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STATIONARY-PHASE PHYSIOLOGY

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■ **Abstract** Bacteria enjoy an infinite capacity for reproduction as long as they reside in an environment supporting growth. However, their rapid growth and efficient metabolism ultimately results in depletion of growth-supporting substrates and the population of cells enters a phase defined as the stationary phase of growth. In this phase, their reproductive ability is gradually lost. The molecular mechanism underlying this cellular degeneration has not been fully deciphered. Still, recent analysis of the physiology and molecular biology of stationary-phase *E. coli* cells has revealed interesting similarities to the aging process of higher organisms. The similarities include increased oxidation of cellular constituents and its target specificity, the role of antioxidants and oxygen tension in determining life span, and an apparent trade-off between activities related to reproduction and survival.

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INTRODUCTION

Most bacterial species that have been isolated, cultivated, and analyzed in the laboratory reproduce continually as vegetative cells. The speed at which this vegetative growth and reproduction can take place is breathtaking. For example, the process of producing an entire functional *Escherichia coli* cell from a suitable carbon source, amino acids, and some minerals takes less then 20 min at 37°C. As pointed out by Postgate (65), one single bacterial cell can produce a biomass equivalent to the mass of earth in about three days. Not surprisingly, the physiological and molecular mechanisms behind this remarkable efficiency of metabolism and reproduction have been a major subject matter of bacteriology. Indeed, it can be said that studies of growth and how its processes are coordinated serve as the core of bacterial physiology. However, bacterial growth cannot proceed ad infinitum. Eventually, a required nutrient becomes exhausted (or the concentration of toxic waste products becomes too high), resulting in the cessation of reproduction and growth. The bacteria are then said to enter stationary phase. During transition from exponential growth to stationary phase, growth becomes unbalanced, i.e., the synthesis of different macromolecules and cell constituents do not slow down synchronically. For example, the DNA/protein ratio usually increases during transition to stationary phase and because cell division continues after the cessation of most macromolecular syntheses, the cells in stationary phase become smaller.

Stationary phase is an operational definition and does not describe a specific and fixed physiological state or response of the bacteria. Cells of a stationary-phase culture differ chemically from the time of sampling, and cells that have entered stationary phase because of phosphate rather than, for example, glucose exhaustion are vastly different in composition and pattern of gene expression. Unfortunately, a confusing definition of stationary phase has recently entered microbiology that states that stationary phase is a specific developmental process and a physiological response. Thus, one can come across statements to the effect that mutant X failed to enter stationary phase, which, when using the correct definition of stationary phase, means that the mutant fails to stop growing in the absence of nutrients! This is of course unfeasible and the author rather means that the mutant failed to express a certain gene or failed to exhibit specific alterations in cellular constituents that the author regards a hallmark of the stationary-phase response. This confounding definition of "stationary phase" should be avoided and is not used in this review.

BACTERIAL CELL AGE AND DEATH

A philosophically interesting peculiarity of exponentially growing bacterial populations is that there is no adult form of bacterial cells and the population is not age structured. The raison d'être is that binary fission proceeds in a symmetrical fashion with a nonconservative dispersion of both undamaged and damaged constituents. This has led to the conclusion that bacteria are essentially immortal.

In other words, their capacity for reproduction appears limitless as long as the environment supports growth. However, the statement that prokaryotes are examples of organisms that have evolved indefinitely long life spans (70) is misleading because it can be argued that the bacterium as an individual ceases to exist when cytokinesis is completed. Thus, we can logically analyze and discuss an exponentially growing cell's generation time, but the concept of a life span for such dividing individual bacteria is obscure.

On the other hand, the terms life span, longevity, and bacterial age can be useful when considering cells entering the stationary phase. Progressive physiological alterations can be followed as a function of the chronological age of individual stationary-phase cells, and the life span of such cells is far from indefinitely long. For example, the average life span, measured as the time of sustained reproductive ability, of stationary-phase *E. coli* cells is around 3 to 5 days when starved for exogenous carbon (4, 19, 47). This loss in plating efficiency has been described in microbial textbooks as the death phase of the bacterial growth cycle, and such death has been argued to be the nearest bacteria come to a "natural" death of the kind familiar to higher organisms (64). Even so, it is important to stress that it is a form of accidental death conceptually distinct from mandatory aging in higher organisms. Therefore, the term conditional senescence was proposed to make such a distinction (52–55). All the same, it has turned out that many molecular alterations occurring during conditional senescence of stationary-phase bacteria are similar to those of aging organisms (54). These alterations and their physiological consequences are summarized in this review.

STATIONARY-PHASE MORPHOLOGY

The size of most bacteria is considerably reduced upon entry into stationary phase. This size reduction is the result of two discrete processes referred to as reductive division and dwarfing.

