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Review

Endurance of extremely prolonged nutrient prevention across kingdoms of life

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SUMMARY

Numerous observations demonstrate that microorganisms can survive very long periods of nutrient deprivation and starvation. Moreover, it is evident that prolonged periods of starvation are a feature of many habitats, and many cells in all kingdoms of life are found in prolonged starvation conditions. Bacteria exhibit a range of responses to long-term starvation. These include genetic adaptations such as the long-term stationary phase and the growth advantage in stationary phase phenotypes characterized by mutations in stress-signaling genes and elevated mutation rates. Here, we suggest using the term "endurance of prolonged nutrient prevention" (EPNP phase), to describe this phase, which was also recently described in eukaryotes. Here, we review this literature and describe the current knowledge about the adaptations to very long-term starvation conditions in bacteria and eukaryotes, its conceptual and structural conservation across all kingdoms of life, and point out possible directions that merit further research.

INTRODUCTION

Searching for the term "growth curve" in bacteriology textbooks, for example, in Todar's Online Textbook of bacteriology (Todar, 2015) or even in Google Images, reveals the "classic" four-stage curve. This curve consists of the "lag phase," where bacteria adapt to the different environment. For example, this step may take a few hours for *Escherichia coli* (Figure 1). Next is the "log phase" where cells divide exponentially. For *E. coli*, this step likewise takes a few hours. The log phase comes to an end due to several possible reasons, including exhaustion of nutrients, accumulation of toxins, and growth-inhibiting quorum-sensing signals. In rich media such as Luria–Bertani medium (LB), *E. coli* cells and many other bacteria may grow to concentrations of about 10^9 bacteria/ml. The next growth phase is the "stationary phase," where cell division ceases in a regulated manner and can remain in their maximal concentration for days (Figure 1). This step is followed by the "death" phase, also termed "the decline" phase of growth. This phase is characterized by massive cell death – within a few days, the concentration of live cells drops many folds until a fraction of the original culture (exact number varies and depends on exact conditions) remains viable (Pletnev et al., 2015).

The common conception about the death phase used to be that it terminates with the eradication of the culture. However, usually, this is not the case. At this point begins a very interesting and important, yet underexplored phase in the life of microorganisms. This phase is termed "long-term stationary phase" (LTSP), describing one similarity of this phase to the stationary phase. However, since this phase also has many differences with a stationary phase such as active division and death of cells and cycles of growth, we now suggest using the term "endurance of prolonged nutrient prevention (EPNP phase)," which we will use throughout this manuscript.

In general, organisms from all branches of life can use several strategies when starvation occurs. Initially, as an immediate reaction, they execute transcription-based and protein-based programs that allow them to face starvation (Pletnev et al., 2015). Examples of such programs are the starvation transcriptional factor SigmaS in bacteria, TOR in eukaryotic cells, autophagy, and the use of energy storage molecules such as fat and glycogen in multicellular organisms (Yang et al., 2019). A second common strategy is to differentiate into specialized long-term survival forms which are nondividing and have reduced metabolic activity. Examples of this strategy are spores of bacteria (Huang and Hull, 2017; Paul et al., 2019), fungi (Park and Yu, 2012), protozoa (Corliss, 2001), and seeds of plants (Fenner, 2000). The third approach is reducing metabolism to a minimum in a dormant cell form. Examples of this strategy are persistent cells in bacteria

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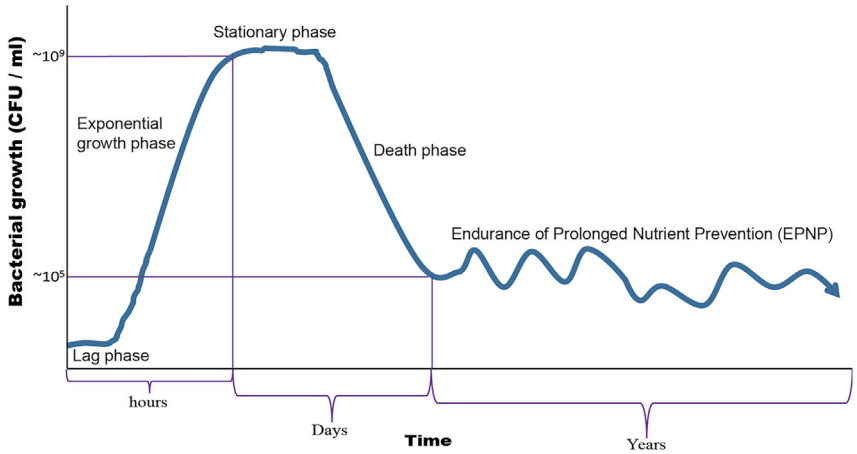

