Trends in Microbiology

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Review

Microbial life in slow and stopped lanes

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Microbes in nature often lack nutrients and face extreme or widely fluctuating temperatures, unlike microbes in growth-optimized settings in laboratories that much of the literature examines. Slowed or suspended lives are the norm for microbes. Studying them is important for understanding the consequences of climate change and for addressing fundamental questions about life: are there limits to how slowly a cell's life can progress, and how long cells can remain viable without self-replicating? Recent studies began addressing these questions with single-cell-level measurements and mathematical models. Emerging principles that govern slowed or suspended lives of cells – including lives of dormant spores and microbes at extreme temperatures – are re-defining discrete cellular states as continuums and revealing intracellular dynamics at new timescales. Nearly inactive, lifeless-appearing microbes are transforming our understanding of life.

Highlights

Unlike in most laboratory settings, microbes in natural habitats often lack essential nutrients and withstand widely fluctuating or extreme temperatures; thus, laboratory studies fail to capture the widespread phenomenon of slowed or suspended lives of microbes manifested as, for example, in dormancy and quiescence.

Quantitative, single-cell-level measurements and mathematical modeling are overcoming the challenges of observing and understanding slow dynamics of individual cells at long timescales.

New conceptual, quantitative frameworks have re-defined and clarified microbes' suspended states, with dormancy defined as a continuous spectrum and antibiotic-induced growth-arrest defined into distinct states.

Studying slowed and suspended growth in microbes revealed fundamental constraints to remaining viable without growing and limits to slowing a cell's life.

Importance of seemingly lifeless microbes

Living systems remain dynamic – they move, grow, or self-replicate – for much longer than their constituent molecules' lifetimes. Schrödinger eloquently encapsulated this trait in his thought-provoking book, '*What is Life?*', by describing living systems as entities that resist 'the decay to equilibrium' [1]. By contrast, non-living systems exhibit relatively short-lived dynamics that thermally equilibrate. Although life's dynamic nature serves as the critical distinction between living and non-living systems, the distinction blurs when we consider cells that appear static – cells that appear lifeless – without being dead. Indeed, dormant spores, like those of *Saccharomyces cerevisiae* and *Bacillus subtilis*, seem lifeless but are viable: they self-replicate when essential nutrients appear. What properties make lifeless-looking cells, like dormant spores, viable? (Figure 1A). Researchers recently began to rigorously address this question with quantitative modeling and experiments on microbes that often exist in nature as lifeless-looking cells. Surprisingly, recent studies revealed that some lifeless-looking microbes exhibit intracellular processes that maintain their viability. These processes are difficult to detect and therefore had eluded discovery until recently [2].

Loosely speaking, a microbe is said to 'slowly grow' if its growth rate, the inverse of the doubling time, is much smaller than the value corresponding to growing in nutrient-rich media at a growth-optimized temperature – a fast-growth condition that virtually all laboratories use (e.g., 30°C for *S. cerevisiae* and 37°C for *Escherichia coli*). Although this comparison makes slow growth seem unusual, the faster growth in laboratories is, in fact, unusual; microbes in natural habitats often lack the essential nutrients found in laboratory media and withstand widely fluctuating or extreme temperatures (Figure 1B,C). Consequently, much of the microbiology literature does not examine the slowed or suspended lives of microbes in natural habitats such as winter environments, glaciers, and marine sediments [3–5]. The underexplored topic of slowed and suspended lives of microbes is both timely and important, given its relevance to natural habitats and the ongoing threats of climate change.

Their practical importance aside, slowed or suspended lives of microbes pose conceptual questions that transcend microbiology. One of these is whether cells can self-replicate arbitrarily slowly, taking 1

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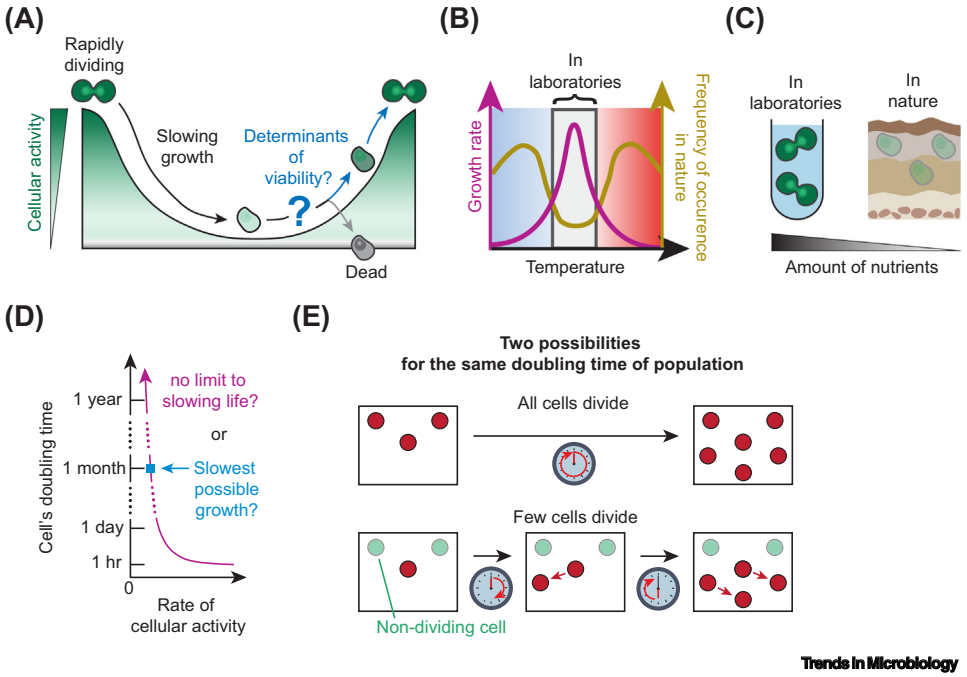

