Kinetics and Mechanisms of the Low pH-Induced Inactivation of *Escherichia coli*

by

Janelle L. Brown B.Agr.Sc.(Hons.)

Submitted in fulfilment of

the requirements for the degree of

Doctor of Philosophy

University of Tasmania, October 2002

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person except where due reference is made in the text of the thesis.

J.L. Brown

October, 2002

This thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968*.

J.L. Brown

October, 2002

ABSTRACT

Newly emerged foodborne pathogens with low infectious doses and remarkable capacities to tolerate environmental stress have prompted the use of food processing strategies that not only restrict the growth of contaminating microorganisms but also effect their inactivation. The rational design of such processing technologies depends on the accurate prediction of microbial responses to lethal constraints and hence on an appreciation of the mechanism(s) of microbial inactivation. This thesis describes the process of the low pH-induced inactivation of *Escherichia coli* populations by low pH and attempts to provide a mechanistic interpretation that is consistent with the kinetic data presented.

To present an unambiguous account of the low pH-induced inactivation of *E. coli* those uncertainties associated with defining and reporting microbial death were addressed. An operational definition of microbial viability, and a lack thereof, was established, and the methods employed to enumerate viable cells (traditional culture-based methods) were optimised for the recovery of low pH treated *E. coli*. In addition precautions were taken to eliminate kinetic artefacts.

Initial experiments provided evidence of two distinct phases of inactivation in low pH-treated exponential phase populations of *E. coli* - an initial phase of rapid inactivation whose rate is influenced both by the stringency of the low pH treatment imposed and by temperature, and a protracted phase of much slower inactivation whose rate is independent of the severity of the lethal agent employed, and of temperatures ≤ 25°C. Subsequent studies illustrated that a third phase of inactivation, characterized by the rapid and complete loss of population viability, is observed if population viability is monitored over a sufficiently long period of time.

A considerable degree of 'day to day' kinetic variability was observed among exponential phase populations of *E. coli* prepared from stationary phase inocula. That variability was attributed to small differences in the initial viable counts and the number of residual stationary phase cells in individual populations, and precautions were taken to minimise that variability in subsequent experiments. Those experiments indicate that the low pH tolerance of *E. coli* decreases with increasing cell density in purely exponential phase populations, that it increases with the physiological age of populations whose initial viable counts exceed 1×10^8 $cfu.ml^{-1}$ reaching a maximum when purely exponential phase population are cultivated for thirty-two generation time equivalents at 25°C, and that it declines conspicuously in populations cultivated for longer periods of time.

Hypotheses concerned with the basis of the shape of non-linear inactivation curves were introduced and critically evaluated in light of published data, kinetic data obtained in initial experiments, and data obtained from new experiments designed specifically to test alternative hypotheses. A case for the inherent differential resistance of individuals within a population is argued but not promoted as a comprehensive interpretation of the kinetic data presented.

Finally, an *E. coli* mutant carrying an unmarked deletion in *cfa*, the gene encoding cyclopropane fatty acid (CFA) synthase was constructed, and employed with its parental strain to evaluate the role of CFAs as molecular mediators of low pH tolerance in *E. coli*. The results obtained indicate that CFAs play no role in mediating the intrinsic or inducible low pH tolerance of *E. coli*.

ACKNOWLEDGEMENTS

The assistance and encouragement of those around me made the work completed herein possible. I owe my sincerest thanks to:

My supervisors *Dr Tom Ross* and *Professor Tom McMeekin* - for their enthusiasm, their sound advice, and for demonstrating their confidence in me

Dr June Olley – for sharing her time and thoughts during preparation of the thesis manuscript

Professor Ian Booth, Dr Lorna Ness, and other members of the IRB lab - for providing advice with molecular techniques

Dr Gordon Niven and *Dr Bernard Mackey* – for 'showing me the ropes' of DSC

Students and staff from the School of Agricultural Science, particularly:
Mr *Bill Peterson* – for the maintenance of laboratory equipment
Kirsty Presser – for providing growth rate data for *E. coli* FT1 and *E. coli* YYC1106
Lyndal Mellefont and *Craig Shadbolt* – who were always willing to take a sample or to provide late night entertainment

The Latte Ladies – for sharing experiences and friendship

My Family – for their unending patience, support and love

The financial support of Meat and Livestock Australia, and the Australian Society of Microbiology, is also gratefully acknowledged.

TABLE OF CONTENTS

INTRODUCTION AND OBJECTIVES

The reliable provision of a safe high quality food supply requires the control of microbial growth and survival throughout the production chain. Typically this has been achieved by manipulating the physical and chemical properties of foods. Microorganisms exhibit growth only over a relatively restricted range of environmental conditions and consequently de-optimising one or more of these parameters restricts the potential for microbial growth (Booth and Kroll, 1989).

As primary hurdles to microbial growth temperature, water activity and pH have been employed for centuries in the preservation of foods. Primitive societies observed that dried meat and salted fish resisted decay and that soured milk resisted further decomposition and remained palatable (Tortora et al., 1992). Many of the basic strategies used to prevent the spoilage of foods, including salting, drying and microbial fermentation, thus arose empirically (Booth and Kroll, 1989). In recent years a substantial amount of quantitative information concerning the behaviour of foodborne microorganisms exposed to individual or combined environmental constraints has been collected and modelled (Ross and McMeekin, 1994; Ross, McMeekin and Baranyi, 2000). While this information has improved our ability to predict and manipulate microbial behaviour and consequently has enabled industry to refine food preservation strategies, outbreaks of foodborne illness continue.

Between 1995 and 1999 the Australian National Centre for Disease Control reported an annual mean of almost 22,000 notified cases of illness among ~ 20 million consumers (NCDC, 2000). Utilising assumptions made by the US Centres for Disease Control, notably the proportion of gastrointestinal disease attributable to

foodborne transmission, the degree of under-reporting, and the contribution to foodborne illness from non- notifiable bacterial and viral disease, Sumner et al. (2000) estimate that this translates to between 1 and 2 million cases of foodborne illness occurring in Australia annually. Recent per capita estimates for the United States, Western Europe, Japan, and New Zealand indicate that Australia is not unique with respect to the incidence of foodborne illness and that, despite the development and implementation of strategies designed to limit the spread of foodborne pathogens, the incidence of foodborne illness in developed countries is increasing (Maurice, 1994; CDNANZ, 1997; Käferstein et al., 1997; Hall, 1999).

A report issued recently by the World Health Organisation explains that progress in combating foodborne illness has largely been offset by other global trends (WHO, 2000). Changing human demographics, notably increasing populations and numbers of 'high risk' (elderly, immunocompromised or malnourished) individuals, and the intensification and globalisation of food production and distribution have contributed to the observed rise in food related illness (Gerba et al, 1996; Morris and Potter, 1997; Miller et al., 1998). Concomitant with these trends new pathogens have emerged, "old" ones re-emerged, and existing ones spread (Desmarchelier, 1996; Alterkruse et al., 1997; Tauxe, 1997). Smith and Fratimico (1995) suggest that the acquisition of new virulence and/or drug resistance factors, changing food production and handling practices, and new routes or vehicles of transmission have facilitated the emergence, persistence and spread of foodborne pathogens. Consumer preference for a less processed preservative free product (Zink, 1997) has compounded the issue by prompting food processors to utilise marginal treatments.

With non-industrialised countries bearing the brunt of the problem (Käferstein, 1997), foodborne illness undoubtedly accounts for a substantial measure of human

suffering and financial loss on a global scale. In developed nations the ill health and morbidity precipitated by foodborne pathogens is typically accompanied by medical expenses, lost income and productivity (through absenteeism, business closure and/or product recall), and investigative and regulatory costs that affect individuals and communities. At a national level outbreaks of foodborne illness have a negative influence on both tourism and trade (Buzby and Roberts, 1997; Käferstein, F.K., 1997). The microbial safety of food supplies has consequently become a major global public health issue (Alterkruse and Swerdlow, 1996; Desmarchelier, 1996).

Confronted by an increase in the prevalence of foodborne illnesses, the emergence of highly virulent pathogens with remarkable capacities to tolerate environmental stress, and the challenge of complying with both product safety specifications and consumer demands the food industry is desperately in need of an innovative approach to food safety. The rational development of novel processing strategies designed to preclude the growth and/or survival of contaminating microorganisms depends on the accurate prediction of microbial responses to those physicochemical parameters defining the microenvironment. An increasing awareness that many such responses are non-intuitive has led to the realisation that reliable predictions can only be made from a knowledge of the mechanisms underpinning microbial growth and survival responses. Knøchel and Gould (1995) suggest that a strong argument exists for a change of emphasis in our approach to food preservation and safety. Specifically, they highlight the need for a move away from the traditional empirical approach to one based on an appreciation of the physiology of foodborne microorganisms. Unless an understanding both of microbial responses to environmental constraints and of the molecular mechanisms underpinning those responses is developed, our past failure to couple observation with understanding

will undoubtedly impede the rational design of novel food preservation technologies and consequently our efforts to stem foodborne illness.

Within the above context the aim of this thesis is to expand understanding of the mechanism(s) whereby low pH effects the inactivation[1] of *E. coli*, specifically by:

i) reviewing the literature concerned with *E. coli*, its responses to low pH (within the growth permissive and non-permissive ranges), and the molecular basis of those responses [Chapter 1]

ii) establishing methods that permit the loss of viability in populations of *E. coli* exposed to growth non-permissive low pH constraints to be quantified accurately [Chapter 2]

iii) describing the kinetics of the low pH-induced inactivation of *E. coli* and the influence of various environmental and physiological parameters on those kinetics [Chapter 3]

iv) developing and critically evaluating mechanistic interpretations of the kinetics of the low pH-induced inactivation of *E. coli* [Chapter 4]

v) evaluating the role of cyclopropane fatty acids as molecular mediators of low pH tolerance in *E. coli* [Chapter 5].

From a practical viewpoint, the project attempts to extend the framework available for the development of strategies designed to ensure microbiologically safe yet minimally processed foods.

---

[1] Note that the term inactivation is employed throughout this thesis to describe irreversible loss of viability.

CHAPTER 1 LITERATURE REVIEW

1.1 ABSTRACT

Literature concerned with *E. coli*, its responses to low pH (within the growth permissive and non-permissive ranges), and the molecular basis of those responses was reviewed. Evidence suggests that for microorganisms such as *E. coli*, encounters with low pH are inevitable. The growth and survival of *E. coli*, and consequently its ability to precipitate illness will, therefore, be determined in part by its capacity to tolerate low pH. As a foodborne bacterium of both historical and contemporary clinical significance, *E. coli* has been employed as the organism of choice in many classic studies concerned with cell biochemistry, physiology and molecular biology. Nonetheless, knowledge of this species, of its responses to environmental constraints, and of the molecular basis of those responses is fragmentary. In recent years, a substantial amount of quantitative information relating the growth response characteristics (lag phase duration, growth rate, cell yield and growth/no growth interface) of *E. coli* to the primary physicochemical parameters controlling bacterial growth, including pH, has been collected and modelled. By contrast, relatively little effort has been made to systematically describe the response characteristics of populations of *E. coli* exposed to analogous lethal constraints, or to develop an appreciation of the mechanism(s) whereby low pH constraints influence the growth and/or survival of *E. coli*. Without this knowledge our ability to reliably predict the responses of *E. coli* to known environmental constraints, and hence to develop processes designed to ensure microbiologically safe yet minimally processed foods, is limited.

1.2 LOW pH – DECONTAMINANT, PRESERVATIVE AND BARRIER TO MICROBIAL INFECTION

The unicellularity of bacteria renders them directly susceptible to a complexity of environmental parameters that govern microbial growth. Among the primary physicochemical parameters controlling microbial growth is pH, a measure of hydrogen ion concentration. Low pH equates to a high concentration of hydrogen ions and is often referred to as an acidic pH. Increasingly acidic conditions reduce the potential for microbial growth and ultimately bring about microbial inactivation.

Low pH is one of the most common stresses encountered by foodborne microorganisms (Archer, 1996; Bearson et al., 1996). In the unprocessed state many foods including meat, fish and vegetables are slightly acidic while most fruits are moderately to highly acidic, their pH typically falling below 4 (Booth and Kroll, 1989). The pH of many foods is also deliberately lowered during processing, either by the addition of an edible acidulant or through fermentation (Brown and Booth, 1991). The efficacy of such treatments, designed to prevent the growth and/or survival of contaminating microorganisms, depends both on the final pH achieved and on the nature of the acidulant (Rompf and Jahn, 1999).

Typically, acids have been classified on the basis of their potential to dissociate in solution. The pK_a of an acid, that is the pH at which the concentration of dissociated and undissociated species is equal, is constant. Those acids with low pK_a values dissociate readily and have been described as strong acids. The antimicrobial action of strong acids derives entirely from their capacity to generate a high concentration of protons in the microenvironment. In contrast, weak acids have relatively high pK_a values and remain incompletely dissociated at the pH

values typical of foods (Corlett and Brown, 1980). In their undissociated form weak acids are lipophilic and permeate the cell membrane readily. Inside the cell they dissociate according to their pK_a and acidify the cytoplasm. While the antimicrobial activity of weak acids undoubtedly depends on their ability to lower the cytoplasmic pH (Booth, 1985), it has been suggested that additional inhibitory effects may be elicited by the undissociated acid or its anion (Salmond et al., 1984).

In some instances the low pH of a food will preclude microbial growth and/or survival in the absence of additional preservative factors. In many cases, however, low pH is employed in combination either with a weak acid or with some other physical or chemical constraint (Brown and Booth, 1991). The stability and safety of uncooked fermented meat products, for example, depends on the synergistic effects of low pH and low water activity (Bacus, 1997). Acidic sprays or washes used for the decontamination of carcass meats rely on the bactericidal effects of organic acids at low pH (Cutter and Siragusa, 1994; van Netten, 1996; Dorsa, 1997). Likewise, the canning industry routinely employs low pH to potentiate the affects of thermal processing (Corlett and Brown, 1980). The decontamination and preservation of many foods is effected, at least in part then, by low pH (Brown and Booth, 1991; Archer, 1996).

Pathogenic microorganisms not eliminated from the food supply during processing or storage have the potential to elicit illness when ingested by a potential host. Before an infection can be established, however, invading microorganisms must overcome those challenges imposed by the host's defence systems. Gastric acidity, which is primarily pH-hydrochloric-acid-dependent, provides a first line of defence against foodborne pathogens ingested by humans. To reach the large intestine, the primary site of infection, enteric pathogens must survive passage through the acidic environment of the stomach (Gianella et al., 1972). Likewise, low pH is used by the

immune system to counter microbial infection. In this instance temporal pH changes within the phagocytic vacuole of polymorphonuclear leukocytes facilitate the inactivation and digestion of invading microbes (Jensen and Bainton, 1973). Low pH thus plays an important role in determining the outcome of microbial invasions (Peterson et al., 1989).

For foodborne microorganisms encounters with low pH are inevitable; in the natural environment, in processed foods, and/or in the human body. The growth and survival of such organisms and consequently their ability to precipitate spoilage or cause illness will, therefore, be determined in part by their capacity to tolerate low pH. Knowledge of the low pH growth and survival responses of common spoilage-causing and pathogenic bacteria is thus essential to the strategic use of low pH in treatments designed to ensure the microbial stability and safety of foods.

1.3 *ESCHERICHIA COLI* – HARMLESS COMMENSAL & EMERGING PATHOGEN

Described as a facultatively anaerobic, Gram negative rod, *Escherichia coli* was named after Dr Theodor Escherich who first described this bacterium in 1885 (Ørskov, 1984). Representing a wide cluster of biotypes and designated a member of the family *Enterobacteriaceae*, *E. coli* has been known as a common inhabitant of the gastrointestinal tract of humans and other large mammals since the early days of bacteriology. This observation gave rise to the notion that *E. coli* was no more than a harmless commensal (Bettelheim, 1996a). Studies conducted during the 1920s and 1930s, however, suggested that some strains of *E. coli* could cause diarrhoea in humans, a finding that gained acceptance during the 1940s (Doyle and Padhye, 1989). Since then epidemiological, clinical and laboratory investigations

have clearly established the pathogenicity of *E. coli* and coupled this bacterium to a range of clinical illnesses. Neonatal meningitis, urinary tract infections and septicemia are described as the three primary extraintestinal illnesses precipitated by *E. coli* (Ørskov, 1984). Gastrointestinal disturbances ranging from uncomplicated diarrhoea to several life threatening clinical syndromes have also been associated with infections caused by pathogenic *E. coli.* Six classes of diarrheaogenic *E. coli* differentiated by distinct clinical syndromes, differences in epidemiology, and well defined serological properties are now recognised: enteropathogenic (EPEC), enterotoxigenic, (ETEC), enteroinvasive (EIEC), enteroaggregative (EaggEC), diffusely adherent (DAEC) and enterohaemorrhagic (EHEC) (Buchanan and Doyle, 1997; Jay, 2000).

EHEC, the most recently described yet well known group of pathogenic *E. coli,* emerged following the implication of *E. coli* O157:H7 as the etiological agent responsible for two outbreaks of haemorrhagic colitis in 1982 (Riley et al., 1983). Recognised as one of the most important groups of emerging pathogens within the developed world (ANZFA, 1999; Parry and Palmer, 2000), EHEC have frequently been associated with foodborne disease resulting from the consumption of undercooked ground beef or raw milk (Griffin and Tauxe, 1991; Doyle, 1994). The EHEC group is characterised by production of the shiga-like toxins SLT I and/or SLT II, also referred to as verotoxin 1 and verotoxin 2 (VT1/VT2) (Bettelheim, 1996b; Jay, 2000), and by the clinical symptoms it precipitates (Buchanan and Doyle, 1997). Haemorrhagic colitis (HC), a bloody diarrhoea accompanied by severe abdominal pain is the primary syndrome caused by EHEC. Complications arising from HC include Haemolytic uremic syndrome (HUS), Thrombotic Thrombocytopenic Purpura (TTP), and in some instances death (Riley, 1987; Tarr, 1995).

A further characteristic of the EHEC group is their low infectious dose (Tilden et al., 1996; Buchanan and Doyle, 1997; Desmarchelier and Grau, 1997; Tuttle et al., 1999). The implication of EHEC in outbreaks of illness associated with the consumption of low pH foods (Besser et al., 1993; Morgan et al., 1993; Zhao and Doyle, 1994; CDC, 1995; Chivell, 1995; CDC, 1997) has led to speculation that the low infectious dose of these pathogens may stem from an enhanced tolerance to low pH (Miller and Kaspar, 1994; Semanchek and Golden, 1996; Buchanan and Doyle, 1997). Although a large number of studies have illustrated the survival of EHEC in acidic foods (Glass et al., 1992; Miller and Kaspar, 1994; Weagant et al., 1994; Riordan et al., 1998; Ryu and Beuchat, 1998; Roering et al., 1999) little evidence has been presented to substantiate the notion that EHEC are more acid tolerant than other pathogenic or commensal isolates (McClure and Hall, 2000). In fact several recent studies have illustrated that considerable strain dependent variability exists within the enterohaemorrhagic, non-enterohaemorrhagic-pathogenic and non-pathogenic groups of *E. coli* with regard to physical and chemical stress tolerance (Gorden and Small, 1993; Benjamin and Datta, 1995; Brown et al., 1997; Kaur et al., 1998; Benito et al., 1999).

Perhaps because of its perceived lack of virulence or because of its accessibility and ease of culture, *E. coli* was used in many early cell biology studies. Neidhardt (1996) suggests that the knowledge of this organism accumulated through these studies became a driving force that established *E. coli* as the organism of choice in many classic studies concerning cell biochemistry, physiology and more recently molecular biology. Despite the wealth of information that has been collected concerning *E. coli* our understanding of this species, its responses to environmental constraints, and the molecular basis of those responses, is still quite rudimentary.

1.4 CHARACTERISING THE INFLUENCE OF LOW pH ON THE GROWTH & SURVIVAL OF *E. COLI*

1.4.1 The Growth Response Characteristics of *E. coli* as a Function of pH & H^+ Concentration

For any given environmental parameter a microbial species will exhibit growth only over a relatively restricted range of values (Booth and Kroll, 1989), with a well defined optimum value for growth often being apparent. Deviation from this optimum tends to restrict microbial growth, increasing the lag phase duration and decreasing the growth rate and cell yield of populations until growth is finally precluded (Brown and Booth, 1991). As a neutralophile, literally a 'neutral loving' organism, *E. coli* exhibits a broad pH optimum for growth centered about neutrality (Cohen, 1922; Ingraham and Marr, 1996). The growth characteristics of this organism (lag phase duration, generation time and cell yield) are largely independent of pH for one to two units either side of neutrality varying only when the pH drops below 5 (Figure 1.1, IA-IC). Lag time and generation time increase in a curvilinear manner below pH 5 and cell yield declines rapidly until the pH becomes non-permissive for growth. Brown and Booth (1991) have pointed out the value of examining the low pH growth characteristics of *E. coli* as a function of hydrogen ion concentration. pH is logarithmic and consequently large changes in hydrogen ion concentration are masked by small changes in pH. As a function of hydrogen ion concentration the growth characteristics of *E. coli* are typically linear (Figure 1.1, IIA-C). Lag phase duration and generation time increase linearly with increasing hydrogen ion concentration while cell yield, which has been regarded as a measure of the metabolic efficiency of a culture (Krist et al., 1998), decreases as the hydrogen ion concentration increases.

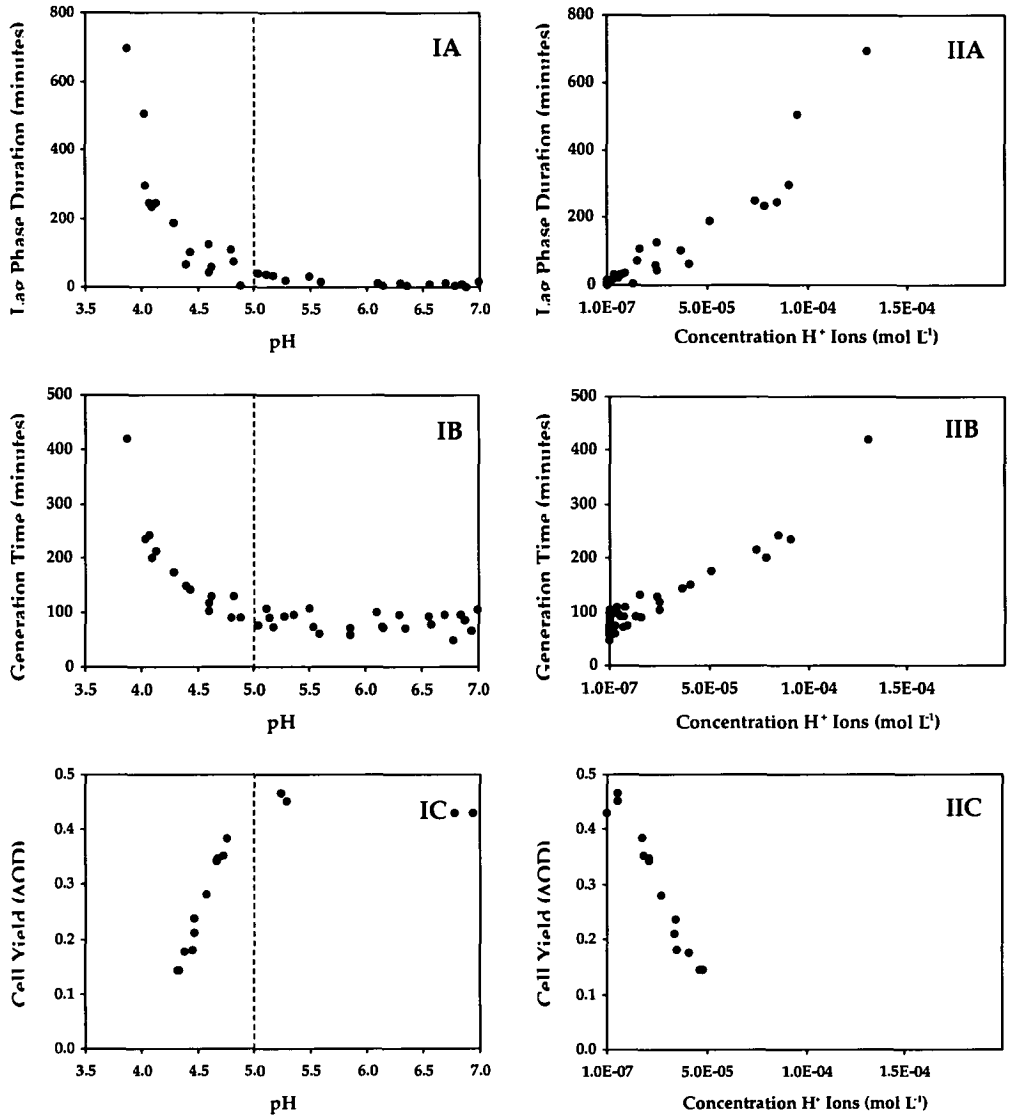


Figure 1.1 Influence of pH (I) and hydrogen ion concentration (II) of the growth characteristics of *E. coli* M23: lag phase duration (A), generation time (B) and cell yield of substrate limited cultures (C). Data from K.A. Krist (1997) and K.A. Presser (2001), used with permission.