Figure 1. The four widely-described phases of microorganism growth in culture

Lag phase-adapting to the new conditions, log phase-active cell division and cell cycle, stationary phase-exhaustion of nutrients to the point of cell division-controlled arrest and maintenance of cell numbers, death phase-loss of cell viability down to 1/10,000 of the original live cell density. These four phases are followed by a fifth, less known, and longer, endurance of prolonged nutrient prevention (EPNP) phase, where cell death and cell division balance each other, and where different mutants occasionally take over the culture in typical waves. Different phenotypes such as resistance to stress, mutations in stress signaling genes, elevated mutation rate, and increase in diversity also appear at this stage (see text).

(Fisher et al., 2017) and fungi (Wuyts et al., 2018), the "viable but not culturable" state of bacteria (Ramamurthy et al., 2014), and hibernation (Gregory, 1982; Storey, 2003), and estivation (Geiser, 2010) of multicellular organisms.

A fourth strategy, which we hypothesize to be highly abundant in nature and conserved in both prokaryotes and eukaryotes (see below), yet paradoxically one of the least explored, is the EPNP phase, where cell cultures intriguingly maintain their dividing and metabolic activities at a relatively low level in the absence of new nutrients. This is the predominant response in cases of prolonged starvation beyond a few days. Actually, most of the cells on earth are likely in the EPNP phase, as evident from the following observation: Ocean water contains about 10^5 cells/ml (metabolically inert and live cells). However, the addition of nutrients, for instance, rich medium like LB, to seawater, will increase cell concentration to about 10^9 cell/ml, indicating that the reason the cells were not present in their maximal capacity was the lack of nutrients and their EPNP phase strategy.

Another growth phase that was not well studied but, like the EPNP phase, is starting to be appreciated as important, is the lag phase. In the lag phase, cells are arrested in the division and are adapting to a change in the environment or medium, before embarking on exponential growth. Like in the EPNP phase, lag phase cells are in preparation for active growth and are activating regulatory mechanisms of several types to overcome the stress of the new environment. Therefore, the interesting similarities and differences between the lag phase and the EPNP phase could provide an important area of research and provide hints for global strategies to cope with stress (Bertrand, 2019; Swinnen et al., 2004; Vermeersch et al., 2019).

It is important to note that bacterial cultures can take several of the four survival strategies simultaneously. For example, this was shown for cultures of *Bacillus*, the classical model of sporulation, that sporulated while also entering the EPNP phase during prolonged starvation (Gray et al., 2019). Intriguingly, laboratory experiments demonstrated that cells in the EPNP phase can survive for years.

Here, we review current knowledge about the EPNP phase (LTSP) longer than 10 days in bacteria and eukaryotic microorganisms. Moreover, we argue that EPNP phase may exist in cells of multicellular organisms.

EPNP PHASE IN BACTERIA

As mentioned above, the growth of bacteria is characterized by four phases (Figure 1) that vary in length of time, sets of genes expressed, phenotypes that emerge, and the metabolic processes taking place in them.

Ironically, our knowledge is negatively correlated to the time bacteria spend in each phase. The number of studies on bacteria at the logarithmic and stationary phases, which takes only hours to days, is immeasurably greater than the number of studies describing bacteria in the EPNP phase, which may last for years.

Initial observations published as early as 1939 (Steinhaus and Birkeland, 1939) showed that the death phase of many kinds of bacteria does not persist until total eradication of the culture but rather that the concentration of bacteria stabilizes and remains steady for long periods (Table S1). In fact, the question of how long a culture of *E. coli*, for example, can survive under starvation until total eradication is still open. Remarkably, some studies show that bacterial cell cultures retain their viability and division ability for at least 10 years (!) (Gagliardi et al., 2016). However, the majority of previous studies on the EPNP phase (LTSP) have concentrated on genetic and phenotypic effects between 10 days in culture, when the sharp decline of viable cells occurs (death phase), and two years in culture (see Table S1 for details of known studies) (Steinhaus and Birkeland, 1939).

