Starvation, cessation of growth and bacterial aging

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Several components of different *Escherichia coli* regulons are integrated to prevent premature oxidative deterioration of starving cells. The interconnected regulation of these regulons encompasses oxidation signalling, sigma factor competition, and possibly also the use of sigma factor inhibitors. Recent data demonstrate that stasis-induced oxidation targets both DNA and protein and that some enzymes are specifically susceptible to oxidative attack.

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Current Opinion in Microbiology 1999, **2**:214–219

http://biomednet.com/elecref/1369527400200214

 ISSN 1369-5274

Abbreviations

E	core RNA polymerase
ROS	reactive oxygen species

Introduction

Gerontologists have proposed that the ubiquitous progressive decline in the functional capacity of aging eukaryotes may be a consequence of the accumulation of oxidative damage caused by reactive oxygen species (ROS) produced by normal metabolism. This is the postulation of the free radical hypothesis of cellular aging. The study of the mortality of bacteria and how they survive starvation-induced growth arrest has raised the question of whether the free radical hypothesis of aging is relevant also for explaining the progressive deterioration of growth arrested bacterial cells. This issue seems relevant in the light of the well documented increase in the levels of oxidative stress defence proteins in bacteria starved for any of a variety of nutrients. It is, however, unclear whether these stress proteins are really involved in allowing the cells to better survive stasis rather than enhancing the cell's ability to survive cataclysmic oxidative stresses that they may encounter in the future. Recent data support the idea that oxidative stress defence proteins prevent stasis-related accumulation of oxidative damage caused by ongoing metabolic activities (respiration) in the growth arrested cell and further point to a role of oxidation signalling in developmental induction of stress regulons. These data are reviewed in relation to the recent findings that bacteria possess chromosomally encoded proteins that could potentially elicit programmed cell death during starvation.

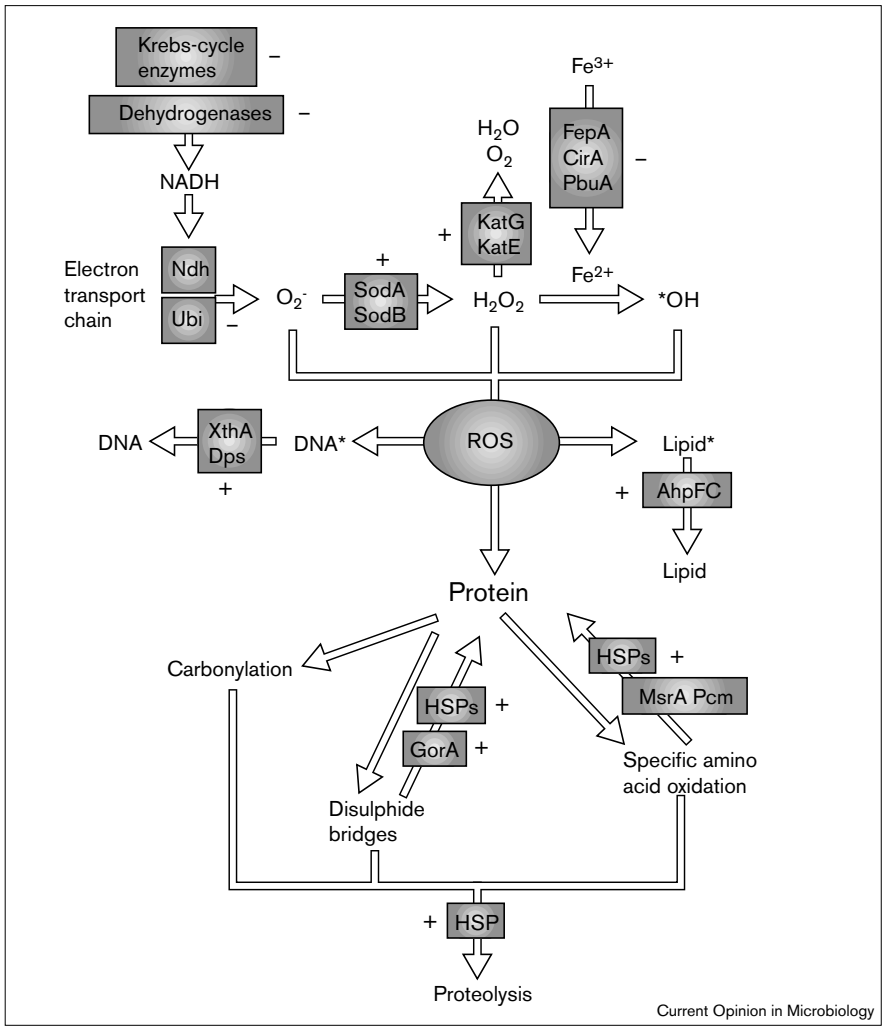
Oxidative damage and the fight against aging

