Genetic Determinants of Growth Arrest Survival in the Bacterial Pathogen *Pseudomonas aeruginosa* and the Role of Proteases

Thesis by

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I have learned a whole lot about a whole lot over the course of my Ph.D. But some of the most significant lessons were on the topic of failure. I learned not only how to fail, but that I

should expect to fail, and fail repeatedly. Although it took longer than I would have liked, I finally embraced the fact that failure in science, and in life, is inevitable. I realized that my negative outlook on failure was arresting my growth, not just as a scientist, but as a human being. In the process of learning how bacteria respond to adversity, how they react when the future looks bleak, I was also learning important lessons about my own responses to hardship. I learned that resilience is mandatory for success, and just like in microbiology, I learned that it is essential to not just focus on the good times, but to acknowledge the times in life when nothing seems to be going right, when it feels like what once nourished us has become depleted. It is only when we refuse to ignore our inevitable struggles and failures, and instead confront them head on, are we truly capable of growth. And with growth comes a surprising realization: "we've never failed at all, we've just found 10,000 ways it won't work."

This thesis is dedicated to the few people in my life who never once asked me when I was going to graduate.

ABSTRACT

Growth arrest is the dominant mode of microbial existence on the planet, yet the molecular mechanisms that underpin survival during growth arrest remain far less studied than other growth states. A better understanding of these mechanisms would provide valuable insight into the activity of microbial communities in both biogeochemical and clinical contexts, including the treatment of chronic infections. This thesis investigates the genetic requirements for survival of the bacterium *Pseudomonas aeruginosa*, a metabolically versatile opportunistic pathogen that thrives in diverse environments in which growth arrest is often caused by energy limitation. After reviewing our current knowledge of the strategies used by growth-arrested bacteria to adjust metabolism, regulate transcription and translation, and maintain the chromosome, I perform a functional genomic screen to identify genes that promote fitness of *P. aeruginosa* during growth arrest caused by carbon or oxygen starvation. I find that *P. aeruginosa* can survive for days to weeks in these energy-starved conditions by maintaining a reduced steady-state level of ATP, and that many functional classes of genes are required for fitness. Intriguingly, a majority of genetic fitness determinants differ between carbon and oxygen starvation, despite the common endpoint of reduced ATP levels in these two conditions. Among the few genes generally required for fitness are the stress response sigma factor encoded by *rpoS* and the heat shock protease encoded by *ftsH*. Using independently-generated deletion strains, I show that mutants in distinct functional categories exhibit temporal fitness dynamics during oxygen starvation: regulatory genes generally manifest a phenotype early during growth arrest, whereas genes involved in cell wall metabolism are required later. Building on these findings, I investigate the functional role of FtsH during growth arrest more deeply and find a surprising negative genetic interaction between *ftsH* and *rpoS*, with mutations in *rpoS* alleviating the fitness defects of Δ*ftsH* during growth arrest. I also find that FtsH functions coordinately with the other conserved heat shock proteases to maintain cellular integrity and delay aging of *P. aeruginosa* during growth arrest. Finally, I investigate the role of FtsH and the other heat shock proteases in a novel N-terminal protein degradation pathway and find that the molecular details of this pathway likely differ between *E. coli* and *P. aeruginosa*. Together,

these findings uncover essential molecular processes that promote fitness of an important bacterial pathogen during growth and survival.

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D.W.B. conceived the study and performed the experiments. D.W.B., D.A.A., and D.K.N analyzed the results and D.W.B. wrote the manuscript.

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D.W.B. participated in collecting and reviewing the literature and wrote the section on bacterial metabolism.

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INTRODUCTION

"The mathematics of uncontrolled growth are frightening. A single cell of the bacterium E. coli *would, under ideal circumstances, divide every twenty minutes. That is not particularly disturbing until you think about it, but the fact is that bacteria multiply geometrically: one becomes two, two become four, four become eight, and so on. In this way it can be shown that in a single day, one cell of* E. coli *could produce a super-colony equal in size and weight to the entire planet Earth."*

-Michael Crichton (1969) *The Andromeda Strain*.

Since its inception, the discipline of microbiology has been predicated on our ability to grow bacteria in the laboratory. In an effort to study their physiology, microbiologists have gone to great lengths to engineer pristine, ideal environments with just the right temperature, the right pH, the right amount of oxygen, and the right concoction of an amply nutritious food source in order to coax their bacterium to grow. For many bacteria, it doesn't take much coaxing, because in contrast to multicellular organisms like ourselves, bacteria are opportunistic dividers. When conditions are favorable, a bacterium will invariably grow and divide to form two copies of itself. As Michael Crichton elegantly illustrates in the opening quote, the truth is that many species of bacteria have evolved to grow *fast*. In fact, they've evolved to grow so fast that the rate-limiting step is often the time it takes to fully replicate their genome so that it can be partitioned equally into each daughter cell. This is the state in which microbiologists generally prefer to study their bacterium of choice: when it is happy, healthy, and growing so quickly that it might forget to include a copy of its genome in each new cell. We have derived much of our insight into the physiology of the bacterial cell, as well as that of our own cells, by studying these fast-growing bacteria in the prime of their lives and at the top of their game.

Pseudomonas aeruginosa is one well-studied example of these fast-growing bacteria. Because of its metabolic versatility, *P. aeruginosa* is happy to make a living in diverse environments, sometimes with a food source that could be considered meager at best. These environments vary widely: from

open ocean or fresh water streams, to the soil beneath our feet, to the biofilm growing in our hot tub. Unfortunately, its versatility also allows *P. aeruginosa* to thrive in more sinister environments, like the lungs of a patient with pneumonia or cystic fibrosis, or a wound caused by a severe burn or surgical procedure.

But in all of these environments, from lake to lung or test tube to toilet bowl, we know that *P. aeruginosa* can not grow indefinitely. At some point it will exhaust a key nutrient and cease its growth. What happens to *P. aeruginosa* after it stops growing? What happens to any bacterial community, for that matter, when the food or air it was happily consuming is all used up? Most of our study of bacterial physiology ends here, at the "stationary" or "growth arrest" phase of the bacterial life cycle. Microbiologists generally pay less attention to bacteria in this phase. For some, it is simply an "in between" phase, *i.e.*, in between bouts of fast growth, and in between where their interests lie. This is an understandable sentiment. Studying growth arrest can be mechanistically tricky and highly variable from one experiment or organism to the next. In fact, when studying a growth-related process, it's a good idea to minimize the emergence of growth-arrested cells altogether. Among the few valiant souls who devote their time and energy to try and better understand the physiology of growth arrest, I am certain all will readily admit that it's HARD! Studying growth arrest is just hard—a point that I hope will resonate with the reader of this thesis.

Because of the difficulty of its study, growth arrest is still very much a black box in our understanding of bacterial physiology. While we have developed a relatively detailed and exquisite picture of the many highly-conserved processes required for bacterial growth, an analogous framework does not exist for growth arrest. The question "what must cells do to grow?" is much more readily answered than "what must cells do to survive?" Most of the literature that has addressed the latter question has made it clear that bacteria do not simply "hang out" during periods of growth arrest, in what might be considered a "dormant" state. Rather, they must coordinately activate and maintain regulatory and metabolic responses to ensure their survival. These responses often differ greatly depending on the organism and the type of growth arrest—a concept that will be emphasized throughout this thesis.

Whether or not we care about bacterial growth arrest (although there are many reasons we should care, as will be made clear in this thesis) certainly the bacteria care. After all, they're the ones who

are starving. And the fact is the real world is not like the laboratory, where a friendly scientist is always on standby to replenish nutrients. The real world is harsh. And more often than not, the time to feast is over and famine has befallen the land. How well bacteria can survive these periods of nutrient scarcity determines who will still be around when nutrients once again become available. This thesis is about acknowledging the resilience of bacteria and appreciating what it takes for them not just to grow, but to survive. My hope is that by the end of this thesis the reader will also join me in acknowledgement and appreciation.

Overview

In Chapter 2, together with Megan Bergkessel, a postdoctoral scholar in the Newman Lab, I review much of our recent knowledge on how diverse organisms survive growth arrest, with emphasis on their metabolic, transcriptional, and translational responses, and the mechanisms they use to maintain genomic integrity. This chapter describes some of the recent technology that has facilitated the study of growth arrest, including techniques to measure the dynamics of protein synthesis, assess metabolic heterogeneity at the single-cell level, and screen for genetic fitness determinants on a genome-wide scale.

Chapter 3 utilizes one of these techniques, called Tn-seq, to identify genes required for fitness of *P. aeruginosa* when it is growth arrested due to energy limitation. I show that *P. aeruginosa* is capable of extended survival when limited for organic carbon as an electron donor or oxygen as a terminal electron acceptor, and that the genes required for survival vary greatly depending on the type of energy limitation. I also show that many of these genes are temporally important for survival, suggesting that growth arrest encompasses a more dynamic state than was previously appreciated.

Building on the findings in Chapter 3, Chapter 4 uncovers a central role for heat shock proteases in promoting survival of *P. aeruginosa* during carbon starvation. Using Tn-seq and multiple knockout strains, I show that protein turnover is a fundamental problem for *P. aeruginosa* during growth arrest, and that a highly redundant genetic system is in place to delay what might be considered “aging” of growth-arrested cells.

Chapter 5 explores a possible connection between the heat shock proteases of *P. aeruginosa* and a novel degradation pathway that recognizes the N-terminal formyl group of bacterial proteins. This project resulted from a collaboration with the Alexander Varshavsky Lab at Caltech, who first described this degradation pathway in *E. coli*. Together with Tri Vu, a postdoctoral scholar in the Varshavsky Lab, we show that none of the major heat shock proteases in *P. aeruginosa* appear to play a role in degrading N-terminally formylated proteins *in vivo*. However, the possibility remains that the mechanistic details of this degradation pathway are not conserved between *E. coli* and *P. aeruginosa*.

Finally, Chapter 6 provides some concluding remarks and a few suggested experiments to further build on the findings of this thesis.

An appendix is also included that details a protocol I developed to generate highly-saturated transposon libraries in *P. aeruginosa* strain UCBPP-PA14. This protocol could prove useful to those seeking to make their own transposon libraries in *P. aeruginosa*.

Chapter 2

THE PHYSIOLOGY OF GROWTH ARREST

This chapter is adapted from:

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Abstract

Most bacteria spend the majority of their time in prolonged states of very low metabolic activity and little or no growth, in which electron donors, electron acceptors and/or nutrients are limited, but cells are poised to undergo rapid division cycles when resources become available. These non-growing states are far less studied than other growth states, which leaves many questions regarding basic bacterial physiology unanswered. In this Review, we discuss findings from a small but diverse set of systems that have been used to investigate how growth-arrested bacteria adjust metabolism, regulate transcription and translation, and maintain their chromosomes. We highlight major questions that remain to be addressed, and suggest that progress in answering them will be aided by recent methodological advances and by dialectic between environmental and molecular microbiology perspectives.

Introduction

Much of our knowledge of the molecular machinery that is responsible for energy generation, gene expression and DNA replication comes from studying fast-growing model organisms, such as *Escherichia coli*, during **exponential phase** in nutritionally complete medium. Under these conditions, a single *E. coli* cell would grow to a population with the mass of the Earth within 2 days. Clearly, this does not occur, but the discrepancy between potential and actual growth underscores that the estimated $5x10^{30}$ bacteria that are on our planet spend the vast majority of their time in energy-limited states and not dividing. Although environmental microbiologists have long

appreciated the importance of extremely energy-limited states (1, 2), their focus has primarily been on exploring theoretical and empirical energetic limits of diverse metabolisms (3, 4). With a few notable exceptions (5-7), molecular microbiologists have largely avoided the study of growth-arrested cells. Consequently, relatively little is known about the mechanisms that underpin the dominant modes of bacterial existence; this gap in our knowledge hinders our understanding of the roles that bacterial communities have in driving global biogeochemical cycles and influencing plant and animal health.

The dearth of information is due, in part, to the challenges in defining, reproducing and measuring non-growing states of bacteria in the laboratory. Microbiologists have traditionally relied on population-level measurements of activity to draw conclusions about molecular mechanisms—an approach that benefits from high levels of biochemical activity and homogeneous populations. The few existing studies of non-growing states have focused on conditions that cause population growth arrest through the severe limitation of at least one basic resource (Box 1). These studies have provided most of our insights into these growth states (referred to as 'long-term **stationary phase**' (6), 'continuous activity stationary phase' (8) or 'starvation–survival' (2)) and have shown that growth-arrested cells synthesize proteins at rates that can be orders of magnitude slower than in exponential phase but stay viable for several days to years, and are usually able to rapidly resume growth when nutrients become available. These populations are neither highly active nor likely to be homogeneous (Box 2), potentially including subpopulations that undergo infrequent division cycles, but we assume, based on their static population numbers, that they represent the best available proxies for non-growing states. A major area for future research, as measurement sensitivity and selectivity continue to improve (Box 3), will be to better understand how these populations are composed of distinct cellular states.

Our focus on non-growing states of relatively fast-growing bacteria purposefully excludes some related states and other survival strategies. We view the responses to nutritional downshift that are part of the transition to stationary phase, which have been well studied and reviewed elsewhere (9, 10), as distinct from the strategies that are used later in stationary phase, and we point out these differences throughout the review (see also Box 1). Another state that we do not address is the endospore—a mode of survival that uses almost no energy for potentially thousands of years—which

has been well reviewed elsewhere (11). Finally, inherent slow growth in bacteria with low-energy core metabolisms is outside the scope of this review. These organisms, which have doubling times of months or much longer, might be viewed as 'non-growing' for substantial periods of time even when doubling at their maximum rates (reviewed in 4). How basic molecular mechanisms function in such contexts is fascinating, and may be related to the growth-arrested strategies of otherwise fast-growing bacteria, but the challenges of direct study remain extreme.

In this Review, we focus on general insights from current research and highlight remaining questions for three fundamental cellular challenges that are encountered during growth arrest. First, how are cellular energy stores maintained and managed during starvation? Second, how is gene expression regulated under extreme limitation for nucleotide and amino acid substrates? Third, how is chromosomal DNA protected in a way that allows occasional replication and low levels of transcription?

Metabolism: maintaining energy supply

The primary goal of growth-arrested organisms is to maintain the supply of the energy and biosynthetic precursors that are required to maintain essential macromolecular components of the cell, sustain active regulatory mechanisms for sensing and responding to the environment, and, perhaps most importantly, preserve the electrochemical gradient of the membrane (12, 13). Preserving this gradient (commonly known as the proton motive force (PMF)) is, in turn, crucial for transporting energetic and biosynthetic substrates into the cell in all bacteria, for flagellar motility in flagellated bacteria and for ATP synthesis in bacteria that are unable to sustain substrate-level phosphorylation (14). Thus, the continual supply of energy and building blocks is highly interdependent on the preservation of the PMF. Many bacteria can re-route metabolism to respond to specific limitations in this supply with impressive flexibility, enabling them to shift to alternative sources of energy and building blocks while balancing flux through central metabolic pathways.

Alternative sources of substrates for energy and biosynthesis.

Severe substrate limitation is often the underlying cause of growth arrest, as it leads to reduced rates of both **anabolic** and **catabolic** metabolism, and forces cells to rely on alternative sources of energy

and biosynthetic substrates, including internal stores. Indeed, cellular components themselves can be catabolized, which solves two problems that arise from severe substrate limitation: it provides nutrients and it removes the burden of maintaining cellular machinery that has become dispensable as overall cellular activity decreases. The result of this catabolism is a large reduction in cell size and volume (4), which increases the surface area-to-volume ratio of the cell and thus, in theory, could increase the efficiency of substrate transport (see below). Catabolism in growth-arrested bacteria has been shown most clearly to target ribosomes (see below) and membrane phospholipids. For example, genetic studies in *E. coli* found that derepression of genes that are involved in β-oxidation, and thus fatty acid degradation, is required for long-term survival in stationary phase (15). *Vibrio cholerae* also undergoes a substantial decrease (>99%) in total lipids within 7 days of starvation, despite an increase in viable cell count due to **reductive divisions** (see below), which led to the proposal that membrane phospholipids are an endogenous energy source to maintain viability in these cells (16). A recent microarray analysis of carbon-starved *Vibrio harveyi* cells supported this hypothesis, showing that genes that are involved in fatty acid β-oxidation were upregulated during starvation, concomitant with a decrease in cell size. Genes that have important roles in metabolic reactions that require acetyl-CoA, which is the major metabolite that is generated from the degradation of fatty acids, were also upregulated; many of these genes are part of the **glyoxylate shunt**, which is an **anaplerotic** pathway that bypasses most of the tricarboxylic acid (TCA) cycle, which is substantially downregulated during starvation (17) (Fig. 1).

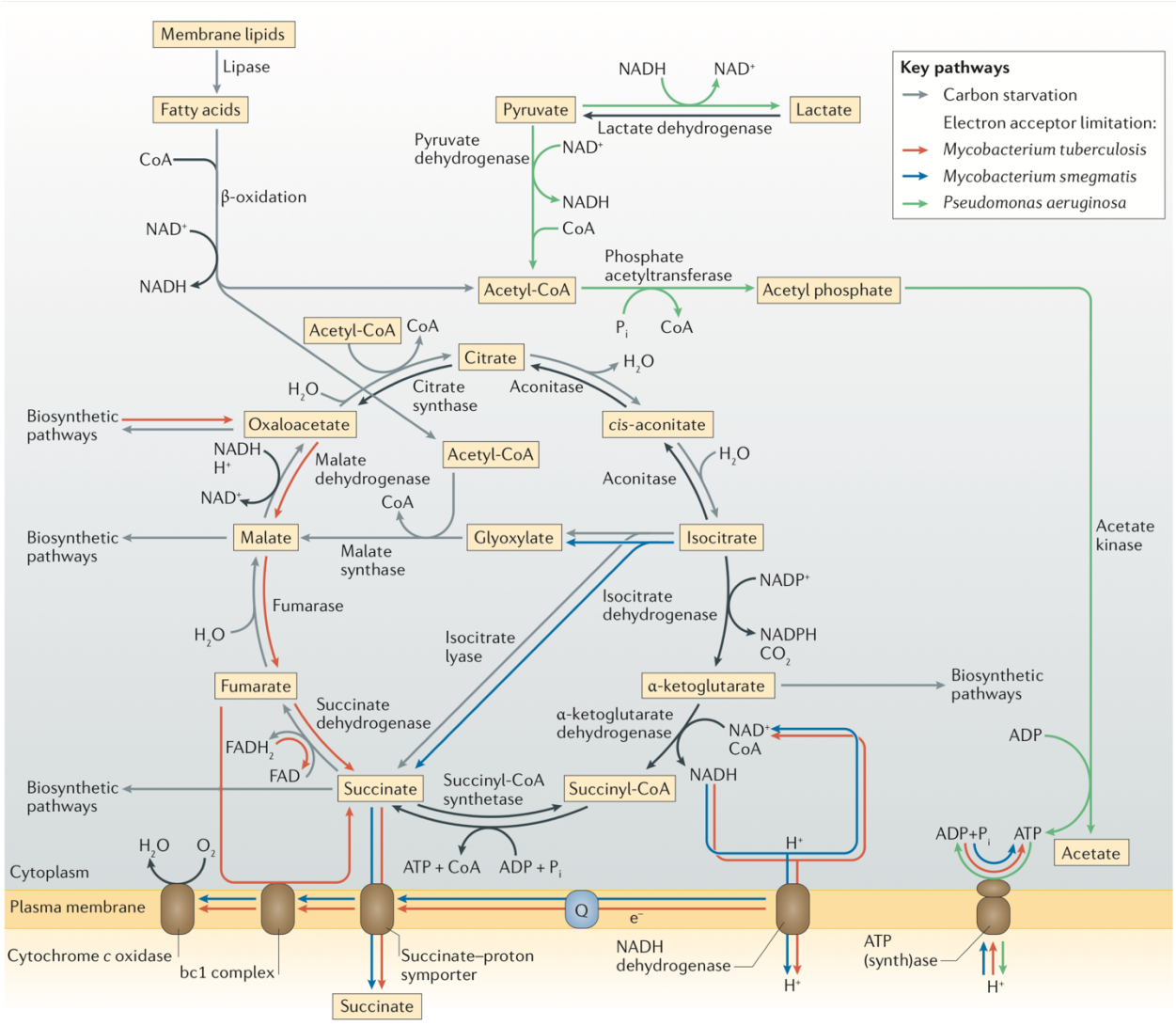


Figure 1. Metabolic rewiring during growth arrest. Different organisms use distinct metabolic strategies to meet cellular requirements under growth-limiting conditions. Both *Mycobacterium* spp. and *Pseudomonas aeruginosa* must adjust their strategies for maintaining their membrane electrochemical gradient under oxygen-limited conditions. *Mycobacterium* spp. continue to use the electron transport chain, using either low levels of oxygen or fumarate as the terminal electron acceptor, and also contribute to the membrane electrochemical gradient by secreting succinate, which is generated through reversal of the tricarboxylic acid (TCA) cycle in *Mycobacterium tuberculosis* and by the glyoxylate shunt in *Mycobacterium smegmatis*. In *P.aeruginosa*, fluxes through the TCA cycle and electron transport chain drop close to zero under anoxic conditions, and substrate-level phosphorylation generates ATP to run the ATP synthase in reverse, thus pumping protons outward across the membrane. Under carbon-limiting conditions, fatty acid β-oxidation generates acetyl-CoA, which can be fed into biosynthetic pathways through the glyoxylate shunt; this generates the TCA cycle intermediates that are most useful as precursors without overproducing other intermediates. P_i, inorganic phosphate; Q, quinone.

In addition to catabolism of internal stores, increased active acquisition of exogenous nutrients is an important survival strategy during nutrient limitation. In heterotrophs, limitation of organic carbon causes shortages of both energy and biosynthetic precursors. Many heterotrophs respond to this shortage by upregulating high-affinity transporters to scavenge carbon from the environment. For example, *Vibrio* sp. strain Ant-300 effectively depletes nanomolar concentrations of arginine from its environment and, interestingly, only exhibits chemotaxis toward arginine after starvation (18). Both high-affinity and low-affinity transport depend on respiration rather than ATP hydrolysis for energy, which suggests that the maintenance of the PMF is crucial for uptake by these transporters. Limitation of phosphate, nitrogen, sulfur and other nutrients also induces the upregulation of scavenging mechanisms in diverse organisms (19-21).

The evolutionary significance of competitive scavenging for resources is illustrated by the dynamics of populations of *E. coli* in batch culture. During long-term stationary phase in rich complex medium, in which the 'growth advantage in stationary phase' (GASP; Boxes 1,2) phenotype arises, *E. coli* mutants arise that take over the population. These mutants are able to outcompete 'naive' wild-type cells in stationary phase competition experiments (22). Strikingly, the majority of the GASP mutations that have been studied result in improved amino acid uptake and catabolism, by affecting genes such as those that encode leucine-responsive regulatory protein (Lrp; a **nucleoid**-associated protein) or the **sigma factor** RpoS (see below), or genes that are located in the amino acid transport locus *ybeJ–gltJKL* (6, 23, 24). Increased amino acid uptake ability is particularly important in the long-term stationary phase that is associated with the GASP phenotype, as the breakdown products of dead and dying cells are the major sources of nutrients.

Re-routing metabolic pathways.

A final strategy by which cells respond to nutrient limitation is to re-route metabolic fluxes to maintain acceptable levels of affected intermediates. For example, the preferentially aerobic pathogens *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* have very different responses to the hypoxic conditions that they experience during human infections (25). As oxygen is preferred by these bacteria as a terminal electron acceptor, hypoxia causes decreased flux through the electron transport chain, which, in turn, causes a decrease in the PMF and an accumulation of reducing

equivalents (primarily NAD(P)H). In *M. tuberculosis*, hypoxic conditions lead to a decreased but stable level of ATP, the maintenance of which requires the ATP synthase and some degree of PMF across the membrane (26). The PMF is maintained by the forward fluxes through the TCA cycle and the electron transport chain that can be sustained by the trace amounts of oxygen available to accept electrons, supplemented by **electrogenic secretion** of succinate. This succinate can be produced by one or both of two pathways: a reversal of the reductive branch of the TCA cycle through the upregulation of phosphoenolpyruvate carboxykinase, malic enzyme and fumarate reductase (which also re-oxidizes NADH); and the glyoxylate shunt, which bypasses steps of the TCA cycle that produce reducing equivalents (27-29) (Fig. 1). Interestingly, *M. tuberculosis* preferentially generates succinate by reversing the TCA cycle, whereas *Mycobacterium smegmatis* uses the glyoxylate shunt and *Mycobacterium bovis* uses both pathways (29), which shows that the re-routing of metabolic pathways in response to the limitation of terminal electron acceptors can vary, even between closely related bacterial species. *M. tuberculosis* can use metabolic reorganization as part of a strategy to survive hypoxic conditions for years without observable growth (30, 31); indeed, even when oxygen is completely absent, *M. tuberculosis* has been suggested to survive by using endogenously generated fumarate as an alternative terminal electron acceptor for oxidative phosphorylation (26) (Fig. 1). In contrast to mycobacteria, *P. aeruginosa* dispenses with oxidative phosphorylation altogether when oxygen is severely limited, and can instead maintain the PMF anaerobically by reversing the reaction that is catalysed by the ATP synthase (32), with ATP supplied by substrate-level phosphorylation using pyruvate or arginine as a substrate (33, 34) (Fig. 1). Under these conditions, fluxes through the TCA cycle and the electron transport chain are presumably close to zero. Together, these examples illustrate the flexibility of metabolic networks in maintaining crucial metabolic parameters during severe nutrient and energy limitation.

Further questions.

Although recent work has advanced our understanding of the metabolic strategies that are used to survive growth arrest, much is still unknown. At the level of single cells, questions remain about the absolute minimal energy requirements for viability, and how these limits vary according to the organism and environment, despite progress in investigating these boundary conditions (4). At an environmental level, complexities such as co-limitation for different substrates in a changing

environment and interaction with other microorganisms are likely contributors to energy dynamics in the natural world and significant forces in the evolution of strategies to regulate metabolism (35, 36). For example, in at least some cases, including low-energy environments, different species of microorganism can form cooperative metabolic interactions—an arrangement known as **syntrophy** (37). Indeed, mutually beneficial metabolic interactions may be more common than is currently appreciated; for example, redox-active phenazine pigments that are synthesized by *Pseudomonas* spp. were recently shown to support substrate-level phosphorylation by *P. aeruginosa* (32). Furthermore, studies have suggested that other organisms may also benefit from the presence of phenazines produced by *P. aeruginosa* in some contexts (38). Understanding metabolic network connectivity, even within one organism, still presents a research challenge, and we are just beginning to probe the metabolic interactions of microbial communities. Identifying new energy yielding pathways within community contexts is a priority for future research.

Regulation of gene expression

Limitation for energy, nucleotides and amino acids are common features of non-growing states that probably impose general constraints on gene expression, although the precise identities of upregulated and downregulated genes vary depending on the organism and the underlying causes of growth arrest. The constraints on gene expression may differ in non-growing states from those imposed by the better understood challenges of exponential growth and the initial transition to stationary phase, and recent technical advances have made feasible the study of how gene expression might adapt to the constraints that are imposed by growth arrest at a molecular level.

Regulatory paradigms of different growth states.

During exponential growth in nutritionally complete media, most gene expression is directed towards the biosynthesis of ribosomes, which are the principal mass constituent of each new cell being produced and are the drivers of the biosynthesis of all other proteins (reviewed in (39); Fig. 2a). Under these conditions, rates of translation elongation are limited by the intrinsic properties of the ribosome rather than the availability of amino acids (40). However, the unlimited availability of amino acids is short-lived even in rich medium batch culture. Thus, some resources will soon become limiting, at which time global regulatory systems will coordinate a transition away from maximum

growth. Two global regulators of this transition that are phylogenetically widely distributed but best characterized in *E. coli* are the sigma factor RpoS, which is induced by stresses such as heat and osmotic shock as well as nutrient downshift, and the **stringent response** alarmone guanosine pentaphosphate ((p)ppGpp), which is synthesized in response to signals of nutrient limitation (reviewed in (9) and (41), respectively). RpoS has relaxed sequence specificity compared with the housekeeping sigma factor RpoD, thus favoring the expression of a **regulon** of stress-adaptive genes with non-consensus promoters, but also drives lower levels of transcriptional activity (42). (p)ppGpp has pleiotropic effects on many cellular processes, and, together with the co-regulator DksA, strongly represses rRNA and ribosomal protein gene expression (43, 44). Together, RpoS and (p)ppGpp lead to a reduction in the total rates of gene expression, but also lead to a redirection of biosynthetic capacity away from ribosome biogenesis and towards more urgent concerns, such as preventing or repairing DNA damage (see next section), osmoprotection, refolding damaged pro- teins or increasing the synthesis of missing biosynthetic intermediates (Fig. 2b).

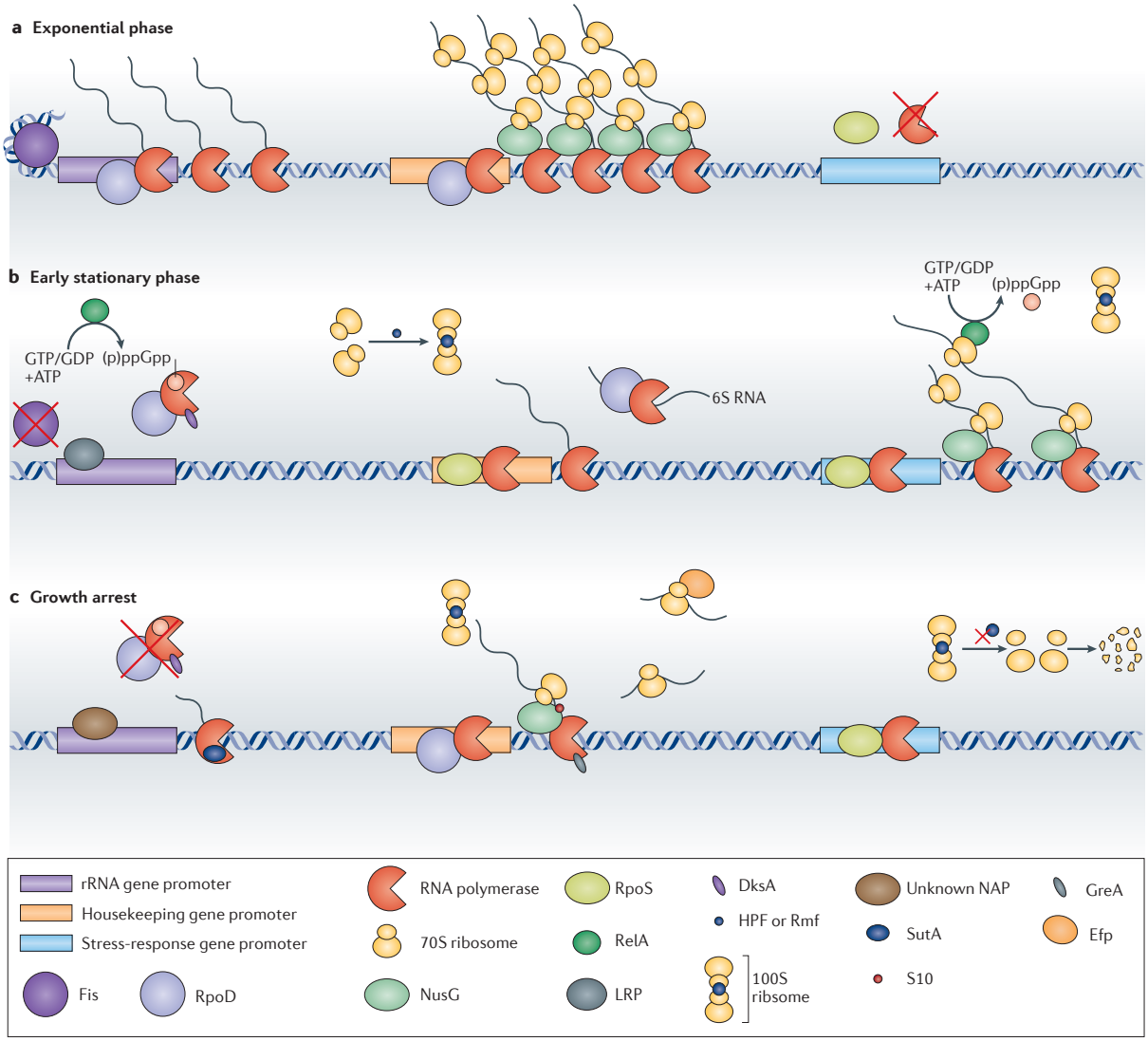


Figure 2. Transcription and translation during different growth phases. a | During exponential phase, rRNA genes are among the most highly transcribed in the cell, as ribosome biogenesis is a top biosynthetic priority. In addition, genes with promoters that have strong consensus sequences for RpoD binding are highly expressed and efficiently co-transcriptionally translated, aided by the transcription elongation factor NusG, which helps physically associate a ribosome with the RNA polymerase. The stationary phase sigma factor RpoS is synthesized to some extent but fails to compete with RpoD for RNA polymerase; consequently, stress-responsive genes with promoters that do not match the RpoD consensus sequence are not efficiently expressed. **b** | In the transition to stationary phase, limitation for amino acids activates RelA, which senses uncharged tRNAs and synthesizes the alarmone guanosine pentaphosphate ((p)ppGpp). (p)ppGpp, in conjunction with DksA, represses the transcription of rRNA by destabilizing the rRNA open promoter complex. The decrease in abundance of the nucleoid-associated protein Fis and the increase in abundance of leucine-responsive regulatory protein (Lrp) also contribute to rRNA repression. Hibernation promoting factor (HPF) and ribosome modulation factor (Rmf) are upregulated and lead to the dimerization of ribosomes to 100S complexes that are inactive for translation. RNA polymerase complexes with RpoD are selectively sequestered through several mechanisms, including binding to a small RNA (6S

RNA), and levels of RpoS are also increased, which leads to increased transcription of stress-responsive genes in the RpoS regulon. RpoS can also drive transcription of housekeeping genes that have RpoD-consensus promoters, but does so at much lower levels than transcription of these genes that is mediated by RpoD. **c** | During growth arrest, overall gene expression activity is much lower than in exponential phase or the transition to stationary phase. Although much remains to be elucidated about how these very low levels of activity are regulated, the observations that several global regulators change in abundance suggest some possible mechanisms. Regulators that are important during the transition to stationary phase, such as DksA, (p)ppGpp, RpoS and HPF, seem to be downregulated during growth arrest. Also, the complement of nucleoid-associated proteins (NAPs) changes substantially, which probably affects the expression of rRNA and other genes, although details remain to be explored. In *Pseudomonas aeruginosa*, some factors that are thought to contribute to transcription and translation elongation (GreA, S10 and elongation factor P (Efp)) were upregulated, possibly suggesting that they could help buffer against pausing and arrest in severely substrate-limited conditions. Although some ribosomes are catabolized, with the dual benefit of decreasing the number of ribosomes that are competing for amino acid substrates and liberating nutrients to be used for energy and maintenance, the newly identified transcriptional regulator SutA, which is upregulated during growth arrest in *P.aeruginosa*, enhances rRNA and ribosomal protein gene expression, which suggests that some repair and replacement of ribosomes may also be important.

