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Review

Bacterial suicide through stress

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Abstract. Outside of the laboratory, bacterial cells are constantly exposed to stressful conditions, and an ability to resist those stresses is essential to their survival. However, the degree of stress required to bring about cell death varies with growth phase, amongst other parameters. Exponential phase cells are significantly more sensitive to stress than stationary phase ones, and a novel hypothesis has recently been advanced to explain this difference in sensitivity, the suicide response. Essentially, the suicide response predicts that rapidly growing and respiring bacterial cells will suffer growth arrest when subjected to relatively mild stresses, but their metabolism will continue: a burst of free-radical production results from this uncoupling of growth from metabolism, and it is this free-radical burst that is lethal to the cells, rather than the stress per se. The suicide response hypothesis unifies a variety of previously unrelated empirical observations, for instance induction of superoxide dismutase by heat shock, alkyl-hydroperoxide reductase by osmotic shock and catalase by ethanol shock. The suicide response also has major implications for current [food] processing methods.

Key words. Bacteria; sublethal injury; stress; suicide response; free radical; cell death.

What is meant by suicide through stress?

At some point in their life cycle, bacteria will experience stress—an environmental condition that lies outside of the normal parameters for growth. Indeed, the human race has developed technologies to apply exquisite stress to bacteria so as to control their numbers in sensitive locations such as food products or even the human body itself. However, although a stress may lie outside of a bacterium's normal experience, it need not be so extreme as to be lethal—hence the term 'sublethal stress'. Moreover, there is a grey area at the border between sublethal stress and lethal stress, where a lone cell may prove susceptible or resistant to a given level of stress, depending upon its growth phase or metabolic state. This grey area is the area of concern in this review.

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We have recently advanced a new hypothesis in an attempt to explain the many empirical observations relating to the greater sensitivity of cells in exponential growth phase compared with those in stationary phase: this hypothesis is the suicide response hypothesis [1]. Essentially, bacterial cells are predicted to kill themselves through production of free radicals as a result of uncontrolled metabolic activity subsequent to the application of a relatively mild (sublethal) stress. The suicide response proposes that when actively growing and aerobically respiring bacterial cells are subjected to a sublethal stress, they suffer a growth arrest but their metabolism continues to function for a time. As a consequence of this uncoupling of energy production from energy utilisation, a burst of excess free radicals occurs, and it is this burst of free radicals which is lethal to the cell, not the action of the stress per se [1].

Development of the suicide response hypothesis

As a research group, our interest has been in the survival of food-borne pathogens after injury associated with processing stresses, such as thermal and osmotic shocks. Previously, we noted that a competitive microflora, at a density of 10^8 colony forming units (CFU) ml^{-1} or greater, could significantly enhance the survival of an exponential phase population of 10^5 CFU ml^{-1} *Salmonella typhimurium* when subjected to heat shock or to freeze–thaw injury [2, 3]. A cell density of 10^8 CFU ml^{-1} is associated with the transition into stationary phase, and the initial suggestion was that the competitors were initiating an early stationary phase in the *Salmonella* population. The increased resistance observed in these experiments could then be explained by the known greater resistance of cells in stationary phase to thermal and osmotic stress. In *Escherichia coli*, entry into the stationary phase of growth leads to the acquisition of a range of resistance mechanisms, the genes for a number of which have been shown to be regulated by the alternative sigma factor, σ^s (RpoS) [4–7]. Such genes include ones for thermotolerance (*otsAB* and *treA*) [8], and resistance to osmotic and oxidative stress (*otsAB* and *katE*) [4, 8]; induction of such genes in the stressed *Salmonella* could therefore explain the observed increase in resistance.

Using a real-time biosensor for intracellular RpoS activity (*spvRA′::luxCDABE*) [9], we demonstrated that increased RpoS activity was induced earlier in *Salmonella* in the presence of 10^8 CFU ml^{-1} live competitor bacteria, leading to earlier expression of RpoS-regulated genes. However, onset of this induced RpoS activity took 2 h, whereas the increased resistance of the *Salmonella* was effectively instantaneous on mixing with competitor cells. Moreover, in an *rpoS* negative background, the increased resistance afforded by competitor cells was still evident. Hence, the elevated stress resistance afforded to the salmonellae by the competitors was not a consequence of increased RpoS activity [3]. This meant that another mechanism must have been in action, and the fact that it was so rapid militated against the induction of a genetic response as the explanation. Thus, a physiologically based change could be the key.