Reductive Division

Reductive division results from the fact that initiated rounds of DNA replication and cell division are completed in the absence of further growth upon entry of cells into stationary phase. The number of replication sites per cells increases with the growth rate, and accordingly, the number of cells produced during reductive division is determined by the growth rate of cells achieved prior to the depletion of a required nutrient. In some bacteria, a new round of DNA replication appears to be initiated also during the early stationary phase and this further amplifies the number of cells produced during reductive division (56). However, stationary-phase cells do not divide and segregate their genetic material until all cells end up with one single chromosome. In fact, with respect to chromosome content, a stationary-phase *E. coli* population is highly heterogeneous. When *E. coli* cells are grown in a rich complex medium (i.e., Luria broth), the stationary-phase cells end

up with 1, 2, 4, or 8 chromosome equivalents, with cells containing 2 or 4 the most common (3). Cells grown in a glucose minimal medium, on the other hand, end up with mostly 1 or 2 chromosome equivalents in stationary phase (3). The reason for chromosome content heterogeneity may be that some cells have not reached a sufficient distance between the nucleoids during growth to allow the cell division machinery to act or that replication termination and decatenation are adversely affected in growth-arrested stationary-phase cells (3). Whatever the reason, the fact that the cells of a stationary-phase population harbor different quantities of genetic material, i.e., different gene copies, should be considered when discussing stationary-phase survival, mutation frequencies, and genome instability.

The cells produced during reductive division are often coccoid in their appearance. This morphology depends, at least in part, on the *bolA* morphogene, which is increasingly expressed in early stationary phase in an *rpoS*-dependent manner (42, 71). BolA is most likely affecting cellular morphology by controlling transcription of *dacA* (encoding penicillin binding protein 5, PBP5), *dacC* (encoding PBP6), and *ampC* (encoding a class C β-lactamase; 71). However, the role of BolA in controlling cell morphology does not seem restricted to stationary phase (71).

Dwarfing

Reductive division is an adaptive process that may be beneficial during starvation conditions because the surface/volume ratio of the cells is increased. Even so, it should be stressed that reductive division is not a starvation-induced activity, because the division of cells during the early stages of stationary phase is merely a completion of events initiated during growth (56). Dwarfing, in contrast, is a phenomenon triggered by starvation. Dwarfing is the continuous size reduction of cells observed after the completion of reductive division. It is the result of degradation of endogenous material and this degradation includes the cell envelope, especially the cytoplasmic membrane and cell wall. In some gram-negative bacteria, e.g., *E. coli*, the outer membrane, in contrast to the cytoplasmic membrane, is not digested and does not shrink in stationary phase and this results in an enlarged periplasm (69). Other gram-negative rods, e.g., *Pseudomonas* and *Enterobacter*, produce and release outer-membrane vesicles during dwarfing and the outer membrane and cytoplasmic membrane shrink to the same extent. Thus, the size and integrity of the periplasm is maintained in these bacterial species (36).

STATIONARY-PHASE AUTOPHAGY

Dwarfing is a form of self-digestion and is the bacterial equivalent to autophagy in eukaryotes. Autophagy, which in part works through the sequestration and delivery of cargo to the lysosomes, is the major route for degradation of long-lived proteins and organelles in eukaryotic cells. It is an integral part of the response of both lower and higher eukaryotes to starvation: For example, dauer formation (starvation-induced developmental arrest) in *Caenorhabditis elegans* requires the

activity of several conserved autophagy genes. In addition, proper autophagy activity is essential for life span extension of aging *C. elegans* (48) and in delaying leaf senescence in plants (29). Similarly, self-digestion is an important survival mechanism of starved bacteria.

FadR-Dependent Autophagy

The increased expression of genes of the FadR regulon during entry of cells into stationary phase (20) suggests that this regulon, apart from being required for growth on exogenous long-chain fatty acids, may be involved in providing the growth-arrested bacteria with endogenous carbon and energy during digestion of membrane constituents. The *E. coli fadR* gene product functions as a repressor of many unlinked genes and operons encoding proteins involved in long-chain fatty acid transport, activation, and β-oxidation. When acting as a repressor, FadR binds specific operator sites upstream of the fatty acid degradative (*fad*) gene-coding sequences to turn off transcription (13). Long-chain acyl-CoA thioesters (C_{14}–C_{18}), generated from exogenous fatty acids, are the effector molecules that regulate fatty acid metabolism by binding directly to FadR (13). The FadR-acyl-CoA thioester complex is unable to bind the operator sequence, resulting in derepression of the *fad* genes (13).

The derepression of the regulon would make physiological sense also during carbon starvation in that fatty acids generated from degradation of membrane lipids during dwarfing could be scavenged by the activity of the acyl-CoA synthetase (product of the *fadD* gene) to generate acyl-CoA. Acyl-CoA is further catabolized by the β-oxidation enzymes (encoded by *fadBA*, *fadE*, *fadFG*, and *fadH*) to generate acetyl-CoA, a source of carbon and energy. In support of such a physiological role for β-oxidation and the FadR regulon during stasis, Spector et al. (75) showed that *fadF* (encoding the acyl-CoA dehydrogenase) mutants, in which β-oxidation levels are undetectable, survive carbon starvation poorly.