Decreased availability of nucleotides and amino acids probably begins to affect transcription and translation rates during this transition phase, but (p)ppGpp and DksA function, in part, by further sensitizing the initiation of transcription to the availability of nucleotides—a strategy that makes sense during a transition from high to low nucleotide availability, but perhaps not if limiting nucleotides become a long-term challenge. Indeed, several lines of evidence suggest that RpoS and (p)ppGpp govern a transient, active adjustment to dynamic conditions rather than survival of a long-term non-growing state. DksA and (p)ppGpp have been shown to decrease to low levels in late stationary phase in *P. aeruginosa* and *E. coli*, respectively (45, 46), and two studies have suggested that levels of RpoS in *P. aeruginosa* may actually be lower in the non-growing state than in mildly nutrient-limited states (47, 48). In addition, mutations in *rpoS* that decrease function cause a GASP phenotype and provide a selective advantage during continuous culture at the lowest possible dilution rates, which suggests that prolonged RpoS activity may be maladaptive (7, 49). Although gene products of the RpoS regulon have important roles in growth-arrested states, the many layers of regulation that affect levels of RpoS suggest a delicate balance between preparing for growth arrest and actually surviving a prolonged non-growing state (50-52).

Nucleotide and energy levels continue to decrease after growth is arrested (53), potentially affecting both the priorities and mechanisms of gene expression regulation. As discussed, the priorities that are reflected by upregulated genes include using alternative energy and nutrient sources, and carrying out essential repair and replacement of cellular components. The mechanisms of expression

regulation are less clear—the biochemical implications of severe nucleotide and amino acid shortages are of great interest. Studies of *E. coli* in exponential phase and early stationary phase suggest that even moderate nutrient downshifts lead to profound changes in the stability of **open promoter complexes** and the tendencies of both polymerases and ribosomes towards pausing, permanently arresting or terminating (40, 43, 44, 54) (see (55, 56) for reviews). A successful regulatory strategy for non-growing states must favor the expression genes that are essential for survival, but must also ensure that the transcriptional and translational activities of the cell are matched to the available resources, and that relevant cellular machinery can handle slow elongation rates and long pauses (Fig. 2c).

Tuning gene expression capacity and rate to match limited substrates.

One strategy for tolerating shortages of nucleotides and amino acids is to limit the number of active polymerases and ribosomes so that fewer of these complexes are competing for the limited supply of substrates. Indeed, both RNA polymerases and ribosomes are sequestered at the entry to stationary phase, which reduces the number of active complexes. RNA polymerases bound to RpoD are sequestered by binding to a small RNA, at least in *E. coli* (57), and ribosomes are sequestered by hibernation promoting factor or ribosome modulation factor (or both, depending on the organism; reviewed in (56)), which mediate the formation of inactive ribosome dimers. In *Listeria monocytogenes*, the abundance of hibernation promoting factor peaks at the entry to stationary phase, and then decreases (58). Further into growth arrest, ribosome degradation begins to have an important role in the regulation of the number of ribosomes, and has the dual benefit of both limiting the number of active ribosomes and converting unused ribosomes to nutrients (58, 59). In *E. coli*, studies have shown that individual 30S and 50S subunits are preferred substrates for degradation during starvation; therefore, ribosome dimerization at the entry to stationary phase may help delay degradation until it is essential (60). Many bacteria also encode additional ribosome-binding proteins that are upregulated in stationary phase and that may have distinct roles in modulating the activity, sequestration or degradation of ribosomes during growth arrest. For example, RsfA, which is a highly conserved factor that has been shown to prevent subunit joining in *E. coli* (61), is upregulated during anaerobic survival in *P. aeruginosa* (48). Understanding how these factors coordinate to affect the fates of ribosomes during growth arrest will require further study.

Another strategy may be to tune the transcription and translation machineries to be less sensitive to pausing and substrate limitation, through changes in the levels of accessory factors. Detailed *in vivo* studies of such factors have not, for the most part, been conducted in non-growing bacteria, but some possible mechanisms are suggested by considering the proteins that are upregulated during anaerobic survival in *P. aeruginosa* in light of their functions in other growth states. One such factor is an RNA polymerase-binding protein, SutA, which enhances transcription of rRNA and ribosomal protein genes during slow growth. SutA may decrease the sensitivity of these genes to shortages of nucleotides and amino acids, and thus enable some repair and replacement of ribosomes during growth arrest (48). Although homologues of SutA are found only in a subset of Gammaproteobacteria (not including *E. coli*), another RNA polymerase accessory factor that may have a similar function is CarD, which is widely distributed outside the Gammaproteobacteria. CarD has been shown to be upregulated during nutrient limitation (62) and to enhance the transcription of rRNA genes (63) in *M. smegmatis,* which suggests that this type of regulatory response may be of broad importance. Two additional factors that are known to have roles in transcription elongation, at least in growing cells, and that are upregulated in *P. aeruginosa* during anaerobic survival are the ribosomal protein S10, which can 'moonlight' as a transcription elongation factor through its interaction with NusG (64), and GreA, which can help to rescue RNA polymerases that are arrested in 'back-tracked' states (known as **backtracked RNA polymerases**) (65). Finally, upregulation of the translation factor elongation factor P (Efp) may help buffer translation against stalling-induced arrest (66).

Further questions.

Although the observations described here imply that the non-growing state is actively regulated, the molecular details of the specific mechanisms that are responsible remain largely unknown, and there are hints that the solutions to fundamental problems of non-growing states may be quite diverse between phylogenetically divergent bacteria. This means that researchers must broaden the scope of inquiry to include more model organisms, including ones that are less adapted to laboratory growth conditions, and indicates that comparative and functional genomics techniques may become increasingly useful for identifying and understanding regulatory paradigms. Exploring molecular mechanisms in low-activity, heterogeneous states is challenging, but new applications for doing so

have shown promise, notably those that are based on next-generation sequencing or proteomics techniques (Box 3).

Maintenance and replication of DNA

A defining feature of non-growing states is the repression of DNA replication and cell division, and accompanying changes to the nucleoid have mostly been studied in *E. coli* and other model organisms as they transition towards stationary phase. The most observable feature of the stationary-phase nucleoid is a condensed, even crystalline, morphology when viewed by electron microscopy (67), which is thought to protect the DNA from damage and confer a survival advantage.

The generation times of exponentially growing *E. coli* cells are shorter than the time that is required to replicate the chromosome, which means that a new round of replication must start before the previous one completes. However, this process must be regulated to ensure a yield of exactly one replicated chromosome per cell division (Fig. 3a). One of the main factors in the regulation of the initiation of chromosome replication is DnaA, which is the ATPase that binds to the **origin of replication**. To prevent new rounds of initiation as the cell transitions to a non-growing state, the level, availability and activity of DnaA are markedly downregulated by several mechanisms (reviewed in (68, 69)). During this transition to stationary phase, ongoing rounds of replication can still be completed, but the accompanying cell divisions result in small progeny because ribosome biosynthesis and other biosyntheses are suppressed. Recent work suggests that the cell division machinery in *E. coli* may be capable of directly sensing the nutritional status of the cell through interactions between the membrane glycosyltransferase osmoregulated periplasmic glucans H (OpgH; also known as MdoH) and the cytokinesis regulator FtsZ. Poor nutrient status leads to lower levels of OpgH, which removes a block to FtsZ assembly and cytokinesis, permitting reductive divisions during nutrient starvation (70) (Fig. 3b).

The nucleoid: condensation and protection from DNA damage.

As cells progress towards growth arrest, the nucleoid undergoes increasingly extreme morphological changes. In general, the nucleoid becomes more condensed, while clusters of bound, transcribing RNA polymerase dissipate and ribosomes move from the central region of the cell, which they had

shared with the nucleoid, to the poles (71). Some of these changes are driven by the global changes in gene expression that are discussed above; in particular, the substantial decrease in rRNA transcription can sufficiently alter the distribution of RNA polymerase to affect the gross morphology of the nucleoid (72) (Fig. 3b, c). However, a crucial factor that drives changes in the nucleoid is the more specific transcriptional changes that occur in the expression of individual nucleoid-associated proteins (NAPs), which are proteins that bind to DNA with low or no sequence specificity and can give rise to higher-order structures. Most organisms encode several NAPs, which range in specificity from nearly universal to species specific (reviewed in (73)). The most abundant NAP shifts from factor for inversion stimulation (FIS) (74) during nutrient upshift and early exponential phase (75) to DNA-binding protein from starved cells (Dps) in stationary phase—a change that is driven, in part, by the regulatory activity of RpoS (76, 77). Dps, which has homologs in many bacterial species, can condense DNA through ordered self-aggregation, both *in vivo* and *in vitro* (67, 78), and transmission electron micrographs and atomic force microscopy have shown that nucleoid condensation in stationary-phase *E. coli* is dependent on Dps (79, 80) (Fig. 3c). Condensation of the nucleoid by DPS separates the DNA from potentially damaging reactants in the cytoplasm, holding it in a low-energy equilibrium state that is distinct from the dynamic disequilibrium of the nucleoid in exponential phase (79). In addition to protecting DNA by this spatial separation, Dps may protect DNA from oxidative damage (81). Dps has structural homology to ferritin (82) and can oxidize ferrous iron to its ferric, insoluble form (81). This reaction is proposed to compete with the **Fenton reaction**, thus protecting cellular DNA from oxidative damage by decreasing the production of oxidizing free radicals (76).

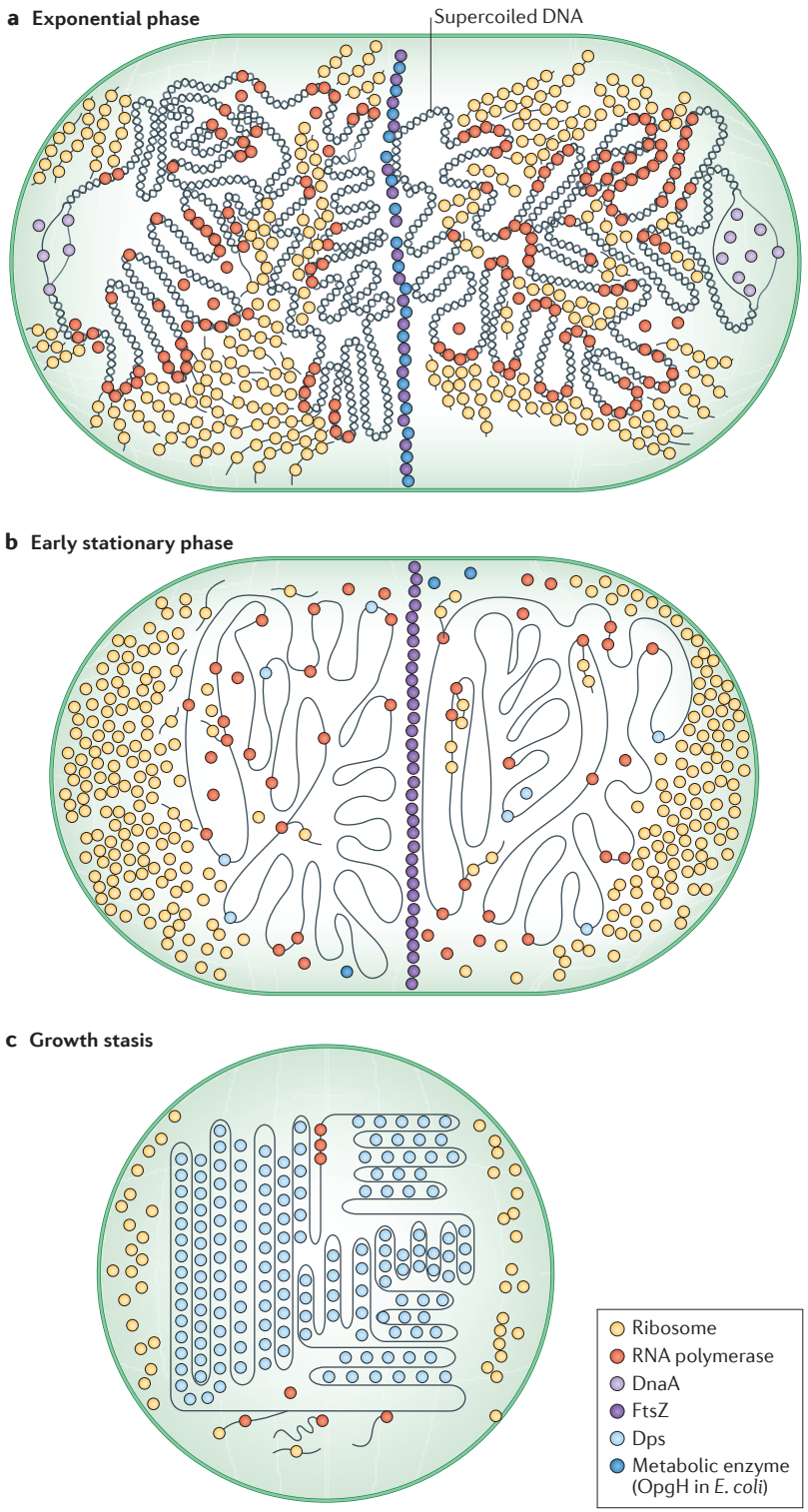


Figure 3. Overview of cellular morphology with emphasis on nucleoid. Cells undergo gross morphological changes in transitions between different growth states. **a** | In exponential phase, the chromosome has a high degree of negative supercoiling, owing to large

amounts of active transcription. RNA polymerase is mostly bound to DNA and is gathered in large clusters of highly transcriptionally active genes, which tend to migrate to the periphery of the nucleoid region. Ribosomes are observed in the central portion of the cell, sharing space with the nucleoid. New rounds of replication, initiated by DnaA, begin even before previous rounds have completed. Cytokinesis is inhibited by interaction of an abundant metabolic enzyme (for example, OpgH in *Escherichia coli*) with FtsZ, thus maintaining a larger cell size. **b** | In the transition to stationary phase, decreases in total transcription, and rRNA transcription in particular, lead to less supercoiling of the chromosome but a more condensed overall morphology, with fewer RNA polymerases and ribosomes associated with the nucleoid region in the centre of the cell. New rounds of replication are inhibited by the decreased abundance and activity of DnaA, but cell division to segregate chromosomes that have already been replicated can still take place (facilitated, in part, by a decrease in OpgH, which releases FtsZ), leading to progeny with a small cell size. The abundance of the nucleoid-associated DNA-binding protein from starved cells (Dps) begins to increase, owing to transcriptional upregulation by the stationary phase sigma factor RpoS. **c** | During growth arrest, an extremely high abundance of Dps leads to a highly condensed, crystalline appearance of the nucleoid region. Reductive divisions in stationary phase, combined with catabolism of ribosomes and membranes, leads to much smaller cell sizes. Transcription and translation still occur, despite reduced ribosome abundance, but at greatly decreased rates.

Despite the potentially protective effects of Dps, studies suggest that repairing DNA damage during growth arrest is still a priority for bacterial cells. In a detailed study of their expression, all three DNA polymerases that are responsible for error-prone repair (PolII, PolIV and PolV) were upregulated at various times during long-term stationary phase, and combinatorial deletions of these genes conferred competitive fitness disadvantages (83). In addition, microarrays of *V. cholerae* entering a viable-but-nonculturable (VBNC) state showed that *polB*, which encodes PolII, was the most upregulated gene in the transcriptome (84), and proteomics of *P. aeruginosa* during anaerobic survival showed upregulation of PolA (the polymerase that fills in gaps in the lagging strand) and DNA ligase (48), both of which have functions that could contribute to DNA repair. The sources of DNA damage during growth arrest are probably diverse and could include increases in free radicals and oxidative damage that may arise from challenges in maintaining flux through the electron transport chain; the possible accumulation of ongoing abiotic damage over long periods of time; and the double-stranded breaks that could arise if the cell fails to efficiently complete replication.

Further questions.

Many questions regarding the maintenance of the nucleoid during growth arrest remain unaddressed. For example, how does the condensed nucleoid interact with enzymes that mediate transcription, replication and repair? Several possible scenarios seem plausible. The distribution of NAPs along the chromosome in non-growing states has not been investigated in detail, and some chromosomal

regions may remain free of NAPs and thus be less condensed, even in growth states in which NAPs are some of the most abundant proteins in the cell. NAPs may also be capable of translocating along the chromosome or temporarily dissociating from the DNA, possibly through a mechanism that takes advantage of the high sensitivity of Dps–DNA interactions to concentrations of divalent cations and pH (85).

Another question is, how do other NAPs contribute to nucleoid organization and protection during growth arrest, both in *E. coli* and in other organisms? Work in *E. coli* has shown that the NAPs curled DNAbinding protein A (CbpA), integration host factor (IHF), histone-like nucleoid-structuring protein (H-NS), heat-unstable protein (HU) and Lrp all interact with the chromosome during stationary phase, despite being less abundant than Dps (73, 86). These proteins can substantially affect gene expression, as exemplified by the role of Lrp in regulating the transport of amino acids (see above), and rates of mutagenic break repair (87); therefore, the mechanisms by which they interact with specific regions of the chromosome during growth arrest could be important. Furthermore, different organisms have different complements of NAPs encoded in their genomes (73), and even homologous NAPs seem to have different regulatory parameters in different organisms. For example, in *Staphylococcus aureus*, Dps is induced by oxidative stress and not by the stationary phase as observed in *E. coli* (88), and in *P. aeruginosa*, the only NAP that is upregulated during anaerobic survival is HU (48).

Finally, little is known about how and why individuals of a non-growing population might occasionally engage in cycles of replication and cell division. An important insight from studies of the GASP phenotype is that the ‘evolution’ evident in the selective sweeps is possible at all; this proves that a numerically stable population of viable cells does in fact experience replication and division (89). Can genetic changes in a seemingly non-growing population be explained by relatively normal division cycles that occur infrequently in a small number of cells? Or does DNA replication and repair instead occur in larger numbers of cells but at very slow rates? What are the mechanisms? As new techniques facilitate the investigation of the non-growing state in diverse organisms, such questions should become easier to address.

Concluding remarks

Although the characterization of molecular mechanisms in non-growing states of bacteria remains in its infancy, observations from diverse fields and organisms suggest that these states are actively controlled to ensure surprisingly robust survival in the face of deprivation. Gene expression programs in these states reflect the priorities of catabolizing internal energy stores and scavenging trace nutrients from the environment; re-routing metabolism to integrate these alternative sources into central pathways; protecting and repairing DNA; and maintaining biosynthetic capacity for replenishing other essential molecular components of the cell. Furthermore, the gene expression machinery itself may adapt to the shortages of nucleotides and amino acids that are encountered. A better understanding of these priorities and mechanisms will open new doors for investigation of contexts in which non-growing states are probably required and regulated, such as symbiotic associations with plants and animals, and stable, structured microbial communities in both natural and applied settings (90, 91).

Towards this understanding, we have highlighted specific unanswered questions regarding changes in gene expression and two other important aspects of the biology of non-growing states: the maintenance of metabolic energy and the replication and maintenance of DNA. In doing so, we emphasize that fundamental questions about growth-arrested states are still not fully understood, even in model organisms. The examples that are cited in this Review are predominantly from the Gammaproteobacteria, owing to the biases of the authors and the literature, but further exploration of the profound diversity across bacterial species should be highly encouraged. We have focused on the potentially unifying characteristics that are observed in various non-growing bacterial populations as a starting point and the sample of studies discussed indicate the presence of general principles that transcend individual organisms—but also of diversity in the mechanisms by which these principles are applied. We expect that broader investigations into non-growing states of diverse bacteria will uncover substantial deviations from the picture that has emerged based on the information that is currently available. Further complexity arises from the heterogeneity observed in growth states, even for clonal populations in the laboratory. Such heterogeneity is probably central to the function of microbial communities, and deciphering how it arises and what it contributes to population fitness will require attention to be paid to both the intracellular mechanisms and the

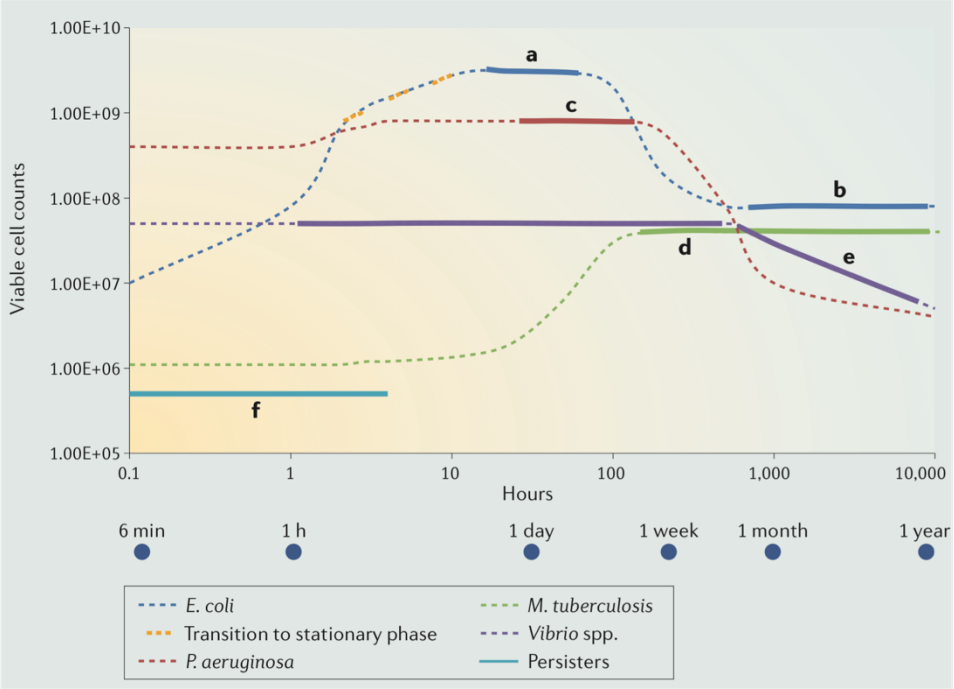
environmental factors that constrain non-growing states. Growth arrest is becoming ever more amenable to laboratory or *in situ* study with the development of tools that enable sensitive and specific measurements of subsets of (or even individual) cells and their microenvironments, and the increasing ease of creating genetic systems in diverse organisms. We hope that a piqued interest in the cellular and molecular biology of non-growing bacteria will help bridge a gap between environmental and molecular microbiology perspectives, and ultimately reveal the molecular underpinnings of the most dominant mode of microbial existence on the planet.

Box 1: Non-growing states

Several approaches have been used to study bacterial cells in non-growing states, and for comparison to each other and to other familiar growth states, non-growing states of interest can be shown as portions of representative, idealized growth curves (see the figure, solid lines).

Stationary phase.

Perhaps the simplest and most intuitive approach is to grow a batch culture until carbon and/or nitrogen sources are depleted. In 1971, this approach was used in *Escherichia coli* to show that, after an initial drop at the entry to stationary phase, the **adenylate energy charge** (AEC) decreased very slowly for days (see the figure, region a) before it began to decrease further and cells began to lose viability (53). These results are consistent with a much more recent study that used a microfluidic device to track protein synthesis by single cells during and after the transition into stationary phase, which found that after an initial large decrease, protein synthesis rates remained fairly stable during several days of starvation. This insight into the population heterogeneity of a growth-arrested state showed that, at least for the initial period of the stationary phase that lasted several days, non-growing status and protein synthesis rates are reasonably uniform across the majority of cells (8).



Box 1 Figure.

Long-term stationary phase (GASP).

Following this initial period, many species exhibit a large decrease in viable cell counts. However, at least in batch cultures of *E. coli*, the cells that survive can use nutrients that are released from dead cells, such that the population enters into a state of balanced cell death and growth that can continue for years without any new input of nutrients (see the figure, region **b**). During this long-term stationary phase, mutants arise that take over the population, a phenomenon that is referred to as 'growth advantage in stationary phase' (GASP). The heterogeneity that underlies GASP poses some challenges for study (Box 2). Studies of GASP have focused on genetic changes, which have shown that the functions that are under the strongest selective pressure include amino acid uptake and catabolism and modulation of RpoS activity (22, 89). In addition, the selective sweeps of beneficial mutations that are observed in GASP populations have confirmed that cell division is occurring, although this must be infrequent because the population numbers remain static. We consider the 'long-term stationary phase' condition in which GASP phenotypes are observed a reasonable proxy for non-growth, albeit a different one than the 'continuous activity stationary phase' (see the figure, regions b and a, respectively).

Oxygen limitation.

In the nutrient-rich environments inhabited by *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and other bacteria that are responsible for causing chronic infections, oxygen is much more likely than nutrients to become limiting, which can lead to growth arrest as oxygen is preferred for oxidative phosphorylation. For *P. aeruginosa*, the cell density of a culture that is incubated in an anaerobic chamber with either pyruvate or arginine as a carbon source remains relatively constant over a period of 1–2 weeks before cells begin to lose viability, and some cells survive anaerobic conditions for periods of at least several months (33, 34) (see the figure, region c). For *M. tuberculosis,* the slow depletion of oxygen from a stirred, closed culture flask, which models the latent stage of infection, induces a long-term non-replicating survival state in which the cell density remains stable for at least several months (30) (see the figure, region d).

VBNC.

It remains possible that some cells that seem to lose viability during growth-arrested states are actually entering a 'viable-but-nonculturable' (VBNC) state, which is characterized by an inability to form colonies on rich media but the continued maintenance of the proton motive force (PMF). First observed in *Vibrio cholerae* (see the figure, region e) and *E. coli* (92), but subsequently in phylogenetically diverse bacteria (93), the VBNC state can often be induced by stresses that overlap with stationary phase, such as prolonged starvation or high osmolarity, which suggests that it may be part of the 'growth-arrested state' continuum (94). The widespread existence of VBNC states in the environment, and a lack of understanding of the triggers for emerging from them, has been proposed as one of the reasons why many bacteria remain uncultured in the laboratory (95).

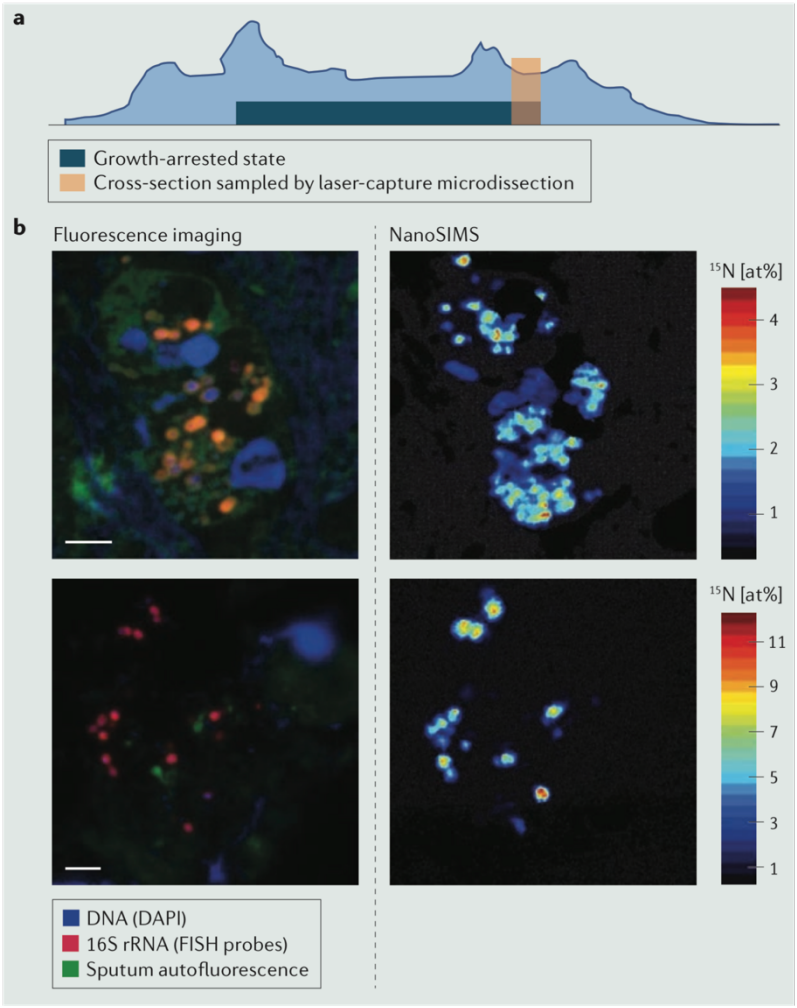
Persisters.

A different state in which cells remain growth-arrested despite an abundance of nutrients is the persister state that is associated with **antibiotic tolerance** (see the figure, region f). **Persisters** exist in a growth-arrested state in exponential phase batch cultures, in which most of the population is dividing at the maximum rate, which results in a substantial heterogeneity (Box 2) in replication rates (96). Entry into the persister state seems to be stochastic and reversible, and the isolation of mutants

that increase the rate of formation of persisters has suggested mechanisms that may be responsible, such as toxin–antitoxin systems that target the translational machinery and the stringent response (97, 98). Thus, the mechanisms by which cells impose growth arrest in the context of high nutrient availability might share components with the mechanisms by which cells enter growth arrest in response to nutrient deprivation (97, 98).

Box 2: Heterogeneity and growth arrest

Population heterogeneity is clearly present in many of the contexts in which growth arrest has been investigated, including the 'growth advantage in stationary phase' (GASP) phenotype, persisters and biofilms. Phenotypic heterogeneity in cultures that exhibit a GASP phenotype (6) probably helps determine which individuals survive the initial loss of viability; much later, genetic heterogeneity also develops in these populations (89). Perhaps the best-studied example of phenotypic heterogeneity in growth rates is that of persisters. Persisters are not mutants; indeed, isolated persisters give rise to a new population of exponentially growing cells that again has a tiny minority of persisters (99). Finally, heterogeneity in growth rates is clearly observable and important in biofilms. Laser-capture microdissection of cross-section samples of *Pseudomonas aeruginosa* biofilms (47, 100) has shown that, although cells at the edges of biofilms, which have the best access to nutrients and/or oxygen, have gene expression signatures that are similar to cells that are entering stationary phase, cells at the interior of biofilms show much lower rates of metabolic activity and different gene expression profiles, which is consistent with growth arrest (see the figure, part a). Different regions of these biofilms also exhibit different antibiotic sensitivities and rates of respiration (47, 100), which provides further evidence that cells in the different regions are in different growth states. Recently, it has been shown using time-lapse microscopy that *Bacillus subtilis* biofilms can also exhibit temporal heterogeneity in growth states, at least under some defined growth conditions, with cells at the periphery cycling through pulses of growth and non-growth; the authors propose that the non-growing periods provide the cells in the interior with better access to nutrients (101).



