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The phenomenon of microbial uncultivability

SS Epstein

Most of the microbial diversity on our planet cannot be cultivated, and remains inaccessible. To bring the missing species into culture, microbiologists have introduced over the past decade a number of innovations aiming to meet the demands of new microbes and better mimic their natural conditions. This resulted in a significant increase in microbial recovery yet the real reasons why so many microbes do not grow on artificial media remain largely unknown. The recently proposed scout model of microbial life cycle may provide a partial explanation for the phenomenon. It postulates that transition from dormancy to activity is a stochastic process originating in noise-driven bistability. The model helps explain several otherwise perplexing observations, and informs the future cultivation efforts.

Addresses
Department of Biology, Northeastern University, Boston, MA 02115, USA

Corresponding author: Epstein, SS (s.epstein@neu.edu)

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Introduction to the problem

The microbial richness of the biosphere is large [1], and yet its accessible, cultivable fraction is low, less than 1% [2]. This remarkable gap, 'The Great Plate Count Anomaly' [3•], was noted at the dawn of microbiology [4] and researched by the finest microbiologists of the past (e.g. [5–7]), but has not been closed. The implication is that after nearly two centuries of microbiology as a science, we know remarkably little about the overwhelming part of microbial diversity on our planet. Accessing this missing diversity is important for two key reasons: it likely plays significant roles in the function of the biosphere, and quite possibly represents an untapped mine of novel bioactive compounds [8]. Not surprisingly, learning the nature of the 'missing' diversity is widely recognized as one of the most important challenges facing microbiology [9]. This review will synthesize recent findings about the nature of 'uncultivable' microbial diversity, aiming to provide at least a partial explanation for the phenomenon.

Experiences gained

Traditionally, the cultivation microbiologist would attempt to grow novel species by manipulating the macronutrients and micronutrients in the medium, and changing cultivation conditions. While the success of this approach is undeniable, the rate of microbial discovery it affords is low: just over 7000 valid species have been described to date [10], out of perhaps millions that existed in the samples used. The last decade has seen a renaissance of novel cultivation approaches, all striving to bridge the gap.

One success story is cultivation of SAR11, a ubiquitous marine clade that until 2002 had no cultivable representatives [11•]. This study employed two ideas: dilution to extinction to minimize the influence of fast growing weeds and the use of natural seawater to facilitate growth of oligotrophs [12]. The approach was validated by successful follow up applications, and development of a higher throughput modification of the method [13,14]. A different high throughput cultivation approach based on co-incubation of cells individually encapsulated into microdroplets, under low flux nutrient conditions, also considerably increased microbial recovery [15]. Likewise, lowering nutrient concentration of standard media enabled longer incubation and resulted in isolation of species that did not appear to grow otherwise [16].

Departing from conventional thinking, Bruns *et al.* [17] explored whether addition of signaling compounds could trigger microbial growth and showed that supplementation of growth media with cAMP and homoserine lactones did increase microbial recovery. This suggests that standard media components may be necessary but not sufficient for growth of some species.

This finding is in line with the old idea that metabolites of other species may be the key to growth of many microbes. Significance of commensalisms and mutualisms in microbial world has been a popular explanation as to why many microbes refuse to grow in isolation [18], but the explicit use of co-cultivation to increase microbial recovery is a recent development. D'Onofrio *et al.* [19] directly showed that some marine microorganisms will not grow unless paired with other species, and that the critical growth factors exchanged are siderophores. How general this observation is remains to be confirmed. There are indications that, at least if co-cultivated in Petri dishes, hundreds of pairs of microbes may be required to detect partners exhibiting positive interactions (Epstein, unpubl.), especially in species from the human microbiome (K. Lewis, pers. comm).

