Bacteria Grow Swiftly, Live Thriftly

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Summary

Bacteria have evolved numerous strategies to use resources efficiently. However, bacterial economies depend on the physiological context of the organisms, whether they are growing, non-growing or reinitiating growth. We discuss some of the features that make bacteria efficient under these different conditions and during the transitions between them. We point to the fact that much still needs to be investigated regarding the physiology of non-growing bacterial cells. We also examine how efficiency is apparent in both the mode and tempo of bacterial evolution.

Introduction

Over the course of the four billion years that there has been life on Earth, bacteria (and microbes in general) have evolved to utilize resources efficiently. In this essay, we outline how bacteria find and take-up these resources and then partition the products appropriately to generate the energy and biomass required for their growth and survival. Importantly, even an oversimplified look at their lifecycles reveals that bacteria exist in very different states. When resources are abundant, they typically grow and divide exponentially. As soon as resources become scarce, or when they are exposed to other severe stresses, bacteria stop growing and differentiate into much sturdier forms. When resources become available again or when other stresses cease, bacteria restart growth. These different states impose very different metabolic requirements on the organisms. Thus, what it means for bacteria to "live thriftly," that is use resources efficiently, depends strongly on the environmental conditions that the cells experience. There are many adaptations that a bacterium can use when rapid growth ceases. Initially there will inevitably be a transient energy limitation that will adapt the cell for survival under extremely slow growth. If limited resources continue, cells will adapt further to withstand long-term energy limitation. A striking feature of most bacteria is their ability to exit either transient or long-term energy limitations and resume rapid growth when resources once again become available. While a cell's prior history greatly influences the transition to rapid growth, bacteria are particularly good at "jump starting their economies." Bacterial growth is thus highly nuanced but for the sake of simplicity we discuss these transitions in three all-

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encompassing stages: plentiful resources with no energy limitation, starvation (or stress) leading to energy limitation and resumption of growth. In natural settings, resource scarcity is likely to be the condition that most bacteria encounter most of the time. We thus also discuss how those conditions might influence the evolutionary process in bacteria.

When resources are plentiful - bacterial strategies during growth

When bacteria find themselves in the presence of plentiful nutrients in non-stressful environments they grow and divide unrestricted. Yet, the way they use the resources available is by no means haphazard. Like all other organisms, bacteria have evolved elaborate regulatory mechanisms that lead to very efficient utilization of nutrients. Regulating the expression of the genes required for the utilization of different resources is the most common strategy observed. Whether regulation is the optimal strategy depends on how often a particular resource is encountered. Conditionally shutting off a set of genes is worth it when the corresponding resource is not encountered very often. Otherwise, constitutive expression is more efficient than the cost of maintaining the regulatory mechanism[1]. The fact that the utilization of most resources is tightly regulated is indicative that most bacteria have evolved in environments where the availability of essential nutrients is constantly and rapidly changing. In this regard, it is important that future work in this area precisely define the microscale environmental changes that bacteria experience in their natural setting if we are to better understand the physiology of bacteria in their natural contexts.

Regulatory mechanisms also allow bacteria to prioritize which resources they use to be most efficient. The way *Escherichia coli* prioritizes the use of different sugars for its growth is a clear example of this. The now classic studies on lactose utilization – the first demonstration of regulation of gene expression– demonstrated this bacterium's efficiency. When grown in the presence of both glucose and lactose, it first uses up the glucose and only when that sugar is depleted does it invest the resources needed to be able to use lactose[2].

When we look closer at the way *E. coli* grows on glucose in the presence of oxygen, we learn it does not make the most efficient use of the substrate to generate energy via respiration and oxidizing the glucose completely. Instead, it opts for growing fermentatively and excretes acetate. At first glance this response, referred to as "overflow metabolism" appears wasteful. But it is not. We now know that in doing so, the rapidly growing cells are indeed optimizing resource utilization by not using the respiratory pathway. This is because under those conditions, the cost of making the proteins needed for respiration exceeds the cost of making the proteins for fermentation[3]. And the excretion of acetate, is that a waste? Not necessarily, because acetate can be used after the glucose is exhausted. It can be seen as a savings strategy, akin to putting money on the bank, so long as there are no other species around that can steal the savings.