Box 2 Figure.

Although population heterogeneity has not been addressed in most studies of gene expression during growth arrest, it seems likely that cells are heterogeneous to some extent in their molecular physiology, given that prolonged growth arrest often leads to a loss of viability in some, but not all, cells. Substantial heterogeneity can pose serious problems for the interpretation of measurements at the population level. Even the question of whether cells are really growth arrested becomes difficult to answer. In the context of emergence of the GASP phenotype, the number of viable cells in the culture remains stable, but cell division is clearly occurring, at a low rate and/or among a subset of cells, as shown by the observation of a selective sweep of beneficial mutations through the population. In this heterogeneous population, the population numbers are static because growth is

balanced by death. The same might be true in other examples cited in this Review. In terms of understanding molecular mechanisms, this distinction is very important: in the absence of heterogeneity, the mechanisms proposed must account for very low levels of average activity, occurring in all cells of the population. If the populations that are under growth arrest are instead nearly always heterogeneous, then it is possible that the cells accounting for all of the observed activity are working at rates much closer to those observed for cells in exponential phase, but the population average is substantially lowered by the majority of nearly dormant cells. The increasing availability of methods for measuring single cells (Box 3) should help to address this question and enable further exploration of the mechanisms that underlie heterogeneity. For example, nanoscale secondary ion mass spectrometry (NanoSIMS) was used to investigate heterogeneity of microbial metabolic activity, as measured by the incorporation of ^{15}N, in sputum collected from a patient with cystic fibrosis and incubated in the presence of ^{15}N-labelled ammonium, which showed that metabolic activity is heterogeneous and not well correlated with the abundance of 16S rRNA in matched cells. DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization. Fluorescence microscopy and NanoSIMS images in part b courtesy of S. Kopf, University of Colorado Boulder, USA.

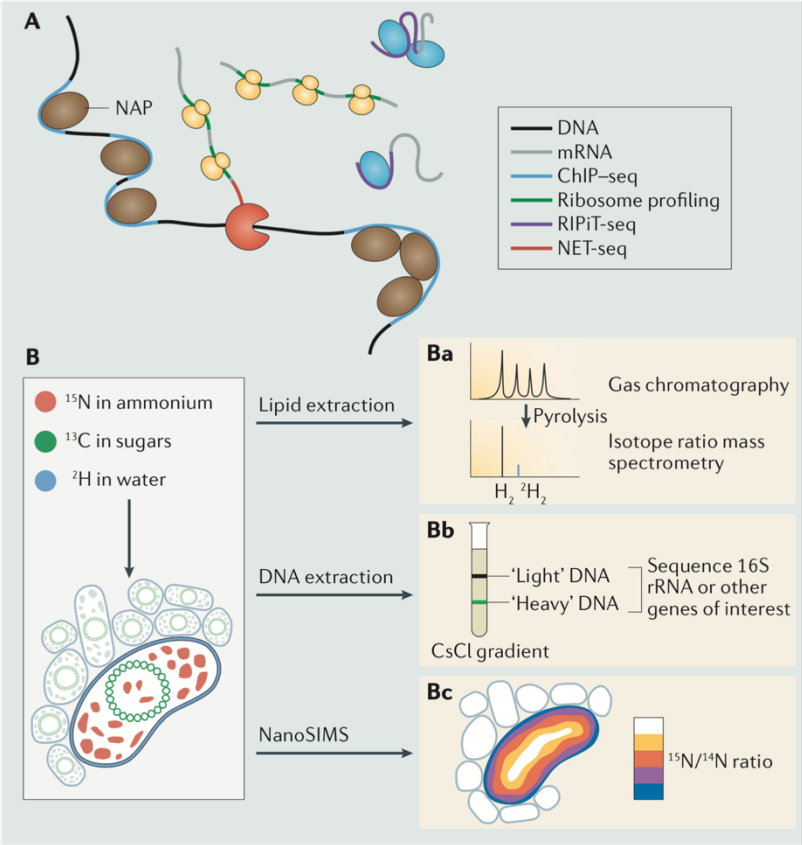
Box 3: New tools for studying non-growing states

The key technical challenges that are associated with studying growth arrest are the low rates of metabolic activity; the low intensity of 'signals' of newly synthesized macromolecules compared with the 'noise' of the pre-existing background; the contribution of degradation to changes in steady state levels, which could be comparable to that of new synthesis; the probable high level of heterogeneity in populations; and the difficulty in maintaining constant or known conditions in laboratory cultures for long periods of time. Many advances in the development, modification and accessibility of methods for measuring the levels and activity rates of macromolecules, both in populations and at the single-cell level, are improving the feasibility of accurately observing non-growing states.

Sensitive and selective population measurements.

Retentostat cultivation methods have been used by environmental microbiologists to overcome the challenge of maintaining constant conditions that arrest growth (102). Similarly to chemostats, retentostats maintain a flow of growth medium at a defined rate, but unlike chemostats, they retain all biomass, thus enabling the study of very slow or non-growing states. Such devices could be used to culture organisms for the study of the molecular physiology of minimum energy states.

Bio-orthogonal non-canonical amino acid tagging (BONCAT) uses click chemistry to attach biotin or fluorescent tags to amino acid analogues that are incorporated into proteins (103). For example, pulse labelling of L-azidohomoalanine, which cells can naturally incorporate in place of methionine, was used to study anaerobic survival by *Pseudomonas aeruginosa* (48). Incorporation of L-azidonorleucine, which requires a mutated tRNA synthetase, can be used to measure the proteomes of a subpopulation of cells by placing the mutant synthetase under the control of a promoter that is specifically active in that subpopulation.



Box 3 Figure.

Next-generation sequencing has numerous applications (see the figure, part a) that, in many cases, could be straightforwardly applied to non-growing cells, owing to the generally low quantity of starting material that is required. Direct sequencing of genomes has been used to detect mutations that arise during chronic infection (104), whereas transposon sequencing (Tn-seq) determines mutations in a transposon mutagenesis screen that affect fitness during exposure to a stress (105), and RNA sequencing (RNA-seq) has been widely used to study transcriptomes under various conditions (106). Other applications include chromatin immunoprecipitation followed by sequencing (ChIP–seq), in which regions of the genome bound by a factor of interest are captured by immunoprecipitation (107); ribosome profiling, which uses nuclease degradation to remove sequences of mRNAs that are not physically protected by ribosomes (108); native elongating transcript sequencing (NET-seq), in which nascent elongating transcripts are captured by immunoprecipitation of transcribing RNA polymerases (109); and RNA–protein

immunoprecipitation in tandem sequencing (RIPiT-seq), which reveals RNA–protein interactions through the sequencing of mRNAs that are captured by immunoprecipitation of RNA-binding proteins (110).

Population isotope labelling can be used in microbiology to trace the metabolic incorporation of substrates that are composed of heavy isotopes with low natural abundance (such as ^{15}N ammonium, ^{13}C glucose or ^{2}H water). Such an approach was recently combined with the extreme sensitivity of gas chromatography–pyrolysis–isotope ratio mass spectrometry (GC–pyrolysis–IRMS) to measure growth rates of *Staphylococcus aureus* in cystic fibrosis sputum (111) (see the figure, part Ba). Calculating isotope enrichment in labelled samples provides a measure of biosynthetic turnover that is independent of changes to total biomass, which makes this method applicable to a wide range of growth states. Stable isotope labeling (SIP) can also be used to separate the DNA of metabolically active cells from that of inactive cells in a mixed population, as metabolic incorporation of a stable heavy carbon isotope can lead to sufficient changes in DNA density for separation on a cesium chloride gradient (112) (see the figure, part Bb).

Single-cell measurements.

Microfluidic devices enable the isolation and cultivation of single or small numbers of cells for study by microscopy, sequencing or other techniques (113).

Microscopy is inherently well suited to observations of single cells, and can be combined with fluorescent markers that might be relevant in the study of growth-arrested states, such as engineered protein 'biosensors' of redox state or ATP levels (114, 115) and fluorescent dyes that detect membrane permeability and defects in membrane polarization, or even distinguish between the two (32).

Single-cell isotope methods that use radioactive isotopes have, for many years, been used by environmental microbiologists in conjunction with fluorescence *in situ* hybridization (FISH) to identify individual metabolically active cells in mixed populations (116). New methods include nanoscale secondary ion mass spectrometry (NanoSIMS), which provides sufficiently high resolution to accurately investigate bacterial metabolism on a cellular, and even subcellular, scale.

NanoSIMS uses stable, heavy isotope substrates, which, following metabolic incorporation and sample fixation, yield distinct secondary ions on bombardment with a cesium ion beam. A sensitive detector can determine the ratio of heavy to light isotopes present in the sample with high spatial resolution and this ratio gives insight into single-cell rates of metabolism (111) (see the figure, part Bc). Raman spectroscopy, in which the wavelengths of photons that travel through a sample are shifted by characteristic amounts as they interact with different chemical bonds, is also sensitive enough to distinguish between heavy and light stable isotopes and can be used to make rapid, high-throughput measurements of substrate uptake for individual cells (117).

Key points

Most bacteria in the environment are not actively growing most of the time, but the molecular mechanisms that govern growth-arrested states are not well understood.

Growth arrest has been studied by depleting nutrients or oxygen, which leads to large changes in metabolism, nucleoid state and the regulation of gene expression compared with exponential growth. Metabolism shifts during growth arrest to the use of alternative sources of energy and biosynthetic precursors, including internal stores.

The regulation of gene expression in non-growing states seems to differ from regulation during the entry to stationary phase and prioritizes successful expression of maintenance and survival functions.

The nucleoid is highly condensed during growth arrest through the activities of nucleoid-associated proteins, which not only protect DNA but also modulate gene expression.

Progress in understanding the physiology of stasis requires work to identify the key environmental factors that constrain microbial growth *in situ*, and, informed by this knowledge, laboratory studies that use emerging tools to reveal the molecular mechanisms that sustain cells through periods of growth arrest.

Glossary

Exponential phase – Microbial population growth that can fit to the exponential equation $N(t) = N_0 e^{kt}$, where $N(t)$ is the population size at time t, N_0 is the starting population size, e is the base of the natural logarithm, and k is a constant. Exponential growth is generally assumed to occur when no resource is limiting, to be balanced and at steady state.

Stationary phase – A growth phase of microbial populations that occurs after at least one resource becomes limiting for growth. At the transition to stationary phase, the population continues to increase in size, but the rate of increase decreases; in stationary phase, the population size stops increasing.

Anabolic – Metabolic reactions that construct larger macromolecules from smaller substrates.

Catabolic – Metabolic reactions that break down macromolecules into smaller components for the generation of energy or for recycling.

Reductive divisions – Cell divisions that are uncoupled from biosynthesis and growth, leading to progeny that are smaller in size. These contribute to the decrease in cell size that is observed during stationary phase.

Glyoxylate shunt – An alternative to the standard tricarboxylic acid (TCA) cycle in which steps that generate reduced NAD(P)H are bypassed to enable succinate, fumarate, malate and oxaloacetate to be produced for biosynthetic reactions without generating reducing equivalents. The glyoxylate shunt is useful in the context of limitation for terminal electron acceptors or the catabolism of lipids.

Anaplerotic – Reactions that replenish key intermediates of central metabolic cycles to compensate for their use by other biosynthetic pathways.

Electrogenic secretion – Symport of a substrate (with its chemical gradient) and a proton (against its chemical gradient) that results in a net increase in the proton motive force across a membrane.

Syntrophy – A mutually beneficial metabolic interaction between two (or more) species of microorganism.

Sigma factor – A protein that recruits RNA polymerase to a specific set of promoters on DNA. Some sigma factors have large regulons, whereas others drive expression from only a few loci.

Stringent response – A conserved regulatory mechanism that coordinates the responses of bacteria to nutrient downshift. The response is mediated by the small-molecule alarmone (p)ppGpp, the synthesis of which from ATP and GDP or GTP is stimulated by uncharged tRNAs or disrupted lipid biosynthesis.

Backtracked RNA polymerase – Transcribing RNA polymerases that slip backwards along a template after pausing, which causes the RNA–DNA hybrid at the 3′ end of the nascent transcript to unwind.

Nucleoid – The chromosome and associated proteins.

Fenton reaction – A metal-catalysed free radical chain reaction in which Fe^{2+} is oxidized by H_2O_2 to produce OH^- and $OH^{\bullet}$, which is a highly reactive radical species.

Adenylate energy charge (AEC) – A value based on the ratio of high energy phosphate bonds in ATP and ADP molecules to the total amount of adenylate in the cell.

Antibiotic tolerance – The survival of cells that are exposed to high doses of antibiotics for periods of time that would usually be lethal. Antibiotic tolerance extends the length of time that a cell survives exposure to the drug, whereas resistance enables a cell to survive an increased concentration of the drug.

Persisters – A subpopulation of cells that exhibits antibiotic tolerance in a population in which other cells are killed by the same dose and length of exposure to a drug. Persisters were first noted in an exponential-phase culture that was treated with high doses of antibiotics for extended periods of time.

Regulon – The group of genes that is regulated by a specific regulatory factor.

Open promoter complexes – The intermediate in transcription initiation in which RNA polymerase has bound to a promoter and unwound the double-stranded DNA, which allows the template strand of the DNA to pass through the active site of the polymerase.

Origin of replication – The site on the bacterial chromosome, determined by its sequence, where the two strands of DNA are unwound to enable replication of the chromosome to begin.

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Chapter 3

FITNESS DETERMINANTS DURING ENERGY-LIMITED GROWTH ARREST IN *P. AERUGINOSA*

This chapter is adapted from:

Basta D.W., Bergkessel M., Newman D.K. (2017) Identification of fitness determinants during energy-limited growth arrest in *Pseudomonas aeruginosa*. *MBio* 8(6). doi:10.1128/mBio.01170-17.

Abstract

Microbial growth arrest can be triggered by diverse factors, one of which is energy limitation due to scarcity of electron donors or acceptors. Genes that govern fitness during energy-limited growth arrest, and the extent to which they overlap between different types of energy limitation, are poorly defined. In this study, we exploited the fact that *Pseudomonas aeruginosa* can remain viable over several weeks when limited for organic carbon (pyruvate) as an electron donor or oxygen as an electron acceptor. ATP values were reduced under both types of limitation, yet more severely in the absence of oxygen. Using transposon-insertion sequencing (Tn-seq), we identified fitness determinants in these two energy-limited states. Multiple genes encoding general functions like transcriptional regulation and energy generation were required for fitness during carbon or oxygen limitation, yet many specific genes, and thus specific activities, differed in their relevance between these states. For instance, the global regulator RpoS was required during both types of energy limitation, while other global regulators such as DksA and LasR were required only during carbon or oxygen limitation, respectively. Similarly, certain ribosomal and tRNA modifications were specifically required during oxygen limitation. We validated fitness defects during energy limitation using independently-generated mutants of genes detected in our screen. Mutants in distinct functional categories exhibited different fitness dynamics: regulatory genes generally manifested a phenotype early, whereas genes involved in cell wall metabolism were required later. Together, these results provide a new window into how *P. aeruginosa* survives growth arrest.

Importance

Growth-arrested bacteria are ubiquitous in nature and disease yet understudied at the molecular level. For example, growth-arrested cells constitute a major subpopulation of mature biofilms, serving as an antibiotic-tolerant reservoir in chronic infections. Identifying the genes required for survival of growth arrest (encompassing entry, maintenance, and exit) is an important first step towards understanding the physiology of bacteria in this state. Using Tn-seq, we identified and validated genes required for fitness of *Pseudomonas aeruginosa* when energy-limited for organic carbon or oxygen, which represent two common causes of growth arrest for *P. aeruginosa* in diverse habitats. This unbiased, genome-wide survey is the first to reveal essential activities for a pathogen experiencing different types of energy limitation, finding both shared and divergent activities that are relevant at different survival stages. Future efforts can now be directed towards understanding how the biomolecules responsible for these activities contribute to fitness under these conditions.

Introduction

Microbiologists have long appreciated that most microbes on our planet spend much of their lives in a growth-arrested state due to limitation for an essential nutrient, inhibition by a toxic agent, or stalled regulatory adjustment to a new growth condition (1, 2). Importantly, cells remain viable in this state and are capable of regrowth once the limiting nutrient is replenished, the toxic inhibition relieved, or the regulatory adjustment made. Growth arrest is important in a variety of contexts, including antibiotic tolerance and persistence (3–5), establishment of mature biofilms (6–8), and ecological biodiversity (9). For example, longer durations of antibiotic exposure selects for *Escherichia coli* mutants that spend proportionally more time in growth arrest (5), *Pseudomonas aeruginosa* cells in the interior of biofilms have reduced metabolic activity and little to no growth compared to cells at the periphery (7, 8), and "seed banks" of dormant microbes contribute significantly to species richness in nutrient-poor ecosystems (10). Studies of spontaneous mutant growth during prolonged periods of starvation, referred to as the growth advantage in stationary phase (GASP) phenotype, have revealed how resourceful bacteria can be under periods of apparent nutrient limitation by recycling nutrients from their dying relatives (11). Yet the survival strategies that permit cells to cope when nutrients are truly scarce is poorly understood.

Microbes growing in dense communities like biofilms can quickly exhaust their electron donors or acceptors and enter growth arrest due to energy limitation. Most investigations into this state have focused on changes in cellular morphology and composition or global gene and protein expression. These studies demonstrate that energy-limited cells undergo wholesale reductions in DNA, RNA and protein synthesis, as well as reductions in cell size and volume (12–15) and shift their regulatory landscape away from active growth to one of survival and metabolic efficiency (16). However, the functional importance of many of these structural and regulatory changes remain unclear.

While all heterotrophic microbes must contend with limiting amounts of organic carbon as an energy source, the depletion of oxygen as a terminal electron acceptor is an energy limitation specifically important for opportunistic pathogens like *P. aeruginosa*. Reduced oxygen levels have been measured in *P. aeruginosa* colonized biofilms, cystic fibrosis sputum, and chronic wounds, and likely contribute to the slow growth and antibiotic tolerance of this organism during infection (7, 17–24). In the absence of oxygen or nitrate as a terminal electron acceptor, *P. aeruginosa* enters a growth-arrested state but can survive for days to weeks if provided with pyruvate or arginine as a fermentable energy source (25, 26).

Despite the relevance of energy-limited growth arrest for *P. aeruginosa* physiology in the environment and in chronic infections, only a few studies have identified or examined genes functionally important during this state (25–31). These studies have revealed crucial metabolic pathways essential for ongoing energy generation and maintenance of the proton motive force (PMF) (25, 26) and characterized novel regulators with widespread effects on gene expression (30). However, no studies have attempted to identify genes required for fitness of *P. aeruginosa* during carbon or oxygen limitation at a genome-wide scale. A systematic investigation to identify these genes is important not only for a better understanding of basic microbial physiology, but also to determine the factors that might contribute to chronic infections caused by this organism.

In this study, we performed a functional genomic screen using transposon-insertion sequencing (Tn-seq) to identify fitness determinants in *P. aeruginosa* when energy limited for organic carbon (pyruvate) as an electron donor or oxygen as an electron acceptor. Our screen reveals divergent and

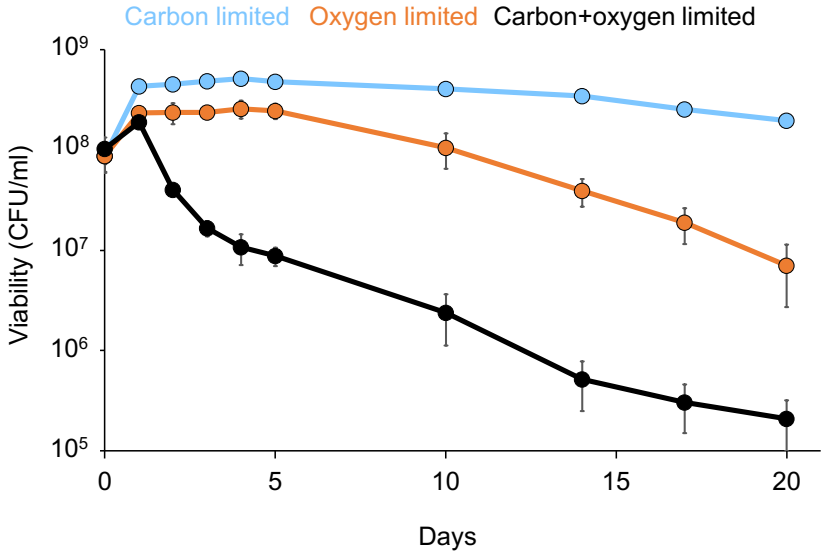
overlapping activities required for fitness during both types of energy limitation and highlights the value of a functional-genomics approach for studying the physiology of growth arrest.

Results

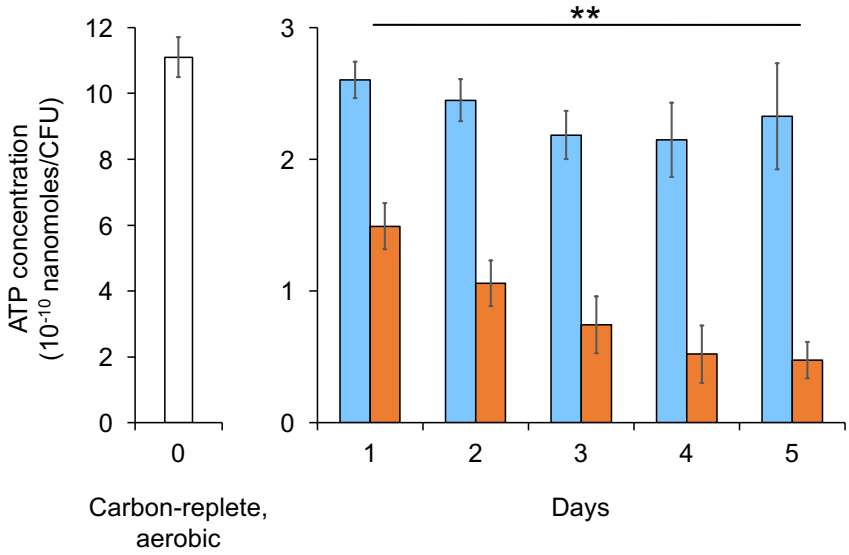
Viability and energy levels of P. aeruginosa *during carbon or oxygen limitation.*

We began our study by measuring the survival dynamics of *P. aeruginosa* during growth arrest caused by carbon or oxygen limitation. We chose pyruvate as the sole exogenous carbon and energy source for our experiments because *P. aeruginosa* is capable of aerobic growth as well as anaerobic survival on this substrate (25, 26). We grew cultures of PA14 wild type (WT) aerobically to exponential phase in minimal medium with 40 mM pyruvate, then pelleted the cells and resuspended them in fresh medium with either 1 mM pyruvate or 40 mM pyruvate. The cultures resuspended in 1 mM pyruvate were shaken aerobically for 20 days (carbon limited) while the cultures resuspended in 40 mM pyruvate were transferred into an anoxic chamber and incubated without shaking in the absence of any terminal electron acceptor for 20 days (oxygen limited). We also incubated cultures anaerobically without pyruvate to assess survival in the absence of both electron donors and acceptors (both carbon and oxygen limited). Under our experimental conditions, *P. aeruginosa* maintained viability for nearly 20 days during carbon limitation and 10 days during oxygen limitation, whereas its viability rapidly declined when limited for both carbon and oxygen (Fig. 1A). To confirm that pyruvate was completely consumed during carbon limitation, we measured its concentration using high-performance liquid chromatography (HPLC) and observed that the pyruvate concentration became undetectable by day 1 of survival, coinciding with growth arrest of the population. Addition of 40 mM pyruvate to the carbon-limited cultures on day 5 promoted outgrowth to high cell density, further confirming that pyruvate was indeed limiting for growth in our experiment (data not shown).

A


Carbon limited
Oxygen limited
Carbon+oxygen limited
Viability (CFU/ml)
10^9
10^8
10^7
10^6
10^5
0
5
10
15
20
Days

B


ATP concentration (10^{-10} nanomoles/CFU)
12
10
8
6
4
2
0
3
2
1
0
**
0
1
2
3
4
5
Carbon-replete, aerobic
Days

C

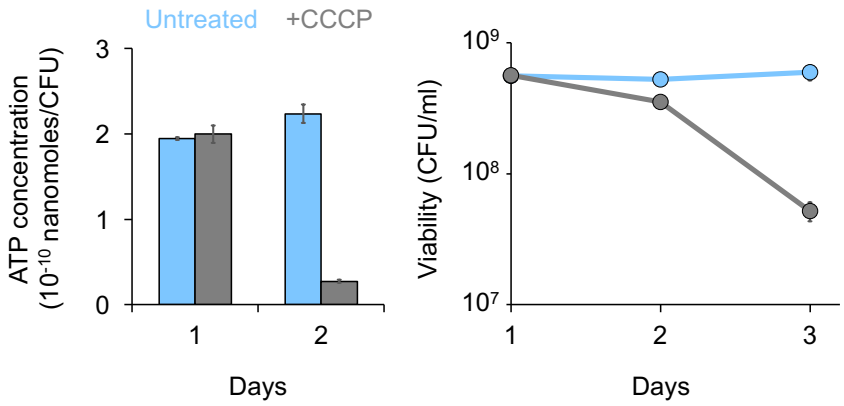

Untreated
+CCCP
ATP concentration (10^{-10} nanomoles/CFU)
3
2
1
0
1
2
Days
Viability (CFU/ml)
10^9
10^8
10^7
1
2
3
Days

Figure 1. *P. aeruginosa* maintains viability at a reduced level of ATP during energy-limited growth arrest. (A) Viability of *P. aeruginosa* cultures as measured by colony forming units (CFU) over 20 days of carbon, oxygen, or carbon and oxygen limitation. (B) Steady-state ATP concentration per CFU over the first five days of carbon or oxygen limitation. (C) Viability and ATP concentration of carbon-limited cells treated with 25 μM CCCP on day 1 of growth arrest. Error bars show the standard deviation of biological replicates (n = 3). The double asterisk indicates a significant difference in ATP concentration between carbon and oxygen-limited cells (paired Student's *t* test, *P* value <0.005).

The difference in viability between carbon and oxygen limitation suggests that a lack of oxygen is a more severe growth-arresting trigger. We hypothesized that energy limitation might be more extreme for oxygen-limited cells because they are constrained to using the low-energy yielding acetate kinase-phosphate acetyltransferase (AckA-Pta) pathway (25), whereas carbon-limited cells can potentially generate more energy by respiring alternative endogenous or exogenous carbon sources, such as fatty acids and amino acids derived from lipid and protein degradation, respectively (16). To test this hypothesis, we measured the steady-state ATP level during both types of energy limitation (Fig. 1B). We observed a greater than 4-fold reduction in ATP levels between actively growing (carbon-replete, aerobic) cells (day 0) and growth-arrested (carbon- or oxygen-limited) cells (days 1-5), confirming that carbon- or oxygen-limited cells are indeed energy limited. As predicted, ATP levels fell to an even greater extent for oxygen-limited cells by day 1 of growth arrest, and continued to decline over the following days, reaching nearly 5-fold lower levels compared to carbon-limited cells by day 5. After day 5, the viability trajectories of carbon- and oxygen-limited cells diverged, with lower viability correlated with the difference in ATP levels for these populations on day 5. To directly link ATP levels with viability, we treated carbon-limited cells with CCCP, an ionophore that dissipates the PMF (Fig. 1C). After 1 day of CCCP treatment (day 2 of growth arrest), ATP levels were reduced 8-fold compared to untreated cells. This was followed by a nearly 8-fold reduction in viability on day 3. Together, these results indicate that viability is dependent on the steady-state ATP level and suggest that the reduced viability of oxygen-limited cells is due to their lower level of ATP.

Tn-seq experimental approach.

To identify fitness determinants during carbon or oxygen limitation, we performed a genomic screen using Tn-seq. Tn-seq uses the power of massively parallel sequencing to quantify changes in relative abundance of insertion mutants in a transposon mutant library under a condition of interest (32). These changes in abundance approximate the contribution of the mutated region of DNA to growth

and survival under the relevant condition. Insertion mutants with a fitness advantage increase in relative abundance while insertion mutants with a fitness disadvantage decrease in abundance. Insertion mutants in neutral regions show no change in abundance. Tn-seq has been optimized for use in diverse bacteria and offers a high throughput, unbiased, semi-quantitative approach to interrogate the genome of an organism for fitness determinants under a variety of conditions (33).

We generated a transposon library in PA14 containing ~150,000 unique mutants using the randomly inserting Tn5-based transposon T8 (34). This transposon is designed such that only the gene into which it inserts is transcriptionally silenced, *i.e.* polar effects on downstream genes are avoided (34). We subjected replicate aliquots of our library to either carbon or oxygen limitation for 10 days and compared the reads per gene following aerobic outgrowth of these energy-limited samples to control samples grown only under carbon-replete, aerobic conditions (Fig. 2A; see Materials and Methods for details). The majority of mutated genes had a mean read ratio of ~1 between the energy-limited and control samples, indicating that most insertions had a neutral effect on fitness (Fig. S1). There were no unique insertions in any of our energy-limited samples with a read count greater than 0.4% of the total reads in the population (where the average was ~0.0008%). Importantly, those mutants with the highest proportional read counts were present at equally high rates in all replicates of both the energy-limited and control samples. This indicates that their increased abundance was not due to an overwhelming fitness advantage caused by either the transposon insertion or a spontaneous mutation, but rather due to an insertion "hotspot" in the initial pooled library. Together, these data strongly suggest that no significant population turnover occurred throughout the duration of our experiments due to GASP mutations.

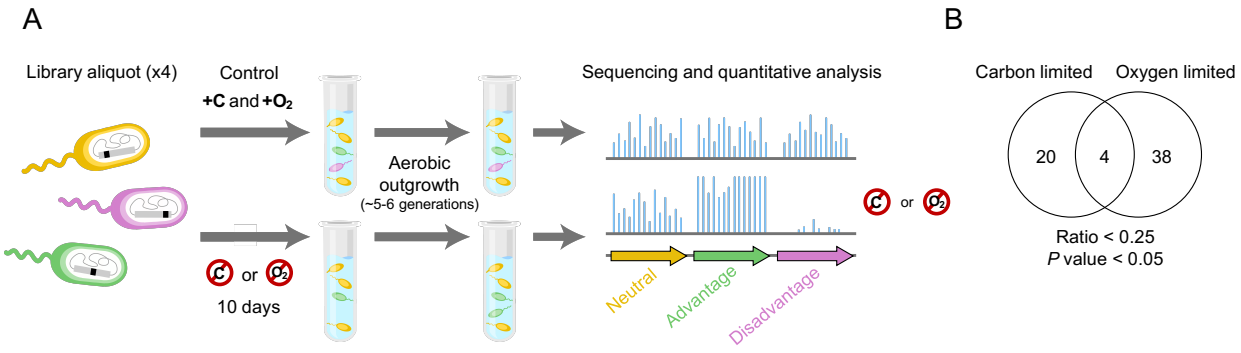


Figure 2. Tn-seq identifies fitness determinants during energy-limited growth arrest. (A) Diagram of the experimental approach. Each experiment was performed in duplicate. Control samples were grown only under carbon-replete, aerobic conditions, while energy-limited samples were incubated without carbon or oxygen for 10 days followed by carbon-replete, aerobic outgrowth. (B) Number of genes identified as having a greater than 4-fold significant fitness defect defined as a mean read ratio <0.25 and a combined *P* value <0.05.

