Cultivation strategies for growth of uncultivated bacteria

Sonia R. Vartoukian [a]

[a] Barts and The London School of Medicine and Dentistry, Queen Mary University of London, UK.

Centre for Immunobiology, Blizard Institute, Queen Mary University of London, 4 Newark Street, London, E1 2AT; E-mail: s.vartoukian@qmul.ac.uk

Corresponding author:

Dr. Sonia R. Vartoukian
Centre for Immunobiology
Blizard Institute
Queen Mary University of London
4 Newark Street
London
E1 2AT
Tel: 44 20 7882 2304
E-mail: s.vartoukian@qmul.ac.uk

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.

Keywords: culture, isolation, microbiome, bacteriology

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*.

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 unfavorable 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 neighboring 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 signaling 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 neighboring bacteria is probably the single most important factor that prevents the in-vitro growth of bacteria in isolation. Auxotrophy, the inability of bacteria to synthesize 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 characterized 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 sediment [41, 42]. Further development resulted in the application of this method for the highly-parallel simultaneous culture of multiple 'uncultivables' with the aid of the 'ichip', a device which is made up of hundreds of miniature diffusion chambers [39]. Using a similar principle and separating culture / simulated natural environment by a transwell insert with a microporous membrane, previously uncultivated bacteria were also successfully cultured and isolated from soil [43, 44].

Other inventive systems for the in-situ cultivation of fastidious bacteria in natural environments include the I-tip method [45] and the hollow-fiber membrane chamber (HFMC) device [46]. Schematic diagrams of these novel devices are included in the respective papers. The I-tip method appears to be primarily targeted to motile organisms since it relies on the movement of small organisms from the freshwater sponge natural environment in which the I-tip is placed, across glass beads of

various sizes to the agar culture medium within the I-tip. The HFMC on the other hand – a system of multiple, 0.1 µm-pore, hollow-fiber tubes within which heavily-diluted microbial samples are injected, and which are placed *in situ* in natural or simulated natural environments for the influx of natural chemical compounds – is perhaps more universally applicable. The authors compared the cultivation performance of the HFMC device against that of traditional agar-based culture for environmental samples ranging from tidal flat sediment to activated sludge from water treatment plants, and reported the recovery of a higher proportion of novel isolates (within the total) with the HFMC than with conventional culture in petri dishes, despite the 16S rRNA gene sequence similarity threshold for identification being set at 97%, a value lower than the 98.5% or 99% cut-offs used in HOMD and elsewhere [14, 47], which could have resulted in an underestimation in the prevalence of novel taxa. Furthermore, the results also indicated higher Shannon-Weaver and Simpson diversity indices for samples processed with the HFMC, confirming the efficacy of the method.

Bacteria of interest may also be exposed to signals or nutrients from the natural habitat, or engineered versions of the latter, after encapsulation of single cells or subsets of the microbial community as microdroplets using microfluidic devices. Zengler *et al* [48] encapsulated single bacterial cells in gel microdroplets, incubated them in amended sterile versions of the natural environmental medium, detected growth as microcolonies in the microdroplets using flow cytometry, and successfully isolated and cultured several novel bacterial strains following transfer to microtitre plates. The process of diluting mixed samples down to single cells prior to attempted cultivation, termed ‘dilution-to-extinction’, eliminates the potential drawback of competition between bacteria in the community and results ideally in pure cultures. Furthermore, the confinement of single cells to a small volume using microfluidics,

results in a favourable cell density to volume ratio, which may initiate quorum sensing-dependent growth [49].

Growth-stimulatory chemical factors present in bacterial communities in the natural setting may derive from neighboring bacteria. In addition, such bacteria may modify the environment in such a manner as to make it more favorable for growth of dependent strains. On the basis that bacteria within communities cooperate closely in these two key ways, a number of researchers have attempted to cultivate uncultivated bacteria using community culture or bacterial co-culture techniques. As an example of the latter, Park et al [50] co-cultured pairs of dependent organisms within oil-coated microdroplets – since the two organisms in each symbiotic pair were auxotrophic for a different amino acid, growth in minimal medium was achieved only by co-cultivation, indicative of cross-feeding. Nichols *et al* [51] enabled pure growth of the previously uncultivated marine strain *Psychrobacter* sp. MSC33 by co-culturing it in close proximity to, but separated from, its 'helper' strain MSC105 using a two-compartment chamber incorporating a tissue culture insert. Likewise, Ueda and Beppu [52] co-cultured the syntrophic bacterium *Symbiobacterium thermophilum* and the *Bacillus* strain on which it depends for growth, in a two-compartment flask with dialysis membrane separating the two. In another innovative example, included here although it relates to the human gut flora rather than to environmental microorganisms because it uses a similar principle to the above, pairwise symbiotic relationships were detected between co-cultivated bacteria growing in soft agar on either side of a 0.22 μm membrane filter [53]. Morris *et al* [23, 24] demonstrated that *Prochlorococcus* taxa are dependent for growth on neighboring hydrogen peroxide-scavenging bacteria for protection from oxidative stress and cellular damage.