While Figure 1.1 illustrates the pattern of the influence of pH on the growth characteristics of *E. coli*, the absolute values describing those characteristics shift in response to environmental variables other than pH (Jay, 2000). The number of variables significantly influencing the rate of microbial growth in foods is relatively small, however, with temperature, pH and water activity identified as the primary

controlling variables (Roberts, 1990). This observation, together with an appreciation of the power of predictive microbiology as an aid to microbial food safety and quality assurance, stimulated the development of a number of mathematical models that relate the growth rate of foodborne microorganisms to the primary environmental variables controlling their growth (Ross et al., 2000). While many of the models published (Buchanan and Klawitter, 1992; Buchanan et al., 1993; Buchanan and Bagi, 1994; Rosso et al., 1995; Sutherland et al., 1995; Presser et al., 1997) successfully describe the growth rate of *E. coli* as a function of pH, either alone or in combination with other environmental parameters, few attempt to quantify the influence of pH on the lag phase duration of this organism. Unlike growth rate and cell yield, lag phase duration is influenced by physiological parameters including phenotype, growth phase and inoculum density (Buchanan and Cygnarowicz, 1990; McMeekin et al., 1993; Baranyi and Roberts, 1994; Mellefont, 2001). Development of models that accurately describe the lag phase duration of microbial populations as a function of pH thus presents a substantially greater challenge than the development of corresponding growth rate models (Robinson et al., 1998).

In addition to the growth characteristics discussed above, the pH value below which growth is precluded is pertinent to a description of the growth responses of an organism cultured at low pH. Like growth rate, lag phase duration and cell yield, the lower pH limit for growth (i.e. the growth/no growth boundary) is influenced by the acidulant employed (Booth and Kroll, 1989; Jay, 2000) and by interacting environmental parameters including temperature and water activity (Presser et al., 1998). In the absence of secondary environmental constraints the lower pH limit for the growth of *E. coli* is generally regarded to be 4.4 (Desmarchelier and Grau, 1997; Rompf and Jahn, 1999). Presser et al. (1998) have,

however, confirmed the growth of *E. coli* M23 in liquid media acidified with hydrochloric acid at pH values as low as 3.9.

1.4.2 Extending the Picture Below the Boundary for Growth – Kinetic Studies in the Growth Non-Permissive Low pH Range

When environmental pH drops below the boundary for growth microbial cells begin to lose viability (Ahamad and Marth, 1989). In contrast with the time and resources that have been invested in describing the response characteristics of *E. coli* within the growth permissive low pH range, relatively little effort has been made to systematically describe the response characteristics of populations of *E. coli* exposed to analogous lethal constraints (Ellsion et al., 1994). In addition to the inherent properties of *E. coli* that determine its capacity to withstand inimical low pH treatments (i.e. genotype derived peculiarities) multiple physical and chemical parameters influence the low pH tolerance of this species (Table 1.1), directly (during the low pH challenge), indirectly (during preparation of the population exposed to the low pH challenge), or apparently[1] (during the recovery of survivors). A particularly well known example of the influence of an environmental parameter on the low pH-induced loss of viability of *E. coli* is that of acid habituation, the phenomenon in which cells exposed to mildly acidic conditions during their growth phenotypically acquire the ability to survive low pH conditions that would otherwise be lethal. Unfortunately many of the studies that have identified physiological and environmental parameters which influence the low pH tolerance

[1] Note that the observed, or apparent, viability of a dying population is actually the resultant measure of the true viability at the time of sampling supplemented by the mortality that occurs during the recovery process (see Chapter 2). Those parameters that influence the low pH tolerance of *E. coli* 'apparently' do so through influencing the mortality observed during the recovery process.

Table 1.1 Physiological and environmental parameters that influence the tolerance of *E. coli* to inimical low pH constraints. Each parameter is described as having an [A]indirect, [B]direct, or [C]apparent influence on the low pH tolerance of this organism.

Parameter		Reference
Genotype	*Within Species*	Gorden & Small, 1993; Lin et al., 1995*; Brown et al., 1997*; Tsai & Ingham, 1997*; Benito et al., 1999*
	Within Serotypes	Arnold & Kaspar, 1995; Benjamin & Datta, 1995; Buchanan & Edelson, 1996*; Wang & Doyle, 1998; Jordan et al., 1999a; McKellar & Knight, 1999
Growth Phase of Inoculum		Small et al., 1994; Arnold & Kaspar, 1995; Benjamin & Datta, 1995; Benito et al., 1999*; Datta & Benjamin, 1999; Jordan et al., 1999b*
Temperature	*Preparation*[A]	Arnold & Kaspar, 1995; Cheng & Kaspar, 1998; Wang & Doyle, 1998*
	Challenge[B]	Tsai & Ingham, 1997*; Cheng & Kaspar, 1998*
pH	*Preparation*	Rowbury et al., 1989*; Raja et al., 1991a; O'Hara & Glenn, 1994*; Benjamin & Datta, 1995; Leyer et al., 1995*; Lin et al., 1995*; Brown et al., 1997*; Tsai & Ingham, 1997*; Chang & Kaspar, 1998; Ryu & Beuchat, 1998*; Wang & Doyle, 1998*; Chang & Cronan, 1999*; Diez-Gonzalez & Russell, 1999; Jordan et al., 1999b*
	Challenge	Poynter et al., 1986; Rowbury & Goodson, 1993*; Benjamin & Datta, 1995; Ryu & Beuchat, 1998*; Jordan et al., 1999a
Water Activity	*Preparation*	Brown, 1996*; Cheng & Kaspar, 1998
	Challenge	Shadbolt et al., 2001*
	Recovery[C]	Ryu and Beuchat, 1998*; Jordan et al., 1999c
Aerobicity	*Preparation*	Small et al., 1994; Cheng & Kaspar, 1998
Surface Attachment	*Challenge*	Poynter et al., 1986*
Acidulant/Weak acid	*Challenge*	Poynter et al., 1986; Benito et al. 1999*; Buchanan & Edelson, 1999; Diez-Gonzalez & Russell, 1999; Jordan et al., 1999a*
Richness of Medium	*Preparation*	Lin et al., 1995; Rowbury, 1995; Buchanan and Edelson, 1996*
	Challenge	Rowbury et al., 1992; Leyer et al., 1995*; Lin et al., 1995; Diez-Gonzalez and Russell, 1999
Inhibitors	*Preparation*	Raja et al., 1991a; Rowbury, 1995
	Recovery	Roth & Keenan, 1971*; Przybylski & Witter, 1979*; Buchanan & Edelson, 1996*
Ethanol	*Challenge*	Semanchek and Golden, 1996*; Jordan et al., 1999a*
Presence of Phosphates	*Challenge*	Rowbury & Goodson, 1993
Presence of Metabolites	*Preparation*	Rowbury and Goodson, 1999

*Kinetic data is provided, but note that in many instances this data is fragmentary.

of *E. coli* fail to illustrate the manner in which these parameters influence the time-dependent loss of viability of populations of *E. coli* exposed to growth non-permissive low pH constraints. On the whole kinetic data pertaining to the low pH-induced loss of viability of *E. coli* is scarce and where it is presented the data is often fragmentary. Knowledge of the kinetics of inactivation of populations of *E. coli* exposed to inimical low pH constraints, and of the influence of physiological and environmental parameters on the kinetics of low pH-induced inactivation, thus remains limited.

1.5 MECHANISTIC INTERPRETATIONS CONCERNING THE INFLUENCE OF LOW pH ON THE GROWTH & SURVIVAL OF *E. COLI*

Conventional wisdom implies that the optima, limits and rates of microbial growth are determined by the stability and activity of enzymes and other biologically important molecules. The stability of such macromolecules is influenced by physical and chemical parameters of the molecular environment, including pH (Lamanna and Mallette, 1959). Changes in local pH alter the protonation of biomolecules and thereby elicit changes in their conformation and activity (Booth and Kroll, 1989; Montville, 1997). Most biologically active molecules have a narrow pH range of optimum stability and activity that falls close to neutrality (Padan et al., 1981; Ingraham and Marr, 1996). Accordingly, a microorganism's ability to maintain its intracellular pH (pH_i) within this range determines its potential for growth in low pH environments (Salmond et al., 1984; Zilberstein et al., 1984). *E. coli* displays a remarkable ability to regulate its intracellular pH around the value of 7.6. Over an external pH (pH_o) range of 4.5 to 7.9 the pH_i of *E. coli* is reported to vary by less than 0.1 unit per unit change in external pH (Slonczewski et al., 1981; Hill et al., 1995; Slonczewski and Blankenhorn, 1999). This constancy of pH_i in spite of external pH change is termed 'pH homeostasis' (Booth, 1985) and is thought to

derive from both constitutively expressed and inducible regulatory systems (Foster, 2000).

Although the mechanisms of pH homeostasis in *E. coli* remain poorly understood (Slonczewski and Foster, 1996) the regulation of cytoplasmic pH is generally regarded to be an energy requiring process (Kroll and Booth, 1983; Booth, 1985; Montville, 1997; Shabala et al., 2002). The inverse linear relationship that exists between the hydrogen ion concentration of the growth milieu and the cell yield of substrate limited cultures (Figure 1.1, IIC) lends support to this notion. That cell yield should also be a function of the growth rate of a microbial culture was suggested by Pirt (1965) who explains that the amount of energy diverted from growth to maintenance (i.e. into functions other than the production of new cell material) increases as the growth rate of a culture declines. Krist (1997) demonstrated such a relationship for *E. coli* concluding that the efficiency of substrate conversion to biomass is reduced as pH_o becomes increasingly stringent.

Exposed to an external pH shift, populations of *E. coli* exhibit a growth lag – a period of no growth during which the cells adapt to their new environment (Montville, 1997). Robinson et al. (1998) have suggested that the duration of microbial lag phases may be regarded as a function of two hypothetical quantities: the amount of work that a cell must perform to adapt to its new conditions and the rate at which it can perform that work. When *E. coli* experience a shift in external pH, their intracellular pH is transiently perturbed (Slonczewski et al., 1981; Zilberstein et al., 1982) and must be restored to a value close to neutrality before growth will be resumed (Salmond et al., 1984; Zilberstein et al., 1984). The lag phase duration of populations shifted to an acidic pH will depend, therefore, on the degree to which pH_i is perturbed and on the rate at which the cell can effect the restoration of pH_i. Zilberstein et al. (1984) indicate that the recovery of pH_i depends

on the load imposed on the system, stating that "the capacity of the pH homeostatic mechanism appears to be a function of both the span of the shift in pH_o and the rate at which the change occurs". The authors speculate that larger shifts in external pH will not only result in a greater perturbation of pH_i, but that they will also precipitate more extensive cellular damage thereby impeding the rate and capacity for restoration of pH_i. Accordingly the lag phase duration of *E. coli* lengthens as the magnitude of the shift in pH_o, and hence the perturbation of pH_i, increases (Figure 1.1, IIA).

Under extremely acidic conditions (pH_o less than 4.5) *E. coli* begin to lose the ability to restore their intracellular pH (Hall et al., 1995; Foster and Moreno, 1999). A small decline in pH_i will be tolerated (Booth and Kroll, 1989), but growth is precluded when the pH of the cytoplasm falls below about 6.8 (Salmond et al., 1984). As conditions become increasingly stringent *E. coli* begin to lose viability. Two hypotheses concerning the mechanism of low pH-induced growth inhibition and inactivation are presented in the literature, although rather informally. The first draws on the notion that biomolecules exhibit a pH sensitivity, and implies that growth inhibition and loss of viability under acidic conditions stem from an inability to regulate pH_i and the consequent destabilisation of biomolecules (Hall et al., 1995; Small et al., 1994). Zilberstein et al. (1984) suggest that ".... the need for pH homeostasis during growth is not due to a general pH sensitivity of cytoplasmic proteins, but that there is a specific pH-sensitive function". With the aid of a mutant defective in pH homeostasis they examined various aspects of cellular physiology including viability, growth rate, energy transduction, respiration, ΔpH (the difference between pH_o and pH_i), $\Delta\psi$ (the membrane potential), protein synthesis, and DNA synthesis under growth non-permissive pH conditions. In this manner Zilberstein and his co-workers attempted to pinpoint possible intracellular-pH-sensitive functions that might arrest growth. Of all the parameters examined

only cell division was both immediately and completely inhibited when intracellular pH became non-permissive, leading them to conclude that a pH-sensitive process of cell division must be responsible for growth arrest. While a specific pH-sensitive molecule responsible for cell division has not been identified, the reversible nature of microbial stasis suggests that the process leading to this phenomenon should also be reversible.

Emerging from the notion that the mechanisms of pH homeostasis are energy demanding (Slonczewski et al., 1981; Kroll and Booth, 1983; Booth, 1985), the so called 'energy diversion hypothesis' (Pirt, 1965; Knochel and Gould, 1995; Krist, 1997) provides an alternative mechanistic explanation for the inhibition of microbial growth and loss of microbial viability witnessed at low pH. According to this hypothesis, cells exposed to sub-optimal growth constraints channel available energy from biosynthetic reactions into maintenance functions (including the turnover of macromolecules and pH homeostasis). The energy demands of maintenance under sub-optimal conditions are thought to reduce the cell's growth potential and result in microbial death when they exceed the cell's energy-producing capacity (Montville, 1997; Leistner, 2000).

Attempts to elucidate specific mechanism(s) whereby a high extracellular concentration of hydrogen ions precludes the growth and/or effects the inactivation of *E. coli* (or any other microorganism) have enlisted the attention of only a small number of investigators. As a result, much of what we 'know' concerning the mechanistic basis of low pH-induced microbial growth inhibition, stasis and inactivation remains largely speculative (Ingraham and Marr, 1996).

1.6 MOLECULAR SYSTEMS THAT FACILITATE THE GROWTH AND SURVIVAL OF *E. COLI* UNDER ACIDIC CONDITIONS

The growth and survival of *E. coli* at external pH values below the pH optimum of many of its constituent biomolecules is possible because of its remarkable ability to regulate pH_i (Ingraham and Marr, 1996). This section of the review deals with what is known concerning the molecular systems that enable *E. coli* to maintain pH homeostasis, to avoid acidic conditions if that is an option, and to protect and repair critical biomolecules from low pH-induced damage. Many of the molecular systems described below are depicted in Figure 1.2.

1.6.1 Constitutive Homeostatic Systems

Although the mechanisms underpinning pH homeostasis in bacteria have been studied for some time, they remain poorly understood (Kobayashi et al., 2000). Typically, *E. coli* is thought to rely on constitutively expressed systems for the homeostasis of pH_i at pH_o values up to one pH unit either side of its optimum value (Slonczewski and Foster, 1996; Foster, 2000). Described either as passive or active contributors to pH homeostasis (Booth, 1985) these systems include the cell membrane, cytoplasmic buffering, and regulated membrane-bound proton/cation antiport systems (Hall et al., 1995; Booth, 1999).

Impeding the uncontrolled movement of hydrogen ions between the external milieu and the cytoplasm, the cell membrane is regarded as a passive contributor to pH homeostasis (Raven and Beardall, 1981). Comprised of a phospholipid bilayer embedded with proteins it defines the boundary between the cytoplasmic and extracellular environments, and retards the passage of ions by virtue of its hydrophobic interior (Campbell, 1990). Compounds that compromise the low proton permeability of the cytoplasmic membrane (eg. H^+-specific ionophores or

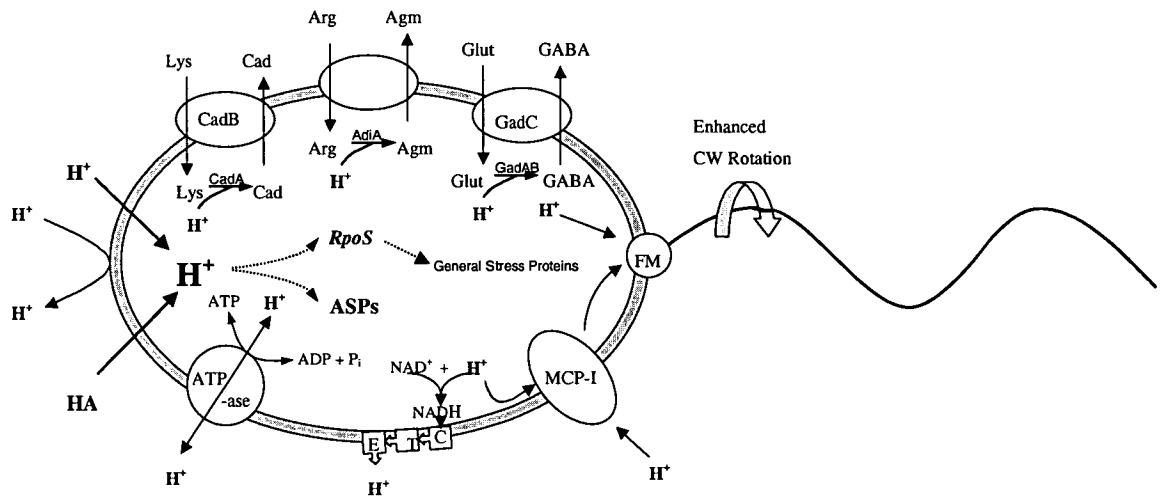


Figure 1.2 Schematic representation of the constitutive and inducible molecular systems thought to enable *E. coli* to persist in low pH environments. Lys, lysine; Cad, cadaverine; Arg, arginine; Agm, agmatine; Glut, glutamate; GABA, gamma-amino-isobtyrate; FM, flagellar motor; MCP I, methyl-accepting protein I; ETC, electron transport chain; ASPs, acid shock proteins. Adapted from Abee and Wouters, 1999. See text for details.

weak acids) lead to intracellular acidification, restricting and ultimately precluding microbial growth in low pH environments (Harold and van Brunt, 1977; Salmond et al., 1984). Although an intact cell membrane is essential for pH homeostasis it is important to note that biomembranes are not entirely impermeable to protons, their conductance to hydrogen ions increasing as the external pH decreases (Rius et al., 1995). Consequently, external pH shifts transiently perturb the intracellular pH of *E. coli* (Slonczewski et al., 1981; Zilberstein et al., 1982). Cytoplasmic buffering and the controlled transport of hydrogen ions are among the molecular systems that

promote the restoration and subsequent maintenance of pH_i following its perturbation.

Capable of offsetting limited amounts of acidification cytoplasmic buffering is, like the cell membrane, regarded as a passive contributor to pH homeostasis (Booth, 1985). The buffering capacity of the cytoplasm derives from the phosphates and carboxylates of various metabolic species (Booth, 1999), and for *E. coli* is estimated to be of the order of 400 nmol H^+/pH unit/mg protein in the neutral to slightly acidic pH range. Increasing as the external pH decreases, the buffering capacity of the cytoplasm almost doubles in response to an external pH shift from 7 to 4.5 (Rius et al., 1995).

Active pH homeostasis in *E. coli* is thought to rely upon the controlled removal of hydrogen ions from the cytoplasm via membrane-bound transporters. Information concerning the exact mechanism of this system remains limited although a requirement for concomitant potassium uptake, and for a high respiratory rate or available ATP, has been demonstrated (Kroll and Booth, 1983; Booth, 1985). *E. coli* possess various membrane-bound proton translocators including the cytochrome oxidases Cyt D and Cyt O, and an F_1F_0 ATPase (Cotter et al., 1990; Kasimoglu et al., 1996). Driven by electron transport (NADH oxidation) and the hydrolysis of ATP respectively, these membrane-bound transporters can function as proton pumps (Slonczewski et al., 1981; Rowbury, 1997; Fillingame and Divall, 1999). Kroll and Booth (1981) proposed a model in which proton extrusion achieved through such transporters, and electroneutrally balanced by the uptake of potassium, would lead to alkalinisation of the cytoplasmic pH. While such a mechanism has been shown to facilitate the regulation of intracellular pH in some microorganisms (Kobayashi et al., 1982; Serrano, 1984), the contribution made by membrane-bound proton

transporters to pH homeostasis in *E. coli* remains unclear (Slonczewski and Foster, 1996).

1.6.2 Systems that Facilitate Avoidance 'Behaviour'

When exposed to acidity *E. coli* exhibit a chemotactic response that facilitates their migration towards more favourable environments. Described as a primitive 'behavioural' strategy (Slonczewski et al., 1982) this response indicates that *E. coli* can both sense and actively respond to low pH, a notion that was also supported by the work of Kroll and Booth (1983). A mechanistic basis for the phenomenon of bacterial chemotaxis is described by Kihara and Macnab (1981). They explain that a shift in the probability of alteration of the flagellar motor between counterclockwise (CCW) and clockwise (CW) rotational states causes bacterial cells to exhibit either translational motion (swimming) or chaotic angular motion (tumbling). Repellent tactic stimuli increase the probability of CW flagellar rotation, and hence the occurrence of re-orientation events. In turn, the frequency of re-orientation determines the probability of the cell encountering more favourable conditions.

Both weak acids and a low pH *per se* elicit a repellent tactic response in *E. coli* that was initially thought to be mediated solely by pH_i (Kihara and Macnab, 1981; Repaske and Adler, 1981; Slonczewski et al., 1981). Analysing the chemotactic response of mutants lacking functionality in one of three methyl-accepting chemotaxis proteins (MCP I, MCP II or MCP III), the central components of three pathways that exist for the flow of information between various receptors and the flagellar motor, Repaske and Adler (1981) demonstrated that changes in the intracellular pH of *E. coli* alter the rotational state of the flagellar motor (increasing the probability of CW rotation) via a sensory transduction pathway which involves demethylation of MCP I. Subsequently, Slonczewski et al. (1982) illustrated that

MCP I is sensitive to both intracellular and extracellular pH, and that pH_i may also elicit a direct (non-MCP-related) effect on the flagellar motor. In this manner the migration of bacterial cells towards more favourable environments is promoted (Kihara and Macnab, 1981) and the growth inhibitory or lethal effects of an acidic environment may be avoided.

1.6.3 Low pH Inducible Systems

When external pH conditions deviate from the optimum for growth by more than one pH unit *E. coli* begin to invoke a series of inducible (i.e. expressionally regulated) molecular systems that enhance their potential for survival and growth (Hall, 1999; Foster, 1999). Designed to reinforce extant pH homeostasis systems, to protect specific biomolecular structures from low pH damage, and/or to repair acid damaged macromolecules these systems facilitate the acid tolerance (ATR), acid resistance (AR), and acid habituation responses of *E. coli* (Goodson and Rowbury, 1989; Raja et al., 1991a; O'Hara and Glenn, 1994; Lin et al., 1995; Lin et al., 1996).

While it was demonstrated as early as 1940 that *E. coli* elicit inducible molecular responses to low pH (Gale, 1940), little research in this area occurred before the early 1990s when it became apparent that *E. coli* and other significant foodborne pathogens exposed to mildly acidic conditions phenotypically acquire the ability to survive low pH conditions that would otherwise be lethal (Goodson and Rowbury, 1989; Foster and Hall, 1990; Kroll and Patchett, 1992). As the clinical and industrial implications of this revelation became apparent, a number of studies attempting to elucidate the molecular basis of the adaptive acid tolerance response(s) of *E. coli* were initiated. A global approach to the study of pH-regulated polypeptide expression based on polyacrylamide gel electrophoresis of total or fractionated cell proteins was employed in several laboratories. Such analyses reveal that under

sub-optimal pH conditions the biosynthesis of multiple polypeptides is altered with respect to their level of expression under neutral conditions, the biosynthesis of some being induced and others repressed (Heyde and Portalier, 1990; Hickey and Hirshfield, 1990; Raja et al., 1991a; Jordan et al., 1999b; Slonczewski and Blankenhorn, 1999). Comparative analysis of protein profiles from cells grown at low pH with those from cells exposed to alternative forms of environmental stress has revealed that some of the proteins induced under low pH conditions may also be induced in response to other physical and chemical stress factors (Olson, 1993). For example, Heyde and Portalier (1990) reported that of sixteen polypeptides induced by a shift to acidic conditions four corresponded to well known heat shock proteins (GroEL, DnaK, HtpG and HtpM), and three to proteins previously identified as stress proteins induced by high osmolarity, anaerobiosis or low temperature. It is now well recognised that an overlap exists between the proteins induced by low pH and those induced by a range of thermal and non-thermal environmental constraints (Slonczewski and Blankenhorn, 1999). Those proteins induced specifically by low pH have been termed acid shock proteins (ASPs) (Heyde and Portlier, 1990).

Many of the proteins regulated by low pH are, as yet, unidentified although N-terminal sequence analysis is being employed in an attempt to establish the identity of these polypeptides (Slonczewski and Blankenhorn, 1999). Likewise, the function of many of the pH regulated proteins remains unknown. Fractionation of cell proteins has revealed that at least seven of the low pH-regulated proteins of *E. coli* are membrane associated (Jordan et al., 1999b). Two membrane-associated proteins known to be regulated by low pH are the membrane porins OmpC and OmpF, which are induced and repressed respectively in response to acidification of the growth medium (Heyde and Portalier, 1987; Thomas and Booth, 1992). Jordan et al. (1999b) have suggested that regulation of the protein complement of the membrane

may alter its permeability to protons, thereby enhancing the cell's tolerance to low pH.

An examination of the physiological properties of acid adapted cells has shed some light on the possible functions of other low pH-induced proteins. Raja et al. (1991a; 1991b) demonstrated that acid adapted cells sustained less DNA damage when exposed to acidic conditions and that they were better able to repair low pH-induced DNA damage than their non-adapted counterparts. The latter finding is supported by the work of Hickey and Hirshfield (1990) which indicates that DNA polymerase I, the *polA* gene product that functions in DNA repair, is induced by low pH. Heyde and Portalier (1990) showed that several chaperone proteins are also induced by low pH. Molecular chaperones act to prevent the misfolding and aggregation of cellular proteins and to promote refolding and proper assembly (Gatenby, 1992; Mayhew and Hartl, 1996) and may, therefore, enhance repair of acid-damaged proteins.

While it seems that molecular systems facilitating the repair of acid damaged macromolecules will be induced in response to a decreasing pH, Hengge-Aronis (1999) suggests that pH-regulated proteins induced as part of the general stress response (see below) enhance the resistance of cells by preventing damage rather than by repairing it. As indicated above, Raja et al. (1991b) demonstrated that acid adapted cells sustain less low pH-induced damage than their non-adapted counterparts. Raja and colleagues (Raja et al., 1991a) postulated that adapted cells have an enhanced ability either to accumulate compounds which protect their DNA from acid stress or to maintain their pH_i near neutrality. The latter hypothesis was supported by Jordan et al. (1999b) who used proton flux measurements to demonstrate that adapted cells accumulate protons at a much slower rate than non-adapted cells, although they were unable to demonstrate whether this was the

result of an enhanced ability to exclude protons from the cell or an increase in the active efflux of protons.