Most of the data about bacteria in the EPNP phase was gained from *E. coli* using the colony-forming unit method (Table S1), even though this bacterium is not considered a specialized survivor which can tolerate extreme conditions such as *Bacillus* spores (Robador et al., 2019). However, survival in the EPNP phase is not limited to *E. coli*, and examples of cultures in the EPNP phase were shown in many bacterial and archaeal species (Winters et al., 2015) (Table S1), demonstrating its wide-spread conservation among prokaryotes (Katz et al., 2021; Ratib et al., 2021; Steinhaus and Birkeland, 1939).

PHENOTYPES OF CELLS IN EPNP PHASE

In 1993, groundbreaking work by Kolter's laboratory from Harvard showed that surviving *E. coli* cells in the EPNP phase (LTSP) have a growth advantage over "young" cells, reflected in the observation that "aged" cells take over in competition experiments where both cell types are growing in a mixture (Zambrano et al., 1993). They termed this phenotype growth advantage in stationary phase (GASP), which became a significant characteristic feature of EPNP phase cells (Navarro Llorens et al., 2010; Sewell et al., 2011). GASP was mainly studied in liquid cultures; however, several studies demonstrated its involvement in biofilms as well (Elabed et al., 2013; Kraigsley and Finkel, 2009). It seems that the ability of EPNP phase cells to take over the culture is not a fundamental characteristic of the EPNP phase. For example, as a *Campylobacter jejuni* culture survived long-term starvation, its cells became more resistant to various stresses such as aeration, peroxide challenge, and heat. Yet in competition experiments with "young" cells, they failed to take over (Martinez-Rodriguez et al., 2004) (see discussion below).

Surprisingly, cells that were isolated from "aged" cultures were often more resistant than their "young" ancestors to various stresses which were irrelevant to the conditions of the EPNP phase; for instance, more resistant to cold stress after starvation in ambient temperatures (Kram et al., 2020). This observation strongly suggests that EPNP phase cells tend to gain mutations in general stress regulators, as was indeed shown in genome sequencing studies (See below).

Other common phenotypes described for EPNP phase cells are changes in cell morphology such as size, shape (rods or spheres) (Alvarez et al., 2008; Gray et al., 2019; Winters et al., 2015), and motility (Elabed et al., 2013). Morphological changes were also observed in the shape, size, and surface structure of EPNP phase bacterial colonies (Machreki et al., 2019; Tavares et al., 2020). However, these changes in cell and colony morphology are species and even strain- and condition-specific and it is difficult to generalize conclusions from these specific examples.

Conversely, several reports describe that despite the prolonged time pathogenic bacteria spent in a liquid media without supplemental nutrients and any contact with a host, they still maintained their pathogenicity and their ability to form biofilms (Chapartegui-Gonzalez et al., 2018).

The diversity in phenotypes is likely attributed to the mutator effect found in many clones of the EPNP phase (Katz et al., 2021) (see below).

THE LINKS BETWEEN CHANGES IN STRESS SURVIVAL AND CHANGES IN COMPETITIVE FITNESS

The finding that cells that are exposed to long periods of stress become stress-resistant and, at the same time, increase their competitive fitness and raises the possibility that these two phenomena are linked. The discovery that an important mutation in the EPNP phase occurs in the *rpoS* sigma subunit, the same subunit induced in starvation and other kinds of stress strengthens this hypothesis (Zambrano et al., 1993) (see below). However, the links between increased stress survival and competitive advantage in culture have been explored in other situations, and the picture seems to be much more complex than initially perceived. Some studies support a strong link between the two (Bruggeman et al., 2020; Schinner et al., 2020; Schirrmacher, 2021). Other studies have failed to find a simple correlation and have attributed this to the specific system they were exploring (Bleibtreu et al., 2013). Yet, more studies have sought and found specific parameters in stress behavior that predict fitness. One study has looked at the behavior of single cells and integrated it into a model (Mathis and Ackermann, 2016). Another study has measured entropy in stress resistance as a predictor for bacterial fitness (Mathis and Ackermann, 2016). Overall, it seems that there may indeed be specific parameters in behavior under stress that could predict how well the organism will adapt to various conditions but that the two phenomena are not completely correlated. The complex nature of this correlative relationship also emerges from two studies performed on long-term starvation in *E. coli* (see below) (Katz et al., 2021; Ratib et al., 2021). This raises the possibility that such is the situation in all species.