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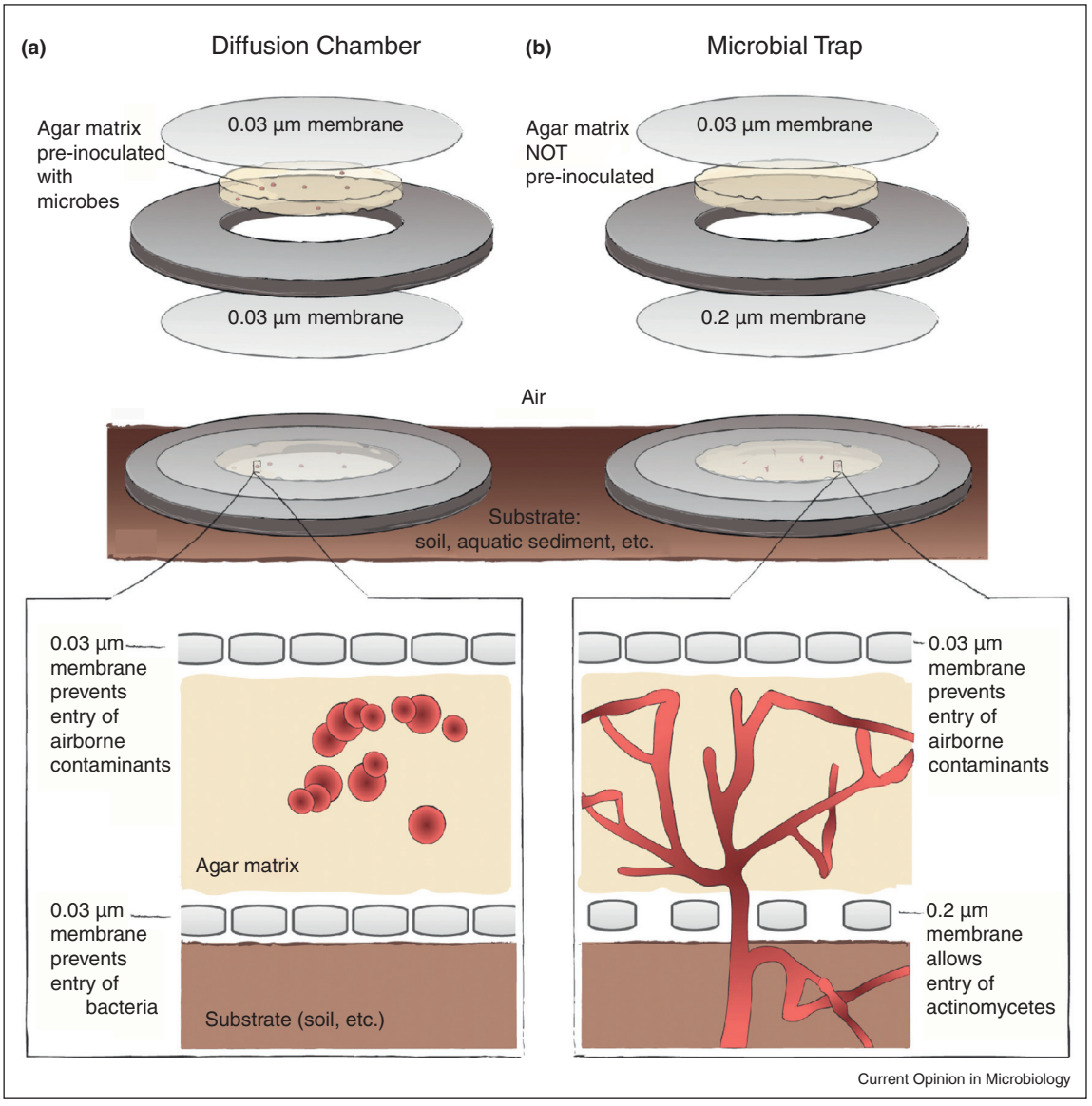
Figure 1



The principle of *in situ* microbial cultivation. **(a)** Diffusion chamber. Environmental cells targeted for cultivation are mixed with agar, sandwiched between two 0.02-µm pore-size membranes, and returned to the natural environment of the cells for cultivation. **(b)** Microbial trap. Superficially similar to the diffusion chamber, the principle is different. The membranes have larger pores, and it is not inoculated with environmental cells, and is incubated in the environment with sterile agar inside. The pore size above 0.2 µm allows microorganisms to penetrate into the inner space, and form colonies. Both methods are based on the expectation that diffusion through membranes will establish conditions inside the device that closely mimic the natural conditions, allowing strains with unknown requirements to grow and be isolated. The trap method is more selective and enriches for filamentous microorganisms.
Reprinted from [27].

