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Advancing gut microbiome research using cultivation

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Culture-independent approaches have driven the field of microbiome research and illuminated intricate relationships between the gut microbiota and human health. However, definitively associating phenotypes to specific strains or elucidating physiological interactions is challenging for metagenomic approaches. Recently a number of new approaches to gut microbiota cultivation have emerged through the integration of high-throughput phylogenetic mapping and new simplified cultivation methods. These methodologies are described along with their potential use within microbiome research. Deployment of novel cultivation approaches should enable improved studies of xenobiotic tolerance and modification phenotypes and allow a drastic expansion of the gut microbiota reference genome catalogues. Furthermore, the new cultivation methods should facilitate systematic studies of the causal relationship between constituents of the microbiota and human health accelerating new probiotic development.

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Introduction

For centuries microbiologists relied on cultivation to study microorganisms, including the microorganisms that colonize the human body. These efforts provided the foundation for microbiology and the identification of pathogens responsible for a wide range of infectious disease. However, comparison of microscopic cell counts with the number of colony forming units growing on nutrient agar plates from the same sample highlighted that a large number of microorganisms are recalcitrant to culture. With the advent of molecular taxonomy it became clear that this 'plate count anomaly' resulted from vast amounts of unknown organisms. Potential reasons for the limited ability of researchers to cultivate all microorganisms from a given environment include complex cross feeding relationships, differential nutrient requirements or very slow growth rates. This finding spurred interest in studying this potentially significant new biology, which so far had gone by unnoticed. A new era of microbiology began with metagenomics focused on the enumeration and characterization of this previously unknown microbial diversity. Since then complex communities have been characterized in virtually every environmental niche [1,2].

Over the past decade evidence has been accumulating regarding the influence of the gut microbiome on human health [3]. Studies have identified correlations between gut microbiome composition and several disease states [4]. The causal relationship between the gut microbiota as a whole and various diseases has been supported by germ-free animal studies in which fecal/cecal transplantations from different hosts lead to distinct phenotypes in recipient animals [5,6]. Recently, some findings of these animal studies have been verified in humans through landmark clinical studies [7,8]. However, in spite of the progress made by culture-independent approaches and fecal transplantation studies, definitive and causal links between specific strains or microbial communities and disease states remain limited. Furthermore, the underlying interactions between specific strains in the microbiome cannot be definitively mapped using culture-independent approaches.

To map microbial interactions and to narrow down on specific constituents of the microbiome that have a causal relationship to disease states, individual strains or communities must be cultivated and interrogated experimentally. Accordingly, there is a renewed and growing interest in cultivation methods to study the gut microbiota [9]. This interest has resulted in several new approaches to mine for new bacteria and these approaches have the potential to propel our understanding of the gut microbiome and its causal relationship to human health states. Furthermore, these new cultivation methods have also revealed that culture-independent approaches have their biases and that polyphasic approaches to study the gut microbiome are needed to further our knowledge of the incredible diversity living on and within the human body [10••,11••,12••]. Cultivation-based approaches are likely to dramatically expand our knowledge of the gut microbiome and open new avenues for the development of next-generation probiotics.

Novel approaches for cultivation of the gut microbiota

A number of advanced cultivation methods have been developed in order to study difficult to culture organisms

over the past decades, these include encapsulation of bacteria into microdroplets or gel particles [13,14], diffusion chambers simulating the natural environment of the samples [15], microfabricated cultivation chips [16]. These techniques enable cultivation of novel species; however, they require access to complex microfluidic or microfabrication technology. In this article focus is on recent simple cultivation methodologies that can be readily implemented in most biology laboratories.

Most simple cultivation procedures have been developed to cultivate specific anaerobic microorganisms from the gut microbiota [17]. However, a couple of pioneering studies compared the species recovered using culture-independent and culture-dependent methods from human fecal samples [18–20]. These studies showed that 30–60% of live cells identified by microscope counts could in fact be cultured as determined by colony counts on solid growth media. Yet, due to the limited sequencing capability available at the time, accurate comparisons of the microbial diversity covered by each method was challenging. With the advent of next-generation sequencing and mass spectrometry based phylotyping it became possible to phylogenetically characterize thousands of isolates with a reasonable effort [21]. The novelty of the recent gut microbiota cultivation approaches arises in large part through the integration of such phylogenetic profiling methods with new cultivation conditions (Figure 1).