CONDITIONS AFFECTING SURVIVAL IN THE EPNP PHASE

Most of the studies published on the EPNP phase are descriptive, comparing the effects of growth conditions on the EPNP phase (Table S1). Clearly, and perhaps not surprisingly, environmental conditions of the culture in terms of media richness, salinity, pH, and temperature before starvation determine whether and for how long, the culture would survive in the EPNP phase (Table S1). Also, adaptation in one medium affects fitness in other media (Westphal et al., 2018). Nevertheless, it is difficult to draw general conclusions from these comparisons because as with all other growth phases, the effect of the environmental conditions is bacterial species-specific. Moreover, when new nutrients are introduced, the new conditions of the culture are also important for the degree of culture recovery (Xavier et al., 2014).

FUNDAMENTAL DIFFERENCES BETWEEN SHORT- AND LONG-TERM SURVIVAL TECHNIQUES

An important question that arises when looking at the strategy that cells use upon long-term starvation is the similarity of this program to the reaction to short-term starvation. Short-term starvation in prokaryotes has been widely studied and many genetic networks and other types of response (e.g. metabolic, morphological) have been characterized in some detail (Shen and Chou, 2016; Shimizu, 2016; Switzer et al., 2018). The end points of those strategies, that is the phenotype of the cells with regards to the ability to withstand shortage of nutrients are certainly similar. In both processes, the cells are not only resistant to starvation but also other types of stresses such as heat shock. In both types of strategy, the cells change their metabolism and their morphology to adapt to the stressful environment. However, the mechanisms used to achieve these phenotypes vary greatly between short-term and long-term starvation. The key distinction between the mechanisms leading to both strategies is that most reactions to short-term starvation are transient and reversible in nature. However, in long-term starvation, considerations of energy conservation come into play and changes tend to occur as mutations that are not transient or easily reversible. For example, as detailed below, a common reaction to stress caused by short-term starvation in bacteria is the activation of different types of sigma subunits of the RNA polymerase (Paget, 2015), while in long-term starvation, mutations occur in Sigma factors which enable the culture to develop the phenotype referred as GASP (Zambrano et al., 1993) (Paget, 2015; Zambrano et al., 1993).

GENETIC STUDIES OF EPNP PHASE

An overall look at studies that examine genetic components involved in survival during the EPNP phase and the evolvement of GASP (Table S1) raises several clear conclusions. Perhaps most striking is the observation that despite the selective conditions which usually lead to the dominance of the fittest strain over the others, diversity and the richness of mutations in the EPNP phase, and the cohabitation of multiple mutants increases over time (Avrani et al., 2017; Chib et al., 2017; Katz et al., 2021). This incremental diversity is achieved by a mutator effect originating from mutations or alteration in the expression in DNA damage

repair factors (Avrani et al., 2017; Chib et al., 2017), error-prone SOS-induced DNA polymerases (Avrani et al., 2017; Corzett et al., 2013; Yeiser et al., 2002), oxidative stress factors such as OxyR, and upregulation of genes coding for insertion sequences and putative transposases (Arunasri et al., 2014). Moreover, the appearance of the mutations is dynamic and they are highly transient (Avrani et al., 2020; Katz et al., 2021). To conclude, it seems that a diverse culture containing many different genotypes is a key factor in EPNP phase survival. As a result, the mutation variants which provide adaptation might be different in each experiment, even when conditions are identical.