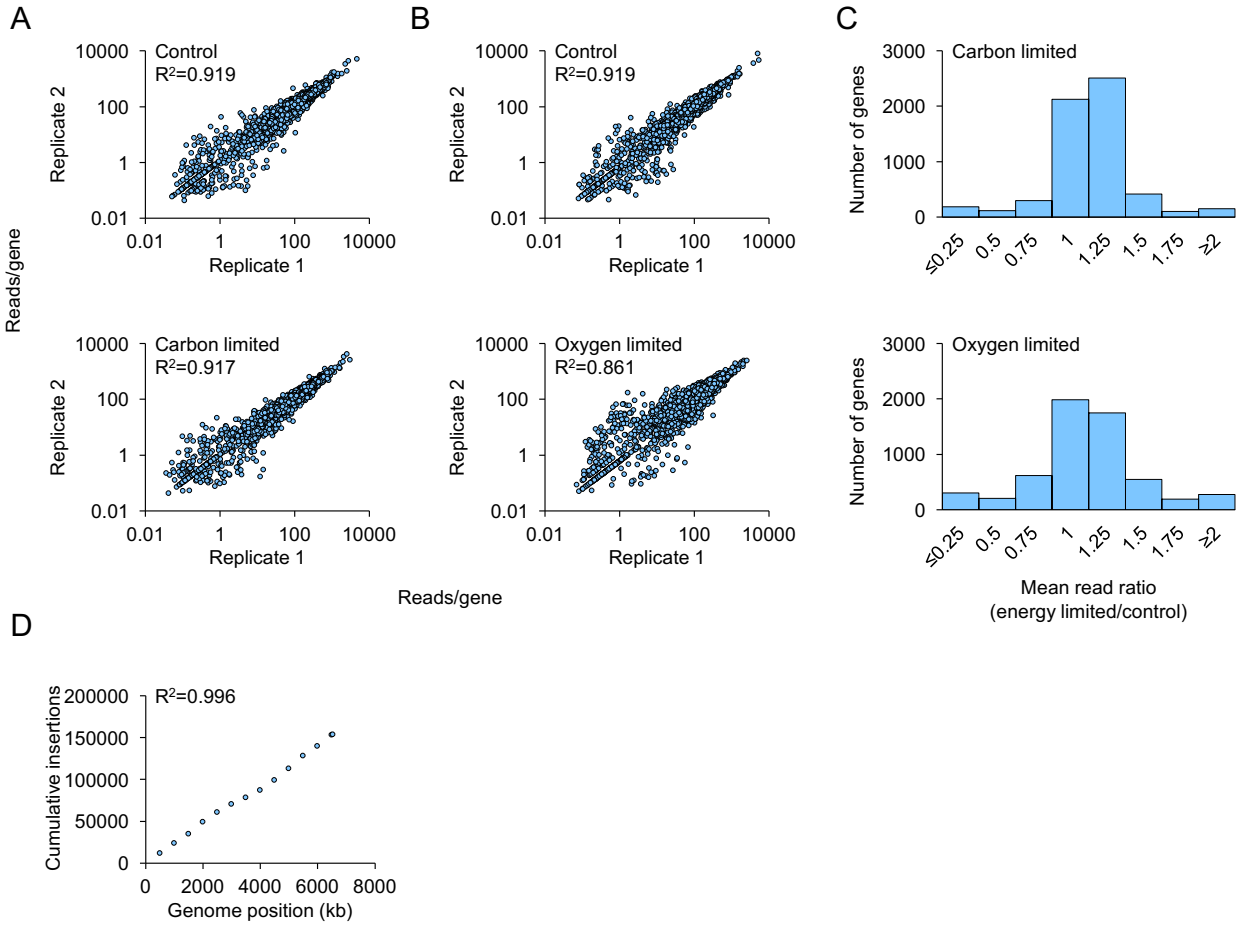


Figure S1. General Tn-seq results. Correlation of total reads per gene between the replicates for control and carbon-limited samples (A) and control and oxygen-limited samples (B). Reads are displayed in reads per kilobase per million mapped reads (RPKM). Each point represents a single gene. For display on a logarithmic scale, genes with zero reads were given a value of one before RPKM conversion. (C) Frequency of read ratios between the energy-limited samples and the corresponding controls for each gene. The geometric mean of the read ratios for each replicate is plotted. (D) Cumulative number of unique insertions in our PA14 transposon library. A linear pattern indicates even distribution of insertions across the genome.

We defined a gene as being required for fitness during carbon or oxygen limitation if the mean read ratio of the replicates was <0.25 and the combined *P* value <0.05. This represents mutants with a greater than 4-fold fitness defect following aerobic outgrowth after 10 days of carbon or oxygen

limitation, and encompasses genes required for entry, maintenance, and reemergence from growth arrest. We picked these stringent criteria to limit the number of potential hits and increase the likelihood of identifying genes that would exhibit a strong phenotype; Data Sets S1 and S2 in the supplemental material show read ratios and *P* values for genes and intergenic regions across the entire genome. Based on our criteria, we identified a total of 62 required genes. Of these genes, 20 were required specifically during carbon limitation, 38 specifically during oxygen limitation, and 4 during both types of energy limitation (Fig. 2B). We did not identify any mutants with a fitness advantage during carbon limitation based on a mean read ratio greater than 4 and a combined *P* value less than 0.05. However, 25 mutants were identified as having a fitness advantage during oxygen limitation based on these criteria (Fig. S2).

A

Locus	Gene	Protein description
PA14_25160	*lexA*	Transcriptional regulator
PA14_26590		GntR family transcriptional regulator
PA14_44780		Transcriptional regulator
PA14_62530	*cbrA*	Two-component sensor
PA14_11560	*ispA*	Geranyl diphosphate/farnesyl diphosphate synthase
PA14_40640	*cti*	Cis/trans isomerase
PA14_29920	*nuoI*	NADH dehydrogenase subunit I
PA14_29970	*nuoF*	NADH dehydrogenase subunit F
PA14_29990	*nuoD*	NADH:ubiquinone oxidoreductase subunit C/D
PA14_45290	*ccmH*	Cytochrome c-type biogenesis protein
PA14_45310	*ccmF*	Cytochrome c-type biogenesis protein
PA14_45370	*ccmB*	Heme exporter protein
PA14_57570		Cytochrome c reductase, iron-sulfur subunit
PA14_61280		Epimerase
PA14_65740	*thiC*	Thiamine biosynthesis protein
PA14_03680	*cysT*	Sulfate transport protein
PA14_22350	*actP*	Acetate permease
PA14_31680	*tamA*	Translocation and assembly module subunit A
PA14_31690	*tamB*	Translocation and assembly module subunit B
PA14_44440		Cation-transporting P-type ATPase
PA14_60950	*cstA*	Carbon starvation protein
PA14_66980	*tatC*	Sec-independent protein translocase
PA14_30230	*clpA*	ATP-dependent Clp protease, ATP-binding subunit
PA14_41220	*lon*	ATP-dependent protease
PA14_65280	*hflK*	Protease accessory factor

B

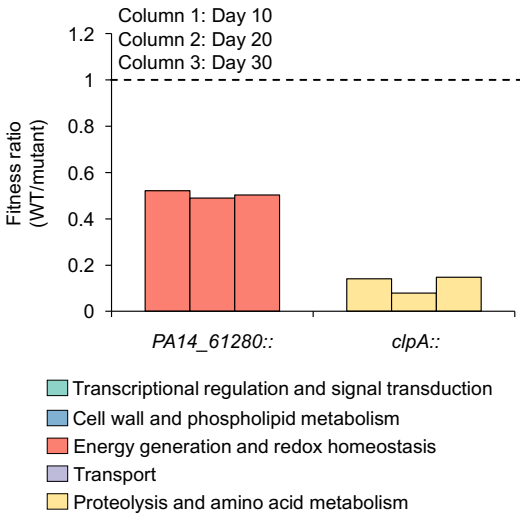


Figure S2. Mutants with a fitness advantage during oxygen-limited growth arrest. (A) Functional categories of genes identified as having a greater than 4-fold significant fitness advantage defined as a mean read ratio >4 and a combined *P* value <0.05. (B) Fitness of WT relative to two mutants at days 10, 20, and 30 of oxygen limitation. The double colon indicates a transposon mutant.

Identification of known fitness determinants during carbon or oxygen limitation.

We identified *rpoS* as required for fitness during carbon or oxygen limitation, as expected (Fig. 3A). This conserved stress-response regulator is activated upon entry into stationary phase, and known to directly or indirectly regulate hundreds of genes in *P. aeruginosa* (35, 36). The functional importance

of RpoS during carbon limitation has been demonstrated previously for *P. aeruginosa* using glucose as the limiting carbon source (27, 28). Finding that RpoS is also required under our experimental conditions is not surprising, and thus provides an important validation of our approach.

We also identified most of the loci previously shown to have a survival defect during oxygen limitation. These include the *ackA-pta* operon, encoding acetate kinase-phosphate acetyltransferase; *ldhA,* encoding D-lactate dehydrogenase; *anr*, encoding an anaerobic transcriptional regulator; and *uspK*, encoding a universal stress protein (25, 26, 29) (Fig. 3A). We did not detect a fitness defect for insertions in the integration host factor encoded by *ihfA* and members of the *arcDABC* operon required for arginine fermentation, although both loci were previously shown to have an anaerobic survival defect on pyruvate (25, 29). However, in our screen *ihfA* appeared to be essential for growth of PA14 on pyruvate, showing few reads in the aerobically-grown controls compared to the initial pooled library (see Materials and Methods and Data Sets S3 and S4). This precluded a determination of conditional fitness because there were too few reads to compare between the energy-limited and control samples. Additionally, a strain deleted in the entire *arcDABC* operon was used in previous survival experiments (29). Because Tn-seq only assesses the fitness of single gene mutants this could explain why we did not detect a fitness defect in any of the *arc* genes individually. Overall, we conclude that our experimental approach is sensitive to detect individual, conditionally-required genes during carbon or oxygen limitation.

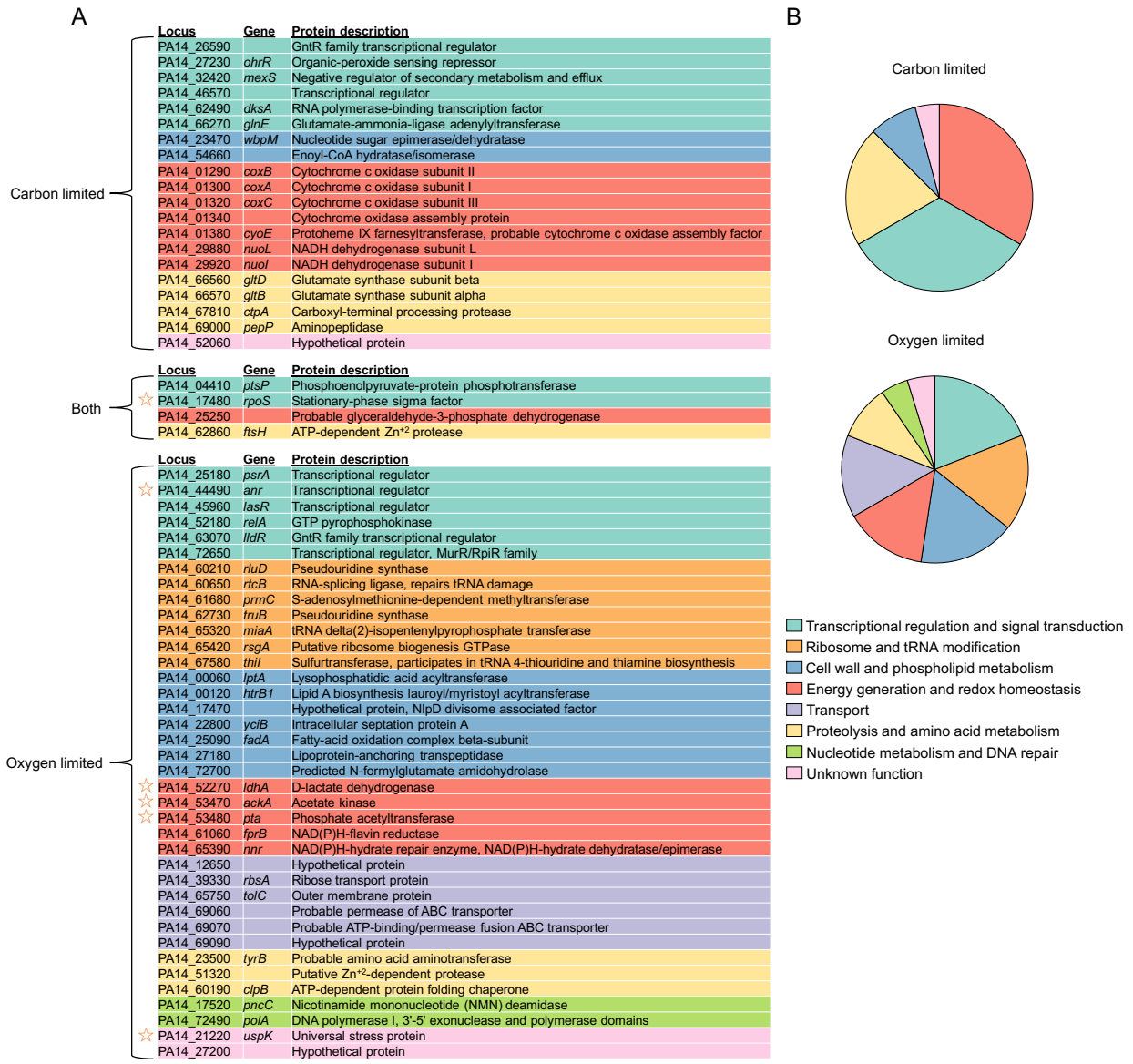


Figure 3. Functional categories of genes required during energy-limited growth arrest. (A) The locus and description of required genes. "Both" represents genes required during carbon or oxygen limitation. Stars indicate genes that were previously described as important for fitness during energy-limited growth arrest in *P. aeruginosa*. (B) Pie chart representing the genes listed in (A).

Functional classification of fitness determinants.

We grouped genes required for fitness into eight categories to get a better sense of the activities required during energy-limited growth arrest (Fig. 3A and B). Genes were assigned to the different categories based on previous functional characterization in *P. aeruginosa* or similar annotation to a characterized gene in *E. coli*. We found that many categories were common to both types of energy

limitation whereas the specific genes in each category mostly differed. For example, among genes required for "energy generation and redox homeostasis" only *PA14_25250*, encoding a putative glyceraldehyde-3-phosphate dehydrogenase, was required during carbon or oxygen limitation. The *coxBAC* gene cluster, encoding subunits of the low affinity aa_3-type cytochrome c oxidase, and *nuoL* and *nuoI*, encoding subunits of the proton-pumping NADH dehydrogenase I (NDH-1) were specifically required during carbon limitation. Other members of the *nuo* operon had milder defects during carbon limitation that did not meet our fitness criteria (Data Set S1). On the other hand, the *ackA-pta* and *ldhA* loci, the NAD(P)H oxidoreductase encoded by *PA14_61060*, and the NAD(P)H-hydrate dehydratase/epimerase encoded by *nnr* were all specifically required during oxygen limitation. Other categories with this pattern included "transcriptional regulation and signal transduction", "cell wall and phospholipid metabolism", "proteolysis and amino acid metabolism" and "unknown function". Notably, in the "transcriptional regulation and signal transduction" category, *dksA* was specifically required during carbon limitation, the global regulators *psrA*, *anr*, *lasR*, and *relA* were specifically required during oxygen limitation, and *rpoS* and *ptsP* were required during both types of energy limitation.

Unexpectedly, we found that the categories of "ribosome and tRNA modification", "transport", and "nucleotide metabolism and DNA repair" were specifically required during oxygen limitation. Among the first category were genes encoding for the pseudouridine synthases RluD and TruB, the thiouridine synthase ThiI, and the isopentenyltransferase MiaA. The second category included a homolog of *E. coli tolC*, encoding an outer-membrane efflux protein, and an operon encoding a putative ABC transporter (*PA14_69060, 69070* and *69090*). The third category comprised *pncC,* encoding a nicotinamide mononucleotide deamidase, and *polA*, encoding DNA polymerase I.

Experimental validation of Tn-seq results.

To validate our Tn-seq results, we made strains with unmarked deletions of the *coxBAC* (including the open reading frame (ORF) *PA14_01310*), *ackA-pta*, and *rpoS* loci, required during carbon limitation, oxygen limitation, or both, respectively (Fig. 4A), and co-cultured them with a fluorescently-labeled WT strain to simulate the competitive environment in our screen. As predicted, a fitness defect was observed for Δ*coxBAC* specifically during carbon limitation, Δ*ackA-pta*

specifically during oxygen limitation, and Δ*rpoS* during carbon or oxygen limitation (Fig. 4B). The fitness defect for each strain was rescued by complementing the deleted locus at the *att*Tn7 site on the chromosome. These results indicate that our experimental approach is both robust and specific in its detection of genes required for fitness during energy-limited growth arrest.

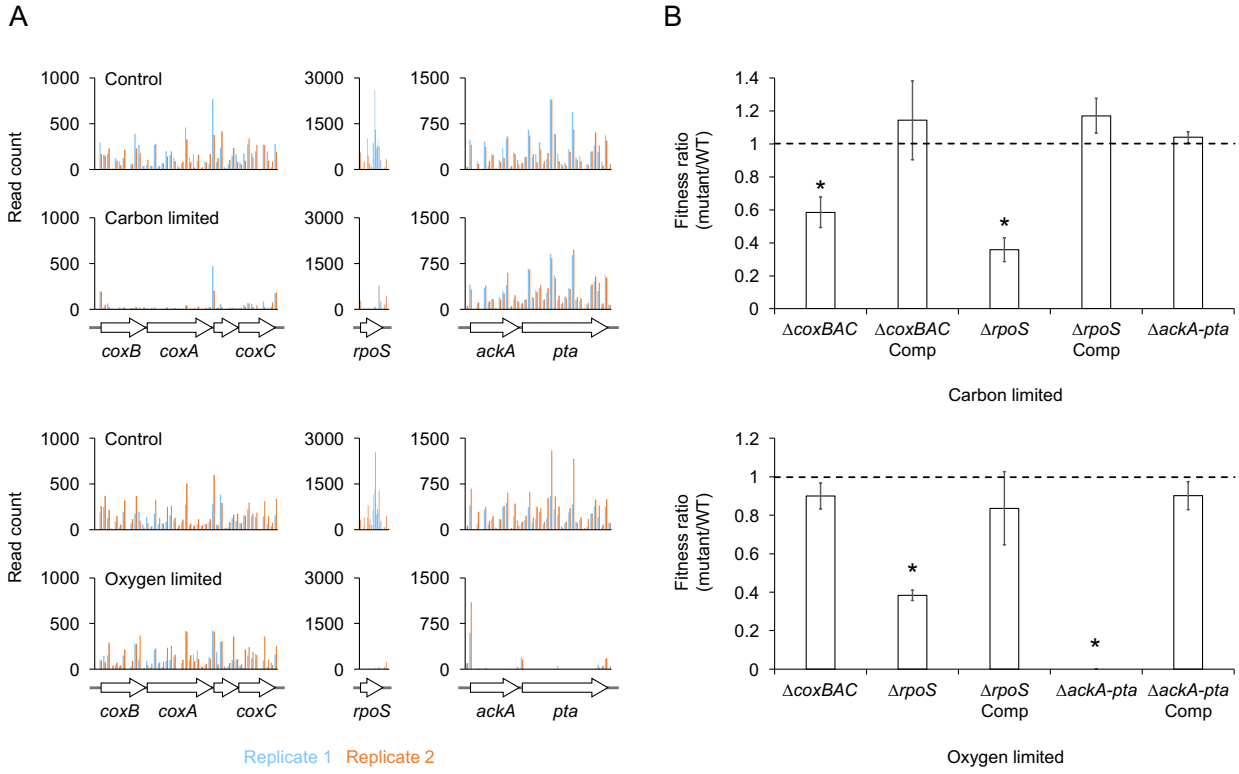


Figure 4. Validation of fitness determinants identified by Tn-seq. (A) Read counts for insertions across the *coxBAC*, *ackA-pta*, and *rpoS* loci required during carbon limitation, oxygen limitation, or both, respectively. Each locus is divided into 100 bp windows and the cumulative number of insertions in each window is plotted. (B) Fitness of the corresponding deletion mutants and the complemented strains relative to WT after 13 days of carbon limitation or 10 days of oxygen limitation. Error bars show the standard deviation of biological replicates (n = 3). The asterisk indicates a significant fitness disadvantage relative to WT (paired Student's *t* test, *P* value <0.05).

Fitness dynamics of mutants during oxygen limitation.

We chose to investigate genes required during oxygen limitation in more detail based on the relevance of this condition to *P. aeruginosa* physiology in biofilms and chronic infections (20–24). We retrieved transposon mutants (37) or made unmarked deletions of representative genes from each functional category required during oxygen limitation. We then competed these mutants against our

fluorescent WT under oxygen-limited conditions and confirmed that most (17 of 19) mutants had a fitness defect; representative strains from different functional categories are shown in Fig. 5A. We noticed that some mutants had either a mild or no defect after 10 days of competition, but were outcompeted by days 20 and 30. In contrast, nearly all insertion mutants within each gene showed a fitness defect at day 10 in our screen (Fig. 5B). This suggests that our screen identified mutants with fitness defects earlier than might be detected by direct, one-on-one competition with the WT strain, further highlighting the sensitivity of our experimental approach.

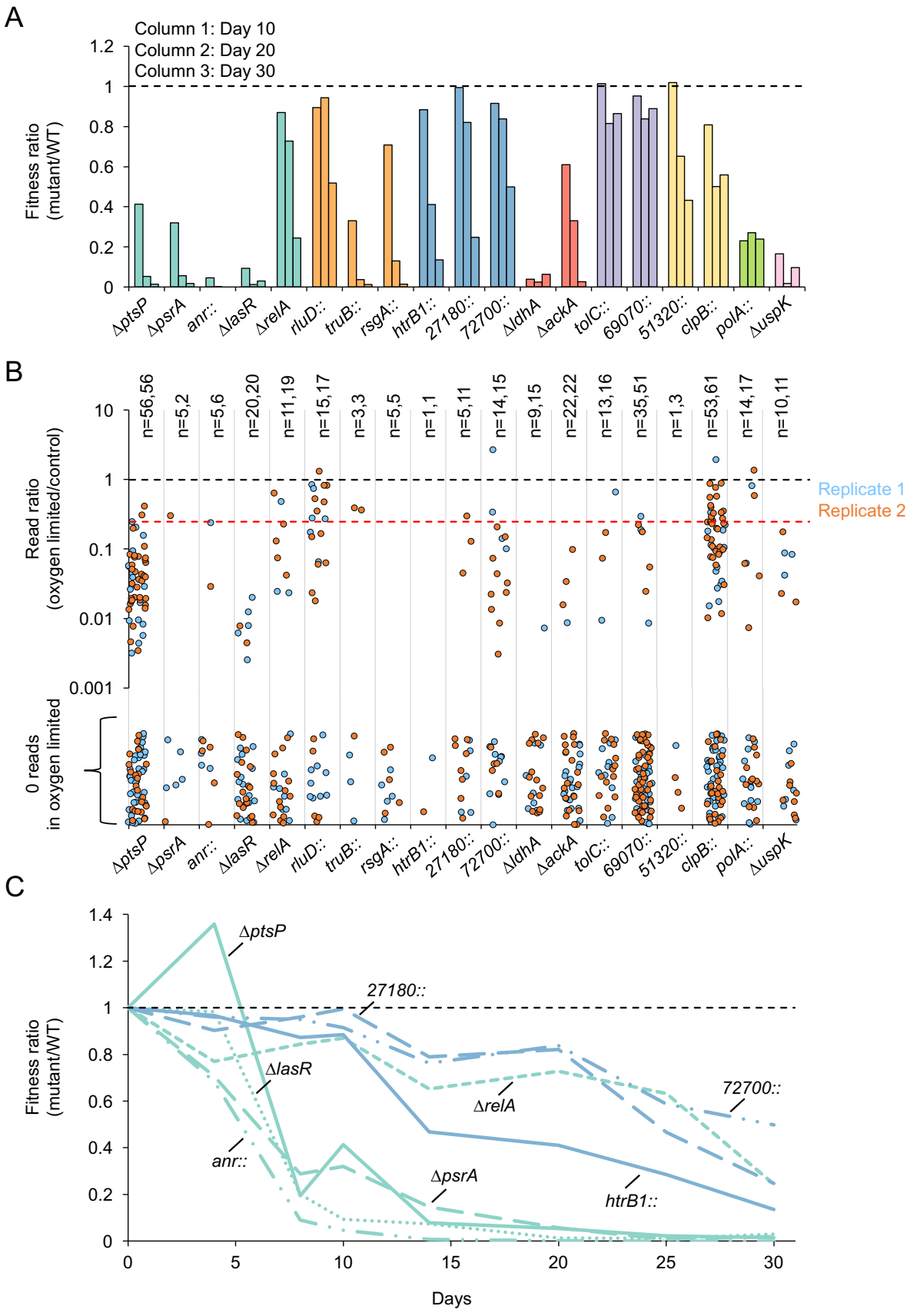

A
Column 1: Day 10
Column 2: Day 20
Column 3: Day 30
Fitness ratio (mutant/WT)
1.2
1
0.8
0.6
0.4
0.2
0
ΔptsP
ΔpsrA
anr::
ΔlasR
ΔrelA
rluD::
truB::
rsgA::
htrB1::
27180::
72700::
ΔldhA
ΔackA
tolC::
69070::
51320::
clpB::
polA::
ΔuspK
B
Read ratio (oxygen limited/control)
10
1
0.1
0.01
0.001
n=56,56
n=5,2
n=5,6
n=20,20
n=11,19
n=15,17
n=3,3
n=5,5
n=1,1
n=5,11
n=14,15
n=9,15
n=22,22
n=13,16
n=35,51
n=1,3
n=53,61
n=14,17
n=10,11
Replicate 1
Replicate 2
0 reads in oxygen limited
ΔptsP
ΔpsrA
anr::
ΔlasR
ΔrelA
rluD::
truB::
rsgA::
htrB1::
27180::
72700::
ΔldhA
ΔackA
tolC::
69070::
51320::
clpB::
polA::
ΔuspK
C
Fitness ratio (mutant/WT)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ΔptsP
27180::
ΔlasR
ΔrelA
72700::
anr::
ΔpsrA
htrB1::
0
5
10
15
20
25
30
Days

Figure 5. Competition between select mutants and WT during oxygen limitation. (A) Fitness of mutants relative to WT at days 10, 20, and 30 of oxygen limitation. Colors correspond to the functional categories described in Fig. 3. A double colon indicates a transposon mutant. (B) Read ratio of insertions in the oxygen-limited Tn-seq samples relative to the control for each mutant tested in (A). Each point represents a unique insertion within the middle 80% of the gene. Ratios are shown for insertions with a minimum of 20 reads in the control. n = the number of unique mutants in replicates 1 and 2, respectively. The dashed red line indicates a read ratio of 0.25. (C) Time course of mutant fitness relative to WT for genes in the "transcriptional regulation and signal transduction" and the "cell wall and phospholipid metabolism" categories.

Some mutants we tested did not have an observable fitness defect in competition with WT. Of note, the two "transport" mutants showed little to no defect even at day 30 of competition (Fig. 5A, purple columns). We found this particularly surprising for *PA14_69070*, which is part of a three-member operon encoding subunits of a putative ABC transporter (*PA14_69060*, *69070*, and *69090*). According to our selection criteria, all three genes in this operon were required during oxygen limitation (Fig. 3A) and all independent insertions within the middle 80% of the *PA14_69070* ORF had a fitness defect (Fig. 5B). To determine if the lack of a fitness defect for *PA14_69070* in our competition experiment was due to the specific transposon mutant used, we made an unmarked deletion strain of the entire transporter operon and competed this strain against our fluorescent WT. To our surprise, the unmarked deletion strain also did not have a fitness defect (data not shown). This discrepancy could be a false positive detected in our screen or a consequence of some environmental difference between the screen and competition experiment that influences a gene's importance. One difference is that each mutant represents a tiny fraction of the population in the screen, whereas it is closer to half the population in the competition experiment. This difference in the mutant proportion could influence community dynamics in an unforeseen way that cannot easily be captured in one-to-one competitions.

We noticed that the magnitude of the fitness defect at each time point in our competition experiment varied between the different categories (Fig. 5A). Although all the genes we tested had a greater than 4-fold fitness defect in our screen, this observation suggested that some functional categories might be more important for fitness than others, or they might be important at different stages during oxygen limitation. To test this hypothesis, we performed the competition experiment with mutants in the "transcriptional regulation and signal transduction" and "cell wall and phospholipid metabolism" categories and took more frequent time points to monitor fitness with greater temporal resolution (Fig. 5C). As suggested by Fig. 5A, most regulatory genes were required earlier in growth

arrest, being reduced to a small fraction of the population by day 10, and in the case of the *anr* mutant becoming undetectable by day 15. In contrast, genes involved in cell wall metabolism maintained nearly 100% fitness at day 10 and began to gradually decline only later in growth arrest. The exception to this general trend is Δ*relA*, which showed a milder defect compared to the other regulators. We conclude that genes belonging to multiple functional categories are required during energy-limited growth arrest and likely contribute to fitness at different stages and to varying extents.

Discussion

Tn-seq is a powerful method for identifying fitness defects of individual mutants in a genetically heterogeneous population. Importantly, Tn-seq can detect these fitness defects independent of the pattern of gene expression, which is useful under stress conditions where significant post-transcriptional regulation can occur (30, 38). Previous studies have used Tn-seq to identify fitness determinants in bacteria under different growth-arresting conditions. These studies have expanded our knowledge of genes required for survival of ionizing radiation (39, 40), reactive oxygen and nitrogen species (41), antibiotics (42), and the host immune system (43, 44). To our knowledge, however, no studies have systematically identified fitness determinants during growth arrest caused by long-term energy limitation.

In this study, we used Tn-seq to identify the genes required for fitness of *P. aeruginosa* when limited for organic carbon as an electron donor or oxygen as an electron acceptor. The companion study by Pechter et al. in this issue identifies fitness determinants in *Rhodopseudomonas palustris* under carbon limited but energy-replete conditions (45). We found that multiple general functions were important during carbon or oxygen limitation *in P. aeruginosa*, despite substantial differences in the specific genes required. Our results highlight the fundamental challenges cells face in the context of energy-limited growth arrest: maintaining the PMF and redox homeostasis, conserving ATP, efficiently repairing and synthesizing macromolecules such as proteins and DNA, and regulating these activities in a fine-tuned and concerted way. Only when these challenges are met can cells properly enter into, endure, and reemerge from growth arrest, as established for yeast over a decade ago (46).

Two methods to maintain the PMF and ATP synthesis during energy-limited growth arrest.

The importance of continued, efficient respiration during carbon limitation is underscored by the requirement for multiple genes in the electron transport chain (ETC). The subunits of NADH dehydrogenase I (NDH-1) encoded by *nuoL* and *nuoI*, and the low affinity aa_3-type cytochrome c oxidase encoded by the *coxBAC* gene cluster (47), were required specifically during carbon limitation. In contrast, mutations in *nuo* and the cytochrome biogenesis operon *ccm* resulted in a significant fitness advantage during oxygen limitation, suggesting that ongoing activity of the ETC is deleterious in the absence of a terminal electron acceptor (Fig. S2). Unlike NDH-2, the multi-subunit NDH-1 complex catalyzes proton-coupled electron flow from NADH to ubiquinone, thereby contributing to the PMF. NDH-1 mutants of *E. coli* have a fitness defect in stationary phase (48), and the need for this complex in our study indicates that its proton-pumping activity may be crucial for survival during aerobic carbon limitation. The *coxBAC* locus is normally repressed under nutrient-replete growth conditions and induced in an RpoS-dependent manner upon starvation for carbon, nitrogen or iron (49). The aa_3 oxidase is the most efficient proton pump of the five terminal oxidases encoded by *P. aeruginosa* (50), and was recently shown to be required for fitness during carbon limitation (51). These results suggest that enhanced respiratory efficiency is essential to maintain the PMF and ongoing ATP synthesis during carbon limitation, a condition in which there is a shortage of electron donors for respiration.

In contrast to the importance of respiration during carbon limitation, pyruvate fermentation becomes an essential means of energy generation in the absence of a terminal electron acceptor. The *ackA-pta* operon, encoding acetate kinase and phosphate acetyltransferase, and *ldhA,* encoding lactate dehydrogenase, showed specific fitness defects during oxygen limitation. AckA and Pta are required for ATP generation via anaerobic pyruvate oxidation, while LdhA is required for regenerating NAD^+ consumed in the reaction (25, 26). The ATP produced by this pathway is then used by the F_1F_0-ATPase to pump protons across the membrane (26). The importance of NAD^+ regeneration is underscored by the requirement for the putative NAD(P)H oxidoreductase encoded by *PA14_61060* and the NAD(P)H-hydrate dehydratase/epimerase encoded by *nnr*. The latter acts to repair hydrated, nonfunctional NAD(P)H using ADP as a phosphoryl donor (52). Together, these results indicate that redox homeostasis and substrate-level phosphorylation are critical for PMF maintenance and

ongoing ATP synthesis during oxygen limitation, in the absence of alternative electron acceptors for respiration.

Functional categories required specifically during oxygen limitation.

Genes involved in ribosome and tRNA modification were required during oxygen limitation but not carbon limitation (Fig. 3A and B). This could be due to the lower energy levels in oxygen-limited cells relative to carbon-limited cells (Fig. 1B). Protein synthesis is one of the most energy-intensive processes that occurs during bacterial growth, and is substantially downregulated upon growth arrest (16). Ribosomal and tRNA modifications could promote the fidelity and efficiency of translation during oxygen limitation by a variety of mechanisms. In *E. coli* for example, the methyltransferase PrmC is required for efficient translation termination (53, 54) and the pseudouridine synthase TruB functions as an essential tRNA chaperone (55, 56). These different activities could become important at the more reduced energy levels encountered during oxygen limitation, where inefficiencies in translation might have a proportionally greater impact on fitness. Another possibility is that these ribosome and tRNA modifications promote translation of specific proteins required during oxygen limitation. One example of this type of regulation is the requirement of the tRNA isopentenyltransferase MiaA for efficient RpoS expression in *E. coli* (57). Both genes are required during oxygen limitation in our screen, suggesting that MiaA may function as an activator of RpoS in *P. aeruginosa* as well. Whatever the exact roles of these ribosome and tRNA modifying enzymes, they highlight the importance of ongoing protein synthesis during energy-limited growth arrest.

