MICROBIOLOGY

REVIEW
Nandy, *Microbiology* 2022;168:001195
DOI 10.1099/mic.0.001195

MICROBIOLOGY SOCIETY
OPEN ACCESS

The role of sigma factor competition in bacterial adaptation under prolonged starvation

Pabitra Nandy[1,2,*]

Abstract

The study of adaptive microbial evolution in the laboratory can illuminate the genetic mechanisms of gaining fitness under a pre-defined set of selection factors. Laboratory evolution of bacteria under long-term starvation has gained importance in recent years because of its ability to uncover adaptive strategies that overcome prolonged nutrient limitation, a condition often encountered by natural microbes. In this evolutionary paradigm, bacteria are maintained in an energy-restricted environment in a growth phase called long-term stationary phase (LTSP). This phase is characterized by a stable, viable population size and highly dynamic genetic changes. Multiple independent iterations of LTSP evolution experiments have given rise to mutants that are slow-growing compared to the ancestor. Although the antagonistic regulation between rapid growth and the stress response is well-known in bacteria (especially *Escherichia coli*), the growth deficit of many LTSP-adapted mutants has not been explored in detail. In this review, I pinpoint the trade-off between growth and stress response as a dominant driver of evolutionary strategies under prolonged starvation. Focusing on mainly *E. coli*-based research, I discuss the various affectors and regulators of the competition between sigma factors to occupy their targets on the genome, and assess its effect on growth advantage in stationary phase (GASP). Finally, I comment on some crucial issues that hinder the progress of the field, including identification of novel metabolites in nutrient-depleted media, and the importance of using multidisciplinary research to resolve them.

INTRODUCTION

Bacteria are known to inhabit some of the harshest conditions on this planet, and are ubiquitous in our internal and external environments. In order to understand these organisms better, efforts to culture bacterial strains within the laboratory began in the early nineteenth century. In this regard, Louis Pasteur is credited with the formulation of liquid growth media, and Robert Koch with the invention of solid growth media [1]. Although a handful of species could be cultured in the optimized media, most bacteria isolated from natural habitats like soil, ocean beds, and within hosts still remain unculturable in the laboratory [2, 3], mostly because of the lack of carbon, phosphate and nitrogen sources [4–6]. In natural niches, bacteria are compelled to spend most of their lifetime under nutrient limitation – a significant selection pressure shaping microbial genomes [7–9]. Under nutrient limitation, different strategies are adopted by diverse microbes, such as, the formation of resilient dormant structures like endospores [10], the formation of various morphological/functional forms resulting in the division of labour [11], and diversification into variants as seen in long-term stationary phase (LTSP) [12, 13]. The long-term evolutionary trajectories of strains under resource limitation were initially based on *Escherichia coli*. However, an increasing number of microbes across prokaryotes such as *Geobacter sulfurreducens* [14], *Vibrio cholerae* [15], *Listeria monocytogenes* [16], *Pseudomonas aeruginosa* [17] and euckaryotes such as *Saccharomyces cerevisiae* [18] are being probed under strenuous, nutrient-limiting environments, revealing novel adaptive strategies to grain growth advantage in stationary phase (GASP).

Bacterial growth rate is the only essential indicator of adaptive fitness under ideal, resource-abundant conditions, as demonstrated by decades-long evolutionary experiments (long-term evolution experiment, or LTEE) with *E. coli* [19–21]. However, in the wild, acute scarcity of resources to build up biomass and competition from other organisms heavily limit bacterial growth. Hence, the

Received 22 December 2021; Accepted 06 May 2022; Published 20 May 2022
Author affiliations: [1]National Centre for Biological Sciences (NCBS-TIFR), Bangalore, India; [2]Max Planck Institute for Evolutionary Biology, Plön, Germany.
***Correspondence:** Pabitra Nandy, nandy@evolbio.mpg.de
Keywords: LTSP; slow growth; SPANC; sigma factor competition.
Abbreviations: D, dilution rate; GASP, growth advantage in stationary phase; LTEE, long-term evolutionary experiment; LTSP, long-term stationary phase; NAG, N-acetyl glucosamide; NAM, N-acetyl muraminic acid; SCV, small-colony variants; SPANC, self-preservation and nutritional competence.
001195 © 2022 The Authors

This is an open-access article distributed under the terms of the Creative Commons Attribution License. This article was made open access via a Publish and Read agreement between the Microbiology Society and the corresponding author's institution.

dynamics of satisfying the two opposing selection pressures – (i) to maximize growth and (ii) to stay dormant and evade stress via the general stress response – dictate adaptive fitness in natural environments [22, 23].

Although some of the genetic mechanisms enabling adaptation of fast-growing bacteria to LTSP have been reviewed in the past [12, 24], a consummative evolutionary driver behind LTSP adaptation is lacking. In this review, I synthesize recent advances regarding the evolutionary roots of the adaptative strategies demonstrated by fast-growing bacteria (primarily based on, but not limited to, *E. coli*) under resource limitations, one of which is the slowdown of growth. I establish sigma factor competition as a critical determinant of fitness during LTSP. Then, I discuss the mechanisms by which major genetic regulators affect this competition, and leave the readers with some important, unresolved questions in the field.

Slow growth of fast-growing bacterial mutants emerging from starved cultures

A large fraction of microbes occupying natural, nutrient-limited niches like soil, sections of the mammalian gut, and permafrost are slow-growing [25–28]. Slower growth in bacteria has been shown to be advantageous under carbon starvation [29]. Within the confines of the laboratory, long-term evolution experiments conducted under prolonged starvation have also led to the emergence of slow-growing mutants in both non-pathogens [7, 30, 31], and pathogens [32]. This trend suggests positive selection for slow growth under long-term starvation. Very slow or near-dormant growth states have been demonstrated to increase fitness by enhancing tolerance to different stressors, such as, antibiotic tolerance – a strategy exhibited by persister pathogens [33–35]. Hence, slow growth is viewed as an essential bet-hedging strategy deployed by microbial populations to survive dynamic and stressful environments.

Under high stress, some bacteria such as *Bacillus* spp. can form resilient structures called spores and remain in a non-growing, dormant state until favourable conditions are restored, while other non-sporulating bacteria such as *E. coli* exhibit extremely slow growth under stress [7, 36]. In *E. coli,* the growth rate is controlled by the antagonistic regulation balancing rapid growth and self-preservation (also called self-preservation and nutritional competence or SPANC balance) [22]. Whenever the ambient nutrient availability becomes sub-optimal, resources are allocated to express stress-response proteins instead of proteins assisting rapid growth [37], thereby reducing the growth rate.

Small-colony variants in diverse microbes and their importance in public health

A fraction of slow-growing bacteria are observed to form small-sized colonies on solid agar plates – a phenotype ubiquitously observed in specific isolates of both natural and laboratory-maintained strains. The first documented report of small colony variants (SCVs) dates back to 1910 [38]. Over time, more studies have reported the emergence of small colonies upon exposure to chemicals including copper sulphate [39], phenol [40] and antibiotics such as gentamycin [35]. SCVs also poses a threat in the public health sector, as multiple groups have demonstrated key pathogens to form SCVs at the site of infection, establishing SCV as a dominant phenotype among pathogens [27]. *Staphylococcus aureus* remains the most well-characterized species to form SCVs [41]. However, other pathogens like *Pseudomonas aeruginosa* [35, 42], *Staphylococcus epidermidis* [43], *E. coli* [34], *Serratia marcescens* [44] and *Neisseria gonorrhoeae* [45] are also known to form small colonies. A reduced respiration rate and pigmentation are characteristic of pathogenic SCVs [27]. They consistently demonstrate slow growth primarily through metabolic mutations – either via thymidine auxotrophy, or deficiency in the electron transport chain pathway. Due to their near-dormant metabolic state, SCVs may avoid immune responses from the host and multiple externally administered antibiotics [27] and persist within host niches for prolonged periods, driving chronic diseases [34]. Enhanced tolerance to numerous stressors renders SCVs a public health challenge. In order to specifically target and eliminate infectious SCVs, further investigations into the regulatory origin of this phenotype are required both in laboratory and clinical settings.

