analyses, confirmed that there were indeed a large number of novel phylotypes without corresponding cultivated strains [4–8]. It was therefore apparent that certain bacteria might not be readily cultured *in vitro*.

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The terms 'uncultivated' and 'uncultivable', often used interchangeably in the literature, will be used for the purposes of this review to describe bacteria that have not previously been cultivated in isolation on artificial media. Previously-uncultivated bacteria that have ultimately been purified *in vitro* are frequently found to require special strategies for cultivation, are fastidious and unable to grow using conventional methods; these will be termed 'difficult-to-culture'.

Based on previous estimates, it is thought that approximately 99% of all bacteria on Earth are 'uncultivable' [9]. Likewise, the proportion of uncultivated bacteria from environmental habitats is estimated to be around 99% [10]. The uncultivated proportion is somewhat less for human-associated microbial communities, probably as a result of a concerted effort to study the microbiota in these ecosystems. For example, approximately 60–70% of bacteria from the human intestinal tract are uncultivated [11,12]; and based on the Human Oral Microbiome Database (HOMD) [13,14] release 13, 700 or so bacterial taxa have been found in the human oral cavity, of which roughly a third are known only as uncultivated phylotypes.

There are at least 38 bacterial phyla without any cultivable members [15], despite their widespread detection in samples from a variety of environments. Other phyla are comprised both of clusters of bacteria that are readily cultivated by standard methods, and clusters with no, or very few, cultivable representatives. A prime example is the phylum *Synergistetes*, proposed in 2009 [16,17]. The oral cavity harbours *Synergistetes* taxa from two main phylogenetic clusters, A and B [18] – the latter is comprised of cultivated species, whereas the former (more recently known as the genus *Fretibacterium*) is, despite frequent detection of representative phylotypes in oral samples by molecular methods, represented predominantly by 'uncultivable' taxa, there being only one cultivated species, the 'difficult-to-culture' *Fretibacterium fastidiosum* [19,20].

The absence of cultivated taxa from the genus *Fretibacterium* is clearly not due to a low prevalence; rather, there will be specific reasons for an apparent resistance to in-vitro culture. Fastidious bacteria may have specific growth requirements including temperature, pH, oxygen availability, nutrient sources and be unable to grow unless these requirements are stringently met in the laboratory. Furthermore, faced with an unfavourable growth environment with associated stress factors, bacteria may, as a survival strategy, enter a 'viable but non-culturable' or dormant state whereby cells are alive but no longer dividing [21,22] and be only able to revive when external conditions become more favourable or when appropriate growth factors and signals are provided. The growth-inhibitory effect of reactive oxygen species such as hydrogen peroxide, which leads to oxidative stress and cellular damage, has been well documented, with growing evidence in recent years for significantly reduced growth efficiency of 'difficult-to-culture' and 'uncultivated' bacterial taxa as a result of hydrogen peroxide generated within artificial growth media [23–25]. Bacterial growth may also be inhibited by the high concentration of nutrients present in the nutrient-rich media typically used to cultivate human pathogens, as well as by bacteriocins or other inhibitors produced by neighbouring bacteria in mixed cultures. On the other hand, members of bacterial communities in natural habitats, particularly those occurring as biofilms, often show a significant degree of inter-bacterial cooperation and interaction [26] through intercellular signalling via small peptides or quorum sensing, and the sharing of nutrients or essential metabolites such as iron-scavenging siderophores [27,28]. In line with this, bacteria in dental plaque biofilm have been shown to form precise and reproducible structural associations with each other, implying a defined functional interaction between individual bacteria within consortia [29]. Consequently, when attempts are made to isolate bacteria in purity, away from the host community and its beneficial interactive networks, they may not grow. Dependence for growth on signals and chemical factors produced by neighbouring bacteria is probably the single most important factor that prevents the in-vitro growth of bacteria in isolation. Auxotrophy, the inability of bacteria to synthesise various essential metabolites, has been shown to be associated with gene loss [30]; representatives of various Candidate bacterial phyla with no cultured members, such as Candidatus *Saccharibacteria* (formerly TM7), SR1, WWE3 and OD1, have small genomes lacking genes for certain key biosynthetic pathways [15]. As a result, such bacteria may survive only in very close association with – living on the surface of or inside – 'helper' organisms. Examples of such bacteria include the recently-cultivated *Saccharibacteria* strain, TM7x, which leads an obligately symbiotic relationship with the bacterium *Actinomyces odontolyticus* [31] and the intracellular pathogen *Tropheryma whipplei* [32], both of which have reduced genomes deficient in biosynthetic pathways for various essential amino acids. Clearly, the culture of such dependent organisms in isolation presents a significant challenge.

Bacterial culture remains indispensible as a microbiological method despite significant developments in recent years in molecular and 'meta-omic' techniques. Indeed it is only through the study of pure cultures of bacteria that phenotype and genotype may be characterised in full. Several uncultivated or 'difficult-to-culture' bacteria, such as the recently-cultivated taxon *Anaerolineae* bacterium HOT-439 from the phylum *Chloroflexi* [33], *F. fastidiosum* of the *Synergistetes* phylum, TM7 phylotype HOT-356 from Candidatus *Saccharibacteria*, *Peptostreptococcaceae* bacterium HOT-091, and the intracellular pathogens *T. whipplei* and *Coxiella burnetii*, have been found to be associated with human disease processes, including the oral disease periodontitis [34–38] and the systemic diseases Whipple's disease and Q fever; evaluation of virulence potential of these putative or confirmed pathogens and assessment of their role in disease relies on having a pure culture in the laboratory. In light of the importance of bacterial culture in modern day microbiology, the quest to isolate and culture uncultivated bacteria remains a high priority.

The aim of this review is to describe a range of strategies for the cultivation of uncultivated bacteria, along with the various rationales on which these methods are based.

2. Cultivation strategies for uncultivated bacteria

2.1. Approaches used in environmental microbiology

The significant majority of environmental bacteria found in habitats such as soil and seawater is uncultivated [39]. Hence a number of innovative methods for the culture of uncultivated bacteria derive from environmental microbiology.

Several of the approaches that have been developed are based on the principle that bacteria growing naturally in mixed communities depend on interaction with other members of that community, as well as on signals and nutrients present within the natural habitat.

Kaeberlein et al. [40] were amongst the first to propose the 'simulated natural environment' concept. Briefly, they designed diffusion chambers within which organisms were inoculated. The chambers were incubated under conditions mimicking the natural environment, allowing the passage of growth-stimulatory chemical factors from the external environment across semi-permeable (0.03 μm-pore) membrane walls of the chambers and resulting in the growth, and ultimately pure culture, of previously-uncultivated bacteria from the marine environment. This method was later also successfully applied to samples of fresh water and subsurface

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