Personalized culture collections

In a remarkable study Goodman *et al.* sparked renewed interest in cultivation-based approaches for studying the gut microbiome [10••]. The authors developed a new gut microbiota medium (GMM) and cultivated individual strains of the gut microbiota by diluting fecal samples such that only single cells are added to each culture well (Figure 1a). Individual cells are cultured in liquid media separately in microtiter plates and cultures are collected after growth. The resulting culture collections are comprised of isolated strains that were all derived from the original sample. Notably, >50% of the species identified from a sample using culture-independent methods were covered by the isolates in the culture collections. Furthermore, personalized culture collections were used to inoculate germ-free animals along with uncultured fecal samples and the authors showed that the microbiome response to dietary changes were similar between the two groups. These results challenged the hypothesis that uncultivated microorganisms played a key role in the dynamic responses of the gut microbiome to perturbations.

Culturomics

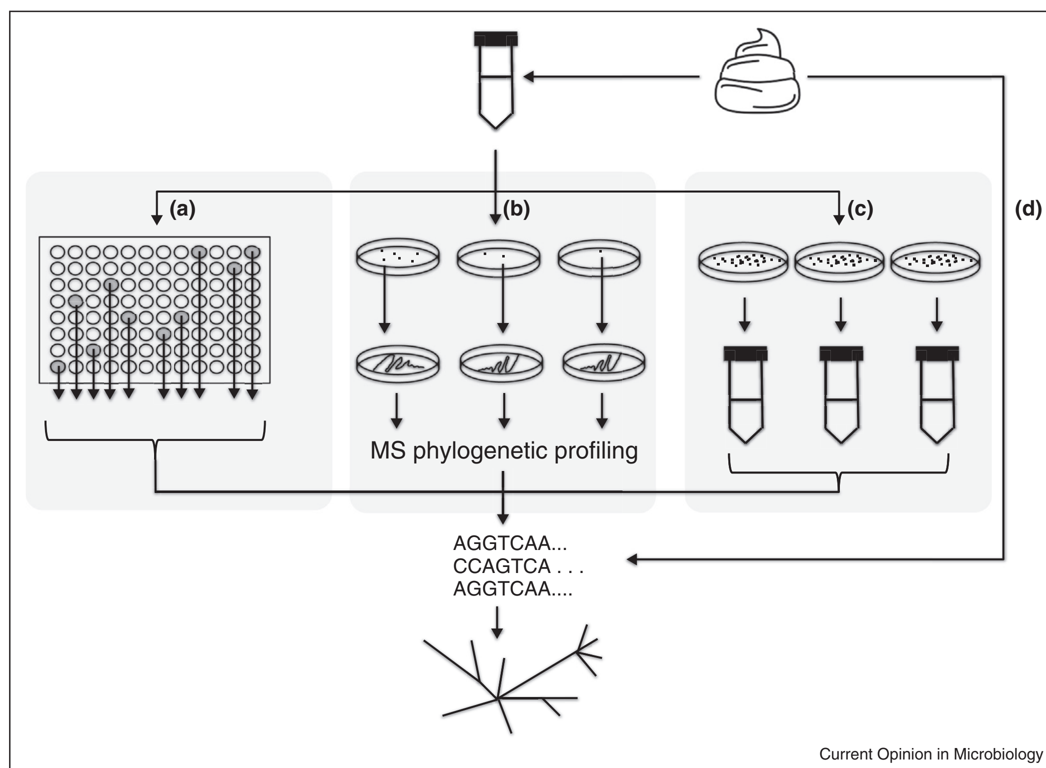
A next major step was the development of a parallel cultivation setup coupled to rapid taxonomic identification termed culturomics (Figure 1b). In a key study Lagier *et al.* used 212 different cultivation conditions chosen to mimic biological niches relevant to the gut environment to isolate bacteria from human fecal samples [11••]. The authors isolated over 30,000 colonies comprising over 300 different species. Notably, over half of the species identified in this study had not previously been identified in the human gut, including a number of entirely new species and genera. The authors compared the taxonomy of the cultured isolates to that resulting from 16S rDNA sequencing of uncultured samples and found that only 51 out of a total of 571 species identified overlapped between the two approaches highlighting the need for using both cultivation-based and cultivation-independent approaches to study the gut microbiome. Culturomics has since been applied to diverse samples as well as to cultivate eukaryotic organisms [22–24].