These concepts were modeled by Austin's group, who used an approach of evolutionary game theory, demonstrating that the GASP phenotype resulted from a combination of cooperative and selfish behaviors. Another group of mutations confer adaptation to the EPNP phase and are responsible for the GASP phenotype, which was paradoxically found in factors that are responsible for the regulation of short-term starvation (Table S1). When bacteria face starvation, they simultaneously express a large set of operons and genes, scattered across the chromosome, allowing them to cope with the situation. This global response is coordinated by modular sigma subunits of the RNA polymerase that replace the housekeeping Sigma^{70} and are responsible for the recognition of the promoter sequence of stress-relevant genes. In starvation, the levels of SigmaS, encoded by *rpoS,* are increased, and the levels of Sigma^{70}, the housekeeping sigma, are decreased (Paget, 2015). Interestingly, in EPNP phase cultures, one of the most significant mutations (and the first discovered mutation to contribute to the EPNP phase) is *rpoS819* which also by itself leads to the formation of the GASP phenotype (Zambrano et al., 1993). In addition to SigmaS, mutations that lead to the downregulation of genes coding for stress and stationary phase response were identified (Arunasri et al., 2014). These kinds of mutations probably arise because a constant activation of the stress response for a long period becomes energetically costly and more of a burden than a benefit. A reduction in stress response at this stage seems to provide benefits. To use an analogy, the bacteria turn off their alarm when the state of emergency continues. Several more mutations that contribute to adaptation to EPNP phase were found using the *rpos819* mutation as a genetic background for a genetic screen, including mutations in the regulators H-NS (Chib and Mahadevan, 2012) and *lrp* (Zinser and Kolter, 2000) and in the *sga* genes (Zinser and Kolter, 1999). All these mutations were found to provide growth advantages in various media with a different subset of amino acids. However, it is important to note that mutations in *rpoS* are not obligatory in the EPNP phase, and *rpoS*-independent pathways leading to GASP were also described in *rpoS* deleted mutants (Helmus et al., 2012; Martinez-Garcia et al., 2003; Westphal et al., 2018).

In addition to these, mutations conferring adaptation to the EPNP phase were found (Al Dahouk et al., 2013) in various metabolic stress proteins (Gagliardi et al., 2016; Lostroh and Voyles, 2010; Tavares et al., 2020), chaperones, cell envelope genes (Lostroh and Voyles, 2010), amino acid metabolism genes, transport, and outer membrane proteins (Al Dahouk et al., 2013; Ben Abdallah et al., 2010; Bruscolini et al., 2014) and other cellular functions (Al Dahouk et al., 2013; de Maat et al., 2020). These mutations also include the conserved polyamine spermidine (Elabed et al., 2013) and fatty acids (Lagha et al., 2015). Quorum-sensing regulation machinery components were also found to be mutated in the EPNP phase (Takano et al., 2017).

Two recent studies have genetically characterized EPNP phase cells most comprehensively up to date (Katz et al., 2021; Ratib et al., 2021). Both studies have looked at cultures sampled for over 3 years and have mapped the genetic landscape of mutations in several parallel sampled cultures. The mutational landscape uncovered is complex, but several conclusions emerge from both studies. One main conclusion concerns the appearance of different types of mutations, including large deletions and amplifications, although the major type of mutation that occurred was a base substitution creating SNPs. Both studies saw the fixation of specific mutations, including mutations that were described before such as the mutations in *rpoS*. One of these studies (Katz et al., 2021) characterized the appearance of mutators during this type of evolution. Interestingly, this phenotype did not appear in all clones but lead to a different mutational landscape than in non-mutator clones. In general, these two studies confirmed previous studies on the subject but have provided more details in an unprecedented manner.

A significant gap in knowledge and a possible direction for future studies is that most of the studies on EPNP phase (LTSP) so far were performed using single species of bacteria in liquid culture, after the cells were grown to stationary phase, with concentrations of about 10^9 cells/ml. These conditions hardly

resemble the natural conditions that bacteria face. In contrast, only a few studies explored the EPNP phase in solid cultures or biofilms (Castegnier et al., 2006; Elabed et al., 2013; Kraigsley and Finkel, 2009). Therefore, in our opinion, it is now time to move forward to the next step of studies, using methods from the field of systems biology and omics to directly view natural niches, multispecies cultures, and biofilms comprehensively. These global-view studies will assist in better understanding the most natural state of bacterial life around us.