Emergency Derepression

The fact that *fad* genes in both *E. coli* and *Salmonella typhimurium* are derepressed in stationary phase led to the assumption that FadR is inactivated as a repressor during stasis (20, 75). However, it was later shown that FadR is active in carbon-starvation-induced stationary phase and that the *fad* genes are rather induced by a mechanism that overrides FadR repression (40). This mechanism, called emergency derepression, requires ppGpp programming of the RNA polymerase and, for some *fad* genes, concomitant cAMP/CAP activation (40). Emergency derepression is an essential regulatory mechanism because in its absence the β-oxidation pathway is not induced in starved cells, leading to accelerated die-off (20, 75). It is not clear exactly how a ppGpp-programmed RNA polymerase overcomes repression by FadR, but this ability of the polymerase depends on the strength of the FadR operator preceeding the structural gene (40). Emergency derepression cannot overcome repression at the strongest FadR operators (40).

The fact that FadR remains active during starvation strongly argues against the accumulation of long-chain fatty acyl-CoAs in the stationary-phase cell. Instead, the likely substrates for β-oxidation during autophagy are short- or medium-chain fatty acyl-CoAs. Short- and medium-chain fatty acyl-CoAs do not bind or inactivate FadR, but the emergency derepression mechanism allows the stationary-phase wild-type cells to overcome this physiological dilemma.

Catabolic Control

Apart from derepression of *fad* genes, dwarfing of carbon-starved *E. coli* cells is accompanied by an increased synthesis of glycolysis enzymes, and pyruvate formate lyase, phospho-transacetylase, and acetate kinase concomitantly with a reduced production of TCA cycle enzymes (51). The two-component response regulator ArcA is required for the decreased synthesis of NADH/$FADH_2$-generating TCA cycle enzymes in carbon-starved stationary-phase cells; an *arcA* deletion mutant fails to decrease the synthesis of malate dehydrogenase, isocitrate dehydrogenase, lipoamide dehydrogenase E3, and succinate dehydrogenase during stasis, whereas the increased production of the glycolysis enzymes phosphoglycerate mutase and pyruvate kinase is unaffected (57). The ArcA-dependent response of glucose-starved cells results in a reduced respiratory activity (57). This modulation in catabolic activities appears important, since cells lacking ArcA fail to perform reductive division early during starvation and lose viability at an accelerated rate after a few days in stationary phase (57).

Gene expression profiling analysis from Conway's laboratory demonstrated that repression of aerobic metabolism upon stationary phase correlates with upregulation of *arcA* (10). Further, ArcA, via its sensor component ArcB, may be activated in stationary phase by a mechanism encompassing redox control of the quinone pool. ArcB monitors the oxidation/reduction status of the cell by interacting with the membrane quinone pool; under conditions suitable for aerobic respiration the quinone pool is primarily oxidized and this exerts a negative effect on ArcB activity (25). Interestingly, the gene encoding quinone oxidoreductase, *qor*, is markedly upregulated in stationary-phase cells. It was argued that this may result in an increased ratio of reduced to oxidized quinones, which in turn could activate the ArcB/ArcA system and result in repression of the ArcA regulon (10).

The role of an activated ArcB/ArcA system in stasis survival may be severalfold. First, the reduced production and activity of the aerobic respiratory apparatus during starvation may prevent an uncontrolled drainage of endogenous reserves during autophagy. The rate of degradation of endogenous carbon energy sources, such as membrane lipids, is most likely feedback regulated by the activity of the catabolic apparatus. Thus, an uncontrolled respiratory activity, as seen in the Δ*arcA* mutant, may drain these sources and seriously debilitate the membrane, resulting in loss of cell integrity.

Second, the reduced production of respiratory substrate and components of the aerobic respiratory apparatus during starvation may be a defense mechanism

mustered by the cell to protect itself against potentially damaging effects of reactive oxygen species (ROS) produced by the electron transport chain. This notion is supported by data demonstrating that the accelerated die-off of Δ*arcA* mutants starved for glucose could be suppressed by overproducing the superoxide dismutase SodA (57).

Peptidase-Dependent Autophagy

Work in the Matin laboratory demonstrated that the response of *E. coli* to starvation-induced growth arrest encompassed de novo protein synthesis in the absence of exogenous carbon (28). The amino acids required for this de novo protein synthesis are provided by peptidase-dependent autophagy, and mutations that reduced the peptidase activity drastically reduced stationary-phase survival (69). In accordance with the results obtained, Matin (47) proposed that ongoing protein synthesis is a prerequisite for starvation survival and that the amino acids required are provided by protein turnover. The fact that cells lacking functional ClpAP or ClpXP proteases exhibit an accelerated die-off during extended stationary phase further supports the role of protein degradation in starvation survival (80).

A question of interest is whether the peptidase-dependent autophagy of stationary-phase *E. coli* cells affects specific proteins in a manner similar to eukaryotic autophagy involving the delivery of proteins to the lysosomes. One possible mechanism for such degradation of specific polypeptides in *E. coli* might be the oxidation of specific proteins in stationary-phase cells, which renders the oxidized protein more susceptible to proteolytic attack (14; and see below).