There have been substantial efforts to cultivate previously uncultivated bacteria by modifying culture media and growth conditions. For example, Tamaki *et al* [54, 55] compared the growth of bacteria from freshwater sediment on gellan-gum-solidified and conventional agar-solidified media. They reported a significantly greater recovery of novel previously uncultivated isolates on gellan-gum than on agar media, as well as faster growth on the former of strains that were able to grow on both. Although they did not offer any explanation for the benefit of gellan-gum, Pham and Kim [56] later suggested, in a review paper, that gellan-gum plates are 'clearer' than agar and therefore more suitable for the detection of tiny colonies. More recently, evidence has started emerging that the generation of hydrogen peroxide in conventionally-prepared agar media has an inhibitory effect on bacterial growth [25] and this may perhaps explain the inferiority of standard agar over gellan-gum. Tanaka *et al* [25] found that agar medium plates prepared by autoclaving phosphate and agar separately, then mixing and pouring, resulted not only in higher numbers of CFUs, but also a higher recovery of novel isolates, compared to standard agar media where all components were autoclaved together. Whereas hydrogen peroxide was detected in the standard agar media plates, none was detectable in media prepared by separate autoclaving of phosphate and agar. Furthermore, the growth-inhibitory effect of standard agar media was successfully reversed by adding catalase (an enzyme for the decomposition of hydrogen peroxide) to the surface of plates post inoculation. Clearly hydrogen peroxide has a detrimental effect on the growth of bacteria, including difficult-to-culture organisms, and there appear to be a number of ways to overcome this.

Another strategy for stimulating growth of uncultivated bacteria is the supplementation of media with chemical compounds that are likely to be required by the bacteria in question. D'Onofrio et al [10] determined the identity of growth factors

produced by ‘helper’ strains that were responsible for growth of dependent uncultivated organisms from marine sediment, and were able to stimulate growth of the latter by supplementing media with the growth factors, namely iron-scavenging siderophores. Indeed there is evidence from many years previously [57] as well as more recently [33] – see section 2.2 – for the growth-promoting effect of siderophores on uncultivated and ‘difficult-to-culture’ bacteria. It has been suggested that the ability to autonomously produce siderophores may have been lost in uncultivated bacteria [10, 58]. Other growth-stimulatory factors include the quorum-sensing molecules acylhomoserine lactones (AHL), which have been shown to facilitate the growth of an AHL-degrading novel proteobacterium [59], and trace elements at levels matching those found in the source environment [60].

Genomic analysis of bacteria targeted for cultivation may provide information on specific aspects of an organism’s metabolism, such as potential nutrient sources that are likely to be utilized or the absence of genes related to a particular metabolic pathway, indicating auxotrophy. These insights may help inform the design of customized culture media. Kawanishi *et al* [61] have used this principle for their ‘selective medium-design algorithm restricted by two constraints’ (SMART) strategy, which evaluates carbon source requirements and antimicrobial sensitivity in order to devise highly selective media for target bacteria. This method resulted in the successful selective culture of specific environmental bacteria from mixtures of 10 species. Related examples, though unconnected to environmental microbiology, include the design of customized media for human intracellular pathogens such as *T. whipplei*, which have reduced genomes and metabolic pathway deficiencies, by supplementation with the particular amino acids for which the bacteria are auxotrophic [32, 62].

296 It is clear from the methods described above that there are a variety of highly
297 sophisticated strategies available for the attempted cultivation of uncultivated
298 bacteria. Yet, Browne *et al* [63] have suggested that, at least in the case of human
299 faecal microbiota, a large proportion of the bacterial community can be cultivated and
300 purified on a single broad-range culture medium provided that sufficient numbers of
301 colonies are harvested for isolation and identification. Based on their hypothesis that
302 the human intestinal microbiota is comprised of a high proportion of ethanol-resistant
303 spore-forming bacteria, they cultured ethanol-treated and -untreated faecal samples
304 on YCFA medium supplemented with sodium taurocholate to facilitate germination of
305 spores, and of the 2000 or so colonies picked and isolated, they identified 45 novel
306 previously-uncultivated taxa. It would appear that in certain circumstances, the yield
307 maybe relative to the time and effort expended.

