# FACTORS AFFECTING THE EMERGENCE OF PATHOGENS ON FOODS

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## ABSTRACT

Recent times have seen the emergence of a number of new or emerging pathogens. Research is needed to establish the mechanisms underlying their emergence in foods, and their interactions with traditional food production processes. The paper reviews the general relationships between environmental stresses and pathogen emergence. Non genetic mechanisms of pathogen adaption to stress are considered, focusing on acid resistance as an example of particular importance to pathogen survival in food and the human host. The influence of environmental factors in genetic adaptation in stationary phase cells are considered in relation to the development of non-directed antibiotic resistance i.e. development of resistance when antibiotics are not present in the environment. The paper examines the molecular mechanisms underliying such genetic processes and their enhancement during environmental stress. The implications of these mechanisms for food safety and pathogen emergence are discussed.

## INTRODUCTION

In the 13th century a large proportion of the inhabitants of Dublin city died as a result of bubonic plague, probably Yersinia pestis. At that time Dublin was a walled city with narrow streets in which people lived very closely together. Ships arriving at Dublin port brought rats from other countries which were infected with the plague organism. The houses became infested with rats who spread the plague to the human population through fleas. The fleas were further spread to other areas of the country in hay and straw. The population in the city was ill-equipped to deal with the plague due to the presence of famine, a constant threat in the middle ages. With crowded living conditions, poor sanitation, host deprivation, and the presence of suitable pathogen vectors, bubonic plague emerged in Ireland with devastating consequences (Guinan, 1997).

In modern times, the emergence of pathogenic bacteria is controlled by a different sets of factors, but their success is still fundamentally dependent on conditions which favour their growth and spread. In advanced societies, such factors include the introduction of new food types (sous vide, modified atmosphere packs, cook-chill), changes in animal production and health maintenance, relocation of animal production systems to warmer climates, changes in animal slaughter and processing methods, and changes in consumer eating habits (Miller et al., 1997). While these societal and environmental factors can have profound influences on the success of emerging pathogens, bacteria may also employ a number of strategies at the cellular level that play a significant role in emergence (Archer, 1996). Thus humans are in what Lederberg (1997) describes as " a type of race, enmeshing our ecological circumstances with evolutionary changes in our predatory (bacterial) competitors."

Pathogenic bacteria may be subjected to a wide range of stresses when growing on foods or invading a host, and must be capable of adapting to each of these in order to survive and cause disease. Success in dealing with the varied stresses that organisms encounter on foods and on subsequent ingestion by humans indicates that they are capable of adapting to survival in a wide range of hostile environments (Table 1).

According to a recent report, research is needed to determine how bacteria become tolerant to antimicrobials and traditional food-safety controls, such as heat, cold, low pH, high salt and disinfectants (Anon., 1997). It also recommends the investigation of those factors in processing which may influence the development of resistance, as well as the acquisition of data on the physiology and genetics of these organisms, to facilitate new strategies to prevent their emergence as pathogens.

The above statement recognises that cells can respond to stress by temporary changes, and can also undergo mutation which introduces permanent genomic changes that favour their survival. According to Lederberg (1997) the responses of bacteria to stress is poorly understood and mutations may themselves be a response to stress. While mutations are relatively rare events in nature, there is increasing evidence that in stationary phase cells, mutation may occur more frequently (LeClerc et al., 1996).

**Table 1: Products, processes and environmental niches in food production and the human body which stress pathogenic bacteria**

| FOODS / PROCESS | STRESS | HUMAN BODY |
|---|---|---|
| Mayonnaise, fermented foods, Pepperoni, salami | Acid | Stomach / small intestine colon/ phagosomes |
| Cooking / processing sous vide | Heat | Temperature upshift-Intracellular environment |
| Fish, brines, marinades(salt solutions) | Osmolarity | Stomach |
| $H_20_2$ in foods | Oxygen/oxidation | Oxidative properties of phagocytes |
| Refrigeration | Cold | Temperature downshift on excretion |
| Surfaces in food Plants | Starvation | Nutrient dilution in water on defecation/ macrophages |
| Sous vide -Vacuum packaging of meat | Anaerobiosis | Phagosomes |

It has also been suggested that mutations in cells may have a significant influence on the rates of exchange of genetic material between organisms. Such genetic transfer can lead to the development of organisms with radically different characteristics, facilitating the emergence of new pathogens. The classic example is *Escherichia coli* 0157:H7 which, according to Whittam (1996), originated from an 055:H7 ancestor through horizontal transfer and recombination. The 055:H7 ancestor was most probably an enteropathogenic *E. coli* (EPEC), which had plasmid associated adherence factors, such as fimbriae, and genes (eae A) for the production of attaching and effacing lesions located on the chromosome (Donnenberg and Kaper, 1992). This ancestral EPEC pathogen acquired cytotoxic genes from *Shigella dysenteriae* type 1 as a result of phage infection and the subsequent transfer of the Shiga-like toxin genes during transduction (Wachsmuth et al., 1997). These phages are widespread in nature and can attach to a number of related enteric bacteria (Whittam, 1996). Such transfers may not be rare, as a similar transfer has been suggested in *Citrobacter freundii*, recently noted to have acquired related cytotoxins and considered to be an emerging pathogen (Miller et al., 1997).

Environmental stress can also lead to bacterial adaptation which may influence virulence (Archer, 1996). It is well recognised that the induction of bacterial stress reactions, such as increased acid resistance, enhances both survival and virulence in food borne pathogens (Foster, 1995). Thus there is the danger that inadequate application of food preservation systems designed to suppress the growth of undesirable bacteria may lead to the emergence of hardier, more virulent pathogens.