Now recognised as molecular systems designed to facilitate pH homeostasis in *E. coli*, several amino acid decarboxylases were shown to be induced under acidic conditions as early as 1940 (Gale, 1940). Subsequently Gale (1946) identified six different amino acid decarboxylases induced by *E. coli* in response to low pH: arginine decarboxylase, lysine decarboxylase, glutamate decarboxylase, ornithine decarboxylase, histidine decarboxylase and tyrosine decarboxylase. Of one hundred and fifty one *E. coli* strains examined none were found to possess all six decarboxylases. The majority, however, produced the arginine, lysine, and glutamate decarboxylases. Encoded by *adiA*, *cadA*, and *gadAB* respectively, these amino acid decarboxylases are co-expressed with membrane bound antiporters (AdiA:?[1], CadA:CadB, GadAB:GadC) that facilitate uptake of the appropriate amino acid and excretion of the basic polyamine that results from their action (Meng and Bennett, 1992; Smith et al., 1992; Stim and Bennett, 1993; Castanie-Cornet et al., 1999). Consuming one cytoplasmically derived proton per amino acid molecule processed the amino acid decarboxylases effect an increase both in the cytoplasmic pH and in the pH of the growth medium (Gale and Epps, 1942; Meng and Bennett, 1992). Bearson et al. (1997) have suggested that these 'emergency pH homeostasis systems' induced by mild acidity may function to maintain pH_i above a critical value while more elaborate molecular systems are invoked.

Although a number of studies designed to elucidate the regulation of the acid inducible genes of *E. coli* have been published (Slonczewski et al., 1987; Auger et al., 1989; Meng and Bennett, 1992; Schellhorn and Stones, 1992; Thomas and Booth,

[1] The gene encoding the arginine/agmatine antiport remains unidentified.

1992; Watson et al., 1992; Stim and Bennett, 1993; Neely et al., 1994; Loewen et al., 1998) many of the regulatory pathways remain unknown or incompletely known. Many of the genes induced by low pH appear to be under the control of the regulator of the general stress response, RpoS (Small et al., 1994; Cheville et al., 1996). RpoS, an alternative sigma subunit of RNA polymerase recognises and binds to certain promoter regions of the primary DNA sequence, mediating selective gene transcription (Lange and Hengge-Aronis, 1991a; Hengge-Aronis, 1993; Hengge-Aronis, 2000). The molecular stability of this alternative sigma factor is regulated at multiple levels. Schellhorn and Stones (1992) indicate that weak acids promote both the transcription and translation of RpoS while Hengge-Aronis (2000) indicates that low pH enhances the molecular stability of this alternative sigma factor by preventing its degradation by the protease ClpXP. In turn, RpoS enhances the expression of a suite of genes encoding proteins designed to protect the cell from a range of environmental stresses including low pH (Hengge-Aronis, 2000). Three other proteins known to play important roles in regulation of the acid inducible molecular responses of *E. coli* are the transcriptional activator CysB, and two proteins that facilitate DNA bending, H-NS and the integration host factor IHF (Rowbury, 1997).

Rowbury (1997) points out that the process of elucidating low pH signal transduction pathways is often quite arduous and that a major difficulty in dissecting these pathways is that a number of diverse but interdependent physiological parameters are altered when the external pH changes. Intracellular pH, ΔpH, $\Delta\psi$, ΔP (the proton-motive force) and the concentration of other ions (eg. K^+, Na^+) all vary with pH_o (Olson, 1993). Many bacterial signal transduction pathways are two component systems that involve a sensor/transmitter protein and a receiver or target protein (Gross et al., 1989). Rowbury (1997) explains that initially the sensor protein must be located and identified. Weak acids are

particularly useful for locating a pH sensor as either periplasmic or cytoplasmic since a weak acid at a high pH_o will elicit a response if the sensor is cytoplasmically located but not if the sensor is in the periplasm. Although some of the outer membrane proteins aid proton passage to the periplasm (eg. phosphoporin PhoE), none have been shown to act as pH sensors (Rowbury, 1997). Interestingly, Rowbury and Goodson (1999) reported the occurrence of an extracellular stress-sensing protein involved in the low pH-inducible acid habituation response of *E. coli*. Once a sensor has been located and identified, and the manner in which the stimulus alters the sensor leading to the production of a signal has been determined, the transcriptional region regulated must be mapped and its interaction with the signal molecule demonstrated. Other components that affect the response must also be found and their role in the response demonstrated. As a result of their complex nature, few of the low pH signal transduction pathways of *E. coli* have been fully elucidated (Rowbury, 1997).

In conclusion, *E. coli* possess an array of overlapping molecular systems that facilitate growth under acidic conditions. Under mildly acidic conditions, extant and somewhat non-specific systems tend to be employed. As the magnitude of the stress increases a move towards more elaborate, but probably also more energetically expensive protective systems, is witnessed (Hill et al., 1995). Much remains to be learned about the nature of the response of *E. coli* to growth non-permissive low pH constraints, about the molecular systems that enable *E. coli* to survive such conditions, and about the mechanism(s) whereby low pH effects growth inhibition and inactivation.

CHAPTER 2 CHARACTERISING LOSS OF VIABILITY IN MICROBIAL POPULATIONS – PRELIMINARY CONSIDERATIONS

2.1 ABSTRACT

The notion that microbial viability is synonymous with culturability was explored and traditional culture-based methods of enumeration were embraced as an appropriate means of assessing the viability of populations of *E. coli* exposed to growth non-permissive low pH constraints. By extending the empirical evaluation of culture materials and procedures employed for the enumeration of acid injured *E. coli*, steps were taken to ensure that accurate accounts of the low pH induced loss of viability of *E. coli* would be presented in the chapters that follow. To this end, the influence of the physical structure and composition of bacteriological growth media, and of incubation temperature, on the recovery of acid injured *E. coli* was investigated. Using media supplemented with pyruvate, the physical structure of the growth medium employed was found to have little influence on the level of recovery achieved. Four of five solid media utilised for the recovery of acid injured *E. coli* facilitated comparable levels of recovery while the fifth, by comparison, performed poorly. Two incubation temperatures, one close to that maximising the metabolic efficiency (25°C), and one close to that maximising the growth rate (35°C), of *E. coli* were employed but no difference in their ability to facilitate the recovery of acid injured *E. coli* was apparent. The results presented provide a basis for the selection of culture materials and procedures to characterise the kinetics of the low pH induced inactivation of *E. coli*.

2.2 INTRODUCTION

Fissile vegetative microorganisms do not grow old and die in a manner comparable to macroorganisms. Instead individuals are replaced as they progress through a division cycle that gives rise to twin progeny of equal youth. The replacement of extant microorganisms continues indefinitely, albeit slowly in many instances, unless environmental constraints preclude growth. Under such circumstances populations of otherwise 'immortal' microorganisms begin to lose viability (Postgate, 1976).

While the term 'viability' can be employed to describe the proportion of viable microorganisms in a population, it can also be applied to individual microbes in an 'all-or-none' sense. Thus microorganisms exposed to growth non-permissive constraints have been classified as viable or non-viable, states traditionally assigned on the basis of a microorganism's ability to grow to detectable levels when provided with favourable conditions, for example on an agar plate, in slide culture or in a broth (Postgate, 1969). Operationally viability has generally been regarded as synonymous with culturability, and as a property that can only be ascribed to a microorganism retrospectively (Postgate, 1976).

Conceptually, however, the classification of a microorganism as either viable or not on the basis of its culturability has been called into question. Kell et al. (1998) point out that on one hand the majority of microorganisms in the biosphere are yet to be cultured axenically. Consequently they cannot be recognised as 'alive or dead' on the basis of the culturable/non-culturable dichotomy outlined above. On the other hand, recent advances in our understanding of the physiology of readily culturable microorganisms have made us aware that such microorganisms, when exposed to environmental stress, can temporarily exhibit non-culturability on some or all of the

media that normally support their growth. The possibility of such an occurrence undoubtedly warrants consideration since inaccurate reports concerning the loss of viability of microbial populations exposed to inimical constraints will have practical implications in areas of applied research and will confound the interpretation of published data.

To present an unambiguous and accurate account of the loss of viability in populations of *E. coli* exposed to growth non-permissive low pH constraints, this chapter describes an evaluation of the use of culturability as a measure of viability (for *E. coli*), and extends the empirical evaluation of culture materials and procedures employed for the enumeration of *E. coli* surviving inimical low pH challenges.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Strains, Bacteriological Media, Chemical Reagents & Equipment

Details of the bacterial strains, bacteriological media, chemical reagents and equipment employed throughout this study, together with the methods employed for bacterial maintenance and recovery, are presented in Appendix A.

Note: *E. coli* M23, a non-pathogenic laboratory strain, was employed in each of the experiments described in this chapter[1]. In the absence of secondary environmental constraints this strain was found to exhibit a lower pH growth limit of 3.9 (Presser et al., 1998) and a high level of intrinsic tolerance when exposed to inimical low pH constraints [pH 3.0] (Brown et al., 1997).

[1] This strain was also employed in all of the experiments presented in Chapters 3 and 4.

2.3.2 General Methods

2.3.2.1 *Preparation of Exponential Phase Populations*

Individual colonies of *E. coli* were inoculated to 60 ml volumes of Tryptone Soya Broth (TSB) and incubated overnight at 37°C with shaking. Exponential phase cultures were subsequently prepared by diluting these overnight cultures 1:1000 in fresh TSB then incubating the dilute cultures as described above until an optical density [OD_{540}] of 0.26, which corresponds to a viable count of *ca.* 1×10^8 cfu.ml^{-1}, was attained.

2.3.2.2 *Low pH Treatments and the Construction of Survival Curves*

Populations of *E. coli* were exposed to growth non-permissive low pH treatments by direct acidification of the growth medium with 10M HCl. The pH of each culture was rapidly (within 1 minute) adjusted to the desired value, and the cultures were subsequently incubated in oscillating waterbaths maintained at the temperatures specified. The viability of each population was estimated by cultural methods (described below) immediately prior to, and at intervals throughout, the low pH treatment. Survival curves were constructed by plotting the logarithm of the number of colony forming units recovered per milliliter against time.

2.3.3 Evaluation of Culture Materials & Procedures Used for the Enumeration of Viable Cells from Populations of *E. coli* Exposed to Low pH

2.3.3.1 *Physical Structure of the Growth Medium*

The number of viable cells in an exponential phase population (2.3.2.1) of *E. coli* M23 exposed to a low pH treatment (2.3.2.2) [pH 3.0, 35°C] was estimated by the Most Probable Number (MPN) technique following recovery in Tryptone Soya Broth amended with 0.1% sodium pyruvate (TSB-P), and from plate counts

afforded by the same medium solidified with 1.5% agar (TSA-P). For MPN determinations samples were serially diluted in TSB-P, and five 0.1 ml volumes of the three most appropriate dilutions were delivered to 1.5 ml eppendorf tubes containing 0.9 ml of TSB-P. Following static incubation of the tubes at 37°C for 24 hours the tubes were scored +/- for growth. A loopful of broth from tubes positive for growth was plated to Eosin Methylene Blue Agar (EMB) and incubated at 37°C for 24 hours to verify the presence of *E. coli*. The MPN for each combination was estimated according to the formula presented by Thomas (1942) which states that:

$$\text{MPN/ml} = \frac{\text{no. of positive tubes}}{\sqrt{(\text{ml sample in negative tubes} \times \text{ml sample in all tubes})}}$$

For plate counts, samples were serially diluted [1:10] in a 0.1% peptone/0.85% sodium chloride solution and surface plated to TSA-P with a spiral plater. The plates were incubated at 37°C for 14 hours and the viable counts were determined manually.

2.3.3.2 *Composition of the Growth Medium*

The number of viable cells in an exponential phase population (2.3.2.1) of *E. coli* M23 exposed to a low pH treatment (2.3.2.2) [pH 3.0, 35°C] was estimated from the plate counts afforded by five solid media types, each amended with 0.1% sodium pyruvate: Brain Heart Infusion Agar (BHA-P), Eosin Methylene Blue Agar (EMB-P), Luria-Bertani Agar (LBA-P), Tryptone Soya Agar (TSA-P), and Tryptose Phosphate Agar (TPA-P). Samples withdrawn from the experimental culture were serially diluted in 0.85% sodium chloride dissolved in 0.1% peptone water and surface plated with the aid of a spiral plater. The plates were incubated at 37°C for 18 ± 2 hours and viable counts were determined manually.

2.3.3.3 *Incubation Temperature*

The number of viable cells in an exponential phase population (2.3.2.1) of *E. coli* M23 exposed to a low pH treatment (2.3.2.2) [pH 3.0, 35°C] was estimated from the plate counts obtained at two incubation temperatures. Samples withdrawn from the experimental population were serially diluted in a 0.1% peptone/0.85% sodium chloride solution and surface plated, in duplicate, to TPA-P. One plate from each pair was incubated at 37°C for 14 hrs, the other at 25°C for 36 hrs. Viable counts were then determined manually.

2.4 RESULTS

2.4.1 Evaluation of Culture Materials & Procedures Used for the Enumeration of Viable Cells fom Populations of *E. coli* Exposed to Low pH

2.4.1.1 *Physical Structure of the Growth Medium*

Figure 2.1 illustrates the survival curves obtained when viable cells withdrawn from an exponential phase population of *E. coli* M23 exposed to low pH [pH 3.0, 35°C] were enumerated in a non-selective liquid medium (TSB-P) and on the same medium solidified with agar (TSA-P). While the viable count estimates achieved in the liquid medium were often lower than those obtained on the solid medium they were not consistently so, and the differences noted did not exceed the confidence limits, indicated by Jarvis (1989) [i.e. $\pm$ 0.3 log CFU.ml^{-1}], of the methods employed.

2.4.1.2 *Composition of the Growth Medium*

Figure 2.2 illustrates the survival curves obtained when viable cells withdrawn from an exponential phase population of *E. coli* M23 exposed to low pH constraints [pH 3.0, 35°C] were enumerated on five solid media routinely employed for the study of this organism. Most noticeable among the datasets is that obtained when

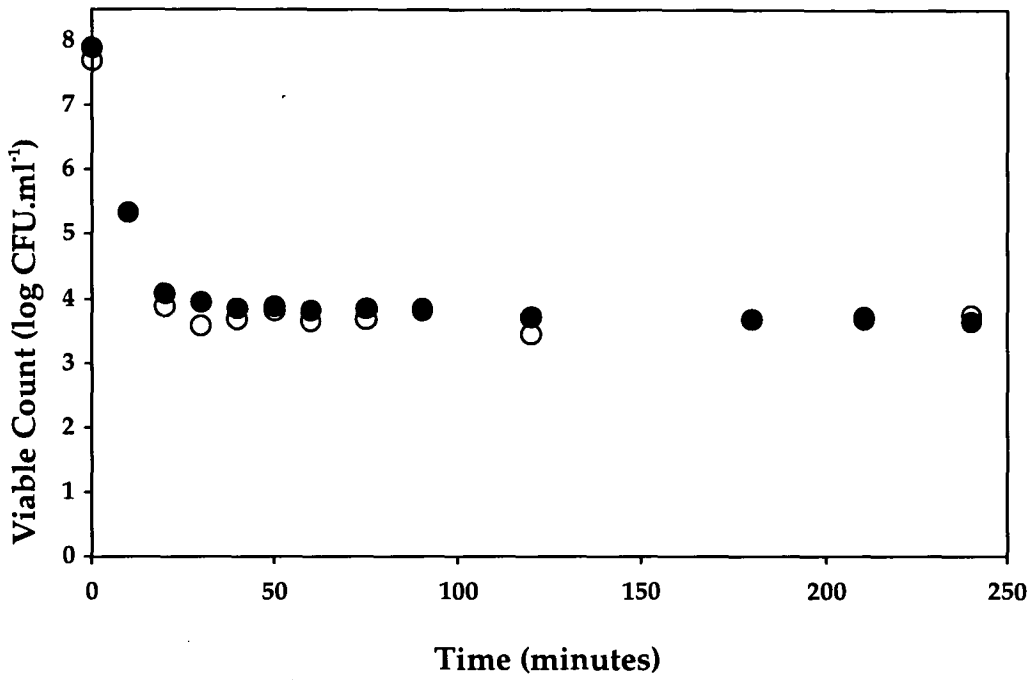


Figure 2.1 Viable count estimates afforded by the enumeration of viable cells in TSB-P (○) and on TSA-P (●) for an exponential phase population of *E. coli* M23 exposed to low pH [pH 3.0, 35°C].

EMB-P was employed for the enumeration of viable cells. With the exception of the viable count obtained prior to the low pH treatment (T_0), the viable counts obtained on EMB-P were consistently lower than those obtained on the other media typesemployed. This trend became increasingly apparent as the duration of the low pH treatment increased, with no viable cells being recovered on EMB-P at the conclusion of the experiment (T_{240}). In contrast with EMB-P, the numbers of viable cells recovered throughout the low pH treatment on BHA-P, LBA-P, TPA-P and TSA-P varied by less than half a log at each sample point, and no one growth medium consistently recovered higher numbers of cells.

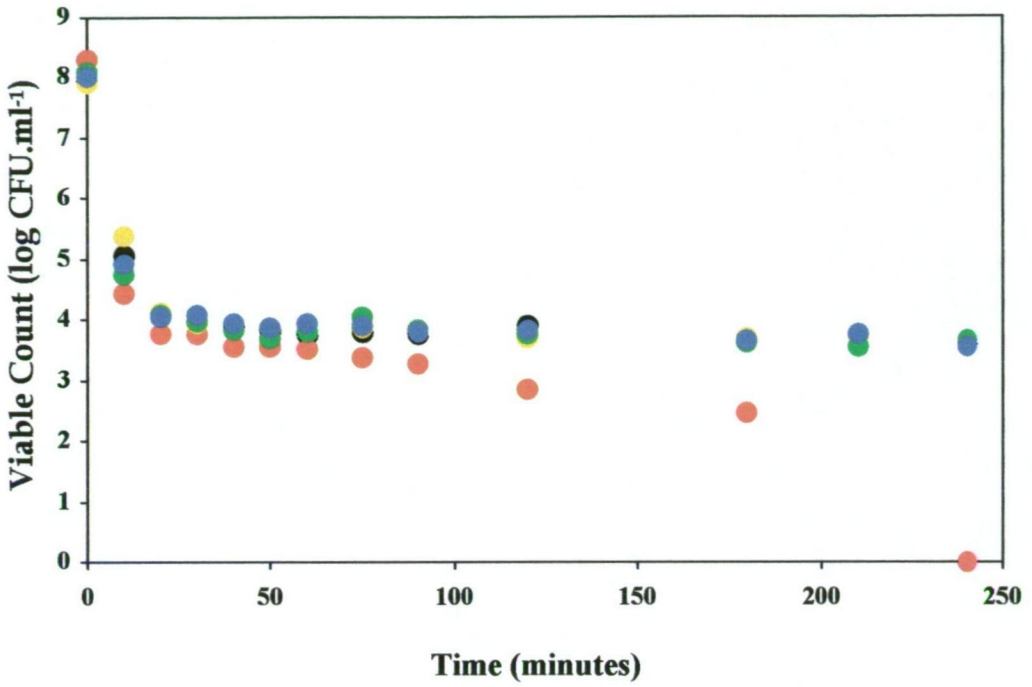


Figure 2.2 Viable count estimates afforded by the enumeration of viable cells on BHA-P (●), EMB-P (●), LBA-P (●), TPA-P (●), and TSA-P (●) for an exponential phase population of *E. coli* M23 exposed to low pH [pH 3.0, 35°C].

2.4.1.3 *Incubation Temperature*

Figure 2.3 illustrates the survival curves obtained when viable cells withdrawn from an exponential phase population of *E. coli* M23 exposed to growth non-permissive low pH constraints [pH 3.0, 35°C] were enumerated on TPA-P incubated at 25°C and 37°C. Differences in the viable counts achieved at the two temperatures were not consistent and did not exceed the confidence limits, estimated by Jarvis (1989), of the method employed.

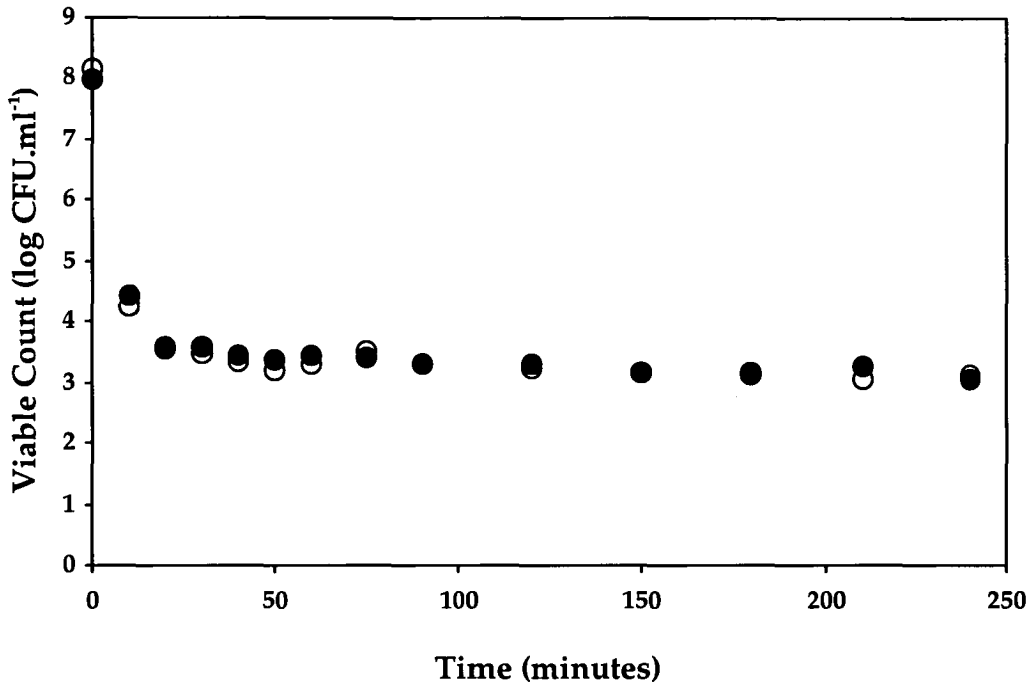


Figure 2.3 Viable count estimates afforded by the enumeration of viable cells on TPA-P incubated at 25°C (O) and 37°C (●) for an exponential phase population of *E. coli* M23 exposed to low pH challenge [pH 3.0, 35°C].

2.5 DISCUSSION

2.5.1 Evaluating the Use of Culturability as a Measure of Viability for *E. coli*

Demonstrating that *E. coli* and *Vibrio cholerae* incubated in artificial seawater became non-recoverable on both selective and non-selective media routinely employed for their culture yet retained physiological responsiveness, Xu et al. (1982) provided the first direct experimental evidence that readily culturable microorganisms, exposed to environmental stress, may exhibit temporary non-culturability on all media that normally support their growth. Further reports demonstrating the stress induced loss of culturability of both pathogenic and non-pathogenic bacteria followed that of Xu and his colleagues, and gave rise to the

description of a 'viable but non-culturable' (VBNC) state of bacterial existence. While some authors cited measurable metabolic activity (e.g. respiration, inducible enzyme activity, substrate responsiveness, membrane potential) and/or the maintenance of stable cellular structures (as indicated by fluorescence microscopy) as 'proof' of the viability of cells that were demonstrably non-culturable (Oliver, 1993; McDougald et al., 1998), others claimed that putative VBNC bacteria could be returned to a state of culturability (resuscitated) (Roszak et al., 1984; Jones et al., 1991; Nilsson et al., 1991; Oliver et al., 1995; Oliver and Bockian, 1995; Colwell et al., 1996). As a result the VBNC phenomenon emerged, embracing cells with "... various and often poorly defined physiological attributes but which, nonetheless, could not be cultured by methods normally appropriate to the organism concerned" (Kell et al., 1998).

Despite the lack of agreement between authors concerning the physiological basis of a VBNC state of bacterial existence the practical implications arising from the possibility of bacteria adopting such a state were immediately apparent. Studies suggesting that non-culturable bacterial pathogens can retain their ability to express virulence factors and hence their potential to elicit illness (Rahman et al., 1996) underscored the public health significance of putative VBNC bacteria, and heightened concerns about the reliability of culture-based methods as means of determining the distribution of pathogens and their responses to antimicrobial agents. In turn, these concerns provided an impetus for the development of new methods for assessing the viability of microorganisms.

While numerous 'rapid' cytological assays exploiting criteria other than replication as the basis for differentiating viable from non-viable bacterial cells have been proposed, no single method of generally accepted validity has emerged (Roszak and Colwell, 1987; McDougald et al., 1998; Kell et al., 1998; Breeuwer and Abee,

2000). Central to non-culture based methodologies is the assumption that cellular activity or integrity can be equated with cellular viability (Barer, 1997). Studies indicating that some bacteria exhibit dormancy, a reversible state of non-culturability in which cells display negligible amounts of metabolic activity (Kaprelyants et al., 1993), and that non-viable bacterial cells can retain both integrity and signs of metabolic activity (Villarino et al., 2000), clearly negate this assumption. It must be argued, therefore, that culturability in the 'ultimate' sense remains the only reliable indicator of microbial viability and that the VBNC phenomenon can only be verified by demonstrating the resuscitation of non-culturable cells (Kell et al., 1998).