THE STATIONARY-PHASE PROTEOME

Sigmas and Stationary-Phase Cross Protection

Genes expressed early during starvation are the likely candidates required for starvation survival (47, 68). This notion has been confirmed experimentally and many networks and individual genes of importance for stasis survival are now genetically identified (30, 31, 37, 42, 50, 57, 58). Many of these genes encode proteins with specific roles in protecting the cell against external stresses, e.g., heat, oxidants, osmotic challenge, and exposure to toxic chemicals (16, 33, 47). Consequently, growth-arrested cells are highly resistant to a variety of secondary stresses, a phenomenon known as stasis-induced cross protection (33, 47). Work in Hengge's laboratory demonstrated that this cross protection largely relies on one single regulator, the sigma factor σ^S (42). The σ^S transcription factor accumulates, binds, and directs the RNA polymerase to over 50 genes upon conditions of cellular starvation and stress (31). The members of the regulon are a diverse set of proteins that functions relate to stress management, central metabolism, rearrangements of cell morphology, and virulence (31). Mutants lacking σ^S exhibit accelerated senescence during conditions of growth arrest (42), and markedly elevated levels of oxidized proteins (15, 16).

Other global regulators of transcription, including the heat shock sigma factors σ^H and σ^E and the oxidative stress response regulators OxyR and SoxRS, work in concert with σ^S during stasis. In *Salmonella*, both σ^S and σ^E are required for protection against oxidative damage in stationary phase. In addition, nearly all cells of a *Salmonella* population lacking both σ^E and σ^S lose viability within the first 24 h of stationary phase, but the plating efficiency of these mutants is completely preserved under anaerobic stationary-phase conditions (78). Also, the loss of viability of wild-type *E. coli* cells during the first 10 days of stasis can be completely counteracted by omitting oxygen (16). In addition, disruption of *acnA* encoding aconitase enhanced the survival of stationary-phase *Staphylococcus aureus* cells about 100-fold, a phenomenon suggested to result from a reduced oxidative load (74). Thus, the accumulated data suggest that oxidation damage may be the Achilles' heel of stationary-phase bacterial cells and that there is an increased demand for oxidation management in cells subjected to nutrient starvation. Indeed, a large number of genes and regulons induced by stasis are part of such an oxidative defense machinery (17, 31, 47, 55, 66, 67, 79).

Oxidative Modification of the Stationary-Phase Proteome

The oxidative defense machinery obviously fails to fully combat starvation-induced oxidation, because oxidative modifications of proteins, such as carbonylation and illegitimate disulfide bond formation, have been demonstrated to increase during stasis in wild-type *E. coli* cells (15, 16). Stasis- and stress-induced oxidation affects specific *E. coli* proteins, e.g., the Hsp70 chaperone DnaK, the histone-like protein H-NS, the universal protein UspA, elongation factors EF-Tu and EF-G, glutamine synthetase, glutamate synthase, aconitase, malate dehydrogenase, and pyruvate kinase (15, 16, 77). Some of these proteins are specifically carbonylated also in oxidation-stressed yeast cells (8), aging flies (72, 84), and in Alzheimer's disease brain (9). On the basis of the identity of the oxidized proteins, several different cell processes are targets for stasis-induced damage; these functions include peptide chain elongation, protein folding and reconstruction, large-scale DNA organization, gene expression, central carbon catabolism, and general stress protection (15, 16). The molecular basis for the apparent sensitivity of some proteins to oxidation is not known. Metal-catalyzed oxidation may be an intrinsic problem for proteins containing, for example, iron and manganese, and some proteins (e.g., enzymes of Krebs cycle and electron transport chain) may be oxidized mainly because they are located near sites generating ROS.

In contrast to the oxidation of relatively few specific target proteins in cells that have progressed into stasis-induced senescence, the early stage of stasis is characterized by a general and sudden burst of oxidation of a large number of proteins (4). The reason for this immediate and general oxidation of proteins is beginning to be understood and highlights a hitherto unknown link between ribosomal proofreading and protein oxidation. Proteomics demonstrated that this sudden increase in protein oxidation is strongly associated with the production of aberrant

protein isoforms, seen as protein stuttering on two-dimensional gels (4). The phenomenon protein stuttering is the result of erroneous incorporation of amino acids into proteins and can be detected on autoradiograms of two-dimensional gels as satellite spots with molecular weights similar to the authentic protein but separate from it in the isoelectric focusing dimension. The level of protein carbonylation increases also upon treatment of cells with antibiotics, e.g., streptomycin and puromycin, and mutations, causing increased mistranslation (14). During these treatments, the rate of superoxide production and the activity of the superoxide dismutases and catalases were unchanged and the expression of oxidative stress defense genes did not increase (14). In other words, protein oxidation of aberrant proteins does not appear to be sensed by the oxidative defense regulons and does not require increased generation of ROS.