The present paper will consider the general relationship between environmental stress and pathogen emergence. Mechanisms of pathogen adaptation to stress are considered, focusing on resistance to acid, an example of particular importance to pathogen survival in food and in the human host. Consideration will also be given to pathogen adaptation as a result of mutation during stationary phase and the implications of these processes on virulence.

## ADAPTIVE RESPONSES IN BACTERIA

### 1.The acid stress response as an example of nongenetic adaptation

Pathogenic bacteria develop systems that assist them to survive and adapt to environmental stresses such as heat, cold or acid shock, in a variety of ways. These responses entail the production of protective proteins, some of which offer protection to more than one type of stress. This indicates that the genes involved in the production of stress proteins are common and operate within regulons (operons under the control of a common regulatory protein) and/or stimulons (regulons which respond independently to the same environmental stimulus) (Neidhardt, 1987), to control stress responses (McCann et al., 1991; Dorman and Ni Bhriain, 1992; Farr and Kogoma, 1991). While bacteria are capable of adapting to stresses to allow them to overcome an immediate environmental problem, these responses are temporary in nature and when a response is not required, the genes involved are switched off (Archer, 1996). The acid stress response, as one of the most comprehensively investigated, will provide an illustration of a non-genetic adaptive response.

Bacteria are known to exhibit a wide range of acid stress responses, including a number of acid tolerance responses (ATRs). These are among the most widely recognised and investigated, occurring in minimal or complex media, during exponential

growth and stationary phase. Recent reports suggest that there are at least 11 ATRs, induced under a range of conditions (Rowbury, 1997). These should not be confused with the acid responses (AR) which, as discussed by Lin et al., (1995) only occur in complex media during stationary phase. There is also a generalised stress response (GSR) where acid tolerance is expressed in stationary phase in the absence of acid induction (Matin, 1991). The following examples are among the most clearly elucidated.

**ATR during exponential growth**

This process typically involves the exposure of cells to a pH between 4.5 and 6.0 for a period of 20 mins or more, and is always accompanied by protein synthesis (Rowbury, 1977). In order to induce acid tolerance, cells are grown to about mid exponential phase in minimal or complex media at near neutral pH (Foster & Hall, 1990). The pH of the medium is then adjusted to about 5.0, usually by the addition of HCl. During this time acid shock takes place and is manifested by the formation of acid shock proteins. The number of acid shock proteins formed will vary with the pH of the inducing medium, and they generally appear during the period required for a doubling of the cells in that medium (Foster, 1995). The induced or adapted cells are then challenged at a low pH (2.5 - 3.5) for a specified time (1 to 4 hr) and the percentage survival measured. The acid challenge selects cells which have become acid resistant through the prior acid induction. Exposed cells can subsequently survive pH conditions of between pH 2.5 and 3.5, whereas non-adapted cells cannot survive below pH 4 (Foster, 1995).

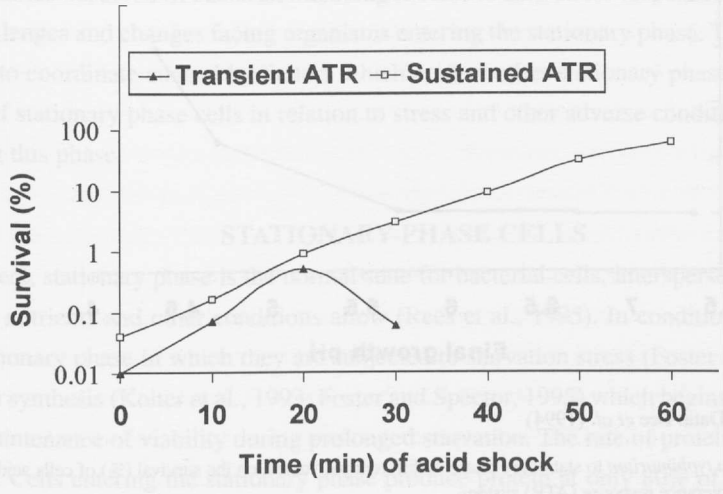
**Types of exponential ATR**

Exponentially growing cells can display a number of acid tolerance systems. Early studies by Foster and Hall (1990) established that Salmonella typhimurium cells adapted at pH 5.8 displayed "pre-shock ATR" in which a small number of acid shock proteins were synthesised. Subsequent investigations revealed the operation of an acid adaptation system at pH 4.4, which involved the production of 50 acid shock proteins. Two forms of this system are presented in Fig. 1, which shows that cells of S. typhimurium can have a sustained or transient ATR. If cells with a functioning stress response system are grown at pH 7.7 and acid shocked at pH 4.4, a sustained ATR response is observed. Mutations with a defective stress response system exhibit a transient or negligible ATR (Foster, 1995). Log phase ATR has been demonstrated in E. coli strains, Salmonella species and Listeria monocytogenes but not in Shigella flexneri (Davis et al., 1996; Lin et al., 1995) (Table 2).

**Table 2: The effect of medium, acid induction and growth phase on the acid resistance of enteric pathogens**

| Response | Medium | Phase | Induction | *Escherichia coli* | *Salmonella typhimurium* | *Shigella flexneri* |
|---|---|---|---|---|---|---|
| ATR (pH 3.3) | MM | Log | AI | + | + | - |
| ATR (pH 3.0) | MM | Stationary | AI | ? | + | - |
| GSR (pH 3.0) | MM | Stationary | NAI | + | + | + |
| AR (pH 2.5) Oxidative | CM | Stationary | AI | + | - | + |
| AR (pH 2.5) Fermentative | | | | | | |
| (a) Glutamate | CM | Stationary | (1) NAI | + | - | - |
| | | | (2) AI | - | - | + |
| (b) Arginine | CM | Stationary | AI | + | - | - |

ATR- acid tolerance response MM- Minimal Medium AI - acid induced
AR - acid response CM - Complex Medium NAI - not acid induced
Data: Lin et al. (1995)