Figure 1. Microbes with slowed or suspended lives are abundant in nature, but not in laboratories, and pose questions about fundamental limits to life. (A) Illustration with the key questions. When a cell's growth drastically slows down or stops, which factors determine whether the cell remains viable? What determines whether a lifeless-looking cell remains alive or dies? (B) Illustration of a cell's growth rate (purple curve) and frequency of occurrence of each temperature in nature (brown curve), both as a function of temperature. Growth-optimizing temperatures inside laboratories (gray zone). Frigid temperatures (blue zone) and high temperatures (red zone) in nature that slow or stop microbial growth. (C) Illustration showing culture conditions in laboratories with abundant nutrients (test tube) and natural habitats for microbes in soil that lacks nutrients. (D) Illustration of a fundamental question about life: can a cell's doubling time (purple curve) increase without an upper bound by decreasing the rates of all intracellular activities to an arbitrarily low value? (E) Illustration showing a challenge to studying slowed growth and the need for single-cell-level measurements. Scenario 1 (top row): all three cells divide to form six cells. Scenario 2 (bottom row): red cells divide with half the doubling time of the red cells in the top row. Green cells do not divide. After the same amount of time as in the top row, three cells become six cells.

year or 100 years, or however long we want. Do the laws of chemistry and physics impose an upper bound on a cell's doubling time and, if so, what sets it? Chemical reactions cannot occur arbitrarily rapidly; every cell's doubling time thus has a lower bound. By contrast, we lack similarly general arguments – such as, that chemical reactions cannot occur arbitrarily slowly – that impose an upper bound on a cell's doubling time. Apparently, slowing down growth by, for example, gradually decreasing temperature should boundlessly increase the cell's doubling time based solely on mathematical logic (Figure 1D). However, researchers recently revealed that upper bounds on doubling times do exist for yeast and that one will likely find others for various other cell types [6]. Another conceptual question is whether every cell grows slowly in a barely growing population. A population's growth rate is the average of the growth rate of every cell in that population. But aside from rapidity, the rate of any cellular process, averaged over every cell, also represents the probability of that process occurring in a cell within some time-window. Hence, a population can grow slowly because only a tiny percentage of its cells ever divides, but each of these cells can divide as rapidly as in growth-optimized conditions (Figure 1E). Thus, for understanding slow growths, one must examine individual cells – use single-cell methods – to find the potentially rare, replicating cells and quantify stochastic processes by monitoring large numbers of cells, one by one, for long durations that are unusual for microbiology. For example, a recent study monitored individual *S. cerevisiae* cells for months and found that, at 1°C, ~10% of the cells divided and that they took ~3 months to grow

Glossary

Dormancy: dormancy and quiescence, both being loosely defined, are often used interchangeably. Both refer to a cell with reversibly halted dynamics of self-replication. Removing stress or encountering nutrients restarts the cell's self-replicating dynamics. Spores are prime examples of dormancy. Many consider dormancy to be a 'deeper' form of quiescence with virtually no intracellular activities occurring because quiescent cells can exist while nutrients are abundant. Given this, quiescent cells have measurable intracellular activities, whereas dormant spores – existing without any nutrients – have no measurable activities for the most species, with a recently discovered exception being *S. cerevisiae* spores.
Lag phase: a cell-population is in lag phase when its extracellular environment drastically changes (e.g., appearance of a new type of nutrient). During this phase, the population grows negligibly but its cells are metabolically active and are switching their gene expression to adapt to the new environment (e.g., by expressing genes for metabolizing a new form of nutrient). The lag-phase duration, after which a log-phase growth occurs, continues to attract much interest.
Persistence: when an antibiotic halts the growth of an isogenic bacterial population and this population contains a subpopulation of cells that can withstand the antibiotic for much longer than the other cells, the subpopulation is said to be composed of persister cells. A hallmark of an isogenic population with persisters is a biphasic killing curve in an antibiotic's presence: the number of viable cells decays exponentially over time by following an exponential function but, after some time, decays slower by
following another exponential function.
Quiescence: quiescence, also called 'G0' part of the eukaryotic cell cycle for *S. cerevisiae* and mammalian cells, is loosely defined. No universally agreed upon definition currently exists. But one generally views quiescent cells to be more active than dormant cells. Quiescent cells have detectable intracellular processes, albeit at drastically reduced rates compared with those of vegetative cells.
Stationary phase: as cells deplete nutrients, a microbial population's growth slows and eventually stops, entering a stationary phase in which, per unit time, the number of cells dividing

and divide [6]. With nascent tools for trapping and monitoring single cells [7–13] and mathematical modeling [6,14–23], researchers are unraveling quantitative principles that govern the microbes' slowed and suspended lives. We now summarize these principles and discuss promising future directions.

Classifying states of slowed or suspended growth

Familiar states of slowed or suspended growth include **quiescence** (see Glossary) [24–26], **dormancy** [2,27–34], starvation-induced **stationary phase** [35,36], and growth-arrested states in antibiotics like **persistence** [37–40]. Several challenges exist in understanding these and other states. One difficulty is determining whether truly no cell in a population ever divides, since one typically does not monitor individual cells for a sufficiently long timescale. In fact, it is unclear how long this timescale should even be in many contexts. For example, it may take up to 2 weeks for a microscope camera to detect changes in a yeast cell's size at 1°C [6]. Another challenge is that an isogenic cell population can harbor multiple states of slowed or suspended growths that only single-cell-level measurements can detect. For example, in an isogenic population of starved *S. cerevisiae*, some cells become quiescent, some become stationary, and some sporulate [25]. In fact, with single-cell-level measurements, recent studies have revealed that there is a continuous gradient of non-growth 'states' within an isogenic population such that each cell has a quantifiably distinct phenotype [2,32,40].