Many starvation-inducible genes have been identified as *bona fide* members of known stress response networks including the RpoH-, RpoS- and OxyR-dependent regulons [1–6]. The functions of the induced genes indicate that a significant part of the starving cell's transcriptional and translational machinery is devoted to making proteins with specific roles in oxidative stress defence. Indeed, the increased levels of catalases, superoxide dismutases, L-isoaspartyl protein methyltransferase, peptide methionine sulfoxide reductase, glutathione, glutathione reductase, and heat shock proteins [1–3,7,8,9••,10] strongly suggest that the growth-arrested cell increases its ability to deal with oxidative denaturation and the spontaneous aging of proteins (Figure 1). In addition, the ArcA-dependent repression of Krebs-cycle enzymes and respiratory components during stasis may be part of an oxidative defence system aimed at minimising unnecessary harmful respiration and self-inflicted oxidative damage [11].

Recent data more directly support the idea that stasis-induced deterioration of proteins may be a problem in growth-arrested cells; a clear correlation exists between the age of a stationary phase *Escherichia coli* culture and the levels of isoaspartyl residues, which are formed by spontaneous damage of aspartate and asparagine residues [12], illegitimate oxidative disulphide bond formation, and protein oxidation producing aldehyde or ketone groups (protein carbonylation) [9••]. The RpoS and OxyR regulons are both important in slowing down this protein oxidation during growth arrest [9••]. A number of proteins are specifically susceptible to stasis-induced oxidation including DnaK, H-NS, UspA, EF-Tu, glutamine synthetase, glutamate synthase, pyruvate kinase, PtsI, FabB and several Krebs-cycle enzymes [9••]. Thus, the growth-arrested cell may experience problems in performing peptide chain elongation, protein folding and reconstruction, central carbon catabolism, and nitrogen assimilation. *E. coli* cells appear to experience similar problems during conditions of iron overload and hydrogen peroxide, and superoxide radical exposure [13•].

ROS generated during starvation (in this case after growth on plates [14]) appears to target DNA as well as proteins, since introduction of plasmids expressing singlet oxygen scavengers (carotenoids) significantly reduce the yield of mutants generated during starvation [14]. The reversion to prototrophy of amino acid auxotrophs occurring when *E. coli* is starved of the required amino acid results in many cases from the accumulation of oxidative damage to guanine residues in DNA. In the experimental set-up of Bridges and Timms [14], however, RpoS, which is required for stationary phase induction of several genes involved in DNA protection, does not seem to contribute to the protection of DNA against oxidative attack nor does this attack on DNA appear to limit viability during the starvation conditions studied. Overproducing the mismatch repair protein MutL, like overproducing carotenoids, reduces the mutation frequency in stationary phase but not

