Title: Regulation of Protein Biosynthetic Activity During Growth Arrest

Megan Bergkessel

e-mail: mbergkessel001@dundee.ac.uk

postal address: University of Dundee, School of Life Sciences, Dow St., Dundee DD1 5EH, Scotland, UK

Division of Molecular Microbiology, School of Life Sciences, University of Dundee

Abstract: Heterotrophic bacteria grow and divide rapidly when resources are abundant. Yet resources are finite, and environments fluctuate, so bacteria need strategies to survive when nutrients become scarce. In fact, many bacteria spend most of their time in such conditions of nutrient limitation, and hence they need to optimise gene regulation and protein biosynthesis during growth arrest. An optimal strategy in these conditions must mitigate the challenges and risks of making new proteins, while the cell is severely limited for energy and substrates. Recently, ribosome abundance and activity were measured in these conditions, revealing very low amounts of new protein synthesis, which is nevertheless vital for survival. The underlying mechanisms are only now starting to be explored. Improving our understanding of the regulation of protein production during bacterial growth arrest could have important implications for a wide range of challenges, including the identification of new targets for antibiotic development.

What are the causes of growth arrest?

Many heterotrophic bacteria can grow and divide rapidly if their nutritional needs are met, but this condition is the exception, not the rule. Even in nutrient-rich environments, bacterial growth itself quickly depletes local resources and causes accumulation of waste products that inhibit further rapid growth. For example, many species form dense biofilm communities, which are held together by self-produced matrices, and which quickly become self-limiting. Experimental measurements and modelling in Pseudomonas aeruginosa biofilms suggest that at thicknesses greater than about 40-70 microns, metabolism of the biofilm cells completely depletes oxygen at the base of the biofilm, leading to near-zero growth rates[1,2]. Depending on the organism and environment, other macronutrients (e.g. carbon[3], nitrogen[4], or phosphorus[5]), or micronutrients (such as iron[6]) may become limiting first, but in all cases the lack of an essential substrate for new biosynthesis causes growth to stop. Outside of biofilms, heterotrophic bacteria often exist in low-nutrient environments that cause frequent growth arrest[7]. Other environmental stresses, such as reactive oxygen species, high osmolarity, and unfavourable temperature, can suppress growth by directly inhibiting ATP and/or protein synthesis[8-10]. Finally, bacteria (as well as fungi, plants, and animals) have evolved a wide array of weapons that specifically target the energy conservation or biosynthetic machinery of competing bacteria in order to inhibit their growth (for example, see [11]). All of these challenges would hinder growth whether there were an adaptive response from the challenged bacteria or not, but decades of work strongly suggest that complex regulation is in place to coordinate stopping replication, repressing expression of new biosynthetic machinery, and redirecting resources toward functions needed for survival (reviewed in [12,13]). Still, many questions remain about the mechanisms that allow ongoing adjustments to protein biosynthetic rates and priorities during protracted growth.
arrest – adjustments that, though small in magnitude, may make the difference between survival and death.

**Risks and benefits of protein biosynthesis during growth arrest**

Regulation operating during growth arrest must weigh the risks of consuming extremely limited resources against the benefits of making potentially useful new proteins. Many studies of multiple organisms in different growth-arrest contexts have found that growth-arrested bacteria continue to make new proteins at low rates[14-20]. However, protein biosynthesis requires networks of interconnected metabolic activities to supply the energy and substrates required, and, especially if flux through these networks is inconsistent, activity can increase the vulnerability of the bacteria to damage (Fig. 1A). For example, the redox reactions of the electron transport chain can release reactive oxygen species, resulting in damage that is difficult to repair with limited resources[3,21]. Stalled, transcribing RNA polymerases can increase the likelihood of some types of DNA damage by leaving DNA-RNA hybrids that can be recognised improperly by DNA synthesis machinery[22-24], and ongoing translation under starvation conditions can lead to protein aggregation[25], requiring the activity of heat-shock proteases to maintain viability[26]. The presence of antibiotic compounds exacerbates the risks associated with activity, as these compounds are largely subvert biosynthetic processes. Mutants that tolerate antibiotics better often show slower growth or increased lag times[27]. Indeed, the bacterial form best suited to long-term survival of harsh, resource-depleted conditions is the spore, with metabolic activity very close to zero (reviewed in [28]).