EPNP PHASE IN EUKARYOTIC CELLS

Strategies to cope with long-term starvation, resistance to drug treatment, stress, and tolerance mechanisms are universal traits of all cells, rather than only in bacteria. Indeed, expression of sets of stress genes (Vihervaara et al., 2018), dormancy (Recasens and Munoz, 2019), persistence (LaFleur et al., 2006), sporulation (Huang and Hull, 2017), and hibernation (Mohr et al., 2020) was described in many eukaryotic cells and organisms including yeast, spores of filamentous fungi, seeds of plants, and even whole plants and animals. Persistence, for example, was exhibited in *Candida albicans* cells (LaFleur et al., 2006) where biofilms of the fungus were exposed to the drug Amphotericin B. Persister cells have also been shown to exist also in human tumors (Ravindran Menon et al., 2020; Yu et al., 2018). For example, in targeted therapy to T-cell-acute-lymphoblastic leukemia by γ-secretase inhibitors (GSIs), some transiently resistant cells have been found. This study links these persistent cells to mutations enhancing the activity of several chromatin regulators, among them the gene BRD4, which modulates the activity of MYC and BCL2. This study also found that using inhibitors against BRD4, together with chemotherapy, was effective against persister formation. In another example, researchers have shown that melanoma cells switch into a slow-cycling state in reaction to stress. This switching led to the upregulation of multiple melanoma stem cell markers (Ravindran Menon et al., 2015). Another interesting example of a stress response in eukaryotes is long-term dormancy. Specifically, dormancy has been described in tumors. For example, interesting recent research has shown that dormant cancer cells utilize the NRF2 antioxidant transcription factor as a mechanism to change the cell's metabolism and sustain high levels of stress (Fox et al., 2020). Eukaryotic adaptation to stress mechanisms also includes whole-organism or whole-tissue responses. One such example is the formation of stress-resistant differentiated forms such as seeds in plants. Another interesting response is putting the whole organism through an energy conservation mode through a hard season-a response termed hibernation. Hibernation affects the aging rate of the organism and has different physiological effects that affect the reproductive behavior of the animal. This strategy is thus programmed as a way to maximize the fitness of the whole organism through harsh conditions (Bieber et al., 2019).

Despite much research into different stress adaptation in eukaryotes described above, the study of genetic-based adaptation to extremely long-term stress, such as EPNP phase (LTSP) and GASP-related phenotypes, were barely studied and described in eukaryotes.

Yeast has been known to adapt to stress genetically, as evident from the existence of specialized domesticated *Saccharomyces cerevisiae* strains for making beer or wine-processes which include growth in stressful conditions (Adamczyk et al., 2016; Borneman et al., 2011; Gallone et al., 2016). Some important contributions that paralleled experiments discussed above in *E. coli* were made by evolution experiments in *S. cerevisiae*. Among others, the origin of sex and the rate of mutation under normal growth conditions were tested by long-term evolution experiments (Zeyl, 2006). However, studies looking at genetic adaptation to long-term starvation (*e.g.*, GASP-like phenotypes) or other long-term stress conditions are lacking. We have investigated the adaptation process that *S. cerevisiae* cells undergo after two years of starvation in a stressful environment of a beer bottle (Aouizerat et al., 2019a). We found striking lines of similarity between characteristics of GASP in bacteria and yeast. Among others, we found that yeast increase resistance to general stress, show higher mutation rates, and disrupt stress signaling pathways while still maintaining relatively high rates of cell division under prolonged starvation and stress conditions (Aouizerat et al., 2019a). Specific molecular changes that we found are mutations in the TOR pathway, which is known to be involved in many aspects of resistance to stress (Cebollero and Reggiori, 2009), and many DNA repair pathways. Generally, we find that like in GASP, the resulting strains, after two years of prolonged stress, are more adapted to the stress than the original parent. Support for this notion is the finding of yeast strains that survived for thousands of years in the micro-pores of clay vessels that contained beer. We managed to revive these yeast cells and rebrew beer from them (Aouizerat et al., 2019b). Although the exact mechanisms that lead to the survival of these cells remain a mystery (including the question of whether yeast

sporulation was involved), GASP-like phenotypes and adaptation to stress could contribute to this phenomenon.

