Nonculturable bacteria: programmed survival forms or cells at death's door?

Thomas Nyström

Summary

Upon starvation and growth arrest, *Escherichia coli* cells gradually lose their ability to reproduce. These apparently sterile/nonculturable cells initially remain intact and metabolically active and the underlying molecular mechanism behind this sterility is something of an enigma in bacteriology. Three different models have been proposed to explain this phenomenon. The first theory suggests that starving cells become nonculturable due to cellular deterioration, are moribund, and show some of the same signs of senescence as aging organisms. The two other theories suggest that genetically programmed pathways, rather than stochastic deterioration, trigger nonculturability. One ''program'' theory suggests that nonculturability is the culmination of an adaptive pathway generating dormant survival forms, similar to spore formation in differentiating bacteria. The other ''program'' theory states that starved cells lose viability due to activation of genetic modules mediating programmed cell death. The different models will be reviewed and evaluated in light of recent data on the physiology and molecular biology of growth-arrested *E. coli* cells. *BioEssays* 25:204–211, 2003. © 2003 Wiley Periodicals, Inc.

Introduction

Cells of an *Escherichia coli* population entering stationary phase as a result of nutrient depletion gradually lose their ability to recover and reproduce on standard nutrient plates. This loss in plating efficiency has been described in microbial textbooks as the death phase of the bacterial growth cycle (e.g. Ref. 1). Thus, the loss of culturability has been assumed to reflect bacterial death; a consequence of stochastic, starvation-induced deterioration. The death phase following starvation was, in fact, used by some groups in the 1960s and 1970s as a model system to study how and when bacteria die when subject to no overt stress. The loss of bacterial culturability was argued to be equivalent to senescence and analogous, in some respects, to aging and death in higher organisms (e.g. Refs. 2,3). Matin's group at Stanford followed this line of thinking and demonstrated that senescing *E. coli* cells do not give up without a fight.[4,5] It was shown that proteins were synthesized for an extended period of time in cells held in the absence of exogenous carbon, nitrogen, or phosphate. Inhibitors of protein synthesis accelerated the loss of culturability in the starving *E. coli* population,[4] as did mutations that reduced the peptidase activity of the cells.[5] Thus, it was proposed that ongoing protein synthesis is a prerequisite for starvation survival and that the amino acids required were provided by protein turnover.[6] It was suggested also that proteins belonging to the early class of starvation proteins were the likely candidates required for fighting senescence,[4,6] a notion that was later confirmed.[7–11]

Several of the genes required in the alleged defence against starvation-induced deterioration have now been identified and have been argued to give insights into the phenomenon that Postgate[3] and others referred to as bacterial senescence.[11,12] However, this view has been challenged by theories advocating that starvation-induced sterility is the culmination of a programmed pathway. There are two alternative programming theories; one suggests that nonculturable cells are dormant survival forms similar to spores of differentiating bacteria (the theory of viable but nonculturable [VBNC] cells) whereas the other claims that nonculturable cells are the products of a genetically orchestrated death program. The different views on nonculturable cells will be scrutinized in this review and the conclusion reached from this exercise is that it is not yet time to obliterate the concept of a bacterial 'death phase' and senescence from textbooks in microbiology.

Department of Cell and Molecular Biology, Microbiology, Göteborg University, Box 462, 405 30 Göteborg, Sweden.
E-mail: Thomas.Nystrom@gmm.gu.se
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Acronyms & abbreviations: VBNC, viable but non-culturable cells; TA; toxin-antitoxin; σ, sigma factor.

The theory of VBNC cell formation

On the one hand, the models for nonculturability can, as stated above, be divided into one stochastic and two programming theories. On the other hand, one could also divide the theories based on the proposed cytological outcome of starvation. The stochastic deterioration theory and programmed death theory

both agree that the nonculturable cells are moribund and, if starvation and growth arrest prevails, will eventually die. The VBNC theory, in contrast, argues for nonculturable cells being genetically programmed survival forms awaiting appropriate conditions for re-growth. We will start by looking closer at this latter theory and its origin.