Utilising this premise, Kell et al. (1998) critically evaluated those studies reporting the resuscitation of dormant or non-culturable bacteria. Demonstrating that most of the reports claiming a return to culturability failed to exclude the possibility of the growth of a small (non-detectable) number of cells which had never lost culturability, these authors indicate that sufficient evidence to support a reversible state of non-culturability has been demonstrated in only three instances. Kell (2000, pers.comm.) explains that in fact no evidence exists to suggest that *E. coli*, or bacteria other than several gram positive species, truly become VBNC. Employing a 'mixed culture recovery' method designed specifically to investigate the possibility of bacteria exhibiting a reversible non-culturable phenotype, Bogosian et al. (1998) demonstrated that the apparent 'resuscitation' of five enteric microorganisms was consistently due to the growth of a non-detectable number of culturable cells. They conclude, like Kell and his colleagues, that enteric bacteria such as *E. coli* almost certainly do not enter the VBNC state as originally suggested.

Not to be confused with the VBNC phenomenon, sub-lethal injury may also cause otherwise readily culturable bacteria to exhibit non-culturability. Unlike VBNC

and dead cells which exhibit temporary and permanent non-culturability, respectively, on all types of media normally appropriate for their culture, the loss of culturability of injured cells is incomplete. While they tend to be more exacting in their nutritional requirements, and to exhibit a greater sensitivity to secondary stresses than their non-injured counterparts, injured cells will repair cellular damage and grow normally if provided with a suitable environment (Mackey, 1999).

Bacterial cells can thus exhibit non-culturability as a consequence of several fundamentally different physiological processes: injury, dormancy, and death (Kell et al., 1998). For species that exhibit a true VBNC phenotype (as demonstrated by the resuscitation of non-culturable cells), simple[1] culture based methods of assessing viability will underestimate population viability, and consequently overestimate the efficacy of any inimical treatment to which that species is exposed. For species that do not exhibit a true VBNC phenotype, culturability undoubtedly remains the most reliable indicator of viability. Based on evidence (discussed above) which suggests that *E. coli* does not exhibit a true VBNC phenotype, and unconstrained by the need to use a selective medium for the recovery of this organism, the notion that viability is synonymous with culturability is deemed appropriate for the purposes of this study. Having said this, the potential limitations of employing culturability as a measure of viability are acknowledged, and must be addressed.

Central to culture based viability assays is the assumption that, exposed to favourable conditions, all of the viable cells within a sample will multiply to a detectable level. Postgate (1967) has pointed out, however, that bacteria do not

[1] The term 'simple' indicates the absence of resuscitation steps in the enumeration procedure

necessarily multiply the moment they are given the opportunity to do so, but rather that it is usual for them to exhibit a period of repair and/or adjustment to their new environment, prior to division, which he terms the division lag. Whether an individual microorganism was dead at the time of sampling or whether it died during the division lag, which for convenience is assumed to include the 'transfer' period (i.e. the period between which a sample is withdrawn from the parent culture and introduced to the growth environment), cannot be ascertained. Consequently, an assessment of the viability of a dying population will actually be the resultant measure of two theoretical viabilities: the viability at the time of sampling supplemented by the mortality that occurred during the division lag.

While the latter is often trivial it may, under certain circumstances, assume importance (Postgate, 1967). Consider, for example, microbial populations exposed to inimical physical or chemical conditions. A proportion of the viable cells within such populations often sustain sub-lethal injury which, as previously indicated, renders them hypersensitive to secondary stresses. Should the transfer of a sample to a growth permissive environment, or in fact the growth environment itself, expose injured cells to a secondary stress, the viability of the parent culture at the time of sampling may be significantly underestimated. In turn, the efficacy of any inimical treatment to which that population is exposed will be overestimated. Consequently, the process of optimising enumeration protocols should be regarded as a fundamental aspect of studies concerned with the loss of viability of microbial populations exposed to inimical constraints. Mossel and Van Netten (1984) point out that the success of any enumeration protocol will depend on the nature of the stress imposed and of the organism concerned, and consequently that generalisations, with regard to the enumeration of viable cells, cannot and should not be made. Instead they suggest that the suitability of enumeration procedures be evaluated empirically, case by case. With this in mind, experiments designed to

extend the evaluation of cultural materials and procedures employed for the enumeration of viable cells from populations of *E. coli* exposed to growth non-permissive low pH constraints were performed, and it is to the results of these experiments that the discussion now turns.

2.5.2 Evaluation of Culture Materials & Procedures Used for the Enumeration of Viable Cells from Populations of *E. coli* Exposed to Low pH

Although the process of optimising culturability as a measure of viability should be regarded as a fundamental aspect of survival studies, a review of the literature concerned with the survival of *E. coli* at low pH reveals few studies that evaluate the relative merits of various media or the influence of other methodological variables on the outcome of culture-based enumeration protocols. Noteworthy exceptions include several studies indicating that the growth of *E. coli*, following low pH exposure, is inhibited in media containing bile salts (Roth and Keenan, 1971; Przybylski and Witter, 1979), and a study published by Jordan et al. (1999c) in which the suitability of various diluents employed in the enumeration of acid injured *E. coli* is evaluated. The experiments presented above extend the evaluation of enumeration protocols used to assess the viability of populations of *E. coli* exposed to growth non-permissive low pH constraints.

2.5.2.1 *Physical Structure of the Growth Medium*

As previously noted, the suitability of any growth medium used to assess the viability of a bacterial population is likely to depend both on the nature of the bacterium studied and on the nature of the stress imposed. That the outcomes of studies concerned with the influence of the physical structure of bacteriological growth media on the recovery of injured cells are conflicting, then, comes as no surprise. While Roberts (1970) reported no significant difference in the suitability

of media employed in a solid or liquid state, Dabbah et al. (1969), Abshire et al. (1980), and Mackey and Derrick (1982) reported that the recovery of injured bacteria was greater in liquid media than on the same media solidified with agar. Observations to the contrary were reported by Hewlett and Hall (1911), Rosenstein and Levin (1935), Falk et al. (1939), and Cook et al. (1964).

Dealing specifically with the recovery of injured *E. coli* Hershey (1939), Roberts and Aldous (1949), and Milbauer and Grossowicz (1959) illustrated respectively that liquid media facilitated the recovery of higher number of cells injured by high temperature, ultraviolet irradiation, and chlorine than did the same media solidified with agar. By contrast, the results of the present study (Figure 2.1) illustrate little difference in the efficacy of a liquid medium (TSB-P) and it's solidified counterpart (TSA-P) for the recovery of *E. coli* injured by exposure to growth non-permissive low pH constraints. Such an outcome may imply that those conditions responsible for the poor recovery of *E. coli* injured by high temperature, ultraviolet irradiation, or chlorine on media solidified with agar, do not impair the recovery of *E. coli* injured by low pH. Such conditions may include sub-optimal water availability (Hershey, 1939) and/or a sub-optimal rate of diffusion of nutrients and toxic by-products to and from cells (Miller and Davey, 1965; Abshire et al., 1980). Alternatively, it may be that a component (pyruvate) of the recovery medium employed in the present study but not in previous studies concerned with the influence of the physical structure of bacteriological growth media on the recovery of injured *E. coli*, has masked a difference that would be observed in the absence of this component. Investigating the influence of the physical structure of growth media on the recovery of *Salmonella* Typhimurium, Mackey and Derrick (1982) illustrated that the recovery of cells exposed to high temperature was consistently higher in nutrient broth than on nutrient agar. They also illustrated, however, that the difference in the recovery facilitated by the two media was

eliminated by the addition of catalase or pyruvate, known scavengers of reactive oxygen species. This finding led them to speculate that a difference in the concentration of reactive oxygen species encountered in a liquid medium and on the same medium solidified with agar might explain the influence of the 'physical structure' of the growth medium on the recovery of *S.* Typhimurium. Bearing Mackey and Derrick's study in mind, the results of the present study cannot simply be interpreted as an indication that *E. coli* injured by low pH do not respond to changes in the physical structure of growth media used for their recovery as do *E. coli* injured by high temperature, ultraviolet irradiation, or chlorine.

Numerous studies have illustrated that the addition of pyruvate to solid media improves the recovery of bacterial cells injured by various physical and chemical means (Martin et al., 1976; McDonald et al., 1983; Lee and Hartman, 1989; Calabrese and Bissonnette, 1990; Sartory, 1995; Czechowicz et al., 1996). With regard to acid induced injury, Leyer and Johnson (1992) reported that the addition of sodium pyruvate to Luria-Bertani agar increased the viable counts of *Salmonella* spp. 1000-fold. Likewise, unpublished data from this laboratory indicates that the presence of sodium pyruvate in Luria-Bertani agar improves the recovery of acid stressed *E. coli.* From the outset of this study, then, sodium pyruvate was added to all growth media used to assess the viability of populations of *E. coli* exposed to growth non-permissive low pH constraints. In hindsight, this may have masked the true influence of the physical structure of growth media on the recovery of *E. coli* injured by low pH.

2.5.2.2 *Composition of the Growth Medium*

Although it is generally accepted that a nutritionally rich medium will facilitate the recovery of bacterial cells injured by chemical or physical means, Leyer and

Johnson (1992) noted a degree of variability in the outcome of experiments concerned with the low pH induced inactivation of *Salmonella* spp. precipitated by the use of two different complex media. Such findings reinforce the notion that assumptions, regarding the suitability of media employed for the enumeration of cells surviving a growth non-permissive low pH challenge, cannot and should not be made. They also illustrate the problems that can be associated with the comparison of data generated by enumeration protocols employing different media.

The viable count data presented in Figure 2.2 reveals little difference in the relative performance of BHA-P, LBA-P, TPA-P and TSA-P employed for the enumeration of *E. coli* surviving a low challenge. By contrast EMB-P performed poorly, a result perhaps not unexpected since EMB is a selective medium. Other selective media including Violet Red Bile Agar (VRBA) and Sorbitol MacConkey Agar (SMA) have previously been shown to be poor media for the recovery of acid stressed *E. coli* (Roth and Keenan, 1971; Przybylski and Witter, 1979; Abdul-Raouf et al., 1993; Silk and Donnelly, 1997). In these instances, however, an increased sensitivity to bile salts has been held responsible for the inability of injured cells to repair injury and subsequently grow (Mackey, 1999). Unlike VRBA and SMA, EMB does not contain bile salts which may imply that this medium is nutritionally inadequate for the repair of acid injured *E. coli* or that such cells exhibit an enhanced sensitivity to eosin and/or methylene blue. In either case, the results presented here suggest that viable cell counts will be underestimated if EMB is employed in the enumeration of acid stressed populations of *E. coli*. In instances where the recovery of such cells is critical, as it is in process development and evaluation in the food industry, this medium should be reserved for confirmatory analysis rather than for detection or enumeration.

2.5.2.3 *Incubation Temperature*

Although temperatures close to those which optimise the growth rate of *E. coli* are often employed for the purpose of enumerating cells surviving growth non-permissive non-thermal environmental challenges, little evidence exists to indicate that such temperatures are optimal for the repair of injured *E. coli*. Recent studies by Presser et al. (1998) and Salter et al. (2000) have indicated that the potential for *E. coli* to resist low pH and low water activity stresses is maximised at temperatures between 25°C and 30°C. As Salter and his colleagues pointed out, this temperature range corresponds to that which was demonstrated by Krist et al. (1998) to maximise the yield of substrate limited cultures of *E. coli*. Declining gradually at temperatures within the normal physiological range and abruptly beyond this range, cell yield has been interpreted as a measure of the metabolic efficiency of bacterial cultures (Krist et al., 1998). Ross (1999) speculated that the influence of temperature on the yield of *E. coli* may be interpreted in terms of an interplay of the effects of temperature on the rate of biochemical reactions and on the conformation and activity of macromolecule(s) essential for growth.

That the resistance of *E. coli* to non-thermal environmental constraints is maximised at temperatures which maximise the metabolic efficiency of this organism is an interesting observation, and one that has fueled speculation that such temperatures might also be optimal for the recovery of *E. coli* injured by exposure to growth non-permissive non-thermal environmental constraints. While Stapleton et al. (1953) presented data to this effect, invoking a hypothesis similar to that presented by Ross (1999) to explain the influence of temperature on the recovery of injured cells, the results presented in Figure 2.3 do not illustrate that the recovery of acid injured *E. coli* is greater at temperatures which maximise the metabolic efficiency of this organism than at temperatures close to those which maximise its rate of growth.

Findings such as this illustrate the difficulties associated with defining what may, or may not, constitute a stress capable of inhibiting the repair of injured cells.

2.5.3 Conclusions

While numerous 'rapid' cytological assays exploiting criteria other than replication as the basis for differentiating viable from non-viable cells have been proposed as means of assessing the viability of microbial populations, culturability remains the most reliable indicator of viability for species such as *E. coli* that do not exhibit a true VBNC state of existence. The notion that viability is synonymous with culturability is therefore appropriate for the purposes of this study. Having said this, culture-based methods of assessing microbial viability are not without potential limitations. Unless steps are taken to ensure that the materials and procedures employed to enumerate viable cells do not introduce secondary stresses that impair the ability of injured cells to repair damage, the viability of populations containing injured cells may be significantly underestimated. In turn, the efficacy of any inimical treatment to which that population is exposed will be overestimated. The process of optimising enumeration protocols must therefore be regarded as a fundamental aspect of studies concerned with the loss of viability of microbial populations exposed to inimical environmental constraints.

Since what constitutes a stress for injured cells can be difficult to define, the materials and procedures employed to enumerate cells surviving growth non-permissive environmental challenges must be evaluated empirically. Data presented in this study illustrate that, in the case of media containing pyruvate, the physical structure of the growth medium employed for the enumeration of *E. coli* surviving a low pH challenge has little influence on the level of recovery achieved. It was also shown that four solid media routinely employed in the study of *E. coli* (BHA-P, LBA-P, TPA-P, and TSA-P) facilitate comparable levels of recovery of *E.*

coli injured by low pH. A fifth solid medium (EMB-P) performed poorly by comparison and should not, therefore, be used to assess the viability of populations of *E. coli* exposed to growth non-permissive low pH constraints. Results presented also show that the recovery of acid injured *E. coli* at a temperature which optimises the metabolic efficiency, but not the growth rate of this organism (25°C), is no greater than that achieved at a temperature close to those which maximise the growth rate of *E. coli* (35°C). This study provides a basis for the selection of protocols employed in subsequent chapters to assess population viability.

CHAPTER 3 CHARACTERISING THE INFLUENCE OF pH STRINGENCY, TEMPERATURE, AND PHYSIOLOGICAL AGE ON THE KINETICS OF LOW PH-INDUCED INACTIVATION

3.1 ABSTRACT

The kinetics of inactivation of *E. coli* populations exposed to inimical low pH treatments, and the influence of pH stringency, temperature, and physiological age on the kinetics of low pH-induced inactivation were characterised. Initial viability datasets generated in this study illustrated that exponential phase populations of *E. coli* M23 exhibit two distinct phases of inactivation – an initial phase of rapid inactivation, whose rate is influenced both by the stringency of the low pH treatment imposed and by temperature, and a second protracted phase of much slower inactivation whose rate is independent of the severity of the lethal agent employed, and of temperature at temperatures ≤ 25°C. Viability datasets obtained subsequently, however, demonstrated the occurrence of a third phase of inactivation. This phase of inactivation was marked by an increase in the rate of inactivation and persisted until the viable count fell below the limit of detection. During the course of investigations designed to evaluate a novel hypothesis concerning the occurrence of the triphasic inactivation kinetics observed, two unanticipated observations were made. The first of these was that exponential phase populations, although typically exhibiting triphasic inactivation kinetics occassionally exhibited atypical patterns of inactivation in which the viable count fluctuated dramatically. It is postulated that the growth of a series of subpopulations, arising from the growth of survivors that acquire novel mutations, may explain the occurrence of such atypical curves. The second unanticipated

observation was that 'identical' populations exposed to identical low pH treatments exhibited considerable variability in their low pH tolerance. Subsequent investigations illustrated that a large proportion of those cells surviving into the second phase of inactivation are residual stationary phase cells and that consequently, the use of 'overnight' inocula for the preparation of exponential phase populations is not sufficiently rigorous to prevent considerable 'day-to-day' variability in the responses of 'identical populations'. New methods designed to improve the comparability of stationary phase inocula, and to rid exponential phase populations of residual stationary phase cells were thus implemented. The low pH tolerance of purely exponential phase populations (as the resulting populations were designated) decreased as physiological age increased. While the low pH tolerance of more mature populations increased with increasing physiological age initially (i.e. amongst populations aged 8-24 hours), it declined amongst populations aged 30-42 hours. Kinetic data pertaining to the influence of phsyiological age on low pH tolerance, and differential scanning calorimetry data pertaining to the influence of physiological age on ribosome stability, imply that a continuum of physiological states exists not only between, but also within, the exponential and stationary phases. The results presented are discussed in terms of their implications for the design, conduct and interpretation of survival studies, and for the development of strategies designed to enhance the microbiological safety of acidic foods.

3.2 INTRODUCTION

Early studies concerned with the loss of viability of microbial populations illustrated that the logarithm of the number of viable cells exposed to an inimical treatment decreased, as a function of time, in a linear manner (Krönig and Paul, 1897 cited by Chick, 1908; Madsen and Nyman, 1907 cited by Withell, 1942; Chick,

1908; Chick, 1910; Lee and Gilbert, 1918; Cohen, 1922; Watkins and Winslow, 1932; Hollaender et al., 1951). Consequently, log linear inactivation kinetics described mathematically by the expression $N = N_0e^{-kt}$ (where N_0 is the initial number of viable microbes, N is the number of survivors, t is the time of exposure and k is a rate constant) were employed widely to determine the comparative resistance of microbial populations to lethal agents, and as a basis for the development and evaluation of sterilisation procedures employed both in the food processing industry and the medical profession (Ball and Olson, 1955; Vas and Proszt, 1957; Jawetz et al., 1958; Stumbo, 1973; Cerf, 1977). As the study of microbial inactivation progressed, however, deviations from the logarithmic order of death were frequently noted (Falk and Winslow, 1926; Rahn, 1930; Jordan et al., 1947; Vas and Proszt, 1957; Jacobs, 1960; Sykes, 1963; Moats et al., 1971; Skinner and Hugo, 1976). Now, just over one hundred years since the kinetics of microbial inactivation were first investigated, the literature is replete with log survivor-time plots defining curvilinear (concave or convex), biphasic (with a distinct shoulder or tail), or more complex patterns of inactivation. An examination of the literature, however, yields no consistent association between either the lethal agent employed or the microorganism examined, and the kinetics observed. Three factors have undoubtedly contributed to this apparent inconsistency, particularly in relation to the kinetics of inactivation of *E. coli* by non-thermal means: a lack of systematic research concerning kinetic responses to specific non-thermal environmental constraints; the fragmentary/sparse nature of many kinetic datasets; and a lack of methodological consistency.

McClure and Hall (2000) suggest that in some respects the situation concerning study of the survival of *E. coli* is analogous to the situation some years ago for growth. They explain that much of the published work focuses on the survival of pathogenic strains, and in particular O157:H7, in foods with few studies attempting

to systematically identify, describe and characterise individual factors that determine the fate of this organism or its non-pathogenic counterparts. Moreover, many of the studies that have identified those parameters which determine the fate of *E. coli* fail to illustrate the manner in which they influence the time-dependent loss of viability (i.e. the kinetics of inactivation). Instead of generating multiple log survivor-time plots many investigators studying the low pH-tolerance of *E. coli* (e.g. Raja et al., 1991a; Gorden and Small, 1993; Arnold and Kaspar, 1995; Benjamin and Datta; 1995; Lin et al., 1995; Cheng and Kaspar, 1998; Rowbury and Goodson, 1998; Wang and Doyle, 1998; Datta and Benjamin, 1999; Diez-Gonzalez and Russell, 1999) have adopted methods in which the relative viability of control and test cultures exposed to an inimical low pH challenge of fixed duration is regarded as an indicator of the significance of the test parameter in determining the low pH tolerance of *E. coli*. While this type of comparative study has obvious advantages over systematic kinetic studies, which have often been put in the 'too hard' category ".... owing to the labour and length of time that would be occupied by any one set of experiments" (Chick, 1908), it yields little information concerning the process, or the mechanism(s), of inactivation.

As previously mentioned (see Table 1.1), the low pH tolerance of *E. coli* is determined not only by inherent genotypic peculiarities but also by a number of physiological and physicochemical parameters. In studies where kinetic data pertaining to the low pH-induced loss of viability of *E. coli* has been generated, often the data is scanty or a lack of methodological consistency between laboratories renders the comparison of data from different studies inappropriate. Consequently, knowledge of the kinetics of the low pH-induced loss of viability of *E. coli* is poor. In fact, information concerning the kinetics of inactivation of populations of *E. coli* exposed to any non-thermal, inimical physicochemical constraint, is limited (Ross and Shadbolt, 2001). Peleg and Cole (1998) point out

that to compare the equivalence of different processing regimes accurately one must be able to reliably estimate the effect of different processing parameters on the numbers of dead, or surviving, microorganisms. Without a knowledge of the kinetics of the non-thermal inactivation of foodborne microorganisms of public health significance, and of the manner in which they are influenced by physiological and physicochemical parameters, the food industry's ability to refine existing and/or develop new processing strategies designed to ensure minimally processed yet safe products will be limited. The development of an appreciation of the mechanism(s) whereby low pH effects microbial inactivation will, likewise, be hampered by a lack of data describing the process(es) of low pH-induced inactivation.

The work presented in this chapter was thus undertaken with the aim of expanding knowledge of the time-dependent low pH-induced loss of viability of *E. coli*, of characterising the influence of pH stringency, temperature and growth phase on the kinetics of low pH-induced inactivation, and of gaining insights into the mechanism(s) whereby the inactivation of *E. coli* is effected by low pH.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Strains, Bacteriological Media, Chemical Reagents & Equipment

Details of the bacterial strains, bacteriological media, chemical reagents and equipment employed throughout this study, together with the methods employed for bacterial maintenance and recovery, are presented in Appendix A.

3.3.2 General Methods

3.3.2.1 *Low pH Treatments and the Construction of Survival Curves*

Populations of *E. coli* were exposed to growth non-permissive low pH treatments by direct acidification of the growth medium with 10M HCl. The pH of each culture was rapidly (within 1 minute) adjusted to the desired value, and the cultures were subsequently incubated in oscillating waterbaths maintained at the temperatures specified. Immediately prior to, and at regular intervals throughout the low pH treatment, the viability of each population was estimated. Samples were withdrawn from the experimental populations, serially diluted in 0.85% sodium chloride dissolved in 0.1% peptone water, and surface plated to TPA-P with a spiral plater. When it was anticipated that the viable count of a population would be < 3 log cfu.ml^{-1}, four 250 µl volumes of undiluted sample were plated. After incubating the plates at 37°C for 14 ± 2 hours an image scanner and analysis software (CASBA™4) were employed to enumerate the colony forming units recovered from each sample. Survival curves were constructed by plotting the logarithm of the number of colony forming units recovered per milliliter against time. For convenience the logarithm of a non-detectable number of survivors was plotted as zero, which equates to < 1 cfu.ml^{-1}.

3.3.3 An Analysis of the Kinetics of the Low pH-Induced Inactivation of Exponential Phase Populations of *E. coli*

Three exponential phase populations[1] (2.3.2.1) of *E. coli* M23 were exposed to an

[1] When used without clarification the phrase 'exponential phase' is used to describe populations prepared by incubating dilute [1:1000] stationary phase cultures at 25°C until viable counts of *ca.* 1 x 10^{8} cfu.ml^{-1} were attained. These populations are also described as 1^{0}Exp populations for clarity.

inimical low pH treatment [pH 3.0, 25°C] (3.3.2.1). The viability of each population was assessed by culture-based methods immediately prior to, and at regular intervals throughout, the low pH treatment (3.3.2.1).

3.3.4 Characterising the Influence of pH Stringency and Temperature on the Kinetics of Low pH-Induced Inactivation

Duplicate exponential phase populations (2.3.2.1) of *E. coli* M23 exposed to one of three growth non-permissive low pH treatments [pH 3.5, 3.0, or 2.5] (3.3.2.1) were incubated in oscillating waterbaths maintained at 4°C, 15°C, 25°C, 35°C, 45°C and 50°C. Culture-based methods of assessing microbial viability were employed to monitor population viability throughout the low pH treatments (3.3.2.1) and Ultrafit 3.0 software was employed to fit a model (the 'Death Curve Model') describing a two phase concave reduction in log numbers to the viability datasets obtained. The fitted parameters (A and B) of the 'Death Curve Model' were utilised as objective estimates of the rates of inactivation. They were also employed to generate estimates of the duration of the first phase of inactivation and of the loss of viability observed during this phase. This model is described in Appendix B.

3.3.5 Examining the Possibility of an Injury-Recovery Phenomenon in Populations of *E. coli* Exposed to Inimical Low pH Treatments

Duplicate exponential phase populations (2.3.2.1) of *E. coli* M23 were exposed to an inimical low pH treatment [pH 3.5, 25°C] (3.3.2.1). Immediately prior to, and at regular intervals throughout, the low pH treatment samples [0.1-1ml] were withdrawn from each population, serially diluted in 0.85% sodium chloride dissolved in 0.1% peptone water, and surface plated to both TPA-P and TPA containing 0.15% Oxoid Bile Salts No.3 (NuBile). The plates were incubated at 37°C

for 14 ± 2 hours and the colony forming units recovered on each media type were enumerated with an image scanner and analysis software (CASBA™4). Differences in the viable counts achieved on the two media types were regarded as measures of the proportion of each population sustaining membrane-associated injuries.