Repeated emergence of slow-growing variants of *E. coli* during adaptive lab evolution in LTSP

Batch cultures of bacteria have been shown to remain viable for years without the addition of external nutrients in a paradigm called LTSP [12, 46–48]. In contrast to evolution under one, or a set of, pre-defined stressor(s), the evolving population is exposed to a dynamic set of stress factors during prolonged starvation. Under such conditions, batch cultures experience cycles of 'feast-and-famine' in their environment akin to bacteria in natural habitats such as eukaryotic hosts, river bed sediment, or soil [4–6]. Feast-and-famine alludes to the altering abundance of ambient resources. Most bacteria in nature are thought to spend long periods under minimal nutrient availability (famine), interjected with short periods of nutrient abundance (feast). To maximize survival under such dynamic environments, mechanisms enabling rapid shifts between exponential growth and dormancy (stress-survival) are essential. Multiple independent studies have shown that the bacterial population increases in genotypic and phenotypic diversity during evolution in batch cultures for prolonged periods [13, 46, 49, 50]. Slow-growing variants have also been observed to emerge specifically during LTSP across different studies [13, 30].

This trend raises several questions: Why are slow-growing variants so common across naturally occurring microbiota? Why are small colony variants repeatedly found to emerge from prolonged starved culture independent of ancestral genotypes? Although slow-growing strains are ubiquitous in natural and lab-based populations under nutrient stress, this correlation has not been

reviewed from a gene-regulatory and evolutionary perspective. Pursuance of this question deserves merit because most pathogens as well as other natural isolates are generally slow-growing, and the evolutionary driver behind this trend might hold insights into challenges like antimicrobial resistance and persistence – which are downstream effects of slow growth. This review describes the existing knowledge regarding gene regulation under nutrient deficiency and focuses on sigma factor competition as a genetic mechanism that orchestrates bacterial growth under dynamic stress environments akin to those encountered in LTSP.

BACTERIAL GROWTH DYNAMICS UNDER PROLONGED NUTRIENT LIMITATION

Natural habitats are characterized by long periods of nutrient scarcity interjected by short stints of resource abundance termed the 'feast- and-famine' lifestyle. The evolutionary effects of this lifestyle have been best characterized in *E. coli*. This metabolic, environmentally decided cycle of nutritional abundance and dearth is thought to be one of the key selection pressures in bacterial evolution [4–6, 12]. Within their natural niche, bacteria spend most of their lifetime dividing at very low rates under limited availability of carbon and nitrogen sources, as opposed to extreme scenarios like the complete lack of growth (dormancy) or exponential growth [7, 51]. For instance, *E. coli* inhabiting the intestinal mucosa of the host are limited by carbon and nitrogen source and utilize complex sugars like N-acetyl glucosamide (NAG) and N-acetyl muraminic acid (NAM) to derive energy [52]. Standard methods for growing bacteria in rich media are unable to capture the genetic and transcriptional regulation that occurs under such nutrient-limited conditions [23]. Bacteria have evolved multiple regulatory circuits to control their growth rate by constantly monitoring available resources under carbon-, nitrogen-limited and starvation conditions [53]. Rapid growth is facilitated by the housekeeping sigma factor RpoD (σ^D), whereas the starvation response comes under the general stress response regulated by stationary-phase sigma factor RpoS (σ^S). Although this review focuses on the role of *rpoS* in starvation, the role of RpoS in responding to other stressors (e.g. temperature, DNA damage/UV stress, carbon, phosphate and magnesium deficiency) are well-known [54–56] and have already been reviewed previously [54, 55, 57]. Ferenci and colleagues have shown that in *E. coli*, the hunger response and starvation response are distinct and regulated based on the ambient nutrient levels [23]. The σ^S-controlled starvation response is triggered when the concentration of the carbon source in the media falls below 10^{-7} M, and is absent in both nutrient-excess (e.g. early and mid-exponential growth phases) and nutrient-limited (e.g. early stationary and prolonged stationary phases) conditions [22, 23, 58].

In this manner, bacterial cells constantly monitor the nutrient status of their external environment and accordingly regulate their growth. In the following sections, I will discuss some of the mechanistic details that make the aforementioned regulation feasible.

Competition between *E. coli* sigma factors controls growth rate based on ambient nutrient status

Initially, Gourse and others reported different instances of gene regulation being controlled by growth rate, primarily via regulation of the expression of *rrn* operons that control the intracellular ribosome levels [53, 59, 60]. The ambient growth rate modulates global gene expression in two ways – (i) by controlling proteins involved in biomass increment and cell division, (ii) by repressing irrelevant gene expression by sequestration of the core RNA polymerase via various sigma factors [53]. Diverse sigma factors regulating rapid growth and general stress response compete to occupy the core RNA polymerase [61, 62]. As a result of this competition, different gene sets are expressed based on which sigma factor is active or bound to the core RNA polymerase [53, 63]. Hence, the binding of a particular sigma factor to the core RNA polymerase leads to upregulation of its own targets and, at the same time, downregulation of the targets of other competing sigma factors.

After bacterial cells exit the lag phase, gene expression is primarily modulated by σ^D, promoting rapid growth because of the high ambient nutrient levels. As the population grows, carbon and nitrogen sources in the media steadily decline, compelling the bacterial cell to scavenge nutrients. This 'scavenging response' is activated in two parts: (i) *changing the permeability of the outer membrane* through selective expression of porin channels OmpF (higher permeability) or OmpC (lower permeability) via the transcriptional regulator OmpR, and (ii) *overexpression of ABC type transporters* (encoded by *mgl, mal, lamB*) via an increase in cAMP concentration [23]. This 'early stationary phase' response is prevalent at sugar concentrations ranging from $3*10^{-3}$ M, when the growth rate is 70% of the maximum growth rate, to 10^{-6} M when growth becomes negligible [22, 23]. When the carbon source concentration drops below a threshold of 10^{-7} M, the growth-stress response balance cannot be maintained by regulating the membrane porosity alone, as most of the porins and transporter proteins are saturated at very low sugar concentrations. At this point, the σ^S-mediated starvation response is triggered. This response induces an array of changes like the compaction of the genome [64], reduction in the cell membrane permeability by expressing the low porosity porin OmpC [65], an increase in the production of osmoprotectants like trehalose by upregulation of *osmY* and *treA* transcription [51], and direct repression of all growth-promoting genes that helps bacteria to adapt to different stress factors in the stationary phase [66].

The ability of a bacterial cell to mount the σ^S-mediated response is not devoid of cost, especially under prolonged periods of slow growth. The effects of this cost are evident from evolutionary dynamics in two different paradigms – (i) a rapid decline of $rpoS^{WT}$ carrying populations evolving in chemostats under low growth rates (dilution rate $<0.3\ hr^{-1}$) [67, 68], and selection of functionally attenuated *rpoS* mutants in populations maintained in LTSP [69].

