Cultivation of recalcitrant microbes: cells are alive, well and revealing their secrets in the 21st century laboratory

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Any talk of the demise of *in vitro* cultivation as a useful mechanism for revealing many of nature's past and present secrets appears to be unfounded and premature. The first years of this century have been as productive in the cultivation of physiologically novel, environmentally abundant and phylogenetically distinct microbes as were the first years of the 20th century. The diversity of organic and inorganic electron donors and acceptors known to be used during microbial energy metabolisms continues to grow, expanding our appreciation for the niches that may be, or historically may have been, filled by microbes in the biosphere. Either guided and instigated by, or independent of, the results of gene inventories representing diverse environmental settings, significant advances are constantly being made in the isolation of bacteria and archaea, demonstrating either strikingly rich phylogenetic diversity or significant activity and abundance in their respective environments. The potential synergisms between molecular ecological analyses and innovative *in vitro* growth studies are real and should be embraced, rather than treated as dueling agents in some zero-sum game.

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Abbreviations
AQDS anthraquinone disulfonate
AHDS anthrahydroquinone disulfonate
SSU small subunit

Introduction: is cultivation research stagnant?

The past 25 years have yielded unprecedented advances in our appreciation for the breadth and depth of microbial diversity. These advances are mainly derived from the development of high-throughput, nucleic-acid-based technologies that have served to help quantify the striking abundance and genetic diversity of microbes in all of life's recognized reaches. For anyone truly interested in microbial diversity and function, the field of molecular ecology can only be embraced with excitement and continued anticipation. Certainly, many aspects of microbial diversity have now been revealed definitively, through gene inventories and related approaches.

Despite the triumphs of molecular systematics and ecology, the suggestion that *in vitro* growth studies of organisms are passé or irrelevant to reaching an understanding of the true diversity and function of microbes in nature is unjustified. Almost all involved in attempting to cultivate new organisms recognize it is a daunting task; one that is constantly reinforced with each publication of a gene inventory. Reassuringly, however, the past 25 years of cultivation-based studies have led to as many significant advances in our understanding of microbes as has modern molecular ecology over the same period.

There have been a number of breakthroughs in environmental physiology since the 1970s: for example, the cultivation of phenotypically and genetically widespread sulfate-reducing bacteria [1]; the isolation of diverse hyperthermophilic bacteria and Crenarchaeota [2]; the cultivation and demonstration of the broad ecological importance of iron and other metal reductions [3]; the discovery of the physiology of reductive dehalogenation [4]; and the sequencing of the first archaeal genome [5]. This sequence was published within a year of that for the first bacterium, using a thermophilic strain that had, at the time, only relatively recently been isolated from the newly discovered deep-ocean hydrothermal vent communities.

When a phylogenetic tree of life, constructed in 2000, is compared with one constructed in 1987, a remarkable increase in the number of major, recognized bacterial lineages is observed [6]. These can, depending on the authority, be called divisions, kingdoms, or phyla (the latter of which, even if overly conservative, is the accepted term in *Bergey's Manual of Systematic Bacteriology*). The phylogenetic tree constructed in 1987 contained 12 phyla, all of which were identified by the sequencing of small subunit (SSU) ribosomal RNAs (rRNAs) obtained from cultivated organisms (several of them, e.g. thermophiles identified by Stetter, had only been isolated around 1980). The tree of life published in 2000 is remarkable in that the number of known, identified phyla has tripled since 1987. Several of these phyla, along with the substantial number of significant subdivisions within them, are known to us only through SSU rRNA-encoding genes cloned from environmental samples. But is the glass half full or half empty? Of the 27 new phyla identified since

Box 1 Recognized bacterial phyla.