Frameshifting (5, 82), missense errors (59), and stop codon readthrough (4) increase in response to stasis in *E. coli* cells. This fact, together with results showing that aberrant proteins are more susceptible to oxidation, raises the possibility that the swift carbonylation in nonproliferating cells may be caused by an increased mistranslation. In line with this notion, protein carbonylation is drastically attenuated in the early stages of stationary phase in *rpsL141* mutants, which harbor intrinsically hyperaccurate ribosomes (4). Thus, the elevated oxidation of proteins in growth-arrested cells may be due to an increased availability of substrates (aberrant proteins) available for oxidative attack. These substrates surge during stasis owing to a reduced fidelity of the translational apparatus. It is not clear why aberrant proteins are more susceptible to carbonylation, but it is possible that a slight misfolding of the corrupted polypeptide exposes oxidation-sensitive targets that are normally hidden during the coupled translation-folding process.

One consequence of protein carbonylation is that the targeted protein becomes susceptible to proteolysis (14). Thus, one could argue that carbonylation may act as a mechanism, intimately connected to the peptidase-dependent autophagy, that targets proteins no longer required during conditions of growth arrest. However, this notion may be incorrect because carbonylation targets proteins such as UspA, DnaK, and HN-S, all of which are important for stationary-phase survival. On the other hand, protein degradation affects specific proteins in stationary-phase cells. For example, some proteins, including LeuA, TrxB, GdhA, GlnA, MetK, and alkyl hydroperoxide reductase, are significantly more abundant in stationary-phase cells lacking proteases ClpAP or ClpXP (80). These proteins may be substrates for ClpAP or ClpXP in stationary-phase cells. In fact, Weichart et al. (80) suggest that the abundance of most major growth-phase-regulated proteins in *E. coli* is regulated by the activity of the ClpAP and ClpXP proteases.

Protein Oxidation and Metabolic Activity

The rate of living hypothesis, in its updated form, predicts that the higher the metabolic activity (i.e., respiration) in a nongrowing system, the higher the protein oxidation and the shorter the life span. However, data concerning nonproliferating

E. coli and yeast G_0 cells do not support this notion because the correlation between respiratory activity and protein oxidation in growth-arrested stationary-phase cells is poor or nonexistent in the set of starvation experiments performed (1, 4). For example, phosphate-starved cells exhibited the highest rates of respiration during growth arrest, yet protein oxidation is only marginally increased. In addition, the culture half-life is longer in the phosphate-starved cultures despite the continued high metabolic activity in these nonproliferating cells (4). Again, this result is at odds with the rate of living hypothesis but not the free radical hypothesis of aging, since phosphate-starved cells exhibited very low levels of oxidized proteins. Thus, the rate of respiration in a nongrowing microbial system does not, per se, determine the degree of oxidative damage to the proteins of the system. Interestingly, the translational fidelity of phosphate-starved cells was higher than that of carbon- and nitrogen-starved cells, i.e., the levels of oxidation in *E. coli* cells starved for different nutrients correlated with translational errors rather than metabolic activity (4).

Protein Oxidation and Error Propagation

Orgel (60) suggested that an error feedback loop in macromolecular synthesis might cause an irreversible and exponential increase in error levels, leading to an "error catastrophe." It was argued that errors in the sequences of proteins, which themselves functioned in protein synthesis (e.g., ribosomal proteins, elongation factors), might lead to additional errors and an inexorable decay of translational accuracy, and as a result, the cell or organism ages. The hypothesis is based on the assumption that aberrant proteins escape degradation and are incorporated into functional ribosomes. However, the use of *E. coli* as a model system has shown that increased mistranslation does not cause a progressive decay in the proofreading capacity of the ribosomes (23). The susceptibility of mistranslated proteins to carbonylation may provide a molecular explanation for this, because oxidized proteins are more susceptible to proteolytic degradation than their nonoxidized counterparts (7, 14). Thus, the carbonylation of mistranslated proteins may denote a quality control mechanism that effectively reduces incorporation of erroneous proteins into mature machines (e.g., ribosomes, and RNA and DNA polymerases) involved in information transfer. The reduced translation fidelity of growth-arrested cells is most likely the result of ribosomes being increasingly starved for charged tRNAs (empty A-sites are slippery) rather than being intrinsically error-prone.

Protein Oxidation and Life Expectancy

In a few cases, the levels of oxidatively damaged proteins are associated with the physiological age or life expectancy of an organism rather than with its chronological age. For example, carbonyl levels are higher in crawlers (low life expectancy) than in fliers in a cohort of house flies of the same chronological age (73). Similarly, using in situ detection of protein oxidation in single *E. coli* cells and a density gradient centrifugation technique to separate culturable and nonculturable cells of the

same chronological age, Desnues et al. (12) demonstrated that protein oxidation is highly heterogeneous in the population and that proteins of the nonculturable cell population exhibited markedly higher levels of irreversible oxidative damage than culturable ones.