308

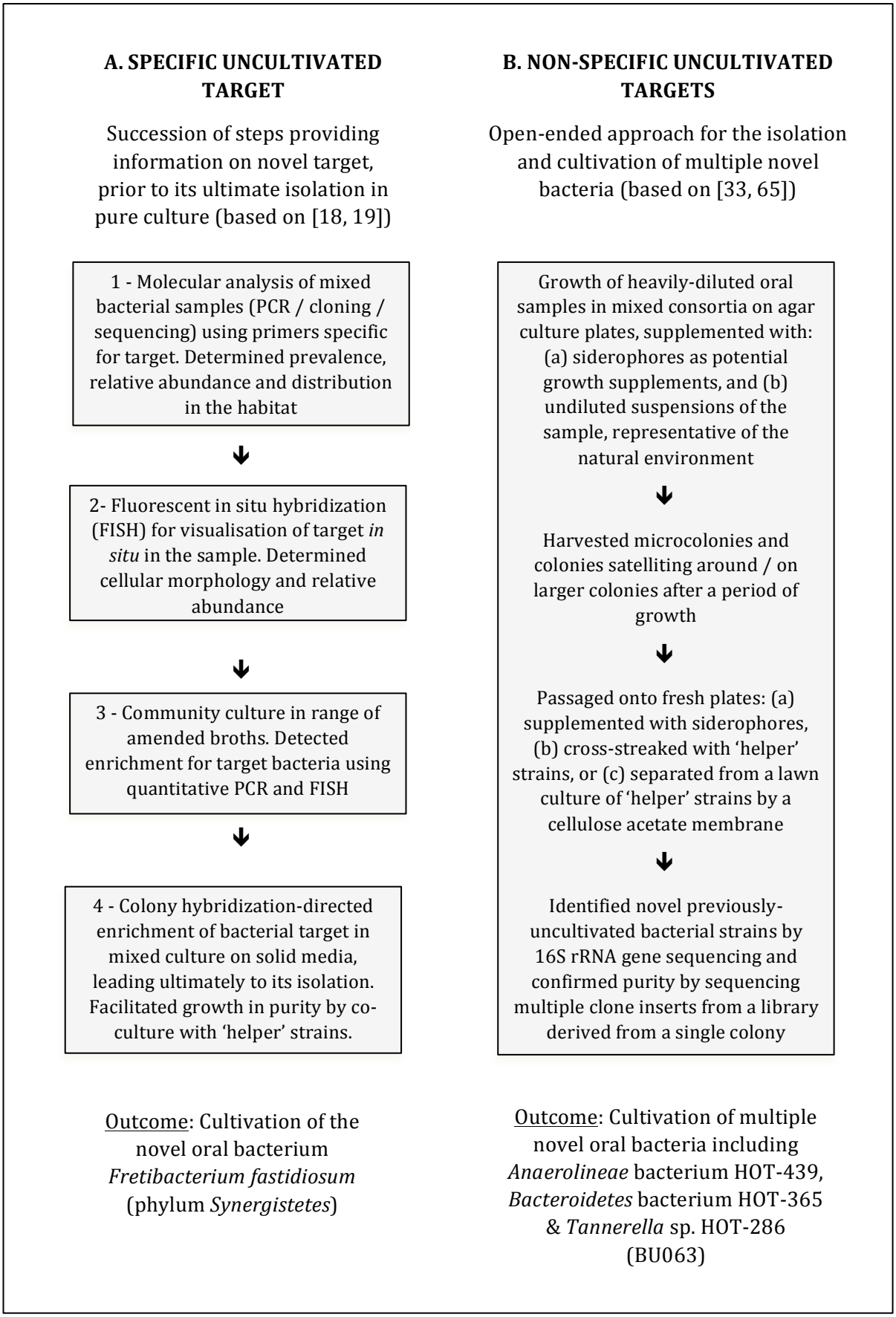
309 **2.2 Approaches used in oral microbiology**

310

311 In comparison with the high level of research activity in environmental microbiology
312 for cultivation of 'uncultivable' bacteria, efforts to culture previously uncultivated oral
313 bacteria have been relatively limited.