Represented by pure cultures
1. *Acidobacteria*
2. *Actinobacteria*[*†]
3. *Aquificae*[*]
4. *Bacteroidetes*[*]
5. *Chlamydiae*
6. *Chlorobi*[*]
7. *Chloroflexi*[*]
8. *Chrysiogenetes*
9. *Coprothermobacteria*
10. *Cyanobacteria*[*]
11. *Deferribacteres*
12. *Dehalococcoides*
13. *Deinococcus-Thermus*[*]
14. *Dictyoglomus*
15. *Fibrobacteres*
16. *Firmicutes*[*†]
17. *Fusobacteria*
18. *Nitrospira*
19. *Planctomycetes*[*]
20. *Proteobacteria*[*]
21. *Spirochaetes*
22. *Synergistes*
23. *Thermodesulfobacteria*
24. *Thermomicrobia*
25. *Thermotogae*[*]
26. *Verrucomicrobia*

Cultivated, but not-yet-pure
27. Candidate OP10
28. Candidate TM7

Not-yet-cultivated
29. Candidate marine group A
30. Candidate OP3
31. Candidate OP5
32. Candidate OP8
33. Candidate OP9
34. Candidate OP11
35. Candidate OS-K
36. Candidate termite group 1
37. Candidate TM6
38. Candidate WS1
39. Candidate WS6

[*]Phylum assemblages recognized by 1987, in most cases under different names. [†]Gram-positives are now considered to comprise two distinct phyla. Adapted from [6,38].

1987, at least 16 are represented by strains that have been cultured *in vitro*, doubling the number from that in 1987 (see Box 1). Given the often-cited estimates that anywhere from 90% to 99% of all microbial species have yet to be cultivated, it seems remarkably fortunate that only 11 of the currently recognized 37 bacterial phyla remain entirely elusive to our cultivation attempts.

A recent discussion of microbial systematics, including the current state of cultivation-based studies, has been published elsewhere [6].

In this review, I highlight a sample of the most recent cultivation successes, providing examples of how newly recognised microbes continue to be coaxed into laboratory-controlled settings at a rapid pace and with the promise of affecting research for decades to come.

Why do so many microbes remain recalcitrant to cultivation?

There are two overarching reasons for why most microorganisms remain recalcitrant to *in vitro* cultivation techniques. First, many microbes will not grow in the laboratory, primarily because we have an insufficient knowledge or imagination of the chemistry of their native, extracellular milieu, and so are unable adequately to recreate viable laboratory conditions for them. This is the case even for conditions required to grow 'obligately intraeucaryal cellular' organisms, such as *Rickettsia* and *Chlamydiae*. Second, the impatient laboratory scientist might have overlooked the fact that an organism has actually grown under his or her very watch, because obvious turbidity or colonies had not developed. In both cases, it is paramount that we endeavour to entertain a given microbe on its own, well-evolved terms if we are to cultivate it successfully. It is fortunate for the continued development of the fields of microbiology, biology and even earth systems studies that many colleagues have the interest and patience.

Enrichment and isolation of novel physiotypes

The list of electron donors and acceptors successfully used for microbial growth continues to increase and reveal previously unknown microbes (Table 1). The design of media formulations involving the combination of non-traditional growth substrates is therefore a major driving force in cultivating and isolating new microbes.

An anaerobe capable of autotrophic growth via the oxidation of phosphite (+III) as a sole electron donor has been described [7••,8]. It is frequently, but naively, stated in many texts that there is no biological redox cycle for phosphorous, making this finding even more significant.

Table 1

Growth-supporting reactions of relevance to the recent cultivation of novel organisms.

	References
1. $4\ Fe(II) + O_2 + 4\ H^+ \rightarrow 4\ Fe(III) + 2\ H_2O$	[13]
2. $2\ H_3AsO_3$ (Arsenite) $+ O_2 \rightarrow HAsO_4^{2-} + H_2AsO_4^- + 3H^+$	[9]
3. $H_2AsO_3^- + NO_3^- \rightarrow H_2AsO_4^- + NO_2^-$	[10]
4. $4\ HPO_3^{2-}$ (Phosphite) $+ SO_4^{2-} + H^+ \rightarrow 4\ HPO_4^{2-} + HS^-$	[8]
5. $4\ HPO_3^{2-} + 2\ CO_2 + 2H_2O \rightarrow 4\ HPO_4^{2-} + \text{Acetate}^- + H^+$	[7••]
6. $5\ AHDS^{2-} + 2\ NO_3^- + 2\ H^+ \rightarrow 5\ AQDS^{2-} + N_2 + 6\ H_2O$	[15]
7. C_6H_6 (Benzene) $+ 6\ NO_3^- + 6\ H^+ \rightarrow 6\ CO_2 + 3\ N_2 + 6\ H_2O$	[16]