The foregoing examples focus on cells' efficiency considering them as separate, disconnected individuals. This knowledge is gathered from studying growing bacterial populations and

assuming that all cells behave similarly when in "balanced growth." But if there is something key about bacterial populations that we have learned in recent decades it is that they are extremely heterogeneous even when clonal[4]. When bacteria grow as biofilms on surfaces, say a colony on an agar plate, their metabolic activity generates microscale gradients of the local conditions[5]. Individuals then adapt locally, and this leads to the appearance of differentiated subpopulations[6]. Once co-existing, each cell type might carry out a different specialized function. Thus, by dividing labor the cells can achieve synergistic interactions such that their collective phenotype is more than the sum of the parts. This is the case, for example, when colonies of *Bacillus subtilis* expand using sliding motility to achieve the ecological gain of occupying and exploiting new territory. In this case, some individual cells produce a friction-reducing surfactant. These cells, however, do not migrate efficiently. Another cell type does not produce surfactant but forms long filaments that bundle and growth. Yet, these bundles cannot migrate without the surfactant. The synergistic interactions between the two cell types permits the expansive migration of the colony[7].

Starvation and severe stress - no growth and slow responses

The foregoing examples refer to growing bacteria, and often growing quite rapidly. But in their natural settings bacteria most often find themselves under conditions where the resources needed for growth are extremely limited[8]. Thus, their metabolic rates are very slow and, consequently, their generation times are extremely slow. This radically slower metabolism implies that the energy conservation and utilization, as well as anabolic reactions need to be regulated in ways that are dramatically different from the way a rapidly dividing cell would do it (Figure 1). The rate at which a growing cell alters the concentrations depends on both synthesis and degradation but also on dilution as the cell volume increases[1]. This is not the case in non-growing cells. This means that when a slowly growing or non-growing cell encounters a rapid change in environment or a stress-induced damage, it will not have the capacity to change its proteome as quickly as a growing cell. The way non-growing bacteria handle this is by preparing in advance. As they sense a decrease in their growth rate due to the onset of starvation, they differentiate into sturdier forms able to better withstand diverse environmental insults[9].

Differentiation into sturdier forms: spores

For many bacteria, the evolutionary solution to survive when resources dwindle is to enter a dormant or almost dormant state through the formation of spores[10]. In some cases, as in endospore formation by *B. subtilis*, the resulting spores are dehydrated, their DNA highly compacted and inactive, and their metabolism is virtually zero[11]. In other cases of sporulating bacteria, among them many members of the *Actinomycetia*, their spores retain some level of endogenous metabolism despite their being non-dividing cells[12]. Whether completely or partially dormant, spores offer an increased chance of survival under hard times both because of their lower energy expenditure and, importantly, their increased resistance to environmental stresses. Future studies should attempt to understand the reasons behind the evolution of

these two different modes of bacterial thriftiness. What were the differences in ecological settings and natural selection that led some lineages to evolve complete dormancy and others only partial dormancy?

Spores can remain in place to wait it out until conditions once again become propitious for growth, but they can also disseminate towards "greener pastures." Migrating in search of better environments is a common feature of most organisms, from bacteria to humans. But exactly how efficient migrations are varies widely. For spores, the processes of locating habitats where they can germinate, and re-initiate vegetative growth can range from very active to completely passive. The spores of a few species of bacteria have flagella and can thus swim in search of environments suitable for growth[13]. But most bacterial spores rely on other forces to disperse. Because bacterial spores are too small and too close to the ground to be easily dispersed by wind, they often rely instead on being transported by other organisms, *e.g.,* insects or pollen grains. The fully dormant endospores of *Bacillus subtilis* rely on chance encounter with their mode of transportation. In contrast, *Streptomyces coelicolor* uses a striking strategy to disseminate that requires only a small investment of resources. It produces a small volatile – geosmin – just before completing the process of spore formation. Arthropods (springtails in particular) are attracted to and walk all over the source of the geosmin, inadvertently coating their carapace with spores, carrying them to far off lands[14]. There may be something in this behavior for the arthropods as well. In coating themselves with *S. coelicolo*r, they gain a companion which could be a source of protective antibiotics. Which brings up the tantalizing question of why humans, despite their relatively poor sense of smell, retain a remarkable sensitivity to detect geosmin (threshold odor concentration of ~1 ngL^{-1}). Is the reason we find the smell of soil, *i.e.*, geosmin, pleasant the fact that contact with that soil will coat our skin with beneficial antibiotic-producing bacteria? Think about this next time you take a walk in the woods, barefoot. It would indeed be an economical prophylaxis for humans.