3.3.6 Investigating the Possibility that Exponential Phase Populations Contain Residual Stationary Phase Cells

An 8 ml volume of TSB contained in a 15 ml sterile plastic tube was inoculated from a 14 hour plate culture of *E. coli* M23 and incubated statically for 12 hours at 37°C. An exponential phase population with a viable count of *ca.* 1×10^8 cfu.ml^{-1} was subsequently prepared by diluting the 12 hour broth culture 1:1000 in fresh TSB and incubating for 5.5 hours in an oscillating waterbath maintained at 25°C. A small volume of this exponential phase culture, designated the primary exponential phase population (1^0Exp), was employed as the inoculum for a second exponential phase population (2^0Exp). The 2^0Exp population was prepared by diluting 60 µl of the 1^0Exp population 1:1000 in fresh TSB and incubating the culture thus prepared in an oscillating waterbath maintained at 25°C until a viable count of *ca.* 1×10^8 cfu.ml^{-1} was attained. In an analogous manner the 2^0Exp population served as an inoculum for a third exponential phase population (3^0Exp), and the 3^0Exp population as an inoculum for a fourth exponential phase population (4^0Exp). In the latter instance, however, parallel exponential phase populations were prepared, one being incubated until a viable count of *ca.* 1×10^7 cfu.ml^{-1} was attained (4^0Exp-LD[1]) and the other until a viable count of *ca.* 1×10^8 cfu.ml^{-1} was attained (4^0Exp). All five of the populations were exposed to a low pH treatment [pH 3.5, 25°C] and

[1] LD designates low population density compared to the 4^0Exp population.

survival curves depicting the time-dependent loss of viability that occurred in each population were constructed (3.3.2.1).

3.3.7 Characterising the Influence of Physiological Age on the Kinetics of Low pH-Induced Inactivation

Primary (1^0Exp) and secondary (2^0Exp) exponential phase populations of *E. coli* M23 were prepared as described above (3.3.6). A series of cultures that differed in their 'physiological age'[1], and in some instances their cell density, was subsequently prepared incubating dilute [1:1000 in fresh TSB] 2^0Exp cultures in an oscillating waterbath maintained at 25°C for periods of 4, 6, 7, 8, 10, 12, 15, 24, 36, or 42 hours. Each experimental population was exposed to a low pH treatment [pH 3.5, 25°C] and the time-dependent loss of viability that resulted was determined by culture-based methods (3.3.2.1). Two models were fitted to the datasets obtained-the 'Death Curve Model' to datasets describing two phases of inactivation and the 'Three Phase Death Model' to datasets describing three phases of inactivation. The models were fitted using Ultrafit 3.0 software and the fitted parameters of the models employed (A & B in the case of the 'Death Curve Model' and A, B and K in the case of the 'Three Phase Death Model') were utilised as objective estimates of the rates of inactivation. The fitted paramters N_o, N_l and I were employed to generate estimates of the duration of the first, second, and third phases of inactivation, and of the loss of viability that occurred during these phases. The 'Three Phase Death Model' is described, and its use is exemplified, in Appendix B. It should also be noted that for populations displaying only two phases of

[1] The term 'physiological age' was employed to describe that period of time between the dilution of a 2^0Exp population and the low pH treatment of individual populations prepared by incubating the dilute 2^0Exp populations at 25°C.

inactivation, the duration of the second phase and the loss of viability that occurred during this phase were estimated by extrapolating that line fitted through the second phase to the x-axis (i.e. to 1 cfu.ml^{-1}).

3.3.8 Growth and its Influence on the pH of the Culture Medium

Primary and secondary exponential phase populations of *E. coli* M23 were prepared as previously described (3.3.6). A dilute [1:1000 in fresh TSB] 2^{0}Exp culture was subsequently prepared, and this culture was incubated at 25°C. By determining the viable count of this culture at regular intervals, its growth was monitored. For viable count determinations the enumeration protocol described in section 3.3.2.1 was employed. At each sample time, an additional volume of the culture was withdrawn and transferred to a sterile well plate for pH determination.

3.3.9 Determining the Influence of Physiological Age on Ribosomal Stability

Primary and secondary exponential phase populations of *E. coli* M23 were prepared as previously described (3.3.6). An experimental population was then prepared by diluting the 2^{0}Exp population 1:1000 in fresh TSB and incubating the culture thus prepared in an oscillating waterbath maintained at 25°C. At regular intervals the optical density [OD_{450}] and the viable count of the population was determined, the latter by means of the enumeration protocol described in section 3.3.2.1. Once the optical density of the population reached 0.1, the thermal stability of the ribosomes of cells withdrawn at regular intervals was examined by differential scanning calorimetry (DSC). For this purpose cells were collected by centrifugation (12,000 *g* for 4 minutes), the sample volume being adjusted such that centrifugation yielded cell pellets of approximately the same size despite the culture density, and resuspended in a minimum volume of the supernatant. The resulting suspensions

(maximum volume 20 µl) were sealed in pre-weighed aluminium DSC sample pans. DSC thermograms were obtained using a Perkin-Elmer DSC 7 differential scanning calorimeter, the data being acquired and the calorimeter controlled by a Perkin-Elmer 3600 data station. The dry box and DSC head were purged with oxygen-free nitrogen and the calorimeter was calibrated with indium metal (99.99% pure; m.p. = 156.6°C and $\Delta H = 28.45\ J.g^{-1}$) at a scan rate of 10°C min^{-1}. Thermograms were generated during sample heating (from 30°C to 110°C at 10°C min^{-1}), and an empty pan was used as a reference. After the initial analysis, samples were rapidly cooled in the machine and re-scanned. The second thermogram was subtracted from the first to remove consistent baseline fluctuations. This process also removed a broad shallow peak at 55-80°C which corresponded to reversible RNA melting (Mackey et al., 1991).

3.4 RESULTS

3.4.1 Kinetics of the Low pH-Induced Inactivation of Exponential Phase Populations of *E. coli*

The survival curves presented in Figure 3.1 illustrate loss of viability in three exponential phase populations of *E. coli* M23 during exposure to an inimical low pH treatment [pH 3.0, 25°C]. In each population an initial phase of rapid decline in viability (Phase 1, P1) was followed by a phase of much slower decline (Phase 2, P2) that persisted for extended periods. These inactivation kinetics are described as biphasic, and hereafter are characterised in terms of the rates of inactivation (R1 and R2 signifying the rates of inactivation observed during Phase 1 and Phase 2 respectively), the loss of viability or the magnitude of the kill observed during Phase 1 (K1), and the duration of Phase 1 (D1).

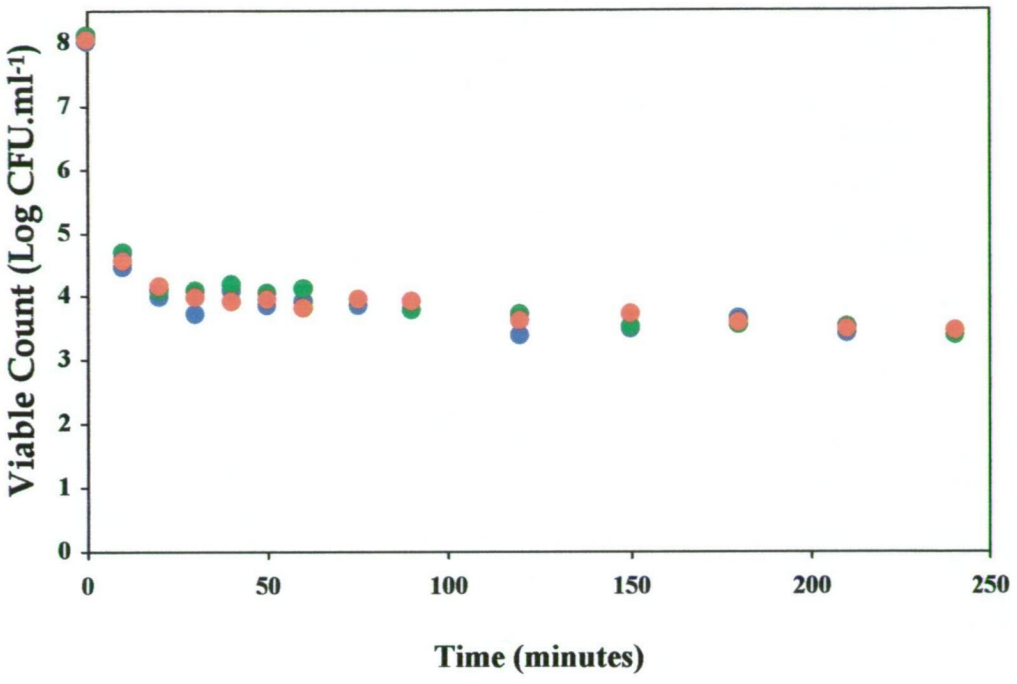


Figure 3.1 Loss of viability in exponential phase populations of *E. coli* M23 exposed to an inimical low pH treatment [pH 3, 25°C]; Rep1 (●), Rep2 (●), and Rep 3 (●).

3.4.2 Influence of pH Stringency and Temperature on the Kinetics of Low pH-Induced Inactivation

Figure 3.2 depicts the time-dependent loss of viability of exponential phase populations of *E. coli* M23 exposed to inimical low pH treatments of varying pH stringency [pH 3.5, 3.0, and 2.5 at 25°C]. It illustrates the influence of pH stringency on the kinetics of the low pH-induced inactivation of *E. coli*. Figure 3.3, on the other hand, illustrates the time-dependent loss of viability of exponential phase populations of *E. coli* M23 exposed to an inimical low pH treatment of fixed pH stringency [pH 2.5] at 4°C, 15°C, 35°C, 45°C and 50°C. It exemplifies the influence of temperature on the kinetics of the low pH-induced inactivation of *E. coli*, and illustrates that the first two phases of inactivation may be followed by a third phase in which the viable count declines rapidly below the limit of detection.

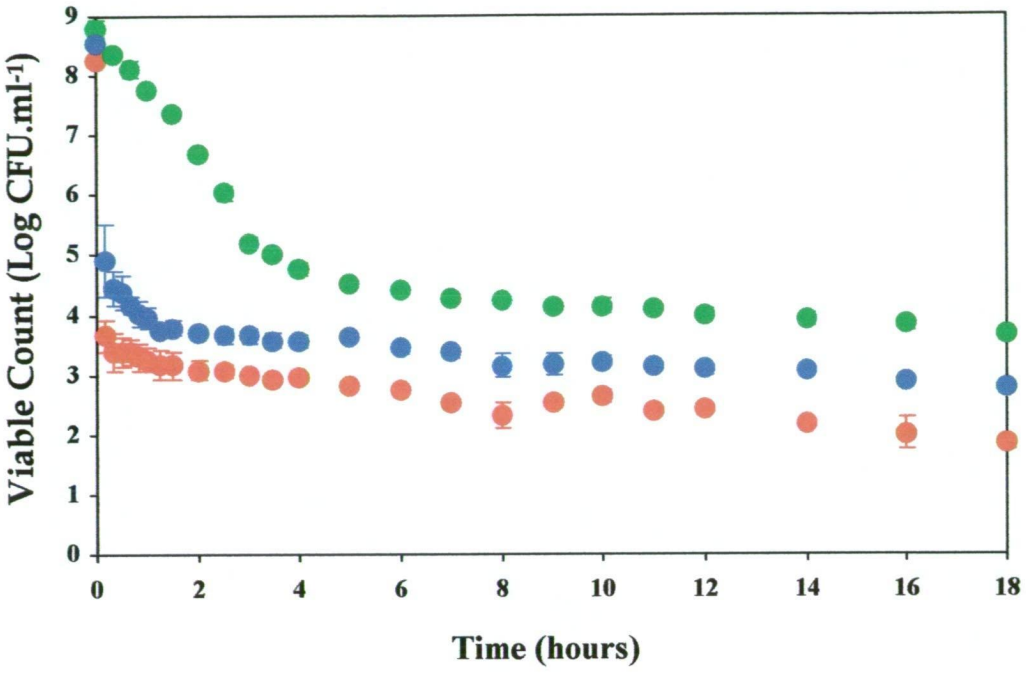


Figure 3.2 Loss of viability in exponential phase populations of *E. coli* M23 exposed to low pH treatments of varying stringency - pH 3.5 (●), pH 3.0 (●), and pH 2.5 (●) - at 25°C [mean ± SD; n = 2].

A summary of inactivation characteristics (R1, R2, D1 and K1) derived from individual datasets describing the loss of viability of exponential phase populations of *E. coli* for each variable combination of pH and temperature is presented in Figure 3.4. The data shown are mean values obtained from studies performed in duplicate, and they illustrate several trends. Firstly, an increase in the stringency of the lethal agent employed (i.e. pH) precipitated an increase in the rate of inactivation that occurred during P1. pH-mediated increases in R1 were greater for half unit decreases in pH from 3.5 to 3.0 than from 3.0 to 2.5. By contrast with R1, R2 was largely independent of the stringency of the lethal agent employed. The magnitude of the decline in population viability that occurred during P1 was also influenced little by the pH, although this characterisic increased with decreasing pH at temperatures above 35°C. D1, the duration of the first phase of inactivation,

decreased as the stringency of the lethal agent increased and as with R1, pH-mediated shifts in this characteristic were more pronounced for half unit shifts in pH from 3.5 to 3.0 than from 3.0 to 2.5.

Like pH, temperature influenced the rate of inactivation that occurred during P1. In general, R1 increased with temperature although the relationship between the two was not linear. The magnitude of the increase in R1 effected by a ten degree temperature shift increased, in most instances, with increasing pH. The shift from 4°C to 50°C thus resulted in a 10-fold increase in R1 at pH 2.5, a 24-fold increase in R1 at pH 3.0, and a 103-fold increase in R1 at pH 3.5. By contrast with R1, R2 was influenced noticably by temperature only at temperatures exceeding 25°C. Above this temperature R2 increased rapidly with temperature. K1, which increased with temperature when it exceeded 35°C, was independent of both temperature and pH

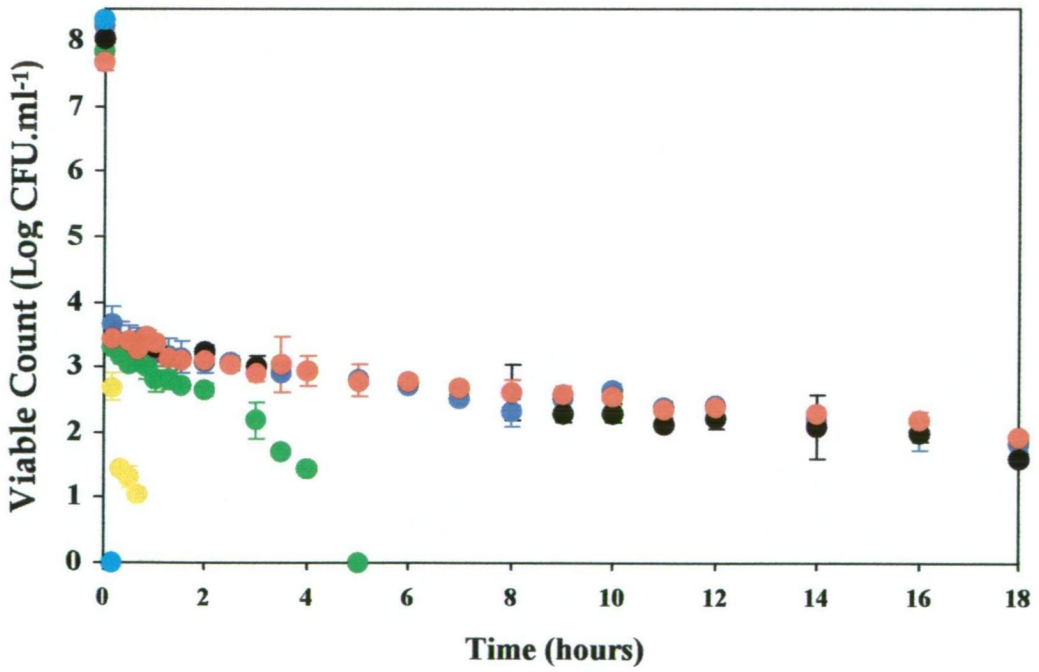


Figure 3.3 Loss of viability in exponential phase populations of *E. coli* M23 exposed to an inimical low pH treatment [pH 2.5] at 4°C (●), 15°C (●), 25°C (●), 35°C (●), 45°C (●), and 50°C (●) [mean ± SD; n = 2].

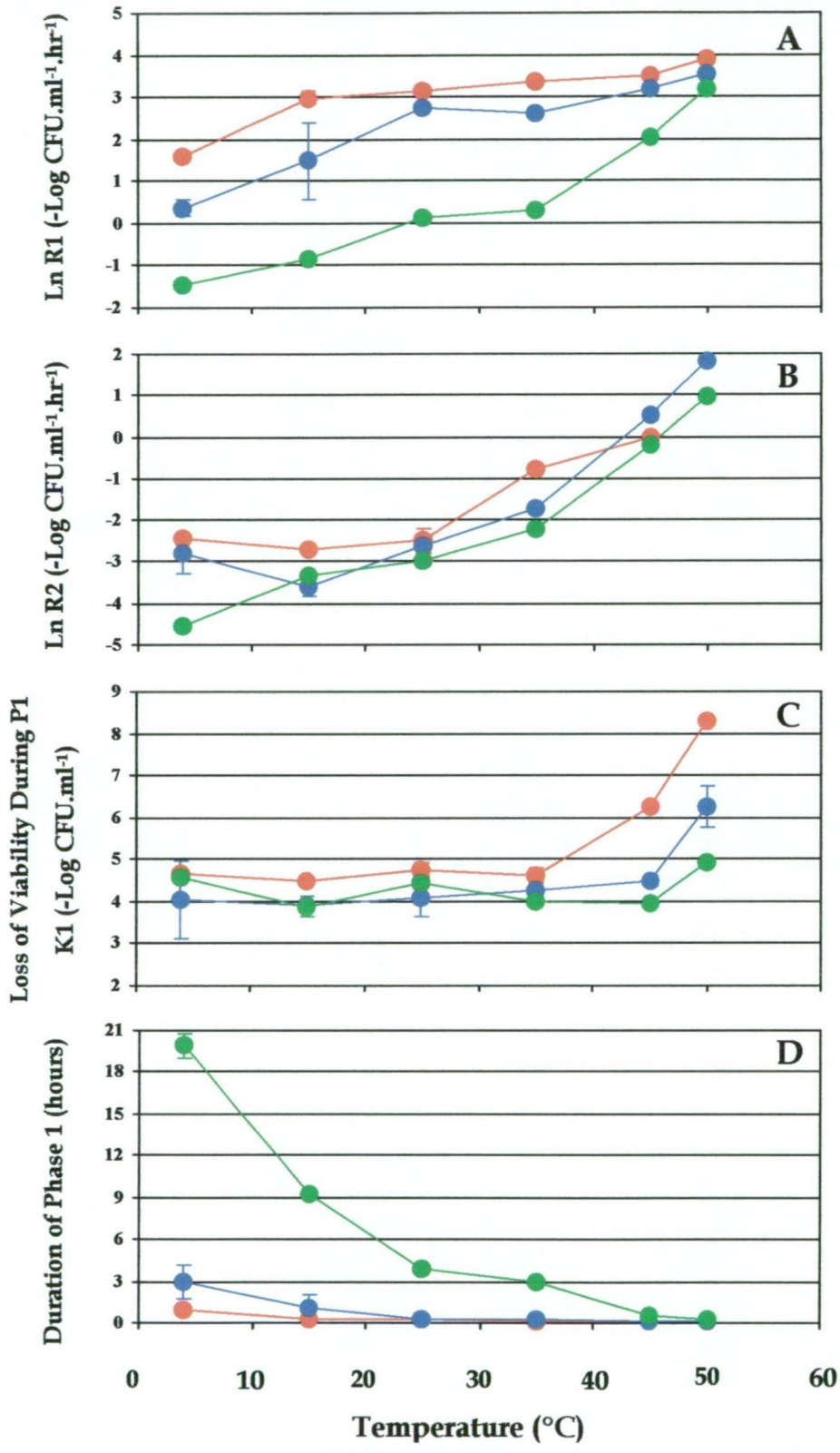


Figure 3.4 Influence of temperature and pH stringency on the rate of inactivation during phase 1 (A) and phase 2 (B), the loss of viability during phase 1 (C), and the duration of phase 1 (D) exhibited by exponential phase populations of *E. coli* M23 exposed to low pH - pH 3.5 (●), 3.0 (●), and 2.5 (●) [mean ± SD; n=2].

at temperatures below 35°C. By contrast, D1 was influenced by temperature across the entire range employed. This inactivation characteristic decreased with increasing temperature, and this trend was more pronounced when the stringency of the lethal agent was low than when it was high.

3.4.3 Injury in Populations of *E. coli* Exposed to Inimical Low pH Treatments

Figure 3.5 and Figure 3.6 illustrate the counts obtained when viable cells withdrawn from exponential phase populations of *E. coli* M23 (designated population A and population B) exposed to an inimical low pH treatment [pH 3.5, 25°C] were enumerated on TPA-P and TPA containing bile salts (NuBile). The kinetics of the inactivation of population A were, as expected, triphasic (Figure 3.5). During the first phase of the inactivation of this population, equal numbers of cells

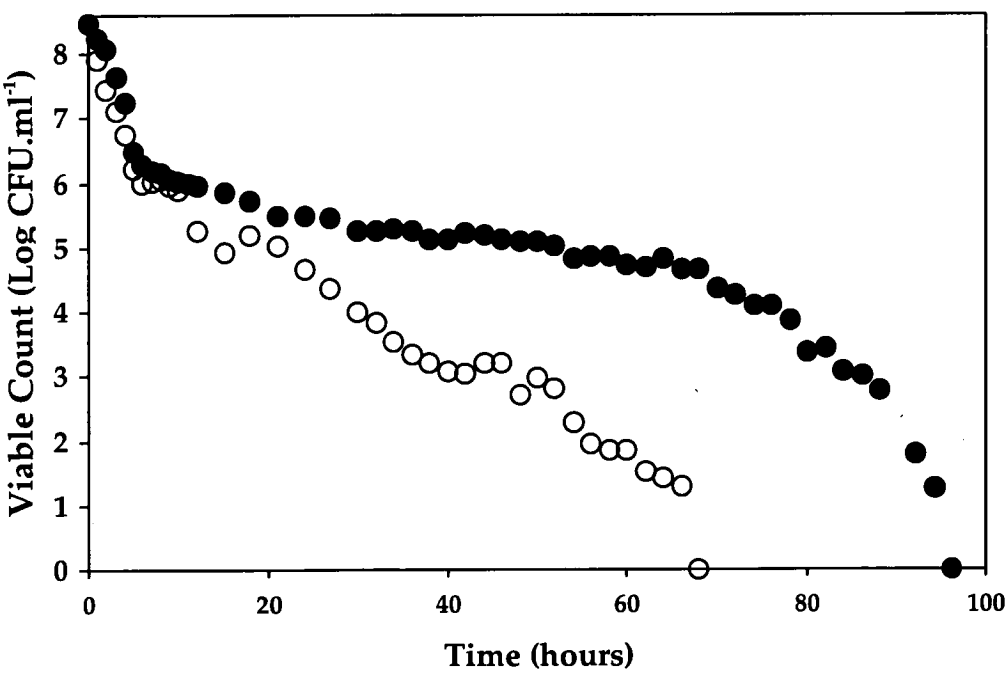


Figure 3.5 Counts obtained when viable cells withdrawn from an exponential phase population of *E. coli* M23 (designated population A) exposed to an inimical low pH treatment [pH 3.5, 25°C] were enumerated on TPA-P (●) or NuBile (○).

were recovered on TPA-P and Nubile. During the second phase of inactivation, however, a disparity emerged with the number of cells recovered on NuBile dropping below the number recovered on TPA-P. The difference in the viable counts afforded by the two media increased with the duration of the low pH treatment, and by the onset of the third phase of inactivation no viable cells were recovered on NuBile. By contrast with population A, population B behaved atypically (Figure 3.6). Upon exposure to low pH the viable count of this population declined for a period of about forty hours. Thereafter the viable count fluctuated. Fluctuations were also evident in the counts obtained by the enumeration of viable cells on NuBile. No consistent difference, or trend in the difference (other than that recovery on NuBile was always lower), in the viable counts afforded by the two media was apparent in this instance.

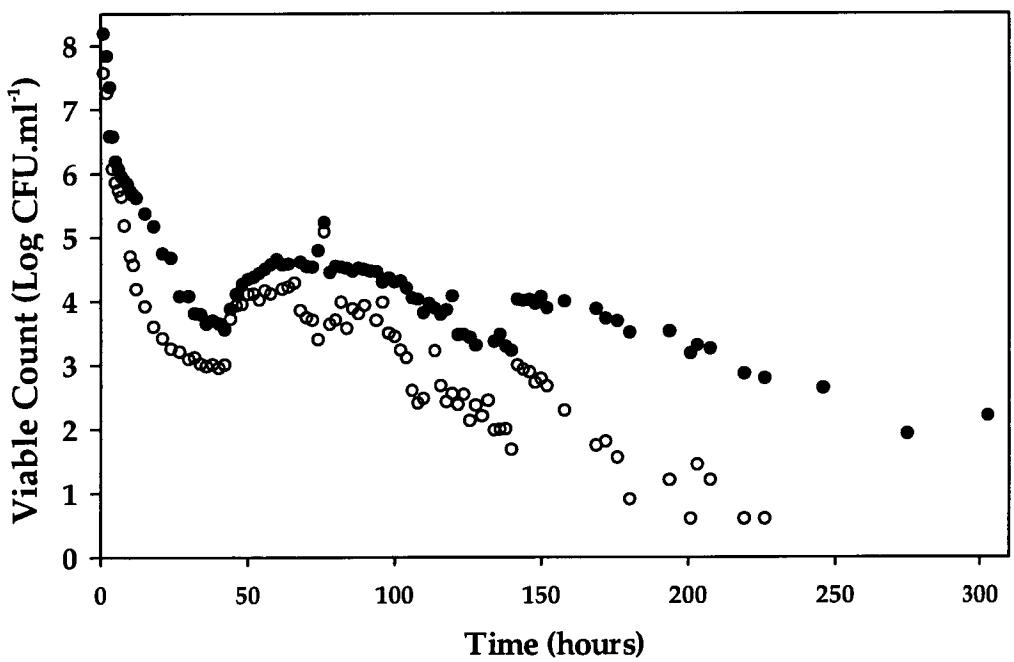


Figure 3.6 Counts obtained when viable cells withdrawn from an exponential phase population of *E. coli* M23 (designated population B) exposed to an inimical low pH treatment [pH 3.5, 25°C] were enumerated on TPA-P (●) or NuBile (O).