The capacity of self-replication and colony formation on nutrient agar plates is usually used for operational reasons in the experimental determination of bacterial life or death state. It is a simple and often reliable method to evaluate the live/dead fraction of a bacterial population. However, the failure of a bacterial cell to produce a colony on a standard nutrient plate may not necessarily mean that the cell is dead (or was dead at the time of sampling). Indeed, work in Colwell's and Kogure's laboratories suggested that bacterial cells subjected to adverse conditions, such as starvation, remained metabolically active but became incapable of reproducing on nutrient agar plates that normally allow colony formation of the bacteria.[13,14] The phrase "viable but non-culturable" (VBNC) was coined to describe these sterile but intact bacteria, and it was argued that nutrient starvation, low temperatures, high pressure, and changes in pH or salinity are some of the conditions that commonly render bacterial cells VBNC.[15] Further, Roszak and Colwell[16] proposed that the formation of VBNC cells is analogous to the spore formation of differentiating bacteria. In their view, then, the VBNC state is not primarily a cytological condition but a genetically orchestrated strategy for survival of bacteria in aquatic environments.[15,17] Indeed, it has been suggested that the ability to enter the VBNC state is a recognized inducible, genetically programmed, capacity of cells to ensure survival under adverse environmental conditions that are non-conductive to cell division and biomass increase.[15]

The possible formation of VBNC bacteria has been a major concern in public health risk assessment since many pathogenic Gram-negative bacteria, such as *Vibrio cholerae*, *Vibrio vulnificus*, and *Escherichia coli*, have been suggested to enter a VBNC state from which they "escape" detection and are able to resuscitate to the infectious state following, for example, temperature upshifts or animal passage.[18,19] However, critical scrutiny of reported resuscitations of alleged VBNC cells have attributed such results to the presence of low levels of already culturable cells that simply grow in response to the "resuscitation" conditions.[20–24]

Ravel et al.[25] is often cited as a critical paper in support of VBNC formation being a genetically programmed phenomenon. In this work, transposon mutagenesis was used to obtain mutants with an altered VBNC response. The screening procedure was designed to isolate mutants of *Vibrio cholerae* with an accelerated loss of culturability during prolonged incubation in stationary phase. One mutant (JR09H1) isolated was argued to exhibit a more rapid entry into the VBNC state based on the fact that it lost its capacity for colony formation earlier and at an accelerated rate during entry into stationary phase.[25] Also, the mutant cells remained intact and appeared to be metabolically active as judged by the fact that they became elongated when treated with a mixture of nutrients and the inhibitor of replication, nalidixic acid.[25] No information is available on whether the JR09H1 mutant is able to resuscitate under some conditions or where the mutation maps on the chromosome. Nevertheless, it has been suggested that JR09H1 is the first bacterial isolate exhibiting an altered VBNC response and that this denoted the first step towards an understanding of the genetic control of the VBNC state.[15,25]

It could be argued that a more proper approach to obtain evidence for a genetic control of VBNC formation would be to isolate mutants that fail to enter the VBNC state, i.e. mutants with a retarded rather than accelerated loss of culturability in stationary phase. If, as the VBNC hypothesis states,[15] VBNC formation is a physiological adaptation similar to spore formation, then such mutants could be selected. The VBNC hypothesis predicts that such mutants would become nonculturable later than their isogenic parent but should eventually, after long-term starvation, be worse off. No such mutants have been reported. In addition, work in Bogosian's laboratory has recently shown that long-term starvation of *Vibrio vulnificus* cells, which have been extensively used as model organisms in VBNC research, gives rise to oxidation-sensitive cells, which fail to reproduce on standard agar plates unless the plates are provided with agents that eliminate hydrogen peroxide.[23] Thus, the *V. vulnificus* cells that have previously been regarded as VBNC were, in fact, shown to be a subpopulation of the culture that failed to reproduce due to starvation-induced hydrogen peroxide sensitivity. Moreover, it was demonstrated that the reported resuscitation of VBNC cells during incubation at high temperatures[26] was the result of growth of a small fraction of already culturable, but initially hydrogen peroxide-sensitive, cells.[23] Growth of the cells on constituents leaking from their dead siblings restored their resistance to hydrogen peroxide and thus their ability to grow on standard nutrient plates.[23]