Figure 1



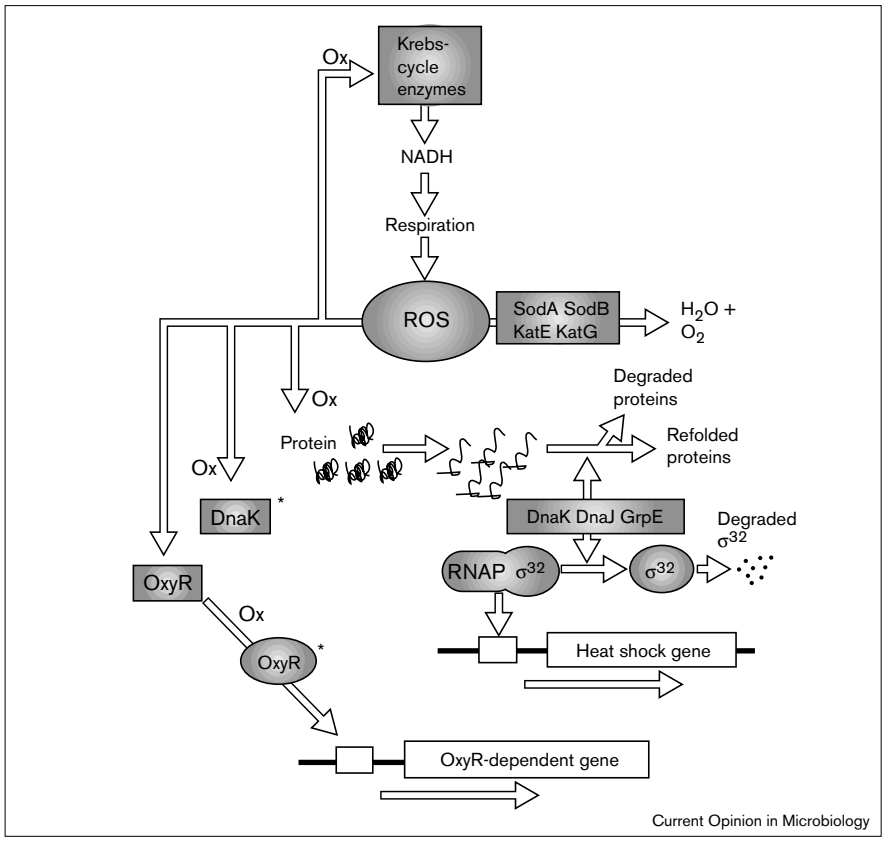
Schematic representation of the stationary phase bacterial defence against oxidation derived aging of macromolecules. Several genes/proteins responding to starvation appear to form an integral part of a defense system aimed at avoiding the damaging effects of ongoing respiratory activity during growth arrest. This defence system includes (i) an ArcA-dependent reduced production of respiratory substrates and components of the aerobic respiratory apparatus, (ii) an increased production of primary detoxification proteins, such as Sod and Kat, (iii) the silencing of genes, including *fepA, cirA,* and *pbuA,* involved in iron uptake possibly to avoid generation of hydroxyl groups via the Fenton reaction, and (iv) an increased production of proteins, including HSPs, Pcm, MsrA, GorA, XthA, and AhpFC, involved in repairing damaged proteins, DNA and lipids. Plus and minus signs denote induction and repression, respectively, of the corresponding genes during stationary phase. Stars indicate oxidised macromolecules. AhpFC, alkyl hydroperoxide reductase; Dps, DNA-binding protein stationary phase; FepA, CirA, and PbuA are outer membrane iron uptake proteins; GorA, glutathione reductase; HSPs, heat shock proteins; Kat, catalase; MsrA, peptide methionine sulfoxide reductase; Ndh, NADH dehydrogenase 1; Pcm, L-isoaspartyl protein methyltransferase; Sod, superoxide dismutase; Ubi, ubiqinone: XthA, exonuclease III.

during growth [15], indicating that ROS in concert with a limiting mismatch repair system may be a key player in stationary-phase mutations. Oxygen attack of DNA is most likely intimately connected to the production of hydroxyl radicals, which is stimulated by the presence of Fe^{2+} (e.g. [16]). Moreover, the crystal structure of the stationary phase DNA-binding protein Dps, which is involved in protecting DNA against oxidative attack, strongly suggests that Dps functions by sequestrating Fe ions [17•]. Interestingly, overexpression of the stress protein UspA drastically shuts off expression of genes (*cirA*, *fepA* and *pbuA*) involved in iron uptake and this may be an additional attempt by the aging cell to minimise generation of hydroxyl radicals [18].

Recent research has focused less on stasis-induced lipid peroxidation but it is likely that stasis-inducible genes have a role also in protecting membrane lipids. In fact, members of the OxyR regulon, including KatG and AhpFC (Figure 1), have been demonstrated to prevent accumulation of lipids modified by oxidation [19]. In addition, it is known that protein carbonylation, as seen during starvation-induced stasis [9••], can occur via reactions with aldehydes produced during lipid peroxidation [20]. A schematic representation of stasis-induced defence against oxidative macromolecular damage is depicted in Figure 1.

A large number of *E. coli* mutants that are specifically hypersensitive to various oxidative agents are also impaired in surviving starvation [21,22], which fits with the notion that endogenously generated oxidative stress may lead to accelerated bacterial aging as suggested by Eisenstark and colleagues [22]. These results highlight the fact that many, or most, oxidative stress proteins are not solely emergency response proteins but mediate homeostatic regulation of the ROS levels produced by normal aerobic catabolism; this has been stressed by Gonzalez-Fletcha and Demple [19] who also have shown that, similar to eukaryotic mitochondria, most of the superoxide anions are generated at the NADH dehydrogenase and ubiquinone sites at least during 'normal' aerobic growth conditions [23].