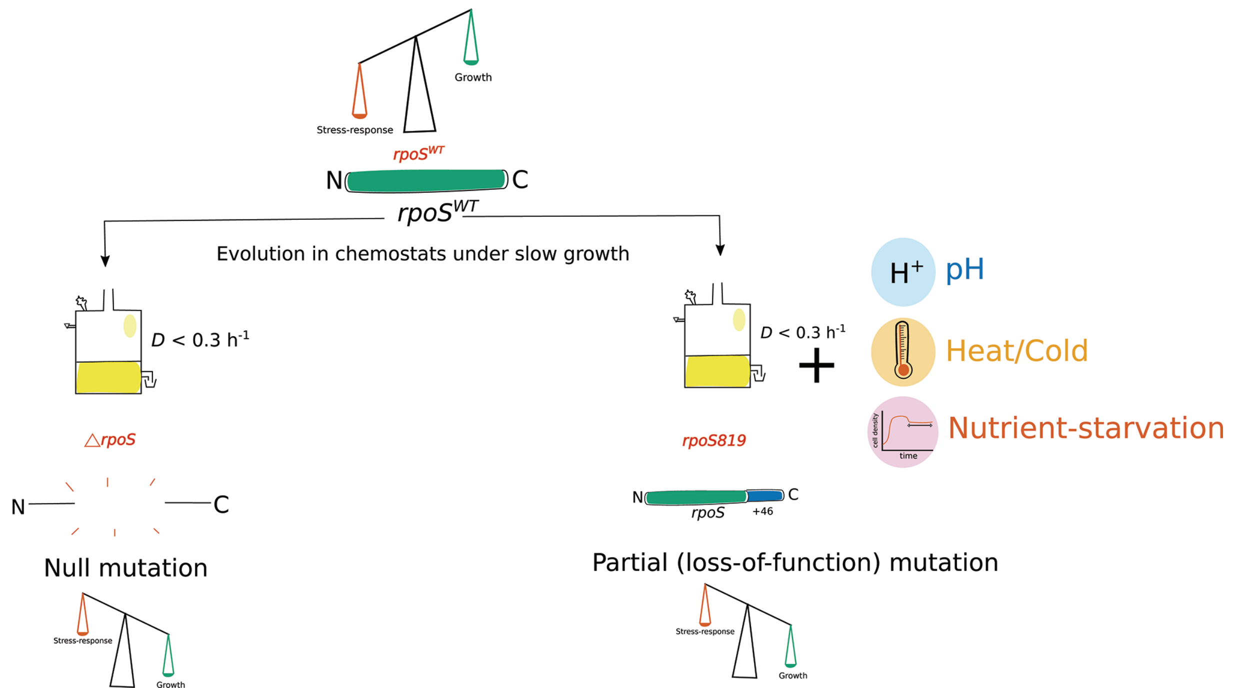

Fig. 1. Change in the balance between growth and stress response modules in fast-growing bacteria based on the evolution paradigm. Slow growth without additional stress factors results in complete loss of the stationary phase sigma factor RpoS. The presence of additional stress factors leads to the emergence of attenuated rpoS mutants calibrating sigma factor competition.

Balance between rapid growth and stress response through sigma factor competition

Because of the dynamic nature of nutrient availability and environmental stressors, a constant balance has to be maintained between increasing biomass for rapid growth and self-preservation (sometimes termed the SPANC balance). Maintenance of this balance is crucial because over-optimizing rapid growth makes the population sensitive to even minute perturbations in the environment, while excess weightage on self-preservation/stress resistance results in loss of fitness.

Core RNA polymerase concentration is limiting for *in vivo* transcription [66]. Across different phases of bacterial growth, competition for the core RNA polymerase ensues between σ^D and σ^S to initiate transcription from specific promoters by integrating environmental cues [37, 66]. Due to this competition, a change in the intracellular levels of either sigma factor can indirectly affect the expression of genes under the control of the other [61]. In *E. coli,* intracellular levels of σ^D have been shown to remain roughly constant across growth phases, whereas σ^S concentration increases sharply during the onset of the stationary phase [70].

It has been observed that strains carrying wild-type *rpoS* can utilize a lower number of carbon sources as compared to attenuated and null *rpoS* mutants [22, 58]. Hence, σ^S acts as a 'necessary evil', presenting a particular cost – lower flexibility in carbon utilization – under nutrient-rich conditions, but becoming essential under stress (schematically shown in Fig. 1). It is not surprising that bacteria have evolved multiple regulatory circuits to control the expression and activity of σ^S at all levels (comprehensively reviewed in [54]).

Regulation of RpoS expression and activity in fast-growing bacteria

In unpredictable environments, phenotypic heterogeneity can aid survival of a population (i.e. acts as a bet-hedging strategy) [71–73]. This phenotypic variability can be caused by heterogeneity in basic processes like gene expression (both at the levels of transcription [74, 75] and translation [76]), gene regulation [77, 78], as well as protein–protein interactions [79]. It has been shown both theoretically and experimentally that particular types of environmental bottlenecks can cause specific variants to be selected, which might have an individual fitness cost. This finding sheds some light onto the emergence of slow-growing variants in LTSP (discussed in section titled '*Repeated emergence of slow-growing variants of E. coli during adaptive lab evolution in LTSP*') [79].

At the sequence level, *rpoS* varies widely across species [54, 67, 68, 80]. Many of these mutants are selected from two separate evolutionary paradigms – (i) evolution in LTSP, and (ii) batch cultures maintained in chemostats under low growth rates and an additional ambient stressor. The ubiquitous observation of the emergence of *rpoS* mutants from several different evolution experiments underlines the utility of *rpoS* under diverse conditions [22]. Transcription of *rpoS* is controlled by multiple regulators,

a key inducer being the small-molecule stress alarmone guanosine pentaphosphate or *(p)ppGpp* [37, 81, 82]. This regulatory role of *(p)ppGpp* has broader implications for the competition between RpoS and RpoD, described later in this review. Other known modulators of *rpoS* transcript levels are the cAMP-CRP complex [83, 84], and ArcA, the regulator of the ArcAB phosphotransfer system, which can positively and negatively regulate RpoS levels [84, 85]. ArcAB complex regulates the aerobic-anaerobic shift, repressing *rpoS* expression by sensing the ambient oxygen and energy status. It does so by the autophosphorylation of ArcB, which in turn phosphorylates ArcA and RssB [86].

A stem-loop structure formed by the 5′ UTR region of the *rpoS* mRNA reduces its translation efficiency [87]. A central role in the post-transcriptional modification and regulation of RpoS is played by small RNAs like DsrA, RprA and ArcZ, which bind to the 5′ UTR region. An adaptor protein called Hfq is necessary for these small RNAs to stabilize the nascent *rpoS* transcript [54]. A fourth sRNA, OxyS, is known to negatively regulate *rpoS* levels, likely by occupying Hfq and out-competing other sRNAs [88]. Many other genes like the *cspA* family [89], HU [90] and *csdA* [91] have been implicated in regulating *rpoS* mRNA stability by binding to the 5′ UTR.

Several elegant mechanisms have been evolved to control intracellular RpoS levels by regulating its degaradation rate. *E. coli* has a dedicated protease called ClpX to degrade RpoS [92]. An adaptor protein called RssB is essential to identify RpoS and help in the function of the protease [93]. RssB is the limiting factor for the degradation of RpoS, and its intracellular level is tightly controlled with the help of three anti-adaptor proteins – IraP, IraM and IraD [94, 95]. These anti-adaptors are induced in response to environmental perturbations like DNA damage and cold shock, primarily via the *(p)ppGpp*-mediated stringent response [54].

Heterogeneity in *rpoS*: nutrient scavenging as an active strategy to survive nutrient limitation in *E. coli*

Initial studies characterizing long-term cultures primarily relied on metabolic readout via growth rates and phenotypic annotation of various facets of the bacterial lifestyle. In recent years the real-time monitoring of the mutation frequencies over prolonged periods has been enabled by high-throughput sequencing. Identification of multiple subpopulations with unique genomic signatures is now possible by combining adaptive lab evolution with periodic population sequencing of evolving isolates [48, 50, 96–98]. Across these experiments, *rpoS* has emerged as a prime target for mutation, allowing bacteria to adapt to different stress factors in their environment.

rpoS mutations that emerge under slow growth in chemostats

Chemostats allow the continuous monitoring of cultures growing with a specific growth rate by regulating the influx and efflux of nutrients via the dilution rate (*D*). Mutations in multiple global regulators like *rpoS* are found in fast-growing strains evolved in chemostats under glucose limitation [99]. In another study, mutations in *rpoS* quickly accumulated in *E. coli* growing at rates of $\leq 0.3\,h^{-1}$, and the proportion of cells carrying wild-type *rpoS* declined in carbon- and nitrogen-limited media [51]. Both attenuated and null *rpoS* mutants emerged, based on the period and composition of stress exposure, with slow growth under carbon and nitrogen limitation giving rise to loss-of-function ('null') mutants, while nutrient limitation coupled with other stress factors (like pH, reactive oxygen species) selecting for attenuated mutants [66, 67] (Fig. 1). This trend highlights the role of the ambient nutrient status in directly shaping bacterial genomes by selecting mutations in global regulators that regulate a large number of smaller regulatory circuits.