Until results are available on whether VBNC formation is a genetically programmed pathway and the functions of the genes involved have been identified and characterized, the concept of VBNC as an adaptive strategy remains obscure. In addition, it should be noted that the VBNC semantics as such have been heavily debated and the phrase "viable but nonculturable" has been called an oxymoron.[20] For refreshing in-depths reviews on these issues the reader is referred to e.g. Kell et al.[21] Barer and Harwood,[20] and Bogosian and Bourneuf.[24]

The theory of programmed cell death

It has been recognized that many bacteria harbor toxin-antitoxin (TA) loci. These TA loci, or addiction modules, were shown to be 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 TA loci most often consist of a downstream toxin gene and an upstream antitoxin gene organized in an operon. 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. The cells are thus said to be addicted to the antitoxin and the plasmid carrying it because it prevents the lethal action of their cognate toxins.[27] Many antitoxins appear to neutralize their cognate toxins by direct protein–protein interaction and the targets for some toxins have been identified. For example, CcdB of the F plasmid inhibits DNA gyrase whereas Kid of R100/R1 inhibits initiation of replication at *oriC* by interacting with the DNA helicase subunit DnaB.[28,29]

More recently, it has been recognized that bacteria harbor TA loci also on the chromosome. The chromosome of *E. coli* codes for five TA loci; three *relBE* and two *chp* (*mazEF*) operons.[30] It is difficult, to say the least, to accommodate these chromosomally located TA loci in an addiction scenario and instead it has been proposed that they mediate programmed cell death upon nutritional stress and growth arrest.[31,32] 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.[31] Ectopic overproduction of MazF effectively reduced the viable counts of the population suggesting that MazF is a bona fide toxin. Moreover, it was demonstrated that artificial elevation of ppGpp levels, the alarmone of the stringent response, reduced transcription of the *mazEF* operon. Based on these results, it was argued that programmed cell death is triggered whenever conditions, like nutrient starvation and growth arrest, elicit ppGpp accumulation, which will block further production of MazEF and allow the more stable toxin, MazF, to express its killing function.[31] In a sense, this will be analogous to losing an episome carrying a TA locus. Aizenman et al.[31] proposed that the *mazEF* operon provide the cells with a system for altruistic cell death during starvation conditions in that programmed deterioration of part of the population may enable the rest to survive or even grow on constituents leaking out of dead siblings.

This is an intriguing idea, which, if correct, would call for a re-evaluation of the stringent response and its proposed beneficial role in starvation survival. However, before doing this, a number of questions concerning the concept of altruistic cell death in unicellular organisms and the exact regulation and function of the chromosomally TA loci needs to be clarified. For example, the proposed benefits of cell death for the population as a whole have not been convincingly shown either experimentally or theoretically. The altruistic death hypothesis also has the weakness of many group selection theories (even though in this case it can be argued that the population is clonal) in that it is difficult to understand how such a response might have evolved through natural selection. Also, the loss of viable counts elicited by the TA systems has most often 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,[31] this operon is induced in a wild-type context during conditions eliciting stringency.[33] Based on these problems, the possibility was raised 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.[11] It was argued that, when artificially expressed in an exponentially growing culture, they may inhibit a required process, leading to cell death or the loss of culturability.[11] Indeed, a recent publication from the Gerdes group[34] has given experimental support for this notion and clarified many of the issues described above. Most importantly, it is demonstrated that the MazF (ChpAK) and the RelE toxins do not, in fact, kill the cell. Overproduction of the toxins rather elicits a bacteriostatic condition, which can be fully reversed by ectopic production of the cognate antitoxins.[34] In other words, elevated levels of the toxins locks the cells in a growth-arrested, G_0-like, state, which is incompatible with colony formation on nutrient agar plates unless the cognate antitoxin is similarly elevated to counteract this state. The RelE toxin was shown to primarily 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 (Fig. 1; Ref. 34). As such, the TA modules may serve as a back-up system to the stringent response and check superfluous macromolecular synthesis in a ppGpp-independent fashion during stasis. The Pedersen paper[34] undermines the theory of programmed and altruistic cell death by TA loci and it contradicts its notion that some members of the stringent response network have evolved to promote starvation survival while others promote killing:[31] a not-so-trivial regulon conflict.