A question of interest is how this asymmetry in population damage is generated. Recent publications demonstrate that the expression of more genes than previously anticipated is affected during progression through the bacterial division cycle (43, 81). A sudden arrest of growth at a time in the cycle when specific gene products, e.g., superoxide dismutases A and B, are present at low levels could generate a subpopulation of cells experiencing increased damage during prolonged stasis. Alternatively, the difference in chromosome numbers, and consequently gene dosage, in stationary-phase cells (3) could result in different levels of stress defense proteins. There is no direct evidence for these ideas but it is interesting to note that the abundance of Sod is much lower in the nonculturable *E. coli* cells and that the pattern of protein carbonylation is similar in these cells and cells lacking cytoplasmic Sod activity. For example, self-inflicted oxidation of proteins is enhanced in both *sod* mutants (16) and nonculturable cells (12). Moreover, the target specificity of protein oxidation (e.g., H-NS, GltD, and FabB) is similar in *sod*-deficient cells and nonculturable wild-type cells. Also, the elevated expression of specific stress regulons in nonculturable cells can be mimicked by a diminished Sod activity (12). Last, *sodA sodB* mutants lose culturability at an elevated rate during stasis (6, 16). Thus, it is possible that the loss of reproductive ability of some cells entering stationary phase is linked to the abundance of Sod in these individual cells. If so, the longevity of stationary-phase *E. coli* cells, similar to that in aging fruit flies (76), might be associated with the cellular availability of Sod.

TRANSCRIPTIONAL TRADE-OFFS

Evolutionary models of aging in animals propose that there is a trade-off between the resources an organism devotes to reproduction and growth and those devoted to cellular maintenance and repair (35, 61, 70). As a result, an optimal life history, inevitability, entails an insufficient ability to resist stress. There are examples of such a trade-off also in *E. coli*. For example, Kurland & Mikkola (38) found that, in general, natural and laboratory *E. coli* isolates exhibiting fast growth and efficient ribosomes died more rapidly in stationary phase. Continuous cultivation in chemostats effectively selected for cells with faster growth rates and increased efficiency of translation. However, the trade-off for this increased rate of reproduction was a reduced ability to withstand starvation-induced stasis (38).

Sigma Factor Competition

More recently, the trade-off, or antagonism, phenomenon of *E. coli* has been linked to the status of the *rpoS* gene. *rpoS* is polymorphic in many natural and laboratory *E. coli* strains, and there is a selective advantage of losing σ^S function during

growth under carbon-limited conditions in a chemostat (22, 49). This loss of σ^S is accompanied by an elevated expression of genes contributing to fitness, e.g., genes encoding glucose-uptake systems that require the housekeeping sigma factor σ^{70} (22, 49). However, increased fitness is traded for stasis survival and stress resistance, since σ^S is a master regulator required for these functions. This is an example of antagonistic pleiotropy, in which mutations that are beneficial for reproduction may be harmful during old age, stasis, or stress. This kind of antagonistic pleiotropy has been suggested to be a major factor in the evolution of aging (83).

A molecular model for this antagonism, or trade-off, in *E. coli* has recently been suggested that includes sigma factor competition for RNA polymerase binding (34, 44, 55). This model explains how the quality of the environment can be sensed and translated to intracellular signals that control the allocation of resources between reproductive and maintenance activities (55). The model argues that the conflict between proliferation activities and maintenance could stem from the fact that RNA polymerase may be limiting for transcription and that sigma factors compete for polymerase binding. Even a subtle overproduction of σ^{70} effectively shuts down transcription from genes requiring σ^S, and the cells become stress sensitive (21). Also, overproduction of σ^S attenuates the expression of genes requiring σ^{70} (21). The antagonism between sigma factors is highly regulated and is dictated by the nutritional quality of the environment and the nucleotide ppGpp (34). Mutants lacking ppGpp fail to induce σ^S-dependent genes upon the imposition of stress and starvation, a phenomenon that can be explained by the fact that σ^S requires ppGpp for its own production (24, 41). However, σ^S-dependent genes require ppGpp even in the presence of wild-type levels of σ^S (39), indicating that ppGpp is required for both σ^S production and activity. This activity appears to be linked to ppGpp, facilitating the ability of σ^S to compete with σ^{70} for RNA polymerase binding (34). Thus, ppGpp is priming the RNA polymerase in accordance with environmental signals so that the transcriptional apparatus is primarily occupied with transcription of σ^{70}-dependent housekeeping genes as long as the ppGpp levels are low, which signals that the nutritional status of the environment is favorable for reproduction. When conditions are less favorable, ppGpp levels are elevated and this allows the alternative sigma factors to work in concert with σ^{70} by shifting their relative competitiveness (34). In the sigma factor competition scenario, the antagonistic pleiotropy observed by Notley-McRobb et al. (49) and the acquisition of *rpoS* polymorphism (22) could be explained by the fact that more σ^{70} proteins are allowed to bind RNA polymerase core in the absence of competing σ^S, and more resources are thereby directed toward growth and reproduction-related activities. A similar effect could be achieved by mutations lowering ppGpp levels in the cell, which would favor σ^{70}-dependent activities related to reproduction. Indeed, a recent study of gene expression after 20,000 generations of evolution in *E. coli* demonstrated that a nonsynonymous mutation in *spoT* occurred during evolution and that this mutation increased fitness (11). The data are consistent with reduced levels of ppGpp in this mutant, although this was not measured directly (11).