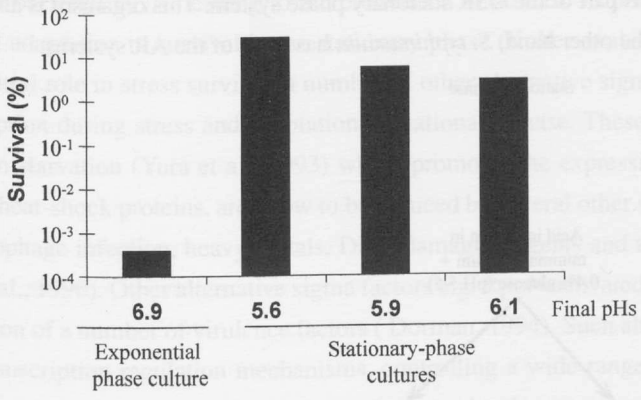

Data: Foster (1995)

Fig.1. Sustained and transient acid tolerence response (ATR) in *Salmonella typhimurium* grown at pH 7.7, after acid shock at pH 4.4 and acid challenge for 2 hours at pH 3.3 (transient) and pH 3.0 (sustained)

### ATR during stationary phase

There are two systems capable of inducing the acid tolerance response in stationary phase cells in minimal media.

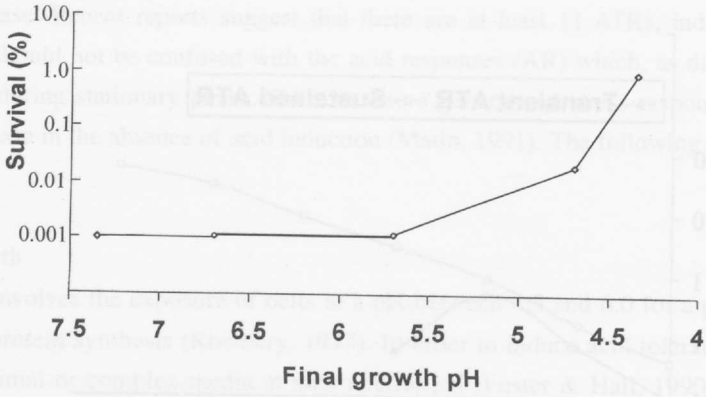
(i) Acid resistance in stationary phase cells can be induced as a result of the general stress resistance mechanism (GSR), in which acid induction is not required prior to acid challenge. Fig 2 shows the survival (%) of L. monocytogenes cells grown to stationary phase in a complex medium, unbuffered (final pH 5.6) or buffered (5.9 or 6.1), and subsequently challenged at pH 3.0 for 1.5h. The survival [%] of an exponential phase culture is included for comparison. Exponential phase cells did not survive this type of acid shock treatment, but stationary phase cells exhibited significant survival. As discussed later, specific transcription factors (i.e. alternate sigma factors such as RpoS) have been identified as controlling such survival in a number of organisms. As yet no specific control factor has been identified in the case of L. monocytogenes, although one may exist (Davis et al., 1996). In S. typhimurium a similar acid tolerance pH independent GSR system has been demonstrated and is associated with the presence of alternate sigma factor, RpoS (Lee et al., 1994), differentiating it from the following acid inducible RpoS independent system.




Data: Davis *et al.* (1996)

Fig. 2. The effect of growing *Listeria monocytogenes* to stationary phase at different pH values, on the survival (%) of cells acid challenged at pH 3.0 for 1.5 hours. This is the pH independent general stress response (GSR) system

(ii) The second type of stationary phase ATR involves an acid induction step similar to that necessary for exponential phase ATR, and is illustrated in Fig. 3. Cultures were grown to stationary phase in minimal glucose medium to final pHs between 7.4 and 4.3, and subsequently acid challenged at pH 3.0 for 4 h. Survival of cells induced at pH 4.3 was 1000 times greater than cells induced at 7.4.




Data: Lee *et al.* (1994)

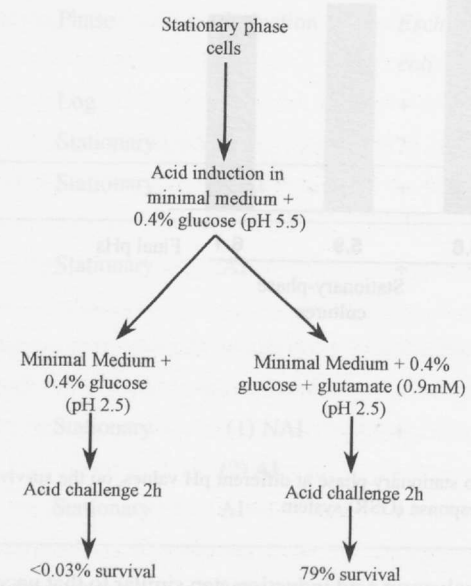
Fig. 3. The effect of growing *Salmonella typhimurium* to stationary phase at different pH values, on the survival (%) of cells acid challenged at pH 3.0 for 4 hours. This is the low pH dependent acid tolerance response (ATR) system

ATR induction in log phase is rapid (within 20 min) in contrast with ATR induction in stationary phase which takes 2 hours (Rowbury 1997). These differences reflect the dissimilar physiological states of the cells in these phases (Lee et al.,1994; Rowbury, 1997).

**Acid resistant (AR) systems**

A number of AR systems have been identified (Lin et al., 1995; 1996). These are physiological systems, requiring oxidation or fermentation in the presence of a protective component such as glutamate or arginine, and they may or may not include acid induction (Lin et al., 1995). An example of the AR in *E. coli* utilising glutamate is presented in Fig. 4. Stationary phase cells were put in minimal glucose medium (MMG) adjusted to pH 5.5 (acid induction) and subsequently challenged at pH 2.5 for two hours in (i) MMG plus glutamate or (ii) MMG without glutamate. Acid induced cells showed high levels of survival if glutamate was present in the acid challenge medium, but very low levels of survival if this protective agent was absent. Arginine can also protect *E coli* during acid challenge. However, in this case, *E. coli* required the acid challenge to be presented in a complex medium, such as Lauria broth, to achieve an AR response. At least some of these AR responses are independent of alternative sigma factor (RpoS) control (Lin et al., 1995).