The theory of stochastic deterioration

Genes induced early upon cellular growth arrest have been recognized as the most important ones in the bacterial fight against stasis-induced loss of culturability (e.g. Refs. 6,8,35). Many of these genes encode proteins with specific roles in protecting the cell against external stresses, e.g. heat, oxidants and osmotic challenge. As a consequence, growth-arrested cells are highly resistant to a variety of secondary stresses, a phenomenon known as stasis-induced cross protection.[6,8] This cross protection relies, to a large extent, on one single regulator, the sigma factor σ^S.[8] The σ^S transcription factor accumulates, binds, and directs the RNA polymerase to more than 50 specific genes upon conditions of cellular starvation and stress.[8] The members of the regulon are a diverse set of proteins, the functions of which overlap

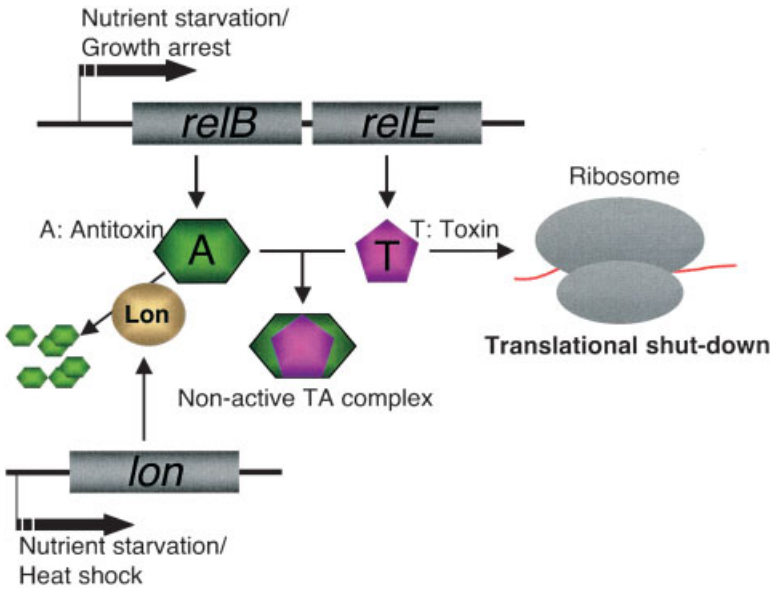

Figure 1. Schematic representation of the RelBE toxin-antitoxin (TA) loci. The *relB* gene encodes the antitoxin (A) and the *relE* gene the toxin (T). The RelE toxin appears to specifically inhibit the process of translation by a hitherto unknown mechanism, which may work in concert with the ppGpp-dependent downregulation of ribosome biosynthesis during stasis. Upon starvation and growth arrest the operon is induced in a ppGpp-independent fashion.[33,34] The antitoxin is degraded by the heat-shock protease Lon (other toxins are degraded by ClpP), which is increasingly produced during a variety of adverse conditions including starvation, elevated temperatures and conditions causing reduced translational fidelity. Possibly, the elevated production of Lon and ClpP during growth arrest allows some toxins to "escape" binding to their cognate antitoxins leading to downregulation of superfluous macromolecular biosynthesis.[11,34]