Despite this, even in organisms that can form spores as a response to extreme resource limitation (such as *Bacillus subtilis*), a substantial subpopulation will instead maintain a vegetative state that is metabolically and biosynthetically active at low rates[29]. Furthermore, researchers have observed that despite the risks of protein synthesis during growth arrest, inhibiting it decreases survival[14,30,31]. What benefits outweigh the risks of biosynthesis during resource limitation? Essential proteins sustain damage over time and must be replaced[32]. Ongoing activity also allows defensive responses against specific environmental threats, e.g. by upregulation of efflux pumps or DNA repair enzymes[31,33]. And even in conditions of extreme resource limitation, cells encounter small amounts of useful nutrients (from lysis of other bacteria, for example); taking advantage of them requires uptake and incorporation via biosynthesis[17,34]. Finally, when favourable conditions return, bacteria must be prepared to rapidly resume growth or risk being overrun by competitors.

Because growth-arrested bacteria are often exposed to fluctuating environments with opportunities and threats varying unpredictably over time, an ability to alter protein synthesis activity in response to the environment while still remaining in a growth-arrested state would likely be advantageous (Fig. 1B).

**Ribosome dynamics during growth and growth arrest**

Ribosomes are the engines of protein synthesis, so their abundance and activity are important points of control of biosynthetic capacity, and accordingly, their regulation changes dramatically as nutrient availability drops (Fig. 2). Elegant work over several decades has shown how *Escherichia coli* tunes resource allocation to maximise ribosome biogenesis when nutrients are plentiful, and diverts resources toward acquiring nutrients when those
nutrients become limiting ([35-37] and references therein). Earlier work compared ribosome
production and protein synthesis at fast (doubling time ($\tau = 20$ minutes) and slow
($\tau = 100$ minutes) growth rates, but recently these quantitative analyses have been extended
to conditions that impose extremely slow ($\tau = 1440$ minutes, in chemostats) or no growth
(e.g. stationary phase or carbon starvation)[18,19,38].
Several interesting observations emerge from these studies. First, quantitative comparisons
across this expanded range of growth conditions highlight the striking magnitude of the
changes as cells enter growth arrest. The rate of new protein synthesis in growth arrest is
estimated to be approximately 1500-fold lower than during optimal growth (Fig. 2A), and the
average number of active ribosomes per cell approximately 100, compared to 62,000 at the
fastest growth rates (Fig. 2B). Changes of this magnitude have enormous impacts on the
biochemical and biophysical context in which the gene expression machinery operates, but
the mechanisms by which the machinery adjusts to these changes are not well understood.
Second, careful investigation of the translational responses to extreme limitation with
different limiting nutrients has revealed that ribosome dynamics are distinct, suggesting that
finely tuned regulation is important even though new protein synthesis is so strikingly
reduced. In carbon starvation, a large fraction (80%) of the ribosomes are sequestered in an
inactive state, while nitrogen limitation more dramatically represses the translation
extension rate, and phosphorus limitation strongly reduces cells' ribosome content, at least
in part by degradation of ribosomes[18,38,39]. Growth-limiting osmotic and oxidative
stresses also strongly repress translation elongation rates, requiring adjustments to
ribosome activities or amounts[8,9]. Maximising survival of growth arrest in a dynamic
environment likely requires complex regulation to identify and recycle damaged ribosomes,
safely sequester excess functional ribosomes, and occasionally produce new ribosomes as
conditions require. Consistent with this notion, multiple studies have shown that genes
involved in ribosome biogenesis are expressed during carbon- or energy-limited growth
arrest, and that they are important for survival[14,15,40].
Ribosome dynamics have mostly been observed at the population level, so the distribution
of active and inactive ribosomes across individual cells is not well studied. Multiple
mechanisms exist for sequestering ribosomes upon growth arrest, which could be used by
each cell to inactivate a consistent fraction of its ribosomes (reviewed in [41]). However, it is
also possible that translational activity is unequally distributed across the bacterial
population. Toxin-antitoxin systems have in fact been proposed to drive heterogeneity, when
stochastically arising imbalances allow the toxin to escape antitoxin control and target
biosynthetic machinery, including ribosomes, tRNAs, EF-Tu, and mRNAs (reviewed in [42]).
Two studies have investigated protein synthesis rates (in E. coli) and ribosome levels (in P.
aeruginosa) in single cells during starvation-induced growth arrest, to directly assess their
distributions[16,43]. In both cases, after initial large drops in ribosome abundance[43] or
protein synthesis rates[16] at the entry to growth arrest, the new levels were maintained by
most cells for several days. However, both studies also found outliers of high and low
ribosome abundance and activity, suggesting that heterogeneity does arise and may play an
important role. Within dense communities of sibling bacteria, selective pressure could
conceivably favour the uneven distribution of protein biosynthetic activity across individual
cells to maximise survival of the population. Examples of metabolic coordination in biofilms
mediated by gated ion channels[4] and redox-active secondary metabolites[2] have been
described, but further explorations of mechanisms contributing to such coordination and their impacts on protein biosynthesis are still needed.