314

315 A selection of approaches used to culture novel oral bacteria is described in Fig 1. In
316 brief, targeted approaches were used for the cultivation of specific uncultivated
317 targets [19, 64] and open-ended approaches were used for cultivation of multiple
318 uncultivated bacteria, in anticipation of a higher-throughput yield [33, 65].



319 Figure 1. Targeted and open-ended approaches to the in-vitro culture of uncultivated oral

320 bacteria

The cultivation of the first representative of the oral cluster A *Synergistetes* (described in Fig 1 A) [18, 19] is of particular interest. As mentioned previously, the oral *Synergistetes* community comprises of two main clusters, A and B, where cluster A (genus *Fretibacterium*) has had no cultivated members despite frequent detection of phylotypes using molecular methods [17]. In view of the dearth of information on this new bacterial lineage and potentially important group of organisms, primers and probes specific for *Synergistetes* were designed, validated, and used to detect this bacterial target in mixed bacterial samples. Targeted PCR/cloning/sequencing analysis of oral samples revealed a high taxon-richness (12 cluster A *Synergistetes* taxa, of which 5 were novel) and high prevalence in the oral cavity, particularly in subgingival plaque harvested from sites of periodontal disease. Having gained some insight into the prevalence, diversity and distribution in the oral cavity of the uncultivated cluster A *Synergistetes*, group-specific fluorescent in situ hybridization (FISH) probes were used to visualize cells *in situ* in subgingival plaque for the first time, confirming the presence of intact cells that were large curved bacilli and prominent members of the plaque bacterial community, making up on average 8% of the total population. A first attempt to cultivate these bacteria *in vitro* followed on from this. On the hypothesis that these bacteria are not readily cultivated outside the natural habitat due to a dependence for growth on interaction with other bacteria within the community, they were first 'cultivated' in consortia, in plain and supplemented 'cooked meat medium' broths incubated anaerobically for up to 33 days. Monitoring for cluster A *Synergistetes* and total bacteria by quantitative PCR and FISH indicated the successful culture and significant enrichment of target bacteria in community culture, particularly in serum- and mucin- supplemented media. The final and chief aim was to attempt to cultivate cluster A *Synergistetes* in purity. With this in mind, the colony-hybridization method was used to probe for and detect colonies of target cluster A *Synergistetes* on mixed-culture plates of subgingival plaque on solid culture media. Briefly, colonies were lifted from culture plates onto nylon membranes, bacterial target was probed for and resultant digoxigenin-labelled probe/anti-digoxigenin antibody hybridizations were detected by a colorimetric reaction. Subculture of regions of cells matching positive

hybridisation detections on membranes did not initially lead to isolation of target cluster A *Synergistetes*, therefore the colony hybridization process was sequentially repeated on subculture plates. This led to the enrichment of the bacterial target within low-complexity mixed cultures and ultimately, after eight passages and growth stimulation by cross-streaks of other bacteria present within the original community, to the first isolation and cultivation *in vitro* of a member of cluster A *Synergistetes*, the 'difficult-to-culture' bacterium, since named *Fretibacterium fastidiosum* [20]. This species has been found to be a slow-growing fastidious bacterium, dependent for growth on helper strains including species present within the host community. Furthermore, evidence is emerging for an association of this bacterium with periodontal disease [35-38].

Thompson *et al* [64] also used a targeted colony-hybridization technique to successfully isolate the difficult-to-culture bacterium *Lachnospiraceae* HOT-500 from subgingival plaque following a sequence of steps involving first the culture of the sample as a biofilm in a Calgary Biofilm Device, followed by transfer to plates of proteose-peptone-agar, a customised medium rich in nutrients. Like *F. fastidiosum*, this novel bacterium was found to depend for growth on 'helpers' present in the original host community, namely *Parvimonas micra* and *Veillonella dispar*.