Differentiation into sturdier forms: non-growing cells

As a mechanism to cope with limited resources, the generation of dormant (or almost dormant) spores is the exception rather than the rule for bacteria. Most bacterial species do not sporulate. Yet, they almost universally undergo some form of differentiation that results in hardier cells able to survive for extremely long periods growing very slowly or even in the absence of growth[15]. As bacteria transition from rapid growth into slow growth or the cessation of growth due to the lack of a carbon source, they turn on the expression of numerous genes whose products ensure a more resistant cell type. The molecular processes that underlie this sort of differentiation are reasonably well understood. The now classic example is the case of the RpoS regulon of *E. coli*[16]. Numerous environmental signals converge on the activation of a key transcriptional factor (RpoS or σ^s). An active RpoS then redirects the cell into a gene expression program that may appear costly at first, but which prepares the cell for the eventuality of encountering numerous stresses. Thus, many bacteria display this thrifty strategy: they invest during the entry into a non-growing phase to be able to save themselves during the uncertain times of resource scarcity.

Investment in survival

The mechanisms at play that allow starved cells to maintain some level of metabolism and thus remain viable for extremely long times are much more poorly understood. Yet, it is important to understand such cells if for no other reason that they are likely more representative of most of the bacteria on the planet. There are some features of non-growing cells that we do know, and which exemplify their remarkable ability to optimize the use of limited resources. They obtain some energy and anabolic substrates by degrading cellular components that are plentiful. For example, cells that start with many ribosomes which they will not be needing degrade most of them without any deleterious consequences[17]. Utilization of lipids, through fatty acid β-oxidation, is another way to aid in survival[15]. The same cannot be said of the cell's DNA, which must be protected in its entirety. The protection and compaction of DNA with the protein Dps, without sacrificing its ability to be expressed, seems to be a widespread strategy that bacteria employ to keep the integrity of DNA while still being able to carry out maintenance metabolism[18]. Because everything that these cells do, they do at rates that are orders of magnitude slower than those observed in growing cells, most of the methods and approaches that work for rapidly growing cells fail when applied to non-growing cells. A lack of sensitivity is usually a problem. How does one measure metabolism that is many orders of magnitude lower than that of a growing cell?

On the need to develop methods to study and model non-growing cells

The rates of cellular processes in non-growing cells, including metabolism and gene expression, are orders of magnitude lower than those observed in growing cells. This means that classical protocols developed to characterize the non-growing cells often lack sensitivity. Moreover, non-growing bacterial populations often feature larger phenotypic heterogeneity[15]. Thus, population-averaged measurements are hard to interpret because they cannot report on the variability among cells. Altogether, this means that single-cell techniques developed recently to measure growing bacteria with single-cell resolution constitute very promising tools to characterize non-growing bacteria[19]. In this context, methods that utilize the incorporation of stable isotopes and multi-isotope secondary ion imaging mass spectrometry (NanoSIMS) as well as parallel sequential fluorescence in situ hybridization (par-seqFISH) are particularly powerful because they permit assessment of physiological responses of individual cells over myriad environmental conditions, including extremely slow growth[20,21]. These approaches are highly sensitive and extremely informative since they are used to measure individual cells, and they allow measuring distributions over populations of cells. It is also worth pointing that several of these methods allow the bacteria to control the environment to which they are exposed, which is particularly important to study processes occurring over long time in dense bacterial populations (such as in stationary phase), where bacteria often modify their environment across vast spatial scales, changes that are difficult to measure and hence introduce another confounding factor.