This led to a consideration of other features of stationary and exponential phase cells which may explain their differing resistance to inimical processes, in particular their differences in metabolic potential and growth rate. Stationary phase cells have low metabolic flux associated with halted growth in contrast to the high metabolic flux that accompanies growth of exponential phase cells. Hence, it was suggested that some of the effects of an inimical treatment which are lethal to exponential phase cells but sublethal to stationary phase cells could be due to the difference in metabolic potential between the two populations. Furthermore, it was proposed that it is not the direct physical effect of the treatment per se which kills exponential phase cells but that there is an element of self-destruction by the bacterial cells: this was termed the suicide response.

Thus, when an inimical treatment is imposed on an exponentially growing culture, growth is abruptly arrested while metabolism continues unaffected. Consequently, a burst of free-radical production resulting from an imbalance between anabolism and catabolism causes significant damage to intracellular components, including DNA and proteins, and it is this rather than the inimical treatment itself which leads to death of the cell. Stationary phase cells are protected against this phenomenon first by a reduction in the level of metabolism which is accompanied by substantially reduced free-radical production [7], second by cessation of growth and finally by expression of resistance factors under the control of RpoS [4]. Therefore, when a stationary phase culture is exposed to a mild inimical process, no free-radical burst occurs and the population survives largely uninjured. Using this as a starting hypothesis, the action of the competitive microflora could then be considered.

When 10^8 CFU ml^{-1} of viable competitors are incorporated into the stress experiments, it is proposed that their presence rapidly alters the environment and causes a change in the physiology of the underlying target population so that resistance to the inimical treatment is enhanced. Factors such as alterations to pH or oxygen tension may be considered as a means of producing an environmental switch responsible for altering the resistance of target cells. We have shown that competitor

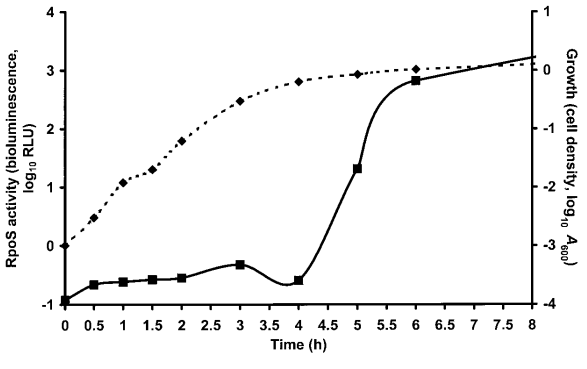

Figure 1. Growth and RpoS activity in a population of 10^5 CFU ml^{-1} *S. typhimurium* LT2[pSB367]. The growth (cell density) of cultures incubated aerobically at 37 °C was monitored spectrophotmetrically (- - -◆- - -, $\log_{10} A_{600}$), whilst RpoS activity was monitored using the reporter plasmid pSB367, which carries *spvRA′::luxCDABE* and produces bioluminescence in response to active intracellular RpoS levels [9] (–■–, $\log_{10}$ RLU).

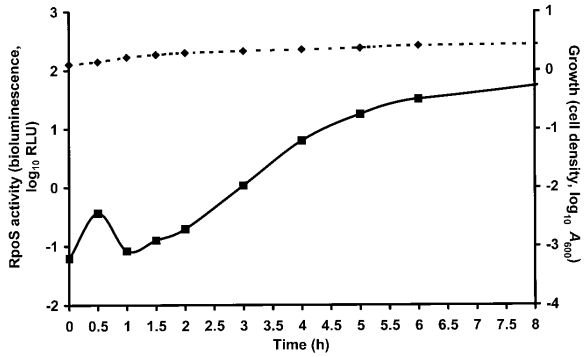

Figure 2. Growth and RpoS activity in a population of 10^5 CFU ml^{-1} *Salmonella typhimurium* LT2[pSB367] mixed with 10^8 CFU ml^{-1} viable competitors. The competitive microflora comprised a mixture of wild-type *E. coli*, *Citrobacter freundii* and *Pseudomonas fluorescens* [3]. Growth (- - -◆- - -, $\log_{10} A_{600}$) and RpoS activity (–■–, $\log_{10}$ RLU) were monitored as described in figure 1.