significantly with those of the *daf-16* regulated genes of *Caenorhabditis elegans*.[36–38] The Daf-16 fork-head transcription factor is a key regulator in the starvation-induced dauer formation and, like σ^S, this regulator directs the transcriptional apparatus to genes involved in protection against heat shock and oxidative agents.[36,37] Overexpression of *daf-16* extends the life span of adult nematodes whereas *daf-16* inactivation accelerates aging and causes an increased oxidative damage of proteins.[38] Similarly, *E. coli* mutants lacking σ^S exhibits accelerated senescence during conditions of growth arrest (8) and elevated levels of oxidatively damaged proteins.[39,40] Apart from σ^S and the primary defence proteins, such as superoxide dimsutases and catalases,[40] glutaredoxin 2 has recently been shown to be required in the combat against protein oxidation, particularly in the stationary phase[41] and another regulatory system, the ArcA regulon, has been suggested to minimize generation of oxygen-free radicals. Indeed, the poor plating efficiency of starved *arcA* mutants can be suppressed by overproduction of superoxide dismutase.[10] In *Salmonella*, both σ^S and σ^E have been shown to be required for protection against oxidative damage in stationary phase. Nearly all cells of a *Salmonella* population lacking both σ^E and σ^S become nonculturable after 24 hours in stationary phase, but the plating efficiency of these mutants is completely preserved under anaerobic stationary-phase conditions.[42] In addition, the loss of culturability of wild-type *E. coli* cells during the first 10 days of stasis can be completely counteracted by omitting oxygen.[40]

Thus, the accumulated data suggest that there is an increased demand for oxidation management in cells subjected to nutrient starvation and a significant number of the genes and regulons induced by stasis are indeed part of such an induced defence machinery (Fig. 2A). However, this machinery obviously fails to fully combat starvation-induced oxidation since oxidative modifications of proteins, such as carbonylation and illegitimate disulfide bond formation, increase during stasis in wild-type *E. coli* cells (Fig. 2B; Ref. 39). Stasis-induced oxidation affects specific *E. coli* proteins; e.g. the Hsp-70 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.[39,40] Some of these proteins have been demonstrated to be specifically carbonylated also in oxidation-stressed yeast cells[43] and aging flies.[44] Based on the identity of the oxidized proteins, it can be concluded that 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.[39,40] It is unclear whether the apparent sensitivity of some proteins to oxidation is the result of design rather than necessity or chance. For example, it is possible that metal-catalyzed oxidation will be an intrinsic problem for proteins containing e.g., iron and manganese.[45] It is known that a number of different reactive oxygen radicals are involved during the course of the protein oxidation process and that transition metal ions can substitute for hydroxyl groups and superoxide radicals in some of these reactions.[46] In addition, it is possible that some proteins (e.g. Krebs cycle enzymes) are oxidized mainly because they are located in the proximity of sites generating reactive oxygen species (ROS).