Regulatory programs to modulate sigma factor competition: insights from studies on *E. coli*

Multiple independent regulatory circuits have been demonstrated to influence competition between σ^D and σ^S, thus regulating the SPANC balance in response to the ambient nutrient and stress status [22, 54]. Some of these regulators increase the efficiency of σ^S binding to the core RNA polymerase, while others interfere with σ^D binding, and repress its activity. In this review, I focus on the role of a few global regulators essential for survival under prolonged nutrient limitations.

(p)ppGpp: the master regulator of sigma factor competition

One of the most important reporters of ambient carbon and nitrogen levels in bacteria is the small molecule stress alarmone *(p)ppGpp* [61]. The intracellular levels of this modified nucleotide govern the balance between rapid growth and self-preservation. During exponential growth, intracellular *(p)ppGpp* concentration remains low due to nutritional abundance. During starvation periods, inactive ribosomes resulting from a lack of amino-acylated tRNAs induce the production of *(p)ppGpp* via the transcription of *relA* and *spoT* [61]. *(p)ppGpp* functions by destabilizing DNA–protein interactions, leading to dissociation of σ^D from the core RNA polymerase. This event increases the availability of core polymerase for RpoS. σ^S- bound holoenzyme can then transcribe from cognate promoters that encode genes involved in stress response [81]. In fact, the expression and activity of σ^S are themselves regulated by *(p)ppGpp,* establishing the latter as the master regulator of sigma factor competition [61, 66, 100].

Role of Crl, *rssB* and 6S RNA in governing sigma factor competition

Crl is a global regulator, active during the transition from exponential growth to stationary phase, that positively affects the activity of σ^S by facilitating the binding of RpoS with the core RNA polymerase [54, 101, 102]. In effect, the sigma factor competition

between σ^S and σ^D for the core RNA polymerase is shifted by Crl in favour of σ^S – firstly by increasing the expression of a large subset of the σ^S regulon under low RpoS concentrations, and secondly by inducing proteolysis of RpoS via increased expression of RssB [93, 102]. Hence regulation by Crl leads to a lesser but more active σ^S protein, which indicates that some σ^S targets are probably expressed during exponential growth [102]. This regulation has implications for the log-to- stationary transit in batch cultures and during the long- term evolution of starved cultures.

6S RNA, a small, non-coding RNA generated from *ssrB* gene, is also instrumental in controlling sigma factor activity [54, 103]. This RNA binds to the σ^D-core polymerase complex by mimicking a promoter and inhibits transcription from several σ^D promoters, and also allows σ^S to occupy the free core polymerase and transcribe from relevant targets [103, 104]. The 6S RNA has been shown to regulate different sets of genes across various growth phases, indicating that its activity is governed by the ambient nutrient status [104]. Another protein, Rsd, upregulates a subset of σ^S regulon by directly sequestering free σ^D and binding to the core polymerase enzyme, effectively shifting the sigma factor competition in favour of σ^S [105]. The crosstalk of these individual regulators enables the cell to fine-tune the SPANC balance by monitoring ambient nutrient and stress states. Rsd and 6S RNA are found to repress each other via transcriptomic analyses in *ΔssrS* and *Δrsd* knockouts [104]. Further 6S RNA also controls the expression of Crl, and regulates the sigma factor competition, likely by altering the levels of free and polymerase-bound σ^D in the cell [104].

Finally, the sigma factor competition is affected by the general nutrient status of the media and the stress factors present in the ambient environment. This competition regulates the pattern of global gene expression in a bacterial cell. Hence, multiple mutations selected under nutrient stress fine-tune these dynamics to orchestrate the balance between rapid growth and stress response.

GASP: conferring fitness by modulating sigma factor competition

In terms of nutrient status and ambient stress factors, the niches naturally occupied by bacteria are closely mimicked by LTSP [49]. During growth in LTSP, younger cultures are outcompeted by aged cultures via the GASP phenotype. This phenotype, primarily studied in *E. coli*, is heavily modulated by environmental factors like the genetic background of the competing strains and the nutrient status of the media [30, 106, 107]. GASP is also controlled by physical factors like the volume and shaking frequency of the culture, and the shape of the culture receptacle [108]. Investigations by different groups on the genetic basis underlying GASP have revealed the involvement of the global regulators like sigma factors, the amino acid metabolism regulator Lrp [47], specific protein carboxyl methyltransferase [109], the iron-binding protein Dpr [110], and the structural subunits of RNA polymerase, encoded by the *rpoABC* operon [30, 50, 111] in the process. However, the first identified and most studied GASP mutation remains an allele with a 46 base-pair duplication at the 3′ end of the *rpoS* gene, termed *rpoS819*, resulting in an elongated sigma factor with attenuated activity [112].

Recently, it was shown that in the initial stages of evolution during LTSP, mutations in global regulators confer a higher fitness advantage – an effect that declines over time, leading to local regulators being mutated at a higher frequency later in the experiment [113]. Evidence for, and the role of GASP have also been detected in populations of vector-borne pathogens. For example, *Xenorhabdus nematophila* balances a trade-off between pathogenicity and the GASP phenotype, with competitively advantageous *lrp* mutants lacking transmissivity and virulence compared to the ancestors [114].

Genetic determinants of GASP: mutations in the stationary phase sigma factor (σ^S) and RNA polymerase complex

Heterogeneity of *rpoS* allele across bacterial species and its variation in the expression levels have been described across multiple studies [67, 68, 80, 115]. Recently, it was shown that the *rpoS819* allele acquires a second, identical duplication of the 46 base-pair segment when passaged further in LTSP, forming a further elongated allele *rpoS92,* which can confer GASP and has efficiency closer to $rpoS^{WT}$ [30, 83].

The basal expression of *rpoS* is optimized based on nutrient availability, the presence of stress factors in the ambient niche, and is observed to differ between different strains of the same species. This variation in basal RpoS levels contributes to phenotypic heterogeneity, acting as a bet-hedging strategy to survive unfavourable conditions [22, 58]. Functional attenuation of RpoS leads to the super-induction of *rpoD* regulon, resulting in the de-repression of specific *rpoS*-induced high-affinity transporters, increasing fitness advantage under carbon limitation [23]. *rpoS* null mutants are selected only under nutrient stress (dilution rate $<0.3\,hr^{-1}$), but when additional stressors (e.g. pH, temperature) are present, mutants with lower expression and/or efficiency are selected [67].

In addition to *rpoS*, LTSP evolution experiments starting with diverse ancestor strains and media have also identified mutations occurring in various RNA polymerase core subunits (ααββ'γω) and global regulators like *crp*, *cpdA* and *fusA* [49, 50, 111]. Although the precise mechanism by which these mutations increase survival in long-term starved cultures is not completely understood, analysis of converging mutations across different ancestral strains reveals selection pressures that are active in LTSP. An evolution experiment maintaining *E. coli* MG1655 in LTSP was carried out for 3 years and the dynamics of different mutations in the first 4 months [49] and over 3 years [13] was reported. Overall, 90% of all isolates sampled over six time points carried a substitution in the *rpo* operon, encoding the structural subunits of RNA polymerase. Most of these mutations were in one of three loci – *rpoB* 1272, *rpoC* 334 and *rpoC* 428. These mutations emerged within the first 4 months of the evolution experiment and persisted until 3 years into starvation [13]. In another LTSP experiment with *E. coli* K-12 *str*. ZK819 as the ancestor, *rpoABC*