3.4.4 Investigations of the Possibility that Exponential Phase Populations Contain Residual Stationary Phase Cells

The loss of viability of 1^0Exp, 2^0Exp, 3^0Exp, 4^0Exp and 4^0Exp-LD populations of *E. coli* M23, induced by an inimical low pH treatment [pH 3.5, 25°C], is illustrated in Figure 3.7. The kinetics of inactivation of the 1^0Exp population were clearly triphasic. During the first hour of low pH exposure the viable count of this population declined dramatically- from *ca.* 1.1×10^8 cfu.ml^{-1} to *ca.* 2.8×10^4 cfu.ml^{-1}. For a period of about sixty hours thereafter the viability of the 1^0Exp population declined at a much slower rate. A third phase of inactivation characterised by an increase in the rate of loss of viability followed, and continued until the viable count of the 1^0Exp population dropped below the limit of detection. The kinetics of inactivation of the 2^0Exp population were also triphasic. The loss of viability that

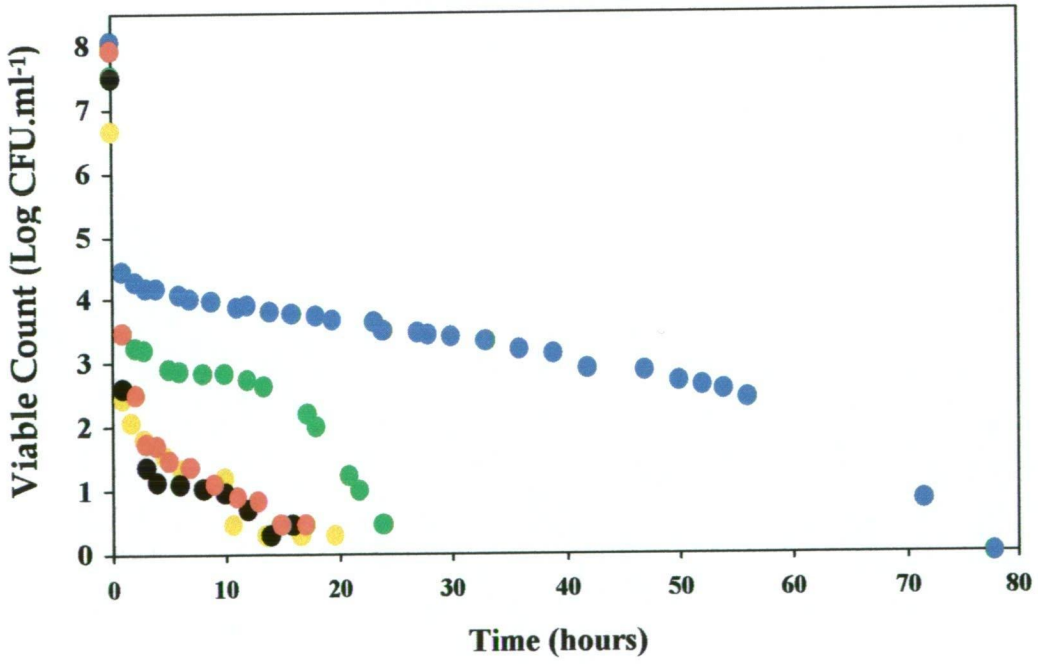


Figure 3.7 Loss of viability in 1^0Exp (●), 2^0Exp (●), 3^0Exp (●), 4^0Exp (●), and 4^0Exp-LD (●) populations of *E. coli* M23 exposed to an inimical low pH treatment [pH 3.5, 25°C]. See text for details.

occurred in this population during P1 was an order of magnitude greater, however, than that which occurred in the 1^0Exp population during the same phase. Furthermore, the onset of the third phase of inactivation occurred much (approximately 45 hours) earlier in the 2^0Exp population than in the 1^0Exp population. The viable count of the 2^0Exp population thus declined below the limits of detection over a much shorter time-frame than that of the 1^0Exp population. By contrast with the kinetics of inactivation exhibited by the 1^0Exp and 2^0Exp populations, the kinetics of inactivation of the 3^0Exp, 4^0Exp and 4^0Exp-LD populations were biphasic in appearance. While the loss of viability which occurred in the 3^0Exp and 4^0Exp populations during P1 exceeded that which occurred in the 1^0Exp population by two-to-three orders of magnitude, the loss of viability in the 4^0Exp-LD population exceeded that of the 1^0Exp by only one-to two orders of magnitude. Thus, viable counts in the 3^0Exp, 4^0Exp and 4^0Exp-LD populations at the onset of phase two were similar.

3.4.5 Influence of Physiological Age on the Kinetics of Low pH-Induced Inactivation of *E. coli*

Eight populations of *E. coli* M23 that differed in their physiological age and their cell density were prepared by incubating dilute (1:1000 in fresh TSB) 2^0Exp phase cultures of the same strain at 25°C for periods of 4, 6, 7, 8, 10, 12, 15 and 24 hours. The time-dependent loss of viability that occurred in these populations as a result of exposure to low pH [pH 3.5, 25°C] is illustrated in Figure 3.8. Tertiary exponential phase populations with viable counts $\leq 1 \times 10^8$ cfu.ml^{-1} (i.e. the 4, 6, and 7 hour cultures) exhibited two distinct phases of inactivation while each of the more mature cultures exhibited triphasic inactivation kinetics. No increase in the 'overall' low pH tolerance of the cultures aged 4, 6 or 7 hours was observed, but the low pH tolerance of populations aged 8-24 hours increased with their physiological

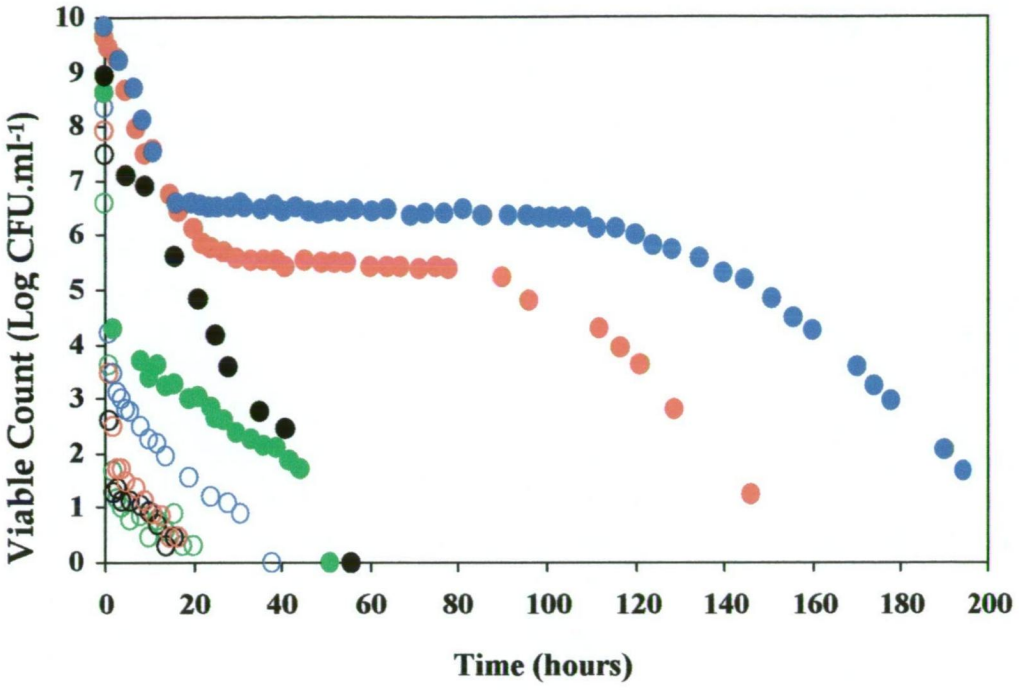


Figure 3.8 Low pH-induced loss of viability in eight populations of *E. coli* M23 that differed in their physiological age at the onset of a low pH treatment [pH 3.5, 25°C]. Populations were prepared by incubating dilute 2^0Exp phase cultures at 25°C for periods of 4 (○), 6 (○), 7 (○), 8 (○), 10 (●), 12 (●), 15 (●) and 24 (●) hours.

age. The low pH-induced loss of viability of two additional cultures, one prepared by incubating a dilute 2^0Exp culture at 25°C for 36 hours and another by incubating a dilute 2^0Exp culture at 25°C for 42 hours, is illustrated in Figure 3.9. These cultures exhibited a greater degree of sensitivity to the low pH constraints imposed than did the 24 hour culture.

For each culture prepared and challenged, those characteristics employed to described the kinetics of inactivation (R1, R2 and R3 signifying the rates of inactivation observed during P1, P2 and P3; D1, D2 and D3 the duration of the three phases of inactivation; and K1, K2 and K3 the loss of viability of kill observed during each of the phases) were estimated. Scatter plots illustrating the manner in which these characteristics varied with the physiological age of the cultures

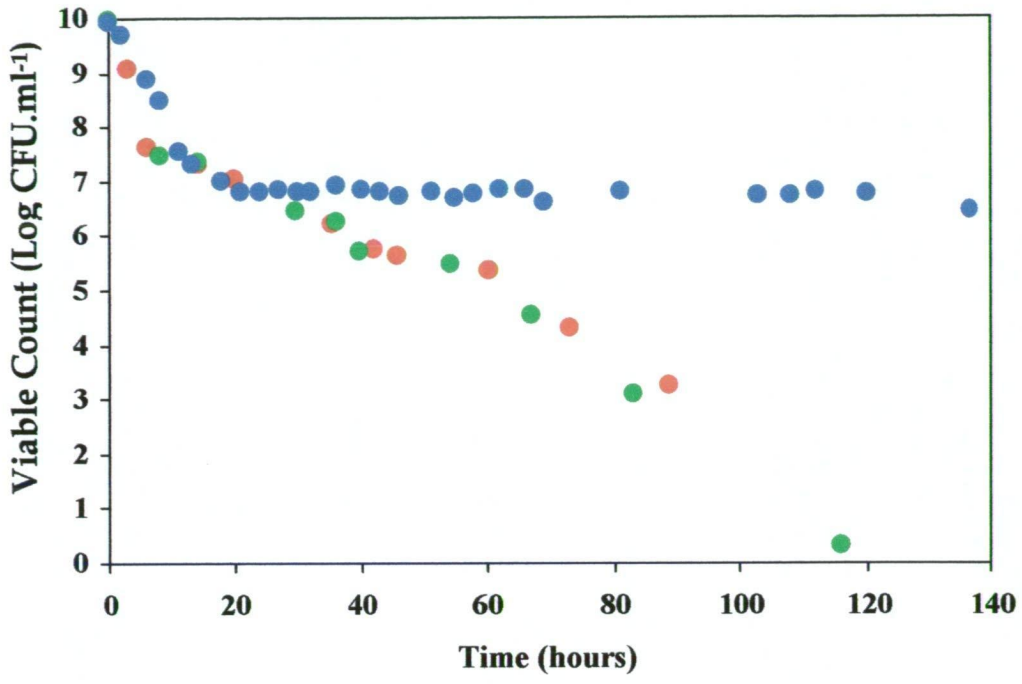


Figure 3.9 Loss of viability of populations of *E. coli* M23 prepared by incubating dilute 2^0Exp cultures of the same at 25°C for 24 (●), 36 (●), or 42 (●) hours, upon exposure to low pH [pH 3.5, 25°C].

prepared are presented in Figure 3.10. A number of these characteristics did not vary in a continuous manner. R1 increased as the cell density of populations aged 4-7 hours increased, before declining markedly with the physiological age of cultures aged 8-15 hours. Little difference was apparent in the value of this inactivation characteristic amongst populations whose physiological age was ≥ 15 hours. D1, which was remarkably uniform amongst cultures aged 4-12 hours, increased dramatically in the 15 hour culture but then declined as the physiological age of the cultures examined increased further. K1 increased with the physiological age of cultures aged 4-7 hours, before declining continuously in more mature cultures. R2 appeared to decrease with physiological age (although this is not evident in Figure 3.9), while D2 increased with the physiological age of cultures aged 4-24 hours then declined in more mature cultures. Amongst those cultures in

which a third phase of inactivation was observed, R3 decreased with physiological age in cultures aged 8-12 hours. In older cultures no further decrease, or change, in this characteristic was observed. D3 increased with increasing physiological age in cultures aged 8-30 hours before declining in more mature cultures, as did K3. These trends are illustrated in the scatter plots presented in Figure 3.10.

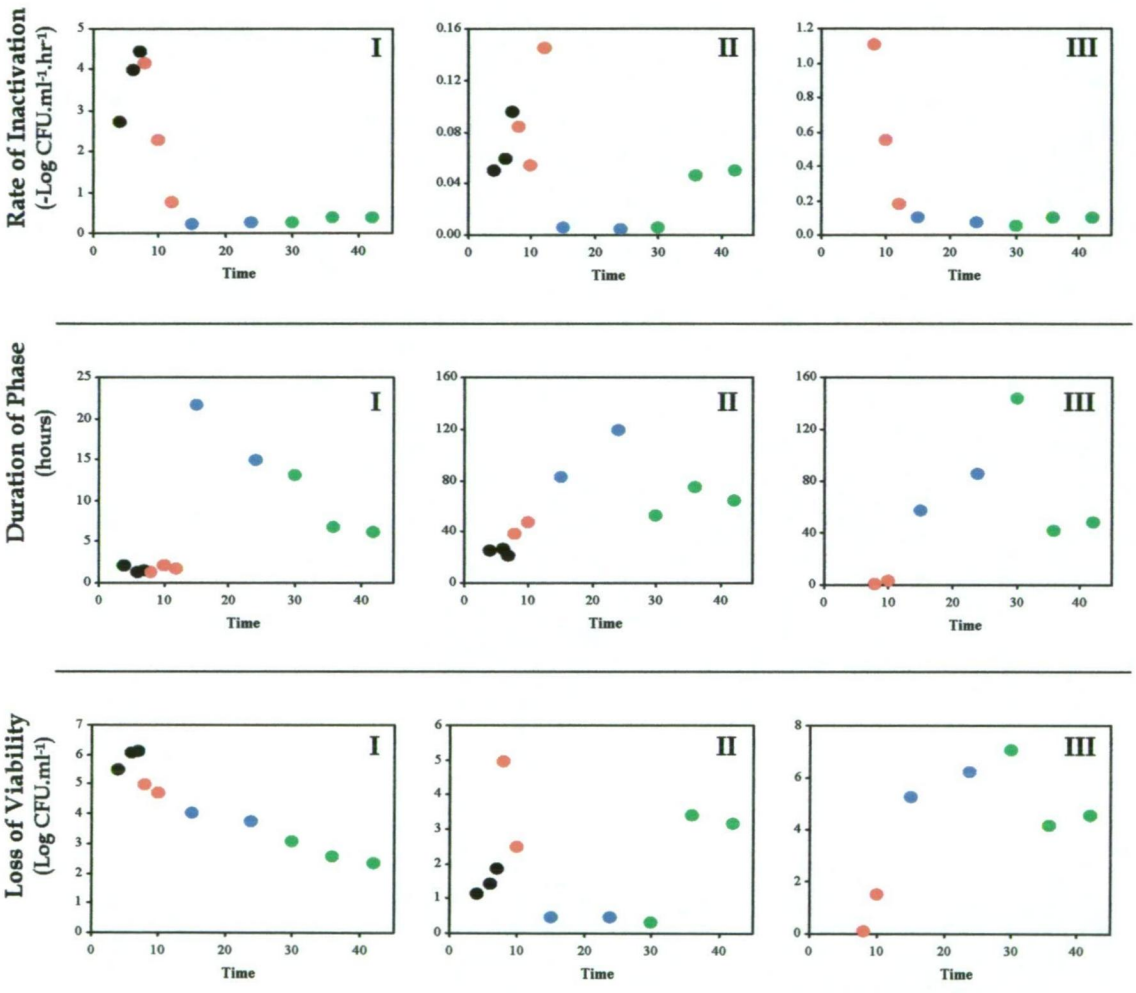


Figure 3.10 Scatter plots illustrating correlations between 'Physiological Age' (i.e. period of incubation since dilution) and those characteristics employed to describe the kinetics of low pH-induced inactivation: the rate of inactivation, phase duration, and loss of viability associated with Phase 1 (I), Phase 2 (II) and Phase 3 (III). Values were derived from the viability datasets obtained for cultures prepared by incubating dilute 2^0Exp cultures for 4-7 hours (●), 8-12 hours (●), 15-24 hours (●), and > 24 hours (●).

3.4.6 Growth and its Influence on the pH of the Culture Medium

Figure 3.11 illustrates the growth (i.e. increase in the viable count) of a dilute 2^{0}Exp phase culture during incubation at 25°C, and the corresponding changes effected in the pH of the culture medium. During the first seven hours of incubation the population grew exponentially. Although the viable count continued to increase for an additional eight hours, the growth rate of the population declined. Fifteen hours after incubation was commenced the viable count of the population began to stabilise at *ca.* 1 x 10^{10} cfu.ml^{-1}. No decline in the viable count of the population was observed before the experiment concluded. The pH of the culture medium, which was 7.0 at the time of inoculation, declined gradually during the first phase of rapid growth then rapidly after the onset of the second slower phase of growth. It reached a minimum value of 6.0 after a twelve hour incubation period then rose rapidly to reach a value of 7.9 after a further twelve hour incubation period.

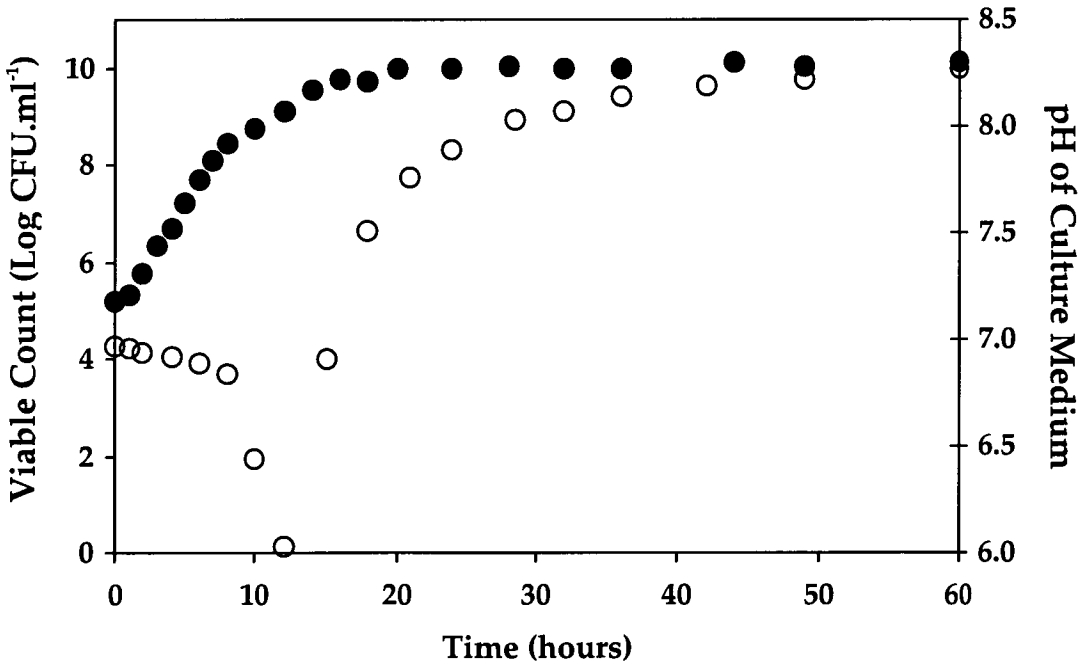


Figure 3.11 Changes in the viable count (●) and pH (○) of a dilute 2^{0}Exp culture during incubation at 25°C.

Beyond this time the pH of the culture medium continued to rise slowly reaching a value of 8.3 at the time monitoring ceased.

3.4.7 Physiological Age and its Influence on Ribosomal Stability

DSC thermograms derived from whole cell suspensions yielded evidence of the occurrence of multiple endothermic events across a temperature range of 50°C to 90°C (Figure 3.12). The thermograms were dominated, however, by a major endothermic peak previously identified by Mackey et al. (1991) as being associated with ribosome melting. Figure 3.13 illustrates the changes observed in the cell density and the peak temperatures of thermograms of samples withdrawn periodically from a dilute 2^0Exp population incubated at 25°C. Both the cell density and the peak temperatures rose with the physiological age of the culture until, after 12 hours of incubation, the cell density began to plateau. The peak temperatures continued to increase, in general beyond this time.

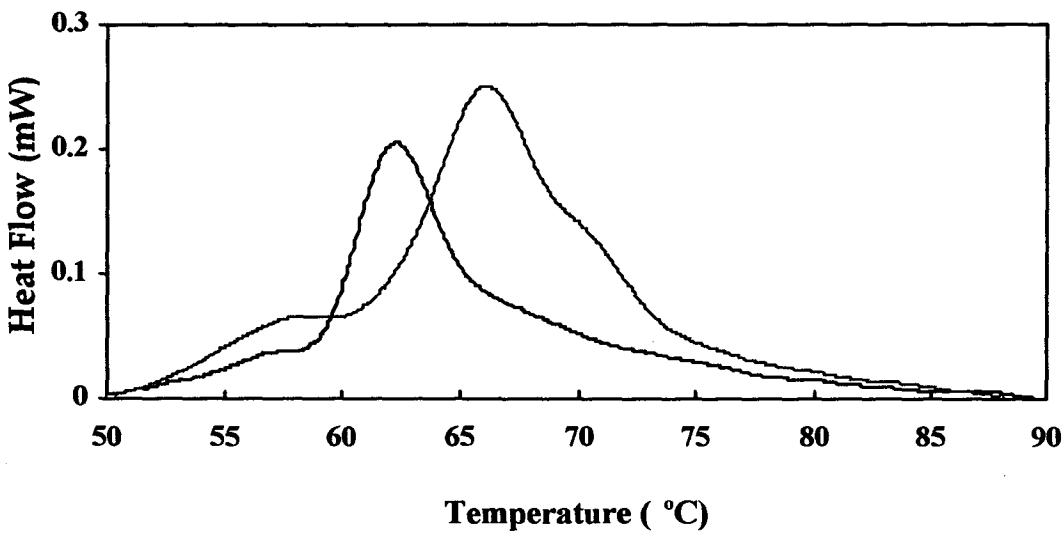


Figure 3.12 DSC thermograms of whole cell suspension derived from a purely exponential phase population (—) and from a mixed phase population (—). The purely exponential phase population was prepared by incubating a dilute 2^0 Exp culture at 25°C for 7 hours, and the mixed phase population by incubating the same culture for an additional 3 hours.

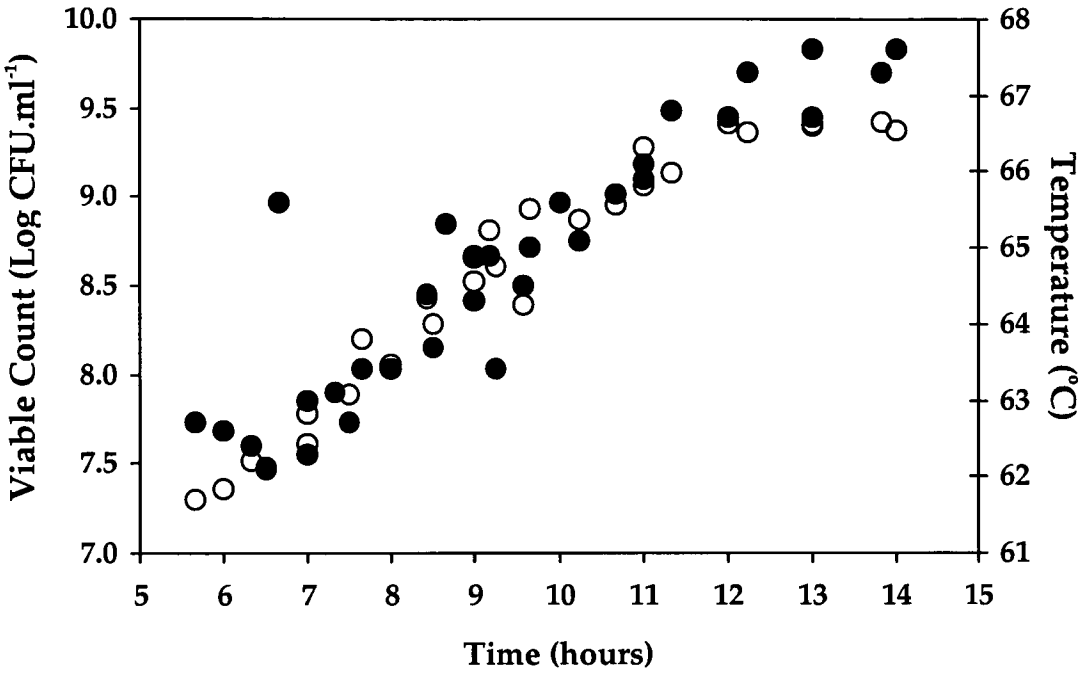


Figure 3.13 Scatter plot illustrating a correlation between the peak temperatures of DSC thermograms of whole cell suspensions derived from two dilute 2 Exp populations during incubation and the viable count of those populations.