**Mechanisms for regulating protein biosynthesis during growth arrest**

Mechanisms to dynamically adjust protein biosynthesis during growth arrest have been studied relatively little, in part because of the difficulty of measuring low activity levels. Most research has focused on the transition from rapid growth to growth arrest as nutrients are depleted. The dominant pattern in this context is strong suppression of ribosome biogenesis and upregulation of genes involved in nutrient acquisition and general stress responses. Key regulators of this transition (which have been recently comprehensively reviewed) include general stress sigma factors, which bias RNA polymerase (RNAP) activity toward stress-adaptive functions,[44] and the modified nucleotides cAMP and (p)ppGpp, which bias RNAP activity away from ribosomal RNA and protein genes.[45,46]. The regulators playing important roles after cells have entered into a protracted growth arrest are much less clear, but (p)ppGpp likely plays an ongoing role even though its levels peak at the entry to growth arrest.[47] Homologs of RelA, which is activated by binding uncharged tRNAs and the ribosome during translation,[48], are major sources of (p)ppGpp in many organisms. Thus, low levels of ongoing translation during growth arrest may contribute to ongoing adjustment of (p)ppGpp levels. Indeed, a Tn-Seq screen in *Rhodopseudomonas palustris* found that both the ability to synthesise (p)ppGpp and genes for ribosome biosynthesis were important for fitness throughout 20 days of carbon-limited growth arrest.[14].

Even though (p)ppGpp has been studied extensively, the fact that its functions are carried out differently in different organisms[49], and that it impacts many core cellular processes simultaneously (reviewed in [50], Fig. 3), has prevented a full understanding of its direct effects. Recent work has shown that these effects are even more widespread than previously appreciated: a careful transcriptomic study showed that (p)ppGpp binding to RNA polymerase in *E. coli* directly impacts expression levels for 757 genes[51], and a crosslinking approach identified 56 direct binding targets of (p)ppGpp, many of which are involved in translation and nucleotide biosynthesis and were previously unknown[52]. The new insights into the direct effects of (p)ppGpp on regulation of nucleotide biosynthesis are particularly interesting and highlight the centrality of the nucleotide pools in cellular metabolic networks (Fig. 3). Levels are tightly controlled, but a notable shift in purine nucleotides toward (p)ppGpp occurs at the entry to growth arrest[47,53], and complex crosstalk and feedback extends these effects to numerous biosynthetic pathways. How cells might adjust gene expression activities in response to the nucleotide pools present in growth arrest, and how they might restore the pools to resume active growth are not known. Within growth-arrested states, perturbations to nucleotide pools appear to impact survival. For example, among antibiotic-exposed persisters in *P. aeruginosa*, mutations inhibiting *de novo* pyrimidine biosynthesis decreased survival, but depleting ATP in those mutants restored survival[54].

New tools to sensitively measure levels of specific nucleotides in single cells and in real time will be extremely useful in dissecting these complex networks.

While mechanisms to downregulate protein biosynthesis during growth arrest are still not fully understood, mechanisms for transiently upregulating biosynthesis in this state are even less explored. In this context, “upregulation” is relative – mechanisms operating during growth arrest (potentially acting with only 100 active ribosomes available per cell) cannot drive high levels of expression of anything, in absolute terms, but instead help cells
overcome challenges to gene expression imposed by nutrient limitation or by the activities of regulators like (p)pGpp. One such challenge is that the substrates needed may not all be present in the environment at once. To take advantage of transient availability, many organisms have storage mechanisms for phosphorus (polyphosphate granules) and carbon (polyhydroxyalkanoate granules and glycogen). Production of these storage compounds is induced at the onset of growth arrest[55,56], and genes for phosphorus and carbon storage are under positive selection in an extremely low-nutrient environment[7]. Nitrogen is abundant in proteins, and proteolysis and reuse of amino acids is important during growth arrest, including both recycling material from dying cells in a starving population[17,20] and degrading aggregated proteins within the cell[26].

Assuming that the substrates for biosynthesis can be scavenged from the environment or intracellular storage, the gene expression machinery must adapt to the repressive regulatory context that occurs with growth arrest. A recent study of the stationary-phase nucleoid-associated protein Dps, which appears to bind the chromosome in a crystalline-like lattice[57], revealed that this growth-arrest adaptation may be less of a barrier to gene expression than previously assumed – it has essentially no impact on RNAP’s ability to access and transcribe DNA[58]. Other work showed that a growth-arrest-specific transcriptional regulator in P. aeruginosa (SutA) can directly enhance transcription initiation of the rRNA genes; the effects were greatest in conjunction with the general stress sigma factor[59]. This observation suggests that the stress-specific holoenzyme might be used for some transcription of housekeeping genes during growth arrest, which could be important if the housekeeping holoenzyme is largely sequestered by binding to the 6S small RNA[60].