The trade-off model also provides a possible explanation for the failure of cells to fully combat stasis-induced deterioration, e.g., oxidative damage to proteins and other macromolecules. The model argues that the housekeeping sigma factor competes with alternative sigma factors even during severe stress and growth arrest. As a consequence, a certain fraction of the cell's resources is therefore allocated to activities related to proliferation rather than survival and oxidation management. Thus, promoters requiring σ^S are working below their maximal capacity even during inducing conditions and a reduction in σ^{70} levels may, at least to some extent, increase σ^S-dependent gene expression and reduce the levels of oxidative damage in growth-arrested cells. Of course, σ^{70}-dependent stress defense genes, such as UspA (58), also contribute to maintenance during stasis, and it may be argued that a ppGpp-dependent reduction in the loading of σ^{70} to RNA polymerase might be counterproductive. However, a 50% reduction in the availability of σ^{70} specifically affected (negatively) the production of proteins involved in translation, i.e., ribosomal proteins and elongation factors, whereas σ^{70}-dependent stress proteins (e.g., UspA) were unaffected (46). Thus, the increased competitiveness of alternative sigma factors during stringency might primarily reduce σ^{70}-dependent functions related to growth. The benefit of retaining residual expression of σ^{70}-dependent proliferation-related genes during stasis might be that the growth-arrested cell maintains the potential to respond rapidly should nutrients become available.

PROGRAMMED CELL DEATH

A question of interest that has been raised recently is whether cellular die-off during stationary phase is due to stochastic degeneration or a genetic program. Many bacteria harbor toxin-antitoxin (TA) loci. These TA loci, or addiction modules, were present on some plasmids (and phage genomes) and contributed to the apparent stability of these episomes by selectively killing episome-free, or cured, segregants or their progeny (27). The toxin protein is usually stable, whereas the antitoxin is unstable and this appears to be the molecular explanation for postsegregational killing of cured progeny.

Bacteria, including *E. coli*, harbor TA loci also on the chromosome, and the loci may mediate programmed cell death upon nutritional stress and growth arrest (2, 18). The origin of this proposition stems from work on the TA loci consisting of the *mazE* (antitoxin) and *mazF* (toxin) genes, which form an operon with the upstream gene, *relA*, of the stringent response (2). Ectopic overproduction of MazF effectively reduced the viable counts of the population, suggesting that MazF is a bona fide toxin. Also, artificial elevation of ppGpp levels, the alarmone of the stringent response, reduces transcription of the *mazEF* operon, and on the basis of these results, it was argued that programmed cell death is triggered whenever nutrient starvation elicits ppGpp accumulation. This blocks further production of MazEF and allows the more stable toxin, MazF, to express its killing function

(2). Aizenman et al. (2) proposed that this is part of an altruistic death program triggered by starvation conditions so that programmed deterioration of part of the population may enable the rest to survive or even grow on constituents leaking out of dead siblings.

However, the proposed benefits of cell death for the bacterial population as a whole have not been convincingly shown either experimentally or theoretically. What is more, the loss of viable counts elicited by the *mazEF* system has only been accomplished by artificial overproduction of the toxin or ppGpp in exponentially growing cells. Despite the fact that artificial elevation of ppGpp reduces expression of the *mazEF* genes (2), this operon is actually induced in wild-type cells during conditions eliciting ppGpp accumulation (26). Moreover, the MazF (ChpAK) and the RelE toxins do not, in fact, kill the cell. Overproduction of the toxins instead elicits a bacteriostatic condition that can be fully reversed by ectopic production of the cognate antitoxins (62). Thus, elevated levels of the toxins lock the cells in a growth-arrested, G_0-like state that is incompatible with colony formation on nutrient agar plates. However, if the cognate antitoxin is elevated simultaneously or subsequently, it will rescue cells from this G_0-like state. Interestingly, the RelE toxin inhibits translation by participating in mRNA cleavage on translating ribosomes (63). As such, the TA modules may serve as a backup system to the stringent response and check superfluous macromolecular synthesis in a ppGpp-independent fashion during stasis. The conclusion that ppGpp elevation does not kill stationary-phase cells via a TA system is also comforting considering that ppGpp is beneficial for stationary-phase survival (50).

THE STATIONARY-PHASE GENOME

Some theories of cellular and molecular aging highlight the central flow of information as a target for deterioration. In other words, aging is argued to be the result of a time-dependent corruption in the flow of information from DNA to RNA to proteins. This could, for example, be due to a diminished ability of the transcriptional and translational machineries to correctly transcribe and translate their templates. There are, however, reasons to suspect that DNA may be the most sensitive target for such a corruption in information transfer. The argument most often used is that there are only two copies of many essential genes in many organisms and often only one in haploids. Therefore, defects in DNA may be more serious than defects in other cellular components, such as proteins, that are present, in general, in multiple copies. The defects arising on DNA could be of several different kinds and discussions have included fragmentation of chromosomes, deletions, point mutations, and transposition. The reason for such an age-dependent accumulation of various DNA damage is ascribed to an inadequate repair system, especially but not exclusively in postmitotic cells. This inadequate repair is suggested to lead to aging, and the major function of meiosis, sex, and outbreeding is to keep the germ line free of damage and corruption in its genetic information.