3.5 DISCUSSION

Initial viability datasets generated in this study illustrated that exponential phase populations of *E. coli* M23 exhibit two distinct phases of inactivation upon exposure to low pH – an initial phase of rapid inactivation [P1] and a second protracted phase of much slower inactivation [P2] (Figure 3.1). While studies concerned with the kinetics of low pH-induced inactivation of *E. coli* have largely utilised stationary phase populations, four studies utilising exponential phase populations (Brown et al., 1997; Booth et al., 1999; Benito et al., 1999; Jordan et al., 1999b) have recently been published. The kinetic observations presented in Figure 3.1 concur with the observations of those studies, and with the unpublished observations of Glover et al. (1998). O'Hara and Glenn (1994) have also published kinetic data pertaining to

the low pH-induced inactivation of exponential phase populations of *E. coli*, however they reported that the kinetics of inactivation were log linear. An examination of their data reveals that loss of population viability was followed through less than 3.5 log cycles. The possibility remains that, had they carried the experiment further, a second phase of inactivation would have been encountered.

Biphasic inactivation kinetics such as those just described are by no means exhibited exculsively by exponential phase populations, by populations of *E. coli*, by populations exposed to low pH, or in model systems. They have been observed for stationary phase populations exposed to low pH (Wang and Doyle, 1998); populations of *Listeria, Salmonella* and other bacterial genera (Benjamin and Datta, 1995; O'Sullivan and Condon, 1997; Phan-Thanh et al., 2000; Samelis et al., 2001); populations exposed to high pressure (Benito et al., 1999), high temperature (Gage and Stoughton, 1906; Moats et al., 1971; Humpheson et al., 1998), and low water activity (Shadbolt et al., 1999); and in foodstuffs (Vanderlinde, 1999; Lahti et al., 2001). In many of these studies the occurrence of the second slower phase of inactivation is described as "tailing", and that fraction of the population which survives into P2 as the "tail". Several theories concerning the cause of tailing have been advanced, the most common being that the tail represents a resistant subpopulation. As Falk and Winslow (1926) point out though, "it is significant to recall that 'resistance' of a cell ... is not an independently measurable factor. Resistance is known only as a function of time .... it represents, therefore, a state whose existence is *assumed* but not *proved*." Whether or not this fraction of the population is especially resistant, or whether the survival of individual cells is simply a stochastic outcome (possibilities discussed in Chapter 4), this tailing phenomenon is undoubtedly a cause of concern for food processors who traditionally have employed log linear inactivation kinetics as a basis for process calculations. While tail cells may be of less importance when, for example, a

reduction in the bacterial load of 10^4 is satisfactory, they will be critical when achieving sterility is the objective.

As previously discussed (1.4.2), the low pH tolerance of *E. coli* is known to be affected by multiple physicochemical parameters including pH stringency and temperature. While Poynter et al. (1986), Rowbury and Goodson (1993), Benjamin and Datta (1995), and Jordan et al. (1999b) indicate that an increase in the stringency of an inimical low pH treatment increases the magnitude, and by inference the rate, of the kill observed, the influence of pH stringency on the kinetics of low pH-induced inactivation of exponential phase populations of *E. coli* has not been described. Likewise, the notion that "...temperature is to be considered an accelerating condition ..." in inactivation processes (Clark and Lubs, 1917) has been accepted for some time, yet the influence of this parameter on the kinetics of low pH-induced inactivation had, prior to the current study, not been demonstrated. Eighteen different pH/temperature combinations were utilised in the present study to investigate the influence of pH stringency and temperature on the kinetics of low pH-induced inactivation of exponential phase populations of *E. coli*.

Increasing the stringency of the lethal agent employed precipitated an increase in the rate of inactivation that occurred during P1 and a decrease in the duration of that phase. R2 was found to be largely independent of the severity of the lethal agent, however, and the magnitude of the kill effected during P1 was influenced noticably by pH only at temperatures exceeding 35°C (Figures 3.2 and 3.4). That R1 increased with increasing pH stringency is consistent with the conventional wisdom regarding the influence of pH stringency on low pH-induced inactivation. The influence of pH stingency on the rate of inactivation that occurred during P2, or rather the general lack thereof, was counter-intuitive however. Shadbolt et al.

(1999) recently investigated the influence of water activity (A_w) and temperature on the kinetics of inactivation of *E. coli* exposed to inimical A_w constraints, and they too reported that the rate of inactivation observed during phase 1, but not phase 2, is influenced by the severity of the lethal agent employed. The results of that study also led the authors to conclude that the size of the kill effected during P1 is proportional to the magnitude of the stress imposed. Jordan et al. (1999b), who studied the low pH-induced inactivation of *E. coli*, also indicate that K1 increases with the magnitude of the stress imposed. By contrast, the results of this study (Figure 3.4) imply that pH stringency influences K1, which may be regarded as a function of R1 and D1, only at temperatures greater than 35°C.

Like pH stringency, temperature was shown to influence the kinetics of low pH-induced inactivation. Increases in this environmental parameter resulted in R1 increasing and D1 decreasing. While temperature also influenced the rate of inactivation that occurred during P2, it did so consistently only at temperatures exceeding 25°C. Above this temperature, R2 increased conspicuously with increasing temperature (Figures 3.3 and 3.4). That R2 was largely independent of temperature at temperatures ≤ 25°C was quite unexpected. Shadbolt et al. (1999) reported that for populations of *E. coli* exposed to inimical A_w constraints, both R1 and R2 increased with increasing temperature for all of the temperature intervals examined between 4°C and 50°C. The influence of temperature on the kinetics of non-thermal inactivation appears to differ, then, for pH and A_w. Ross (2002) suggested that the discontinuity in the influence of temperature on the rate of low pH-induced inactivation observed during P2 might be explained by an 'uncoupling' which results from the influence of temperature on the rate of a passive process (the influx of protons) and the rate of an active process (proton efflux via membrane bound pumps). While the former will be accelerated by increasing temperature, the latter will be reduced by temperatures that exceed that

which is optimal for the conformational stability, and hence activity, of the macromolecule(s) involved.

Several recent studies concerned with the low pH-induced inactivation of *E. coli* (Weagant et al., 1994; Conner and Kotrola, 1995; Tsai and Ingham, 1997; Uyttendaele et al., 2001) have indicated that the level of survival observed at low (e.g. refrigeration) temperatures exceeds that observed at higher (e.g. room) temperatures. Uyttendaele et al. (2001) interpreted these observations as evidence that while making multiple growth factors sub-optimal (i.e. application of the 'hurdle concept' for food preservation) may inhibit the outgrowth of spoilage microorganisms, it may also enhance the survival of pathogenic microorganisms. The results presented in Figures 3.3 and 3.4 illustrate that the validity of this statement is likely to rest on the time frame over which survival is monitored. As previously noted, R1 increased with increasing temperature. K1 was independent of this environmental parameter, however, except under the most stringent conditions employed. As a result the survival curves of populations exposed to low pH at 4°C, 15°C, and 25°C converged during P2 (Figure 3.3). As previously noted (3.2), many of the investigators that have sought to identify the influence of physicochemical and physiological parameters on the low pH tolerance of *E. coli* have employed the relative viability of control and test cultures exposed to a low pH challenge of fixed duration (i.e. point determination methods) as an indicator of the significance of given test parameters in determining the low pH tolerance of *E. coli*. The results presented here illustrate that the two phases of inactivation observed during the low pH-induced inactivation of *E. coli* may be differentially affected by such parameters. Consequently, the outcome of studies employing point determination methods will depend on the time at which the viability of the cultures is assayed. This notion is illustrated in Figure 3.14.

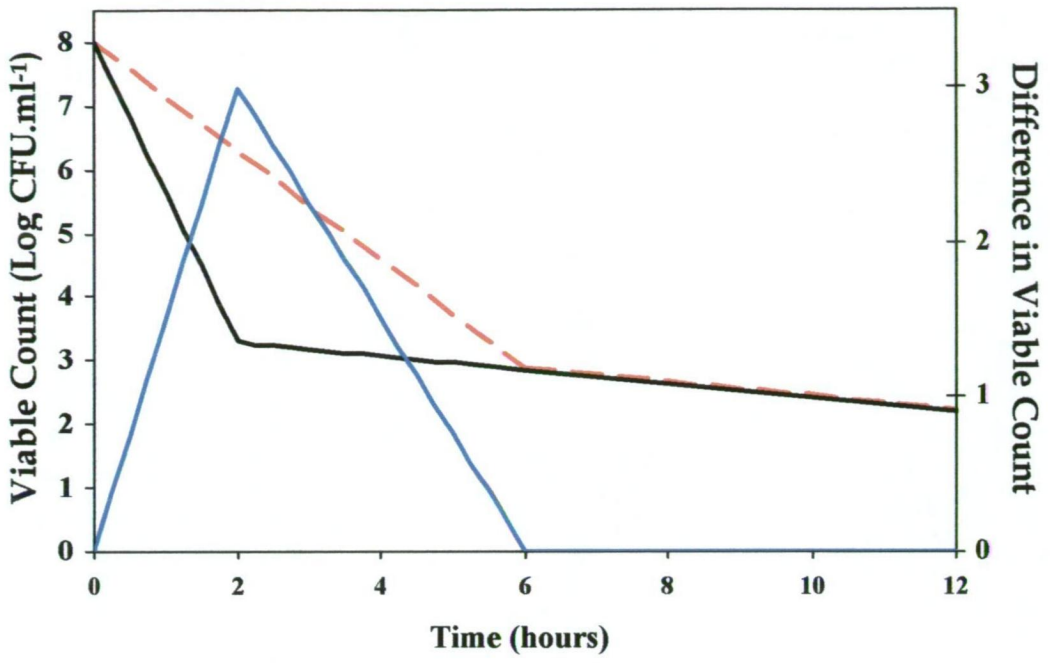


Figure 3.14 Diagrammatic representation of the low pH-induced loss of viability of two populations challenged at different temperatures, where population A (——) is challenged at a higher temperature than population B (— —) and both temperatures are ≤ 25°C. The difference in the viable counts of the two populations (——) over the course of the challenge is also illustrated.

During the course of investigations into the influence of pH stringency and temperature on the kinetics of low pH-induced inactivation, several datasets were obtained that provided evidence of a third phase of inactivation in exponential phase populations exposed to inimical low pH constraints (Figure 3.3). This phase of inactivation, characterised by the rapid and complete loss of population viability, is also evident in a viability dataset published by Moats et al. (1971). That dataset describes the thermal inactivation of a stationary phase population of *E. coli*, and while the authors discuss the tailing phenomenon at some length they neglect to mention the third phase of inactivation. This phase, observed only after long periods of incubation or under quite stringent conditions, is probably missed by

most investigators either because they do not follow the kinetics of inactivation for sufficiently long periods of times or because their data density is insufficient to reveal it. Ross and Shadbolt (2001) point out that this third phase of inactivation, should it occur in foods, would be of "…. great significance as a critical time limit beyond which the product rapidly becomes safer." The acquisition of data pertaining to the influence of pH stringency and temperature of the duration of P2 may thus be pertinent to the development of strategies designed to enhance the microbiological safety of acidic foods.

In seeking to comprehend the mechanisms of non linear inactivation kinetics such as those described in this study, Ross (pers. comm., 1999) postulated that an injury-recovery phenomenon may account for the triphasic inactivation kinetics observed. To explore this possibility, recovery conditions known to inhibit the outgrowth of cells that sustain membrane associated injuries (i.e. media containing bile salts) were employed in an attempt to accentuate any injury-recovery phenomena that might occur during the low pH treatment of exponential phase populations. Implications concerning the mechanisms underpinning non linear inactivation kinetics of the results of this study are discussed in the next chapter of this thesis. For now the readers attention is drawn to two unanticipated observations made during the course of this investigation. The first of these was that exponential phase populations of *E. coli*, although typically exhibiting triphasic inactivation kinetics (Figure 3.5), in some instances exhibited atypical patterns of inactivation in which the viable count fluctuated dramatically (Figure 3.6)[1]. Ryan (1959), and later Postgate and Hunter (1962), observed similar fluctuations in viable counts of

[1] Note that atypical inactivation kinetics such as those illustrated in Figure 3.6 were observed in perhaps four instances during the course of the studies presented in this thesis. For clarity these atypical inactivation curves have been omitted from future chapters.

starved bacterial populations and attributed them to the multiplication of survivors that were metabolising small molecules released by the cells that had died. Unlike the bacterial populations employed by those authors, substrate was not limiting in the bacterial populations employed in the present study. 'Cryptic growth,' as the phenomenon described by Ryan (1959) and Postgate and Hunter (1962) is known, is thus unlikely to explain the behaviour observed in Figure 3.6.

Jacobs (1960), who studied the inactivation of populations of *E. coli* upon their exposure to phenol, also noted marked sudden fluctuations in the viable count of populations whose viability was, in general, declining. He interpreted this as evidence that ".... some cells of a culture can grow in the presence of a concentration of phenol which is lethal to most cells ...." and that "... rapid death must have accompanied the multiplication stage ....". Central to Jacobs argument is his assumption that the inherent ability of a cell to withstand a toxic agent varies with its stage in the division cycle and that newly divided daughter cells are more susceptible to environmental stresses than the parent cell. The latter notion was advanced by Srivasta and Thompson (1965) who demonstrated that the resistance of a synchronised population is at a minimum following cell division. While physiological differences between the individuals of a population may permit some to grow under conditions which are to the majority lethal, this possibility does not appear to provide an entirely satisfactory explanation for the results presented in Figure 3.6 or, in fact, for the results presented by Jacobs (1960). Consider the population whose viability is illustrated in Figure 3.6. In that population the first increase in the viable count occurred almost forty hours after the population was first exposed to low pH. If one assumes that all of the survivors at this time were able to divide, that they had survived and were able to divide for reasons linked to their physiological maturity, and that newly divided cells are more susceptible to the prevailing environmental conditions as Jacobs (1960) suggested, then at most a

doubling (i.e. a 0.3-log increase) in the viable count would be expected before a second phase of decline began. Both Jacobs (1960) and the present study illustrate viable count increases which greatly exceed 0.3-log units.

An alternative explanation for the observed kinetics is the outgrowth of survivors carrying novel alleles that confer increased tolerance to the stress encountered. If one assumes that the increase in the viable count observed does result from the growth of a survivor with a genetic advantage then no subsequent decline in the viable count of the population would be expected until the growth of those cells harbouring the genetic advantage was precluded by some environmental change brought about by their own growth. If growth was possible only as a result of pre-existing mutations just one 'wave of growth', in which that cell with the greatest competitive fitness attained dominance, would be expected. If, on the other hand, survivors from the first wave of growth were able to acquire additional mutations that conferred tolerance to the second growth-preventing factor, then a second wave of growth might be expected. In fact, so long as the population size was large enough and the mutation rate high enough, several waves of growth might be expected before the viable count of the culture declined below the limits of detection. Such a phenomenon has recently been demonstrated by Finkel and his colleagues (Finkel and Kolter, 1999; Finkel et al., 2000). These authors report that over extended periods of time new subpopulations with increased competitive fitness arise in stationary phase cultures through the growth of survivors containing novel mutations. Cells with a particular growth advantage dominate the viable population until they themselves are 'superceded' by cells containing additional advantageous alleles. To the investigator studying viability of the 'macro-population', the growth of new subpopulations is seen only as fluctuations in the viable count.

Interestingly, Finkel et al. (2000) report that during the course of their long-term stationary phase investigations new colony morphotypes, including those described as "opaque" and "mini", were observed. When atypical inactivation kinetics such as those illustrated in Figure 3.6 were observed in the present study, at least two distinct morphotypes were apparent – that regularly observed for *E. coli* M23 and a second smaller morphotype. Bearson et al. (1996) have reported that mutations in *mviA*, a gene whose product is thought to be a component of a signal transduction system responsible for inducing *rpoS* during acid shock, cause overproduction of σ^S and σ^S-dependent acid shock proteins, increased tolerance to acid and other stresses, and significant decreases in colony size. Mutations of a similar nature (i.e. those conferring increased acid tolerance and a new morphotype) may thus explain the occurrence of the viable count fluctuations observed in the present study. An unnerving hypothesis recently tendered by a number of authors is that when subjected to certain nonlethal selections bacteria have some way of producing, or selectively retaining, only the most appropriate mutations (Foster, 1992; Moxon et al., 1994; Cairns et al., 1988; Hall, 1990; Rosenberg, 1994; Rosche and Foster, 2000). A case is readily envisaged where this phenomenon, which has been termed 'directed' or 'adaptive' mutation, might occur in populations exposed to inimical constraints of low stringency[1]. Environmental stress, while playing a vital role in preventing outgrowth of the macro-population, may thus act as a driving force that promotes adaptive mutations that enhance the resistance and virulence of individuals (Rowan, 1999). If true, then the present trend towards minimal processing may have significant implications for the emergence of new pathogens.

[1] It should be noted that atypical inactivation kinetics such as those illustrated in Figure 3.6 were only observed in the present study under the least stringent conditions employed.

The second observation made during the course of studies designed to investigate the potential for injury-recovery phenomena was that in utilising 'identical' populations and conditions on different occasions, substantial variations in the rate of inactivation observed during P1, and in size of the tail, were apparent. The magnitude of the variability observed is illustrated in Figure 3.15 which is a plot of two viability datasets obtained under identical conditions [pH 3.5, 25°C] on different occasions – one during studies investigating the influence of pH stringency and temperature on the kinetics of inactivation (previously illustrated in Figure 3.2), and the other during those studies investigating the potential for injury-recovery phenomena (previously illustrated in Figure 3.5). This degree of variability did not occur between 'identical' experiments performed at the same time (see the datasets presented in Figures 3.1, 3.2 and 3.3). An examination of the

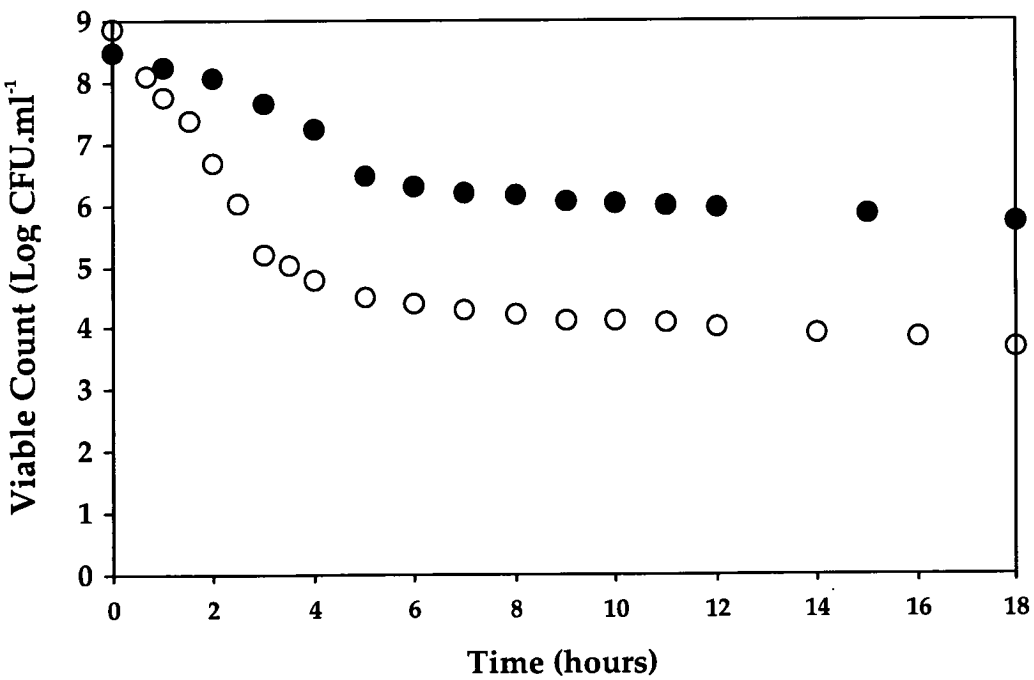


Figure 3.15 Two viability datasets that illustrate the degree of 'day-to-day' variability observed between 'identical' experiments. These viability datasets illustrate loss of viability in exponential phase populations of *E. coli* exposed to low pH [pH 3.5, 25°C] and were presented earlier in Figure 3.2 (O) and Figure 3.5 (●).

literature reveals that 'day-to-day' variability is not a unique feature of the present study. Chick (1910) noted that "My experience confirms that of Eijkmann (1908) who had great difficulty in obtaining uniformity in cultures of *B. coli* .... the species is moreover extremely variable .... and it is necessary ... that for comparison ... the experiments should be carried out on the same day ...". Withell (1942) also reported considerable variation in time-survivor curves when experiments were made as nearly identical as possible, and pointed out that "... often the statement is made that other experiments yield 'similar' results. How far these statements were true, and how far the other experiments were 'similar' can never be ascertained from the published works". An examination of the current 'inactivation' literature suggests that these statements still apply today. For example, Booth et al. (1999) reported that "this [the tail subpopulation] was variable in size between different *E. coli* isolates, but in *general* was consistent for any given isolate in repeat experiments." Datta and Benjamin (1999) present data from a *typical* experiment and indicate that "... the same *basic trend* of the results remained the same"[1]. Rowe and Kirk (1999) on the other hand present data as "... the mean of four replicate runs ..." without providing an indication of the degree of variability observed. An increasing tendency amongst investigators studying the survival of microbial populations to present viability datasets as % survival-time plots may also conceal the extent to which variability is observed between datasets.

While some investigators are ambiguous about the reproducibility of their viability datasets, others quite openly indicate that a considerable degree of variability can be observed. Leyer and Johnson (1993) present data as mean values ± the standard deviation, and in some instances the observed values differed 10-fold or more. Chang and Cronan (1999) indicated that stationary phase cultures challenged more

[1] Italics within the quotations were added, for emphasis, by the candidate.

than one hour after the turbidity of the culture ceased to increase gave "erratic results", and Glover and Malcolm (pers. comm., 1998) indicated that they too observed day-to-day variability of the order of magnitude observed in the present study. Two possible causes for this day-to-day variability were considered and subsequent experiments illustrated that, in fact, both were likely to be contributing factors.

The first possibility was that the widespread view that mid exponential phase batch cultures are in a state of balanced growth from a physiological point of view, and that upon reaching stationary phase[1] the physiological state of a population changes little for some time (until the death phase begins), was not correct. Typically, the bacterial 'growth curve' is divided into four phases – the lag phase, a period during which cells adjust to their new environment and prepare for growth; the exponential or logarithmic phase, a period of rapid balanced growth; the stationary phase, a period in which there is no net increase in cell numbers; and the death phase which is characterised by a decline in population viability (Brock and Madigan, 1991; Pelczar et al., 1993). If not all of the members of an exponential phase population were physiologically 'identical' (for example, some had not begun to multiply and thus retained stationary phase characteristics), and if the physiological state of populations that reach stationary phase continues to change markedly for some time, then the variability observed might be attributable to day-to-day differences in the physiological state of experimental inocula employed for the preparation of exponential phase populations. To test this possibility the kinetics of inactivation of 1^0Exp, 2^0Exp, 3^0Exp, 4^0Exp, and 4^0Exp-LD populations

[1] Note that this statements is not applicable to that period between the exponential and stationary phases of growth (i.e. entry to stationary phase).

were compared, and the influence of physiological age on the kinetics of inactivation was determined.

Glover et al. (1998) have also recently entertained the possibility that exponential phase populations contain cells that retain stationary phase characteristics, and that cells surviving into P2 are those members of the population. While no results were presented by those authors, they did indicate that varying the number of generations between inoculation from an overnight culture and acid challenge did not affect the rate at which a population lost viability or the size of the population surviving into P2. Consequently they concluded that those cells surviving into the tail were not residual stationary phase cells. By contrast, the results obtained in the present study (Figure 3.7) illustrate that the size of the tail of an exponential phase population is reduced substantially by subculturing that population twice. No further reduction in the size of the tail occurred if the population was subcultured a third time[1]. These results imply that a large proportion of the tail of 1^0Exp populations can, in fact, be attributed to the presence of residual stationary phase cells. That subculturing a third time resulted in no further reduction in the size of the tail indicated that some of the cells in 'purely exponential phase[2]' populations may be behaving like stationary phase cells. To determine whether or not a purely exponential phase population was likely to begin entry to stationary phase at a population density of *ca.* 1×10^8 cfu ml^{-1}, a purely exponential phase population was

---

[1] If a stationary phase (SP) population with a viable count of *ca.* 1×10^9 cfu.ml^{-1} is subcultured [1:1000] once, at most 1×10^6 cfu.ml^{-1} residual SP cells will be found in 1^0Exp populations. Subculturing a 2nd time reduces this number to 1×10^3 cfu.ml^{-1}, and a 3rd time to 1×10^0 cfu.ml^{-1}. Consequently, residual SP cells should not be detected in 4^0Exp populations.

[2] The phrase 'purely exponential phase' is used to distinguish 3^0Exp populations, which contain no residual stationary phase cells, from 1^0Exp populations.

grown to a lower cell density than that usually employed (i.e. *ca.* 1×10^7 cfu ml^{-1} instead of 1×10^8 cfu ml^{-1}). That this population displayed a tail whose size was comparable with that of higher density populations indicated that purely exponential phase populations with viable cell counts of *ca.* 1×10^8 cfu ml^{-1} were unlikely to be entering stationary phase. Furthermore, the fact that the tail of purely exponential phase populations did not account for a fixed proportion of the original population (i.e. it increased with decreasing population density) implied that those members of the tail of purely exponential populations survived for some reason linked to the mechanism of inactivation (a notion considered further in Chapter 4).

