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Crystal Ball

Time as a microbial resource

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Microbes need resources for energy and cellular building material. They also need access to clement conditions with liquid water and a cellular damage rate that is lower than repair. When deprived of resources and clement conditions, microbes often enter some form of dormancy (e.g., by ceasing cell division, slowing metabolic rate, or forming an endospore) until they can grow again (Lennon and Jones, 2011). For example, at night, phototrophs wait for the sun to return. In winter, soil microbes wait for warmer temperatures. Microbes that cause diseases like tuberculosis can stay dormant for years, waiting for the cessation of antibiotic or immune system bombardment (Alnimr, 2015). But what about longer timescales? Unlike multicellular life, microbes survive in an extremely broad range of conditions and can access an amazing variety of resources to maintain cellular functions in the absence of cell division (Finkel and Kolter, 1999). This means that they have the potential to be dormant for much longer than a few months or years. There is no theoretical reason microbes cannot survive on maintenance energy for hundreds or thousands of years, or longer, with little to no cell proliferation (Hoehler and Jørgensen, 2013; Lever *et al*., 2015). Given this lack of theoretical constraints on the length of microbial dormancy intervals, two questions arise, (i) is there evidence for the existence of organisms experiencing very long dormancies? And (ii) what could be the advantages of such long wait times?

The only way to be certain about a microbe's physiology is to isolate it from its natural environment and grow it in a laboratory (Overmann *et al*., 2017). Many dormant populations have individual cells that act as 'scouts', coming out of dormancy at random times to periodically sample for the return of conditions conducive to growth (Epstein, 2009; Buerger *et al*., 2012). Some of these scouts can then be given growth factors and cultured. However, there are two problems with relying on vegetative growth to study microbes capable of long-term dormancy. The first is that direct sequencing of DNA from many environments shows that most microbial cells are phylogenetically divergent from those that have ever been cultured by anyone (Lloyd *et al*., 2018; Steen *et al*., 2019a). This may be the driver behind 'the great plate count anomaly', which posits that <1% of cells in many samples can be cultured easily (Staley and Konopka, 1985). It is important to note that not all environments are subject to the great plate count anomaly; environments that undergo rapid environmental change or have copious nutrients, such as the human gut (Lloyd *et al*., 2018) and lakes recently inundated with volcanic ash (Staley and Konopka, 1985) are often dominated by easily cultured cells. However, for many environments under stable conditions, not-yet-cultured microbial clades dominate total cell abundance and the great plate count anomaly applies. There is good evidence to support the notion that the reason these groups have not yet yielded to culture is that they are obligate slow growers that are not easily sped up to grow on normal laboratory timescales. It has long been recognized that many more cultures can be obtained from natural samples if they are incubated for many months (Davis *et al*., 2005). In fact, the biggest recent advances in culturing culture-resistant clades have been in very slow-growing organisms. Highly abundant seawater microbes like *Nitrosopumilus* sp., *Pelagibacter ubiquitans*, and *Prochlorococcus sp*. have doubling times of a day or longer meaning that it takes them up to a month or longer to reach stationary phase (Partensky *et al*., 1999; Rappe *et al*., 2002; Könneke *et al*., 2014). Marine sediment microbial cultures and enrichments operate over even longer timescales, with *Atribacteria* doubling over 5 days, *Lokiarchaeota* doubling over 14–25 days, and uncultured members of the *Methanosarcinales*, called ANME-2, doubling over 7 months (Nauhaus *et al*., 2007; Imachi *et al*., 2020; Katayama *et al*., 2019). These cultures grow so slowly that detailed physiological assessments and genetic manipulations are nearly impossible on human timescales, even though they have technically been cultured. The second problem with relying on cultures to study extremely long-lived microbes is that physiologies in the

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vegetative state may differ greatly than when a microbe is subsisting at low metabolic activity over thousands of years. Therefore, while culturing is essential to be sure about an organism's physiology, it cannot be used to study every type of ultra-slow growing microbe in every type of condition.

For this reason, culturing efforts must be coupled to direct study of ultra-slow organisms in natural samples. However, identifying microbes that are dormant for many years while they wait for infrequent events is challenging in natural samples. To observers on the human timescale, such ultra-slow organisms would appear to be doing nothing. As an analogy, the California coastline is a constantly churning mass of rocks over the geological timescales, but to humans, it is stable enough to build houses on. These houses must be sound enough to withstand the occasional earthquake, but they will not survive the reorientations of land as they are spun, submerged, and exhumed over the course of a few million years. Luckily, modern marine sediments offer a natural laboratory in which to study long-term dormant microbes without either speeding them up in laboratory cultures or waiting thousands of years for changes to occur. Subtle geochemical changes can be quantified over long timescales by comparing changes in concentration of nutrients with the rate of sediment deposition (Berner, 1980). The resulting reaction-transport models show that the total rate of energy delivery to marine sediment microbial communities is often many orders of magnitude lower than that required to support laboratory cultures (Hoehler and Jørgensen, 2013; Larowe and Amend, 2015; Bradley *et al*., 2020). This means that these microbial communities do not have enough energy to maintain a steady rate of cell division. Further evidence that microbial communities buried over many meters in marine sediments are largely in a non-growing state is the fact that very little genetic novelty appears even after timescales over which mutations or ecological competition would arise if the populations were growing normally (Walsh *et al*., 2016; Starnawski *et al*., 2017). Turnover times for these subsisting microbial communities have been calculated to be a few tens of years (Braun *et al*., 2016). This does not necessarily mean that cells undergo traditional replication and cell division once every 30 years. A turnover of biomass can occur by gradually replacing all the cellular material, lipid by lipid, nucleotide by nucleotide, such that over roughly half a century, all the molecules have been replaced. Actual cell division events are likely to take much longer, or possibly never occur until resources return, which could take hundreds to millions of years.