**Implications for antibiotic tolerance and persistence**

One motivation for studying growth-arrested states has been the long-recognised correlation between susceptibility to most commonly-used antibiotics and growth[3,61,62]. It is now appreciated that non-growing cells that persist during antibiotic treatment maintain some metabolic activity but strongly repress protein biosynthesis[63], and that multiple mechanisms affecting biosynthetic activity give rise to complex population dynamics of antibiotic killing[64]. A non-growing persister state also appears to protect Salmonella from the host immune system, and requires some ongoing protein synthesis[65]. Recent efforts to standardise methods for investigating and describing non-growing states and their susceptibilities[66] are helpful, and highlight the importance of developing a deeper understanding of biosynthesis regulation under growth-arrested conditions. Some distribution of biosynthetic activity levels will always exist for any population of bacteria, and below a threshold, they are more likely to tolerate antibiotic exposure. The mean and shape of the distribution, as well as the timescales over which an individual bacterium might sample different regions of the distribution, are characteristics that likely have direct impacts on antibiotic susceptibility patterns. These characteristics are defined by the regulatory networks that govern biosynthetic activity during growth arrest.

A billion years of evolution have shaped these networks to balance the risks of ongoing protein synthesis against the benefits of renewing and adapting the proteome under severe resource limitation. Much still remains to be learned about the mechanisms responsible for this regulation in diverse organisms. By focusing research efforts on mechanisms that favour biosynthesis in addition to those that repress it during growth arrest, and seeking to understand how these mechanisms are coordinated over time and across bacterial
populations, we stand to improve our basic knowledge of bacterial life in natural environments, and also gain useful insights toward addressing the pressing challenges of rising antibiotic resistance.

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References


* This work provides a deeper investigation into mathematical models to describe oscillatory growth and activity that emerges in *Bacillus subtilis* biofilms, dependent on their size and environmental conditions. It is likely that many bacterial communities under nutrient limitation develop activity dynamics, and this system provides an elegant model for investigating parameters that can impact such dynamics.


*This work, along with references 17, 19 and 38, provides quantitative information about the rates of protein synthesis under nutrient-limited conditions. Additionally, it uses a fluorescence *in-situ* hybridisation (FISH) technique to assess transcriptional rates, and finds interesting differences in transcription-translation coupling depending on which nutrient is limiting. While it was not a focus of this manuscript, the method described could also be used to investigate population heterogeneity in transcription kinetics under growth arrest.


**This work clearly demonstrates that dynamic responses during growth arrest are key to survival outcomes in the case of exposure to DNA-damaging fluoroquinolone antibiotics. New synthesis of DNA damage repair machinery must occur prior to resumption of growth in order for growth-arrested cells to maximise survival after exposure to the antibiotic.**


This work, along with references 18 and 19, quantitatively describes adjustments to the translational machinery at very slow growth rates. Chemostat growth was used to set a very slow growth rate, with either carbon, nitrogen, or phosphorus limiting, and different characteristics of the translation machinery were affected by each condition. The quantitative insights allow for a deeper interrogation of the biosynthesis regulation under very limiting conditions than was previously possible.


** This work shows how evolutionary divergence of protein-protein interfaces away from the active sites of purine biosynthetic enzymes can differentially affect the ability of (p)ppGpp to inhibit their activities. Furthermore, it gives detailed insight into the complex mechanisms by which (p)ppGpp can carry out its effects. Understanding this complexity begins to give a sense of how this small molecule might be able to coordinate the behaviors of numerous interrelated biosynthetic pathways in the cell.


**This manuscript identifies and convincingly verifies several new direct targets of (p)ppGpp in *E. coli*. This is of key importance for understanding the regulatory networks operating during growth arrest, because (p)ppGpp is a major regulator of this state in a majority of bacterial species, and its pleiotropic effects had made elucidation of direct targets difficult. Although the direct targets in other species could be different, the method developed could in theory be deployed in other species as well.**


**This work shows that antibiotic-tolerant, non-growing persisters found within macrophages in a mouse model of *Salmonella* infection still maintain sufficient protein synthesis capacity to produce important T3SS effectors needed to manipulate the host cell response. This could be viewed as an example of bacterial cells finding the "sweet spot" of biosynthetic activity in a challenging environment.