Figure 2



Schematic representation of the model for stasis-induced, 'developmental', induction of the OxyR and RpoH regulons. Central to the OxyR model is that the OxyR protein serves as both sensor and regulator of the OxyR regulon and that oxidative disulphide bridge formation between two of its cysteine residues activate the protein (OxyR*) leading to transcription initiation from OxyR dependent promoters [24]. This oxidation of the cysteine residues is suggested to occur during stasis as well as specific oxidative stress (e.g. hydrogen peroxide exposure) [9••]. Central to the RpoH model is that the heat shock proteins DnaJ, DnaK, and GrpE have two different functions: one is to promote refolding of misfolded, aberrant proteins and the other is to participate in the proteolytic degradation of the heat shock regulator σ^{32}. An increase in the cellular level of denatured proteins will sequester the DnaJ, DnaK, and GrpE components allowing stabilization of σ^{32}. During stasis, oxidative damage of target proteins could similarly sequester these heat shock modulators allowing induction of the regulon. This notion is supported by the fact that heat shock induction is markedly suppresses by overproducing SodA during stasis [9••]. In addition, DnaK appears to be specifically sensitive to oxidative carbonylation (Dnak*), which may destroy or reduce its ability to participate in the degradation of σ^{32}. As indicated in the figure, some TCA cycle enzymes appear to be specifically susceptible to oxidative attack and it is tempting to speculate that this is physiologically significant. It may be argued that specific oxidation sensitivity of key enzymes at the TCA cycle will feedback control the rate of respiration so that when the rate of electron transport and ROS production become too high the oxidation, and presumably the loss of activity, of TCA cycle enzymes will reduce respiration by limiting it substrate, NADH. Ox, oxidation.

Developmental regulon control by oxidation signalling

The gradual stasis-induced increase in protein oxidation has recently been suggested to explain starvation induction of the OxyR and RpoH regulons [9••]. OxyR becomes active through the oxidative formation of a disulphide bond between two of its cysteine residues [24••]. It is feasible that cysteine residues on OxyR, similar to a cytoplasmic alkaline phosphatase [9••], may be subjected to gradual oxidation during stasis that eventually may give rise to a high enough titre of oxidised OxyR to activate gene expression. This would mechanistically explain the OxyR-dependent gene expression during growth arrest (Figure 2) [9••,25].

Likewise, developmental induction of heat shock genes may be intimately connected to oxidative attack on target proteins. Inducers of the heat shock response are thought to elicit the response by generating aberrant, partially or completely unfolded proteins. Presumably, the gradual oxidation of proteins observed during stasis would result in increasing levels of denatured or misfolded proteins and the observed oxidation of DnaK itself [9••] may block its ability to participate in the DnaK/DnaJ/GrpE-dependent pathway leading to σ^{32} degradation (Figure 2). In support of this oxidative signalling model, stationary phase induction of heat shock genes is counteracted by overproducing SodA (superoxide dismutase A) and the regulon is superinduced in cells lacking cytoplasmic Sod activity [9••].

Regulon civil war

Stasis-induced expression of *E. coli* genes is the result of the combined effort of at least four different sigma factors: σ^{70}, σ^{s}, σ^{54} and σ^{32} [1,2,4,26•]. Recent data suggest that these sigma factors compete for binding to a limiting amount of core RNA polymerase (E). As a consequence, under- or over-expression of one sigma factor may greatly perturb the binding of other sigma factors to E [26•]. Limitations in E levels would appear to be more severe during stasis, because the concentration of all sigma factors taken together increases markedly during stationary phase (mostly due to the increase in σ^{s} and σ^{32}), whereas E levels decrease by about 33% [27]. Thus, the increase in σ^{s} and σ^{32} and the competition for E binding upon entry into stationary phase may be a mechanism for stasis-induced down-regulation of some genes requiring Eσ^{70}. Promoters

that are poor in recruiting $E\sigma^{70}$ would be expected to be affected greatly by an increased sigma factor competition. Indeed, many $E\sigma^{70}$-dependent genes that are normally down-regulated during stasis fail to be so in *rpoS* mutants [28]. Whether this down-regulation during stasis is due to sigma factor competition leading to reduced levels of active $E\sigma^{70}$ or, for example, expression of specific $E\sigma^{s}$-dependent repressor genes remains to be elucidated.

Moreover, the switching in gene expression during growth arrest may include elevated levels of specific inhibitors of sigma factors. Jishage and Ishihama [29••] identified and isolated a protein known as Rsd, which is produced during stationary phase and forms a complex with σ^{70} both *in vivo* and *in vitro*. The isolated Rsd inhibits transcription from some, but not all, $E\sigma^{70}$-dependent promoters analysed *in vitro* [29••]. It is thus possible that elevated levels of Rsd favour transcription of the $E\sigma^{s}$- and $E\sigma^{32}$-dependent regulons at the expense of $E\sigma^{70}$-dependent genes. In addition, functional interaction of E with inorganic polyphosphate may, under some stationary phase conditions, play a role in promoter selectivity and favour $E\sigma^{s}$-dependent promoters over $E\sigma^{70}$-dependent ones [30]. This is an interesting observation in view of the fact that inorganic polyphosphates appear to play a role in *rpoS* expression [31].