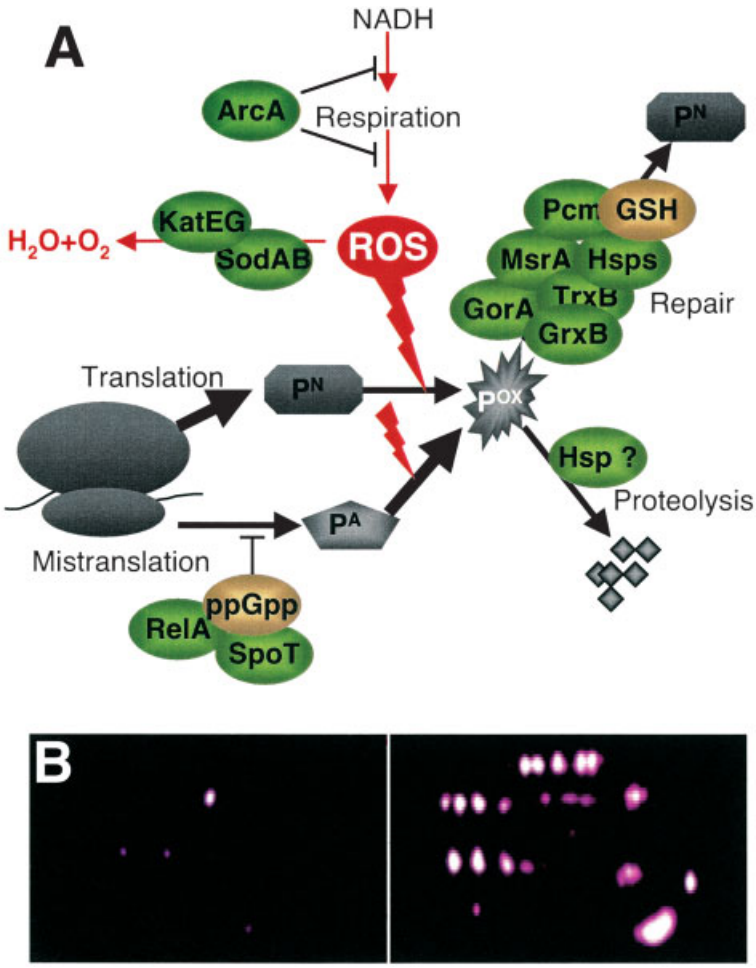

Figure 2. A: Schematic representation of the bacterial defence against starvation-induced oxidative deterioration of proteins. Green ovals denote proteins that are activated or accumulated during starvation whereas GSH and ppGpp (orange ovals) are glutathione and guanosine tetraphosphate, respectively. The defence against oxidation appears to work at different levels. The ArcA-dependent downregulation of reducing equivalent production and respiratory activity is suggested to reduce generation of reactive oxygen species (ROS) during starvation[10] whereas the superoxide dismutases (SodA and SodB), and catalases (KatE and KatG) further reduce ROS levels catalytically. Another line of defence is the elevation of proteins involved in the repair and reduction of oxidized proteins. These proteins include Pcm (L-isoaspartyl protein methyltransferase), MsrA (peptide methionine sulfoxide reductase), GorA (glutathione reductase) in concert with GSH (glutathione), TrxB (thioredoxin), GrxB (glutaredoxin), and HSPs (heat shock proteins). The RelA/SpoT/ppGpp system of the stringent response is suggested to reduce protein oxidation by affecting translation fidelity (aberrant proteins appear to be more susceptible to oxidative attack Ref. 47,49. Finally, oxidized proteins, damaged beyond possible repair (e.g. carbonylated proteins), should be degraded and it has been shown that carbonylated proteins are highly susceptible to proteolytic attack (e.g. Ref. 49). However, the proteases involved in this process have not been identified but may be members of the heat-shock regulon. Abbreviations: P^N, native protein; P^A, aberrant protein; P^{OX}, oxidized protein. **B:** Protein oxidation in growing (left panel) and starving (right panel) *E. coli* cells analyzed by two-dimensional immunodetection of carbonylated proteins (unpublished pictures from the Nyström lab (see also Refs. 47,49).

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.[47] 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. The use of diagnostic proteomics demonstrated that the sudden increase in protein oxidation during the early stages of stasis in *E. coli* is strongly associated to the production of aberrant protein isoforms; seen as protein stuttering on two-dimensional gels.[47] (The phenomenon called protein stuttering has been shown to be the result of erroneous incorporation of amino acids into proteins

and can be detected on autoradiograms of two-dimensional gels as satellite spots with similar molecular weights to the authentic protein but separated from it in the isoelectric focusing dimension.[48] Moreover, the level of protein carbonylation has been found to increase upon treatment of cells with antibiotics, e.g. streptomycin and puromycin, and mutations causing increased mistranslation.[49] 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.[49] In other words, protein oxidation of aberrant proteins does not appear to be sensed by the oxidative defence regulons and does not require increased generation of reactive oxygen species.