Other eukaryotic single-cell organisms have rarely been explored for their response to periods of starvation lasting more than several days. A notable exception is a study of microalgae in prolonged starvation of various nutrients, especially nitrogen. The algae respond to nitrogen starvation by the accumulation of lipids and polysaccharides as carbon storage, and the degradation of several components of the light-harvesting machinery as a nitrogen salvage activity (Lauritano et al., 2019; Zhao et al., 2017). However, the existence of adaptation mechanisms such as the level of resistance to stress and the mutation rate after prolonged starvation was not explored so far in these organisms.

EPNP PHASE IN MULTICELLULAR ORGANISMS

Long-term starvation is not limited to unicellular organisms. However, in most cases, multicellular organisms tightly control the division of their cells by various mechanisms. This partly prevents genetic adaptations such as the EPNP phase and GASP from occurring in multicellular organisms. There are many interesting exceptions to this rule.

Genetic adaptation to starvation in cancer cells: Cancer cells lose organismal control over their division rate. Therefore, cancer cells inside a tumor divide in reaction to external conditions and may react more readily to starvation conditions and genetically adapt to starvation using strategies that resemble GASP. Cancer cells are routinely exposed to low-nutrient environments in the internal part of tumors. The most extreme case tested so far is in glioblastoma (GBM), where the cells in the interior of a large tumor are not exposed to the blood supply, and some of them die and form a necrotic area (Liu et al., 2017). Around the necrotic area, a layer of specialized cells appears which are termed pseudopalisading cells (Rong et al., 2006). These cells have special traits like the secretion of VEGF and interleukin-8 proangiogenic factors and the overexpression of the hypoxia-inducible factor (HIF-1) (Rong et al., 2006). It is not known to what extent pseudopalisading cells genetically adapt to their nutrient-poor, hypoxic environment, and to what extent such an adaptation contributes to the emergence of drug resistance in GBM. If such a mechanism of genetic adaptation indeed occurs, it could partially explain the significantly higher aggressiveness of recurrent tumors in the primary site in comparison to the initial tumor (Lobbezoo et al., 2015). Tumor cells that have survived resection and treatment may have genetically adapted to a nutrient-poor environment inside the tumor and may have acquired a growth advantage which is expressed when they fully mature into a recurrent full-size tumor (Kumar et al., 2019). Further evidence for the possibility that starved cancer cells evolve genetic adaptation mechanisms comes from finding enhanced nonsense mutation readthrough in serum starvation conditions (Wittenstein et al., 2019). In addition, cancer cells with a different chromosome number (diploid vs. aneuploidy) or different mutations show different growth capabilities in a controlled growth experiment (Kurppa et al., 2016; Rutledge et al., 2016). These examples, and many more, show that cancer cells are capable of genetic adaptation to prolonged starvation conditions. Whether they do adapt *in vivo* and whether this genetic adaptation influences clinical features such as tumor recurrence and metastasis are still to be investigated.

Despite the tight control that organisms hold over cell divisions inside their bodies, there are some notable cases of extreme tolerance of whole organisms to prolonged stress and hints in the published literature as to the genetic plasticity and adaptation that occurs during prolonged stress in these organisms. Whether GASP-related phenotypes occur in whole organisms is still a matter of speculation and the aim of possible future studies. Below, we review some of the publications regarding two cases of whole organisms that are known to withstand extreme stress: tardigrades and nematodes.

Tardigrade genetic adaptation to prolonged stress: Tardigrades ("water bears") are fascinating organisms that can survive extreme stresses. Among these stresses, we can find prolonged periods of complete desiccation and starvation, high doses of radiation, and even exposure to outer space (Jonsson et al., 2019; Koshland and Tapia, 2019; Weronika and Lukasz, 2017). Mechanisms that enable these organisms to survive in stress are being studied, and some interesting unique systems have been discovered, such as the use of intrinsically disordered proteins as a "DNA shield" to protect the genome from desiccation and radiation (Boothby et al., 2017; Minguez-Toral et al., 2020). As mentioned, there is no evidence for adaptive genetic mechanisms that operate in tardigrades during stress, but some clues suggest that this is a valid hypothesis. Strong evidence of genetic adaptation appears in the form of unique molecular signatures of RNA