If signaling compounds and metabolites of neighbors are important growth factors, identification of the specific substances critical for growth of the given species is a challenge. One way to minimize the guess work in cultivation is to simply use the naturally occurring chemical milieu for microbial incubation. Environmental cells placed into a diffusion chamber and incubated in their own habitat will have access to the growth components from this habitat (Figure 1). Therefore, if a species grows in nature, it should grow inside the diffusion chamber, enabling cultivation of populations with unknown requirements. This idea was reduced to practice differently by several research groups, and all variations proposed to date showed significant improvement over standard cultivation [20–23,24•,25,26] (for a recent review see [27]). Of note is an interesting observation that

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emerged from these studies: a round of growth *in situ* leads to development of a small number of cultivable variants in the populations that otherwise would not grow *ex situ*. These spontaneously domesticated variants can then readily be grown in the lab, facilitating the study of their properties.

It seems certain that the research of the past decade moved microbial cultivation to a qualitatively different level. In retrospect, it is not surprising that the main lesson learned is that mimicking natural conditions enhances the success of cultivation. The simplest and most typical explanation is that a close match between incubation and environmental conditions provides microbes with the right growth factors at the right concentration. While this is likely correct in many cases, several publications reported intriguing, if not puzzling, observations suggesting that the nature of 'The Great Plate Anomaly' may be more complex. Three selected points deserve special consideration — and explanation.

1. It is tempting to explain the successful cultivation of SAR11 [11•] by the medium used (low nutrient concentration and presence of naturally occurring compounds from sea water). Clearly these conditions were sufficient for some SAR11 cells to grow, but these represented a tiny minority of all SAR11 cells subjected to these very conditions. Why then the overwhelming majority of cells of this clade did not grow?
2. It appears logical that the *in situ* based cultivation techniques are successful because they provide access to critical growth factors supplied by neighboring species. Kaeberlein *et al.* argued [24•] that it is these factors diffusing into the growth chamber from the environment that explain why 300 times more colonies grew in the diffusion chamber than in parallel trials in Petri dishes. The implication is we should expect the newly obtained cultures to be dependent on these factors, and the success of subsequent subcultivation efforts be conditional on the continuous presence of the natural environment as their source. However, this is not the case. Small numbers of cells within diffusion chamber-reared populations spontaneously domesticate and acquire the ability to grow on trivial media. This was readily observed in all species tried and in both marine and groundwater environments [28–30], and independently confirmed by Y. Aoi (personal comm.). Whatever the nature of the process of domestication, if cultivable variants form during *in situ* incubations, they should also form during normal population growth in their natural setting. But, if they do, and such variants are indeed present in the environment, why does 'The Great Plate Phenomenon' exist in the first place?
3. An observation of note is that there appears to be some randomness in how and when a new species gets successfully cultivated. For example, representatives of Verrucomicrobia had been eagerly sought, as the phylum had no cultivable members for a long time. Surprisingly, when the first cultures finally grew [31], they did so on the media extensively used in the past, begging the question why they were not isolated sooner. Others obtained more verrucomicrobial isolates, also using no special cultivation 'tricks' [29]. This seems to indicate that there are factors we may not be aware of that influence how and when microbes initiate growth. Cells from the same population appear to behave differently even if inoculated into identical cultivation vials or Petri dishes; some will not grow, whereas a few will propagate even if little attempt is made to mimic their natural environment.