Frameshifting,[50,51] missense errors,[48,52] and stop codon read-through[47] increase in response to stasis in *E. coli* cells. This, together with results showing that aberrant proteins are more susceptible to oxidation, raises the possibility that carbonylation in non-proliferating cells may be caused by an increased mistranslation. Indeed, it was demonstrated that protein carbonylation is drastically attenuated in the early stages of stasis in *rpsL141* mutants, which harbor intrinsically hyper-accurate ribosomes.[47] Thus, the elevated oxidation of proteins in non-proliferating cells may be due to an increased availability of substrates (aberrant proteins) available for oxidative attack and these substrates surge during stasis due to a reduced fidelity of the translational apparatus. It is not, at present, clear why aberrant proteins are more susceptible to carbonylation. Possibly, a slight misfolding of the corrupted polypeptide exposes oxidation-sensitive targets that are normally hidden during the coupled translation-folding process. This, and other possibilities, await experimental scrutiny.

Conclusion

Proponents of the stochastic deterioration theory argue that aerobic metabolism might be the Achilles heel of starving *E. coli* cells and that the loss of culturability is intimately linked to oxidative damage. It is possible, however, that sterility may be reversible depending on the magnitude of the cellular damage. Nevertheless, if starvation and oxidation damage are allowed to proceed for an extended period of time, the non-culturable cells are argued to be moribund and destined to irreversibly lose their life-supporting activities. This is consistent with results demonstrating that sterility is rapidly followed by membrane leakiness in stationary phase *E. coli* cells.[53] It follows that the reduced culturability of starving e.g. *rpoS*, *rpoE, oxyR, sodAB, katEG*, and *arcA* mutant cells simply means that these mutants die with an accelerated rate. The fact that starvation and nonculturability are intimately associated with several types of oxidation damage, of which one (carbonylation) is highly detrimental and irreversible, strongly favors the stochastic deterioration theory over the VBNC hypothesis. In addition, the VBNC hypothesis does not offer a logical explanation for why VBNC formation is not necessary during starvation under low oxygen tension (cells are culturable for extended periods of time under anaerobic conditions). In addition, the data that are presented to provide evidence for a genetic control of the VBNC state[15,25] are not convincing and no gene conclusively involved in such a program has yet been named or mapped.

So, can bacteria ever exist in a reversible nonculturable mode? Of course, bacterial cells may well become reversibly nonculturable and specific conditions could rescue such cells. For example, cadmium-exposed *E. coli* cells are nonculturable until sufficient DNA repair can catch up with the damage produced by Cd.[54] Ectopic overproduction of the universal stress protein A, UspA, appears to lock stationary phase cells in a growth-arrested state[55] as does artificial expression of toxins of the TA loci until induction of the antitoxins rescues their ability to proliferate.[34] Moreover, work in Kell's laboratory has demonstrated that apparently sterile, starving cells of the Gram-positive organism *Micrococcus luteus* can be truly resuscitated in the presence of Rpf, a protein encoded and produced by the organism itself.[56] However, these, and other examples, do not support the notion that nonculturability is an inducible, genetically programmed capacity of cells to ensure survival under adverse environmental conditions, as stated by the VBNC hypothesis.[15]

The theory of programmed cell death, after being presented as an alternative model for stationary phase death and a novel role of the stringent response in cell suicide,[31] has been challenged by recent work from the Gerdes group. It turns out that the toxins RelE and MazF (ChpAK) of the TA loci do not kill cells but rather lock them in a growth-arrested mode, which can be counteracted by the cognate antitoxins (34). The proposed biochemical function of the toxins points to a beneficial, rather then detrimental, role of these toxins in checking superfluous macromolecular synthesis during starvation. As such, the chromosomally located TA loci have become less mysterious and the name "toxins" may, in fact, be misleading.

Thus, we are left with the notion that the original theory of the bacterial death phase and the concept of bacterial senescence pioneered by e.g. Postgate[3] is the most viable one among contemporary models of non-culturable bacteria. As stated by Postgate in regard to his favorite organism: "During starvation klebsiellae do senesce and die. Some individuals die faster than others. Such death, which seems to be the nearest that they come to a 'natural' death of the kind familiar among higher organisms, is a response to the mild stress of starvation".[57] As reviewed here and elsewhere,[11,12] recent progress on the molecular biology of growth-arrested *E. coli* cells suggest that the pathways of bacterial senescence and mandatory aging in higher organisms may have even more in common than first anticipated.

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