The colony hybridization method is invaluable for attempts at cultivation of specific target bacteria, yet in the long run it results in the isolation of only one bacterium. In an effort to increase productivity, a novel in-vitro cultivation method using an open-ended approach and combination of strategies was devised in the Wade lab [33, 65]. A brief description of the protocol is provided in Fig 1 B. The overall strategy was: (a) to grow mixed bacterial samples in consortia on agar media plates, whilst keeping the source bacterial community in close proximity (in a well in the centre of the plates); (b) to supplement media with siderophores

(pyoverdines-Fe and desferricoprogen) shown previously to stimulate growth of uncultivated bacteria (see section 2.1); and (c) to stimulate growth of dependent strains with potential 'helpers', either cross-streaked across plates or as lawn cultures beneath cellulose acetate membranes, on which the dependent strains were streaked. This work led to the successful isolation of multiple previously-uncultivated bacteria, including *Anaerolineae* bacterium HOT-439, the first oral taxon from the phylum *Chloroflexi* to have been cultivated; *Bacteroidetes* bacterium HOT-365; *Peptostreptococcaceae* bacterium HOT-091; and the much sought after taxon, *Tannerella* sp. HOT-286 (phylotype BU063), which has resisted cultivation efforts by various research groups over a period of over a decade.

Sizova *et al* [66] adapted to the oral environment various approaches previously developed in environmental microbiology, and used a range of complementary strategies in an attempt to cultivate uncultivated bacteria. These included: (a) the 'mini-trap', a modified in-situ version of the diffusion chamber used by Kaeberlein et al [40], which was attached to an intra-oral appliance to enable cultivation of bacteria in the natural environment *in vivo*; (b) single-cell long term cultivation in 96-well plates, with the aim of permitting the growth of 'slow-growers' without competition from faster-growing bacteria; and (c) modified media preparations, such as those containing starch and xylan rather than sugars. Interestingly they found little overlap between the methods in the organisms recovered, which could be explained by divergent rationales – for example, whereas the mini-trap might favour the growth of bacteria dependent on cooperation with other organisms in the natural environment, such bacteria might not do as well using a single-cell cultivation method. The authors concluded that optimal recovery is achieved by using an ensemble of complementary methods.

Davis et al [67] applied an innovative quantitative-PCR-based screening method to evaluate the growth of a selection of specific canine dental plaque bacteria on a range of eight different culture media, and used the results to inform the selection of optimal growth conditions. In conjunction, where necessary, with the use of *Propionibacterium acnes* as a helper strain under nitrocellulose membranes, this method led to the successful cultivation of eight of 11 targeted previously-uncultivated bacteria.

Finally, researchers in the Shi lab [31] recently succeeded in cultivating the first taxon from the candidate phylum *Saccharibacteria* (TM7), strain TM7x from the human oral cavity. They did so by plating saliva samples on a specialised oral culture medium (SHI medium) and targeting the enrichment of TM7 taxa by streptomycin selection. This and their subsequent ground-breaking work [68] have revealed that strain TM7x leads an epibiotic parasitic lifestyle associated with another bacterium, its basibiont *Actinomyces odontolyticus* XH001. Furthermore they confirmed, by transcriptomic and metabolomic analysis, a signalling interaction between the two. With an extremely small cell size and reduced genome of 705 kb lacking the capacity to synthesise any amino acids, strain TM7x represents a classic example of auxotrophy. It is of no great surprise that this organism is dependent on another for survival and growth, and has therefore been recalcitrant to cultivation for so long.

3 Conclusions

Any bacterium must first be cultivated *in vitro* before a comprehensive characterisation can be undertaken, both to study its physiology and phenotype, as well as potentially to better understand how it interacts with other bacteria within the host community. Hence the quest to culture uncultivated bacteria is of paramount importance. Over the years, a host of innovative strategies have been devised and used successfully for this purpose: some have

involved sophisticated devices, while others have relied on relatively simple methods and basic principles. It is clear that the inter-relationships between neighbouring bacteria in mixed communities, particularly those in biofilms, are highly developed; hence several 'difficult-to-culture' bacteria manifest dependencies on other bacteria for growth *ex vivo*. As the relationships between helper and dependent strains are better understood over time, potentially 'universal' growth factors may be uncovered, and this may facilitate the development in the future, of much-needed high-throughput strategies for the cultivation of uncultivated bacteria.

Ethical approval

Not required

Conflict of interest

None

Acknowledgements

This work was supported by The National Institute of Dental and Craniofacial Research of the National Institutes of Health under award R37DE016937. I would like to thank Professor William G. Wade for suggestions to improve the manuscript.

References

[1] Socransky SS, Gibbons RJ, Dale AC, Bortnick L, Rosenthal E, Macdonald JB. The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts and counts of specific organisms. Arch Oral Biol. 1963;8:275-80.

[2] Staley JT, Konopka A. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol. 1985;39:321-46.