The diversity of the ATR and AR systems, is further complicated by differences in the responses of different organisms (Table 2). This shows that there can be significant differences between closely related enteric organisms. For example, the only acid tolerance exhibited by *S. flexneri* in minimal medium is as part of the GSR stationary phase system. This organism is also unique in that its AR glutamate system requires acid induction. On the other hand, *S. typhimurium* has none of the AR systems.




(Data Lin *et al.*, 1995)

Fig. 4. The effect of glutamate during acid challenge at pH 2.5, on the survival (%) of *E. coli* stationary phase cells, after fermentation and acid induction in a minimal medium

Many of the stress responses observed in bacteria, including the above acid stress responses, are related to, and in some cases directly linked to, the challenges and changes facing organisms entering the stationary phase. Thus it is not unexpected that some ATR systems are subject to coordinate control by factors which control other stationary phase activities (Lin et al., 1996).

Given the importance of stationary phase cells in relation to stress and other adverse conditions, it is pertinent to consider the changes that occur during this phase.

## STATIONARY-PHASE CELLS

In the natural environment, stationary phase is the normal state for bacterial cells, interspersed with relatively brief intervals of exponential growth when nutrients and other conditions allow (Rees et al., 1995). In conditions of nutrient deprivation, growth ceases and cells enter stationary phase in which they are subjected to starvation stress (Foster and Spector, 1995). Adaptation to this stress involves protein synthesis (Kolter et al., 1993; Foster and Spector, 1995) which begins as cells enter the stationary phase and is essential for the maintenance of viability during prolonged starvation. The rate of protein synthesis during prolonged starvation changes over time. Cells entering the stationary phase produce protein at only 80% of the rate displayed by exponential phase cells. Protein synthesis during this adaptation is however essential for the development of enhanced tolerance to acid and heat, and resistance to osmotic stress, oxidation and antibiotics (Jenkins et al., 1990). Protein synthesis declines rapidly after about 1 day and after 11 days is continuing at only 0.5% of the rate of a growing culture (Kolter et al., 1993).

The rate of cell death in stationary phase cultures is dependent on the nature of the culture medium. In minimal media, lacking a number of nutrients, cell death is rapid (Siegele et al., 1993). In rich media, cell numbers show an initial small decline and then remain stable for long periods (Zambrano et al., 1996). This pattern, in which significant proportions of initial bacterial populations can survive in nutrient rich environments, has important implications in relation to the persistence of spoilage and pathogenic bacteria in foodstuffs.

No discussion of stationary phase growth would be complete without reference to the importance of transcription regulation, and specifically the central role of RpoS, the alternate sigma factor $\sigma^{38}$ , in gene activation and the physiology of the stationary phase cell. During "normal" exponential growth, transcription of DNA is under the control of vegetative sigma factor $\sigma^{70}$, RpoD) (Dorman and Ni Bhrian, 1997). In this phase of growth, the concentrations of a number of alternative sigma factors, including RpoS are limited by (1) low rates of synthesis and (2) the relatively rapid breakdown of these proteins by the C1pxP protease system (Scheweder et al., 1996). However, as cells enter the stationary phase, starvation conditions provide a signal for greater transcription of rpoS (McCann et al., 1993). Synthesised RpoS redirects RNA polymerase to recognise and transcribe non-standard promoters on the bacterial DNA, leading to the production of at least 30 proteins (McCann et al., 1991; Hengge-Aronis, 1993) involved in cell adaptation to survival in the stationary phase (Kolter et al., 1993; Siegel et al., 1993; Rees et al., 1996). While RpoS has a central role in stress survival, a number of other alternative sigma factors have been identified as modulating the course of transcription during stress and adaptation to stationary phase. These include RpoH (alternative sigma factor $\sigma^{32}$) induced during carbon starvation (Yura et al., 1993) which promotes the expression of a number of proteins which, although formally classified as heat-shock proteins, are know to be induced by several other stimuli, including exposure to hydrogen peroxide, ethanol, bacteriophage infection, heavy metals, DNA damaging agents and the presence of abnormally folded polypeptides (Georgopoulos et al., 1990). Other alternative sigma factors e.g. RpoN activated by nitrogen starvation conditions, are known to control the expression of a number of virulence factors ( Dorman, 1994). Such alternative sigma factors are involved in a number of interlocking transcription regulation mechanisms, controlling a wide range of adaptive activities in Gram negative and Gram positive organisms ranging from heat shock responses to developmental cascades in sporulation and the expression of virulence factors (Totten et al., 1990; Lonetto et al., 1992). These coordinated mechanisms allow bacteria to mount a response to significant and often sudden changes in their environment by producing stress proteins and/or virulence factors.