To summarize, the newly advanced cultivation technologies have undoubtedly produced new species en masse, but it is not quite clear why. The following section will analyze the knowledge gained, consider surprises encountered, and build a model that attempts to accommodate the observations made thus far.

Emerging explanations

In the literature on microbial cultivation and growth patterns, the reader can discern one ordinarily observed trait: often, a small subpopulation is present that is very different in its growth potential than the majority of cells. Out of all SAR11 cells present, only a few grew [11•]. From *in situ* enrichments, only few cells became domesticated [30]. Ageing cultures always contain a few readily viable cells. Populations may be in the viable-but-not-culturable state, but they universally contain a small fraction of 'normal', ready to grow cells (for a recent review, see [32••]). This paints an image of a natural microbial population as consisting of two entities, one small and active, and the other large and dormant. If we postulate that the transition between the two states is regulated by a toggle of sorts, with the switch working stochastically, it may help explaining the perplexity of the three points from above.

The proposal that a stochastic switch may be involved into a microbial 'decision' to divide forms the basis of the 'scout' hypothesis (Figure 2; [33]; for detailed description see [34•]). The gist of the idea is that firstly, dormant cells are able to stochastically wake into activity, and secondly, active cells will explore the available resources (hence the name, scouts). The absence of such resources will of course lead to the scout's demise, but if the awakening is a low frequency event, even a small population will be able to produce new scouts for months and years. Eventually, a new scout will arise under growth permissive conditions, which will reestablish the population. Indirectly, the idea is supported by well-known cases of epigenetic, noise-driven, stochastic bistability [35,36, 37•,38,39]. Accordingly, the scout is thought of as a result of a stochastic change in, for example, the expression or

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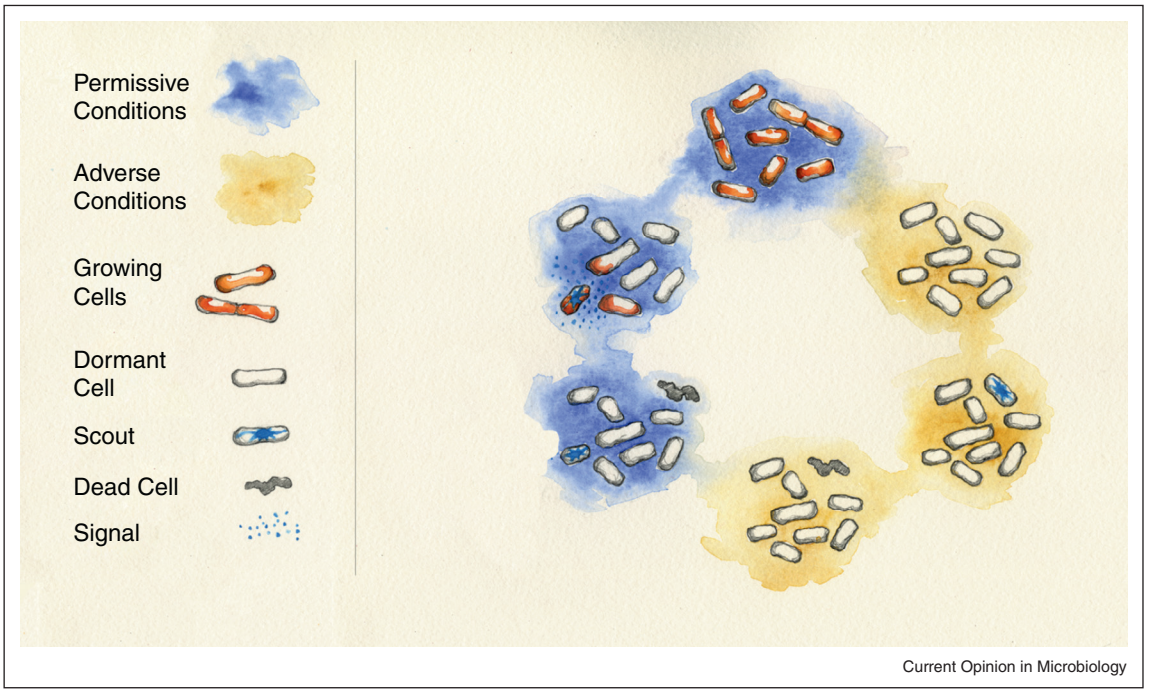
Figure 2