We envision that this ongoing effort of using quantitative biology approaches to study non-growing bacteria will serve at least two important goals. First, it is a long overdue to go beyond the very limited vocabulary used to refer to non-growing bacteria, *e.g.,* 'stationary phase' or 'dormancy'. The physiological states of non-growing cells will no doubt be very different depending on how bacteria reached that state and how long they have been in it. A recent work shows that bacteria exposed to an acute stress can be viewed as entering a disrupted state characterized by a wide single cell heterogeneity, while the same stress applied gradually leads to a very different adaptive state[22]. Systematic quantitative measurements of non-growing cells under various conditions should thus allow to refine the typology of non-growing states. Second, coarse-grained modeling of the metabolism of growing bacteria has pinpointed key organizational principles, in particular regarding resources allocation[23]. In such approaches, modeling that exploits bacterial growth laws can serve to predict the coupling between gene expression and the growth state of the cell in the absence of molecular details. These models can then serve to guide experimental design to understand and manipulate cell behavior. We foresee that modeling efforts along similar lines would be very useful to uncover how non-growing bacteria work, and what variables are relevant to characterize them.

Restarting growth - generation of heterogeneity

When a population of starved, non-growing bacterial cells re-encounters nutrients, growth can re-initiate. Naively, one might imagine that all cells have similar capacity to grow again. However, that is not the case. When populations of starved, non-growing cells once encounter plentiful nutrients – even if they are homogeneously dispersed in liquid – not all cells begin growing again at the same time (Figure 2). As mentioned above, this scenario happens when starvation is either too rapid or too long for the bacteria to deal with[22]. As it turns out, there can be great advantages in the stochastic generation of such heterogeneity. The prime example of this is the observation that in populations that appear to be undergoing balanced growth, i.e., all cells appear to have the same growth rate, there is often a subpopulation of cells that are, in fact, not growing for some period but which can later re-initiate growth. These few "persister" cells represent an interesting case of the population hedging its bets. In the eventuality that the population is confronted with a deadly antibiotic, the non-growing cells will not be affected. In terms of the population economy, such bet-hedging is another example of how bacteria can be thrifty. They gamble and indeed suffer a reduction in fitness by stochastically generating non-growing subpopulations. But the fitness reduction is small and yet the strategy assures the survival of the strain in case they encounter an otherwise total loss.

The clinical consequence of a persister subpopulation is obvious, its members could restart growing after antibiotic treatments are discontinued. Interestingly, most of the observations on antibiotic persistence in fact originate from bacteria that remained non-growing from a previous exposure to stressful conditions[24]. These "triggered persisters" require a previous stress trigger to enter the persister state. Most starters for cultures are taken from an overnight culture, which is a starvation stress and often also a high pH stress. The bacteria that remain

dormant and extend their lag time when transferred to fresh medium can survive antibiotic treatments that do not affect lagging bacteria[25]. One may speculate that exposure to stress is a predictor for future stress and therefore a sub-population remains dormant despite being transferred to conditions supporting balanced growth. In this case, the bet-hedging would depend on the history of the culture. An alternative hypothesis views the persistence phenomenon as an inevitable consequence of glitches and errors and not necessarily a "strategy" selected by evolution[26]. A recent framework shows that the way triggered persisters are generated by acute stress can be understood as a universal feature of the perturbation of the cellular network rather than as an adaptive trait[22].