The energetics of phosphite oxidation to phosphate is quite favorable, using any of the typically tapped terminal electron acceptors. Schink and co-workers [7••,8] demonstrated that the isolate respired sulfate, reducing it to sulfide, or CO_2, reducing it to acetate. High-molar growth yields on phosphite were noted, and the authors propose that the oxidation might involve a unique phosphorylated intermediate in addition to the electron-transport processes. In other studies, arsenite, the arsenical analog of phosphite, has also been shown to support the autotrophic growth of both aerobes and anaerobes [9,10]. An aerobe closely related to *Agrobacterium* spp. was isolated on the basis of its ability to use arsenite as an electron donor; however, its growth yields were quite low [9]. Under anoxic conditions, with nitrate as an electron acceptor (yielding nitrite), arsenite oxidation supports the growth of an alkaliphilic autotroph isolated from Mono Lake sediments in California [10]. Although without phototrophic properties, this strain is reasonably closely related to the halophilic, sulfide-oxidizing phototroph *Ectothiorhodospira*. Perhaps the latter should be examined for the ability to oxidize arsenite during anoxygenic photosynthesis.

The use of reduced iron as a sole electron donor during microaerobic, autotrophic growth at neutral pH continues to increase in significance. Several marine iron-oxidizing strains were isolated using inocula obtained from voluminous iron oxide mats located near the Loihi marine hydrothermal vent site [11•]. The isolates reportedly use no other electron donor, and, although obligate aerobes, are sensitive to oxygen. The microbial use of sulfide emitted from hydrothermal vent fluids is now well established, and widely acknowledged in the lay press. The fact that reduced iron, and not sulfide, is emitted from many vents has neither been adequately reported nor has it been investigated at the cultivation level. This study [11•] revealed that the different isolates formed iron oxides with quite distinctive morphologies, similar to the different iron oxides comprising distinct layers of mat covering the submarine site. The observation of morphologically similar oxides in the fossil record was also cited. One isolate was obtained from a 10^{-7} dilution of iron oxide mat material and so is almost certainly a major player in that particular local environment. Molecular analyses using terminal restriction fragment length polymorphism (T-RFLP) [8] supported this conclusion, as did an earlier finding that one of the dominant ribosomal genes recovered during a molecular inventory of the same site was nearly identical to that of the now-cultivated γ-proteobacterial iron oxidizers [12].

In a companion study, Emerson and colleagues [13] gained further insight into the physiology of iron oxidation at neutral pH. Dissolved, reduced iron typically has a half-life of only a few minutes in circumneutral, aerobic waters. This fact had been used as an argument against the possibility of it being used as an electron donor for growth by microbes, a weak argument that fails to recognize that sulfide, which is widely recognized as a remarkable electron donor for many microbes, is even less stable under similar conditions. Using defined media under controlled micro-oxic conditions, the iron-dependent growth of these autotrophs is now irrefutable. Moreover, the cells oxidized iron at rates that can conservatively account for greater than 50% of the iron oxidation occurring at a site such as the Loihi Seamount.

The relevance of the recent findings of Emerson and colleagues to marine and terrestrial biogeochemistry cannot be overstated, especially in light of the importance of the biological reduction of metals.