cells have no effect upon pH but do reduce the environmental partial pressure of oxygen to virtually nil within 10 s, whilst growth of the salmonellae becomes coordinate with that of the competitors (figs 1 and 2). Thus the *Salmonella* cells are in a state of reduced oxygen tension that would lead to a change from aerobic respiration to anaerobic fermentation and consequently to a reduced risk of free-radical production when subjected to stress [1, 3]. Thus, an explanation for the effect of competitor organisms may be provided by the suicide response hypothesis.

Supporting evidence

Supporting evidence for the proposed free-radical burst associated with the suicide response is provided by our recent work with lucigenin. Lucigenin has been used as a chemiluminescent reporter for free radicals, and using this reagent we have shown that a burst of free-radical activity was evident when exponential phase *E. coli* cells were heat-shocked at 56 °C in the presence of 50 μM lucigenin. In contrast, no free-radical activity (chemiluminescence) was evident from unheated cultures (fig. 3A, B) [10]. Whilst the use of lucigenin as a free-radical reporter can be considered controversial, since at inappropriate concentrations it can act as a superoxide generator in much the same way as paraquat does [e.g. 11, 12], the concentration we have used is sufficiently low as not to cause significant spurious superoxide production [11], and this is clear from the lack of chemiluminescence in the control culture. In addition, there are a number of relatively unconnected observations which support the concept of the suicide response and the production of free radicals as a consequence of exposure to an inimical process. First, the regulator which restricts tricarboxylic acid (TCA) cycle activity in stationary phase cells is ArcAB. Mutants of *E. coli* which are deficient in ArcAB function continue to metabolise at an excessive, exponential phase, rate in stationary phase and continue to utilise oxygen at a high rate; these mutants are much less able to survive prolonged C-starvation unless they overproduce SodA, superoxide dismutase, an enzyme needed for elimination of oxygen radical species [13]. Second, osmotic shock of *Staphylococcus aureus* has been found to induce a homologue of AhpC, an alkyl hydroperoxide reductase whose function is to protect against the action of lipid radicals [14]. Initially, it was unclear why an alkyl hydroperoxide reductase should be expressed in response to osmotic stress; however, if a burst of free radicals is consequent upon the osmotic shock, then the cell may induce oxidative stress

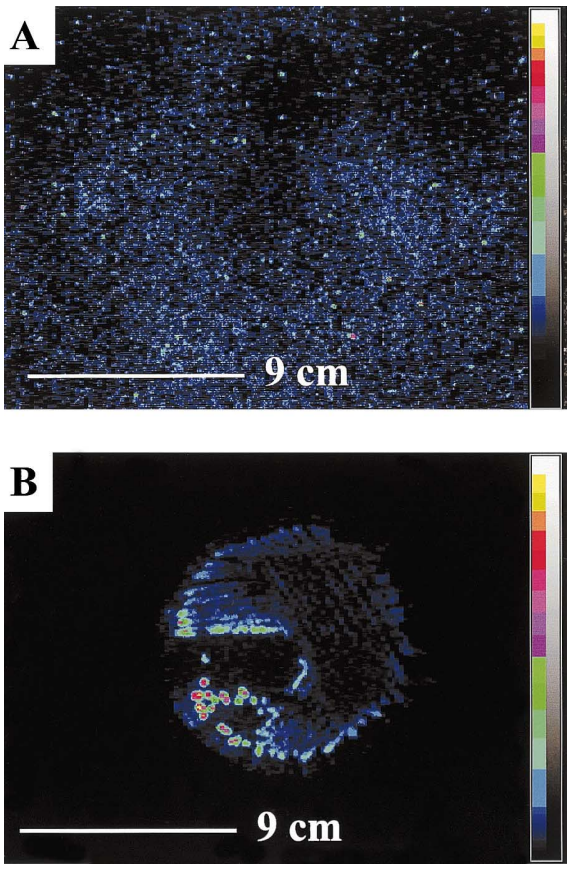

Figure 3. Chemiluminescence from a culture of *E. coli* K12 (grown on LB agar supplemented with 50 μM lucigenin) prior to heat shock (*A*) and after heat shock at 56 °C for 2 min (*B*).