expression during stress in tardigrades. For example, there is a lack of expression of some stress-related genes and transcripts from genes involved in classic non-homologous end joining (Kamilari et al., 2019). Another study reports the loss of an important stress-related transcription factor, HIFα, from a wide portion of the tardigrade evolutionary landscape. An additional assessment showed losses of HIF repression machinery (EGLN and VHL) (Graham and Barreto, 2020). Since alteration of stress signaling capability is one of the GASP's major hallmarks (see above), these findings could hint at the presence of similar stress adaptation mechanisms in tardigrades. Another hallmark of GASP is the loss of genome integrity along with hypermutability. Indeed, tardigrades show double the number of horizontal gene transfers compared to other animals. This elevated rate may assist tardigrades in genetic adaptation to stressful environments (Boothby et al., 2015).

Nematodes: Nematodes are extremely resilient animals. They live in practically every environment on earth, including the poles, and subsurface environments, including deep mines (Borgonie et al., 2011; van den Hoogen et al., 2019). Nematodes have a variety of mechanisms to withstand and survive stressful environments. Studied noted examples include dormancy and quiescence (Carranza-Garcia and Navarro, 2020; Hand et al., 2016; Yen et al., 1995) and a specialized form of a stress-induced durable form called "dauer" in several species including *Caenorhabditis elegans* (Androwski et al., 2017; Hu, 2018). Genetic adaptation to stress for whole worms has not been studied in detail, but several studies have shown that such a phenomenon is possible. Several stress-resistant nematodes show genomic adaptations to stress such as an expanded repertoire of 70-kilodalton heat-shock proteins (Hsp70) and avrRpt2 induced gene 1 proteins, probably acquired through horizontal gene transfer (Guerin et al., 2019; Weinstein et al., 2019). Another study shows genetic adaptations of *C. elegans* wild strains including a region on chromosome III affecting starvation resistance (Webster et al., 2019). An interesting phenomenon also discovered as an adaptive starvation mechanism in *C. elegans* is the processing of mitochondria and the use of nutrients derived as a result of this activity. This activity is termed mitophagy and genetic diversity in this mechanism also affects resistance to starvation (Chen et al., 2017; Galluzzi et al., 2017; Hibshman et al., 2018).

PERSPECTIVES AND FUTURE DIRECTIONS

Mechanisms to overcome stress are widespread and diverse, from cell cycle arrest and quiescence to stress-endurable specialized forms such as spores and seeds in fungi and plants. It is important to understand that while the cells appear to be static during the long stress, genetic adaptation is at play during this stage.

Additional important and underexplored questions are the mechanisms of the transition from nondividing states during short and long starvation to actively dividing cells. For instance, how similar is the known "lag" phase observed at the beginning of culture growth to the division cycles within the EPNP phase?

Genetic adaptation to long-term stress appears to operate in eukaryotic cells. This GASP-related phenotype appears to be a mechanism that can be used by single-cell eukaryotes in reaction to long-term starvation. The characteristics of this GASP-like mechanism show that GASP may be evolutionarily conserved. The exact evolutionary mechanisms used by yeast and other eukaryotes during prolonged stress and starvation are still not clear and future studies will address this mechanism. A related important question is the occurrence of genetic adaptation to prolonged stress of cells that have lost control over their division in multicellular organisms, such as cancer cells. Future studies will address the presence of GASP-like mechanisms in cancer cells.

Whether or not genetic adaptation operates during long-term starvation in whole multicellular organisms is a matter of debate. As more evidence amasses to prove or disprove this theory, defined experiments should address this question in simple model organisms such as *C. elegans*.

Limitations of the study

This manuscript summarizes published work on the subject of long-term endurance of nutrient deprivation. We were limited by span of previously published work and the subjects we decided to cover here. Future perspectives and possible future directions of exploration are detailed above.

Methods

All methods can be found in the accompanying transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102745.

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AUTHOR CONTRIBUTIONS

M.K. and R.H. designed the study and wrote the manuscript. MS has helped with the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare they have no conflict of interest.

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