Surprisingly, genes involved in DNA repair were also specifically required during oxygen limitation. Among these genes, DNA polymerase I, encoded by *polA*, is upregulated during oxygen limitation with an excess of arginine (30). This enzyme may repair DNA damage caused by aberrant anaerobic flux through the ETC or oxidative damage upon re-aeration (16). Although no genes encoding known DNA repair enzymes were required specifically during carbon limitation, we identified the putative glyceraldehyde-3-phosphate dehydrogenase (GAPDH), encoded by *PA14_25250*, as required during both types of energy limitation. GAPDH is canonically thought to be involved in central metabolism as an enzyme of glycolysis, but recent work suggests it can also moonlight as a DNA repair enzyme in *E. coli* (58). Maintaining genomic integrity is critical for survival of growth-

arrested cells (16), and polymerases that respond to DNA damage are required for fitness during long-term stationary phase in *E. coli* (59). Unfortunately, Tn-seq—like any genetic screen with mutants in single loci—is not well suited to detect genes that are important but not required because other genes can play similar roles (60). This might explain why apart from GAPDH we did not detect more DNA repair enzymes as fitness determinants during carbon limitation. Alternatively, multiple non-redundant repair pathways may be operating in parallel during carbon limitation, with a mutation in any one pathway resulting in a mild or stochastic fitness defect not identifiable by our stringent selection criteria (60).

Essentiality of proteolysis.

Multiple proteases were required during carbon or oxygen limitation, including FtsH, which was required during both types of energy limitation. Protein catabolism might be important during energy-limited growth arrest in order to remove aberrant/damaged proteins, relieve the burden of energy-intensive enzymes such as ribosomes, decrease cell mass and thus the cellular maintenance requirement, activate or deactivate regulatory proteins, or provide amino acid substrates as a biosynthetic/energy source (16, 61–64). In regards to the last function, continued protein synthesis is essential during carbon or oxygen limitation (30, 62), and the recycling of amino acids by regulated proteolysis of non-essential proteins can be an energy-efficient way to allow for ongoing synthesis of the proteins required for survival. Furthermore, recycled amino acids can be used to generate energy and may serve as one source of electron donors for continued respiration during carbon limitation (16). Consistent with this notion, *E. coli* mutants more efficient in amino acid uptake display a growth advantage in stationary phase (65).

A central role for RpoS.

The stationary phase sigma-factor RpoS was required during carbon or oxygen limitation, revealing a potentially nuanced interplay between RpoS and other regulators identified in our screen (DksA, RelA, PsrA and LasR). The RNA polymerase-binding transcription factor DksA, which we found to be specifically required during carbon limitation, acts in concert with the stringent-response modulators guanosine tetra- and pentaphosphate [(p)ppGpp] to decrease expression of rRNA in both *E. coli* and *P. aeruginosa* (66, 67), and increase expression of RpoS in *E. coli* (68, 69). This suggests

that DksA might upregulate RpoS in *P. aeruginosa* as well. Regulators required during oxygen limitation with links to RpoS include the stringent-response regulator RelA, which synthesizes (p)ppGpp in response to a variety of stress conditions and is required for RpoS expression and RpoS-dependent gene regulation in *E. coli* (70, 71), the transcription factor PsrA, which directly upregulates RpoS expression in *P. aeruginosa* (72, 73), and the quorum-sensing regulator LasR. The regulon of LasR is highly interlinked with RpoS, with LasR indirectly increasing RpoS expression and RpoS regulating many quorum-controlled genes (35, 74, 75). As mentioned previously, the required tRNA modification enzyme MiaA is also important for efficient RpoS expression (57), although we did not classify it as a regulator in this study. Together, these results indicate that RpoS functions as a central regulator that interacts with distinct, condition-specific co-regulators to tune downstream regulatory output.

In addition to its interplay with other regulators, RpoS might directly or indirectly regulate many genes in different categories important during energy-limited growth arrest. As mentioned above, the *coxBAC* operon is induced by RpoS upon carbon limitation (47). In addition, we found that many genes required during oxygen limitation might also be regulated by RpoS in stationary phase, based on the published data set of a recent study in PA14 (36). These genes include *thiI*, *PA14_00120*, *PA14_27180*, *PA14_72700*, *tolC*, *PA14_69060, 69070* and *69090*, *PA14_23500*, *clpB*, and *uspK*. It thus seems plausible that specific growth-arresting triggers not only influence which regulatory routes lead to RpoS activation, but also which downstream genes are ultimately controlled by RpoS.

Open questions.

An important question raised by our work is to what degree the nutritional environment influences the functions required during energy-limited growth arrest. In our experiments, we limited cells specifically for pyruvate or oxygen and showed that many different functions were required in response to each limitation. However, to what extent the genes required for fitness following limitation for pyruvate or oxygen overlap with required genes following limitation for other carbon or electron-accepting sources remains an open question. For example, while RpoS is required for fitness of *P. aeruginosa* during carbon limitation for glucose or pyruvate, it is dispensable when cells are starved after growth in succinate or LB (28). Clearly, the environmental context plays an

important role in the cellular response to energy limitation, and genes required for fitness will intimately depend on the experimental conditions. It therefore is not surprising that different genes exhibited different fitness dynamics in our experiments: throughout the weeks-long period of our studies, certainly the chemical milieu of our cultures was changing; such changes are known to underpin the selection of different GASP mutants in *E. coli* throughout long-term survival (76), though we do not have evidence that GASP mutants comprised a significant fraction of the population with a fitness advantage.

A complementary question that arises from our work is the generalizability of our findings to other microbes. Studies on the growth-arrest physiology of the respiratory pathogen *Mycobacterium tuberculosis* have revealed both similar and contrasting results to what we observed. Like *P. aeruginosa*, *M. tuberculosis* is capable of long-term survival during carbon or oxygen limitation (77, 78), but dies rapidly when limited for both substrates (79). Similarly, ATP levels are reduced 5- to 6-fold during these two energy-limited states compared to carbon-replete, aerobic conditions (79, 80). However, in contrast to *P. aeruginosa*, *M. tuberculosis* requires continued respiration to drive ATP synthesis during carbon or oxygen limitation, and this respiration is dependent on NDH-2 instead of NDH-1 (79, 80). Additionally, the glyoxylate shunt enzyme isocitrate lyase (ICL) is required for survival of *M. tuberculosis* during these two types of energy limitation (79, 81) but is not required in *P. aeruginosa* under the conditions of our screens. ICL is upregulated during growth arrest in *M. tuberculosis*, where it can be used to bypass steps of the TCA cycle that produce reducing equivalents and can help maintain the PMF via electrogenic succinate secretion (81–83). Together, these data indicate that different microbes can exploit diverse solutions to deal with the common challenges that arise during energy limitation, such as maintaining the NAD^+ pool and the PMF, as well as generating ATP.

Conclusion.

Our findings contribute to a growing body of work revealing the genetic determinants of fitness during energy-limited growth arrest. Future studies will probe the molecular and biochemical bases for these fitness determinants and help us interpret why they are necessary at different stages of survival. The environmental and clinical relevance of these genes can be assessed using *in vitro*

models of biofilm formation and *in vivo* models of chronic infection, complemented by *in situ* gene-expression profiling in patient samples (19). For example, *rpoS* and *fadA*, both required for fitness during oxygen limitation in our screen, are upregulated in the hypoxic sputum of patients with cystic fibrosis (84, 85). Overall, our study validates Tn-seq as a powerful approach for interrogating the genome-wide fitness of *P. aeruginosa* during carbon or oxygen limitation, opening up new targets for studying how this important opportunistic pathogen survives during energy-limited growth arrest.

Materials and Methods

Bacterial strains, plasmids, primers, and growth conditions.

The strains, plasmids, and primers used in this study are listed in Table S1. *E. coli* and *P. aeruginosa* were grown in lysogeny broth (LB) (Difco) or on LB agar plates at 37°C for all cloning and strain construction purposes unless otherwise noted. All growth-arrest experiments were performed at 33°C. This temperature was chosen because PA14 WT survived significantly better anaerobically at 33°C than at 37°C. Isolated transposon mutants retrieved from the PA14 mutant library (37) were verified by colony PCR using primers flanking the annotated insertion site. For the carbon-limited experiments, cultures were shaken at max speed on a standard analog shaker (VWR).

Generation of the transposon library.

The randomly inserting Tn5-based transposon T8 (IS*lacZhah*-tc) was conjugated into PA14 as previously described (34). Briefly, PA14 WT and *E. coli* SM10λ*pir* carrying the transposon-bearing plasmid pIT2 were resuspended in LB from overnight streak plates on LB agar or LB agar plus carbenicillin (100 μg/ml), respectively. The resuspended cultures were mixed in a 2:3 PA14:SM10λ*pir* ratio based on optical density at 500 nm (OD_{500}), spot-plated onto LB agar plates and incubated 2 h at 37°C. Following incubation, spots were pooled from the plates and resuspended thoroughly in LB. The pooled resuspension was diluted to an OD_{500} of ~1.75 and aliquots were plated on LB agar plus tetracycline (60 μg/ml) and chloramphenicol (10 μg/ml) to select for PA14 transposon insertion mutants. The plates were incubated 24 h at 37°C. Following incubation, colonies from all plates were pooled and resuspended in phosphate-buffered saline (PBS)+25% glycerol. The density of the pooled library was adjusted to an OD_{500} of 8 and stored as 1 ml aliquots

at -80°C. The library consisted of ~150,000 unique mutants as determined by the number of pooled colonies and the number of unique insertions identified by sequencing.

Tn-seq sample preparation.

Four aliquots of the transposon library were thawed on ice for 15 min and diluted to a starting OD_{500} of 0.05 in 50 ml of minimal medium (26) supplemented with 40 mM sodium pyruvate (Sigma) as the sole carbon and energy source. The cultures were grown aerobically at 37°C for ~2 generations to an OD_{500} of 0.2. Cells were pelleted and resuspended in 50 ml of minimal medium supplemented with either 40 mM pyruvate for the oxygen-limited samples or 1 mM pyruvate for the carbon-limited samples (two replicates for each condition). Immediately upon resuspension an aliquot of each sample was diluted to a starting OD_{500} of ~0.00625 in 25 ml of minimal medium supplemented with 40 mM pyruvate. These diluted samples served as the control and were grown aerobically at 37°C with shaking for ~5-6 generations to an OD_{500} of 0.2. Following aerobic outgrowth, 10 ml of the control samples were pelleted and stored at -80°C. From the remainder of the resuspended cultures, 25 ml of the oxygen-limited samples were placed in a Balch tube and transferred into a glove chamber (Coy) containing an atmosphere of 15% CO_2, 80% N_2, and 5% H_2. The tubes were stoppered and incubated anaerobically at 33°C. For the carbon-limited samples, 5 ml of the resuspended cultures were placed in a 25 ml test tube and incubated aerobically at 33°C and 50% relative humidity with shaking. On day 10 of incubation an aliquot of each culture, both oxygen-limited and carbon-limited, was diluted to a starting OD_{500} of ~0.00625 in 25 ml of minimal medium supplemented with 40 mM pyruvate. These diluted samples were grown aerobically at 37°C with shaking for ~5 to 6 generations to an OD_{500} of 0.2. Following aerobic outgrowth, 10 ml of the energy-limited samples were pelleted and stored at -80°C. A sample of one thawed transposon library aliquot was also collected to determine the makeup of the initial pooled library.

Sequencing and data analysis.

Genomic DNA was extracted from the pelleted samples using the DNeasy Blood & Tissue Kit (Qiagen) and prepared for Illumina sequencing according to established protocols (86). Briefly, genomic DNA was sheared by sonication to produce 200-500 bp fragments and end repaired using the NEBNext End Repair Module (New England Biolabs). A poly(C) tail was added to the end

repaired DNA using a terminal deoxynucleotidyl transferase (Promega). C-tailed DNA was amplified in two rounds of PCR to enrich for transposon-genome junctions and to add adapters for Illumina sequencing. The amplified DNA was sequenced using 100 bp single-end reads on the Illumina HiSeq 2500 platform at the Millard and Muriel Jacobs Genetics and Genomics Laboratory at Caltech. Sequences were mapped to the UCBPP-PA14 genome sequence using Bowtie (87) and analyzed using the ARTIST Tn-seq analysis pipeline in MATLAB (88). Briefly, total reads mapping to each gene in the carbon- or oxygen-limited samples were compared to their corresponding reads in the control samples using a Mann-Whitney U statistical test (88). After each replicate was analyzed independently, the geometric mean of the read ratio was calculated for each gene in both replicates and the *P* values combined using Fisher's combined probability test (Data Sets S1 and S2). Mutants that dropped out during growth on LB or pyruvate minimal medium overlapped with those previously identified as essential genes in *P. aeruginosa* (Data Sets S3 and S4) (89).

Strain construction.

Unmarked deletions in PA14 were made as previously described (30) with minor modifications. Briefly, ~1 kb fragments immediately upstream and downstream of the target locus were amplified by PCR and joined with the suicide vector pMQ30 (90) (cut with SacI and HindIII) using Gibson assembly (91). The assembled construct was transformed into *E. coli* DH10B and transformants were plated on LB plus gentamicin (20 μg/ml). For all plasmids, a correctly assembled construct was identified by colony PCR using primers flanking the multiple cloning site and then verified by sequencing (Retrogen). Triparental mating was performed to conjugate the construct into PA14 WT. Merodiploids containing the chromosomally integrated construct were then selected on VBMM plus gentamicin (80 μg/ml) (92). Merodiploids were grown to exponential phase in LB and counter-selected on LB agar plates lacking NaCl and containing 10% sucrose. Deletions were identified by colony PCR using primer sets both flanking and internal to the target locus.

To complement the deletion strains, the genomic region of the deleted locus was amplified by PCR and joined with the shuttle vector pUC18T-mini-Tn7T (92) (cut with SacI and HindIII) using Gibson assembly. The assembled construct was transformed into *E. coli* DH10B and transformants were plated on LB plus gentamicin (20 μg/ml). Tetraparental mating was performed to conjugate the

construct into the corresponding deletion strain. Conjugants were selected on VBMM plus gentamicin (80 μg/ml) and chromosomal integration at the *att*Tn7 site was detected by colony PCR as previously described (92).

To make the WT strain constitutively expressing mApple, a 1 kb fragment upstream of the ribosomal-protein encoding gene *rpsG* and a 155 bp fragment immediately downstream were amplified by PCR and joined as flanking sequences to the amplified mApple open reading frame (ORF) (Addgene) in pUC18T-mini-Tn*7*T (cut with SacI and HindIII). The assembled construct was integrated into the *att*Tn7 site in the same manner as described for the complementation strains.

Viability measurements.

CFU were determined over time for WT during carbon and/or oxygen limitation by taking a 20 μl aliquot of the growth-arrested cultures and performing serial dilutions in aerobic pyruvate media. Appropriate dilutions were plated as 10 μl drips on LB agar plates and incubated aerobically at 37°C. Colonies were counted after overnight incubation and the CFU/ml was calculated.

ATP measurements.

Measurement of ATP was performed as previously described (26). Briefly, a 20 μl aliquot of the growth-arrested cultures was added to 180 μl of dimethyl sulfoxide (DMSO). The samples were then diluted with 800 μl of 100 mM HEPES (pH 7.5) and stored at -80°C until analysis. ATP was measured by mixing thawed samples 1:1 with BacTiter-Glo reagent (Promega) in a 96-well opaque white microtiter plate. Luminescence was measured at 30°C using a plate reader (BioTek). A standard curve was generated with each plate measurement using known concentrations of ATP.

Pyruvate measurements.

Samples were collected by centrifuging 300 μl of culture and pipetting 250 μl of the supernatant into a fresh tube. The samples were stored at -80°C until analysis. Thawed samples were mixed 1:1 with 100 mM H_2SO_4 and transferred to an autosampler vial. Pyruvate was measured by HPLC using a Waters Alliance e2695 separations module and 2998 photodiode array detector. Separations were performed using an Aminex HPX-87H column (Bio-Rad) with an isocratic elution of 5 mM H_2SO_4

at 0.5 ml/min and 30°C. The injection volume was 20 µl and the total run time was 40 min. Compounds were detected at 206 nm and 322 nm. Pyruvate was identified by comparing retention times to a pure standard, as well as by a distinctive absorbance peak around 320 nm.

Competition assays.

WT cells constitutively expressing an mApple fluorescent marker and individual markerless mutants were grown aerobically at 37°C in 3 ml of 40 mM pyruvate medium to an OD_{500} between 0.1 and 0.4. Cells were pelleted and resuspended in minimal medium supplemented with either 40 mM sodium pyruvate for the oxygen-limited samples or 1 mM sodium pyruvate for the carbon-limited samples to achieve an OD_{500} of 0.2. After resuspension, the fluorescent WT was mixed with each of the markerless mutants in a 1:3 ratio of WT to mutant. An aliquot of each mixture was diluted to a starting OD_{500} of ~0.00625 in 2 ml of 40 mM pyruvate medium. These dilutions served as time zero for competition and were grown aerobically at 37°C with shaking for ~5-6 generations to an OD_{500} of 0.2. From the remainder of the mixtures, 1 ml of the oxygen-limited samples was placed in an Eppendorf tube and transferred into a glove chamber (Coy) containing an atmosphere of 95% N_2 and 5% H_2. The tubes were incubated anaerobically at 33°C. For the carbon-limited samples, 5 ml of the mixtures were placed in a 25 ml test tube and incubated aerobically at 33°C and 50% relative humidity with shaking. Subsequent time points were taken by diluting an aliquot of the growth-arrested cultures to a starting OD_{500} of ~0.00625 in 2 ml of 40 mM pyruvate medium. These dilutions were grown aerobically at 37°C with shaking for ~5-6 generations to an OD_{500} of 0.2. Following aerobic outgrowth, a small aliquot of the cultures was taken for epifluorescence microscopy using a Zeiss Axio Imager microscope. The ratio of cells with and without fluorescence in each mixed culture was determined for each time point and divided by the ratio at time zero. A markerless WT was also competed against the fluorescent WT strain as a control to normalize the ratios for the WT:mutant mixtures. The fluorescent WT strain had a mild fitness defect in competition with the markerless WT during oxygen limitation.

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Supplemental files accompanying Chapter 3

Table S1. Strains, plasmids, and primers used in this study.

Data Set S1. Read ratios for each gene and intergenic region in the carbon-limited Tn-seq experiment.

Data Set S2. Read ratios for each gene and intergenic region in the oxygen-limited Tn-seq experiment.

Data Set S3. Raw read counts for each gene and intergenic region in the carbon-limited Tn-seq experiment.

Data Set S4. Raw read counts for each gene and intergenic region in the oxygen-limited Tn-seq experiment.

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Chapter 4

HEAT SHOCK PROTEASES DELAY AGING DURING GROWTH ARREST

This chapter is adapted from:

Basta D.W., Angeles-Albores D., Newman D.K. (2019) Heat shock proteases delay aging of *Pseudomonas aeruginosa* during energy-limited growth arrest. *In preparation*.

Abstract

Bacteria must contend with several challenges after nutrients are exhausted and cells become arrested for growth. A primary challenge is continuing to maintain cellular integrity in spite of a reduced capacity for renewal or repair. Despite the significance of this challenge for evolutionary success, our understanding of the underlying genetics and molecular mechanisms that promote survival during growth arrest remains incomplete. Recently, we identified a general requirement for the ATP-dependent protease FtsH in growth-arrest survival of *P. aeruginosa*, an opportunistic pathogen that thrives in a variety of nutrient-limited niches. Here, we show that deletion of *ftsH* causes rapid loss of viability of *P. aeruginosa* during growth arrest, independent of a proposed role in regulating lipopolysaccharide synthesis. Using a genome-wide screen, we identify both aggravating and alleviating interactions between *ftsH* and functionally diverse genes during growth or survival, including an alleviating interaction with the stress response sigma factor encoded by *rpoS* during survival, and aggravating interactions with the other heat shock protease-encoding genes during growth and survival. Systematic deletion of the heat shock protease-encoding genes reveals functional hierarchy during growth arrest survival, with FtsH and ClpXP having a primary, non-redundant role, and HslVU and Lon playing a backup role. This hierarchy is partially conserved during growth at high temperature, suggesting that heat and growth arrest effectively impose a similar type of stress at the cellular level. In support of this inference, protein aggregation occurs more rapidly in protease mutants during growth arrest and correlates with the onset of cell death. Our findings suggest that protein misfolding is a major driver of bacterial aging during growth arrest,

and that coordinated activity of the heat shock response is required to ensure ongoing protein quality control in the absence of growth.

Significance statement

Cellular damage and senescence is a nearly universal feature of aging in eukaryotic cells and is believed to result from the accumulation of misfolded and aggregated proteins. Bacteria must also contend with age-related degeneration when nutrients become limiting for growth—a common occurrence over the cellular life-span of diverse species. Here, we uncover a major, functionally-redundant role for protein degradation in the aging process of a clinically and environmentally important bacterial pathogen. We find that loss of multiple proteases accelerates aging and death of non-growing *P. aeruginosa*, with a concomitant increase in protein aggregation. Our findings have broad implications for the study and treatment of infectious disease and highlight potentially conserved mechanisms for combatting aging from bacteria to humans.

Introduction

Most of our knowledge of bacterial physiology is derived from studying exponentially-growing cells in nutrient-replete environments. While this has provided us with a rich understanding of the complex and diverse processes at play during cellular growth and division, in many ways its scope is limited to what is likely just a fleeting event in the life of a bacterium. The reality is that most bacteria spend a majority of their lives not growing and dividing, as they quickly exhaust the nutrients in their environment and enter into a state of growth arrest (1–3). Comparatively few studies have investigated the physiological processes required for survival in this state, even though they are as much important for evolutionary success as the ability to grow when nutrients once again become available (4). Success is not simply limited to "waiting out" extended periods of starvation, however, as growth arrest itself can be an important driver in the emergence of antibiotic tolerance and resistance (5, 6), as well as in the establishment and persistence of biofilm communities (7–10).

Our limited understanding of bacterial growth arrest is compounded by the fact that most physiological studies of this state have been carried out in just a few model organisms, primarily *E. coli*, despite the fact that growth arrest is commonplace (3, 11, 12). Nevertheless, these studies have

provided valuable insight into the diverse mechanisms bacteria have evolved to survive growth arrest. For example, the challenge of continuing to generate ATP with limited nutrients is overcome in many ways, with different species utilizing varied alternative substrates as a source of electron donors or acceptors, and/or re-routing their metabolic pathways to improve the efficiency of ATP generation (3). This energy is needed to protect the integrity of cellular components, such as nucleic acids and proteins, that cannot easily be replaced by nutrient-limited cells and are essential for survival (2, 3). How the cell efficiently couples maintenance needs in the face of slim energy resources is poorly understood.

To begin to understand the mechanisms underpinning the growth-arrested state, we have been using the opportunistic pathogen *Pseudomonas aeruginosa* as a model organism (13–16). *P. aeruginosa* is well-adapted for survival in a variety of nutrient-poor or otherwise hostile environments, ranging from open ocean and freshwater sources, to the interior of surface-attached biofilms, to chronic wounds and the lungs of patients with cystic fibrosis (17, 18). Despite superficial differences, these environments often share a common physiological constraint: they can be limited for electron donors or acceptors, compelling organisms that survive within them to hone sophisticated strategies to cope with periods of energy-limited growth arrest (19–25).

Recently, we employed an unbiased genome-wide screen to identify genetic determinants in *P. aeruginosa* that confer a fitness advantage during growth arrest (15). From this screen, we identified the ATP-dependent membrane protease FtsH as one of only a few genes that were generally required for fitness of *P. aeruginosa* during energy-limited growth arrest, regardless of the limitation that prompted entry into this state. FtsH has been well-studied in *E. coli* during growth, and is known to perform a variety of functions, including quality control of membrane proteins (26), regulation of the heat shock response (27), and fine-tuning of lipopolysaccharide (LPS) levels by regulated degradation of the LPS biosynthetic enzymes LpxC and KdtA (28–34). This last activity is essential in *E. coli*, as loss of *ftsH* results in unbalanced lipid synthesis and lethal overproduction of LPS (28).

Although many of the growth-related roles of FtsH in *E. coli* are well characterized, little attention has been paid to its role during growth arrest in any organism (35–37). However, a role for FtsH in growth arrest survival recently was posited by two studies describing a novel cell death pathway in

E. coli, in which dysregulation of an outer membrane (OM) signaling system putatively inhibits LpxC degradation by FtsH, resulting in overproduction of LPS and cell death upon nutrient depletion and growth arrest (38, 39). Based on these results, one might posit that FtsH plays a regulatory role during growth arrest by degrading LpxC, yet whether such a role for FtsH could extend to other species like *P. aeruginosa*, in which LpxC levels are not regulated by FtsH (37, 40), is unclear. On the other hand, several studies have pointed to a role for the heat shock response during growth arrest in *E. coli*, suggesting that misfolded or damaged proteins may become a critical problem for non-growing cells (41–46). As a component of the heat shock response in many organisms, we reasoned that FtsH might degrade these misfolded or damaged proteins during growth arrest in *P. aeruginosa*. Indeed, protein aggregation is a hallmark of aging across a wide range of cellular systems—from human to bacteria (47–53), although whether protein aggregation represents a strictly pathological process has recently come into question (54–60). In this study, we sought to distinguish between these possibilities and gain insight into the role of FtsH during growth arrest in *P. aeruginosa* using a genetic and cell biological approach.

Results

FtsH maintains cell integrity during growth arrest.

Throughout this study we use the term "growth arrest" when referring to two states: when cells enter stationary phase after growth in rich medium (lysogeny broth or LB), and when cells growing exponentially in LB are shifted to a buffered minimal medium devoid of organic carbon (carbon starvation medium or CSM). We refer to the former growth-arrested state as "stationary phase" and the latter as "carbon starvation".

To investigate the role of FtsH in growth arrest, an isogenic markerless deletion of the *ftsH* open reading frame (ORF) was constructed in *P. aeruginosa* strain UCBPP-PA14. Although *ftsH* is essential in *E. coli* due to its role in regulating flux through the fatty acid and LPS biosynthetic pathways (28), it is dispensable in *P. aeruginosa* strain PAO1 when grown in LB without added salt (61). We readily deleted *ftsH* in PA14, and in contrast to PAO1, the mutant strain was not more sensitive to the salt concentration in LB (data not shown).

Deletion of *ftsH* did not significantly affect cell size or shape during exponential growth in LB, with a median cell length of 4.8 µm and 4.9 µm for wild-type PA14 (WT) and Δ*ftsH*, respectively (Fig. S1, 3 h). Median cell length decreased gradually as WT and Δ*ftsH* cells entered stationary phase after ~8 h of growth, reaching a significantly different minimum of 2.6 µm and 3.0 µm, respectively (Fig. S1, 8 h). After entering stationary phase, WT cells maintained the same median cell length over the next 20 h while Δ*ftsH* cells slowly increased in length, reaching a median of 3.7 µm after 16 h in stationary phase (24 h of total incubation time) (Fig. S1, 24 h and 28 h).

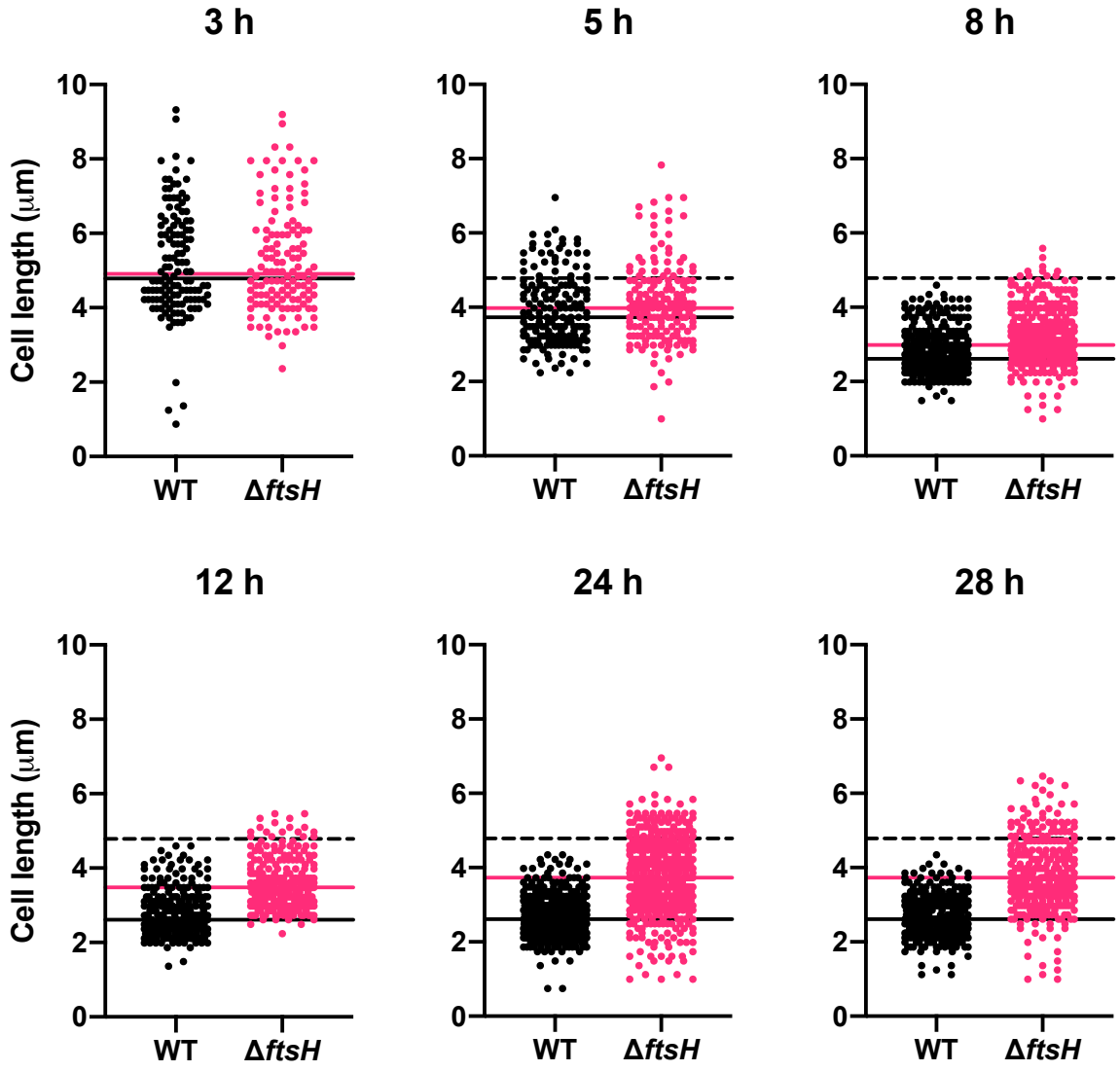

Figure S1. Cell length decreases as WT and Δ*ftsH* enter stationary phase. Overnight cultures were diluted to a starting OD_{500} of 0.01 and incubated at 37°C with shaking. Samples were taken for microscopy at the indicated time points. Cells reached stationary

phase after ~8 h of growth. Time points were taken in conjunction with the viability measurements in Fig. S3C. Solid lines represent median cell length at the indicated time points. The dashed line is median cell length of WT at 3 h.

Cells of Δ*ftsH* began to die after >20 h in stationary phase, becoming effectively unculturable after 60 h of total incubation time (Fig. 1A). WT also died during stationary phase, although the onset of death was delayed by ~16 h and was greatly reduced in magnitude (Fig. 1A). Cells of Δ*ftsH* also began to die after >24 h of growth arrest in CSM, with ~5-to-10-fold reduced viability on day 4 (Fig. 1B) and ~100-fold reduced viability on day 10 (Fig. S2). In contrast, WT maintained full viability after 4 days of carbon starvation (Fig. 1B), and had ~2-fold reduced viability on day 10 (Fig. S2). Integration of the *ftsH* ORF with its native promoter at the *glmS* locus on the chromosome fully rescued the viability defects in growth arrest, confirming the role of FtsH in survival (the complemented strain Δ*ftsH/+ftsH*, Fig. 1A and 1B).