[3] Amann RI, Ludwig W, Schleifer KH, Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 1995;59:143-69.

[4] Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG. Molecular and cultural analysis of the microflora associated with endodontic infections. J Dent Res. 2002;81:761-6.

[5] Munson MA, Banerjee A, Watson TF, Wade WG. Molecular analysis of the microflora associated with dental caries. J Clin Microbiol. 2004;42:3023-9.

[6] Dymock D, Weightman AJ, Scully C, Wade WG. Molecular analysis of microflora associated with dentoalveolar abscesses. J Clin Microbiol. 1996;34:537-42.

[7] Ward DM, Weller R, Bateson MM. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature. 1990;345:63-5.

[8] Giovannoni SJ, Britschgi TB, Moyer CL, Field KG. Genetic diversity in Sargasso Sea bacterioplankton. Nature. 1990;345:60-3.

[9] Wade WG, Spratt DA, Dymock D, Weightman AJ. Molecular detection of novel anaerobic species in dentoalveolar abscesses. Clin Infect Dis. 1997;25:S235-6.

[10] D'Onofrio A, Crawford JM, Stewart EJ, Witt K, Gavrish E, Epstein S, et al. Siderophores from neighboring organisms promote the growth of uncultured bacteria. Chem Biol. 2010;17:254-64.

[11] Hayashi H, Sakamoto M, Benno Y. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol. 2002;46:535-48.

[12] Dave M, Higgins PD, Middha S, Rioux KP. The human gut microbiome: current knowledge, challenges, and future directions. Transl Res. 2012;160:246-57.

[13] Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database (Oxford). 2010;2010:baq013.

[14] Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. J Bacteriol. 2010;192:5002-17.

[15] Kantor RS, Wrighton KC, Handley KM, Sharon I, Hug LA, Castelle CJ, et al. Small genomes and sparse metabolisms of sediment-associated bacteria from four candidate phyla. MBio. 2013;4:e00708-13.

[16] Jumas-Bilak E, Roudiere L, Marchandin H. Description of 'Synergistetes' phyl. nov. and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes'. Int J Syst Evol Microbiol. 2009;59:1028-35.

[17] Vartoukian SR, Palmer RM, Wade WG. The division "Synergistes". Anaerobe. 2007;13:99-106.

[18] Vartoukian SR, Palmer RM, Wade WG. Diversity and morphology of members of the phylum "synergistetes" in periodontal health and disease. Appl Environ Microbiol. 2009;75:3777-86.

[19] Vartoukian SR, Palmer RM, Wade WG. Cultivation of a *Synergistetes* strain representing a previously uncultivated lineage. Env Microbiol. 2010;12:916-28.

[20] Vartoukian SR, Downes J, Palmer RM, Wade WG. Fretibacterium fastidiosum gen. nov., sp. nov., isolated from the human oral cavity. Int J Syst Evol Microbiol. 2013;63:458-63.

[21] Shleeva M, Mukamolova GV, Young M, Williams HD, Kaprelyants AS. Formation of 'non-culturable' cells of Mycobacterium smegmatis in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. Microbiology. 2004;150:1687-97.

[22] Shleeva MO, Kudykina YK, Vostroknutova GN, Suzina NE, Mulyukin AL, Kaprelyants AS. Dormant ovoid cells of Mycobacterium tuberculosis are formed in response to gradual external acidification. Tuberculosis (Edinb). 2011;91:146-54.

[23] Morris JJ, Kirkegaard R, Szul MJ, Johnson ZI, Zinser ER. Facilitation of robust growth of Prochlorococcus colonies and dilute liquid cultures by "helper" heterotrophic bacteria. Appl Environ Microbiol. 2008;74:4530-4.