Coates and co-workers have focused on isolating anaerobes that grow using diverse substrates that until recently were unrecognised or understudied. They describe several new genera of β-*Proteobacteria* capable of respiring the pollutant perchlorate [14]. Investigations into redox reactions centered on the humic compound anthraquinone disulfonate (AQDS) also continue. As well as studying AQDS as an electron shuttle in reductive processes, the use of its reduced form, anthrahydroquinone disulfonate (AHDS), as an electron donor for denitrification was examined [15]. Isolates were obtained from AHDS + nitrate enrichments, initiated using inocula from a range of sources; the isolates oxidized AHDS to AQDS. Strains capable of growth on this previously unexplored mixture of nutrients represented unique lines within the α-, β-, γ-, and δ-*Proteobacteria*. The strains representing unique lines within the δ-*Proteobacteria* were found to affiliate phylogenetically with the myxobacteria, historically viewed as a strictly aerobic microbial group (see below). As it turns out, several of these isolates were capable of using another remarkable physiological electron donor: in an historic finding, one strain was found to be capable of mineralizing benzene under strictly anoxic conditions [16]. Benzene had, for years, remained on the list of substrates that should, in principle, be degraded anaerobically, but such activities had yet to be demonstrated with a pure culture *in vitro*. In addition to many potential ramifications for bioremediation technologies, further studies on this reaction should reveal novel catalytic mechanisms required to activate the benzene ring in the absence of oxygen as a co-substrate.

The enrichment for anaerobic bacteria respiring halogenated xenobiotic compounds has yielded a quite unexpected result: the first unequivocal identification of the long-sought-after anaerobic myxobacterium [17]. Myxobacteria are best known as predatory aerobes that exhibit a complex behavior, multicellular lifestyle and fascinating development (i.e. fruiting body formation). Tiedje and colleagues [17] isolated several strains of a strikingly red-pigmented bacterium capable of respiring nitrate,

fumarate, or chlorinated compounds using hydrogen as the electron donor under strictly anaerobic conditions. With the exception of fruiting body formation, these isolates share phenotypic characteristics of myxobacteria, (e.g. myxospore formation, gliding motility and polar pili). The authors did not address whether the strains are capable of digesting other bacteria or consuming biopolymers for growth, as do many other myxobacteria. Phylogentically, the isolates clustered within the δ-*Proteobacteria*, between the two major myxobacterial lineages. Curiously, these strains, isolated using chlorinated compounds as a sole electron acceptor for respiratory growth, were not obtained from Superfund sites administered by the Environmental Protection Agency (EPA) or other notably polluted sources. Rather, they were obtained from pond and stream sediments, and even household compost, indicating that we have much to learn about the natural distributions of dehalorespiratory organisms.

Niches defined by qualities of light

Specific wavelengths of light define many niches in nature, as potentially, they can be harvested by organisms with specialized photopigments; viable niches are rarely left unfilled. By overlaying the absorption spectra of diverse, cultivated phototrophs, Overmann and co-workers [18] identified a narrow bandwidth at which no organism was known to absorb. They designed specific barrier filters on the basis of this knowledge, such that they could perform enrichments for select anoxygenic phototrophs that could grow at the desired wavelengths. As a result, they cultivated two organisms exhibiting rare absorption maxima at, for example, 911 nm and 963 nm, respectively [18,19]. Evidently, the biosphere remains replete with unexplored niches, filled with organisms awaiting the rational design of selective-enrichment cultivation strategies [20].

Culturing representatives of abundant phylotypes

SAR11 represents a marine α-proteobacterial 'ribotype' believed to account for a staggering 50% of all cells encountered in many open-ocean systems. The organisms encoding the ribotype had remained elusive to all attempts at cultivation for over a decade since their discovery, but now are finally represented in pure culture [21••,22]. The SAR11 organism has been termed '*Pelagibacter ubique*'. The breakthrough followed the development of an elegantly simple, patient and high-throughput method for cultivation and screening of microbes [23•]. The key to cultivating SAR11 appears to be related to the use of dilute growth media (i.e. nothing more than sterilized seawater amended with low amounts of phosphate and ammonium), the ability to recognize very significant (2–3 log) increases in population sizes at 'optical density' thresholds far below the typical detection limit, and the patience to engage a microbe with low yields and slow growth rates (i.e. 30–40 h doubling times).