*This consensus opinion piece is important reading for anyone interested in the mechanisms by which bacterial cells escape killing by antibiotics. Several important distinctions are made, and a key to understanding complex interactions is to ensure that everyone is using consistent definitions to discuss their findings.

**Figure 1. Bacteria must balance the risks and benefits of engaging in protein synthesis while growth arrested. A) New protein synthesis can be beneficial for growth-arrested bacteria as it allows for maintenance and repair of cellular machinery and utilisation of transiently available substrates for catabolism. However, extreme resource limitation can also mean that fluxes through the electron transport chain are interrupted, leading to oxidative damage, or that transcription and translation cannot continue processively. Stalled/aborted translation can leave misfolded proteins that aggregate and must be degraded. Stalled transcription that is uncoupled from translation can leave RNA-DNA hybrids that can be substrates for inappropriate DNA synthesis. Under these conditions, ongoing biosynthetic activity can be damaging, and total dormancy might be a safer state. B) The risks and benefits of adopting an arbitrary level of biosynthetic activity over different environmental exposures during growth arrest. Each column of the table represents the hypothetical fate of a bacterium at a specific activity level during growth arrest, with the lowest activity (a spore) on the left and the highest activity on the right. Each row of the table represents a new set of conditions experienced by the bacterium. The rightmost column depicts a population activity distribution that might be ideal for fitness under the condition in that row. In general, the lowest activity levels lead to the least damage under the stresses of acute starvation and...
antibiotic exposure but are associated with disadvantages in utilising transiently available nutrients, repairing damage, and resuming growth. In this example, a bacterium with a moderate level of activity represents a "sweet spot" that achieves the highest fitness across the series of events occurring, but in theory fitness could be further optimised by changing activity levels in response to changes in conditions throughout growth arrest.

**Figure 2. Ribosome dynamics during growth and growth arrest.** A) Box areas represent total protein synthesis outputs per cell per unit time in *E. coli* at the maximum growth rate (doubling time (t)=20 minutes), a slower growth rate (t=100 minutes) and stationary phase induced by carbon starvation. Rates for fast and slow growth were estimated by multiplying the number of actively translating ribosomes per cell by the average elongation speed, using numbers summarised in [67], and also generally agree with measurements made in [19]. The rate estimate for stationary phase is based on measurements made in [19] and [17]. B) Proteome composition for *E. coli* cells at t=20 minutes, t=100 minutes, and stationary phase. The area of the large box for each growth rate represents all the proteins (by mass) in a cell. Each blue square represents 100 actively translating ribosomes; orange square=100 inactive ribosomes; red circle= 100 RNAPs actively transcribing rRNA; black circle= 100 RNAPs actively transcribing something other than rRNA; grey circle= 100 inactive RNAPs. In this context, "inactive" includes ribosomes that are not yet mature, or are in the process of terminating translation and being recycled to initiate a new protein, or are sequestered, and RNAPs that are non-specifically associated with DNA, or bound at promoters but not yet transcribing, or are sequestered. The area of each symbol is to scale. Numbers for fast and slow growth are from [67]. The ribosome fractions for stationary phase are from [19]. The RNAP number in stationary phase is a rough estimate, by extrapolation from measurements in [68] to stationary phase. The number of active RNAP in stationary phase is not known but is likely small. The reallocation of proteome resources during the transition from fast to slow growth is driven in large part by cAMP [35], while (p)ppGpp is a major regulator of the transition to growth arrest [18,46].

**Figure 3: Nucleotide pools are tightly regulated, impact many biosynthetic processes, and change in growth arrest.** Sizes of boxes represent estimated sizes of nucleotide pools, based on [53,69]. Direct synthesis reactions are represented by black arrows, stimulatory effects are represented by green arrows, and inhibitory effects are represented by red symbols (nucleotides play roles both as regulators and as direct substrates for DNA and RNA synthesis). The amounts of cAMP and the nucleotide sugars were not directly measured at the entry to growth arrest. The synthesis of high amounts of (p)ppGpp can divert a measurable fraction of purine nucleotides away from other processes, and then actively represses new purine synthesis. Pyrimidine biosynthetic enzymes bind (p)ppGpp and may be directly repressed but are also indirectly downregulated by a decrease in purine levels, as purines are positive allosteric effectors. At the same time, consumption of nucleotides by growth-related processes is also reduced, such that pyrimidine pools actually slightly increase. How the cell adapts to the growth-arrest status of nucleotide pools and fluxes, and how it escapes this state to return to active growth, are largely unknown.
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