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Conditional senescence in bacteria: death of the immortals

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Summary

Like ageing insects, worms and mammals, growth-arrested *Escherichia coli* cells accumulate oxidatively damaged proteins. In the early stages of the *E. coli* stationary phase, this oxidation is caused by an increased production of aberrant proteins, which are especially susceptible to oxidative attack. This route of oxidation appears to elude the classical oxidative defence proteins. The failure of growth-arrested cells fully to combat oxidative damage may also be linked to a trade-off between proliferation activities (primarily directed by the housekeeping sigma factor, σ^{70}) and maintenance (primarily directed by σ^{S}). This trade-off is regulated by the alarmone ppGpp such that elevated ppGpp levels allow σ^{S}, and other alternative sigma factors, to work in concert with σ^{70} by shifting their relative competitiveness for RNA polymerase binding. However, even during elevated ppGpp levels and stasis, *E. coli* cells maintain a basal transcription of housekeeping σ^{70}-dependent genes, and resources are thus partly diverted from maintenance and stress defences to activities relating to proliferation. An alternative view argues for ppGpp being involved in programmed cell death upon growth arrest by regulating chromosomally located toxin–antitoxin loci. Thus, models of bacterial senescence, like those dealing with ageing in higher organisms, encompass both stochastic deterioration theories and programming theories. This review summarizes and evaluates these models.

Introduction

Cytokinesis in morphologically simple bacteria such as *Escherichia coli* proceeds in a symmetrical fashion with a non-conservative dispersion of cytoplasmic material. During fission, potentially damaged constituents are supposedly distributed equally to both cells produced, and the two daughters are said to be of exactly the same age (note that this is not formally true when considering the age of the cell poles and DNA strands). As a consequence, *E. coli* cells do not exhibit a mandatory replicative ageing process and are, in principle, immortal creatures. However, *E. coli* cells entering a non-proliferating state (starvation-induced stasis) become unable to reproduce on standard nutrient plates and lose their membrane integrity and life-supporting activities (Ericsson *et al.*, 2000). The fact that the loss of membrane integrity is non-reversible excludes the possibility that the apparent loss of viability is a programmed response in which cells enter a reversible 'viable-but-non-culturable state (Ericsson *et al.*, 2000). In the 1960s and 1970s, the death phase following starvation was argued to be the nearest bacteria come to a 'natural' death of the kind familiar among higher organisms (Burleigh and Dawes, 1967; Postgate, 1976), and this has been referred to more recently as conditional senescence (Nyström, 1999; 2001). The term 'conditional' is used to make a distinction between mandatory ageing in higher organisms and the senescence (loss of culturability and membrane integrity; Ericsson *et al.*, 2000) caused by starvation-induced growth arrest in unicellular systems. Several genes have now been identified as being important in slowing down the rate of stasis-induced senescence, and many of these have specific roles in protecting the cell against heat and oxidative stress (Matin, 1991; Kolter *et al.*, 1993; Hengge-Aronis, 2002; Nyström, 2002). Their expression relies, to a large extent, on the sigma factor σ^{S} (Hengge-Aronis, 2002), although it should be noted that σ^{70}-, σ^{E}- and σ^{32}-dependent genes are required for maximal survival of growth-arrested *E. coli* cells (e.g. Spence *et al.*, 1990; Nyström *et al.*, 1996; Testerman *et al.*, 2002).