An important question is how relatable the various states are to one another as two states can appear to be different but may be the same. Recently, a fresh way of viewing the various states of slowed or suspended growth has emerged: viewing a 'state' – a term that emphasizes discreteness – to be part of a quantifiable continuum. For dormancy, this concept of a continuum stems from the recent, surprising discovery that dormant spores of *S. cerevisiae* – a model for eukaryotic dormancy – can and do express genes without any nutrients (e.g., in water) [2]. This study introduced the concept of a 'dormancy spectrum': a dormant yeast spore's gene-expressing ability without nutrients, measured with the inducible expression of *GFP*, places each dormant spore on a spectrum of GFP levels that the dormant spores can realize. This study discovered that *GFP* expression positively correlates with the amounts of detectable RNA polymerases I, II, and III inside the spore. It thus established that the spectrum is multidimensional and complex. This spectrum connects being dormant (viable) at one of its ends to being dead (nonviable) at its other end (low expression of *GFP* or undetectable levels of RNA polymerases). Placed between the two ends of the spectrum are dormant yeast spores with continually varying levels of gene-expressing ability, with a lesser ability meaning less likely to germinate when nutrients appear and thus more likely to be dead [2] (Figure 2A). This study discovered that yeast spores, while dormant, gradually lose their gene-expressing ability and that once their gene-expressing ability becomes sufficiently low, they cannot germinate when saturating levels of nutrients appear (i.e., they are dead). The study thus revealed how yeast spores, during dormancy, gradually approach death – an irreversible state. The dormancy spectrum quantitatively distinguishes being dormant – being lifeless-looking – from being dead. Similarly, by measuring the re-growth ability of individual *E. coli* cells that transiently encountered an antibiotic, researchers recently proposed the concept of 'dormancy depth' that connects being dead to viable but non-growing. Here, more deeply dormant means taking longer times to recover and regrow after the antibiotic disappears, with being too deep entailing never recovering (i.e., being dead) [40] (Figure 2B). These examples demonstrate that we can revise the notion of discrete states with continuous, quantifiable 'state spectrums' on which we place individual cells, with probabilities or time taken to achieve some process like self-replication determining a cell's location on the spectrum. Isogenic cells in a population can have a range of probabilities or times required for achieving some process and, therefore, span a wide range of locations on the same spectrum. While the ability to express genes without any nutrients,

roughly equals the number of cells dying. Some cells in a stationary-phase population can still self-replicate, albeit slowly, but the population size does not grow given the small numbers and the simultaneously occurring cell deaths.
Vegetative cell: a metabolically active cell that perpetually self-replicates in the presence of nutrients. Much of the biological literature examines vegetative cells in growth-optimized temperature and nutrient-rich environments.

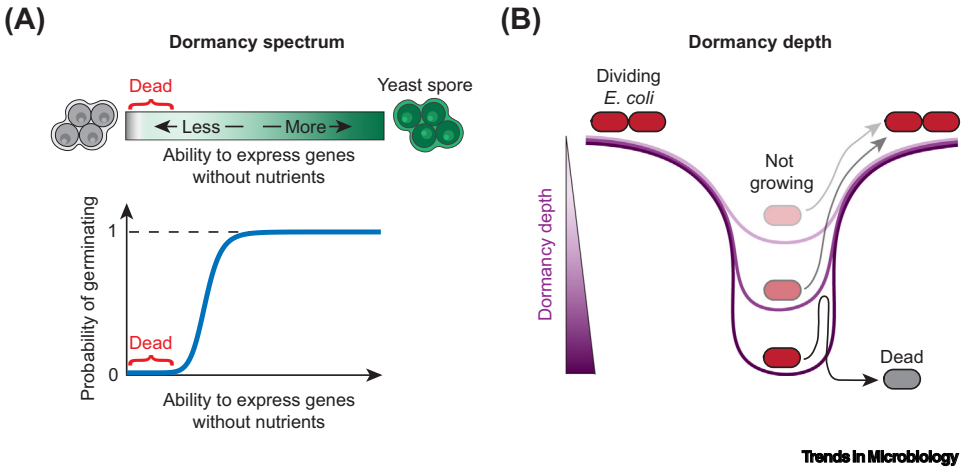

Figure 2. Dormancy as a continuous, quantifiable spectrum. (A) Dormancy spectrum of *Saccharomyces cerevisiae* spores (gray-to-green bar) [2]. Dead spores (gray) are at the left end of the spectrum. At the right end of the spectrum are spores (green) that have 100% chance of germinating in the future when nutrients appear. In between the two ends are spores that have between 0 and 100% chance of germinating in the future. A dormant spore's location on the spectrum is determined by its ability to express genes without nutrients (e.g., in water), as measured by GFP expression in a recent study [2]. Multiple factors, including the amount of detectable RNA polymerases I–III, determine the spore's ability to express genes without nutrients, thereby making the spectrum multidimensional. The blue curve relates a spore's chance of germinating with nutrients to that of a spore's gene-expressing ability without nutrients. (B) Dormancy depth of antibiotic-induced, growth-arrested *Escherichia coli* [40]. Cells that are sufficiently deep in dormancy (bottom trough) cannot grow when the antibiotic disappears, and nutrients appear – they are dead. Cells in the shallower part of the dormancy (upper troughs) are more likely, and take less time, to resume growth after the antibiotic disappears. A recent study showed that cells with less ATP form larger aggregates of proteins – a deeper dormancy – and that when nutrients appear, cells must actively disaggregate the aggregates to resume growth [40]. Too large an aggregate prevents disaggregation, leading to loss of viability.

and the time taken to resume growth can define 'state spectrums', these are likely not the only determinants of a spore's viability and location on a dormancy spectrum. Additionally, other spectrums likely exist, including a spectrum that shows quiescence – the 'G0-phase' of the eukaryotic cell cycle – and dormancy to be quantifiable shades of each other [24,41,42]. Aside from revealing the state spectrums, the probabilistic view of a cell – quantifying the probability of a growth-arrested cell dying in an antibiotic – has recently clarified the different versions of antibiotic tolerance, such as resistance, persistence, and heteroresistance [43].