Bacterial Evolution: Faster dynamics when populations are not growing

Thus far we have described how bacteria undergo phenotypic changes to cope with conditions of limited resources. What do we know about genotypic changes and their selection, *i.e.,* evolution, in starved populations? Bacterial evolution is seen as a slow process where relatively small increases in a bacterium's fitness help its progeny to slowly take over a population. In general, this evolutionary dynamic is observed when following the fitness trajectories of continually evolving E. coli over many thousands of generations[27]. But when an entire bacterial population is unable to grow and some of the cells begin to die, if there are any mutants present able to grow, they can very quickly take over the population. This is what is observed in cultures of bacteria that are kept starved. Mutants able to grow on the detritus of cells quickly take over the cultures[28]. These mutants, said to have a "growth advantage in stationary phase" or GASP phenotype, take over the population simply because they can grow under conditions where the rest of the cells cannot. The dynamics of "GASPing" are interesting. Initially, the selective advantage (or increase in fitness) is great and single GASP mutants quickly take over the population. When cultures are kept starved for extremely long times, the fitness gains of subsequent GASP mutants become lesser and lesser. This leads to the coexistence of different genotypes[29]. They coexist because each genotype can exploit a different nutritional niche from the detritus of the dead and dying cells[30]. Coexistence of multiple genotypes is also observed in long-term evolution experiments where populations were continuously cycled through periods of growth and starvation[31]. Such coexistence could mark the beginning of speciation. In fact, in cases where very similar strains coexist in natural settings, they do so through niche partitioning, where each one of the different species is extremely efficient at utilizing a carbon source that the other species cannot use well[32]. Such niche partitioning is certainly not limited to carbon source utilization. Importantly, all nutrient utilization, e.g., different sources of nitrogen, trace metals, sulfur, etc., could be similarly partitioned.

Bacterial Evolution: Mode and Tempo

Mutations in existing genes within a bacterium's genome, such as described above, might well have served as important players in the early evolutionary history of life on Earth. However, in the more recent history of bacteria, horizontal gene transfer has served as the primary driver of

evolution[33]. As the first genomes took shape, the slow process of gaining new functions was likely happening through gene mutation. A gene duplication might occur and then a mutation in one of the genes gave a slightly different function. But once enough genes had evolved in the planet, a much greater increase in fitness could be obtained by bringing in a gene with a new function by horizontal gene transfer. That is what we see. For example, comparing *Escherichia coli* and *Salmonella typhimurium* – thought to have diverged about 100 million years ago – there are no new functions that arose by gene mutation. All the functions that are different between the two bacteria are due to horizontal gene transfer[34].

Much of how we conceptualize the tempo of bacterial evolution is through what we have learned from laboratory studies done with fast growing cells (at least some of the time) and in cultures containing a single species. Importantly, we tend to visualize horizontal gene transfer events as being rare, occurring much less frequently than cell division. This view may not be an accurate representation of what happens in most bacterial communities in natural settings.

We think it is important to re-think the rates of horizontal gene transfer and thus the tempo of bacterial evolution in the context of non-growing or extremely slow growing cells (Figure 3). Populations of such cells will replicate their DNA and divide only very rarely. Yet, they retain maintenance metabolism. While not replicating, DNA "transactions" still go on. For example, in aging colonies of *E. coli*, sometimes as many as ten percent of the cells experience a precise transposon excision over a period of weeks[35]. Yet, when the same *E. coli* are grown exponentially, that same precise transposon excision is not detectable. Such are the differences between rapidly growing and non-growing cells. We propose that in settings where there are many non-growing or very slow growing cells –an old colony on an agar plate or a biofilm on a rock – DNA replication will be largely halted and there will be little or no cell division. Yet in those settings, recombination – by conjugation, transformation, or transduction – could still be going on relatively frequently. Consequently, individual cells will get a chance to "probe" numerous DNA combinations from their neighbors before they divide. In a pure culture this might not have a tremendous evolutionary impact. But in the context of a multi-species community, the ecological and evolutionary consequence of recombination rates being orders of magnitude higher than division are enormous. Thinking of non-growing populations in this way might also help explain why in so many natural settings, even when sampled at short distances, genetic siblings do not seem to be the norm[36]. It is as if clonal growth does not occur there. Is it because adjacent cells have probed and retained DNA from other strains and/or species? This different view, where recombination rates are higher than division, can also help explain the odd findings that recombination rates, as measured by sequence alignments, vary over many orders of magnitude when comparing individual genes from sympatric *E. coli* strains[37].

Bacterial Evolution: from generalists to specialists - genome reduction, neighbor addiction

Let's set aside the evolutionary process in bacterial assemblages and focus on the ecology of co-existing species in such settings as natural biofilms. It is a well-known fact that from such

settings it is often very difficult to cultivate all the different species in isolation. There is no single answer to this difficulty in cultivation. But one emerging view is that many of the species present absolutely require the presence of the other species to grow. This view is supported by the finding that more species can be obtained as pure cultures if the cultivation is done in the presence of the original community, separated from the isolates by membranes that allow the exchange of secreted metabolites[38]. In the simplest of cases, the dependence turned out to be a cell's inability to make an iron chelator – a siderophore – that it could obtain from a neighbor[39]. Having a reduced genome may make replication less energy intense. But the organism becomes obligately addicted to the presence of neighboring bacteria able to make the siderophore. This "neighbor addiction" could easily become multifactorial, where many different neighbors provide many different requirements to different species. The result: communities where many of the species are obligately interdependent, able to co-exist and remain viable in the context of the community but are not easy to cultivate on their own. Truly a tightly knit community.