Cultivation-based multiplex phenotyping

Rettedal *et al.* tested a variety of individual growth media with the goal of identifying a specific cultivation medium that yielded the best representation of the human gut microbiota as characterized by culture-independent methods. It was found that a modified version of the Gifu anaerobic media introduced a minimal bias in the phylogenetic representation of the isolates cultured from human fecal samples compared to the culture-independent characterization. Furthermore, the media allowed the cultivation of over 30% of the viable cells identified through microscopic observation in the diluted fecal samples. Notably, the taxa making up more than 80% of the gut microbiota as determined by culture-independent methods were in fact cultured on the optimized growth medium. Supplementation of this medium with a variety of antibiotics allowed a rapid assessment of the phylogenetic distribution of antibiotic tolerance phenotypes and offered a direct coupling of tolerance phenotypes to specific taxa (Figure 1c). Furthermore, this information was used to tailor selective media in order to culture previously uncultivated bacteria through the rational combination of specific antibiotics.

It should be noted that the recent cultivation studies differed in their approaches to map bacterial phylogeny. While it remains challenging to arrive at a generally accepted species definition [25], the relative consistency of findings between the recent studies in spite of applying different phylogenetic criteria supports the robustness of the overall conclusion; that a majority of the gut microbiome is amiable to culture. However, since bacterial genomes with identical 16S rDNA sequences can vary significantly in their genomic content and accordingly, the identification of a 16S rDNA tag in a culture condition does not necessarily imply that all strains with this 16S rDNA tag can be cultured. Further studies mapping the genomic content of cultured and uncultured samples are required in order to investigate such potential biases.

Figure 1



Overview of novel methods for cultivation of the human gut microbiota. A fecal sample is diluted into relevant media followed by processing according to one of three cultivation-based techniques (a–c) or using culture-independent approaches (d). **(a)** The approach deployed by Goodman *et al*. to construct personalized culture collections involves performing a limiting dilution of the fecal slurry until only individual cells are added into liquid growth media prepared in microtiter plates. Individual bacteria are grown to saturation and the 16S rDNA is amplified and barcoded according to the position of each saturated culture before next-generation DNA sequencing. As a result the phylogenetic identity of each strain in the culture collection is known enabling researchers to retrieve specific bacteria at will. **(b)** Culturomics as deployed by Lagier *et al*. plates out fecal slurry on a large number of distinct culture conditions designed to capture as many relevant growth media as possible with the goal of obtaining the largest biological diversity in the cultivation experiments. Individual clones are purified and subjected to mass spectrometry based phylogenetic profiling. Clones that cannot be identified based on mass spectra are profiled using 16S rDNA sequencing and in some cases genome sequencing. In this way a diverse set of phylogenetically defined strains are obtained from the original fecal sample. **(c)** Cultivation-based multiplex phenotyping, as developed by Rettedal *et al*., plates out the fecal slurry on several different solid growth media comprised of the same basis medium supplemented with a variety of toxins such as antibiotics. Lawns from solid plates are resuspended and these pooled cultures are subjected to 16S rDNA sequencing in order to associate tolerance phenotypes with specific taxa. **(d)** Culture-independent approaches directly extract DNA from fecal samples and sequence 16S rDNA or full metagenomics to obtain the phylogenetic distribution of the sample. Comparison of phylogenetic distributions obtained using culture based approaches (a–c) with culture-independent approaches (d) reveals that a both approaches capture the abundant taxa of fecal samples, but also that certain taxa are sampled only using culture-dependent (a–c) or independent methods (d). Accordingly, researchers studying the gut microbiota should use polyphasic approaches in order to obtain a better representation of this complex environment.