Slow and rare dynamics in dormancy

Until recently, the predominant view was that no intracellular processes occur in dormant spores. After all, spores lack nutrients – an external source of energy – while spores of some species, like those of *S. cerevisiae*, have a glassy cytoplasm that greatly hinders movements and chemical activities of proteins [29,44–47]. In support of this view, microbes in stationary phase or quiescence – the conventional view is that both states are 'more active' than dormancy – express genes at greatly reduced levels compared with **vegetative cells** [48–51]. Despite this widespread view of dormancy being completely inactive, until recently, there was no definitive proof of dormant spores completely halting their gene expression and all other intracellular activities. This was mainly because it was difficult to unambiguously measure very infrequent and slow, biochemical events occurring inside dormant spores. But first, why is it even important to distinguish between the extremely infrequent from the nonexistent? Infrequent does not mean unimportant; an extremely rare process might be occurring in dormant spores, and after a sufficiently long time, the rare process might have consumed enough of the stored resources that are required for germination such as nucleotides and ATP. The rare process might thus prohibit the spore from germinating in the future when nutrients appear.

Recent studies discovered various factors that determine a dormant spore's lifespan and both its probability of germinating and the time it takes to germinate [2,32–34]. As mentioned earlier, a recent discovery is that the gene-expressing ability of dormant yeast spores (*S. cerevisiae*) determines the spore's lifespan – when it loses viability during dormancy – and its germination propensity (Figure 2A). By contrast, recent studies established that dormant *B. subtilis* spores – a model for prokaryotic dormancy – absolutely cannot express genes, metabolize molecules, and store ATP because they are filled with potassium ions (K^+) and dipicolinic acid that prevent intracellular processes [29–31,44]. Lacking any ATP, *B. subtilis* spores cannot actively sense their environments to detect an appearance of nutrients. Instead, they use receptors that passively bind L-alanine, a nutrient for germination, that triggers an expulsion of monovalent ions and dipicolinic acid from them and lets water in to enable gene expression, metabolism, and, eventually, germination [52,53]. Researchers recently showed how *B. subtilis* spores passively expel their K^+ during transient encounters with nutrients [33]: binding of extracellular L-alanine to its receptor 'squeezes' K^+ from the spores without using ATP, like hands squeezing air out of a balloon while the balloon (spore) remains passive (Figure 3A). After releasing a sufficient number of K^+ molecules, *B. subtilis* spores can finally start gene expression and germinate. The passive nature of *B. subtilis* spores – the complete lack of ATP-consuming activities inside the spores of *B. subtilis* and other organisms – poses conceptual questions that are distinct from those regarding the dormant spores of *S. cerevisiae* and others that maintain ATP-consuming processes. For example, one expects that dormant spores with active processes would have a finite lifespan, given that the active processes would be depleting their stored resources. Yet, given their evolutionary relevance, we expect these dormant spores to remain viable for sufficiently long times. How does a dormant spore with active processes still have the long lifespan that one expects it to have? Answering this question and defining dormancy in less-well studied spores – like those living on glaciers and marine sediments – through quantitative approaches will deepen our understanding of dormancy [3,4,54–56].

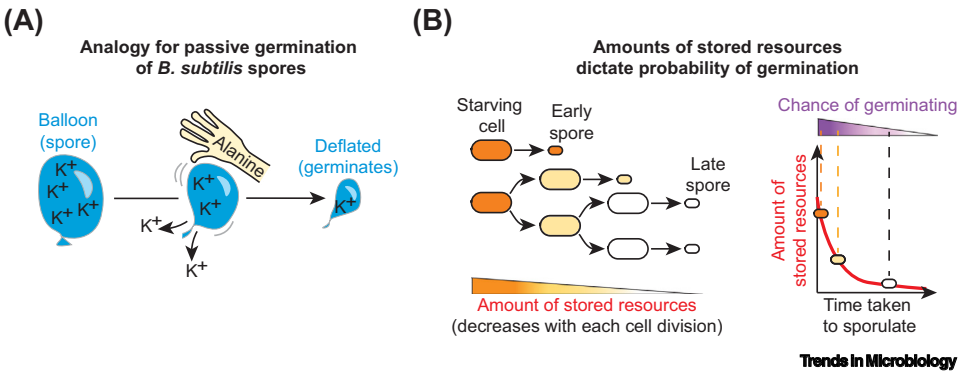

Figure 3. Dynamics of germination revealed by continuously monitoring individual spores over time. (A) Germination without ATP consumption (passive germination) conceptualized as a balloon passively losing its content. A *Bacillus subtilis* spore is conceptualized as a blue balloon filled with dipicolinic acid and potassium ions (K^+) that forbid any intracellular activities (e.g., gene-expression, metabolism, etc.). L-alanine, a nutrient, is conceptualized as the hand. Binding of L-alanine to a passive receptor is conceptualized as the hand pressing on the balloon to squeeze K^+ out of the balloon (spore). The hand does the work, and thus the balloon (spore) does not consume any ATP. After the spore encounters L-alanine for a sufficiently long time – after the hand squeezes the balloon for a sufficiently long time either all at once or in several squeezes – the spore germinates (i.e., the balloon has sufficiently deflated). (B) A recent study revealed that if a starving cell takes a longer time to sporulate, the resulting spore is less likely, and takes a longer time, to germinate than spores that formed earlier [32]. The large ellipse is a starving *B. subtilis* cell and the small ellipse is a *B. subtilis* spore. Black arrows denote cell division. A cell becomes paler orange with each division. The intensity of orange color represents concentrations of stored molecules inside the cell that are required for germination. The graph (red curves) shows the relationships between the chance of germinating, concentrations of germination-aiding molecules stored inside a spore, and the time taken to sporulate.