Closing statement

The ability to utilize resources efficiently, at times during rapid growth but mostly when not growing, has served bacteria well throughout their history on Earth. After four billion years of evolution, bacteria are likely the most diverse and successful organisms in terms of population numbers. Their ubiquity and metabolic diversity point to their foundational role in virtually every one of Earth's ecosystems. While we have limited our discussion to heterotrophic bacteria, we feel the overall general principles apply to other bacteria, for example phototrophs, as well as archaea and microbial eukaryotes. Throughout this essay we have drawn attention to how bacteria are thrifty in their utilization of resources, when they are actively growing but more importantly, when they are not and when they transition between these states. Importantly, we highlight the fact that much still needs to be learned about exactly how non-growing bacteria cope with energy limitation. The application of highly sensitive single-cell assays to study non-growing cells promises to enlighten us in this regard in the coming years. Of equal importance to application of new methodologies, is the need to go beyond prior concepts that have been learned from studying growing cells. Our posited concept of the mode and tempo of bacterial evolution in extremely slow growing bacteria, where many horizontal gene transfer events might happen in the time that it takes a cell to divide, is a conceptual message where even evolution is thrifty in that many genetic changes can happen in a small number of generations.

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Figure Legends

Figure 1. When bacteria are growing the concentration of most cellular components, *e.g.*, proteins, is governed by the ratio of their rate of synthesis over their dilution due to cell volume increase. The degradation rate is less important (shown in grey font to indicate lesser contribution) since most cellular components – mRNA being an exception – are stable relative to their dilution rates. In contrast, during growth arrest the degradation rate often dominates cell component concentration because synthesis rates (now in grey font) drop dramatically and there is no dilution (also in grey font) since the cell volume no longer increases. As a result, growing bacteria can respond much faster to environmental stresses while non-growing bacteria must prepare in advance through cellular differentiation (symbolized by the different color of the non-growing cell) to cope with eventual unfavorable conditions.

Figure 2. Stress triggers some bacteria to go into a non-growing state which subsequently protects them from lethal antibiotic treatments. While growing bacteria (brown) are killed, bacteria that happen to be non-growing during the antibiotic treatment will persist. Once the antibiotic treatment and stress conditions are removed, these triggered persisters may regrow. Whereas persisters that entered a non-growing adapted state (yellow) may regrow fast, persisters that entered the disrupted state (dashed yellow/brown) will re-grow only after an extended and highly variable lag (noted by the dashed arrow).

Figure 3. Evolution may be very different in fast growing versus very slow growing bacteria. Per cell division, the frequency of transpositions, other DNA rearrangements and horizontal gene transfer (HGT, be it via phage, plasmids or naked DNA) is much higher in slow growing cells. When cells grow rapidly, the population is largely clonal, and most cells have nearly identical genomes. In contrast, extremely slow growing cells that have multiple copies of their genome could, over long periods of time (dashed vertical line), have different transpositions, other rearrangements and/or HGT events in separate copies of the genome. When the different genomes eventually segregate into daughter cells, the cells will be genetically different. Thus, very slow growing cells could result in large genetic diversity in populations found in one location (sympatric).