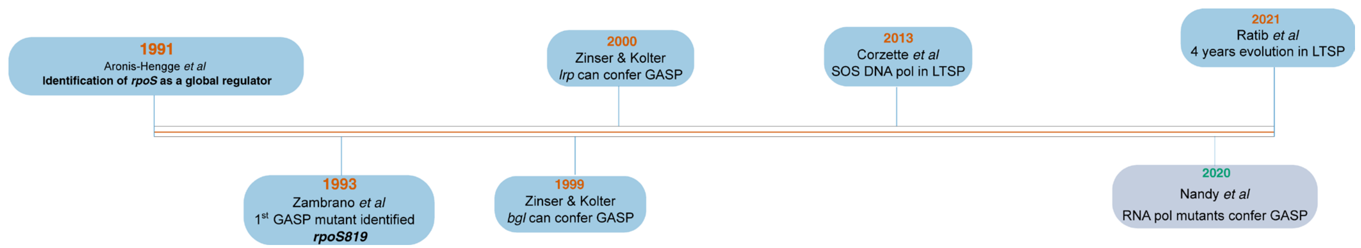

Fig. 2. Timeline showing the key developments in the field of evolution in LTSP and concomittant bacterial gene regulation.

mutations were found to be widespread among the variants that were sequenced over the course of a month [50]. Most of these alleles decrease the growth rate in the exponential phase and hence present a cost to the bearing cell [30, 49]. However, the selection of these mutations across different ancestors and runs of evolution in LTSP point to the obvious advantage of slow growth in nutrient-constricted spent media. Indeed, media age has emerged as a significant determinant of the competitive fitness of LTSP-adapted mutants [30, 46]. The balance between rapid growth and stress response (SPANC) will likely be affected by the growth defect acquired by mutations in the *rpoABC* operon. Diverse strategies to gain fitness under prolonged starvation are developed by the bacterial cell by fine-tuning this SPANC balance (Fig. 1).

Aside from the *rpo* operon, other frequently mutated genes in LTSP-adapted variants include the cyclic-AMP associated global regulated CRP, cAMP phosphodiesterase *cpdA,* and the translational elongation factor *fusA* (encoding EF-G) [13, 50, 83]. All of these genes hold the potential to fine-tune the SPANC balance via controlling sigma factor competition.

In addition to the above-mentioned factors, mutations in other sigma factors like σ^A in *Bacillus subtilis* and σ^N in *E. coli* are also reported to alter the SPANC balance, enabling the bacterial cell to respond to environmental stressors [116, 117]. A theory has been proposed where the binding between σ^D and core RNA polymerase is affected by mutations in σ^D, or concomitant players like alternative sigma factors and core RNA polymerase subunits. This altered binding affinity increases the availability of free core polymerase, allowing it to be occupied by alternative sigma factors, likely via the involvement of small-molecule regulators like *(p)ppGpp* [100]. Evidence for this hypothesis has been provided by work done in different groups across the globe [13, 30, 50].

CONCLUSION: CHALLENGES IN STUDYING LTSP

In this review, I have referred to the altering metabolic status of the environment (feast-and-famine) to situate the positive selection of slow- growing mutants in LTSP. The *rpoS* gene is a common mutation target to confer fitness under prolonged starvation [50, 112]. Such mutations usually affect the regulatory region (e.g. the *rpoS819* duplication interacts closely with the '−35' promoter-binding region), altering the expression and activity of RpoS (*rpoS819* is an attenuated allele, but *rpoS+92* exhibits an almost wild-type level of activity [83]). I have argued how fine-tuning of intracellular *rpoS* levels, either by direct mutations in *rpoS* or indirect mutations in other regulators like *cpdA* [83], *rpoC* [30], shifts the balance of RpoS- RpoD competition, which in turn, affects the balance between rapid growth and self-preservation.

In unpredictably fluctuating environments, fitness is determined by the opposing selection pressures of rapid growth and self-preservation. This balance is orchestrated at the molecular level by the competition between two sigma factors (global regulators σ^D controlling rapid growth and σ^S controlling general stress response) to occupy the RNA polymerase core. Of these two sigma factors *rpoS* presents various attenuated and null alleles that fine-tune RpoS expression and activity, providing evidence of divergent evolution. In this review, I discuss the alteration of σ^S activity as an important strategy to confer fitness under prolonged starvation or GASP.

Resource limitation is the dominant mode of stress experienced by natural isolates across a variety of niches [4, 5]. Studying the effects of resource limitation is notoriously difficult due to the chemical heterogeneity of spent media, and the lack of knowledge of precise selection pressures operating after different periods of starvation. Maintaining strains under LTSP opens a unique window to observe population dynamics under carefully controlled nutrient depletion. However, this field is still riddled with questions.

Although a few gene regulatory mechanisms leading to GASP have been identified – and in some cases, relatively well-studied – their exact metabolic bases are unknown. Most known GASP-conferring mutations map to genes encoding global regulators and cause large-scale alterations across the transcriptome, making it difficult to comment on the metabolic capacities of any sequenced GASP mutant. As previous studies have shown that the GASP phenotype is heavily dependent on the nutritient availability, chemical characterization of differently aged/nutrient-depleted media is essential. However, this has proven difficult to achieve via untargeted mass spectrometric methods because of the inherent variability of rich media [46, 106]. Novel interdisciplinary approaches synthesizing microbial biochemistry with state-of-the-art chemical identification methods

such as mass spectrometry need to be developed to characterize a wide variety of metabolites from rich media. Some novel metabolites (e.g. amino acids) have already been isolated from spent media and characterized [47].

Multiple other looming questions are yet to be answered in this field: What are the mutational landscapes across genomes that enable GASP? How is the emergence of GASP-enabling mutations dependent on genetic contingency and environmental parameters (e.g. pH, temperature, presence of reactive oxygen species)? How do genetically diverse aggregates of microbes or microbiomes behave under long-term resource limitation? From a clinical perspective, many unsolved questions still exist, including the following: What are the exact selection pressures within the host, as experienced by microbiomes and the invading pathogen? How can feast-and-famine conditions impact host–microbiome–pathogen circuits? How are host–pathogen interactions affected by GASP and LTSP-induced stress tolerance? Global increase of interest in the LTSP paradigm and execution of further LTSP evolution experiments with diverse microbial species and assemblies, coupled with an ever-increasing sequencing depth and efficiency, may hold the key to answering these questions.

Finally, LTSP has been demonstrated to be a valuable paradigm for discovering bacterial survival strategies in severely nutrient-depleted environments, akin to what they face in diverse natural niches. Recently the competition between two *E. coli* sigma factors controlling growth and stress response were identified as a strategy to gain a transient competitive advantage in LTSP [30]. In LTSP, the genotypic and phenotypic variation of the founding population increases, generating subpopulations that compete to survive [50, 118] by recycling the debris left by the death of the previous subpopulations [119]. Similarly, persistor subpopulations that can endure antibiotic regimes and drive chronic diseases are formed by pathogens in nutrient-limited host niches [27, 120]. Examples of genetic resistance to novel stress factors have been identified in LTSP evolution experiments [30, 121]. The reason for gaining these novel resistances is not clearly understood, but it might be caused by a global upregulation of the general stress response pathway. The translational utility of the LTSP paradigm can be harnessed by evolving pathogens in conditions resembling specific host niches and identifying strategies developed by the strain to gain fitness [16, 114].

Bacterial gene regulation in the long-term stationary phase is dynamic, complex and spread across multiple subpopulations in an ever-changing environment. The role of external factors in the evolutionary dynamics in this phase is being increasingly recognized. Although the idea of sustained cultivation of microbes is not new, systematic observation of genomic and transcriptomic changes [106, 122, 123] in this survival phase has only begun in the last 30 years. From estimating competitive fitness in 10-day-old *E. coli* cultures [112], we have come a long way to the systemic tracking of *E. coli* populations over 1200 days under LTSP [8] (Fig. 2). LTSP being a temporally open-ended paradigm, the amount of information that could be mined via the emerging high-throughput multi-omics tools is endless. The LTSP paradigm closely resembles the natural evolutionary dynamics in biomes like host gut and soil since it is free from any perturbation by the experimenter. Further developments against current medical challenges like the evolution of multi-drug resistance [35], de novo stress resistance [121], and phenotypic persistence [120] could be made by exploring eco-evolutionary dynamics in LTSP.