## VIRULENCE

The above coordinated approach appears efficient as it has been identified in a wide range of pathogenic species, and suggests that while infection, particularly infection in humans, is important, in broader terms it is just one rather specialised form of ecological interaction. A free living bacterium in coping with an ever changing and frequently hostile environment, faces the same general challenges as a pathogen initiating and maintaining infection in a human or animal host. Therefore it is not surprising

that the external cues which bacteria recognise during adaptation to adverse environmental conditions i.e. stress, starvation etc., and the systems they use during such adaptation may also trigger virulence factors in pathogens adapting to the adverse conditions purposefully presented by host defense systems during infection. Coordinate induction (or repression) of virulence and a range of stationary phase, starvation or stress responses has been demonstrated in a wide range of bacterial species. Mutants which are *rpoS* deficient have been reported to be unable to mount stress responses to a wide range of challenges including ATR, and have reduced ability to cause infections (Wilmes-Riesinberg et al., 1996; Fang et al. 1992) indicating the close linkages between RpoS, virulence and stress resistance. Such linkages have been particularly well established among enteric species (Small and Falkow, 1992; Kowarz et al.,1994; Robbe-Saule et al., 1994), perhaps reflecting a greater knowledge of the overall genetics of this group. It is however becoming clear that RpoS or its homologues play similar if not identical roles in the coregulation of stress responses and virulence in a range of bacteria. Thus RpoS has been shown to regulate virulence in *Y. enterocolitica* (Iriate et al., 1995) and a homologue of RpoS has been associated with the expression of virulence genes in *Pseudomonas* (Tanaka and Takahashi, 1994). The common observation of the induction of virulence genes at the onset of stationary phase in such organisms as *Staphylococcus aureus*, *L. monocytogenes* and Bacillus (Rees et al, 1995) suggest that alternative sigma factors like RpoS are likely to be identified in a very wide range of species, as a common means of controlling the co-ordinate expression of stress and virulence factors.

There is however, already considerable evidence that the development of any one of a number of unfavourable environmental conditions including the onset of stationary phase, can induce the action of alternative sigma factors like RpoS. The RpoS produced can stimulate significant changes in the phenotypic expression of stressed cells, and the development of a coordinated general stress response. It is also clear that such a general stress response can entail the production of a full range of stress proteins and the coordinate expression of a range of virulence factors.

## INFLUENCE OF STRESS ON PATHOGEN EMERGENCE

Table 1 lists a number of potential stresses which bacteria may face, and the possible conditions under which these stresses could occur in foods and during host/pathogen interaction. Effective survival enhancement strategies enable organisms to remain viable under a wide range of adverse environmental conditions including acid, heat, cold, osmotic challenge and the presence of oxidative or inhibitory compounds. Such persistence has significant implications in relation to survival of pathogens in food products.

Many foods and food preservation systems present bacteria with the necessary pH conditions for the expression of log or stationary phase ATR, e.g. mayonnaise (pH 4.0 - 5.0), pepperoni (pH 4.8 - 5.2) and fresh meat (pH 5.4 - 5.5). On these foods pathogens may activate ATR, AR or GSR systems, enabling them to more effectively survive subsequent acid challenge in the stomach (pH 1.0 - 3.0) (Lin et al., 1996). A range of induced acid tolerance systems have been clearly demonstrated. Thus *E. coli* 0157:H7 was noted to activate an AR system which persisted for 25 days at 4°C (Lin et al., 1996), which enabled enhanced survival during subsequent acid challenge. Acid adapted log phase *Salmonella* cells have been shown to demonstrate ATR which gave enhanced survival in a range of cheeses during two months storage at 5°C (Leyer and Johnson, 1992). Stationary phase cells of *E. coli* 0157:H7 were placed in a synthetic gastric fluid (pH 1.5), inoculated onto dry fermented sausage, and stored at 4°C for 21 days. The exposure to synthetic gastric fluid activated a GSR system which gave acid treated cells survival rates which were significantly higher than the survival rates of control cells (Cheville et al., 1996).

Heat processing, a common element in many cooking and food processing systems, is an important and frequent stress challenge to pathogens. Slow heating processes, which do not entail overall temperature/time treatments sufficient to kill pathogens, may in fact enhance their survival. Thus a number of studies have demonstrated that slow heating enhanced the survival of *E. coli* and *S. typhimurium* (Tsuchido et al., 1982; Mackey and Derrick, 1987). Product composition can also influence the survival of pathogens during heat treatments. The higher the carbohydrate content of a product the more heat resistant inoculated bacteria will be (Doherty et al., 1998). A feature of particular interest in terms of food safety is the appearance of resistant subpopulations in foods during heating. These represent the frequently observed 'tailing phenomena' in which a small portion of the flora persists for long periods after the heating process has killed the majority of the initial microflora (Juneja et al., 1997; Doherty et al, 1998). These data emphasise that it is vital to consider the effects of different environmental and substrate conditions in the design of adequately safe cooking processes.

Finally, it has been shown that a number of the above stresses can 'combine' to enhance survival. Thus the acid tolerance of *E. coli* 0157:H7 is further enhanced if the acid induced organism is also subjected to subsequent heat shock (Wang and Doyle, 1998a). It is also suggested that the application of one stress can provide additional protection against a number of other different stresses. In *L. monocytogenes* heat shock has been reported to enhance resistance to ethanol and NaCl, while adaptation to ethanol can increase resistance to lethal doses of acid or $H_2O_2$ (Lou and Yousef, 1997). Such observations pose questions in relation to the potential dangers in the inadequate application of multiple hurdle technology. Thus the application of a number of sublethal stresses may synergistically combine to "stress harden" (Lou and Yousef, 1997) pathogens in treated foods leading to the emergence of organisms with a greater capacity to resist one or more of the stresses applied within current food preservation schemes.

Combined with these concerns in relation to greater survival of organisms, there are also concerns that coregulated virulence factors may therefore be triggered by the use of conditions used to retard bacterial growth (Rees et al., 1995). Concerns in this area are heightened by the increasing use of food preservation systems which function by slowing or inhibiting bacterial growth rather than the more traditional systems, which focused on the permanent inactivation of bacteria. It is possible that such inhibition based processes, may induce stress responses in pathogens on foods, leading to significantly higher levels of persistence and virulence factor expression on stored foods. Thus the simultaneous induction of virulence factors, as part of the overall coordinated stress reactions of such pathogens, will mean that a larger number of persistent organisms may be stimulated to a fuller expression of the virulence factors necessary for the establishment of infection in the human host.