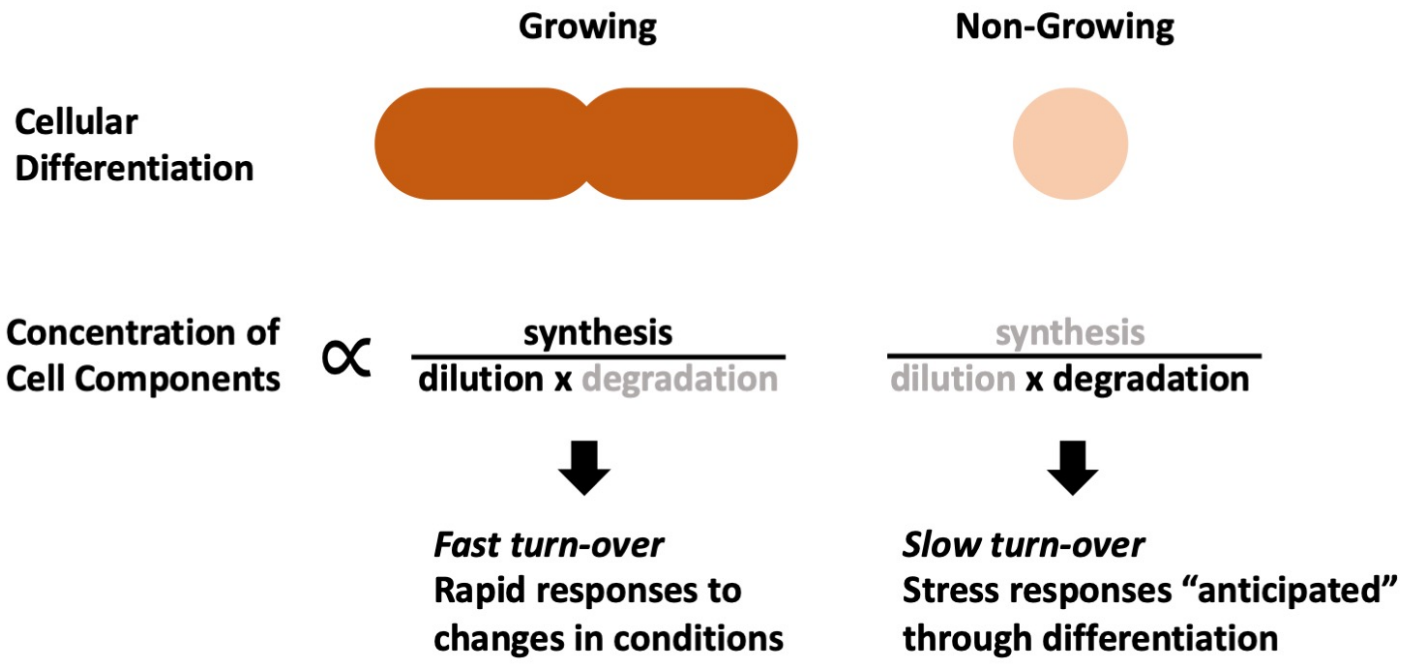
Growing
Non-Growing
Cellular Differentiation
Concentration of Cell Components
∝
synthesis
dilution x degradation
synthesis
dilution x degradation
Fast turn-over
Rapid responses to changes in conditions
Slow turn-over
Stress responses "anticipated" through differentiation

Figure 1

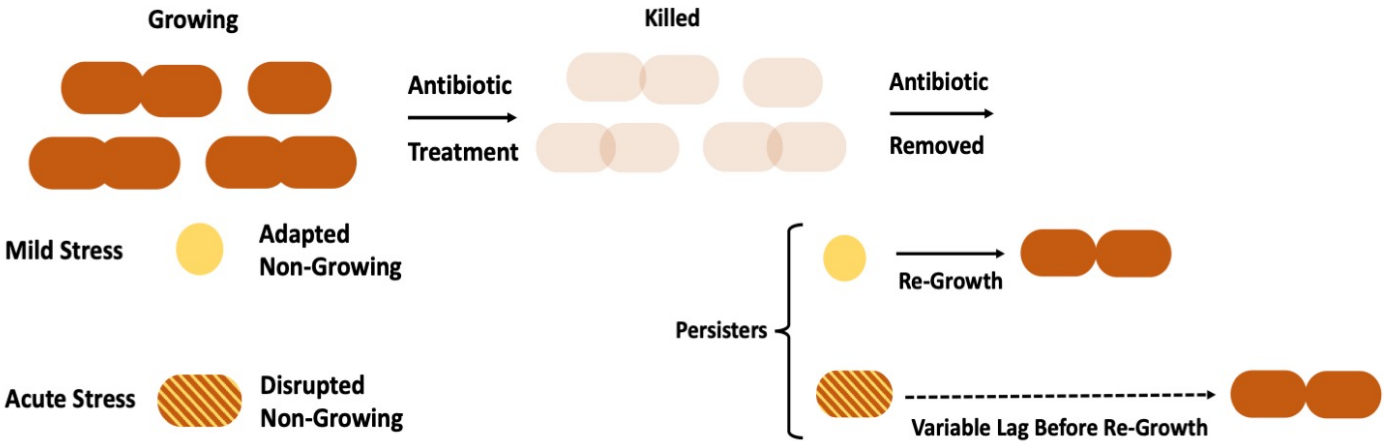
Growing
Killed
Antibiotic
Treatment
Antibiotic
Removed
Mild Stress
Adapted
Non-Growing
Acute Stress
Disrupted
Non-Growing
Persisters
Re-Growth
Variable Lag Before Re-Growth