Funding information

The author received an integrated M.Sc-PhD fellowship from NCBS, TIFR from 2014 to 2020, and is currently supported by the Max Planck Society.

Acknowledgements

The author wants to thank Dr Aswin Sai Narain Seshasayee, Dr Deepa Agashe, Dr Jenna Gallie and Dr Laasya Samhita for providing critical feedback and improving the manuscript.

Author contributions

This manuscript was conceived, drafted, written and edited by P.N.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

1. Sandler T. History and development of microbiological culture media. *J Inst Sci Technol* 2011:10–14.
2. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, *et al*. Culturing of “unculturable” human microbiota reveals novel taxa and extensive sporulation. *Nature* 2016;533:543–546.
3. Lagier J-C, Dubourg G, Million M, Cadoret F, Bilen M, *et al*. Culturing the human microbiota and culturomics. *Nat Rev Microbiol* 2018;16:540–550.
4. Hofer U. Feast and famine: the keys to gut engraftment. *Nat Rev Microbiol* 2018;16:520.
5. Jørgensen BB, Boetius A. Feast and famine--microbial life in the deep-sea bed. *Nat Rev Microbiol* 2007;5:770–781.
6. Himeoka Y, Mitarai N. Dynamics of bacterial populations under the feast-famine cycles. *Phys Rev Research* 2020;2.
7. Gray DA, Dugar G, Gamba P, Strahl H, Jonker MJ, *et al*. Extreme slow growth as alternative strategy to survive deep starvation in bacteria. *Nat Commun* 2019;10:1–12.
8. Ratib NR, Seidl F, Ehrenreich IM, Finkel SE. Evolution in long-term stationary-phase batch culture: emergence of divergent *Escherichia coli* lineages over 1,200 Days. *mBio* 2021;12:e03337-20.
9. Giovannoni SJ, Cameron Thrash J, Temperton B. Implications of streamlining theory for microbial ecology. *ISME J* 2014;8:1553–1565.
10. Higgins D, Dworkin J. Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol Rev* 2012;36:131–148.

11. Zhang Z, Du C, de Barsy F, Liem M, Liakopoulos A, *et al*. Antibiotic production in *Streptomyces* is organized by a division of labor through terminal genomic differentiation. *Sci Adv* 2020;6:eaay5781.
12. Finkel SE. Long-term survival during stationary phase: evolution and the GASP phenotype. *Nat Rev Microbiol* 2006;4:113–120.
13. Avrani S, Katz S, Hershberg R. Adaptations accumulated under prolonged resource exhaustion are highly transient. *mSphere* 2020;5:e00388-20.
14. Esteve-Núñez A, Rothermich M, Sharma M, Lovley D. Growth of *Geobacter sulfurreducens* under nutrient-limiting conditions in continuous culture. *Environ Microbiol* 2005;7:641–648.
15. Paul K, Ghosh A, Sengupta N, Chowdhury R. Competitive growth advantage of nontoxigenic mutants in the stationary phase in archival cultures of pathogenic *Vibrio cholerae* strains. *Infect Immun* 2004;72:5478–5482.
16. Bruno JC, Freitag NE. *Listeria monocytogenes* adapts to long-term stationary phase survival without compromising bacterial virulence. *FEMS Microbiol Lett* 2011;323:171–179.
17. Machreki Y, Kouidhi B, Machreki S, Chaieb K, Sáenz Y. Analysis of a long term starved *Pseudomonas aeruginosa* ATCC27853 in seawater microcosms. *Microb Pathog* 2019;134:103595.
18. Aouizerat T, Gelman D, Szitenberg A, Gutman I, Glazer S, *et al*. Eukaryotic adaptation to years-long starvation resembles that of bacteria. *iScience* 2019;19:545–558.
19. Barrick JE, Lenski RE. Genome dynamics during experimental evolution. *Nat Rev Genet* 2013;14:827–839.
20. Good BH, McDonald MJ, Barrick JE, Lenski RE, Desai MM. The dynamics of molecular evolution over 60,000 generations. *Nature* 2017;551:45–50.
21. Lenski RE. Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. *ISME J* 2017;11:2181–2194.
22. Ferenci T. Maintaining a healthy SPANC balance through regulatory and mutational adaptation. *Mol Microbiol* 2005;57:1–8.
23. Ferenci T. Regulation by nutrient limitation. *Curr Opin Microbiol* 1999;2:208–213.
24. Zambrano MM, Kolter R. GASPing for life in stationary phase. *Cell* 1996;86:181–184.
25. Rappé MS, Connon SA, Vergin KL, Giovannoni SJ. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* 2002;418:630–633.
26. Pulschen AA, Bendia AG, Fricker AD, Pellizari VH, Galante D, *et al*. Isolation of uncultured bacteria from antarctica using long incubation periods and low nutritional media. *Front Microbiol* 2017;8:1346.
27. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, *et al*. Small colony variants: A pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* 2006;4:295–305.
28. Gibson B, Wilson DJ, Feil E, Eyre-Walker A. The distribution of bacterial doubling times in the wild. *Proc Biol Sci* 2018;285:1880.
29. Biselli E, Schink SJ, Gerland U. Slower growth of *Escherichia coli* leads to longer survival in carbon starvation due to a decrease in the maintenance rate. *Mol Syst Biol* 2020;16:e9478.
30. Nandy P, Chib S, Seshasayee A. A Mutant RNA polymerase activates the general stress response, enabling *Escherichia coli* adaptation to late prolonged stationary phase. *mSphere* 2020;5:1–16.
31. Britos L, Abeliuk E, Taverner T, Lipton M, McAdams H, *et al*. Regulatory response to carbon starvation in *Caulobacter crescentus*. *PLoS One* 2011;6:e18179.
32. Aurass P, Düvel J, Karste S, Nübel U, Rabsch W, *et al*. *glnA* Truncation in *Salmonella enterica* results in a small colony variant phenotype, attenuated host cell entry, and reduced expression of flagellin and SPI-1-associated effector genes. *Appl Environ Microbiol* 2018;84:e01838-17.
33. Pontes MH, Groisman EA. Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica Sci Signal* 2019;12:3938.
34. Roggenkamp A, Sing A, Hornef M, Brunner U, Autenrieth IB, *et al*. Chronic prosthetic hip infection caused by a small-colony variant of *Escherichia coli*. *J Clin Microbiol* 1998;36:2530–2534.
35. Wei Q, Tarighi S, Dötsch A, Häussler S, Müsken M, *et al*. Phenotypic and genome-wide analysis of an antibiotic-resistant small colony variant (SCV) of *Pseudomonas aeruginosa*. *PLoS One* 2011;6:e29276.
36. Bachmann NL, Salamzade R, Manson AL, Whittington R, Sintchenko V, *et al*. Key transitions in the evolution of rapid and slow growing *Mycobacteria* identified by comparative genomics. *Front Microbiol* 2019;10:3019.
37. Ramnaresh Gupta K, Chatterji D. Sigma factor competition in escherichia coli: kinetic and thermodynamic perspectives. *Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria, FJ de Bruijn (Ed*) 2016:196–202.
38. Jacobsen KA. Mitteilungen über einen variablen typhusstamm (bacterium typhi mutabile), sowie über eine eigentümliche hemmende wirkung des gewöhnlichen agar, verursacht durch autoklavierung. *Zentralbl Bakteriol* 1910;56:208–216.
39. Colwell CA. Small Colony Variants of *Escherichia coli*. *J Bacteriol* 1946;52:417–422.
40. CLOWES RC, ROWLEY D. Genetic studies on small-colony variants of *Escherichia coli* K-12. *J Gen Microbiol* 1955;13:461–473.
41. Balwit JM, Langevelde P v., Vann JM, Proctor RA. Gentamicin-resistant menadione and hemin auxotrophic *staphylococcus aureus* persist within cultured endothelial cells. *J Infect Dis* 1994;170:1033–1037.
42. Evans TJ. Small colony variants of *Pseudomonas aeruginosa* in chronic bacterial infection of the lung in cystic fibrosis. *Future Microbiol* 2015;10:231–239.
43. Baddour LM, Barker LP, Christensen GD, Parisi JT, Simpson WA. Phenotypic variation of *Staphylococcus epidermidis* in infection of transvenous endocardial pacemaker electrodes. *J Clin Microbiol* 1990;28:676–679.
44. Mowjood M, Miller FE, Schor J, Kocka FE. Small-colony forms of enteric bacteria after exposure to aminoglycosides. *Am J Clin Pathol* 1979;72:79–81.
45. Raven C. Dissociation of the Gonococcus. *J Infect Dis* 1934;55:328–339.
46. Westphal LL, Lau J, Negro Z, Moreno IJ, Ismail Mohammed W, *et al*. Adaptation of *Escherichia coli* to long-term batch culture in various rich media. *Res Microbiol* 2018;169:145–156.
47. Zinser ER, Kolter R. Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. *J Bacteriol* 1999;181:5800–5807.
48. Katz S, Avrani S, Yavneh M, Hilau S, Gross J, *et al*. Dynamics of adaptation during three years of evolution under long-term stationary phase. *Mol Biol Evol* 2021;38:2778–2790.
49. Avrani S, Bolotin E, Katz S, Hershberg R. Rapid genetic adaptation during the first four months of survival under resource exhaustion. *Mol Biol Evol* 2017;34:1758–1769.
50. Chib S, Ali F, Seshasayee ASN, Phase PS. Genomewide mutational diversity in *Escherichia coli* population evolving in prolonged stationary phase. *mSphere* 2017;2:1–15.
51. Notley L, Ferenci T. Induction of RpoS-dependent functions in glucose-limited continuous culture: what level of nutrient limitation induces the stationary phase of *Escherichia coli*? *J Bacteriol* 1996;178:1465–1468.
52. Chang D-E, Smalley DJ, Tucker DL, Leatham MP, Norris WE, *et al*. Carbon nutrition of *Escherichia coli* in the mouse intestine. *Proc Natl Acad Sci U S A* 2004;101:7427–7432.
53. Klumpp S, Hwa T. Bacterial growth: global effects on gene expression, growth feedback and proteome partition. *Curr Opin Biotechnol* 2014;28:96–102.