## 2.Genetic adaptation as a means of surviving adverse conditions

If an organism is subjected to a constant environmental stress, such as the presence of antibiotics or unfavorable acid concentrations, an adaptive induced mutation may arise, making the organism permanently resistant to that stress (Foster and Hall, 1990; Archer, 1996; Moxon et al., 1994). While mutation is normally considered to be a rare event, it has recently been shown to occur at much higher frequencies among pathogenic isolates of *E. coli* and *Salmonella enterica*, i.e. over 1%, which could help explain the rapid emergence of antibiotic resistant mutants (LeClerc et al., 1996). However, Archer (1996) suggests an additional response, in which genetic changes may be induced in response to components of the environment. The latter genetic changes occur in both the exponential and the stationary phase, although they take place by different mechanisms (Harris et al., 1994). In terms of mutagenesis, stationary phase cells are the more important, and will be examined as a model of genetic adaptation.

Although the processes involved in exponential phase and stationary phase mutations may be similar, the outcomes for the population of cells are different. Chance mutations arising during genomic replication in exponential growth may give rise to a large number of identical progeny, although the mutation may not be of any specific benefit to the bacterial population (Foster, 1993; Lenski and Mittler, 1993). Mutations arising during stationary phase in the absence of genomic replication have been grouped as (a) adaptive (directed) mutations i.e. those for which a specific selective agent has been identified, or (b) stationary phase mutations, for which no specific selective agent has yet been identified (Foster, 1993; Symonds, 1994; Cairns et al., 1998).

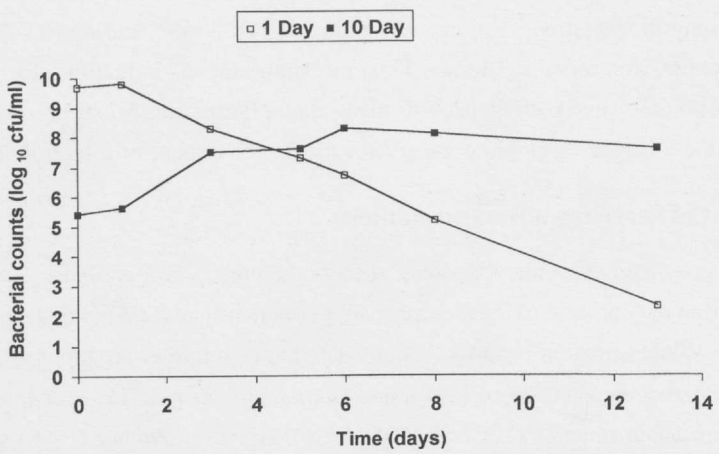
### (a) Adaptive mutations

Adaptive or directed mutations occur in stationary phase cells in the presence of selective agents and are specific to such agents (Foster, 1993). While stationary phase cells are starvation stressed, starvation is not of itself considered to be mutagenic, rather a constituent of the medium (such as lactose or tryptophane) acts as a selective agent, leading to the development of cells capable of metabolising the selective agent. Symonds (1994) considered that the presence of a selective pressure was not essential for adaptive mutation in stationary phase cultures, but that if present, such pressure enhances the rate of adaptive mutation. He also stated that some degree of "directionality" i.e. specific response to specific agents, is involved in adaptive stationary phase mutagenesis. Adaptive mutations occur in stationary phase at higher probabilities than spontaneous mutations occur in growing cells and they are advantageous, rather than neutral, to the survival of the organism (Hall, 1990; Foster, 1993; Harris et al., 1994).

### (b) Stationary phase mutants

As well as the above adaptive or directed mutations, for which selective agents have been identified, other mutations occur in non dividing stationary phase cells. Although these mutations may occur in a similar manner to adaptive or directed mutants, as yet no specific selective agents have been associated with these (unassigned) stationary phase mutations (Foster 1993).

The term "stationary phase" is becoming increasingly recognised as a misnomer. Total cell numbers may remain constant, but it is now clear that the stressed cell populations are undertaking considerable and significant metabolic and genetic activities, as they attempt to adjust to survival and growth under adverse conditions. A number of studies have demonstrated the extent and nature of such adaptations. Zambrano et al., (1993) observed the relative changes in mixed 1day stationary phase (spherical cells) and 10day stationary phase (elongated cells) cultures of E. coli in Lauria broth to final concentrations of log10 9-10 and log10 5.5-6.0 respectively. Fig 5 shows the changes in numbers of the two cell types at 37°C. The aged (10day) culture numbers increased by about 2-3 logs during the first 6 days, while the young (1day) culture numbers declined steadily over the entire period of the experiment. This 'cryptic growth' of aged cells suggests that they were adapted to growth under starvation conditions, while the younger cells were not. Examination of the aged cells established that there had been a mutation in the *rpoS* gene, allowing growth to take place. It was also shown that a null mutation did not confer the growth advantage phenomenon.




Data: Zambrano *et al.* (1993)

Fig. 5. The effect of aged cells (10 days of *E. coli* on the growth of young 1 day old cells in a mixed culture at 37°C

These and other chemostat based studies have established that adaptive mutants, more suited to survival and growth under starvation conditions, appear over time so that the culture changes to become composed of "fitter" cells which grow and predominate (Novick and Szilard, 1950; Kolter et al., 1993). Sub-populations of mutant cells that arise by such dynamic interactions between stationary phase cells and the selective pressure in their environment are referred to as growth advantage in stationary phase (GASP) mutants (Huisman et al., 1996; Torkelson, et al., 1997).