It seems safe to predict that more will be learned about SAR11 organisms over the next 12 months than has been gleaned during intense molecular ecological studies over the past 12 years.

Isolates representing deep phylogenetic diversity

Janssen and co-workers have been making outstanding progress in the cultivation of organisms representing rich, phylogenetic diversity. Their recent triumphs include cultivation of both strict anaerobes and aerobes, making use of straightforward techniques. Using a defined, sulfide-reduced mineral medium with a plant polysaccharide as the energy source, a fermentative biotype belonging to the phylum *Verrucomicrobia* was isolated and found to be extremely abundant in rice paddy soil, occurring at 10^8–10^9 cells per gram of dry weight inoculum [24]. The isolate has been described as a new genus and species, *Opitutus terrae*, and shares less than 80% rRNA sequence identity with the few other examples of cultivated *Verrucomicrobia* [25]. *Opitutus* ferments polysaccharides to propionate, acetate and H_2, and is well adapted to grow syntrophically with methanoarchaea [25].

Janssen *et al.* [26•] continued this patient approach by examining soil organisms that arise as abundant, but slow-to-appear, colonies on gellan-solidified, dilute nutrient broth. By screening plates for colony development over a three-month period (20% of the colonies did not appear until after the second month), 50 isolates comprising five phyla were obtained from a single soil-inoculum. The collection includes the first isolate of a major subdivision within the *Verrucomicrobia*, previously known only through cloned environmental sequences [26•]. In a similar study using a heteropolysaccharide-based xylan, defined medium instead of diluted nutrient broth, the cultivation successes continued [27••]. Again by waiting 12 weeks for colonies to develop, a collection of isolates was obtained that must be among the greatest recoveries of cultivated microbial diversity ever documented in a single publication. The collection includes the first isolates representing subdivision 3 of the phylum *Acidobacteria*, previously known only from environmental clones, plus strains representing six new genera within subdivision 1. Similarly, isolates representing new orders and families of *Actinobacteria*, γ-*Proteobacteria* and α-*Proteobacteria* that had previously only been known through gene-inventory activities are now also represented in culture.

Whereas the existence of many organisms is anticipated in advance of their isolation because of the results of gene-inventory studies, it is still possible to be surprised by the encounter of a creature representing a previously unrecognized and unanticipated phylum. Stetter and colleagues [28•] have added to their considerable success in culturing thermophiles with the humorously worded description of an 'obligately epibiontic archaeon' that

grows attached to the cells of another thermophile, *Ignicoccus*. After first observing the organism during microscopic examination of enrichment cultures, they initially failed in their attempts to clone its SSU rRNA encoding gene. The gene was so distinct from those reported for other archaea that the PCR amplifications were destined to fail using existing 'universal' and 'all archaeal' oligonucleotide primer sets. The sequenced gene shared less than 81% identity with those from all other known archaea. The organism, available as a defined co-culture with *Ignicoccus*, is likely to represent a new phylum within the Archaea. Its abundance and distribution in nature is as yet unknown, but with redesigned oligonucleotides, its distribution and abundance can now be explored. This finding reinforces the correct notion that the design of molecular ecological analyses must be treated as an iterative process, taking into account new cultivation-derived data as soon as they become available. Molecular ecology is not immune to the often-cited limitations of the cultivation approach — many aspects of the true diversity of life can be assumed to continue to escape our current attentions.