Dynamics of exiting dormancy

Nutrients reappearing cause spores to germinate. What are the intracellular steps that lead to germination, and why do some spores germinate while others in the same population do not? Recent studies highlighted nontrivial aspects of germination [2,32,34]. A major, recent finding for *B. subtilis* spores is that spores that took longer times to form during starvation are less likely to germinate once nutrients appear. These later-formed spores arose from cells that divided more times during starvation than the cells that formed earlier-formed spores. The later-formed spores thus have fewer factors that are required for germination than the earlier-formed spores – such as alanine dehydrogenase – and thus have a lower chance of germinating because they arose from cells that depleted or diluted more of these factors during division than the cells that became the earlier-formed spores (Figure 3B) [32]. Complementary studies, by inferring the amounts of resources stored inside *B. subtilis* spores with a luciferase-based luminescence, also showed that spores germinate at different times after encountering L-alanine due to having different amounts of germination-aiding resources such as stored redox potentials, but not ATP [28,30]. To date, researchers have focused on dormant spores' autonomous, germination propensity. An interesting future direction is investigating whether spores can help each other germinate as in the case of the soil bacterium, *Myxococcus xanthus*, whose germinating spores secrete and sense 'germination factors' (e.g., glycine betaine) that helps germination of the other spores in the same population [57].

Dynamics in starved, non-sporulated microbes

Thus far, our discussion has focused on dormancy and germination of spores. But how do microbes that do not, or cannot, sporulate remain alive without nutrients? Like dormant spores, these cells modify their intracellular processes, presumably to reduce their energy expenditure. For example, growth-arrested *Pseudomonas aeruginosa* exhibit slow yet detectable intracellular activities, due to their diminished, non-zero-level activity of RNA polymerases [58,59]. Additionally, some growth-arrested bacteria slowly synthesize proteins and metabolites, including ATP [60–62]. A current focus is on determining which of these molecules and processes are crucial for the cell population to re-enter an exponential growth when ample nutrients appear.

An emerging theme is that distinct growth phenotypes coexist in an isogenic, starved population. This coexistence can enable bet-hedging: a starving population can live longer and more rapidly resume its exponential growth, when nutrients appear, by having some of its cells exist in a phenotypically different state than the rest of the population [23,63]. Examples of this phenotypic variability are the following: only a fraction of cells grow slowly in a growth-arrested, isogenic population; cells within an isogenic population of *E. coli* or *B. subtilis*, during quiescence or stationary phase, have distinct intracellular concentrations of guanosine nucleotides, (pp)pGpp, causing some cells to synthesize more proteins than others [26,64,65]; some nutrient-starved *B. subtilis* cells, instead of sporulating, form a coccoid shape that tolerates antibiotics and divides once every ~4 days, remaining viable for months [66]. Additionally, by trapping individual *E. coli* cells in microfluidic channels and monitoring their growths and expression of metabolic genes [7–10], researchers established that cell-to-cell variations in the expression of nutrient-metabolizing genes creates various bet-hedging strategies. For example, noise in expression of *lac* operon genes causes some *E. coli* to be predisposed to enter an especially long lag-phase during a diauxic shift [67]. Similarly, some cells grow more slowly than their genetically identical neighbors in certain nutrients while growing faster than their neighbors when the nutrient type unexpectedly switches – populations use this bet-hedging strategy to shorten their **lag phase** that occurs when nutrients change from one type to another [65]. By mathematically modeling both bet-hedging strategies and single-cell-level (stochastic) behaviors of starved microbes, researchers also recently revealed optimal strategies that stationary-phase microbes can use for re-entering exponential growth [36,68–71].

The predominant view emerging from myriad studies is that a starved cell's viability is primarily determined by the amounts and types of molecules that were stored in them during starvation. For example, the amount of ATP stored in a growth-arrested phototrophic bacterium, *Rhodopseudomonas palustris*, primarily determines its duration of viability [60]. Likewise, various growth-aiding proteins stored inside the stationary-phase cells of *Salmonella enterica* serovar Typhimurium and *S. cerevisiae* facilitate their re-entrance into exponential growth [72]. As life requires not one but many molecules, it is important to determine which set of molecules truly determines whether or not a growth-arrested, starved cell can resume growing when nutrients appear. Rather than cataloguing a family of growth-aiding molecules one by one – an unmanageably large number of families might exist – a promising direction is to construct a generalizable framework to quantitatively understand how a cell can resume growing – a framework that is agnostic to the identities of the molecules involved, as recently done for bacteria resuming growth after being in an antibiotic-arrested, 'frustrated' state [38]. Another promising direction is to investigate how starved microbes can maintain each other's viability. For example, stationary-phase *E. coli* cells grow and divide through a 'cannibalistic metabolism' – consuming molecules liberated by the lysis of dead cells – and the cell population can thus maintain viable cells longer than if cells autonomously maintained their viability [35].