σ^{S} and its functional analogues in yeast and nematodes

The σ^{S} transcription factor accumulates during stasis and

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directs the RNA polymerase to a large number of genes with diverse functions (e.g. Loewen *et al*., 1998). However, there is a significant bias towards stress defence functions. Interestingly, these functions overlap with those of the *daf-16*-regulated genes of *Caenorhabditis elegans* and the *RAS*/cAMP/PKA-regulated genes in yeast (e.g. Larsen, 1993; Marchler *et al*., 1993; Johnson *et al*., 2000). The Daf-16 forkhead transcription factor is a key regulator in the starvation-induced dauer formation in the nematode and, like σ^S, this regulator directs the transcriptional apparatus to genes involved in protection against heat shock and oxidative agents (e.g. Johnson *et al*., 2000). Overexpression of *daf-16* extends the lifespan of adult nematodes, whereas *daf-16* inactivation accelerates ageing and causes an increased oxidative damage of proteins (Yasuda *et al*., 1999). The *RAS*/cAMP/PKA regulatory pathway of *Saccharomyces cerevisiae* is similarly involved in general stress defence and longevity. Disruption of *RAS* alleviates PKA-dependent repression of genes (e.g. genes encoding heat shock proteins, catalase and CuZn superoxide dismutase) containing a stress response element (STRE) in their promoter region, resulting in increased resistance to oxidative agents and heat (Marchler *et al*., 1993; Martinez-Pastor *et al*., 1996). Moreover, the replicative lifespan of yeast is extended in mutants with low PKA activity, whereas the opposite is true for mutants with constitutive PKA activity (Lin *et al*., 2000). Like the σ^S and Daf-16 pathways, the *RAS*/cAMP/PKA pathway responds to starvation, such that glucose limitation converts Ras to the inactive GDP-bound form, which in turn reduces cAMP levels and elevates the expression of STRE element genes. The σ^S, Daf-16 and *RAS*/cAMP/PKA regulatory systems are thus remarkably similar in their responsiveness and physiological function; they are all responding to starvation, they are all required to mount a general stress defence, and they are longevity determinants.

It is not clear which members of the σ^S regulon are most important in slowing down senescence, but σ^S mutants exhibit elevated levels of oxidatively damaged proteins during stasis, suggesting that oxidative stress proteins, as in *C. elegans* (Yasuda *et al*., 1999), might be key members of the regulon (Dukan and Nyström, 1998; 1999). In addition, the role of σ^S in the survival of stationary phase *Salmonella enterica* serovar Typhimurium has recently been intimately linked to oxidative stress defence. Mutants of this bacterium lacking both σ^E and σ^S lose viability extremely rapidly during stasis, but survival of these mutants is completely preserved under anaerobic stationary phase conditions (Testerman *et al*., 2002). This reinforces the argument that oxidative injury is one of the major mechanisms of reduced microbial viability during periods of nutrient deprivation and that one important, if not primary, role of σ^S in stasis survival is to prevent such damage.

σ^S and a trade–off between survival and proliferation

Some evolutionary models of senescence propose that there is a trade-off between the resources that an organism devotes to reproduction and growth and those devoted to cellular maintenance and repair. As a result, an optimal life history, by necessity, entails an imperfect ability to resist stress. In *C. elegans*, this trade-off can be altered by mutations in *DAF-16* such that transgenic animals carrying *DAF-16* alleles that slow down growth and reproduction live longer and are more resistant to extrinsic stresses (Henderson and Johnson, 2001). There are also examples of such a trade-off in *E. coli.* For example, Kurland and Mikkola (1993) found that, in general, natural and laboratory *E. coli* isolates exhibiting fast growth and efficient ribosomes died more rapidly during starvation-induced stasis. Continuous cultivation in chemostats effectively selected for cells with faster growth rates with a concomitant increased efficiency of translation. However, the trade-off for this increased rate of reproduction was a reduced ability to withstand starvation-induced stasis (Kurland and Mikkola, 1993).

More recently, the *E. coli* trade-off phenomenon has been linked to the status of the *rpoS* gene, encoding σ^S. It is known that mutations in *rpoS* are common in many natural and laboratory *E. coli* strains, and it was demonstrated that there is a selective advantage of losing σ^S function during growth under carbon-limited conditions in a chemostat (Notley-McRobb *et al*., 2002). 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} (Notley-McRobb *et al*., 2002). However, increased fitness is traded for reduced stasis survival and stress resistance, as σ^S is a master regulator required for these functions [it is possible that the starvation-sensitive mutants selected by Kurland and Mikkola (1993) in chemostat experiments were *rpoS* mutants, but this has not be established experimentally]. This is a bacterial example of antagonistic pleiotropy, in which mutations that are beneficial for reproduction may be harmful during old age or stasis. This kind of antagonistic pleiotropy has been suggested to be a major factor in the evolution of ageing (Williams, 1957).