Microbial diversity identified using culture only

Overall these novel approaches to gut microbiota culture have shown that culturing is able to capture a significant subset of the species identified using culture-independent approaches. However, both culture-dependent and culture-independent approaches also capture unique subsets of microorganisms [11••,12••]. While the presence of difficult to culture microorganisms is hardly a surprise, it is worth noting that an equivalent amount of biological diversity may have been overlooked by culture-independent approaches. Indeed, the biological diversity only identified by culturing seems to be at least as significant as that only identified by culture-independent methods. Why is this biological diversity not captured by culture-independent approaches? Analysis of the phylogenetic distribution of species that are isolated using 'culture only' suggests at least one practical explanation. A large majority of the culture only strains belong either to species known for reaching dormant states that are recalcitrant to external exposures such as *Clostridium* that form spores or to species with difficult to lyse cell membranes such as *Enterococcus* and *Staphylococcus* [12••]. Cultivation would allow for germination of spores or give otherwise

difficult to lyse cells the opportunity to grow to larger densities increasing the probability of subsequent detection. Accordingly, organisms with these properties are more readily identified using culturing.

Drug metabolism and its collateral effects on the gut microbiota

Cultivation of gut microorganisms can power discoveries through a targeted effort to identify novel properties of gut microorganisms. One area where cultivation has already had an impact is in the study of drug inactivation by the gut microbiota. By screening individual isolates from the gut microbiota it was shown that some strains of *Eggerthella lenta* are able to reduce the cardiac drug digoxin [26]. Subsequently to this discovery, transcriptomics of cultures of *E. lenta* grown in the presence and absence of digoxin enabled the identification the cardiac glycoside reductase operon, which was shown to be predictive of digoxin reduction in human fecal samples [27•]. This research highlights the potential of cultivating the gut microbiota in order to further our understanding of how it influences drug metabolism. It is expected that studies of this type will be accelerated by the recent developments of improved cultivation techniques.

While the microbiota may metabolize drugs as they are administered leading to potential changes of their pharmacokinetics, drugs can also have significant effects on gut homeostasis with potential impacts on human health. Not only antibiotics, but also other drugs can selectively eradicate species of the gut microbiota causing collateral damage by changing the gut microbial community composition towards a state associated with disease [28]. In order to develop new drugs as well as to improve our understanding of how drug treatment perturbs the gut microbiota we need to understand the tolerance phenotypes of its constituent strains. These tolerance phenotypes are hard to assess from metagenomic data and would require controlled clinical trials in order to infer from the presence and absence of specific species in the treatment and control groups. Instead deployment of cultivation-based multiplex phenotyping can characterize the tolerance phenotypes of the gut microbiota for a range of compounds [12••]. Such data could enable bottom up predictions of the effect of drug treatment on the gut microbiota.

Using novel cultivation approaches to improve reference genome catalogues

Application of cultivation-based approaches and in particular culturomics in which hundreds of distinct media compositions are sampled have revealed a substantial number of species not previously characterized. Lagier *et al.* sequenced 31 novel bacterial genomes and subsequent studies from the same laboratory have yielded several more reference genomes of novel bacterial species [11••,29]. Using a different approach Rettedal *et al.* used the antibiotic tolerance patterns from cultivation-based multiplex phenotyping experiments to design selective growth media with different antibiotics that selected for species not previously cultivated and listed on the Human Microbiome Project's *Most Wanted List* of bacterial species lacking reference genomes [12••]. To assess whether these species were sampled by traditional metagenomic studies, the reads from the MetaHIT metagenomic dataset [30] was mapped to the recently generated reference genomes. The average abundance of these species, quantified based on the fraction of metagenomic reads that mapped to the reference genomes, varied between 0.05 and 1.5% within the individuals characterized in the MetaHIT project. Furthermore, these reads mapped to only 0.5–45% of the assembled genomes. Taken together these findings highlight that these recently cultured strains are not adequately sampled by current levels of metagenomic sequencing and suggests the need for deeper metagenomic sequencing and application of polyphasic approaches to study the gut microbiota.

On the basis of these results it is clear that the novel approaches to cultivating the gut microbiota (Figure 1) can substantially expand reference genome catalogues with rare or new species and possibly also species whose genomes have only been assembled from metagenomic datasets [31]. Systematic efforts of this sort should be undertaken to radically increase the number and diversity of the reference genome catalogues.