Figure 2

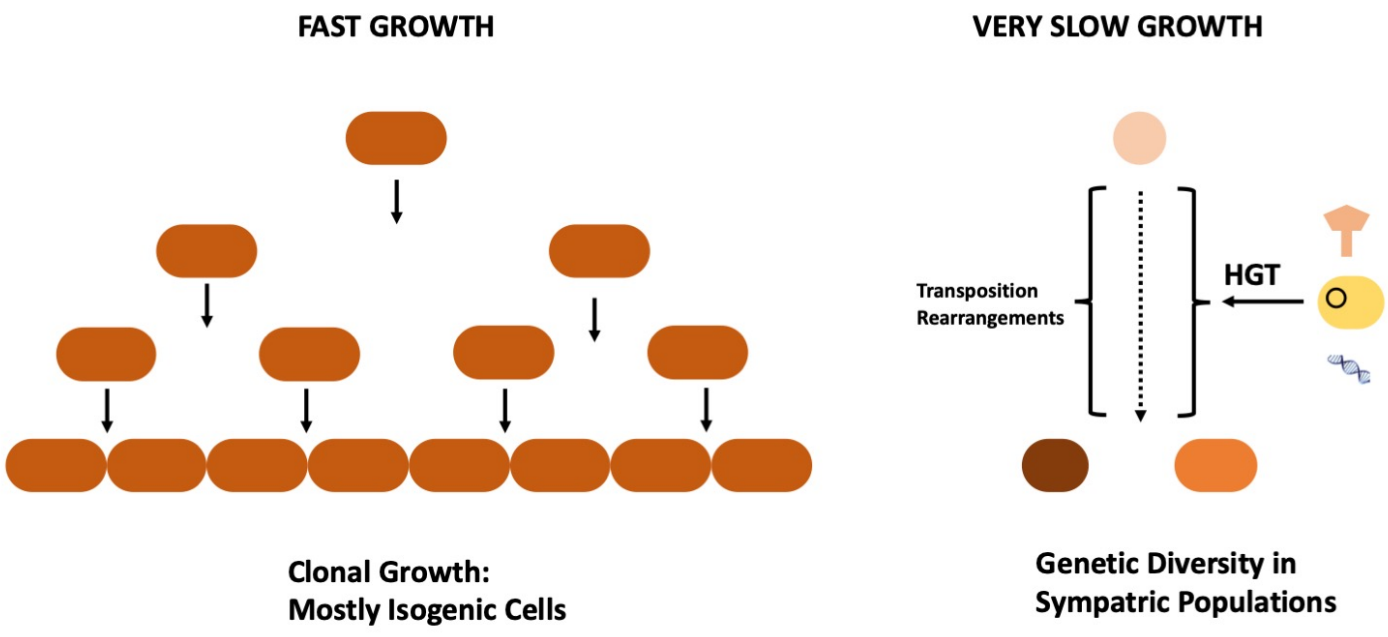
FAST GROWTH
VERY SLOW GROWTH
Transposition
Rearrangements
HGT
Clonal Growth:
Mostly Isogenic Cells
Genetic Diversity in
Sympatric Populations

Figure 3