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 and 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. 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. It has been shown that even a subtle overproduction of σ^{70} effectively shuts down transcription from genes requiring σ^S and the cells become stress sensitive (Farewell *et al.*, 1998). Also, overproduction of σ^S attenuates the expression of genes requiring σ^{70} (Farewell *et al.*, 1998). The antagonism between sigma factors is highly regulated and is dictated by the nutritional quality of the environment and the nucleotide ppGpp (Jishage *et al.*, 2002). Mutants lacking ppGpp fail to induce σ^S-dependent genes upon the imposition of stress and starvation, a phenomenon that was originally explained by the fact that σ^S itself requires ppGpp for its production (Gentry *et al.*, 1993; Lange *et al.*, 1995). However, σ^S-dependent genes require ppGpp even in the presence of wild-type levels of σ^S (Kvint *et al.*, 2000a), 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 (Jishage *et al.*, 2002). Thus, ppGpp is priming the RNA polymerase in accordance with environmental signals such that the transcriptional apparatus will be occupied primarily 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 favourable for reproduction. When conditions are less favourable for proliferation, elevated ppGpp levels allow the alternative sigma factors to work in concert with σ^{70} by shifting their relative competitiveness (Jishage *et al.*, 2002). In the sigma factor competition scenario, the antagonistic pleiotropy observed by Notley-McRobb *et al.* (2002) could be explained by the fact that more σ^{70} proteins are allowed to bind RNA polymerase core in the total absence of any competing σ^S, and more resources are thus directed towards growth and reproduction-related activities.

The trade-off model could also explain why σ^S and its regulon genes are not able fully to combat stasis-induced deterioration, e.g. oxidative damage to proteins and other macromolecules. The model argues that sigma factors work in concert in a ppGpp-regulated fashion and 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. The assumption of the model is that promoters requiring σ^S are working below their maximal capacity even during inducing conditions and that 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. This can be tested experimentally by analysing the global pattern of gene expression/protein production and oxidative modification of proteins during artificial reduction in the levels of σ^{70} (see Magnusson *et al.*, 2003). It should also be noted that σ^{70}-dependent stress defence genes, such as UspA (Nyström and Neidhardt, 1994), 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, it was demonstrated recently that 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 (Magnusson *et al.*, 2002). Thus, the increased competitiveness of alternative sigma factors during stringency might primarily reduce σ^{70}-dependent functions related to growth. The benefit of retaining some residual expression of σ^{70}-dependent proliferation-related genes during stasis might be that the growth-arrested cell maintains the potential to respond rapidly, grow and initiate proliferation should nutrients become available. This is a different strategy from the one that evolved in *E. coli*'s differentiating cousins, the spore-forming bacteria, and may, of course, reflect the difference in the pressures of their environments.

Translation errors and protein oxidation

In addition to sigma competition, a further reason for the failure of the σ^S (and other) regulatory network to combat stasis-induced oxidation of proteins fully is that such oxidation might occur by a route that eludes the classical oxidative defence pathways. The level of oxidized proteins increases upon treatment of cells with antibiotics and by mutations causing increased mistranslation (Dukan *et al.*, 2000). Interestingly, during these treatments, the rate of superoxide production and the activity of the superoxide dismutases and catalases are unchanged, and the expression of oxidative stress defence genes does not increase (Dukan *et al.*, 2000). In addition, it was demonstrated that the increased oxidation during these treatments was primarily the result of aberrant protein isoforms being oxidized (Dukan *et al.*, 2000). In other words, increased protein oxidation can be the result of increased production of aberrant proteins, and this does not appear to be sensed by the oxidative defence regulons and does not require increased generation of reactive oxygen species (Dukan *et al.*, 2000). Moreover, diagnostic proteomics demonstrated that the sudden increase in protein oxidation during the early stages of stasis in *E. coli* is strongly associated with the production of aberrant protein isoforms that appear to be specific targets for oxidative modifications (Ballesteros *et al.*, 2001). This fact, together