Exploring causal relationships using systematic deployment of culture collections

Cultivation of gut microbes is also necessary in order to advance the study of the causal relationships between specific strains or sub-communities of the gut microbiota and disease. Up until now a majority of germ-free animal trials have benefited from fecal/cecal transplantation to implicate the gut microbiota in a specific disease state. For instance it was shown that transfer of cecal microbiota from genetically obese mice to germ free mice led to a greater body weight increase compared to microbiota transfer from non-obese mice [6]. Similarly, changes in the gut microbiota composition have been associated to beneficial effects of gastric bypass surgery [32,33] and several other disease states [2]. These studies have led to a increasing number of case reports as well as a few controlled human clinical trials in which human fecal transplantation is used as a treatment [34]. However, fecal transplantations involve a complex microbial community and only a subset of microorganisms are likely responsible for a particular disease state. In order to prove a causal relationship between a particular disease state and a microbial community or a specific strain it should be tested in isolation.

Currently, the gold standard for proving a causal relationship of a particular strain and a certain disease is through

administration of the strain into either germ-free or colonized animals followed by a monitoring of disease progression or specific biomarkers. This is necessary since a correlation of a particular microorganism to a specific disease state identified from metagenomic data in no way guarantees a causal relationship. This was elegantly demonstrated in a study of inflammatory bowel disease (IBD) where it was shown that commensal strains of *Bacteroides* but not *Escherichia coli* had the capacity to induce IBD phenotypes in an animal model. This was surprising since *E. coli* is correlated to the IBD state and highlights the necessity of controlled testing for defining causality [35•]. In order to do so, researchers must be able to cultivate specific strains that are correlated with disease states. To an increasing extend these efforts will rely on the recently developed cultivation approaches discussed above [10••,11••,12••].

Personalized culture collections represent a particularly promising approach for deciphering the causality between specific disease states and strains or sub-communities [10••]. Using this strategy the causal elements of the microbiota can be identified through the rapid construction of sub-communities selected from the personalized culture collection. Sparse sampling approaches could be used to rationally sample the contribution of several species to a particular disease state using germ-free animal models. In this way, interactions between gut microorganisms could be studied more systematically.

Whereas the throughput of animal studies using defined communities may not be adequate for comprehensively studying microbial interactions, cultivation based approaches could serve as a useful tool. Co-cultivation of strains isolated from the gut microbiome could enable the elucidation of *in vitro* interaction networks as have been demonstrated for soil isolates of *Streptomyces* [36]. *In vitro* interrogation of co-cultures should enable the elucidation of growth promoting (e.g. cross feeding) or growth inhibiting (e.g. production of targeted antibiotics) interactions between gut isolates. Furthermore, as improved microfabrication techniques enable simulations of actual gut environments, it may be possible to identify more physiologically relevant interactions [37]. It is likely that characterization of the interaction networks of gut isolates should enhance our ability to identify strains capable of competing in the complex gut environment.

The majority of currently used probiotics were developed in the pre-metagenomic era. Yet, recent technological advances should aid in the development of the next generation of probiotics. While metagenomic sequence data can be used to identify correlations between microbiome composition and disease states they must be integrated with next-generation cultivation approaches to isolate and cultivate probiotic lead candidates. These lead candidates can be characterized *in vitro* with regards with regards to their interaction networks and strains with beneficial properties *in vitro* can be selected for further testing. Such strains or defined communities can be further assessed in relevant animal models for their efficacy and subsequently move into clinical trials. It is believed that such polyphasic approaches to probiotic discovery and development would open new avenues for microbiome based therapeutics.

Cultivation independent approaches have revealed exciting correlations between gut microbiome composition and disease states; yet, we still need to pinpoint the specific strains and communities that are causative in perturbing human disease and health states. New cultivation methods allow researchers to rapidly expand microbial culture collections opening up new avenues for microbiome research and enabling direct association of specific phenotypic properties to specific strains. Furthermore, such culture collections enable high throughput study of microbial interactions *in vitro* and design of synthetic communities for animal experiments and human interventions. In summary simple cultivation approaches for interrogating the gut microbiome hold significant promise for advancing gut microbiome research.

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