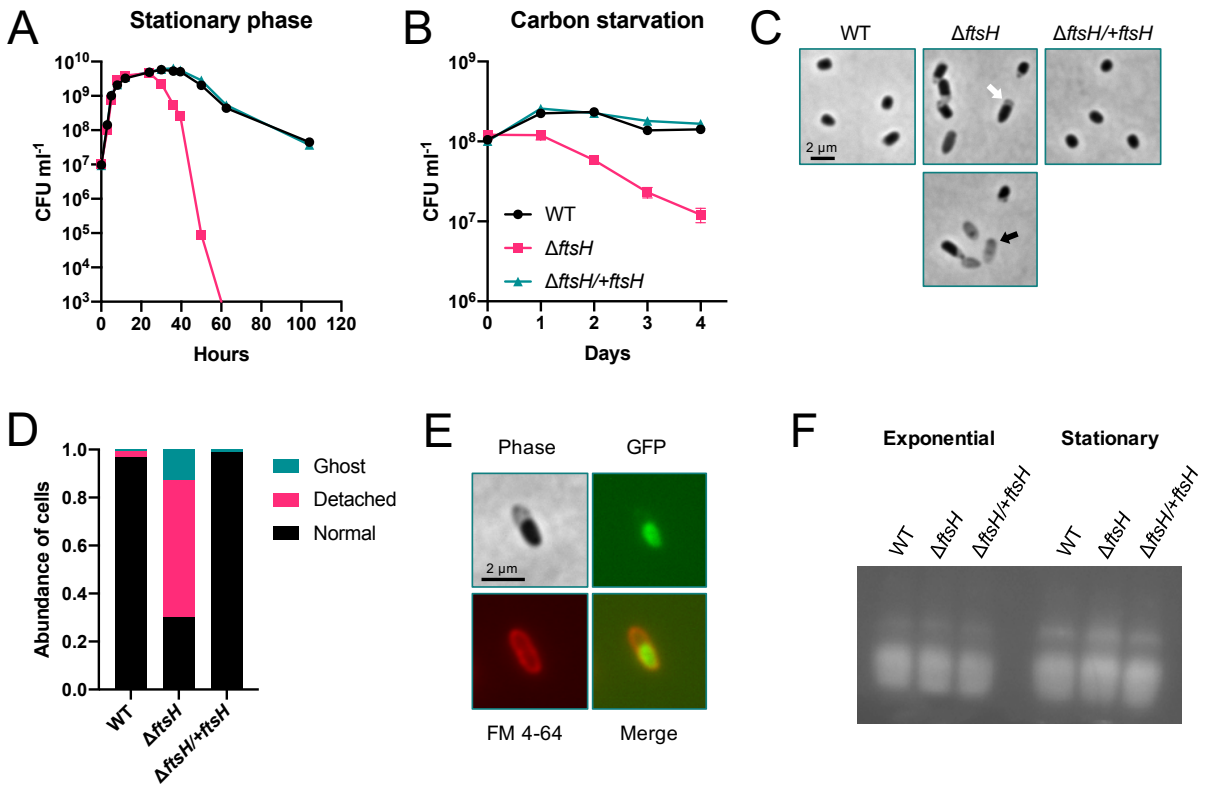

Figure 1. FtsH maintains cell integrity during growth arrest. Cells of Δ*ftsH* lose viability during stationary phase (A) and carbon starvation (B). Viability was below the limit of detection (~3x10^2 CFU ml^{-1}) for Δ*ftsH* at 60 h. Experiments were performed at least three times and representative data are shown. (C) Characteristic morphology of Δ*ftsH* cells after 1 day of carbon starvation. The white arrow indicates a "detached" inner membrane and the black arrow is an example of a "ghost cell". (D) Quantification of the cellular

morphologies described in (C). A minimum of 300 cells were counted for each strain. (E) OM staining with FM 4-64 and cytoplasmic expression of GFP confirms that detachment occurs between the IM and OM. (F) The amount of LPS is similar between WT and Δ*ftsH* during exponential phase and stationary phase.

Most Δ*ftsH* cells lysed following death, as revealed by a decrease in the optical density of the culture, and the appearance of only scattered "ghost" cells by microscopy (Fig. S3D and S3E). Before significant death of Δ*ftsH* cells occurred, however, we observed many intact cells with what appeared to be a "shrunken" or "detached" inner membrane (IM) (Fig. 1C). In contrast, this cell morphology was largely absent in WT and the complemented strain at the same time points (Fig. 1D), and none of the strains displayed this morphology during growth (data not shown). To confirm separation between the OM and IM, carbon-starved Δ*ftsH* cells expressing cytoplasmic GFP were stained with the OM dye FM 4-64 (Fig. 1E). Based on these morphological and viability findings, we conclude that FtsH is required to maintain cellular integrity during growth arrest of *P. aeruginosa*.

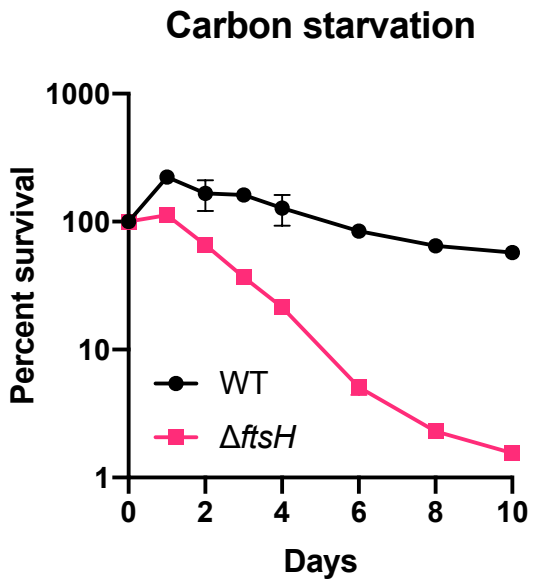

Figure S2. Viability loss plateaus during carbon starvation. 1-2% of Δ*ftsH* cells remain viable during extended carbon starvation, in contrast to complete viability loss in stationary phase. Error bars show standard deviation of biological replicates (n = 3).

Cell integrity of ΔftsH *is not compromised by LPS overproduction during growth arrest.*

The morphology of growth-arrested Δ*ftsH* cells was strikingly similar to a gain-of-function mutant in *E. coli* that also experienced rapid cell death during growth arrest (38). In the cell death pathway

described by Sutterlin *et. al.*, this mutant aberrantly transports phospholipids to the outer leaflet of the OM, which is normally comprised almost exclusively of LPS (38, 62). The accumulation of phospholipids in the outer leaflet triggers compensatory LPS synthesis via increased activity of LpxC, which ultimately destabilizes the OM and leads to vesiculation and loss of OM lipids (28, 38, 39). During growth, lost lipids can be replenished by ongoing synthesis and transport from the IM to the OM. Upon growth arrest, however, *de novo* lipid synthesis ceases while lipid loss from the OM continues unabated. The continual transport of lipids from the IM to the OM results in shrinkage of the IM and ultimately cell lysis (38). Importantly, increased LpxC activity in the gain-of-function mutant is believed to be mediated by inhibition of FtsH proteolysis (38, 39). Thus, deletion of *ftsH* in *P. aeruginosa* could functionally recapitulate its inhibition in *E. coli*, resulting in the morphological and viability defects we observe during growth arrest (Fig. 1).

Although FtsH does not regulate LpxC in *P. aeruginosa* during growth (40), it is possible that temporal regulation might occur upon entry of cells into growth arrest, perhaps ensuring complete shutdown of LpxC activity under conditions where it would be detrimental for survival. In support of this possibility, LpxC degradation has been shown to inversely correlate with growth rate in *E. coli*, highlighting the importance of tightly coordinating LPS synthesis with the cellular demand (63). Based on our experimental findings and the purported role of FtsH in the literature, we performed experiments to test the hypothesis that LPS overproduction in Δ*ftsH* results in cell death during growth arrest.

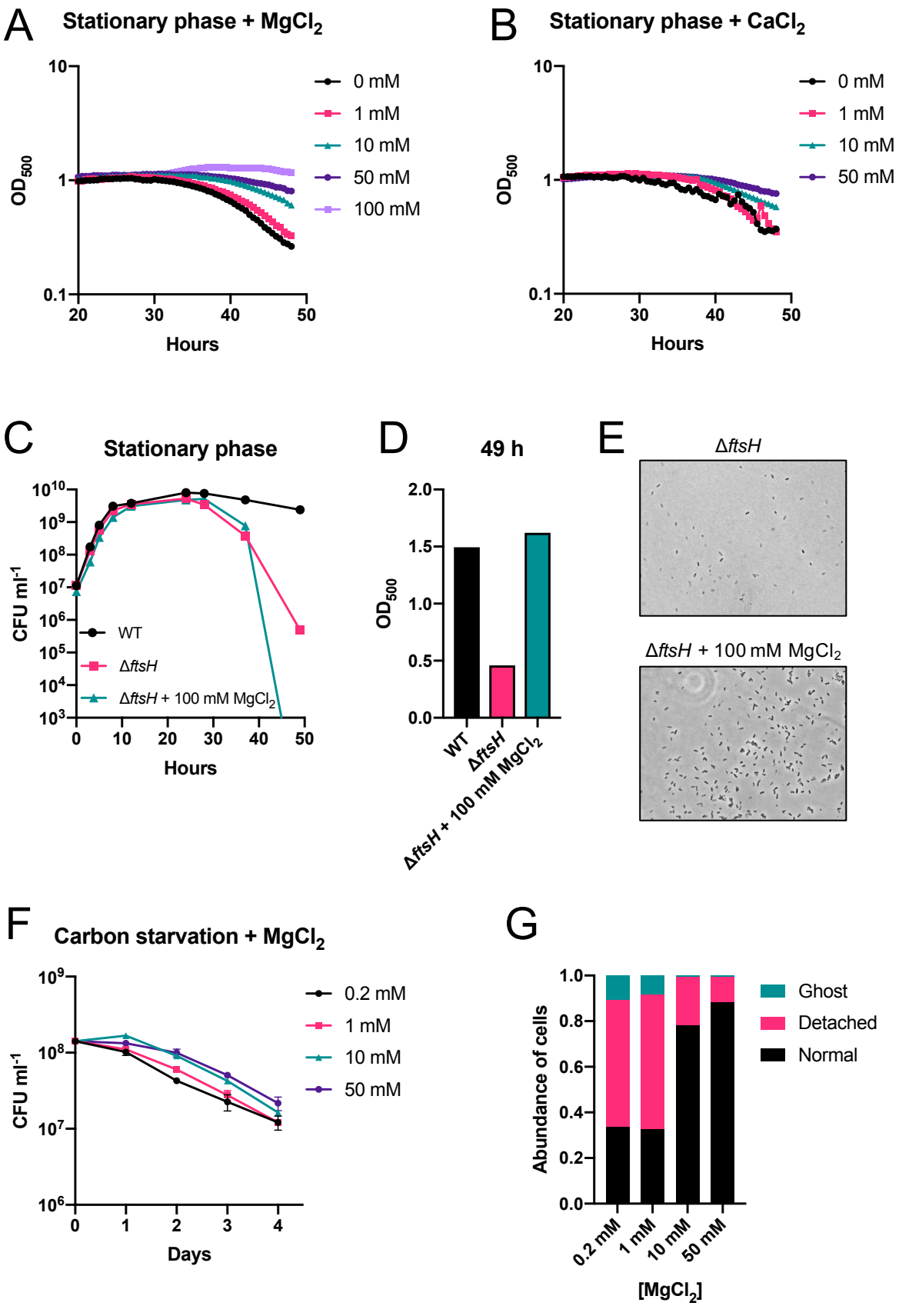
A
Stationary phase + MgCl2
OD500
Hours
0 mM
1 mM
10 mM
50 mM
100 mM
B
Stationary phase + CaCl2
OD500
Hours
0 mM
1 mM
10 mM
50 mM
C
Stationary phase
CFU ml-1
Hours
WT
ΔftsH
ΔftsH + 100 mM MgCl2
D
49 h
OD500
WT
ΔftsH
ΔftsH + 100 mM MgCl2
E
ΔftsH
ΔftsH + 100 mM MgCl2
F
Carbon starvation + MgCl2
CFU ml-1
Days
0.2 mM
1 mM
10 mM
50 mM
G
Abundance of cells
[MgCl2]
Ghost
Detached
Normal

Figure S3. Cation supplementation does not rescue viability of Δ*ftsH* during growth arrest. Addition of $MgCl_2$ (A) or $CaCl_2$ (B) to LB prevents cell lysis of Δ*ftsH* during stationary phase, as measured by optical density. However, 100 mM $MgCl_2$ does not rescue viability of Δ*ftsH* (C) although it prevents cell lysis (D) and (E). Viability was below the limit of detection (~$3x10^3$ CFU ml^{-1}) for Δ*ftsH* at 49 h in (C), (D), and (E). (F) Addition of $MgCl_2$ mildly rescues viability of Δ*ftsH* during carbon starvation, but the effect is transient. (F) $MgCl_2$ partially rescues the morphological defects of Δ*ftsH* on day 1 of carbon starvation. A minimum of 300 cells were counted for each condition. Error bars show standard deviation of biological replicates (n = 3).

Addition of $MgCl_2$ or $CaCl_2$ to LB was previously shown to fully suppress the death of the *E. coli* gain-of-function mutant in stationary phase (38). This is because the positive charge of these cations helps stabilize the negatively-charged LPS, preventing vesiculation and lipid loss (38, 62). Intriguingly, we found that cation supplementation with Mg^{+2} or Ca^{+2} also suppressed cell lysis of Δ*ftsH* in stationary phase in a dose-dependent manner (Fig. S3A and S3B). However, the cation concentration necessary for complete suppression of lysis was much greater than that required in *E. coli* (100 mM $MgCl_2$ vs. 5 mM) (38). The requirement for this unusually high concentration was an artifact, however, as measurement of cell viability in conjunction with optical density revealed that cells still died during stationary phase but no longer lysed (Fig. S3C and S3D). The high cation concentration appears to have simply preserved the "carcasses" of dead cells (Fig. S3E). Furthermore, elevated cation concentrations had a mildly positive, but transient, effect on viability during carbon starvation (Fig. S3F) and only partially reduced the abundance of "shrunken" and "ghost" cells (Fig. S3G). We also treated cells with the LpxC inhibitor CHIR-090 (64) as a means to reduce LPS synthesis, and found that treatment with this inhibitor during growth arrest, or at subinhibitory concentrations during pregrowth of cultures, had no effect on survival (Fig. S4). Finally, we measured LPS levels directly and observed no difference between WT and Δ*ftsH* during exponential growth or stationary phase (Fig. 1F). Taken together, we conclude that elevated production of LPS does not significantly impair cellular integrity of growth-arrested *P. aeruginosa* in the absence of FtsH.

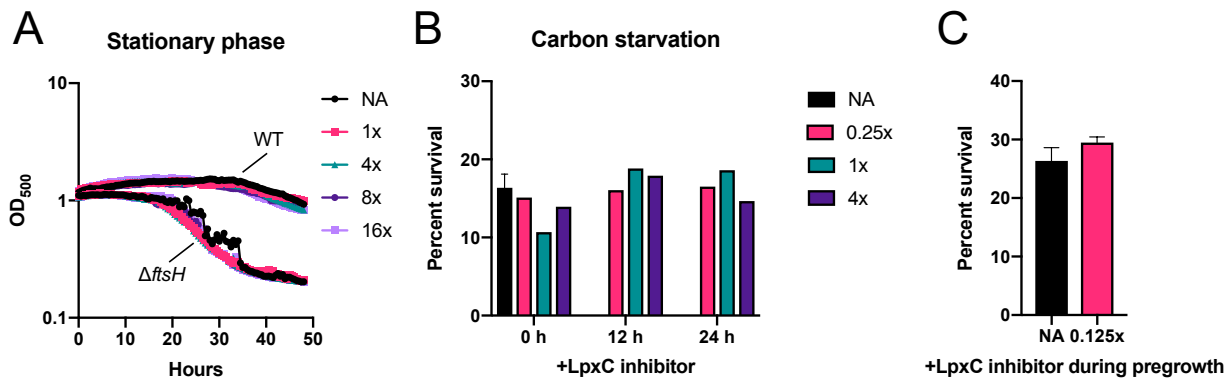

Figure S4. Inhibition of LpxC activity does not rescue viability of Δ*ftsH* during growth arrest. (A) Addition of the LpxC inhibitor CHIR-090 to stationary phase cells of Δ*ftsH* does not prevent cell lysis. The concentration of inhibitor was 1x-16x the minimum inhibitory concentration (MIC) (1 μg ml^{-1}). (B) Addition of CHIR-090 after 0 h, 12 h, or 24 h of carbon starvation does not rescue viability of Δ*ftsH* on day 3 of carbon starvation. NA = nothing added. (C) Addition of a sub-MIC concentration of CHIR-090 to Δ*ftsH* cells during growth does not rescue viability on day 3 of carbon starvation. Error bar shows standard deviation of biological replicates (n = 3).

Identification of genetic interactions with ftsH *during growth arrest.*

We decided to use a functional genetics approach to identify genes that interact with *ftsH*, in the hope that this would shed light on its role during growth arrest. Accordingly, we performed a transposon mutagenesis screen in the Δ*ftsH* background using Tn-seq. Tn-seq is a powerful tool to identify genetic fitness determinants in microorganisms on a genome-wide scale (65). In Tn-seq, a pooled transposon mutant library is grown under a condition of interest and then sequenced at the transposon-genome junction to identify where transposons are inserted and to quantify their abundance in the total population. Mutant abundance is then compared to a control population to determine the relative fitness of insertion at a particular locus under the condition of interest.

A transposon mutant library was generated in the Δ*ftsH* background in the same manner as described previously for the WT parent strain (15). After initial plating on selective LB agar plates at 37°C, the transposon mutants were pooled and stored as frozen aliquots. One library aliquot was thawed and grown for ~3-4 generations in LB at 37°C (pregrowth, Fig. 2A). The pregrowth culture was then pelleted and resuspended in CSM. Immediately upon resuspension, an aliquot of the library was diluted into fresh LB and grown for ~3-4 generations at 37°C, serving as the "growth" arm of the

experiment. The remaining culture was starved in CSM for 4 days followed by dilution of an aliquot into fresh LB and growth for ~3-4 generations at 37°C, serving as the "starvation" arm of the experiment. Samples were collected from the "growth" and "starvation" cultures following outgrowth and prepared for Tn-seq analysis as described previously (15). As a control, the same experiment was performed using a transposon library in the WT background.

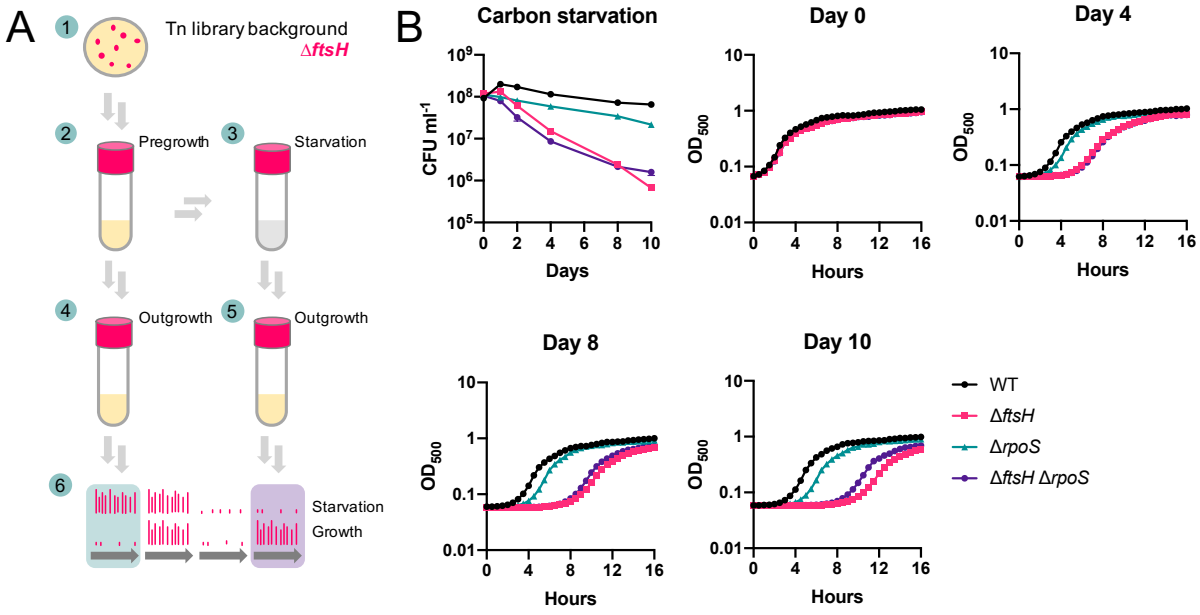

Figure 2. Mutations in σ^S alleviate survival of Δ*ftsH* during carbon starvation. (A) Experimental design of Tn-seq screen. 1: A transposon library was generated in Δ*ftsH* and selected on LB agar plates at 37°C. 2: An aliquot of the library was grown ~3-4 generations in liquid LB to exponential phase. 3 and 4: The culture was washed and resuspended in CSM (3) and an aliquot of the culture was taken for outgrowth at time zero (4) (representing the "growth" arm of the experiment). 5: An aliquot of the culture was taken for outgrowth after 4 days of carbon starvation (the "survival" arm of the experiment). 6: Comparing the read counts of the growth and starvation cultures allows identification of genes that are alleviating or aggravating for survival. (B) Viability was measured in conjunction with outgrowth in liquid LB of the mutant strains at the indicated times.

We identified three genes with reads >10-fold depleted or enriched (P <0.05) following carbon starvation of Δ*ftsH* (*i.e.* aggravating or alleviating for survival, respectively). All three genes were enriched, and encoded the phospholipid acyltransferase LptA (PA14_00060), the ATP-dependent heat shock protease Lon (PA14_41220), and the stress response sigma factor RpoS (σ^S) (PA14_17480) (Data Set S1). In contrast, insertions in these genes did not cause a significant effect on fitness in the WT background at this time point (Data Set S1). As an internal control, we identified

ftsH as one of four genes with insertions causing a significant fitness defect in the WT background (Data Set S1).

One caveat to this screen, however, is that nearly 10-fold fewer reads mapped to the PA14 genome in the Δ*ftsH* "starvation" library compared to the other libraries (~$7x10^5$ vs ~$5x10^6$ reads, respectively). Most reads (~86%) mapped to the donor plasmid carrying the transposon that was used for conjugation (see methods). This is likely because a majority of the population died by day 4 of carbon starvation, which resulted in enrichment of the previously trace amounts of *E. coli* donor carried over from the original conjugation. By day 4 of carbon starvation in the Δ*ftsH* library, ~10% of CFUs appeared to be *E. coli*, as judged by colony morphology, while no *E. coli* colonies were detectable in any of the other libraries (data not shown). All libraries were outgrown in LB plus chloramphenicol to counterselect against *E. coli*. However, the ~3-4 generations of outgrowth was not enough to completely mitigate the *E. coli* background in the Δ*ftsH* "starvation" library, even though no *E. coli* colonies were detectable by CFU plating when samples were collected for Tn-seq analysis.

Irrespective of this caveat, we treated the few genes that interacted with Δ*ftsH* during growth arrest as potential "signals in the noise", reasoning that they likely have real effects on fitness since we could detect them in spite of a reduced signal. We decided to test the role of *rpoS* during growth arrest, as insertions in this gene were most enriched in the Δ*ftsH* starvation library (Data Set S1), and an alleviating effect of *rpoS* inactivation was surprising, considering this gene is generally necessary for growth arrest survival in the WT background (15). One explanation for this counterintuitive finding is that *rpoS* mutants manifest a growth advantage in stationary phase (GASP) phenotype in the Δ*ftsH* background (66, 67). GASP occurs when spontaneous mutants with enhanced nutrient scavenging abilities sweep a growth-arrested population (68), and mutations in a number of genes, including *rpoS*, can elicit GASP in *E. coli* (69). Importantly, GASP arises only after a majority of cells have died and released nutrients into the medium, which could explain why loss of *rpoS* was uniquely beneficial for survival in the Δ*ftsH* background in our experiments. To test this hypothesis, we made an Δ*ftsH* Δ*rpoS* double mutant and compared its survival to WT and either single mutant during carbon starvation. Viability of the double mutant declined similarly to Δ*ftsH* during carbon starvation, with only slightly higher viability (~2-fold) on day 10 (Fig. 2B). The Δ*rpoS* single mutant

had a survival defect compared to WT, in line with our previous results (15), but survived better than Δ*ftsH*, indicating that FtsH is more vital for growth arrest survival than σ^s. In conjunction with viability plating, we also measured outgrowth of the strains in liquid LB, which more faithfully recapitulates the conditions of our Tn-seq experiment. Here, we observed a distinct outgrowth advantage for the double mutant compared to Δ*ftsH* after eight and ten days of carbon starvation (Fig. 2B), even though the average number of viable cells was essentially the same for both strains on day 8 ($2.4x10^6$ vs $2.1x10^6$ for Δ*ftsH* and Δ*ftsH* Δ*rpoS*, respectively). The fact that this outgrowth advantage manifested late in carbon starvation, when ~98% of the population had died, supports the conclusion that loss of *rpoS* confers a GASP phenotype in the Δ*ftsH* strain in our experiments.

Identification of genetic interactions with ftsH *during growth.*

To avoid the problem of *E. coli* contamination during growth arrest, we chose to identify growth-aggravating or alleviating genetic interactions with Δ*ftsH* by comparing insertions between the WT and Δ*ftsH* "growth" libraries after ~6-8 generations of growth in LB at 37°C (Fig. 3A). We identified a comparatively larger number of genes that interacted with *ftsH* during growth, with 36 aggravating interactions that resulted in >10-fold depleted reads in the Δ*ftsH* background relative to the WT background ($P < 0.05$) (Table S2 and Data Set S2). Several functional categories were represented, indicating that loss of FtsH disrupts growth in varied ways. Numerous aggravating interactions were among genes involved in cell envelope biogenesis and outer membrane functions, as well as at the interface of carbohydrate and glycerophospholipid metabolism (Data Set S2 and Fig. S5). Specifically, genes putatively involved in LPS biosynthesis (*ssg*, *wapH*), glycolysis (*tpiA*), fatty acid biosynthesis (*fabF1*), and phospholipid metabolism (*lptA*) were all growth-aggravating in the Δ*ftsH* background. This suggests that although FtsH does not regulate LpxC in *P. aeruginosa*, it could still play an integral role in glycerophospholipid metabolism and cell envelope biogenesis.

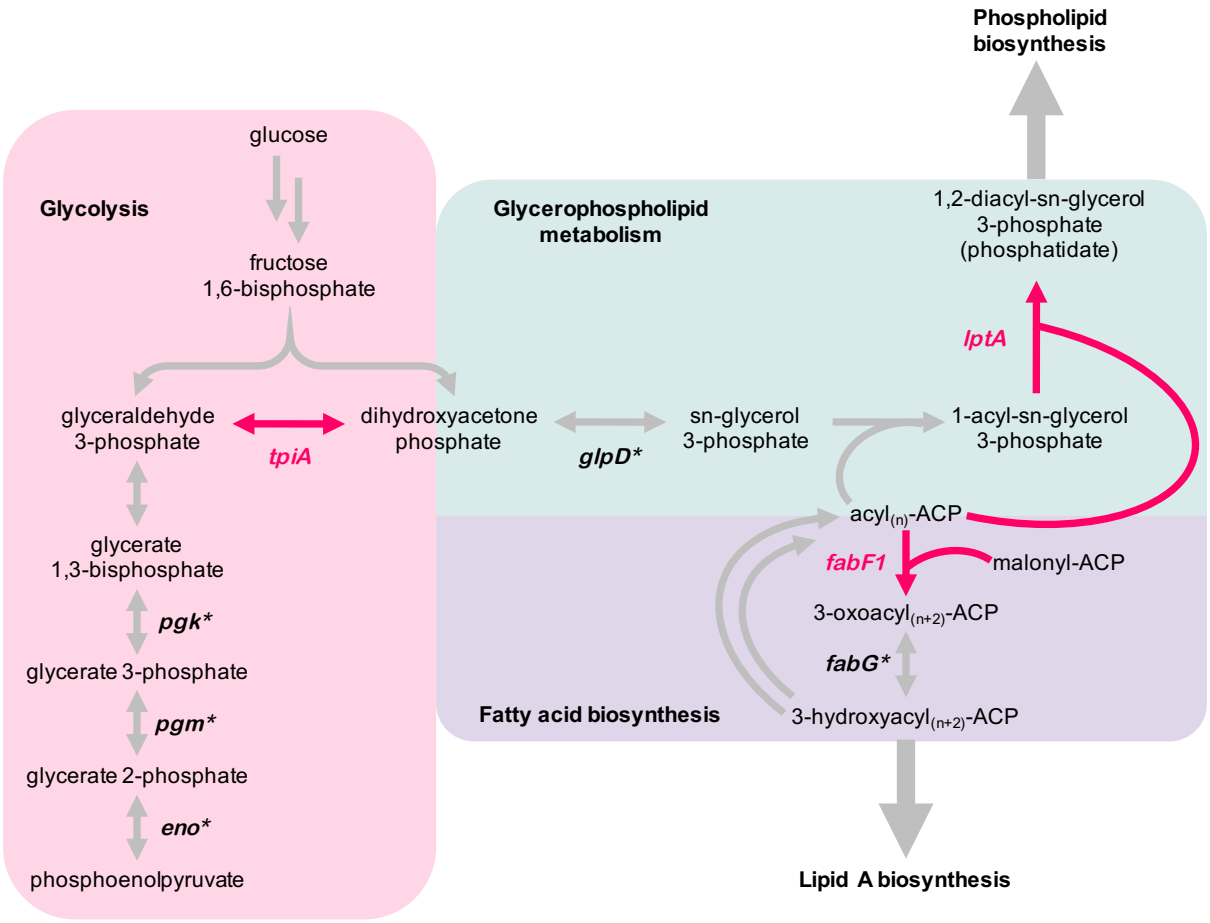

Figure S5. Genetic interactions with *ftsH* at the intersection of glycolysis, fatty acid biosynthesis, and phospholipid metabolism during growth. Genes catalyzing the reactions in red were >10-fold depleted in reads (P <0.05) in the Δ*ftsH* background compared to the WT background. Genes denoted with an asterisk also had a statistically significant depletion in reads, but the depletion was <10-fold (Data Set S2).

Insertions in genes involved in post-translational modification and protein turnover were also aggravating for growth, including the proteases encoded by *hslVU* and *htpX* (70, 71) and the SsrA-binding protein encoded by *smpB* (72). We also detected an aggravating interaction with the membrane-stress responsive two component system (TCS) encoded by *amgRS*, as observed previously in *P. aeruginosa* strain PAO1 (61). AmgRS is a homolog of the CpxRA TCS that regulates the envelope stress response in *E. coli* (73, 74), and loss of *amgRS* or *ftsH* in *P. aeruginosa* results in hypersensitivity to aminoglycoside-induced protein misfolding (61). Notably, *htpX* is regulated by AmgRS in *P. aeruginosa* and contributes to aminoglycoside resistance in conjunction with *PA14_72930*, another AmgRS-regulated gene of unknown function that was also aggravating

for growth in our screen (Table S2 and Data Set S2) (61). These results highlight that loss of FtsH causes hypersensitivity to protein misfolding and the recruitment of multiple protein folding and degradation pathways to help maintain proteostasis.

We made isogenic markerless deletions of growth-aggravating genes from different functional categories to validate their interactions with *ftsH*. To this end, we broadly grouped the genes *tpiA*, *lptA*, and *fabF1* as involved in "carbohydrate and phospholipid metabolism" (Carb+PL metabolism) (Fig. S5) and *hslVU*, *htpX*, and *amgRS* as involved in "protein turnover". As expected, double mutants of *ftsH* and each metabolism gene had a significantly reduced growth rate in LB at 37°C compared to the Δ*ftsH* single mutant (Fig. 3B). The defect was greatest for Δ*ftsH* Δ*fabF1*, with a 63% reduction in growth rate compared to Δ*ftsH*. Double mutants of *ftsH* and the protein turnover genes *hslVU* and *amgRS* also had a reduced growth rate compared to Δ*ftsH*, with the growth rate of Δ*ftsH* Δ*amgRS* 60% reduced (Fig. 3C). However, we did not detect a significant difference in growth rate between Δ*ftsH* and Δ*ftsH* Δ*htpX*. This might be because the growth rate defect was simply too subtle to detect, a possibility that is supported by our previous experience with Tn-seq, in which seemingly strong fitness defects appear much milder when assessed using single mutants (15). The other possibility is that *htpX* is a false positive in our screen, but the fact that its regulator, *amgRS*, interacts strongly with *ftsH* argues against this possibility. Together, these data validate the results of our Tn-seq experiment and confirm a role for FtsH in promoting diverse cellular processes during growth.