54. Battesti A, Majdalani N, Gottesman S. The RpoS-mediated general stress response in *Escherichia coli*. *Annu Rev Microbiol* 2011;65:189–213.
55. Hengge-Aronis R. Survival of hunger and stress: the role of rpoS in early stationary phase gene regulation in *E. coli*. *Cell* 1993;72:165–168.
56. Navarro Llorens JM, Tormo A, Martínez-García E. Stationary phase in gram-negative bacteria. *FEMS Microbiol Rev* 2010;34:476–495.
57. Kolter R, Siegele DA, Tormo A. The stationary phase of the bacterial life cycle. *Annu Rev Microbiol* 1993;47:855–874.
58. King T, Ishihama A, Kori A, Ferenci T. A regulatory trade-off as A source of strain variation in the species *Escherichia coli*. *J Bacteriol* 2004;186:5614–5620.
59. Gourse RL, Gaal T, Bartlett MS, Appleman JA, Ross W. rRNA transcription and growth rate-dependent regulation of ribosome synthesis in *Escherichia coli*. *Annu Rev Microbiol* 1996;50:645–677.
60. Gaal T, Bartlett MS, Ross W, Turnbough CL, Gourse RL. Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. *Science* 1997;278:2092–2097.
61. Jishage M, Kvint K, Shingler V, Nyström T. Regulation of sigma factor competition by the alarmone ppGpp. *Genes Dev* 2002;16:1260–1270.
62. Farrell MJ, Finkel SE. The growth advantage in stationary-phase phenotype conferred by rpoS mutations is dependent on the pH and nutrient environment. *J Bacteriol* 2003;185:7044–7052.
63. Serbanescu D, Ojkic N, Banerjee S. Nutrient-dependent trade-offs between ribosomes and division protein synthesis control bacterial cell size and growth. *Cell Rep* 2020;32:108183.
64. Cosgriff S, Chintakayala K, Chim YTA, Chen X, Allen S, *et al*. Dimerization and DNA-dependent aggregation of the *Escherichia coli* nucleoid protein and chaperone CbpA. *Mol Microbiol* 2010;77:1289–1300.
65. Johansen J, Rasmussen AA, Overgaard M, Valentin-Hansen P. Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol* 2006;364:1–8.
66. Nyström T. Growth versus maintenance: a trade-off dictated by RNA polymerase availability and sigma factor competition? *Mol Microbiol* 2004;54:855–862.
67. Notley-McRobb L, King T, Ferenci T. rpoS mutations and loss of general stress resistance in *Escherichia coli* populations as a consequence of conflict between competing stress responses. *J Bacteriol* 2002;184:806–811.
68. Jishage M, Ishihama A. Variation in RNA polymerase sigma subunit composition within different stocks of *Escherichia coli* W3110. *J Bacteriol* 1997;179:959–963.
69. Helmus RA, Liermann LJ, Brantley SL, Tien M. Growth advantage in stationary-phase (GASP) phenotype in long-term survival strains of *Geobacter sulfurreducens*. *FEMS Microbiol Ecol* 2012;79:218–228.
70. Jishage M, Ishihama A. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of sigma 70 and sigma 38. *J Bacteriol* 1995;177:6832–6835.
71. Kussell E, Kishony R, Balaban NQ, Leibler S. Bacterial persistence: A model of survival in changing environments. *Genetics* 2005;169:1807–1814.
72. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. *Science* 2004;305:1622–1625.
73. Gasperotti A, Brameyer S, Fabiani F, Jung K. Phenotypic heterogeneity of microbial populations under nutrient limitation. *Curr Opin Biotechnol* 2020;62:160–167.
74. Ozbudak EM, Thattai M, Kurtser I, Grossman AD, van Oudenaarden A. Regulation of noise in the expression of a single gene. *Nat Genet* 2002;31:69–73.
75. Raser JM, O'Shea EK. Control of stochasticity in eukaryotic gene expression. *Science* 2004;304:1811–1814.
76. Newman JRS, Ghaemmaghami S, Ihmels J, Breslow DK, Noble M, *et al*. Single-cell proteomic analysis of S. cerevisiae reveals the architecture of biological noise. *Nature* 2006;441:840–846.
77. Paulsson J. Summing up the noise in gene networks. *Nature* 2004;427:415–418.
78. Angeli D, Ferrell JE, Sontag ED. Detection of multistability, bifurcations, and hysteresis in a large class of biological positive-feedback systems. *Proc Natl Acad Sci U S A* 2004;101:1822–1827.
79. Norman TM, Lord ND, Paulsson J, Losick R. Stochastic Switching of Cell Fate in Microbes. *Annu Rev Microbiol* 2015;69:381–403.
80. Chen G, Patten CL, Schellhorn HE. Positive selection for loss of RpoS function in *Escherichia coli*. *Mutat Res* 2004;554:193–203.
81. Traxler MF, Summers SM, Nguyen H-T, Zacharia VM, Hightower GA, *et al*. The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. *Mol Microbiol* 2008;68:1128–1148.
82. Durfee T, Hansen A-M, Zhi H, Blattner FR, Jin DJ. Transcription profiling of the stringent response in *Escherichia coli*. *J Bacteriol* 2008;190:1084–1096.
83. Chib S, Seshasayee AS. Modulation of rpos fitness by loss of cpda activity during stationary-phase in *Escherichia coli*. *biorxiv* 2018:1–24.
84. Lange R, Hengge-Aronis R. The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev* 1994;8:1600–1612.
85. Barth E, Gora KV, Gebendorfer KM, Settele F, Jakob U, *et al*. Interplay of cellular cAMP levels, {sigma}S activity and oxidative stress resistance in *Escherichia coli*. *Microbiology (Reading)* 2009;155:1680–1689.
86. Mika F, Hengge R. A two-component phosphotransfer network involving ArcB, ArcA, and RssB coordinates synthesis and proteolysis of sigmaS (RpoS) in E. coli. *Genes Dev* 2005;19:2770–2781.
87. Cunning C, Brown L, Elliott T. Promoter substitution and deletion analysis of upstream region required for rpoS translational regulation. *J Bacteriol* 1998;180:4564–4570.
88. Zhang A, Altuvia S, Tiwari A, Argaman L, Hengge-Aronis R, *et al*. The OxyS regulatory RNA represses rpoS translation and binds the Hfq (HF-I) protein. *EMBO J* 1998;17:6061–6068.
89. Phadtare S, Inouye M. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in Escherichia coli. *J Bacteriol* 2001;183:1205–1214.
90. Balandina A, Claret L, Hengge-Aronis R, Rouviere-Yaniv J. The Escherichia coli histone-like protein HU regulates rpoS translation. *Mol Microbiol* 2001;39:1069–1079.
91. Resch A, Većerek B, Palavra K, Bläsi U. Requirement of the CsdA DEAD-box helicase for low temperature riboregulation of rpoS mRNA. *RNA Biol* 2010;7:796–802.
92. Schweder T, Lee KHO, Lomovskaya O, Matin A. Regulation of *Escherichia coli* starvation sigma factor (sigma S) by ClpXP protease. *J Bacteriol* 1996;178:470–476.
93. Klauck E, Lingnau M, Hengge-Aronis R. Role of the response regulator RssB in sigma recognition and initiation of sigma proteolysis in *Escherichia coli*. *Mol Microbiol* 2001;40:1381–1390.
94. Bougdour A, Cunning C, Baptiste PJ, Elliott T, Gottesman S. Multiple pathways for regulation of sigmaS (RpoS) stability in *Escherichia coli* via the action of multiple anti-adaptors. *Mol Microbiol* 2008;68:298–313.
95. Bougdour A, Wickner S, Gottesman S. Modulating RssB activity: IraP, a novel regulator of sigma(S) stability in *Escherichia coli*. *Genes Dev* 2006;20:884–897.
96. Deatherage DE, Barrick JE. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods Mol Biol* 2014;1151:165–188.
97. Lenski RE, Winkworth CL, Riley MA. Rates of DNA sequence evolution in experimental populations of *Escherichia coli* during 20,000 generations. *J Mol Evol* 2003;56:498–508.