with results showing that frameshifting (Barak *et al*., 1996; Wenthzel *et al*., 1998), missense errors (O'Farrell, 1978) and stop codon readthrough (Ballesteros *et al*., 2001) increase in response to stasis in *E. coli* cells, raises the possibility that protein oxidation in non-proliferating cells might be caused by increased mistranslation. Indeed, protein oxidation is drastically attenuated in the early stages of stasis in *E. coli* cells harbouring intrinsically hyperaccurate ribosomes (carrying the *rpsL141* allele; Ballesteros *et al*., 2001). Thus, the elevated oxidation of proteins in non-proliferating cells might result from the abundance of substrates (aberrant proteins) available for oxidative attack surges during stasis because of a reduced fidelity of the translational apparatus. Notably, translational error-dependent oxidation that occurs as an immediate response to stasis does not appear to affect the rate of senescence, as the survival of mutants harbouring the *rpsL141* allele and the wild-type parent is indistinguishable (Ballesteros *et al*., 2001).

The σ^S regulon might be ineffective in counteracting such mechanisms of oxidation. In addition, this type of oxidation occurs as an immediate response to growth arrest before σ^S has reached its maximal concentration. It should be noted, however, that the gradual increase in protein oxidation levels observed during prolonged stasis is counteracted by σ^S, as this increase is much more pronounced in *rpoS* mutants (Dukan and Nyström, 1999). It is not clear why aberrant proteins are more susceptible to carbonylation. Possibly, a slight misfolding of the corrupted polypeptide might expose oxidation-sensitive targets that are normally hidden during the coupled translation-folding process.

Senescence as a programmed phenomenon

This review has, so far, argued that senescence is a stochastic deterioration phenomenon and that oxidation damage is a likely culprit in this process, a view that is supported by the fact that the rate of senescence in wild-type *E. coli* cells is drastically retarded by reducing oxygen levels (Dukan and Nyström, 1999). In addition, the fact that starvation-induced senescence is drastically accelerated in ppGpp-deficient cells argues for a beneficial role of this alarmone. Mechanistically, this might be linked to a variety of its activities (Fig. 1): its role in (1) downregulating superfluous macromolecular synthesis (e.g. Chatterji and Ojha, 2001); (2) positively regulating stasis survival genes, such as the *fad* genes and the universal stress protein paralogues (Kvint *et al*., 2000a; Gustavsson *et al*., 2002); (3) allowing alternative sigma factors to compete during stasis (Jishage *et al*., 2002); and (4) reducing translational errors (e.g. O'Farrell, 1978) and, consequently, protein oxidation. However, another view argues that ppGpp mediates programmed cell death upon nutritional stress through its regulation of toxin–antitoxin loci (Aizenman *et al*., 1996).

Many bacteria harbour toxin–antitoxin (TA) loci on some plasmids (and phage genomes) that contribute to the apparent stability of these episomes by selectively killing episome-free or cured segregants or their progeny (Gerdes, 2000). The toxin protein is stable, whereas the antitoxin is unstable, which appears to be the molecular explanation for post-segregational killing of cured progeny. The antitoxins neutralize their cognate toxins by direct protein–protein interaction. The targets for the toxins are involved in macromolecular synthesis, including DNA replication and translation (e.g. Bernard and Couturier, 1992; Ruiz-Echevarria *et al*., 1995; Gotfredsen and Gerdes, 1998; Gerdes, 2000).