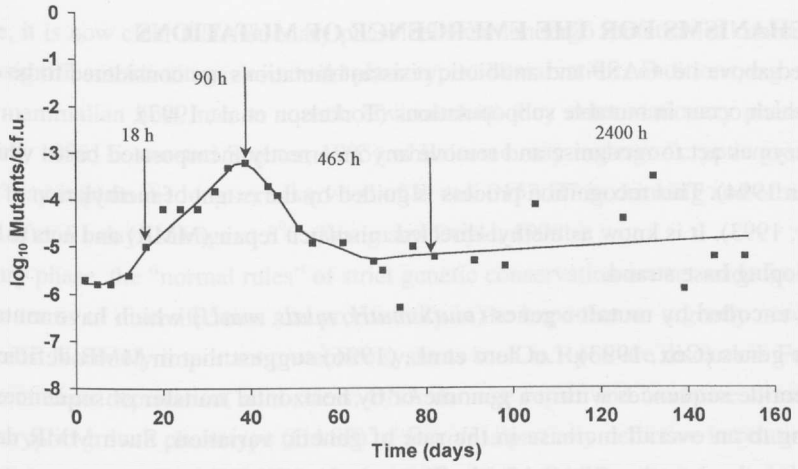
### Mutation to antibiotic resistance

Stationary phase mutations have also be associated with another important form of adaptation among bacteria, i.e. the development of antibiotic resistance.

Thompson et al., (1997) examined the emergence of mutants of *Lactobacillus plantarum* growing in buffered tryptone for up to 120 days in batch and continuous culture in the absence of antibiotics. During prolonged incubation in tubes, antibiotic resistant mutants were detected in the batch cultures from 1 to 120 days at varying rates over time, depending on the antibiotic mutant being detected i.e. mutant growth was stochastic. Mutants resistant to rifampicin, sodium fusidate and streptomycin were detected in the *L. plantarum culture*. Mutants were found to be resistant to only one antibiotic.

During the prolonged continuous culture no increases in cell numbers occurred.

The pattern of isolation of rifampicin resistant mutants in such culture, expressed as the frequency of recovery of mutants in relation to the total viable count ($log_{10}$mutants/cfu) is shown in Fig. 6. The numbers of mutants recovered varied with doubling times and were highest at 90 h. The absolute numbers of mutants increased significantly from 18 h to 90h (Fig. 6). As the doubling times were increased (465 and 2400 h) the numbers of mutants recovered declined. The data suggest that populations of starved bacteria can be genetically dynamic.




The arrows indicate aproximate doubling times.
Data: Thomson *et al.* (1997)

Fig. 6. Frequency of recovery of rifampicin resistant mutants expressed as the log10 of survivig cells of *L. platarum* in slow growing cultures at steady states

Such adaptive mutations in stationary phase cells, for example in culture collections, have been reported (Kolter et al., 1993; Ivanova et al., 1992), and have also been recorded in a range of bacteria including gram negative pathogens. Stored *E. coli* 0157:H7 and *S. enterica* cultures were examined for the presence of mutant strains resistant to rifampicin, spectinomycin and naladixic acid (LeClerc et al., 1996). As above (Thompson et al., 1997) mutants were not selected by exposure to media containing antibiotics. Resistant strains were detected at frequencies up to 1000 times greater than in exponential phase cells (Table 3). The existence of nine mutant strains from a total of 349 isolates examined gave an incidence of 2.6%. Isolates were found to be resistant to only one antibiotic.

**Table 3: Mutation frequency* of mutant clones of E. coli and Salmonella.**

| Isolate | Mutants per $10^8$ cells | | |
|---|---|---|---|
| | [1]Rif | [2]Spc | [3]Nal |
| EC536 (control) | 1.2 ± 0.4 | 0.2 ± 0.1 | 0.5 ± 0.2 |
| EC503 | 114 ± 22 | 31 ± 8 | 403 ± 41 |
| EC535 | 119 ± 23 | 35 ± 7 | 300 ± 57 |
| DEC5A | 425 ± 150 | 26 ± 2 | 195 ± 110 |
| ECOR48 | 441 ± 45 | Spc$^r$ | 177 ± 57 |
| *S. enteritidis* (SL 12) (Control) | 2 ± 08 | 3 ± 0.2 | 0.8 ± 0.7 |
| *S. enteritidis* (C396) | 782 ± 101 | 70 ± 25 | 745 ± 163 |
| *S. berta* (SL78) | 738 ± 289 | 30 ± 3 | 148 ± 13 |
| *S. halmstad* (SL58) | 704 ± 265 | 338 ± 73 | 481 ± 269 |
| *S. arizonae* (S2978) | 798 ± 320 | 407 ± 83 | 199 ± 95 |
| *S. infantis* (SL101) | 981 ± 449 | 172 ± 40 | 113 ± 18 |

[1] Resistance to rifampicin
[2] Resistance to spectinomycin
[3] Resistance to nalidixic acid
Spc$^r$- all cells resistant to spectinomycin

Frequencies ± SD represent three to seven determinations from independent cultures.

Data: LeClerc et al., (1996)

## MECHANISMS FOR THE EMERGENCE OF MUTATIONS

Adaptations of the types discussed above i.e. GASP and antibiotic resistant mutations are considered to be controlled by the presence of hypermutable genes, which occur in mutable subpopulations (Torkelson et al., 1997).

During normal growth, repair enzymes act to recognise and remove any incorrectly incorporated bases which do not match the original DNA template (Dorman 1994). This recognition process is guided by the extent of methylation of the template and newly formed DNA strands (Foster, 1993). It is know as methyl-directed mismatch repair (MMR) and acts to remove any mismatching new DNA from the developing base strand.