98. Tenaillon O, Barrick JE, Ribeck N, Deatherage DE, Blanchard JL, *et al*. Tempo and mode of genome evolution in a 50,000-generation experiment. *Nature* 2016;536:165–170.
99. Maharjan RP, Ferenci T, Reeves PR, Li Y, Liu B, *et al*. The multiplicity of divergence mechanisms in a single evolving population. *Genome Biol* 2012;13:R41.
100. Österberg S, del Peso-Santos T, Shingler V. Regulation of alternative sigma factor use. *Annu Rev Microbiol* 2011;65:37–55.
101. Pratt LA, Silhavy TJ. Crl stimulates RpoS activity during stationary phase. *Mol Microbiol* 1998;29:1225–1236.
102. Typas A, Barembruch C, Possling A, Hengge R. Stationary phase reorganisation of the *Escherichia coli* transcription machinery by Crl protein, a fine-tuner of sigmas activity and levels. *EMBO J* 2007;26:1569–1578.
103. Wassarman KM. 6S RNA: A regulator of transcription. *Mol Microbiol* 2007;65:1425–1431.
104. Lal A, Krishna S, Seshasayee ASN. Regulation of Global Transcription in *Escherichia coli* by Rsd and 6S RNA. *G3 (Bethesda)* 2018;8:2079–2089.
105. Mitchell JE, Oshima T, Piper SE, Webster CL, Westblade LF, *et al*. The *Escherichia coli* regulator of sigma 70 protein, Rsd, can up-regulate some stress-dependent promoters by sequestering sigma 70. *J Bacteriol* 2007;189:3489–3495.
106. Kram KE, Finkel SE. Rich Medium Composition Affects *Escherichia coli* Survival, Glycation, and Mutation Frequency during Long-Term Batch Culture. *Appl Environ Microbiol* 2015;81:4442–4450.
107. Fukuda T, Nakahigashi K, Inokuchi H. Viability of *Escherichia coli* cells under long-term cultivation in a rich nutrient medium. *Genes Genet Syst* 2001;76:271–278.
108. Kram KE, Finkel SE. Culture volume and vessel affect long-term survival, mutation frequency, and oxidative stress of *Escherichia coli*. *Appl Environ Microbiol* 2014;80:1732–1738.
109. Hicks WM, Kotlajich MV, Visick JE. Recovery from long-term stationary phase and stress survival in *Escherichia coli* require the L-isoaspartyl protein carboxyl methyltransferase at alkaline pH. *Microbiology (Reading)* 2005;151:2151–2158.
110. Tsou C-C, Chiang-Ni C, Lin Y-S, Chuang W-J, Lin M-T, *et al*. An iron-binding protein, Dpr, decreases hydrogen peroxide stress and protects *Streptococcus pyogenes* against multiple stresses. *Infect Immun* 2008;76:4038–4045.
111. LaCroix RA, Sandberg TE, O'Brien EJ, Utrilla J, Ebrahim A, *et al*. Use of adaptive laboratory evolution to discover key mutations enabling rapid growth of *Escherichia coli* K-12 MG1655 on glucose minimal medium. *Appl Environ Microbiol* 2015;81:17–30.
112. Zambrano MM, Siegele DA, Almirón M, Tormo A, Kolter R. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* 1993;259:1757–1760.
113. Ali F, Seshasayee ASN. Dynamics of genetic variation in transcription factors and its implications for the evolution of regulatory networks in Bacteria. *Nucleic Acids Res* 2020;48:4100–4114.
114. Cambon MC, Parthuisot N, Pagès S, Lanois A, Givaudan A, *et al*. Selection of Bacterial Mutants in Late Infections: When Vector Transmission Trades Off against Growth Advantage in Stationary Phase. *mBio* 2019;10:e01437-19.
115. Ivanova A, Renshaw M, Guntaka RV, Eisenstark A. DNA base sequence variability in katF (putative sigma factor) gene of *Escherichia coli*. *Nucleic Acids Res* 1992;20:5479–5480.
116. Lee YH, Helmann JD. Mutations in the primary sigma factor σA and termination factor rho that reduce susceptibility to cell wall antibiotics. *J Bacteriol* 2014;196:3700–3711.
117. Szalewska-Palasz A, Johansson LUM, Bernardo LMD, Skärfstad E, Stec E, *et al*. Properties of RNA polymerase bypass mutants: implications for the role of ppGpp and its co-factor DksA in controlling transcription dependent on sigma54. *J Biol Chem* 2007;282:18046–18056.
118. Finkel SE, Kolter R. Evolution of microbial diversity during prolonged starvation. *Proc Natl Acad Sci U S A* 1999;96:4023–4027.
119. Shoemaker WR, Jones SE, Muscarella ME, Behringer MG, Lehmkuhl BK, *et al*. Microbial population dynamics and evolutionary outcomes under extreme energy limitation. *Proc Natl Acad Sci U S A* 2021;118:e2101691118.
120. Singh R, Ray P, Das A, Sharma M. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. *J Med Microbiol* 2009;58:1067–1073.
121. Knöppel A, Näsvall J, Andersson DI. Evolution of Antibiotic Resistance without Antibiotic Exposure. *Antimicrob Agents Chemother* 2017;61:e01495-17.
122. Kram KE, Henderson AL, Finkel SE. *Escherichia coli* Has a Unique Transcriptional Program in Long-Term Stationary Phase Allowing Identification of Genes Important for Survival. *mSystems* 2020;5:e00364-20.
123. Robador A, Amend JP, Finkel SE. Nanocalorimetry Reveals the Growth Dynamics of *Escherichia coli* Cells Undergoing Adaptive Evolution during Long-Term Stationary Phase. *Appl Environ Microbiol* 2019;85:e00968-19.

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as 'excellent' or 'very good'.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.