In addition to being encoded by plasmids and phage DNA, five TA loci are present in the chromosome of *E. coli*: two *relBE*, two *chp* (*mazEF*) operons and one *relB* paralogue with no apparent downstream *relE* paralogue (Gerdes, 2000). It has been proposed that these TA operons mediate programmed cell death upon nutritional stress and growth arrest (Aizenman *et al*., 1996; Engelberg-Kulka *et al*., 1998). The idea stems from work on *mazE* (antitoxin) *mazF* (toxin), which form an operon with the upstream gene, *relA*, of the stringent response. Ectopic overproduction of MazF effectively reduced the viable counts of an *E. coli* population, and artificial elevation of ppGpp levels reduced transcription of the *mazEF* operon. Based on these results, it was argued that programmed cell death is triggered when conditions, such as nutrient starvation, elicit ppGpp accumulation, which will block further production of MazEF and allow the more stable toxin, MazF, to express its killing function (Aizenman *et al*., 1996). The benefits of such a system were suggested to be linked to the fact that *mazEF* provide the cells with a system for altruistic cell death during starvation conditions (Aizenman *et al*., 1996). In other words, programmed deterioration of part of the population might enable the rest of the population to survive or even grow on constituents leaking out of dead siblings. An alternative view suggests that the TA toxins are not really designed to kill the cell but may be involved in checking cellular processes (e.g. translation and replication) that should be downregulated in a growth-arrested cell (Nyström, 1999; Gerdes, 2000). A recent publication (Pedersen *et al*., 2002) has given experimental support for the latter view and clarified many of the enigmas of the TA loci. Most importantly, it was demonstrated that the MazF (ChpAK) and the RelE toxins do not, in fact, kill the cell. Instead, overproduction of the toxins elicits a bacteriostatic condition that can be fully reversed by ectopic production of the cognate antitoxins (Pedersen *et al*., 2002). In other words, elevated levels of the toxins lock the cells in a growth-arrested, G_0-like, state, which is incompatible