genes as a result. Third, heat shock of *E. coli* has been shown to lead to the induction of superoxide dismutase expression, whilst superoxide dismutase negative mutants of *E. coli* are much more susceptible to heat stress in aerobic environments [15, 16]. Constitutive expression of the oxidative stress response regulon, OxyR, also leads to enhanced heat resistance in *S. typhimurium* [17]. Finally, at a more practical level, it has been noted that the heat resistance of a number of food-borne pathogens can be altered by the use of modified atmospheres. The heat resistance of *E. coli* O157:H7, *S. enteritidis* and *Listeria monocytogenes* can be increased eightfold if they are grown, heated and recovered in an anaerobic environment. Even the use of a reduced environmental oxygen partial pressure may enhance the survival of these organisms to some extent, since a 10^6-fold reduction in viability was achieved in 3 min at 59 °C and atmospheric oxygen concentrations, but this was extended to 5–17 min at oxygen concentrations of 0.5–1% and to 19–24 min under anaerobic conditions [18].

Although the suicide response grew from observations with thermally stressed cells, and all of the supporting evidence given so far relates to thermal stress, the literature indicates that exposure to other forms of stress (e.g. ethanol shock) is also experienced by bacterial cells as an oxidative stress. Using lucigenin, we have shown a significant increase in free-radical activity after exponential phase *E. coli* cells were shocked with 20% ethanol [10]. Furthermore, when cells of *E. coli* harbouring a *katG′::lux* construct were exposed to ethanol shock, bioluminescence was expressed, indicating that the catalase was being expressed [19]. As was noted with heat shock, induction of the OxyR regulon can also provide protection against chemical agents such as 5-chloro-2-methyl-isothiazolin-3-one (MCI), an observation which suggested a role for free-radical generation in the antibacterial activity of MCI [20].

The accumulation of adenylylated nucleotides (termed alarmones) in bacterial cells after exposure to either heat shock or ethanol shock has previously suggested a common mode of action for diverse inimical treatments. That these possible alarmones also accumulate in bacterial cells in response to an oxidative stress indicates what that common mode of action might be: that bacterial cells experience diverse stresses as an oxidative stress [21].

These observations together support the idea that bacterial cells experience differing stresses such as thermal, osmotic shock or ethanol shock as an oxidative stress. Evidence that the lethality of some stresses is dependent on metabolic function also exists. For instance, hydrogen peroxide (H_2O_2) can have a lethal action in one of two ways, depending on its concentration: mode I killing occurs at low concentrations of H_2O_2, whilst mode II killing occurs at high concentrations. Whereas mode II killing is due to gross damage of cellular structures by the peroxide and is just as effective against stationary phase cells as exponential phase ones, mode I killing requires the target cells to be metabolically active since starved cells are more resistant than starved cells grown in the presence of glucose [22]. In other words, mode I killing may not be through the action of the H_2O_2 per se, but through the metabolic action of the cell during and after exposure to low concentrations of H_2O_2.

How might suicide through stress occur?

Actively growing and respiring cells use a more hazardous energy production strategy to give them a competitive advantage in growth that leads to their susceptibility to the suicide response. Bacterial cells in the stationary phase of growth have restricted metabolic activity [10] and constitutively express a whole host of resistance factors which essentially preadapt them to stresses [4]. Moreover, bacterial cells which are either growing in an anoxic environment, or which are utilising anaerobic fermentative metabolic pathways, will be producing energy at a much slower rate per mole of carbon source and will not be using oxygen as a terminal electron acceptor.