MMR is controlled by proteins encoded by mutator genes (*mutS mutH, mutL, mutU*) which have mutation rates several hundred fold higher than other genes (Cox, 1993). LeClerc et al., (1996) suggest that in MMR deficient cells, defects introduced by alterations of nucleotide sequences within a genome or by horizontal transfer of sequences between genomes, will not be corrected, leading to an overall increase in the rate of genetic variation. Such MMR defective cells do have an enhanced mutation rate i.e. they are hypermutable. LeClerc et al., (1996) found that nine such hypermutable strains of *E coli* and *Salmonella* had defective MMR genes. Seven strains had defects in *mutS*, while the other two were defective in *mutH* and *mutU*. Examination of the *mutS* gene in the hypermutable strains indicated that a small number of base pairs (212) had been lost as a result of a larger deletion (7449bp) involving the *fhlA, mutS* and *rpoS* genes. These data suggest that mismatch repair is the mechanism underlying hypermutation in stationary phase *E. coli* and *Salmonella*. The close proximity of the *rpoS* gene to the site of the above deletion suggests a possible involvement of an rpoS transcription read-through as an mechanism for *rpoS* control of *mutS* transcription under conditions of environmental stress (LeClerc et al., 1996). A similar mechanism may also underlie the production of mutants in *L. plantarum* (Thompson et al.,1997).

Hypermutability has been linked with the emergence of subpopulations during selection (Hall, 1990), and Boe (1990) has proposed a mismatch repair mechanism for the production of adaptive mutations in *E. coli*. Foster (1993) has expressed some reservations about the MMR model of adaptive mutation, noting that as MMR is an error correction system in newly synthesised DNA, it can only be of significance if DNA synthesis is occurring in stationary phase cells.

Adaptive mutation is also suggested to involve recombination (Rosenberg et al., 1994) by a mechanism involving a lac frameshift. Suppression of MMR which would normally control recombination between divergent sequences (Cox, 1995), would enable cells to transfer recombination defects horizontally from one organism to another and could explain how genes such as eae and slt have come to reside in *E. coli* 0157:H7 (LeClerc et al., 1996). As noted by these authors cells with defective or suppressed MMR become promiscuous.

Other suggested mechanisms for the development of adaptive mutations include "mutational factories". This possibility, outlined by Higgens (1992) suggests that bacterial cells which are no longer capable of dividing, undergo an environmentally triggered acceleration of mutation rate, changing the structure of the genome at a rate of up to 1000 times faster than dividing cells. Altered genes are then transferred by established mechanisms (plasmid or bacteriophage mediated transfer or natural transformation) to cells which are still capable of dividing, fixing any useful mutation into the surviving portion of the population (Dorman, 1994).

The precise significance or mechanisms of control in the above adaptations are not yet clearly understood and there are a number of possibilities in relation to their interactions in hypermutation (Foster, 1993). It is however, generally agreed that sub-populations of hypermutable cells can arise in stationary phase cultures, and that they can display mutations at much higher frequency than the frequency of spontaneous mutations during normal cell growth. The advantages that some of these mutations confer on these sub-populations are generally related to growth in hostile environments, such as in stationary phase, environmental "in vitro" challenge or in the presence of host "in vivo" defense systems.

The examples of stationary phase phenotypic and genotypic adaptation responses, described in the present paper may have far reaching consequences in relation to pathogen emergence. Both confer ecological advantages to stationary phase populations of organisms in terms of persistence and/or growth under stress conditions. The potential impact of such adaptation, mutation and promiscuity is yet to be fully realised, but the emergence of *E. coli* 0157:H7 and other pathogens through horizontal transfer and recombination (Whittam, 1996), gives some indication of the hazards which bacterial adaptation systems may pose.

As noted above, it is now clear that stationary phase cells can undergo mutation of central control mechanism such as rpoS, and thus develop significant advantages over wild phenotypes. In starvation conditions, e.g. when such organisms as *E coli* are expelled from a mammalian host into an aquatic environment, they enter stationary phase or become viable non-culturable (Gordon and Small, 1993; Foster and Spector, 1995) while some may undergo "cryptic growth", at the expense of other cells. Such adaptations may explain the long-term survival of E. coli 0157:H7 in drinking and other waters i.e. a decrease of less than 2.0 logs occurred after 91 days storage at $8^0$C (Wang and Doyle, 1998b).

During stationary phase, the "normal rules" of strict genetic conservation are set aside for the good of the overall population. Cells take risks in terms of diversification and promiscuity in the hope that an urgently needed allele can be made available to a few surviving cells. Normally important protective systems such as RpoS are discarded. For example *rpoS* mutants are defective in relation to nutrient deprivation, acid stress, DNA damage repair and oxidative stress (Fang et al., 1992), and the *rpoS* mutation for the cryptic growth phenotype (GASP) of *E. coli* is partially defective in relation to oxidative stress (Zambrano et al., 1996). Such defects are likely to be offset by the ability of the GASP mutant to produce an increased inoculum level as a result of their growth.

This paper has noted a number of parallels in concepts and mechanisms among phenotypic and genotypic stress adaptation, virulence and the development of antibiotic resistance. It is clear that a more detailed knowledge of how bacteria obtain, and respond to cues of environment change is essential, to the clinician and food microbiologist alike. Many of these systems are under co-ordinate control within the bacterial genome. Perhaps it is time for a co-ordinate approach among those interested in preventing bacterial adaptation and/or survival "in vivo" (in infection) and "in vitro" (in food).

The prolonged use of antibiotics would appear to result in the selection of pathogens that are both resistant and more virulent. Care needs to be taken to avoid the development of a similar pattern in food preservation systems, where suboptimum processing and storage practices induce and select pathogens which are both more resistant to suppression by preservation protocols and potentially more virulent.

It has been suggested that potential control strategies could induce "microbial psychosis" by introducing signals which cause organisms to misinterpret their surroundings, activate the wrong genes at the wrong time and generally mount an inappropriate (and hopefully ineffective) environmental response (Dorman, 1994). Such systems would be of direct and significant application in preventing or reducing the economic and clinical significance of the current and future waves of emerging pathogens.

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