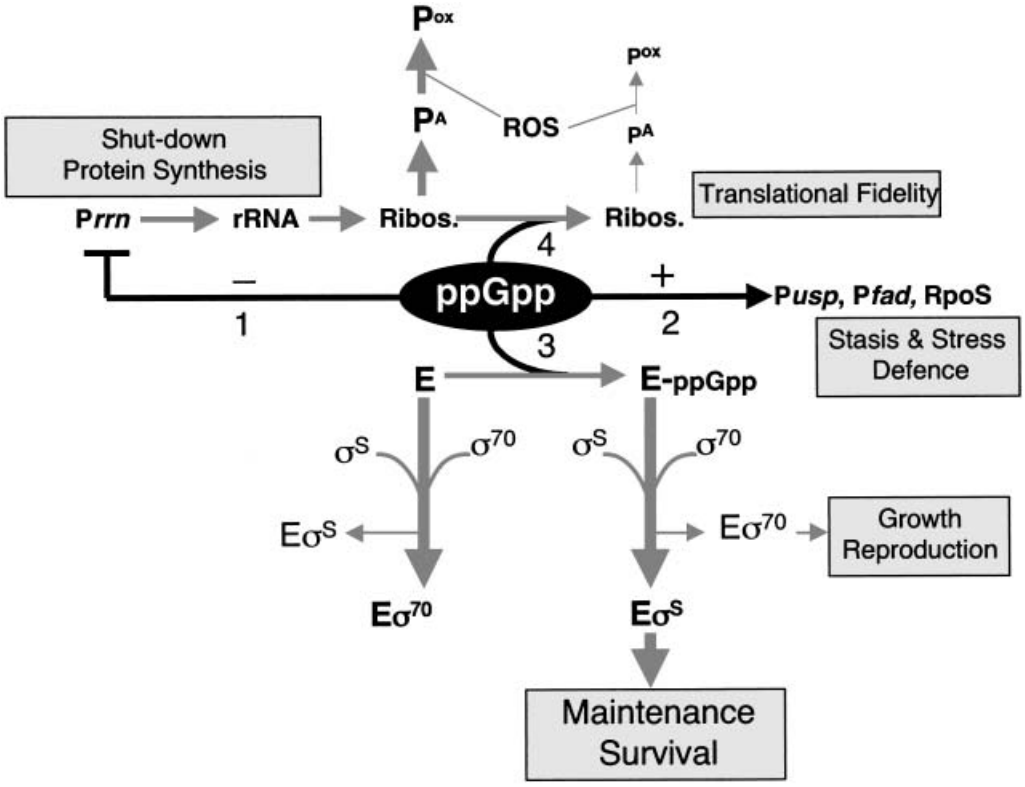

Fig. 1. ppGpp, the Rosetta stone of *E. coli* physiology and stasis survival.
1. Accumulation of ppGpp results in transcriptional shut-down of many genes involved in protein synthesis, notably genes encoding rRNA. This transcriptional shut-down prevents superfluous macromolecular synthesis during growth arrest.
2. Genes of importance for stasis survival, including *rpoS*, *usp* paralogues and *fad* genes involved in fatty acid degradation and β-oxidation are increasingly expressed during stasis in a ppGpp-dependent fashion. The effect on *rpoS* expression appears to be post-transcriptional (Hengge-Aronis, 2002). Notably, the positive effect of ppGpp on *fad* and *usp* expression over-rides negative control by the active repressor FadR, a phenomenon called emergency derepression (Kvint *et al*., 2000b).
3. ppGpp is priming the RNA polymerase in accordance with environmental signals such that elevated ppGpp levels allow the alternative sigma factor σ^S, required for the expression of maintenance genes, to work in concert with σ^{70} by shifting the relative competitiveness of the sigma factors (Jishage *et al*., 2002).
4. ppGpp is required for translational fidelity (O'Farrell, 1978) and, because aberrant proteins are highly susceptible to oxidative modification, the alarmone has a role in mitigating increased oxidation during stasis.

with colony formation on nutrient agar plates unless the cognate antitoxin is similarly elevated to counteract this state. The RelE toxin was shown primarily to inhibit translation, and it was suggested that the function of this 'toxin' is to modulate the global rates of protein synthesis during nutritional stress conditions (Christensen *et al*., 2001; Pedersen *et al*., 2002). On the other hand, it could be argued that the RelE system is involved in a programmed adaptive and reversible response leading to a viable-but-non-culturable (VBNC) state and that the loss of viability observed in starving *E. coli* cultures is the result of such an adaptive mechanism. If so, we have to find a satisfying explanation for why such an adaptive system is not required or triggered under anaerobic conditions (stationary phase viability remains unaffected for long periods of time in the absence of oxygen; Dukan and Nyström, 1999). In addition, it has been shown that starvation of *Vibrio vulnificus* cells, which have been used extensively as model organisms in VBNC research, gives rise to oxidation-sensitive cells that fail to reproduce on standard agar plates unless the plates are provided with agents that eliminate hydrogen peroxide (Bogosian *et al*., 2000). Thus, the cells that have previously been regarded as VBNC were, in fact, shown to be a subpopulation of the culture that failed to reproduce because of starvation-induced hydrogen peroxide sensitivity. These results are also at odds with the adaptive programming theory unless we propose that the programme encompasses an induced sensitivity to external oxidative agents. The role of the TA modules might instead be linked to a control of macromolecular biosynthesis that serves as a back-up system to the stringent response and checks superfluous macromolecular synthesis during stasis. The TA loci appear to be ppGpp independent in the sense that they are induced

during growth arrest regardless of whether the cells are ppGpp deficient or not. In fact, the systems appear to be superinduced in cells lacking ppGpp (Christensen *et al*., 2001; Pedersen *et al*., 2002).

Conclusion

Work on *E. coli* as a model system for senescence has revealed that elevated levels of oxidized proteins might not necessarily stem from an increased production of free radicals or a diminished defence system, but may be caused by increased production of malformed polypeptides. These aberrant proteins are highly susceptible to oxidative modifications, and the number of such polypeptides surges in senescing *E. coli* cells as a result of a decline in ribosome fidelity. This route of oxidation appears to elude the classical oxidative defence systems that are elevated during stasis. In addition, increased oxidation during stasis might stem from the fact that maintenance and stress defence 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 a limiting amount of RNA polymerases in the cell. This limitation in transcriptional capacity results in the antagonism between survival activities and reproduction. The trade-off between these activities is stringently regulated by environmental cues acting through the second messenger, ppGpp, such that RNA polymerase is redistributed from proliferating activities to maintenance when the environment is no longer favourable for growth. Finally, the accumulated data on the molecular biology of growth-arrested *E. coli* cells suggest that bacterial senescence is linked to stochastic deterioration, rather than programmed death pathways, and that self-inflicted oxidative damage may be a causal factor in age-related deterioration of both prokaryotes and higher organisms.

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