Slow and rare dynamics at extreme temperatures

From seafloors to glaciers, microbes inhabit diverse, frigid environments [73,74]. But even in cities, parks, and deserts, microbes endure frigid temperatures during winters and nights. Ongoing efforts to culture, in laboratories, microbes isolated from frigid, wild environments – such as the Canadian Arctic [75] and permafrost [76] – are only starting to reveal the physiology of many cryophiles on Earth [74,77]. A parallel approach for understanding how microbes endure cold environments is studying model organisms at unusually low temperatures. For example, by monitoring individual cells of *S. cerevisiae* for months and building mathematical models, researchers recently discovered that yeast has both a low-speed and a high-speed limit – smallest and largest possible doubling times – for every temperature above 0°C and below 10°C [6]. Here, at each frigid temperature, yeast's viability and speed limits arise from the competition between two opposing processes: production and reduction of reactive oxygen species (ROS). This study showed that the yeast's rate of reducing ROS was primarily determined by the time taken by the yeast to synthesize antioxidants which, in turn, depended on the average time taken for transcription and translation ('gene-expression speed'). The study found that ROS did not affect the gene-expression speed: yeast with a high amount of ROS had the same gene-expression speed as a yeast with a low amount of ROS. Intriguingly, recent studies established that even at high temperatures that hinder growth – for both *E. coli* and *S. cerevisiae* – ROS plays a major role, if not the primary role, in determining a cell's viability [22,78]. Specifically, at high temperatures, *E. coli* stops growing and triggers a 'thermal fuse' – the degradation of MetA required for synthesizing methionine – to pause the synthesis of methionine and other molecules that either create or are damaged by ROS [78]. Furthering the importance of ROS at high and frigid temperatures is that, even without any cells, the typical ingredients of growth media – amino acids and minerals but not sugars – generate ROS [6]. These findings suggest that a cell's ability to reduce ROS sufficiently rapidly – ability to reduce ROS before ROS-induced damages accumulate to some threshold level – is an important, if not the primary, determinant of a cell's viability at extreme temperatures. These findings also suggest an intriguing parallel to those of *B. subtilis* spores described earlier, whereby redox potential is a read-out of a spore's ability to germinate. A promising direction is quantifying how ROS affect the ecological dynamics and survival of microbial communities in the Earth's warming climate [79,80].

Persistent viability while encountering antibiotics

When nutrients disappear or the temperature changes, microbes' programmed responses slow or arrest their growths and prepare them for survival. In contrast to these programmed,

gradual responses, antibiotics often abruptly stop cells' growth by suddenly disrupting vital parts of intracellular machineries in ways that the cells have not been programmed to respond to. Many cells do not instantaneously die the moment they encounter an antibiotic. Thus, antibiotics are tools for studying how cells survive when their processes abruptly halt without being prepared for it. These processes often include cell growth. Thus, antibiotics let us study how cells remain alive when their growths are abruptly interrupted.

In some populations that are encountering an antibiotic, cells can markedly differ in their viability such that the population effectively consists of several subpopulations, each with a distinct half-life. Elucidating how this occurs has attracted much attention [12,18,37,39,43,81]. Genotypic changes, such as mutations or tandemly amplified antibiotic-fighting genes or horizontal gene transfers, can generate such subpopulations [82–85]. But subpopulations with differing half-lives can also arise without genetic changes, as exhibited by a population consisting of two genetically identical subpopulations: persisters and non-persisters – a phenomenon that continues to draw interest [37,43,81]. A persister is viable, on average, for longer than a non-persister in an antibiotic (Figure 4A). Multiple causes yield persisters and, depending on the antibiotic, various mechanisms underly persistence. Often, but not always, persisters in antibiotics exhibit slowed or stalled growth, much like starved cells [86]. Some cells become persisters because – due to stochastic nature of gene expression and partitioning of resources among cells – they acquire a prolonged state of producing elevated levels of antibiotic-fighting machineries such as drug-efflux pumps or enzymes, such as catalase, that activate drugs without stopping their growth [87,88]. Moreover, some cells become persisters by stochastically acquiring a prolonged, quiescence-like state in which intracellular activities that an