On the basis that the regulatory RNA OxyS, a member of the OxyR regulon, represses *rpoS* translation [32], it appears as if the regulon civil war also includes combat between the OxyR and RpoS regulons. This type of regulation, it was argued [32], may prevent redundant induction of oxidative stress genes (the OxyR and RpoS regulons encompass some of the same antioxidant genes).

Toxin–antitoxin proteins: role in programmed cell death or checking superfluous activity during starvation?

Bacterial 'addiction modules' [33] or 'proteic plasmid stabilisation systems' [34] consist of two genes encoding a stable cytotoxin and an unstable antitoxin (usually degraded by the heat shock proteases Lon or Clp (e.g. [34]). The systems are harmless as long as both proteins are produced allowing the unstable antitoxin to neutralise the toxin by forming a tight cytotoxin–antitoxin complex. Recent investigations have shown that *E. coli* possesses chromosomally located toxin–antitoxin systems that may have a role in programming cell death [33]. One such system, the *mazEF* operon, is negatively regulated by guanosine tetraphosphate (ppGpp) and was therefore suggested to have a role in eliciting programmed cell death during different starvation conditions causing stasis [33]. More recently, Gotfredsen and Gerdes [35•] demonstrated that the chromosomal *relBE* genes of *E. coli* encode another cytotoxin–antitoxin system and presented an attractive model explaining the well known but until now mysterious relaxed-control phenotype of *relB* mutants. Elevating RelE levels in the absence of RelB rapidly kills growing *E. coli* cells probably by interfering with translation [35•]. While the presence and regulation of these systems are extremely interesting, they raise the question of the possible benefits of programmed cell death in a unicellular organism. The authors describing the *mazEF* system [33] proposed that this systems may serve as a mechanism for altruistic cell death during starvation conditions in that cell lysis of part of the population may enable the rest of the population to survive or even grow. If so, we would still have to explain the apparent conflict between the regulon members that are assign to enhance survival versus those that promote killing (notably members of the stringent control and heat shock networks [36]).

Another possibility may be that the cytotoxins are not really designed to kill the cell but may be involved in checking cellular processes (e.g. translation and replication) that should be down-regulated in a growth-arrested cell. When artificially expressed in an exponentially growing culture, however, they may inhibit a required process leading, secondarily, to cell death. In other words, the cytotoxins may be conditionally lethal when artificially expressed at the wrong time but may have a beneficial role in stasis physiology.

Conclusions

The recent findings demonstrating stasis-induced oxidation of bacterial proteins and DNA, the poor ability of bacterial cells lacking components of oxidative stress systems to survive stasis, and the role of oxidation signalling in developmental, stasis-induced expression of stress defence regulons, may seem to be in accord with the free radical hypothesis of aging (e.g. [37]). This is, however, a premature conclusion; what this review (and most contemporary papers concerning stationary phase survival in bacteria) has dealt with is the analysis of mutants that are worse off than the wild-type strain and we cannot make the *a priori* assumption that wild-type *E. coli* dies for the same reason as those mutants. Unfortunately, there are, as far as I know, no equivalents to gerontogenes (genes whose alteration causes life extension) identified yet in bacteria with the possible exception of the *Micrococcus luteus rpf* gene, which encodes a protein that when added to a dormant *M. luteus* culture increases the viable cell count at least 100-fold [38•]. Thus, we have to await identification of genes similar to the gerontogenes of *Caenorhabditis elegans* [39] and *Drosophila melanogaster* [37] before we can judge whether the free radical hypothesis of aging is relevant or useful in explaining the longevity of growth arrest in wild-type bacterial cells. The search for such genes may be an important task for the future as is elucidating the physiological role and regulation of the chromosomal toxin–antitoxin systems during stasis. This may establish to what extent stasis-induced bacterial death is accidental or programmed.

Acknowledgements

I thank Kenn Gerdes for enlightening discussions about bacterial addiction and suicide, and Anne Farewell and Sam Dukan for valuable comments on the manuscript. Lars Rutberg and Lars Hederstedt at the Department for Microbiology, Lund University are gratefully acknowledged for providing

support and space in times of need. Work in my laboratory was supported by the Swedish Natural Science Research Council (NFR) and in part by the Swedish Research Council for Engineering Sciences (TFR).

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