High-throughput cultivation of diverse microbes

In addition to the effective approach to cultivation screens developed by the Giovannoni group, one that is easily adoptable by almost any microbiology laboratory [23•], a revolutionary cultivation technique has been developed in the biotechnology industry [29••]. The approach involves the encapsulation of single cells (i.e. from a mixed microbial inoculum), within a 'gel microdroplet', after which the hardened beads are packed into a column through which dilute media is continuously flowed for long periods. As was essentially the case for the cultivation of SAR11-like cells, sterilized seawater alone proved to be remarkably effective for growing forms unrepresented in culture. Following long incubation periods of constant flow of low nutrients, flow cytometry was used to sort gel beads containing cells that had grown into small micro-colonies from those that had not. As the microscope salesmen might say, 'Illumination is everything'. Bead occupancy was sensitively evaluated by plotting the forward scatter data from a bead against its back scatter. Using this technique, increases in cell number corresponding to as few as five to seven cell-doubling events could be rapidly monitored in 1200 separate beads. The results are impressive. Deep lineages of marine *Planctomyces* and *Bacteroidetes* were recovered, along with several other isolates scattered throughout the bacterial tree of life.

Physiological revelations after isolation

Breznak and co-workers have followed up on their initial study [30] detailing the first isolation of termite-gut spirochetes, which are among the dominant microbiota in that environment. In their most recent study and in a recent review of their findings, they detail the isolation of a third strain, the first termite-gut spirochete isolated from a quantitative dilution of gut contents [31,32•]. Although the strain did not immediately reveal itself to be particularly capable, it was ultimately demonstrated to grow via nitrogen fixation, an important function in the termite gut, and the first demonstration of this function in the phylum *Spirochaetes*. A key to unleashing this activity lay in the composition of trace elements: the tungsten that had long been included in media design by this naïve benchworker (i.e. with the hope of culturing additional biodiversity) is actually known to misincorporate into nitrogenase, resulting in an inactive enzyme. By removing tungsten and adding increased amounts of molybdenum to the medium formulation, nitrogenase activity and diazotrophic growth were revealed not only in the gut isolate, but also in several cultivated free-living spirochetes that had been studied for decades. This knowledge was used to reinterpret past molecular ecological studies, which had not predicted this finding (i.e. because of a lack of relevant pure culture sequence data).

Flagan and co-workers also studied the basis for recalcitrant cultivation after isolation and were able to resolve atypical, aberrant growth kinetics [33]. The unusual kinetics were explained after it was realized that the isolate was growing solely on acyl-homoserine, a product of the slow, chemical decomposition of acyl-homoserine lactone, which was the substrate initially provided. When provided with ample amounts of the degradation product at the onset, the organism grew rapidly and exponentially. A kinetic growth model was determined that describes the atypical growth, and might be used by others to interpret or reinterpret aberrant growth data (i.e. when a half-life chemical-degradation event that serves to drive growth has gone unrecognized).

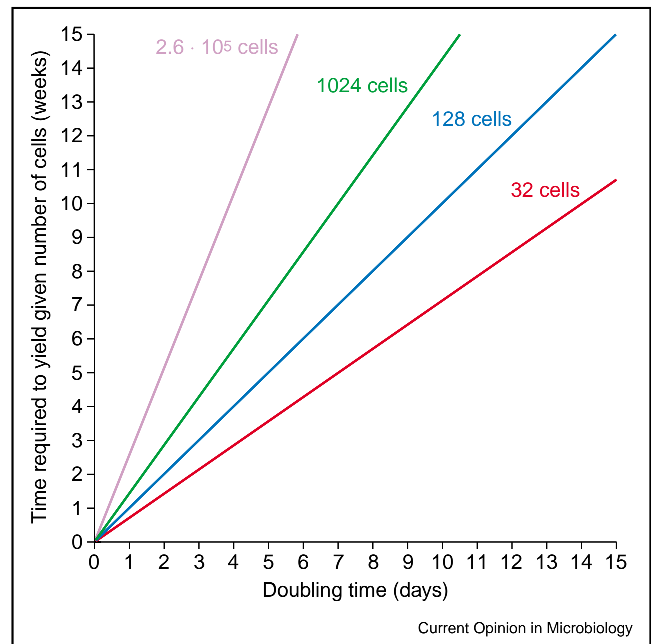
Cultivated but not-yet-isolated organisms