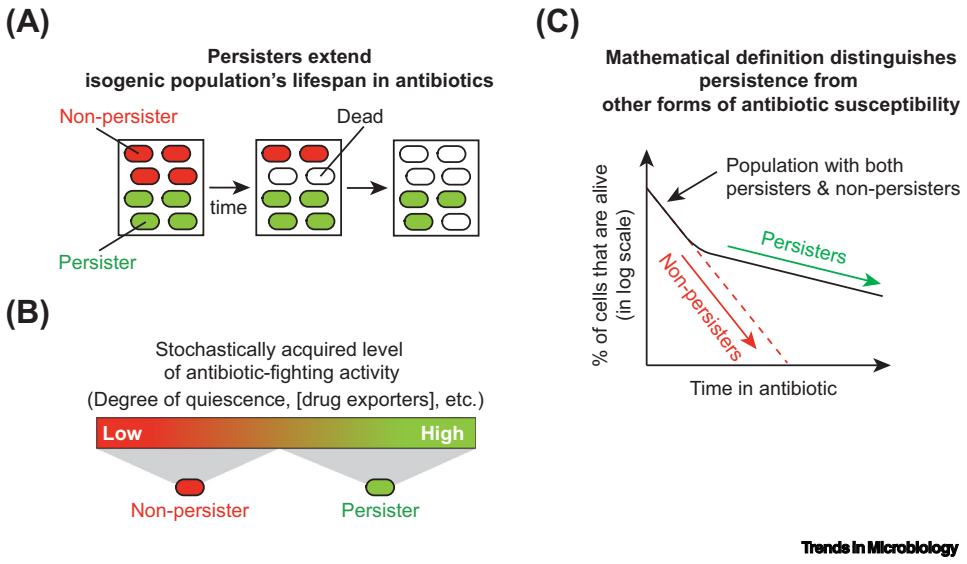

Figure 4. Non-growing persister cells in an antibiotic. (A) Isogenic bacterial population in an antibiotic consists of non-persistent cells (red) and persistent cells (green). As time spent in an antibiotic increases (arrows), both types of cell die (white) but persisters die at a lower rate than non-persisters. (B) Debates exist regarding the various sources for generating persisters. It is clear, however, that persisters arise from some of the isogenic cells stochastically acquiring an elevated, antibiotic-fighting activity that is temporary yet prolonged. The antibiotic-fighting activity, when quantified, can have a wide range of values as shown in the spectrum (bar). A question is how this spectrum yields two distinct subpopulations – non-persisters (red) and persisters (green) – with two distinct, population-level half-lives. (C) Researchers recently used mathematical analyses of population-killing curves, like the one shown here, to reach a consensus on the definition for each form of being susceptible to an antibiotic, including persistence [43]. Black shows a biphasic killing curve that defines persistence. A population consisting of both non-persisters and persisters exhibits the black curve, with the faster decay in the early times – extended as a red line – representing the deaths of non-persisters and the more slowly decaying curve in the later times representing the deaths of persisters in an antibiotic.

antibiotic makes toxic occur at highly reduced rates (Figure 4B) [88]. Recently, with a mathematical analysis of cell killing – how a cell population's size decreases over time – researchers established a quantitative definition of persisters that is independent of the cause of persistence: a population contains some persisters if its size decreases as a function of two summed exponentials: – the population exhibits a biphasic killing curve (Figure 4C) [43]. They established that the biphasic killing distinguishes persistence from other forms of susceptibility to antibiotics. An important future challenge is explaining how stochastically determined, continuous quantities – such as gene-expression level and rate of metabolism – generate two distinct half-lives at the population-level as opposed to more than two half-lives or one half-life that is the average of every cell's lifetime (Figure 4B).

Quiescent bacteria – bacteria with drastically reduced but still-active metabolism – and dormant spores tend to withstand antibiotics for much longer times than fast-growing bacteria. Hence, another important challenge is understanding why some quiescent, stationary-phase-like bacteria are persistent when they are exposed to antibiotics. What is the molecular composition of such cells, and which dynamics occur in them? To help us answer these questions, recent studies established conceptual frameworks based on physics, such as dynamics of frustrated magnets and potential-energy wells to represent growth-arrested cells in an antibiotic [38,40].

Finally, an intriguing, potential connection between antibiotic-induced, stunted growth and other types of growth arrest discussed in the previous sections is through ROS. Antibiotics can depolarize the membrane of growth-arrested, non-sporulated *B. subtilis*, yielding abundant superoxides (ROS) that kills them [89]. By mathematically modeling how ROS determines the probability of a cell dying, we might connect this phenomenon to that of ROS determining the viabilities of *E. coli* and *S. cerevisiae* at extreme temperatures.

Outstanding questions

What are the quantitative principles and molecular mechanisms that determine whether a microbe is viable – and for how long – in non-growing states such as dormancy?

What are the limits to how slowly the life of a microbe can progress?

How does a microbe's lifespan change when its life is slowed or stopped as in frigid temperatures or dormancy?

How similar are the mechanisms that govern slowed and suspended lives of microbes to those governing quiescent vertebrate cells?

What are the implications of slowed and suspended growths in extreme temperatures in the context of climate change?

What are the implications of the suspended growth of microbes in nutrient-deprived and extreme-temperature habitats for the possibility of microbial life outside Earth?

Concluding remarks and future perspectives

An emerging view is that several common principles underlie and unify the diverse studies of slowed and suspended lives of microbes we described. One of these principles is that we can recast the traditionally defined states of slow or suspended growth, such as dormancy, as a continuous spectrum. We can deconstruct a state into infinitely many locations – a 'state spectrum' – or connect two or more states by viewing them as merely different locations on a common spectrum (Figure 5A). On this spectrum, a cell's location is set by a continuous quantity – such as the probability of executing a task like self-replication or the time taken to resume growth – which, in turn, is set by other continuous quantities such as intracellular concentrations of molecules or rates of some processes. The spectrum view revises our perspective: it shifts our thinking away from discreteness of cellular states by conceptualizing cells as occupying a continuous range of locations in an abstract space, thereby naturally explaining the phenotypic heterogeneity among genetically identical cells often seen in slowly growing or non-growing populations. A combination of several factors likely contributes to a cell's location on the spectrum, and these factors may not be the same for all microbial species. A second principle, related to the first, is that heterogeneity is inherent in slowed and suspended growths: two genetically identical cells can have distinct propensities for exiting slowed or suspended growths, and thus different lifespans (Figure 5B). For further exploration and establishment of these principles, single-cell-level measurements and modeling of stochastic events will continue to be invaluable. These approaches reveal a mathematical distribution of a quantitative trait among isogenic cells, such as the doubling times of individual cells at frigid temperatures. The distributions hint at the underlying molecular mechanism that produces the trait. A third principle is that how slowly a cell can progress through its self-replicative dynamics – the cell cycle – without dying is determined by whether the cell's viability-promoting processes, like reducing ROS, can occur at least as rapidly as its viability-demoting processes, like generating ROS. This principle makes it unnecessary to ask whether chemical reactions can