Are these microbial cells that do not replicate for multiple decades, or possibly even hundreds, thousands, or millions of years doing so because they are waiting for an event that occurs over these timescales? An alternative option would be that these microbes are not adapted to ultra-long dormancy, but instead just happen to find themselves in some sort of suspended animation for millions of years before they are eventually subducted under a continent and crushed or scalded to death in a subduction zone. While accidental subsistence in multi-thousand-year stationary phase is an option that must remain on the table, there is some evidence that the organisms who find themselves in this predicament are evolutionarily poised to do so. With increasing depth in estuarine sediments, microbes express enzymes with a higher specificity for the substrates that are available in the subsurface, suggesting that they are adapted for subsurface dormancy with some amount of metabolic activity (Steen *et al*., 2019b). Subsurface microbes also have physiological adaptations for ultra-slow metabolisms and cell divisions (Bird *et al*., 2019). Additionally, the microbial clades found in the subsurface are not just leftovers of pelagic communities that persist rather than perish as they are buried; instead they are distinct from those found in seawater (Teske and Sørensen, 2008; Durbin and Teske, 2012).

Therefore, it is likely that the organisms found in marine sediments are adapted to live in marine sediments, despite not really being able to grow there. But, even if they are well-adjusted and 'happy' during this long period of dormancy, they must grow somewhere – these cells cannot have been dormant since Earth began 4.5 billion years ago. We then must ask question two: What are they waiting for? If we encounter a dormant microbe in soil in winter, we can presume that it aspires to become vegetative in summer. What is the equivalent for a deeply buried marine sediment organism that is dormant for thousands to millions of years? What is their version of summer?

To determine what events cause long-term dormant organisms to regain their vegetative state, we must assume an evolutionary framework where long-term dormancy is an adaptation that has an eventual evolutionary pay-off. The pay-off would be the dormant microbe someday 'wakes up' and produces progeny that receives a survival benefit from having been one of the first to access the resources when they become available. Evidence for this model comes from laboratory cultures that have been studied in stationary phase for many years. When *E. coli* cultures kept in stationary phase for months or years are competed against freshly grown *E. coli* cultures under starvation conditions, the pre-adapted cultures outcompete the freshly grown cultures, a trait that has been named growth advantage in stationary phase (GASP) (Finkel, 2006). If the same is true for microbes living in marine sediments, then they would have an advantage over fresher organisms if they got the chance to compete for meagre resources, like a yogi accustomed to deprivation competing with a glutton during a famine.

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Adaptation to long-term dormancy is likely driven by growth resources that vary with periodicities of equivalently long timescales. Since marine sediment microbes are dormant over hundreds to millions of years, they are likely 'waiting' for events that occur over these timescales. Geological processes occur on a range of timescales long enough to suffice. On the short end of the timescale, microbes could be adapted to multiyear flood, drought, or storm cycles, much like cicadas that undergo a 17-year diapause. But geological events over longer timescale events could also drive dormancy. Dormant microbes in marine sediments could re-enter a vegetative state upon return to the seafloor where nutrients are fresh. Sediments over the upper meter can be exhumed and redeposited on the seafloor with bioturbation, small gravity flows, or extreme storm events (if the water is shallow enough). More deeply buried sediments could be exhumed over much longer timescales and by much larger events. Whole submarine cliffsides can be redistributed by submarine landslides, slumping, or turbidite flows. Over even longer timescales, microbes that have managed to survive burial many hundreds of meters deep into marine sediments could be exhumed when oceanic plates strike other oceanic or continental plates in subduction zones. Here, accretionary prisms or mud volcano eruptions offer potential opportunities for bringing a few of the deeply buried microbes out of dormancy (Hoshino *et al*., 2017). Other environments such as ancient permafrost (Gilichinsky *et al*., 2007; MacKelprang *et al*., 2017; Liang *et al*., 2019) also likely have long-term dormant organisms. The evolutionary pay-off for such organisms could be the end of glaciated periods following Milankovitch cycles, although it is important to note that modern day permafrost is thawing faster than expected due to climate change (Crowther *et al*., 2019).

Just as microbes are not dependent on oxygen, they are also not dependent on achieving a certain growth rate. It is well known that the ability to respire anaerobically increases microbes' environmental range, preventing their imprisonment in oxic environments. Likewise, the ability of microbes to survive long, perhaps extraordinarily long, periods of deprivation enables their habitat expansion into a greater range of timescales. Time itself becomes a resource that microbes can exploit to access new habitats. They can wait for resource-replenishment events that are beyond the temporal reach of organisms that are constrained to faster growth rates. Such ultra-slow microbes could be viewed as K strategists within the classic ecological framework of r vs. K strategies, which have slower reproduction rates, longer lifespans and maintain steady-state populations to maximize use of the carrying capacity of an environment (Pianka, 1970). The caveat, of course, is that ecological paradigms such as this one are designed around multicellular eukaryotes and include predictions about offspring rearing and body size that do not perfectly translate to microbes surviving over geological timescales. The novelty of viewing time as a microbial resource does not, therefore, signify a new ecological paradigm, but a new ecological niche. By actively focusing on how microbes exploit a vast range of timescales, perhaps longer than previously recognized as possible, we can open up new understandings for how microbes and Earth systems interact.

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