Production of the metabolic enzymes of an organism such as *E. coli* is regulated by the ArcAB system [13]. Under low oxygen conditions, production of TCA cycle enzymes is downregulated, whilst fermentative enzymes are expressed. However, when the availability of oxygen increases in the environment, expression of TCA cycle enzymes is upregulated, whilst that of fermentative enzymes is downregulated so that more efficient use may be made of the available energy source. Although the TCA cycle allows more efficient use of resources, it carries with it an increased risk that reactive oxygen species will be produced [23]. Therefore, protective enzymes such as superoxide dismutase and endonuclease IV are also essential to protect against oxidative damage to intracellular macromolecules such as DNA, polypeptides and lipids [24]. The constitutive expression of protective superoxide dismutase enzymes during aerobic respiration is, nevertheless, at a level which is only just sufficient to remove endogenous superoxide ($O_2^{\cdot-}$) that results from respiration. Consequently, any imbalance between the level of superoxide dismutase and the level of $O_2^{\cdot-}$, through stress for instance, will significantly reduce the fitness of the bacterial cell [25]. When DNA is damaged, the SOS response is induced [26], and one of the factors expressed as part of the SOS response is the SulA protein, which stops cell division [27]. This provides a possible explanation for cessation of growth

after exposure to free-radical production by the suicide response since cell division and chromosome replication will be halted to allow DNA repair following induction of the SOS response. Meanwhile, it is possible to envisage continued passage of carbon through the TCA cycle, with concomitant production of free-radical species until oxidative damage to intracellular macromolecules becomes so extreme that the cell cannot continue to function as a viable entity.

Suicide through stress can help to explain some empirical observations

Suicide through stress—the suicide response hypothesis—can help to explain a number of observations which have previously appeared obscure. For instance, Postgate [28] noted a phenomenon which he termed substrate accelerated death. Addition of a substrate to a starved population of Gram-negative bacteria which had previously encountered that carbon source actually accelerated the death of the population, rather than assisting recovery. In contrast, addition of a substrate to which the cells of the starved population had not previously been adapted did not lead to accelerated death. The suicide response provides a potential explanation for this observation: metabolic activity would be initiated upon exposure to a preadapted substrate, and this could lead to lethal free-radical generation. In contrast, exposure to a substrate to which the cells had not previously been adapted would require a period of adaptation which might also provide sufficient time to express antioxidant defences.

This hypothesis could also help to explain the long-accepted observation that injured or starved cells can be recovered much more effectively on minimal media at relatively low temperatures and with limited aeration than on nutrient rich media. The excessive concentration of nutrients present in a nutrient-rich medium coupled with temperatures which promote rapid enzymic activity could quite easily lead to a metabolic imbalance with which the injured cell is ill-equipped to cope. The free radicals generated as a result of this imbalance could then go on to have a lethal effect upon the cells.

What does suicide through stress mean in the real world?

The suicide response hypothesis has serious implications for food-processing technologies. The use of modified atmosphere packaging is becoming more popular amongst manufacturers as a means of stabilising the organoleptic properties of food products (sandwiches, salads, meat and cheeses, for example). However, the suicide response hypothesis predicts that any food-borne pathogens which have survived processing in an injured state will be much more likely to recover in a low-oxygen atmosphere than in an aerobic atmosphere. Indeed, this prediction concurs precisely with the observation of George et al. [18] that the survival of *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* through thermal processing is much greater when a low-oxygen environment is used.

Conclusions

The suicide response brings together a unifying mechanism by which bacteria exposed to sublethal stresses respond, and it reorientates our thinking on the difference in stress resistance between exponential and stationary-phase cells. Whilst stationary-phase cells certainly present an armoury of defence mechanisms against stress, it is their intrinsic difference of being metabolically less active that may present an inherent safety mechanism for the cell. Exploitation of this concept could allow novel approaches to bacterial eradication to be made. Thus, encouraging bacteria to enter a metabolically active phase by the provision of a brief growth period ('good-time window') prior to processing is contrary to current ideas but should increase the efficacy of any applied stress. Such ideas may allow more minimal processing of foods to produce safer products in the future.

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