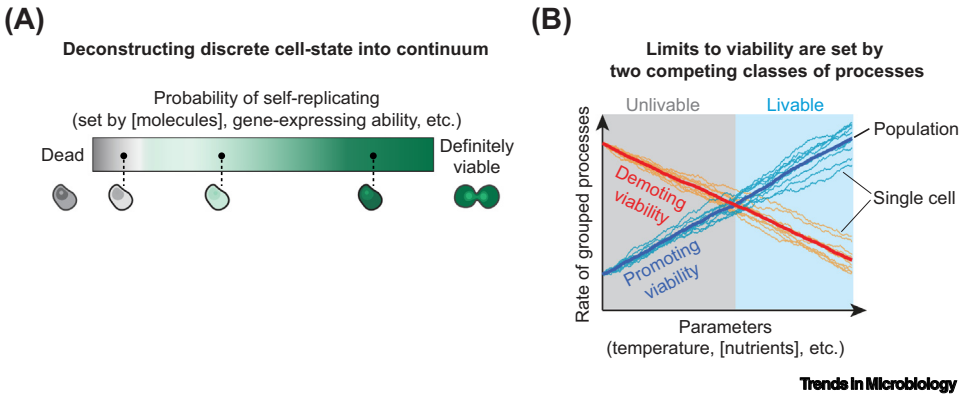

Figure 5. Emerging principles that govern slowed and suspended lives of microbes. (A) One of the emerging principles that underlies multiple studies of distinct organisms described in our review: recasting the traditionally defined states of slow or suspended growth as a spectrum (bar). We can deconstruct a state into infinitely many locations – a spectrum – or connect two or more states such as being dead (left end of bar shown in gray) and being definitely viable (right end of bar shown in dark green) by viewing them as merely different locations – each location having a probability between 0 and 1 of being viable while growing slowly or not growing – on a common spectrum. (B) Another of the emerging principles: we can conceptually lump multiple intracellular and extracellular processes into two groups – those promoting viability (blue) and those demoting viability (red) – and use mathematical modeling and analysis to derive a lumped rate for each class as a function of various parameters, as shown here. Thin lines show the two lumped rates for each cell, with no two cells having an identical blue or red curve, due to the stochastic nature of the processes. Solid red and blue curves are the averages of the thin (single-cell) curves shown here, representing population-level rates. Note that in the gray zone (unlivable for a population), some cells have viability-promoting processes occurring more rapidly than viability-demoting processes, thus being viable while the population that they are in faces extinction.

proceed arbitrarily slowly – how close to zero a reaction rate can be – to determine the longest possible doubling-time for a microbe in any environment. This is because the viability-demoting processes impose a range of rates that the viability-promoting processes must have. We can view the viability-promoting processes outcompeting viability-demoting processes to keep a cell alive as a modern rephrasing of Schrödinger's idea of living systems evading 'the decay to equilibrium' [1,90]. Related to this are the recent revelations, made by the several studies described here, that cells can evade such a decay to thermal equilibrium by helping each other – by extracellularly releasing viability-promoting molecules and then sharing them – and that cells often die at extreme temperatures and nutrient starvations by losing their structural integrity and spatial organization (e.g., ROS-induced damage causing cells to burst) [6,22,35]. These revelations suggest that slowing down cellular processes hinders the cells from maintaining their spatially organized structures and that when cellular processes slow down too much, cells die because they have lost their ability to maintain their spatial organization. This idea is intriguing because, for living systems, being out of thermal equilibrium is fundamentally about generating and maintaining their spatial order. Several studies mentioned here revealed that slowed-down cells can do so by extracellularly releasing and then sharing viability-promoting molecules. We believe that one will discover fundamental limits of life by further investigating this idea experimentally and connecting it to physics-inspired, theoretical investigations of cells collectively generating and maintaining their spatial order [91–93].

The emerging principles mentioned earlier transcend species and molecular identities, making clear that the same quantitative mechanism, formulated in the mathematical language of dynamical systems and stochastic dynamics, governs multiple species, even including slowly growing mammalian cells. For example, the same dynamical system – the same equations – governs the dynamics of self-replication for both *S. cerevisiae* at extreme temperatures and embryonic stem cells during differentiation – a phase of slow or suspended growth for mammalian cells

[94]. In fact, since many cells in humans and animals infrequently divide, and mathematical equations remain unchanged when we change the names of their variables, we expect that continuing the trend of quantitatively studying dormant and slowly growing microbes will reveal unexpected insights into mammalian physiology. Continuing these approaches will also likely address some outstanding questions regarding fundamental limits to slowing and suspending lives of cells, for both microbes and multicellular organisms (see Outstanding questions).

Despite their promise, the quantitative and laboratory approaches for studying slowed and suspended growths – a selection of which we outlined here – have major limitations. First, laboratory settings emulate, but do not truly replicate, the natural habitats in which dormant or slowly growing microbes inhabit. Laboratory studies merely reveal an idealized or limited facet of the slowed physiology of microbes in natural environments. Second, the quantitative approaches to understanding slowed or suspended growths outlined here do not always reveal – and do not even depend on – the specific, molecular factors that yield the slow-growth phenotypes because these are, by design, generalized and abstract formulations of cellular dynamics. An intellectual tug-of-war thus exists between two styles of inquiry: focusing on the specific molecules that yield the slow or suspended dynamics or the more holistic, systems-level mechanisms that quantitative approaches often highlight. We think that both styles are necessary, complementary, and important.

The principles mentioned earlier highlight fundamental questions about life. How slowly can life progress? How does seeming lifeless differ from being dead? Aside from being testbeds for answering these important questions, continued research into the slowed and suspended lives of microbes will offer insights into how microbial communities survive in natural habitats and will be affected by our warming climate [95,96]. Indeed, these seemingly quiet and lifeless microbes are transforming our view of microbiology and life.

Acknowledgments

We thank Caryn Navarro for insightful comments on our manuscript. We also thank the participants of the KITP (UCSB) workshop in 2022 titled, 'Quantitative biology of non-growing microbes', that was organized by Nathalie Balaban, Thomas Julou, and Roberto Kolter for stimulating discussions with one of the authors (H.Y.) on the topics we covered. We apologize to researchers whose work we could not cite due to spatial constraints. This work was supported by the NIH Maximizing Investigator's Research Award to H.Y. (R35GM147508).

Declaration of interests

The authors declare no competing interests.

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