505 [24] Morris JJ, Johnson ZI, Szul MJ, Keller M, Zinser ER. Dependence of the
506 cyanobacterium Prochlorococcus on hydrogen peroxide scavenging microbes for growth at
507 the ocean's surface. PLoS One. 2011;6:e16805.
508 [25] Tanaka T, Kawasaki K, Daimon S, Kitagawa W, Yamamoto K, Tamaki H, et al. A
509 hidden pitfall in the preparation of agar media undermines microorganism cultivability. Appl
510 Environ Microbiol. 2014;80:7659-66.
511 [26] Mihai MM, Holban AM, Giurcaneanu C, Popa LG, Oanea RM, Lazar V, et al.
512 Microbial biofilms: impact on the pathogenesis of periodontitis, cystic fibrosis, chronic
513 wounds and medical device-related infections. Curr Top Med Chem. 2015;15:1552-76.
514 [27] Kummerli R, Jiricny N, Clarke LS, West SA, Griffin AS. Phenotypic plasticity of a
515 cooperative behaviour in bacteria. J Evol Biol. 2009;22:589-98.
516 [28] Kummerli R, Schiessl KT, Waldvogel T, McNeill K, Ackermann M. Habitat structure
517 and the evolution of diffusible siderophores in bacteria. Ecol Lett. 2014;17:1536-44.
518 [29] Mark Welch JL, Rosetti BJ, Rieken CW, Dewhirst FE, Borisy GG. Biogeography of a
519 human oral microbiome at the micron scale. Proc Natl Acad Sci. 2016;113:E791-800.
520 [30] Davis JJ, Xia F, Overbeek RA, Olsen GJ. Genomes of the class Erysipelotrichia
521 clarify the firmicute origin of the class Mollicutes. Int J Syst Evol Microbiol. 2013;63:2727-41.
522 [31] He X, McLean JS, Edlund A, Yooseph S, Hall AP, Liu SY, et al. Cultivation of a
523 human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle.
524 Proc Natl Acad Sci U S A. 2015;112:244-9.
525 [32] Renesto P, Crapoulet N, Ogata H, La Scola B, Vestris G, Claverie JM, et al.
526 Genome-based design of a cell-free culture medium for Tropheryma whipplei. Lancet.
527 2003;362:447-9.
528 [33] Vartoukian SR, Adamowska A, Lawlor M, Moazzez R, Dewhirst FE, Wade WG. In
529 Vitro Cultivation of 'Unculturable' Oral Bacteria, Facilitated by Community Culture and Media
530 Supplementation with Siderophores. PLoS One. 2016;11:e0146926.

[34] Szafranski SP, Wos-Oxley ML, Vilchez-Vargas R, Jauregui R, Plumeier I, Klawonn F, et al. High-resolution taxonomic profiling of the subgingival microbiome for biomarker discovery and periodontitis diagnosis. Appl Environ Microbiol. 2015;81:1047-58.

[35] Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD, et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. Isme j. 2013;7:1016-25.

[36] Galimanas V, Hall MW, Singh N, Lynch MD, Goldberg M, Tenenbaum H, et al. Bacterial community composition of chronic periodontitis and novel oral sampling sites for detecting disease indicators. Microbiome. 2014;2:32.

[37] Marchesan JT, Morelli T, Moss K, Barros SP, Ward M, Jenkins W, et al. Association of Synergistetes and Cyclodipeptides with Periodontitis. J Dent Res. 2015;94:1425-31.

[38] Perez-Chaparro PJ, Goncalves C, Figueiredo LC, Faveri M, Lobao E, Tamashiro N, et al. Newly identified pathogens associated with periodontitis: a systematic review. J Dent Res. 2014;93:846-58.

[39] Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A, et al. Use of ichip for high-throughput in situ cultivation of "uncultivable" microbial species. Appl Environ Microbiol. 2010;76:2445-50.

[40] Kaeberlein T, Lewis K, Epstein SS. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science. 2002;296:1127-9.

[41] Bollmann A, Lewis K, Epstein SS. Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. Appl Environ Microbiol. 2007;73:6386-90.

[42] Bollmann A, Palumbo AV, Lewis K, Epstein SS. Isolation and physiology of bacteria from contaminated subsurface sediments. Appl Environ Microbiol. 2010;76:7413-9.

[43] Moon J, Kim J. Isolation of Paenibacillus pinesoli sp. nov. from forest soil in Gyeonggi-Do, Korea. J Microbiol. 2014;52:273-7.

[44] Van Pham HT, Kim J. Bacillus thaonhiensis sp. nov., a new species, was isolated from the forest soil of Kyonggi University by using a modified culture method. Curr Microbiol. 2014;68:88-95.

[45] Jung D, Seo EY, Epstein SS, Joung Y, Han J, Parfenova VV, et al. Application of a new cultivation technology, I-tip, for studying microbial diversity in freshwater sponges of Lake Baikal, Russia. FEMS Microbiol Ecol. 2014;90:417-23.

[46] Aoi Y, Kinoshita T, Hata T, Ohta H, Obokata H, Tsuneda S. Hollow-fiber membrane chamber as a device for in situ environmental cultivation. Appl Environ Microbiol. 2009;75:3826-33.