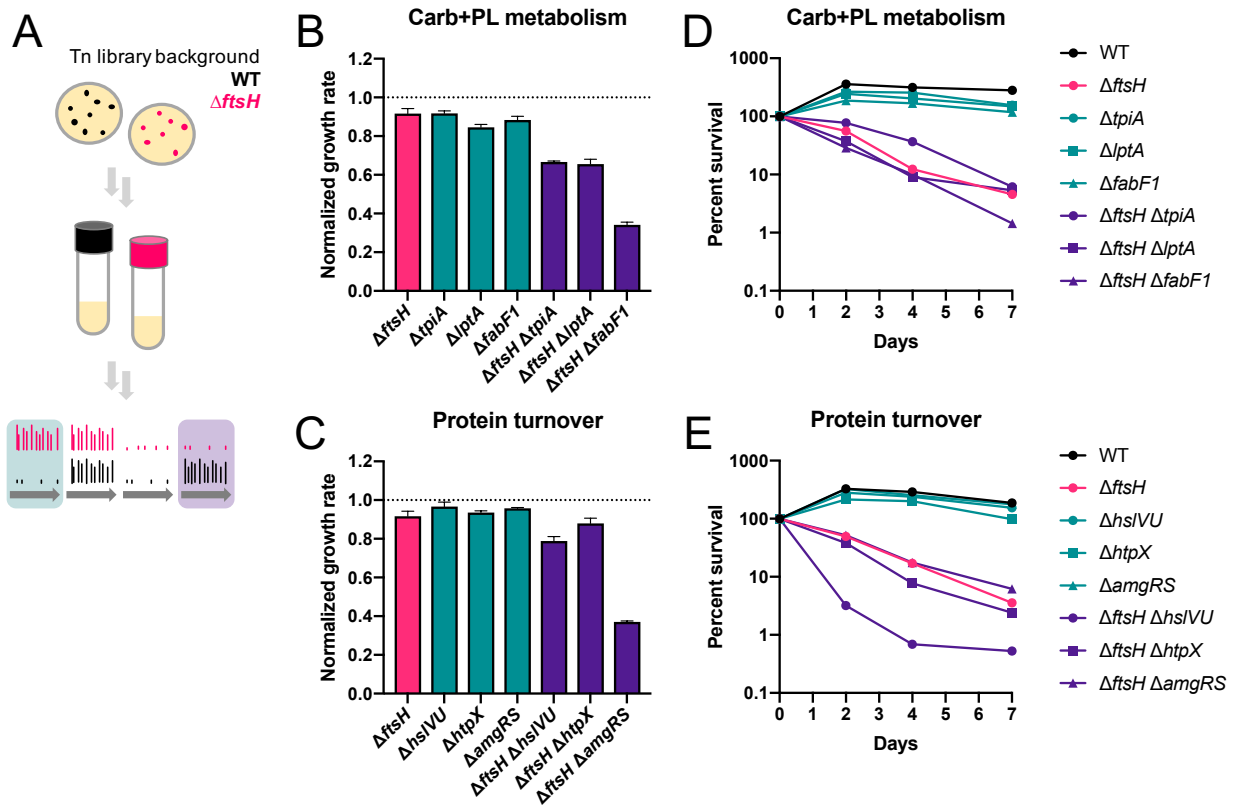

Figure 3. Mutations in metabolic and protein turnover genes aggravate growth of Δ*ftsH*. (A) Experimental design of Tn-seq screen. Comparing the read counts of the WT and Δ*ftsH* growth cultures allows identification of genes that are alleviating or aggravating for growth in the Δ*ftsH* background. Growth rate of carbohydrate and phospholipid metabolism mutants (B) and protein turnover mutants (C) normalized to the WT growth rate in LB at 37°C. Survival of carbohydrate and phospholipid metabolism mutants (D) and protein turnover mutants (E) during carbon starvation. Error bars show standard deviation of biological replicates (n = ≥3).

Deletion of hslVU *exacerbates survival of* ΔftsH *during growth arrest.*

We tested how these two functional categories of genes interact with *ftsH* during carbon starvation and observed surprising discrepancies compared to their growth-dependent interactions. All three Carb+PL metabolism single mutants maintained viability similar to WT after 7 days of carbon starvation (Fig. 3D). Remarkably, the double mutants survived similar to Δ*ftsH* over this time period, indicating that while these genes are important for growth, they do not influence survival in the Δ*ftsH* background. Single mutants of the protein turnover genes also survived similar to WT during carbon starvation, and the Δ*ftsH* Δ*amgRS* and Δ*ftsH* Δ*htpX* double mutants survived similar to Δ*ftsH* (Fig. 3E). Thus, an intact membrane stress response mediated by AmgRS is vital for growth of Δ*ftsH* even

in the absence of aminoglycoside stress, but is dispensable for survival during carbon starvation. In contrast, Δ*ftsH* Δ*hslVU* had a survival defect ~10-fold more severe than Δ*ftsH* after 3 days of carbon starvation (Fig. 3E), indicating that HslVU plays a backup role to FtsH during growth and growth arrest. These results prompted us to systematically investigate the role of ATP-dependent heat shock proteases during growth arrest.

ATP-dependent heat shock proteases cooperatively promote survival during growth arrest.

ATP-dependent proteases classically act in conjunction with protein-folding chaperones to repair or degrade misfolded proteins that form at high temperature (75). This heat shock response also ensures protein quality control in the face of other misfolding stresses, such as exposure to ethanol or heavy metals (76). To more broadly characterize the heat shock response during growth arrest, we measured expression of the major ATP-dependent proteases and chaperones in WT during carbon starvation by quantitative reverse-transcription PCR (qRT-PCR). Following a substantial decrease in expression within the first hour of carbon starvation, we observed mild temporal induction of most heat-shock regulated genes over the next 18 h (Fig. S6). In support of a specific induction of the heat shock response during growth arrest, expression of the growth-related gene *dnaA* did not increase over time.

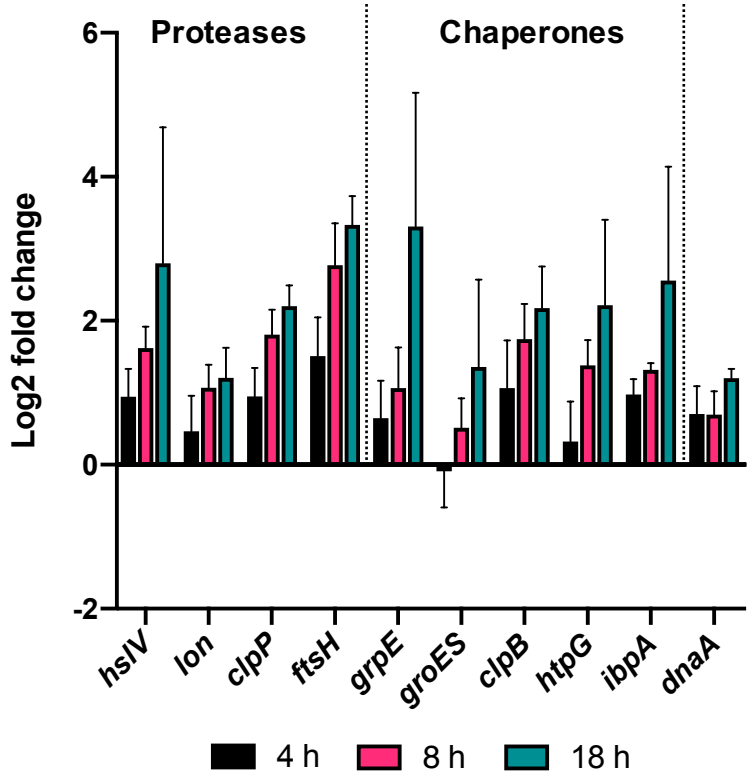

Figure S6. The heat shock regulon is induced during carbon starvation. Gene expression was normalized to *oprI*. Expression of the DNA replication initiator *dnaA* was used as a negative control. Error bars show standard deviation of biological replicates (n = 3).

Based on these results, we predicted that further genetic perturbation of the protease network in *P. aeruginosa* would exacerbate survival defects during growth arrest. To this end, we made all combinatorial deletions of the ATP-dependent protease-encoding genes *ftsH*, *clpXP*, *lon*, and *hslVU*, and assessed survival of the deletion strains during carbon starvation (Fig. 4A). Single deletion of *lon*, like Δ*hslVU*, did not cause a survival defect during carbon starvation, while deletion of *clpXP* caused a similar survival defect as Δ*ftsH* (Fig. 4A and B). We did not detect a fitness defect during carbon starvation for *clpX* or *clpP* in the WT strain using Tn-seq in this study or in our previous study because there were few insertions in these two genes in all transposon libraries (Data Set S3) (15). Although Δ*clpXP* grew significantly slower than WT at 37°C (see below), the growth defect was similar to that of Δ*ftsH*, which contained abundant insertions in the WT transposon library (Data Set S3). However, even though Δ*clpXP* had similar viability compared to Δ*ftsH* during carbon starvation (Fig. 4A and B), we noticed that colonies took longer to appear upon plating (data not shown). Additionally, Δ*clpXP* had a longer lag time compared to the other single mutants after a

nutritional downshift from LB to glucose minimal medium (Fig. S7), as has been observed previously in *E. coli* (77). We speculate that the reduced insertion rate at the *clpXP* locus is due to an extended lag time of mutant cells before beginning to grow, which leads to these mutants being outcompeted early during growth of the transposon libraries. These results emphasize that while Tn-seq is a powerful technique for studying single mutant fitness on a genome-wide scale, it frequently lacks resolving power for studying stress-related phenotypes of mutants with even a minor growth disadvantage under non-stressful conditions (78).

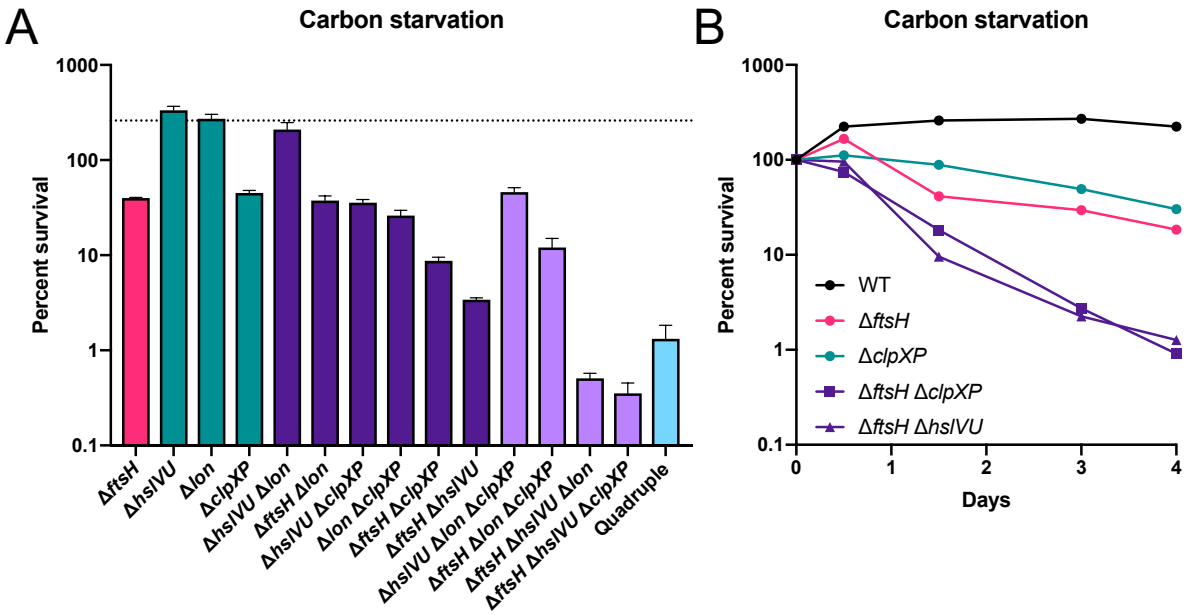

Figure 4. Heat shock proteases overlap functionally to promote survival during carbon starvation. (A) Percent survival of protease mutants on day 3 of carbon starvation. The dashed line indicates the WT survival percentage. (B) Time course of select mutants during carbon starvation. FtsH and ClpXP appear to contribute independently to survival. Error bars show standard deviation of biological replicates (n = 3).

Among the double and triple protease mutants, deletion of *hslVU* did not effect survival in the Δ*lon* or Δ*clpXP* backgrounds, indicating that HslVU functionally overlaps specifically with FtsH (Fig. 4A). Deletion of *lon* exacerbated survival only in the Δ*ftsH* Δ*hslVU* background, indicating that Lon plays a tertiary role in growth arrest survival. This conflicts with our Tn-seq results, where *lon* insertions had a fitness advantage in the Δ*ftsH* background during growth arrest (Data Set S1). We believe this fitness advantage is real but is too subtle to detect in our survival experiments. We

previously found that Tn-seq detects fitness effects during growth arrest more sensitively and much earlier than experiments performed with single mutants, as was also observed with *rpoS* mutants in this study (Fig. 2) (15). Therefore, we chose not to investigate the alleviating effect of *lon* mutation in the Δ*ftsH* background further. Deletion of *clpXP* exacerbated the survival defect of both Δ*ftsH* and Δ*ftsH* Δ*hslVU* (Fig. 4A and B). Thus, *ftsH* and *clpXP* contribute independently to survival during growth arrest.

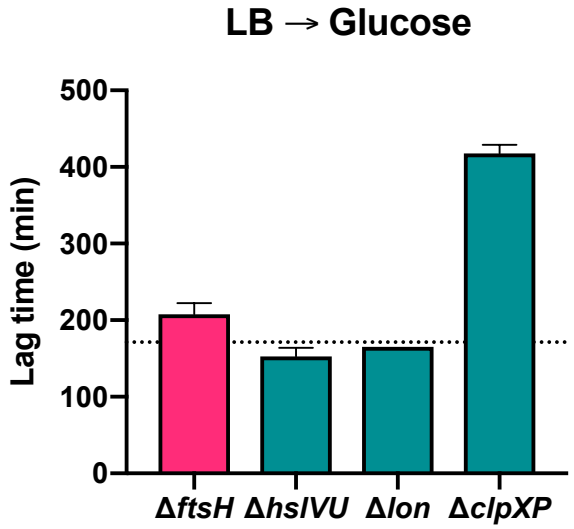

Figure S7. Lag time of protease mutants upon nutrient downshift. Deletion of *clpXP* causes an extended lag phase before growth resumes when cells are shifted from LB to glucose minimal medium.

Finally, the quadruple mutant, in which all four of the major heat shock proteases were deleted, had a survival defect on the order of the more sensitive triple mutants. However, suppressor mutations arose extremely rapidly whenever we cultured this strain, making it difficult to determine an accurate growth rate and survival phenotype for the parent strain.

Heat and alkaline pH exacerbate survival during growth arrest.

The functional overlap of the heat shock proteases during carbon starvation suggests that they collectively act to mitigate a common stress, which we hypothesized was caused by an accumulation of misfolded proteins. Thus, we asked whether the heat shock proteases display a similar pattern of

functional overlap in two other conditions known to cause protein misfolding stress, namely, growth at high temperature or in alkaline pH (79).

Functional overlap during growth at high temperature was roughly similar to that of growth arrest (Fig. 5A). While Δ*ftsH* had only a minor growth rate defect compared to WT in LB at 37°C (~8% reduced growth rate), this defect became more pronounced at 42°C and 44°C (~29% and ~41% reduced growth rate, respectively). Once again, we observed functional redundancy between *ftsH* and *hslVU*, with loss of both genes resulting in no growth at 42°C and 44°C. Unlike in growth arrest, however, the temperature sensitivity of Δ*ftsH* was greater than that of Δ*clpXP*, with the latter displaying temperature sensitivity only at 44°C. The Δ*hslVU* and Δ*lon* single mutants were also temperature sensitive only at 44°C. The pattern of overlap during growth in LB at pH 9 was strikingly similar to that of growth arrest (compare Fig. 5B and 4A), supporting the hypothesis that these two conditions invoke a common stress.

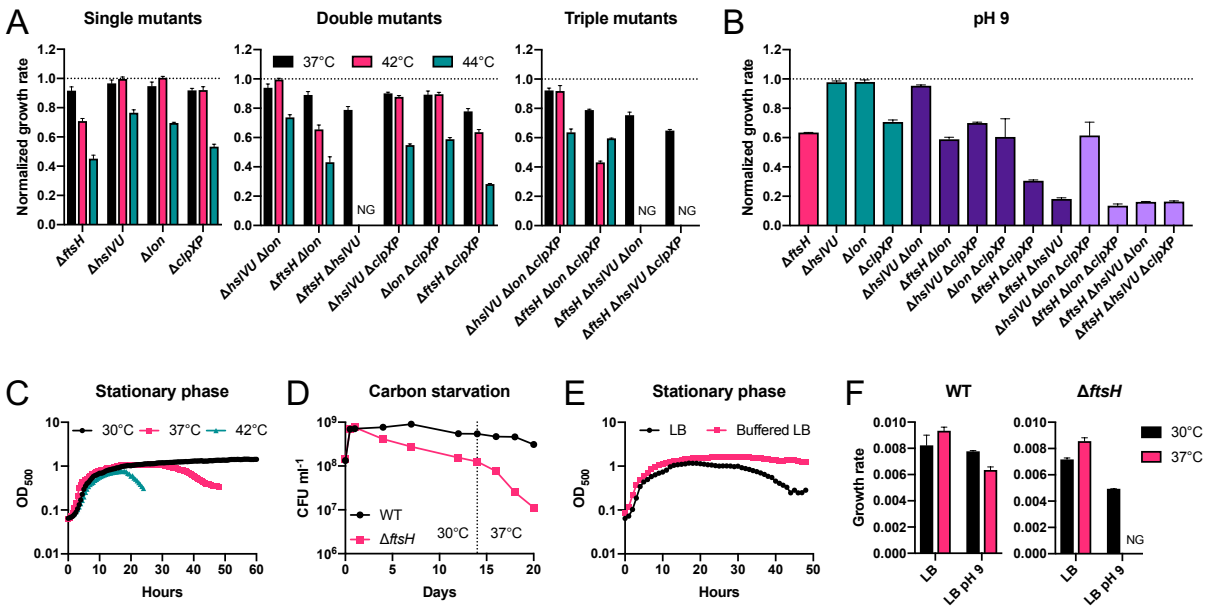

Figure 5. Heat and alkaline pH exacerbate survival during growth arrest. (A) Growth rate of protease mutant strains normalized to the WT growth rate in LB at different temperatures. (B) Normalized growth rate in LB pH 9 at 30°C. (C) Higher temperature correlates with earlier death of Δ*ftsH* during stationary phase. (D) A shift from 30°C to 37°C during carbon starvation accelerates the rate of death of Δ*ftsH*. (E) Incubation in buffered LB mitigates cell death of Δ*ftsH* during stationary phase. (F) Higher temperature exacerbates growth of both WT and Δ*ftsH* in alkaline pH. Note that growth is faster at 37°C than at 30°C for both WT and Δ*ftsH* in

neutral LB, but slower at 37°C than at 30°C in LB pH 9. Error bars show standard deviation of biological replicates (n = ≥3). NG = no growth.

We reasoned that if growth arrest, high temperature, and alkaline pH indeed invoke a common stress, then these conditions should act synergistically to exacerbate survival of protease mutants. Indeed, increasing temperature correlated with earlier death of Δ*ftsH* in stationary phase (Fig. 5C), and a shift from 30°C to 37°C increased the rate of death during carbon starvation (Fig. 5D). Death of Δ*ftsH* in stationary phase was delayed in buffered LB (Fig. 5E), indicating that a combination of growth arrest and alkaline pH likely causes the dramatically greater loss of viability in stationary phase LB cultures compared to carbon starvation in buffered minimal medium (compare Fig. 1A and B).

Higher temperature also exacerbated growth in alkaline pH, as WT grew slower at 37°C than at 30°C in LB pH 9 and Δ*ftsH* failed to grow at 37°C (Fig. 5F). In contrast, both strains grew faster at 37°C than at 30°C in neutral LB. Based on these data, we conclude that growth arrest, high temperature, and alkaline pH are synergistic stresses that require a coordinated response of the ATP-dependent proteases in order for cells to grow and survive.

Heat shock proteases delay protein aggregation during growth arrest.

To directly assess the extent of protein misfolding during growth arrest, we visualized protein aggregates in single cells using fluorescently-tagged IbpA, a small heat shock protein that co-localizes with protein aggregates to form distinct foci (80). IbpA-mVenus was expressed from the chromosome under the control of its native promoter in the WT, Δ*ftsH*, and Δ*ftsH* Δ*hslVU* backgrounds. During exponential growth and as cells entered stationary phase, IbpA expression was low and diffusely distributed throughout the cytoplasm in all three strains, indicating that protein aggregation was minimal in these strains under nutrient-replete growth conditions (Fig. 6A). Importantly, diffuse labeling during growth demonstrates that our reporter is not inherently aggregation-prone—a common artifact in these types of experiments (58, 81). Both Δ*ftsH* and Δ*ftsH* Δ*hslVU* developed 1-2 foci per cell during stationary phase that became brighter and more numerous over time, with an accelerated rate of foci formation in the double mutant. This pattern was also observed in WT, albeit with a delayed progression relative to the protease mutants. For all strains,

the appearance of numerous foci correlated with cell death (Fig. 6B), and following death many cells lost fluorescence. Thus, aggregate formation is delayed, rather than completely prevented, by the activity of ATP-dependent proteases during growth arrest, and aggregate formation correlates with cell death in both WT and the protease-deficient strains.

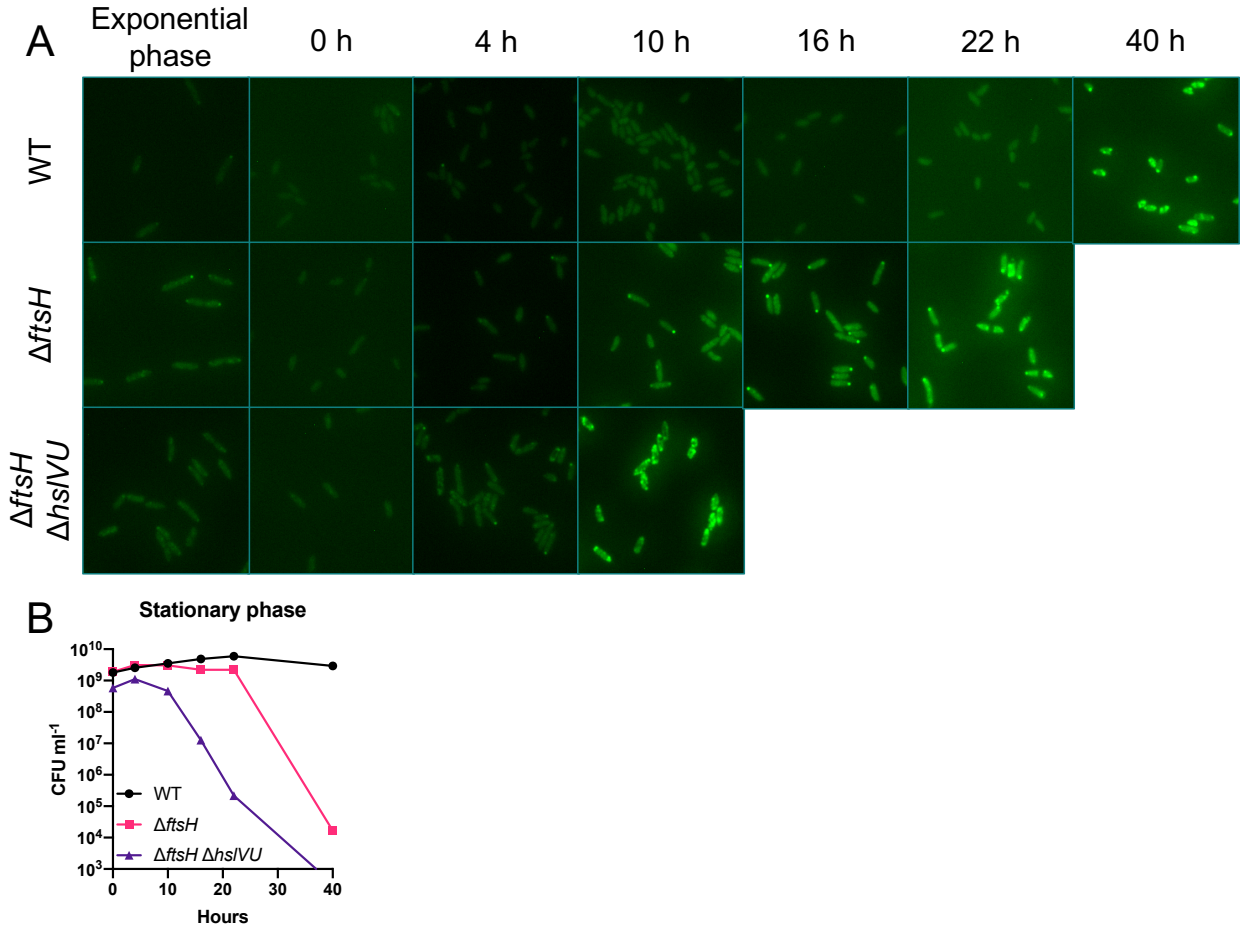

Figure 6. Heat shock proteases delay protein aggregation during growth arrest. (A) Fluorescence measurement of cells expressing the IbpA-mVenus reporter during stationary phase. 0 h corresponds to the start of stationary phase. All images were taken using the same exposure time and are displayed with the same brightness and contrast. (B) Viability measurements corresponding to the time points in (A). Viability was below the limit of detection (~3x10^2 CFU ml^{-1}) for Δ*ftsH* Δ*hslVU* at 40 h.

Discussion

Bacteria must adequately maintain their cellular integrity during extended periods of starvation in order to survive and to be able to resume growth when nutrients become available. In this study, we

describe a role for FtsH and the rest of the heat shock proteases in maintaining cellular integrity of *P. aeruginosa* during growth arrest. Unlike its posited role in *E. coli* (38, 39), our findings suggest that FtsH does not play a regulatory role in growth arrest survival of *P. aeruginosa per se*. Rather, FtsH appears to act in conjunction with the other major ATP-dependent heat shock proteases to ensure ongoing protein quality control in the absence of growth. Protein misfolding appears to become a major problem during growth arrest, and is dealt with by a suite of well-known proteases. These results raise the intriguing hypothesis that challenges associated with growth arrest may constitute a driving evolutionary pressure that has selected for traits that also confer a selective advantage in the presence of more commonly tested stresses, such as heat or pH shock.

We observed discordant interactions between *ftsH* and different functional classes of genes during growth and survival of *P. aeruginosa*, raising the question of whether FtsH has divergent roles in these two states. For example, genetic perturbation of carbohydrate and lipid metabolic pathways exacerbated growth of Δ*ftsH* but had no effect on Δ*ftsH* survival during growth arrest (Fig. 3B and 3D). On the other hand, mutations in heat shock proteases exacerbated both growth and survival of Δ*ftsH* (Fig. 3C and 3E), suggesting a more universal role for proteostasis in bacterial physiology. What is the connection between FtsH and diverse metabolic pathways during growth (Table S2)? One possibility is that cells are more sensitive to protein misfolding and aggregation in the absence of FtsH-mediated quality control, which could interfere with the activity of metabolic enzymes. Evidence for this possibility is found in *Mycobacteria*, where disruption of proteostasis results in misfolding and reduced activity of multimodular lipid synthases (53). Another possibility is that FtsH plays either a direct or indirect regulatory role in one or more metabolic pathways during growth. For instance, FtsH might still control flux through phospholipid and LPS metabolism in *P. aeruginosa* independent of a direct effect on the stability of LpxC. Recent studies have revealed complex crosstalk between phospholipid and LPS metabolism in *E. coli*, and point to a more nuanced role for FtsH in sensing and controlling flux through these pathways (82, 83). Perhaps an analogous role for FtsH exists in *P. aeruginosa*, with the exact regulatory architecture becoming divergent from that of *E. coli* over evolutionary time. Altered regulation of the heat shock sigma factor σ^{32} in the absence of FtsH could also play a role. During growth at moderate temperatures, σ^{32} levels are kept low by FtsH-mediated proteolysis in diverse species (31). Upon heat shock, σ^{32} becomes stabilized

and induces expression of the heat shock regulon. Overexpression of σ^{32} under non-inducing conditions was shown to inhibit growth of *Caulobacter crescentus* by reprogramming gene expression away from a growth-promoting regime towards one favoring repair and maintenance (84). A similar problem could occur in *P. aeruginosa*, where loss of FtsH causes a global change in gene expression indirectly via elevated σ^{32}. In support of this possibility, overexpression of σ^{32} downregulates expression of multiple fatty acid biosynthetic genes in PA14, including *fabF1* (85). If growth-promoting pathways are downregulated across the board in Δ*ftsH*, then further perturbation to these pathways could severely sensitize cells during growth. This could explain why lipid metabolism genes are important for growth of Δ*ftsH* yet dispensable for survival, as fatty acid and phospholipid synthesis would no longer be required once cells have stopped growing. Whatever its exact role may be, it is clear that FtsH activity is intimately tied to numerous aspects of *P. aeruginosa* physiology.

Like FtsH, ClpP has also been implicated in adaptation to and survival of growth arrest in diverse organisms (86–91). Genetic perturbation of protein-folding chaperones and σ^{32} in *E. coli* also causes a survival defect during growth arrest (41, 42). Furthermore, studies from the Nyström lab have described specific induction of heat shock genes in response to oxidatively-modified proteins that accumulate during aerobic growth arrest in *E. coli* (43–46). Together, these findings support a generalized role for the heat shock response in ensuring survival of non-growing bacteria. Outside of these studies, however, the importance of heat shock genes is overlooked in the literature on growth arrest. Most reviews on bacterial growth arrest do not discuss a role for the heat shock response, and most references to heat shock refer mainly to its namesake role in temperature stress. Strikingly, of all the deletion strains of *P. aeruginosa* we have tested, loss of FtsH or ClpP caused by far the most severe survival defect during carbon starvation ((15) and this study). Survival was considerably worse in protease mutant strains than for a mutant missing the stress sigma factor RpoS, which is generally considered one of the principle molecular components required for adaptation to nutrient depletion (Fig. 2B) (92).

In contrast to the situation in bacteria, the importance of the heat shock response and proteostasis in cellular aging is well described and appreciated in eukaryotic organisms, as evidenced by numerous reviews (47, 48, 93–97). Defects in proteostasis are a hallmark of aging in eukaryotes, and our

finding that growth-arrested bacteria sustain similar types of cellular damage as aging eukaryotic cells points to an equally important role for proteostasis in bacterial fitness. While aging in bacteria has been examined in elegant detail in a handful of systems (50, 98–101), these studies focus on "replicative aging" of nutrient-replete, growing cells. This phenomenon occurs when one daughter cell inherits the old cell pole while the other daughter is rejuvenated. While this phenotype is intriguing, its ecological relevance is debated (102, 103). Indeed, it is plausible that outside of the laboratory environment, subtle differences in replicative age resulting from continual growth and division may be overshadowed by aging challenges that arise from extended periods of growth arrest.

Our findings point to proteostasis as an important, yet underexplored, aspect of growth arrest physiology. If growth arrest is as important for bacterial success in diverse ecological contexts as we presume, this should compel us to investigate the role of proteostasis more thoroughly in these contexts, and not simply during growth at high temperature. Indeed, human pathogens like *P. aeruginosa* are unlikely to experience temperature stress much higher than 37°C during infection, a temperature at which *P. aeruginosa* grows optimally. On the other hand, cells can quickly become energy-limited for growth during infection, and what was once an optimal growth temperature could now exacerbate the survival of aging cells. Thus, proteases of the heat shock response could serve as a powerful therapeutic target for the treatment of *P. aeruginosa* and related bacterial infections.

Materials and Methods

Strains and growth conditions.

The strains and plasmids used in this study are listed in Table S1. *E. coli* and *P. aeruginosa* were grown in lysogeny broth (LB) (Difco) or on LB agar plates with appropriate antibiotics at 30°C or 37°C for all cloning and strain construction purposes. All deletion strains in *P. aeruginosa* strain UCBPP-PA14 were made as described previously (15). The LpxC inhibitor CHIR-090 was acquired from ApexBio, and the OM dye FM 4-64 from Life Technologies.

Growth arrest survival assay.

For studying growth arrest caused by carbon starvation, cells were grown in LB at 37°C to an optical density at 500 nm (OD_{500}) between 0.5 and 1, pelleted, and resuspended in carbon starvation medium (CSM), which is derived from a minimal medium without an added carbon source (13). Starved cells were incubated aerobically at 37°C with shaking at 250 rpm and CFU ml^{-1} were determined by viability plating over time.

For studying growth arrest in stationary phase, overnight cultures grown in 5 ml LB were back diluted to an OD_{500} of 0.01 in 5 ml LB and grown at 37°C with shaking at 250 rpm. CFU ml^{-1} were determined by viability plating over time. Most strains reached stationary phase after ~8 hours of growth in LB.