Scout model of the microbial life cycle. (I) Growth under permissive conditions, (**II**) dormancy under adverse conditions, (III) stochastic awakening from dormancy, in the form of 'scout' cells, followed by either their death (IV) or proliferation (V–VI), depending on environmental conditions, and, in case of proliferation, (VII) production and accumulation of signaling compound(s) inducing growth of the remaining dormant cells, concluding the cycle. Reprinted from [34•].

repression of a master regulatory gene. The scout formation is not a result of a genetic change; indeed, it is identical to a typical cell in an actively growing population. Consequently, the scout hypothesis views a clonal population as consisting of two phenotypes: the dormant and active, which alternate in dominance depending on the environmental conditions.

A variant of this model ascribes an additional function to the scout. It is possible that the population formed by the successful scout will accrue, in a quorum sensing [40••] fashion, a growth-inducing factor, which will induce the remaining dormant cells to activity. In a species with this survival strategy, a cell transitions from dormancy to activity either as a result of noise in the gene network, or if induced by a growth factor. Such induction has been shown in a variety of microbial species and may be widespread in nature [30,41,42,43•].

The model has recently gained experimental support [44,45] and provides the following insights into the nature of uncultivated species. Consider the case when the majority of cells in the environment are dormant, or nearly so. For the population to be cultivated, it should be amply represented in the inoculum so as to statistically contain enough active scouts. Alternatively, the incubation should be long enough to give less abundant dormant populations sufficient time to form scouts. Regardless, only a small proportion of cells in a population will form colonies, by necessity producing an image of the rest as 'uncultivable'. If the population in the inoculum is small, the statistical chances of it containing scouts will be minimal, and the entire species may be labeled as 'uncultivable'. This may explain the observations summarized in Point 1 above: it is possible that only selected cultivation trials contained active, 'scouting' SAR11 cells. The idea of stochastic awakening also explains the nature of domestication (Point 2): the few cultivable 'variants' among numerous 'uncultivable' kin may be none other than rare scouts produced by the latter. Note that the same idea also explains why populations of viable but not culturable cells (VBNC, [32••,46•] always have a few culturable cells mixed in. Finally, the random transition between dormancy and activity may be relevant to Point 3 as well. According to the model, the success of cultivation of a species is proportional to the number of scouts present at the time of cultivation. Rare species will have few to no scout present, perhaps requiring replicate cultivation experiments to produce a single colony. Should such experimentation continue for some time, the emergence of this colony may appear random to the observer.