Organisms representing several phyla that were previously considered 'uncultivated' actually have been grown stably as mixed cultures in laboratory bioreactors. When the media used to feed such reactors is synthetic, and when the biota growing in them can successfully be transferred into fresh reactors, it is not at all inaccurate to conclude that the organisms have been cultured. A distinctive organism representing the 'uncultivated phylum' TM7 has been found to grow in a bench-scale reactor community; and it has been assigned a morphotype using *in situ* oligonucleotide hybridization techniques [34]. This TM7 representative is similar in its filamentous morphology to a microbe associated with the problematic bulking of large-scale water-treatment facilities. Because of their dimensions and filamentous nature, cells of the TM7 representative were identified during transmission electron microscopic evaluation of thin sections of bioreactor material. The TM7 representative is sheathed and has the cell wall and membrane

arrangements of a Gram-positive organism, extending the number of bacterial phyla with such an ultrastructure to three. A separate study by Blackall and colleagues [35] revealed that bench-scale reactors, operating on an oscillating oxic–anoxic regime, were not only dominated by representatives of subdivision 4 of the *Acidobacteria* but also the 'uncultivated phylum' OP10. Together, representatives of these two phyla accounted for >85% of the clones recovered during their SSU rRNA-encoding gene inventory. This reactor could accurately be described as being an enrichment culture, and obtaining either a refined co-culture or pure cultures representing OP10 now seems realistic over the short term.

Elsewhere, a simple diffusion gradient device used for the cultivation of microbes from marine sources has been described [36]. Although the cultures obtained did not represent new phylum lineages, the study reaffirms that many microbes can be cultivated in simplified mixed cultures, after which the details of the putatively obligate interaction can be investigated. In transferable, anaerobic batch cultures, a range of microbes related to the phylum currently comprising *Dehalococcoides ethanogenes* have been grown *in vitro* with increased regularity [37]. These organisms appear to have a lifestyle dedicated to the respiration of chlorinated organic compounds. Determination of the nutritional requirements of species growing only as mixed cultures will aid in building new ecological concepts, as well as in increasing the number of known biological cofactors and vitamins that might be applied in the future to media formulations.

Figure 1



The threshold at which population-density increases are resolved, serves to stimulate successes in cultivation. A colony of several hundred bacterial cells is typically about 0.1–1 mm in diameter and, to discern, often necessitates the use of a stereomicroscope. However, an investigator would have to wait approximately five weeks for such a colony to arise from a single cell having a 48 h doubling time — by no means at the extreme of slow growth. By contrast, investigators using high-throughput screening techniques such as cell concentration and blotting of liquid cultures [21••,23•], or flow cytometry of encapsulated ones [29••], are able to discern the growth of a similar cell to a population of around 1000, 100, or even as few as 30 cells in only 1.5–3 weeks. By week five, these investigators would be well on their way to working with a pure culture of a strain representing that first cell type, meanwhile continuing their identification of organisms having substantially slower growth rates.

Conclusions and future directions

One of the most important themes pervading many of the recent cultivation successes is investigator vigilance, either by virtue of instrument design or by brute force, in evaluating cultures over long periods for what might be considered unimpressive signs of growth. Although frustrating, slow growth rates or meagre yields need not discourage those motivated by the well-founded desire to grow important microbes not yet represented in culture. After all, when a single cell divides to become two, and when each of those two clones can be separated into physically distinct cultures, and then each single cell divides just once after each of several successive passages, a pure culture is then in hand. Engaging in such a protocol might seem impractical and tedious, especially when working with an organism such as *Escherichia coli*, which grows rapidly enough on nutrient-rich media to generate a visible colony within a working day. But for a slow-growing strain that might never reach high yields, a tremendous amount of time is saved (and failure avoided) by identifying and sub culturing the microbe of interest at as low a population density as possible (Figure 1).

Observe, recognize, subculture and isolate the microbe of interest first — worry later about whether growth rate and yield might be improved through the efforts of refined studies. This has been the key to many of the successes cited in this review, and, coupled with media reformulations and the identification of unrecognized growth substrates, will continue to spur the *in vitro* cultivation of many more new microbial species with whom we have not yet become acquainted.

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