LPS measurement.

LPS levels were measured as previously described (38). Gels were stained with Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes) according to the manufacturer's protocol and the LPS bands were visualized by UV transillumination.

Generation of the transposon library in ΔftsH.

A transposon library was created in the Δ*ftsH* background in the same manner as described for the WT strain (15). Briefly, the Δ*ftsH* strain of *P. aeruginosa* and the *E. coli* strain SM10λ*pir* carrying the transposon-bearing plasmid pIT2 were resuspended in 1 ml of LB from overnight streak plates on LB agar or LB agar plus carbenicillin (100 μg ml^{-1}), respectively. The resuspended cells were adjusted to an optical density at 500 nm (OD_{500}) of ~50 for *P. aeruginosa* and ~100 for *E. coli*. 100 μL of each OD-adjusted strain was mixed together in an Eppendorf tube and two 50 μL aliquots of this mix were plated on sterile 0.2 μm filter discs placed on an LB agar plate. The conjugation spots were allowed to dry in a safety cabinet with laminar flow for 15 minutes and then the plate was incubated at 37°C for 2.5 hours. Following incubation, the two conjugation spots were resuspended in 3.5 ml of LB and 100 μl aliquots of this resuspension were plated on 33 LB agar plus tetracycline (60 μg ml^{-1}) and chloramphenicol (10 μg ml^{-1}) plates to select for transposon insertion mutants. Each

plate yielded ~4,500 mutants for a total of ~150,000 mutants. The plates were incubated 27 h at 37°C. Following incubation, colonies from all plates were pooled and resuspended in LB plus 15% glycerol. The density of the pooled library was adjusted to an OD_{500} of 10 and stored as 1-ml aliquots at -80°C.

Tn-seq sample preparation and data analysis.

Frozen aliquots of the WT and Δ*ftsH* transposon libraries were thawed on ice for 15 minutes and diluted in 50 ml of LB to a concentration of ~$6x10^6$ CFU ml^{-1}. The cultures were grown at 37°C with shaking at 250 rpm for 3.5 hours, corresponding to ~3-4 cell doublings. Following growth, cells were pelleted and resuspended in 50 ml of CSM. Immediately upon resuspension, 5 ml of each culture was diluted in 50 ml of LB plus chloramphenicol (10 μg ml^{-1}) to a concentration of ~$6x10^6$ CFU ml^{-1}. The CSM cultures and the LB plus chloramphenicol dilution cultures were all incubated at 37°C with shaking at 250 rpm. After 3 hours of growth, corresponding to ~4 doublings, an OD 4 ml^{-1} equivalence of each LB plus chloramphenicol culture was pelleted and stored at -80°C. These were the "growth" samples.

After 4 days of incubation, an aliquot of each CSM culture was diluted in 50 ml of LB plus chloramphenicol (10 μg ml^{-1}) to a concentration of ~$6x10^6$ CFU ml^{-1}. The LB plus chloramphenicol dilutions were incubated at 37°C with shaking at 250 rpm. After 6 hours of growth, corresponding to ~5 doublings for WT and ~3 doublings for Δ*ftsH*, an OD 4 ml^{-1} equivalence of each culture was pelleted and stored at -80°C. These were the "starvation" samples.

Genomic DNA was extracted and prepared for high-throughput sequencing as described previously (15). Sequencing was performed at the Millard and Muriel Jacobs Genetics and Genomics Laboratory at Caltech. Sequences were mapped to the UCBPP-PA14 genome sequence using Bowtie (104) and analyzed using the ARTIST Tn-seq analysis pipeline in MATLAB as described previously (15, 105). The WT "starvation" sample was used as a negative control to identify mutants that had a fitness advantage or disadvantage during carbon starvation specifically in the Δ*ftsH* background.

Growth rate measurements.

All growth rate experiments were performed in 96-well microtiter plates incubated in a BioTek plate reader with medium shaking and reading at OD_{500}. Precultures of each strain were grown at 30°C or 37°C in LB then pelleted and resuspended at an OD_{500} of 0.05 in LB. 150 µl aliquots of the resuspensions were dispensed into the appropriate well and 50 µl of mineral oil was added to each well to prevent evaporation. The growth rate was estimated by calculating the slope of the log-linear region of the growth curve.

Microscopy.

All microscopy was performed using using a Zeiss Axio Imager. An exposure time of 400 ms was used for cells expressing the IbpA-mVenus reporter and images were analyzed using the image-processing software FIJI (106). Cell length in Fig. S1 was measured using SuperSegger (107).

Supplemental files accompanying Chapter 4

Table S1. Strains and plasmids used in this study.

Table S2. Functional categories of genes that have an aggravating interaction with *ftsH* during growth.

Data Set S1. Read ratios for each gene and intergenic region in the WT and Δ*ftsH* "starvation" Tn-seq experiment.

Data Set S2. Read ratios for each gene and intergenic region in the Δ*ftsH* "growth" Tn-seq experiment.

Data Set S3. Raw read counts for each gene and intergenic region in all Tn-seq experiments.

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Chapter 5

ROLE OF HEAT SHOCK PROTEASES IN A NOVEL N-DEGRON PATHWAY

This chapter is based on a collaborative project with Tri Vu, a postdoctoral scholar in the Alexander Varshavsky Lab at Caltech.

Abstract

N-terminal (Nt) formylation of the initiator methionine at the start of protein translation is a conserved process in bacteria, mitochondria, and chloroplasts. However, the functional role of Nt-formylation has remained elusive. Recently, the Varshavsky Lab at Caltech performed experiments in *E. coli* that suggested Nt-formylation serves as a novel tag (N-degron) for protease-mediated degradation of unstable or misfolded proteins. However, the identity of the protease(s) that recognizes this N-degron was not determined. Based on evidence in the literature, the heat shock protease FtsH appeared to be a promising candidate in the degradation of Nt-formylated peptides. In this study, we tested this hypothesis by measuring the stability of Nt-formylated peptides in Δ*ftsH* and other protease-deficient strains of *P. aeruginosa*. Our results suggest that Nt-formylated peptides are not stabilized in the absence of FtsH or any of the other major heat shock proteases when the peptides are expressed from a plasmid, and that the validated reporter system in *E. coli* functions differently in *P. aeruginosa*.

Introduction

Nearly all protein synthesis in bacteria, as well as in the bacterial-derived eukaryotic organelles mitochondria and chloroplasts, begins with an Nt-formylated methionine (fMet) residue specified by the AUG initiation codon of mRNA (1). Formylation is catalyzed by the enzyme methionine formyltransferase (FMT), which attaches a formyl group to the methionine of a charged initiator tRNA pretranslationally to form fMet-$tRNA_i^{Met}$. Upon formylation, fMet-$tRNA_i^{Met}$ is then used to initiate protein synthesis. Although Nt-formylation is universally conserved in bacteria, it is not essential for viability, as strains with a deletion of the FMT-encoding gene *fmt* can be recovered in diverse bacterial species (2–6).

Surprisingly, formylation is a fleeting event in the life of a nascent polypeptide, as the formyl group is cotranslationally removed from the initiator methionine early during protein synthesis, within ~100 amino acid residues from the start of translation (7, 8). Deformylation is performed by a ribosome-associated peptide deformylase (PDF), which in contrast to FMT, is essential for viability in all bacterial species, as removal of the formyl group is required for the proper processing and folding of essential enzymes (9–11). Thus, while peptide formylation is a remarkably transient and non-essential process for bacterial growth, failure to immediately deformylate peptides causes complete inhibition of growth. This raises the question of why cells even bother to formylate their peptides in the first place. Clearly this modification should provide a strong fitness advantage, since otherwise it would not be so broadly conserved. In fact, the production of Nt-formylated peptides may actually be detrimental for bacterial fitness in certain contexts, such as infection, as they can activate the innate immune response of mammalian cells similarly to lipopolysaccharide and peptidoglycan (12, 13). Despite its universality, however, the reason fMet is incorporated at the N-terminus of proteins, as opposed to unformylated Met, which is used for all other methionine codons during peptide chain elongation, is not understood.

Recently, work in the laboratory of Prof. Alexander Varshavsky at Caltech produced convincing evidence that fMet serves as a novel degradation signal (termed fMet/N-degron) in *E. coli* (8). The authors posited that a fraction of N-terminal fMet moieties in nascent polypeptide chains that avoid cotranslational deformylation can serve as fMet/N-degrons for unstable, mistranslated, misfolded, or otherwise abnormal bacterial proteins. Thus, Nt-formylation in bacteria could be functionally equivalent to Nt-acetylation in eukaryotes, in which acetylated peptides (Ac/N-degrons) are recognized by specific E3 ubiquitin ligases (N-recognins) and targeted to the eukaryotic proteasome for degradation (14). Furthermore, a formylation-dependent protein degradation pathway was recently characterized in eukaryotic translation (15). In this pathway, Nt-formylated peptides synthesized by eukaryotic ribosomes are recognized by the Psh1 ubiquitin ligase and tagged for proteosomal degradation.

Unlike the fMet/N-degron pathway of eukaryotes, however, the fMet/N-recognin in bacteria has not been identified. Even so, evidence in the literature raises the intriguing possibility that FtsH may be the protease involved in recognizing and degrading fMet/N-degrons. For example, one FtsH

substrate in *E. coli* appears to preferentially retain its N-terminal formyl group following translation (16), and deletion of *ftsH* sensitizes *P. aeruginosa* to aminoglycoside-induced mistranslation and protein misfolding (17). Based on this evidence, we formally tested the hypothesis that FtsH recognizes and degrades fMet/N-degrons in *P. aeruginosa*.

Results

We utilized an fMet/N-degron reporter system engineered by Piatkov et. al. (8) to test the role of FtsH in degradation of fMet-bearing peptides. This system measures the stability of two FLAG-tagged PpiB reporters that are identical apart from 3 amino acids at their N-terminus. One version of the reporter has an N-terminal amino acid sequence of "MVTF" (Met-Val-Thr-Phe), while the other has an N-terminal sequence of "MDDD" (Met-Asp-Asp-Asp). In E. coli, MVTF and MDDD are either strong or poor substrates for deformylation by PDF, respectively. This is because PDF is relatively inefficient at removing the formyl group of nascent polypeptides with a large, charged amino acid residue (like aspartate) at position 2 in the amino acid sequence (8, 18). Thus, a reporter with an MVTF N-terminus should be more stable in vivo than a reporter with an MDDD N-terminus because retention of the formyl group in the latter makes it a strong fMet/N-degron. Using these two reporters, Piatkov *et. al.* showed that the MDDD reporter becomes stabilized in a Δ*pdf* Δ*fmt* strain of *E. coli*, indicating that reporter stability is formylation dependent (8).

We expressed chromosomally-integrated copies of these reporters using an arabinose-inducible system in a wild-type (WT) and a Δ*ftsH* strain of *P. aeruginosa* to determine the effect of FtsH on reporter stability. If FtsH is required for degradation of Nt-formylated peptides, then the MDDD reporter, which preferentially retains the formyl group, should become stabilized in Δ*ftsH*. Both reporters behaved as expected in the WT strain, with steady-state MDDD levels lower than MVTF, as measured by Western blot (Fig. 1). Remarkably, the MDDD reporter was stabilized relative to MVTF in Δ*ftsH*. As a control, we expressed the MVTF and MDDD reporters in a Δ*pdf* Δ*fmt* strain of *P. aeruginosa*, in which both reporters should be equally stabilized due to the absence of formyltransferase activity. However, chromosomal expression of these reporters was poor in Δ*pdf* Δ*fmt* (Fig. 1), likely due to the slow growth of this strain.

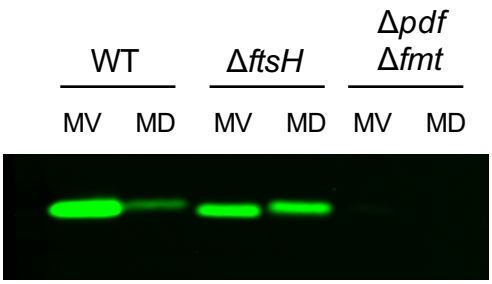

Figure 1. Western blot of fMet/N-degron reporters expressed from the chromosome. Expression was induced for 2 h with 20 mM L-arabinose.

To circumvent the problem of poor expression in Δ*pdf* Δ*fmt*, we expressed both reporters from a high-copy plasmid in the different strains of *P. aeruginosa*. We also included a Δ*hslVU* Δ*lon* Δ*clpXP* strain in these experiments to determine whether any of the other major heat shock proteases might play a role in fMet degradation. Contrary to our previous results, however, expression of a plasmid-encoded MDDD reporter was not stabilized relative to MVTF in Δ*ftsH*, nor was it stabilized in Δ*hslVU* Δ*lon* Δ*clpXP*, indicating that these proteases did not play a role in reporter stability when expressed from a plasmid (Fig. 2). Furthermore, the lower expression of MDDD in Δ*pdf* Δ*fmt* relative to MVTF indicated that this reporter system functioned differently in *P. aeruginosa* than in *E. coli*.

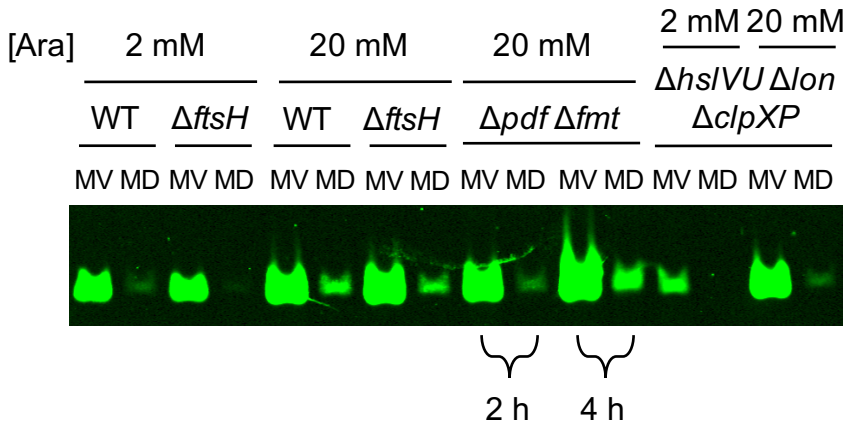

Figure 2. Western blot of fMet/N-degron reporters expressed from a plasmid. Expression was induced for 2 h with either 2 mM or 20 mM L-arabinose in the WT, Δ*ftsH*, and Δ*hslVU* Δ*lon* Δ*clpXP* strains. Expression was induced for either 2 h or 4 h with 20 mM L-arabinose in the Δ*pdf* Δ*fmt* strain.

Discussion

Nt-formylation is universally conserved in bacterial translation, yet our lack of a clear understanding of its role in this process makes it an alluring topic of research. The proposed model by Piatkov *et. al.* (8), implicating Nt-formylation as a marker for protein degradation, provides an intriguing answer to this longstanding puzzle. Indeed, an analogous model was recently described in yeast, with the Psh1 E3 ubiquitin ligase identified as the N-recognin that targets Nt-formylated proteins for proteosomal degradation (15). In yeast, Nt-formylation of cytosolic proteins was shown to increase during stationary phase or upon starvation for specific amino acids, and down-regulation of Nt-formylation caused increased sensitivity of yeast cells to starvation and cold stress. Thus, a physiological role for Nt-formylation in protein turnover has been characterized in both bacteria and eukaryotes.

With this knowledge, we were surprised to find that none of the heat shock proteases appeared to play a role in degradation of Nt-formylated proteins, at least in the plasmid-based system. One explanation for this finding is that degradation is mediated by one or more proteases in *P. aeruginosa* independently of the four major ATP-dependent heat shock proteases tested in this study. Indeed, there are over 70 predicted proteases and protease accessory factors in *P. aeruginosa* (17) and so our finding that FtsH, HslVU, Lon, and ClpXP do not play a role in degradation does not exclude the possibility of protease-dependent degradation altogether. However, if fMet/N-degrons truly exist in bacteria, then the finding that their degradation is independent of these heat shock proteases would be quite unexpected. This is because the heat shock proteases are highly conserved across bacterial phyla, and are also found in mitochondria and chloroplasts. Thus, the possibility that a universally conserved degradation pathway is completely independent of a universally conserved protease network seems unlikely.

An alternative explanation is that the reporter system developed by Piatkov *et. al.* (8) and used in this study functions differently in *P. aeruginosa* than it does in *E. coli*. Our finding that the MDDD reporter was not stabilized in a Δ*pdf* Δ*fmt* strain of *P. aeruginosa* supports this explanation (Fig. 2). Puzzlingly, however, the MDDD reporter was unstable compared to MVTF in both *E. coli* and *P. aeruginosa* (Figs. 1 and 2), indicating that this reporter system displays a similar stability profile in

both organisms, but that stability is formylation-independent in the latter. One explanation for this finding could simply be coincidence. Perhaps the MDDD reporter is unstable in *P. aeruginosa* due to a species-dependent chaperone requirement for proper folding. Another possibility is that MDDD serves as an Nt-degradation signal that is specifically recognized in *P. aeruginosa* irrespective of its formylation status.

Also puzzling is the inconsistency in MDDD reporter stability when expressed from the chromosome *vs*. a plasmid in Δ*ftsH* (Figs. 1 and 2). Stability appeared to be FtsH-dependent when expressed from the chromosome, in line with our hypothesis. However, when expressed from a plasmid, this FtsH-dependent effect disappeared. Of course, one major difference between these two systems is copy number, with each cell having ~1-3 copies in the chromosomal system and ~10s-100s of copies in the plasmid system. However, the expectation is that a degradation pathway is more likely to become overloaded when substrates are expressed from a plasmid, resulting in excess substrate and the loss of a difference in signal, *i.e.* similar expression between the two reporters. This does not explain our results, however, as reporter stability *decreased* in the plasmid-based system compared to the chromosomal system in Δ*ftsH*, whereas the opposite effect would be expected if copy number were the culprit. Perhaps more pertinent than resolving the details of this inconsistency, however, is to first complement Δ*ftsH* in the chromosomal system to confirm that stabilization of MDDD is truly FtsH-dependent.

Although Nt-formylation is not essential for viability, its absence causes severe growth defects in many species, including *P. aeruginosa*. Reduced translational efficiency is no doubt responsible for this effect, but this fails to explain why such a system evolved in the first place. Whether fMet acts as a broadly conserved N-degron in bacteria remains to be determined. One possibility is that Nt-formylation plays different roles in different species, much like the highly conserved proteases tested in this study. Wherever the truth may lie, further research on this enigmatic system should prove fruitful.

Materials and Methods

Construction of fMet/N-degron reporter strains.

The primers, plasmids, and strains used in this study are listed in Table 1. To generate strains expressing the fMet/N-degron reporters from the chromosome, the open reading frames (ORFs) of the MVTF- and MDDD-PpiB reporters were amplified separately from the plasmid pKP458 (8) and joined using Gibson assembly (19) with a pBAD promoter-containing derivative of the shuttle vector pUC18T-mini-Tn*7*T (DKN 1639) that was linearized by PCR (20, 21). The assembled constructs were transformed into *E. coli* DH10B, and transformants were plated on LB plus gentamicin (20 μg/ml). Tetraparental mating was performed to conjugate each construct into *P. aeruginosa* (20). Conjugants were selected on LB agar plus gentamicin (50 μg ml^{-1} for WT or 5 μg ml^{-1} for Δ*ftsH*) and chloramphenicol (10 μg ml^{-1}) plates, and chromosomal integration at the *att*Tn*7* site was confirmed by sequencing.

To generate the strains expressing the fMet/N-degron reporters from a plasmid, the ORFs of the MVTF- and MDDD-PpiB reporters were again amplified separately from the plasmid pKP458 and joined using Gibson assembly with the high-copy plasmid pMQ72 cut with HindIII and SacI (22). The assembled constructs were transformed into *P. aeruginosa* as previously described (23) and transformants were selected on LB plus gentamicin (50 μg ml^{-1} for WT, Δ*pdf* Δ*fmt*, and ΔhslVU Δ*lon* Δ*clpXP* or 5 μg ml^{-1} for Δ*ftsH*).

Sample collection of chromosomally-expressed fMet/N-degron reporters.

All incubations were performed at 37°C, with shaking at 250 rpm for liquid cultures. The WT, Δ*ftsH*, and Δ*pdf* Δ*fmt* strains were streaked onto LB agar plates from glycerol stocks and grown overnight (for WT and Δ*ftsH*) or two days (for Δ*pdf* Δ*fmt*). Cells were resuspended from the plates and diluted to an optical density at 500 nm (OD_{500}) of 0.005 (for WT and Δ*ftsH*) or 0.025 (for Δ*pdf* Δ*fmt*) in 50 ml LB in a 250 ml flask. The 50 ml cultures were incubated for 3.5 h, at which point cells were in the mid-exponential phase of growth. Following incubation, 500 μl of 2 M L-arabinose was added to each culture to achieve a final concentration of 20 mM, and the cultures were incubated an

additional 2 h to induce expression of the reporters. After 2 h of incubation, an OD 10 ml^{-1} equivalence of each culture was pelleted and stored at -80°C to be used later for Western blot.

Sample collection of plasmid-expressed fMet/N-degron reporters.

All incubations were performed at 37°C, with shaking at 250 rpm for liquid cultures. The WT, Δ*ftsH*, Δ*pdf* Δ*fmt*, and Δ*hslVU* Δ*lon* Δ*clpXP* strains carrying the plasmid-expressed reporters were grown overnight in 5 ml LB plus gentamicin (50 μg ml^{-1} for WT, Δ*pdf* Δ*fmt*, and ΔhslVU Δ*lon* Δ*clpXP* or 5 μg ml^{-1} for Δ*ftsH*). Following overnight growth, cells of each strain were washed and diluted to an OD_{500} of 0.5 in duplicate tubes with 5 ml LB. The cultures were incubated for 30 min (1 h for Δ*pdf* Δ*fmt*) and then 5 μl or 50 μl of 2 M L-arabinose was added to one tube of each strain to achieve a final concentration of 2 mM and 20 mM, respectively. The cultures were incubated an additional 2 h to induce expression of the reporters. After 2 h of incubation, an OD 1 ml^{-1} equivalence of each culture was pelleted and stored at -80°C to be used later for Western blot. Samples of the Δ*pdf* Δ*fmt* strains were also taken after 4 h of incubation with L-arabinose.

Western blot.

All Western blots reported in this study were performed by Tri Vu, Caltech. Frozen samples were lysed in 200 μl of lysis buffer (125 mM Tris pH 8, 40% glycerol, 8% LDS, 100 mM DTT, 2 mM EDTA, 0.2% w/v Orange G). Samples were sonicated 3 x for 10 s and centrifuged at 12,000g for 10 min to pellet any precipitate. Samples were then incubated at 70°C for 10 min. Following incubation, samples were fractionated using SDS-PAGE and transferred to either PVDF or nitrocellulose membranes using a ThermoFisher iBlot Dry Blotting System. Membranes were incubated with mouse anti-FLAG M2 antibody (1:10,000) (Sigma) and then with either goat anti-mouse HRP (1:10,000) (Bio-Rad) or goat anti-mouse IRDye800CW (1:10,000) (Li-Cor). Blots were imaged using chemiluminescence (GE healthcare) or using Li-Cor Odyssey Imaging System.

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Chapter 6

CONCLUSIONS

Summary

In this thesis, I utilize functional genetics in tandem with physiological experiments to characterize how *P. aeruginosa* survives during growth arrest. I reveal the genetic determinants of survival under distinct energy-limited states, and show that these determinants can vary depending on the cause of energy limitation, and that they are temporally important over the course of growth arrest. These findings highlight that growth arrest is a dynamic state, with unique challenges that arise under different inducing conditions and at different times. Perhaps among the most pertinent challenges is the accumulation of misfolded proteins, supported by the finding that higher temperature and alkaline pH cause a synergistic effect on cell death during growth arrest. Protein abnormalities during growth arrest could be caused by numerous factors, including misfolding of nascent peptides due to a reduced abundance or activity of co-translational folding chaperones, or misfolding of bulk proteins over time due to sustained and cumulative damage in the absence of growth and turnover. Whatever the exact cause, it is likely that heat shock proteases collectively contribute to maintaining proteostasis during growth arrest. The finding that cells can grow, but not survive, at moderate temperatures in the absence of these proteases indicates that proteostasis becomes an even greater challenge when cells are arrested for growth.

The communal role of these proteases during growth arrest is in contrast to the many varied and specific regulatory roles they play in the physiology of diverse bacteria. Perhaps growth arrest, along with temperature and pH, was an important driver in the evolution of this ancestral proteolytic network to maintain protein quality control. Following its development, this redundant network could have been co-opted by different bacteria to regulate diverse cellular processes unrelated to proteostasis, such as regulation of LPS and phospholipid metabolism by FtsH, the DNA damage response by Lon and HslVU, and cellular replication and division by ClpXP.

Armed with this understanding of the essentiality of proteolysis during growth arrest, and the likely central importance of the growth arrested state in the recalcitrance of many chronic infections, an intriguing avenue for research could be in the development of novel bacterial protease inhibitors. Indeed, the use of therapeutic protease inhibitors has revolutionized the treatment of disease, from HIV to cancer, and novel protease-modulating therapies have recently shown promise in eradicating bacterial infections in animal models. As we contend with the imminent rise of extensively drug-resistant bacterial pathogens, perhaps it is prudent to place greater emphasis on the development of therapies that target this universally conserved class of proteolytic machines.

Future Directions

Functional genomics in multiple protease mutant backgrounds.

My data suggests that multiple heat shock proteases contribute individually to promote survival during growth arrest. However, whether the proteases all perform a common molecular function during growth arrest is not entirely clear. For instance, the functional redundancy of *hslVU* and *ftsH* during growth arrest indicates that these two proteases likely perform a common molecular function, while the non-redundant roles of *clpXP* and *ftsH* makes it difficult to ascertain their functional overlap. One way to answer this question would be to perform Tn-seq in the Δ*ftsH,* Δ*clpXP* and the Δ*ftsH* Δ*clpXP* strain backgrounds to determine the extent of functional overlap between these proteases during growth and growth arrest. Functional overlap and epistasis analysis of interacting genes could identify common molecular functions during growth arrest, as well as reveal novel interactions between these proteases during other types of stress.

Relevance of proteostasis in different ecological contexts.

P. aeruginosa is found ubiquitously in the environment as part of biofilm communities. I have observed a generalized defect for Δ*ftsH* in forming biofilms using different *in vitro* and *in vivo* models. I also observe rapid loss of viability in colony biofilms of Δ*ftsH* following an extended period of growth, highlighting the importance of growth arrest in an environment outside of the test tube. These findings encourage a more thorough characterization of the role of proteases in biofilm formation and persistence.

P. aeruginosa is also a frequent cause of hospital-acquired infections and infects the lungs of patients with cystic fibrosis. With the finding that heat shock proteases are essential for the survival of this organism during growth arrest, an investigation of the role of proteases in infectious contexts could be illuminating.

Relevance of proteostasis in growth arrest survival of different organisms.

One limitation of my work is that the conclusions are all based on experiments performed using a single bacterial strain. Although this does not detract from their importance, these conclusions could be bolstered by demonstrating similar phenotypes using different strains of *P. aeruginosa* as well as different species of bacteria. A key conclusion of this work is that proteostasis is a universal challenge that bacteria must contend with during growth arrest and that proteases are essential for responding to this challenge. Observing similar phenotypes using knockout strains of the heat shock proteases in different organisms would help cement this conclusion. My expectation is that the heat shock proteases will functionally overlap to promote survival regardless of the species background, but that the details of this overlap, *i.e.* which proteases are more or less important for survival, will vary depending on the organism.

Role of ATP in mediating proteostasis during growth arrest.

All four of the heat shock proteases shown to be important for survival act in conjunction with an ATPase protein/domain that recognizes and unfolds protein substrates for degradation. This results in the counterintuitive situation where an energy-requiring process becomes extremely important for survival during an energy-limited state. However, the proteases do not directly require ATP for hydrolysis of the polypeptide chain, and can recognize and degrade some weakly folding substrates independently of an ATPase protein/domain. The possibility exists that there is no absolute ATP-requirement for proteolysis during growth arrest. If so, this would suggest that the proteases act in a nonspecific manner to degrade misfolded proteins (which are more easily accessible for degradation) without the need for an ATPase in the recognition and unfolding process. This could also indicate that the protein folding chaperones like DnaK/J, GrpE, ClpB etc. (which have an absolute requirement for ATP) might be dispensable for survival. A simple experiment to test this hypothesis

would be to make a point mutation in the highly conserved ATPase domain of FtsH and observe its effect on survival.

A p p e n d i x

A PROTOCOL FOR EFFICIENT TRANSPOSON LIBRARY GENERATION IN *P. AERUGINOSA* STRAIN UCBPP-PA14

Abstract

Generation of highly-saturated transposon libraries is critical for comprehensively identifying genetic determinants of fitness using Tn-seq. Here, I outline a simple and rapid protocol for generating a saturated transposon library (~400,000 unique mutants) in *P. aeruginosa* strain UCBPP-PA14. Library generation requires ~3-4 h of hands-on time, with the majority of the time spent plating and harvesting the transposon mutant colonies. The same protocol can be used to generate saturated libraries in different mutant strains of PA14, although the efficiency of mutagenesis should be re-assessed when performing this protocol with any new mutant.

Protocol

Day 1 (Pregrowth).

- Streak a plate of the recipient *P. aeruginosa* strain UCBB-PA14 (or any derivative) from glycerol stocks on LB agar at 37°C.

Day 2 (Pregrowth).

- Start an overnight culture of the recipient strain from the streak plate in 5 ml liquid LB at 37°C with shaking.

- Streak a plate of the donor *E. coli* SM10λ*pir* carrying the transposon-bearing plasmid pIT2 from glycerol stocks on LB agar plus carbenicillin (100 μg ml^{-1}) at 37°C.

Day 3 (Conjugation and plating).

- Place a 0.2 μm filter disc onto an LB agar plate and prewarm the plate at 37°C.

- Resuspend the entire streak plate of *E. coli* SM10λ*pir* in 1 ml LB by pipetting (do not vortex).
- Measure OD_{500} of the SM10 suspension (dilute 10 µl of suspended cells in 990 µl LB). The suspension should be at least an OD of 5.
- Spin the SM10 suspension 5 min at 5,000 g and pipette off the supernatant.
- Resuspend the pellet in 1 ml LB and spin again 5 min at 5,000 g (wash step).
- Pipette off the supernatant and resuspend the pellet in a volume of LB to achieve an OD of 160. For example, if the original 1 ml suspension has an OD of 5, then the pellet should be resuspended in 31.25 µl of LB.
- Measure OD_{500} of the overnight culture of the recipient strain.
- Add an OD 2.5 ml^{-1} equivalence of the overnight culture to a 2 ml tube. For example, if the overnight culture has an OD of 4, then add 625 µl of the culture to the tube.
- Spin the tube 3 min at 16000 g and pipette off the supernatant.
- Resuspend the pellet in 50 µl of LB.
- Mix an equal volume of the concentrated SM10 donor (OD 160) with an equal volume of the concentrated recipient by pipetting. The total volume of the mixture should be at least 50 µl.
- Pipette 50 µl of the donor:recipient mixture as a pooled spot onto the filter disc placed on the prewarmed LB agar plate and dry the spot for ~15 min in a flow hood. Ensure that the spot does not spread excessively across the disc when moving the plate into and out of the flow hood. If possible, pipette the spot onto the plate in the flow hood. It is critical that the mating spot is completely dried on the disc for efficient conjugation.
- After drying, incubate the plate 2.5 h exact at 37°C.

- Following incubation, thoroughly resuspend the mating spot in the desired volume of LB by vigorous vortexing. Assuming ~400,000 ex-conjugants per mating spot, resuspending the spot in 8 ml of LB yields ~5,000 mutants per 100 µl.

- Aliquot 100 µl of the resuspension onto LB agar plates plus tetracycline (60 µg ml^{-1}) and chloramphenicol (10 µg ml^{-1}) and incubate the plates at 37°C for a minimum of 24 h. The tetracycline selects for cells of the recipient PA14 that received the transposon, and the chloramphenicol counter-selects against the donor SM10. 100 µl aliquots of an 8 ml resuspension produces ~80 plates. A more concentrated resuspension can be plated if isolated colonies are not required. This reduces the time spent plating and harvesting cells.

Day 4 (Harvesting).

- Add >100 µl of LB plus 15% glycerol to each plate and resuspend the cells using a sterile cotton swab.

- Pool the resuspended cells from all plates into a 50 ml tube and vortex the tube thoroughly to ensure complete mixing.

- Aliquot 0.5-1 ml of the pooled library into 2 ml tubes and store the aliquots at -80°C. Depending on the volume used for resuspension, a pooled library should produce >50 1 ml aliquots. The pooled library can be diluted or concentrated as desired before aliquotting into the 2 ml tubes.