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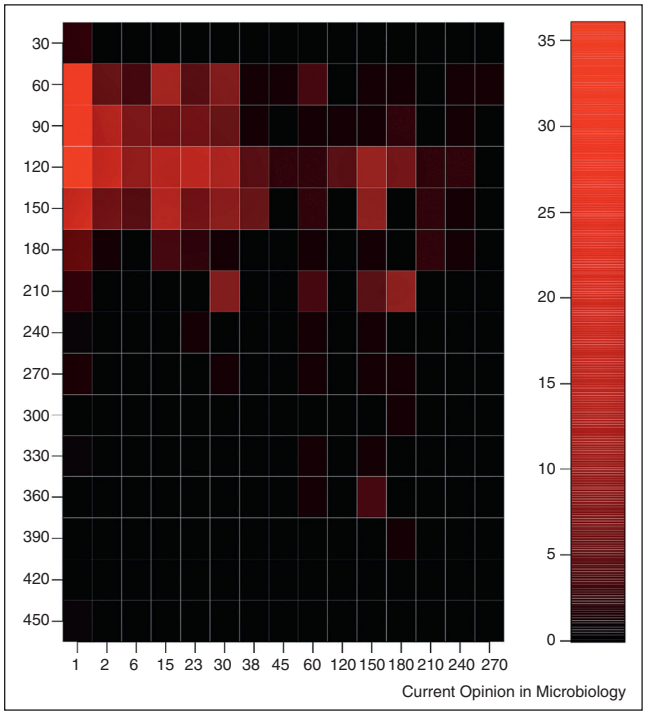
The stochastic awakening of dormant cells into scouts is not the only possible explanation for the apparent 'uncultivable' nature of some species. Scouts that produce growth inducing signals offer an alternative scenario. If the cell's growth depends on activities of the growing kin, its propagation will require a significant presence of such kin. This requirement is met in the environment, at least periodically. If so, it should be met inside environment-incubated diffusion chambers as well. Either way, sooner or later the growing population should form variants that lack, or do not express, the responsible regulatory mechanism. Unlike the majority of their kin, such variants will be able to grow in isolation from the environment and other cells. Note that the same applies to the situation when the required growth factors are supplied not by kin but neighboring (and different) species. Perhaps the cultivated cells of SAR11 [11•], and domesticated cells from [30], were such variants, as are the VBNC. Taking the idea to its logical end, one could see these variants as the colony forming units for some or most of the microbial isolates in the existing culture collections. If so, an interesting question is just why such variants do not take over the populations in nature, what selects against them in the environment, and why growth dependencies on neighboring cells are advantageous.

Conclusions and future directions

A significant number of novel cultivation methods have been introduced over the past ten years, all leading to a significant increase in microbial recovery. However, the reasons why so many species do not grow in the lab are not well understood. The scout idea provides at least a partial explanation for the apparent uncultivability of the microbial majority. It teaches that at least some 'uncultivables' are not fastidious at all: the active component of their populations, the scouts are readily cultivable on existing media — except they may be too rare to capture. This is perhaps the most important lesson learned from the past studies since it informs future cultivation efforts. Three ways appear promising to enhance these efforts. The brute force approach is to simply increase the conventional cultivation effort and geography of sampling. The more targeted approach is twofold. One is to incubate for longer time frames, waiting for dormant cells to wake up and form colonies. One of the longest incubation experiments conducted to date empirically showed that thus obtained colonies are not a pool of specialized 'slow growers'. Instead, they represent 'normal' species, capable of fast proliferation, which happened to have awakened late during initial incubation, in apparently a stochastic fashion (Figure 3; [44]). Incidentally, this means that a larger number of Petri dishes incubated for a limited time should bring as much microbial novelty as a smaller number incubated for longer, which has been confirmed by direct experimentation [45]. Another approach targets the arguably most interesting species: those that are not merely present in the environment but are the active members of the community. Any variant of the *in situ* cultivation methodology will enrich for exactly such species by the nature of the method. The enrichment means an increase in the biomass, and thus in the number of active cells. This in turn will improve the chances of these species' subsequent subcultivation *ex situ*. Armed with these approaches, the cultivation microbiologist should be able to minimize the gap between the microbial richness in nature, and the number of species in culture, for the benefit of both basic and applied microbiology.

Figure 3



Regrowth pattern indicates stochastic awakening of dormant cells. Marine microorganisms were cultivated on a conventional medium for 1.5 years in a single-cell format, and subsequently subcultured. The heat map plots the time required to form visible growth during initial isolation (*Y* axis) and during subculturing (*X* axis), in days. Note that overwhelming majority of isolates observed between 45 and 175 days of the initial incubation re-grew within 24 hours (red squares in the upper left corner of the heatmap). This argues against their slow growing nature and indicates instead prolonged dormancy, followed by stochastic awakening at random time points and fast proliferation thereafter. Reprinted from [44].

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