A recent report points out that the deleterious genomic mutation rate in stationary-phase *E. coli* cells, or a subpopulation of cells, is exceedingly high. In fact, it was calculated that the mutation rate (per genome per day) is over an order of magnitude higher than extrapolations from fast-growing cells (45). It was argued that such high mutation rates may be important for accelerated adaptation, but it is equally possible that deleterious genomic mutations limit the life span of growth-arrested cells. However, earlier reports have shown that *mutT* mutants, which have a 300- to 1000-fold increase in mutation frequency, survive stationary phase as well as wild-type cells do (16). Furthermore, cells lacking *recA*, *dps*, or both do not exhibit a diminished ability to withstand stasis despite the fact that these mutants are seriously flawed in coping with DNA damage (16). Thus, the mutation frequency of stationary-phase cells does not appear to limit cellular life span and DNA does not seem to be the primary target for oxidative deterioration in these cells. In addition, previous measurements of mutation frequencies in stationary phase have shown that the rates of mutation to various types of auxotrophy are unaffected or decreased, as measured by both fluctuation tests and a repeated-streaking procedure (32). Similarly, the rates of reversion to prototrophy of *his* and *lac* nonsense and missense mutations were reported to be unaffected by starvation (32). Clearly, the concept of stationary phase being generally mutagenic needs further scrutiny.

CONCLUDING REMARKS

The progressive decline in the functional capacity of aging eukaryotes may be a consequence of self-inflicted damage caused by ROS. Recent studies of bacterial physiology during starvation-induced growth arrest have raised the question of whether this hypothesis of aging is relevant also for explaining the progressive decline in the viability of stationary-phase cells. This loss of viability triggered by growth arrest seems to be the nearest bacteria come to the natural death of the kind familiar to higher organisms. Similar to aging in higher organisms, life expectancy in a cohort of stationary-phase *E. coli* cells of the same chronological age is intimately correlated to the levels of oxidative damage and the absence of oxygen greatly enhances the cell's ability to withstand starvation. Analysis of oxidation in stationary-phase cells has been concerned primarily with proteins as targets for oxidation. The reason for this is that relatively convenient methods are available to detect such oxidation, but this does not imply that proteins are the major, or most important, targets for oxidation in growth-arrested cells. Another possible target for oxidation damage that needs attention is membrane lipids. These macromolecules will always be in the proximity of sites generating ROS, and the malfunctioning of even a small fraction of the membrane lipids could have pronounced detrimental effects on membrane permeability and cellular homeostasis.

The work on bacterial model systems reveals that there might be a novel and hitherto unrecognized mechanism behind protein oxidation in nonproliferating cells. This oxidation occurs in the absence of an increased oxidative stress but may

instead be due to an increased concentration of substrates available for oxidative attack. Aberrant and misfolded proteins are such substrates, and the concentration of these aberrant proteins increases during starvation owing to a temporarily reduced translational fidelity. It is also possible that increased levels of oxidatively damaged proteins are an inevitable consequence of growth arrest, since erroneous products cannot be diluted in such a system. However, stationary phase elicited by phosphate starvation does not result in significantly elevated levels of oxidized proteins despite a high respiration rate, indicating that elevated damage is not an inexorable consequence of growth arrest and high metabolic activity.

In addition, increased oxidation during stasis might stem from the fact that maintenance and stress defense activities are partly traded for continued transcription of genes involved in proliferation and growth. Sigma factors directing functions related to reproduction on the one hand and stress resistance and survival on the other compete for RNA polymerase binding. This results in the antagonism between survival activities and reproduction. Even during growth arrest, a certain fraction of the cell's transcriptional resources is allocated to activities related to proliferation rather than stress defenses and survival, and as a consequence, stress defense genes are not allowed to operate at their maximal capacity. The sigma factor competition model provides an explanation also for *rpoS* polymorphism by predicting that loss of *rpoS* function and reduced levels of ppGpp increase the fitness of cells in growth-supporting, nonstressful environments.

The concept of programmed cell death via TA modules as an alternative model for stationary-phase death and a novel role of the stringent response in cell suicide has been challenged by data demonstrating that the TA loci do not appear to kill cells but rather lock them in a growth-arrested mode. This mode can be fully reversed by production of the cognate antitoxins. The proposed biochemical functions of the toxins point to beneficial rather than detrimental roles in checking excessive macromolecular synthesis during starvation. However, to what extent the TA systems may be promoting starvation survival remains to be clarified.

In summary, data concerning conditional senescence of stationary-phase bacteria are in some respects in line with the free-radical, antagonistic pleiotropy, and disposable soma theories of aging. In contrast, the data derived from the prokaryotic model systems do not support the rate of living hypothesis or the feedback catastrophe theory of aging. Interestingly, the latter two hypotheses have fallen out of grace also in the field of eukaryotic aging research. The future might, it is hoped, identify the most important bottlenecks in bacterial longevity and answer to what extent the mechanisms underlying stationary-phase death in prokaryotes and mandatory aging in eukaryotes are evolutionary conserved.

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