[47] Stackebrandt E, Ebers J. Taxonomic parameters revisited: tarnished gold standards. Microbiol Today. 2006;33:152-5.

[48] Zengler K, Toledo G, Rappe M, Elkins J, Mathur EJ, Short JM, et al. Cultivating the uncultured. Proc Natl Acad Sci U S A. 2002;99:15681-6.

[49] Boedicker JQ, Vincent ME, Ismagilov RF. Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. Angew Chem Int Ed Engl. 2009;48:5908-11.

[50] Park J, Kerner A, Burns MA, Lin XN. Microdroplet-enabled highly parallel co-cultivation of microbial communities. PLoS One. 2011;6:e17019.

[51] Nichols D, Lewis K, Orjala J, Mo S, Ortenberg R, O'Connor P, et al. Short peptide induces an "uncultivable" microorganism to grow in vitro. Appl Environ Microbiol. 2008;74:4889-97.

[52] Ueda K, Beppu T. Lessons from studies of *Symbiobacterium thermophilum*, a unique syntrophic bacterium. Biosci Biotechnol Biochem. 2007;71:1115-121.

[53] Tanaka Y, Benno Y. Application of a single-colony coculture technique to the isolation of hitherto unculturable gut bacteria. Microbiol Immunol. 2015;59:63-70.

[54] Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumura M, et al. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic

584 lake by molecular and improved cultivation-based techniques. Appl Environ Microbiol.
585 2005;71:2162-9.
586 [55] Tamaki H, Hanada S, Sekiguchi Y, Tanaka Y, Kamagata Y. Effect of gelling agent on
587 colony formation in solid cultivation of microbial community in lake sediment. Environ
588 Microbiol. 2009;11:1827-34.
589 [56] Pham VH, Kim J. Cultivation of unculturable soil bacteria. Trends Biotechnol.
590 2012;30:475-84.
591 [57] Guan LL, Kamino K. Bacterial response to siderophore and quorum-sensing
592 chemical signals in the seawater microbial community. BMC Microbiol. 2001;1:27.
593 [58] Lewis K, Epstein S, D'Onofrio A, Ling LL. Uncultured microorganisms as a source of
594 secondary metabolites. J Antibiot (Tokyo). 2010;63:468-76.
595 [59] Chan KG, Yin WF, Sam CK, Koh CL. A novel medium for the isolation of N-
596 acylhomoserine lactone-degrading bacteria. J Ind Microbiol Biotechnol. 2009;36:247-51.
597 [60] Meyer-Dombard DR, Shock EL, Amend JP. Effects of trace element concentrations
598 on culturing thermophiles. Extremophiles. 2012;16:317-31.
599 [61] Kawanishi T, Shiraishi T, Okano Y, Sugawara K, Hashimoto M, Maejima K, et al.
600 New detection systems of bacteria using highly selective media designed by SMART:
601 selective medium-design algorithm restricted by two constraints. PLoS One. 2011;6:e16512.
602 [62] Ogata H, Claverie JM. Metagrowth: a new resource for the building of metabolic
603 hypotheses in microbiology. Nucleic Acids Res. 2005;33:D321-4.
604 [63] Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, et al.
605 Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation.
606 Nature. 2016.
607 [64] Thompson H, Rybalka A, R M, Dewhirst F, Wade W. In-vitro culture of previously
608 uncultured oral bacterial phylotypes Appl Env Microbiol. 2015;Sept 25.
609 [65] Vartoukian SR, Moazzez RV, Paster BJ, Dewhirst FE, Wade WG. First Cultivation of
610 Health-Associated Tannerella sp. HOT-286 (BU063). J Dent Res. 2016.

611 [66] Sizova MV, Hohmann T, Hazen A, Paster BJ, Halem SR, Murphy CM, et al. New
612 approaches for isolation of previously uncultivated oral bacteria. Appl Environ Microbiol.
613 2012;78:194-203.
614 [67] Davis IJ, Bull C, Horsfall A, Morley I, Harris S. The Unculturables: targeted isolation
615 of bacterial species associated with canine periodontal health or disease from dental plaque.
616 BMC Microbiol. 2014;14:196.
617 [68] Bor B, Poweleit N, Bois JS, Cen L, Bedree JK, Zhou ZH, et al. Phenotypic and
618 Physiological Characterization of the Epibiotic Interaction Between TM7x and Its Basibiont
619 Actinomyces. Microb Ecol. 2016;71:243-55.