Because a large proportion of the tail of 1^0Exp populations could be attributed to the presence of residual stationary phase cells, some variability in the response of those populations to an environmental challenge would be expected unless the experimental inocula (i.e. the stationary phase populations) employed to prepare the 1^0Exp populations were identical. In all but one instance (the experiment just discussed), exponential phase populations had been prepared by incubating dilute [1:1000] overnight[1] cultures at 25°C until viable counts of *ca.* 1×10^8 cfu ml^{-1} were attained (2.3.2.1), and the assumption had been made that once a population reached stationary phase its physiological state would change little for some period of time. To i) test this assumption, ii) evaluate the possibility that small differences in the cell density of populations with initial viable counts of *ca.* 1×10^8 cfu ml^{-1} may alter the kinetics of inactivation substantially (the second possible cause for day-to-day variability considered), and iii) investigate the influence of physiological age on the kinetics of low pH-induced inactivation, datasets describing the low pH-

[1] While no definite period of time is indicated by the term 'overnight' it was a period sufficient for a culture to reach stationary phase, generally a period of 16 ± 4 hours.

induced inactivation of ten cultures that differed in their physiological age, and in many instances their cell density, were prepared. The results obtained (Figures 3.8, 3.9 and 3.10) illustrated two things of importance to the present discussion. First, substantial differences in the survival curves of the four cultures with initial viable counts between 1×10^8 cfu ml^{-1} and 1×10^9 cfu ml^{-1} were apparent (Figure 3.8). This fact illustrated that small differences in the cell density of populations with initial viable counts of *ca* 1×10^8 cfu ml^{-1} may have contributed to the day-to-day variability observed in the kinetic responses of 'identical' populations. The second point of relevance to the present discussion was that the low pH tolerance of those cultures traditionally designated stationary phase was not constant. It increased with physiological age in cultures aged 12 to 24 hours then, unexpectedly, declined (Figures 3.8 and 3.9). In fact, no 'steady state' of resistance was apparent amongst any of the cultures examined. This result implied that a continuum of physiological states exists not only between, but also within, the exponential and stationary phases. The assumption that 'overnight' incubations would yield 'identical' stationary phase populations was thus shown to be invalid, and their use likely to contribute substantially to the day-to-day variability observed in the kinetic responses of 'identical' exponential phase populations.

The use of an 'overnight' inoculum (used typically at 0.1-1%) for the preparation of exponential phase populations, or without dilution as a stationary phase population, is not unique to the present study. Foster and Hall (1990), Kroll and Patchett (1992), Lee et al. (1994), Small et al. (1994), Benjamin and Datta (1995), Leyer et al. (1995), Jordan et al. (1999b), and Jordan et al. (1999c) all report the use of 'overnight' populations. While few problems are likely to be encountered in comparing the survival responses of populations prepared on the same occasion (since stationary phase populations are likely to be prepared over the same time frame), or if precautions are taken to ensure that overnight incubations are of a

fixed duration, it is highly improbable that the exponential and stationary phase populations employed in the studies listed above represent populations in the same physiological state. The comparability of data published in different studies, or within a study in which populations are not prepared with 'due care', must therefore be questioned. To improve the comparability of data generated on different occasions in the present study additional precautions[1] were taken to ensure the uniformity of stationary phase populations employed as inocula.

The results of an investigation of the influence of physiological age on ribosome stability provided further evidence that a continuum of physiological states exists not only between, but also within, the exponential and stationary phases. As noted previously (3.3.7), DSC thermograms derived from whole cell suspensions of *E. coli* are dominated by a major endothermic peak (Figure 3.12) which Mackey et al. (1991) identified as being associated with ribosome melting. An increase in the value of the peak temperature of the major endothermic peak [T_{max}] has thus been interpreted as evidence of an increase in the conformational stability of the ribosomes (Niven and Mackey, pers. comm.., 2000). Data obtained in the present study (Figure 3.13) indicate that T_{max} increases continuously with physiological age during both the exponential and stationary phases. This data implies that the physiological state of a population continuously changes and that there may be no 'balanced state' even amongst purely exponential phase populations of differing physiological age. In the analysis of those data presented in Figure 3.13 it is pertinent to note that the T_{max} values of cells harvested when the cell density of the

[1] Instead of using an 'overnight' stationary phase inoculum for the preparation of 1^0Exp populations, a stationary phase inoculum prepared by inoculating a fixed volume of broth (15 ml) from a plate culture of definite age (14 hrs), and incubating this culture for a fixed period at 37°C, was used.

culture was ≤ 1 x 10^8 cfu ml^{-1} (i.e. when the culture could be described as purely exponential phase) are much (about 5°C) lower than those reported for exponential phase populations by Niven and Mackey (pers. comm., 2000). While other investigators have used only stationary phase populations in the DSC analysis of *E. coli*, Niven and Mackey (pers. comm.., 2000) examined the thermograms of cells derived from both exponential and stationary phase populations to investigate the effects of growth phase on ribosome stability. In that study, exponential phase populations were prepared by incubating a dilute [1:100] stationary phase culture at 37°C until an OD_{600} of 0.25 was attained. Cell suspensions from cultures thus prepared yielded T_{max} values of 68.3 ± 0.2°C - values much closer to the T_{max} values of cells harvested from stationary phase populations (Mackey et al., 1991 [68°C]; Miles et al., 1993 [69°C]; Niven et al., 1999 [69°C]; Niven and Mackey, pers. comm.., 2000 [67.8 ± 0.2°C]; Figure 3.13 [67-68°C]) than of cells derived from purely exponential phase populations in the present study [62-63°C]. It may be that residual stationary phase cells in the exponential phase populations prepared by Mackey and Niven (pers. comm.., 2000) obscured the true T_{max} of exponential phase cells in that study.

While the data presented in Figures 3.8 and 3.9 facilitated resolution of some of the methodological difficulties encountered it also provided a means of investigating the influence of physiological age on the kinetics of low pH-induced inactivation, and it is to the latter topic that the discussion now turns. When precautions were employed to rid exponential phase populations of residual stationary phase cells (i.e. purely exponential phase populations were prepared) just two phases of inactivation were observed – an initial phase of rapid inactivation and a second phase of much slower inactivation that persisted until the viable count dropped below the limit of detection (Figures 3.7 and 3.8). It should be noted that the results

obtained are not necessarily indicative that no third phase of inactivation occurs in purely exponential phase populations. It is possible that the resolution achieved in viable counts below 1×10^1 cfu ml^{-1} was insufficient to enable characterisation of that phase. By contrast, the more mature populations (i.e. those with initial viable counts > 1×10^8 cfu ml^{-1}), including those traditionally defined as stationary phase populations, exhibited inactivation kinetics that were clearly triphasic (Figures 3.8 and 3.9). Published reports concerning the kinetics of low pH-induced inactivation of stationary phase populations of *E. coli* are conflicting. O'Hara and Glenn (1994) reported log linear inactivation kinetics (but as previously noted these authors did not carry their survivor curves through more than 3.5 log cycles), Wang and Doyle (1998) reported the occurrence of two phases of inactivation that are analogous to the first two phases of inactivation observed in the present study (but they carried the relevant survivor curves through no more than 2 log cycles), and Jordan et al. (1999b) reported the occurrence of a small shoulder followed by a phase of rapid inactivation and a protracted period of tailing. Most commonly, however, the kinetics of low pH-induced inactivation of stationary phase populations of *E. coli* are reported to be biphasic with a period of slow or negligible inactivation (generally referred to as the shoulder) preceeding a period of rapid inactivation that persists until the viable count falls below the limit of detection (Semanchek and Golden, 1996; Benito et al., 1999; Uckert, pers. comm., 2001). Data presented in the next chapter indicate that the apparent inconsistency of these observations may be explained largely by methodological differences between the studies.

It is well known that the entry of *E. coli* to stationary phase is accompanied by major morphological and physiological changes, many of them mediated by a global regulatory system, that lead to the acquisition of general stress resistance (Lange and Hengge-Aronis, 1991a; Lange and Hengge-Aronis, 1991b; McCann et al., 1991; Ozaki et al., 1991; Hengge-Aronis et al., 1993; Kolter et al., 1993; Loewen

and Hengge-Aronis, 1994). That the low pH tolerance of stationary phase populations exceeded that of purely exponential phase populations (Figure 3.8) was thus not unexpected. That the low pH tolerance of purely exponential phase populations decreased with increasing physiological age, on the other hand, was quite unexpected. Employing point determination methods Benjamin and Datta (1995) and Jordan et al. (1999b) illustrated that the low pH tolerance of *E. coli* is maximal during the lag phase[1], that it declines as cultures begin to multiply, that it reaches a minimum during the mid exponential phase of growth, and that it increases rapidly as cultures enter stationary phase. Analogous correlations between the age and sensitivity of *E. coli* populations exposed to other forms of inimical stress have also been described (White, 1951; Stapleton, 1955; Lemcke and White, 1959). In those studies, the cultures examined were prepared by dilution of overnight or stationary phase cultures. The low pH tolerance of the exponential phase populations examined would thus be expected to decrease with increasing 'age' as more and more of the residual stationary phase cells resolved their lag and began multiplying. In the present study a purely exponential phase population was employed as the inoculum and still the low pH tolerance of the population decreased with increasing physiological age (Figure 3.8). An increasing proportion of the initial population was inactivated during P1 as the density of the initial population increased. As previously suggested, this observation lends support to the notion that those cells of purely exponential phase populations that survive into the second phase, do so for reasons linked to the mechanism of inactivation.

A second unexpected observation was that after increasing with physiological age in cultures entering stationary phase, the low pH tolerance of populations whose

[1] Data presented in the next chapter provide a potential explanation for the extrem low pH tolerance of lag phase cultures.

physiological age exceeded 24 hours declined. This observation is likely to be of practical significance for the preparation of inocula for challenge studies. A common experimental approach has been to employ cells deemed to be in the most resistant state possible (i.e. to adopt a 'worst case' scenario), which has, on the basis of previous studies, usually been regarded as stationary phase cells. The results of the present study (Figure 3.9) illustrate that great care needs to be extended in the preparation of cells for challenge studies. Cells derived from 'overnight' populations may not, in fact, represent the worst case scenario. While the data presented in Figure 3.9 are for cultures incubated for 24 hours or more, and overnight incubations are unlikely to be that long, the cultures examined were inoculated with cells derived from a purely exponential phase population. Many investigators employ stationary phase inocula and consequently the low pH tolerance of their populations might be expected to peak and decline over a shorter time frame. In fact, an examination of the data presented by White (1951), Benjamin and Datta (1995), and Jordan et al. (1999b) illustrates a sharp peak in the resistance of their cultures after incubation periods of 5.5, 4, and 4 hours respectively. Those authors neglect to comment on that observation, and an examination of the current 'inactivation' literature indicates that it seems to have remained unnoticed.

During their growth, bacterial populations effect change in the medium employed for their culture, depleting nutrients, releasing metabolites, and often altering the pH (Tortora et al., 1992). Should the growth of a population depress the pH of its culture medium sufficiently, that population might be afforded an opportunity to adapt to low pH. Moreover, should the growth of a populations depress the pH of the culture medium, cells of that culture would experience a pH shift of smaller magnitude at the time of the low pH treatment than the cells of populations whose pH remained unchanged. Zilberstein et al. (1984) noted that upon an external pH

shift there is a transient failure of pH homeostasis in the cell, the recovery of pH_i in each case being dependent on both the magnitude of the shift in pH_o and the rate at which the change occurs. Consequently larger pH shifts would be expected to effect a greater amount of injury and death in a population than smaller ones. To examine the possibility that the low pH tolerance of populations of different physiological ages might correlate with the final pH of their culture medium prior to the low pH challenge, the pH (and growth) of a dilute 2^0Exp population was monitored during incubation at 25°C. No correlation between the pH of the culture medium and the low pH tolerance of populations of the corresponding physiological age was apparent, despite the fact that the pH was depressed during growth (Figure 3.11). The pH of the culture medium declined to a value of 6.0 after twelve hours incubation and rose thereafter. The rapidity with which the pH fell from its starting value by more than 0.2 pH units, and subsequently rose to its starting value, may explain the fact that other investigators (e.g. O'Hara and Glenn, 1994) have reported no change in the pH of the culture medium during growth. A 'dip' in the pH of the culture medium would undoubtedly be missed if pH were measured infrequently or only at the beginning and end of the growth period.

The observations presented in this chapter indicate that *E. coli* populations, upon exposure to low pH, exhibit three distinct phases of inactivation - an initial phase of rapid inactivation, a second protracted phase of much slower inactivation, and a third phase of inactivation that is marked by an increase in the rate of inactivation and which persists until the viable count falls below the limit of detection. Failure to observe the three phases of inactivation, in the majority of instances, can be attributed to methodological considerations[1], the scantiness of viability datasets, a

[1] The reader is referred to Chapter 4 for confirmation of this point.

failure to follow the inactivation curve to the limit of detection, or to a lack of resolution in viable counts below 1×10^1 cfu ml^{-1} (although the latter is likely to be applicable only to purely exponential phase populations). The observations presented also illustrate that the three phases of inactivation are influenced differentially, and often in a non-continuous manner, by the stringency of the lethal agent employed (i.e. pH), by temperature, and by the physiological age of a population. Consequently some of the observations made are non-intuitive. The data presented have implications for the design, conduct and interpretation of survival studies. They underscore the need to standardise the conduct of non-thermal resistance determinations, and they emphasise the folly of extrapolation in the absence of a mechanistic appreciation of the phenomena observed.

CHAPTER 4 MECHANISTIC INTERPRETATIONS OF THE KINETICS OF LOW PH-INDUCED INACTIVATION OF *E. COLI*

4.1 ABSTRACT

Hypotheses concerned with the basis of the shape of non-linear log survivor-time plots were introduced and classified into two main classes: those that explain non-linearity as an experimental artefact and those that account for it as a genuine phenomenon. The latter class was subdivided into those that explain the shape of survivor curves in terms of physiological phenomena and those that explain it in terms of physicochemical phenomena. The hypotheses introduced were critically evaluated in light of published data, data presented in the previous chapter, and data from experiments designed specifically to test various aspects of the hypotheses. That process led the author to conclude:

1) that the non-linearity associated with log survivor-time plots presented in this study is not an experimental artefact,
2) that the individuals of a clonal population of *E. coli* are inherently differentially resistant to inimical constraints, and
3) that the shape of survivor curves describing the low pH-induced loss of viability of *E. coli*, while reflecting the distribution of individual resistance states within a population, also reflects the mechanism of inactivation.

A novel hypothesis that explains the kinetics of inactivation exhibited by low pH-treated populations of *E. coli* in terms of both physiological and physicochemical phenomena was advanced.

4.2 INTRODUCTION

One of the classical controversies of quantitative biology is undoubtedly that of the significance of the shape of survival curves of microorganisms subjected to inimical physical or chemical agents (Hiatt, 1964). It began almost a century ago when Madsen and Nyman (1907 cited by Lee and Gilbert, 1918) examining several quantitative experiments published by Kröning and Paul (1897), noted that plotting the logarithm of the number of surviving organisms against time yielded a straight line. Madsen and Nyman (1907) reproduced Kröning and Paul's experiments with different germicides, as did Chick (1908), and in both studies the authors concluded that the inactivation of a microbial population is a gradual process whose rate at any moment is proportional to the number of surviving microorganisms. On that basis the 'logarithmic law of bacterial decline' was formulated, and despite having limited experimental foundations it was widely accepted within the scientific community. The basis of the occurrence of log linear inactivation kinetics, however, developed into an intense debate between two schools of thought known as the 'mechanistic' and the 'vitalistic'.

Proponents of the mechanistic hypothesis asserted that there is a general similarity in resistance between the individuals of a microbial population, and that the gradual nature of the inactivation process is attributable to the fact that only a proportion of the interacting substances ['critical' biomolecule(s) and disinfectant molecules] are in a condition that permits their participation in the 'inactivation reaction' at any given moment. The process of inactivation was thus considered to be analogous to a chemical reaction, the shape of the survivor curve being determined by the fundamental reaction between organism and reagent (Chick, 1908; Paul, 1909 cited by Rahn, 1945; Chick, 1910; Lee and Gilbert, 1918; Winslow and Falk, 1920; Cohen, 1922; Falk and Winslow, 1926; Rahn, 1929; Watkins and

Winslow, 1932; Rahn, 1943; Atwood and Norman, 1949; Hinshelwood, 1951; Charm, 1958). By contrast, advocates of the vitalistic hypothesis maintained that the gradual nature of the inactivation process is a result of differences in resistance between the individuals of a population, the shape of the inactivation curve being determined by the distribution of resistance within a population (Eijkman, 1908 cited by Lee and Gilbert, 1918; Hewlett, 1909; Reichenbach, 1911 cited by Rahn, 1945; Brooks, 1918; Henderson-Smith, 1921; Fulmer and Buchanan, 1923; Withell, 1942; Jordan and Jacobs, 1944; Jordan et al., 1947; Vas and Proszt, 1957; Moats et al., 1971).

During the early 1900s the number of investigators studying the process of microbial inactivation increased markedly. So too did the number claiming allegiance to one school of thought or the other, and while many attempted to justify their positions none were able to fully negate the views held by their opponents. In fact, in seeking experimental evidence that would lend support to their arguments many investigators obtained kinetic datasets that were clearly non-linear and, consequently, the logarithmic law of decline itself became a source of contention. Within both schools of thought some asserted that deviations from linearity were experimental artefacts. Others accepted them as genuine phenomena and presented hypotheses refined to account for the main types of deviations observed. Few of the hypotheses presented were critically evaluated, however, and since it was unlikely that a consensus would be reached rapidly, interest in the study of microbial inactivation dwindled. Within the last ten years that interest has been renewed, presumably in response to the emergence of highly virulent pathogens with low infectious doses. A review of inactivation studies published during that time reveals a propensity amongst investigators to present comparative rather than kinetic data (3.2), however, and where kinetic data has been presented there appears to have been few attempts made to couple observation with

understanding. As pointed out in the introduction to this thesis, that mentality must change if strategies that enable the food industry to comply both with product safety specifications, and with consumer demands, are to be rationally devised.

In view of these comments, the aim of this chapter is to link observation with understanding - to evaluate and refine hypotheses concerned with the basis of the shape of log survivor-time plots, and specifically with reference to those describing the low pH-induced inactivation of *E. coli*.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial Strains, Bacteriological Media, Chemical Reagents & Equipment

Details of the bacterial strains, bacteriological media, chemical reagents and equipment employed throughout this study, together with the methods employed for bacterial maintenance and recovery, are presented in Appendix A.

4.3.2 General Methods

4.3.2.1 *Preparation of Stationary Phase Populations*

8 ml volumes of TSB contained in 15 ml sterile plastic tubes were inoculated from 14 hour plate cultures and incubated statically for 12 hours at 37°C. Primary exponential phase (1^0Exp) populations were subsequently prepared by diluting the 12 hour broth cultures 1:1000 in fresh TSB and incubating the dilute cultures in an oscillating waterbath maintained at 25°C until viable counts of *ca.* 1×10^8 cfu ml^{-1} were achieved. Secondary exponential phase (2^0Exp) populations were prepared from 1^0Exp populations in an analogous manner. Stationary phase populations, in turn, were prepared from 2^0Exp populations by diluting the latter cultures 1:1000 in fresh TSB and incubating the dilute cultures for 24 hours in an oscillating waterbath

maintained at 25°C. Unless otherwise indicated, 60 ml volumes of pre-warmed TSB in 250 ml conical flasks were used for the preparation of both exponential and stationary phase populations.

4.3.3 Evaluating the Possibility of an Injury-Recovery Phenomenon in Populations of *E. coli* Exposed to Inimical Low pH Treatments

4.3.3.1 *Membrane-Associated Injury*

The methods employed to examine the occurrence of membrane-associated injury in populations of *E. coli* M23 are described in Chapter 3, Section 3.3.5.

4.3.3.2 *Lag Phase Duration as a Measure of 'Whole-Cell' Injury*

The time-dependent loss of viability of an exponential phase population (2.3.2.1) of *E. coli* M23 exposed to an inimical low pH treatment [pH 3.5, 25°C] (3.3.2.1) was monitored by culture-based methods (3.3.2.1). Samples were withdrawn from that population (the primary culture) immediately prior to, and at regular intervals throughout, the low pH treatment – not only for the determination of population viability but also as a source of experimental inocula for a series of secondary cultures. For the latter purpose samples were serially diluted in TSB (pH 7) to ~ 1 x 10^4 cfu.ml^{-1} and an aliquot of each inoculum (1 ml) was transferred, upon its preparation, to a conical flask containing 49 ml TSB (pH 7) pre-warmed to 25°C. If it was anticipated that the viable count of the primary culture would be < 1 x 10^4 cfu.ml^{-1} secondary cultures were prepared by inoculating 5 ml of the primary culture to 45 ml pre-warmed TSB. The secondary cultures were incubated in an oscillating waterbath maintained at 25°C, and the viable count of each population was estimated at the time of inoculation and at regular intervals throughout the lag, exponential and early stationary phases of growth (until an OD_{450} of > 1.3 was attained). The enumeration protocol described in section 3.3.2.1 was employed for

viable count determinations and the lag phase of each population was calculated as described, by example, in Appendix C.

4.3.4 Investigating Inherent Differential Resistance as a Basis for Non-Linear Inactivation Kinetics

4.3.4.1 *By the Dilution of Stationary Phase Populations*

A stationary phase population of *E. coli* M23, prepared as described above (4.3.2.1), was diluted 1:1000 in fresh TSB, spent TSB[1], and spent TSB that had been heat-treated (121°C for 15 minutes). The viable count of each population, including the stationary phase population from which the dilute populations were derived, was determined immediately and each culture was then exposed to a low pH treatment [pH 3.5,25°C]. Culture-based methods (3.3.2.1) were employed to monitor the time-dependent low pH-induced loss of viability of each population.

4.3.4.2 *Further Experiments with Dilute Stationary Phase Populations*

Four populations of *E. coli* M23 were prepared by the dilution of a stationary phase population (4.3.2.1). The first [designated population A] was prepared as a 1:100 dilution in fresh TSB (pH 7), the second [B] as a 1:1000 dilution in fresh TSB (pH 7), the third [C] as a 1:1000 dilution in fresh TSB (pH 7) containing nalidixic acid at a concentration of 15 $\mu g.ml^{-1}$, and the fourth [D] as a 1:1000 dilution in fresh TSB that had been adjusted to pH 3.5 with 10M HCl. The viable count of each population was determined immediately after its preparation, and the pH of those cultures prepared in TSB (pH 7) was then adjusted to 3.5. The four populations were incubated in an oscillating waterbath maintained at 25°C, and their viable count

[1] Spent TSB was prepared by removing the cells from a stationary phase (4.3.2.1) culture. Following centrifugation (12 400 g for 10 minutes at 4°C) the broth was filter sterilized.

was monitored periodically by culture-based means (3.3.2.1). Shortly before that time at which the onset of the rapid phase of inactivation was expected, two additional populations [E and F] were prepared by diluting populations A and B, respectively, 1:100 in fresh TSB (pH 7). The viable count of populations E and F was determined immediately, and the pH of those cultures was then adjusted to a value of 3.5. The newly prepared cultures were incubated in an oscillating waterbath maintained at 25°C and their viable count was monitored periodically (3.3.2.1).

4.3.5 Investigating Phenotypically Acquired Resistance as a Basis for Non-Linear Inactivation Kinetics

Exponential phase populations (2.3.2.1) of *E. coli* M23, prepared in duplicate, were exposed to an inimical low pH treatment [pH 3.0, 35°C]. Immediately prior to the low pH treatment, chloramphenicol was added to one population from each pair at a final concentration of 25 $\mu g.ml^{-1}$. The viability of each population was assessed by culture-based methods immediately prior to, and at regular intervals throughout, the low pH treatment (3.3.2.1).

4.3.6 Investigating the Potential for pH Amelioration in Populations of *E. coli* Exposed to an Inimical Low pH Treatment

A series of cultures of *E. coli* M23 that differed in their physiological age and which included purely exponential, mixed exponential and stationary, stationary, and late stationary phase populations was prepared from a 2^0Exp culture as described in Section 3.3.7. Each population was exposed to a growth non-permissive low pH treatment [pH 3.5] by direct acidification of the growth medium (2.3.2.2) and subsequently incubated in an oscillating waterbath maintained at 25°C. At regular intervals an aliquot of each culture was withdrawn for pH analysis.

4.4 RESULTS

4.4.1 Evaluating the Possibility of an Injury-Recovery Phenomenon in Populations of *E. coli* Exposed to Inimical Low pH Treatments

4.4.1.1 *Membrane-Associated Injury*

In investigating the occurrence of membrane-associated injury in populations of *E. coli* exposed to inimical low pH treatments, several unanticipated observations were made. Those observations were relevant to the discussion offered in Chapter 3 and, for that reason, those data pertaining to the occurrence of membrane-associated injury in populations of *E. coli* exposed to low pH were presented and described in that chapter (Section 3.4.3, Figure 3.5). Briefly, the data presented are the counts obtained when viable cells withdrawn from an exponential phase population of *E. coli* M23 exposed to an inimical low pH treatment [pH 3.5, 25°C] were enumerated on TPA-P and TPA containing bile salts (NuBile). As expected, the kinetics of inactivation were triphasic. During the first phase of inactivation equal numbers of cells were recovered on the two media, a fact indicative that all cells sustaining membrane-associated injury were also non-viable. During the second phase of inactivation, however, a disparity in the counts obtained on the two media emerged, indicative that a proportion of the viable population also sustained membrane-associated injuries. That proportion of the population increased throughout the second phase of inactivation and by the onset of the third phase of inactivation, the entire complement of viable cells appeared to have sustained membrane-associated injuries. No recovery from that injury was observed.

4.4.1.2 *'Whole-Cell' Injury*

Figure 4.1 illustrates the low pH-induced [pH 3.5, 25°C] loss of viability of two exponential phase populations (A and B) of *E. coli* M23. Population A was prepared and challenged in advance of population B, the viable count dataset of the former being used to anticipate the time-dependent loss of viability of the latter. Immediately prior to, and at regular intervals throughout the low pH treatment of population B, samples were withdrawn and diluted to fresh TSB (pH 7[1]) in a

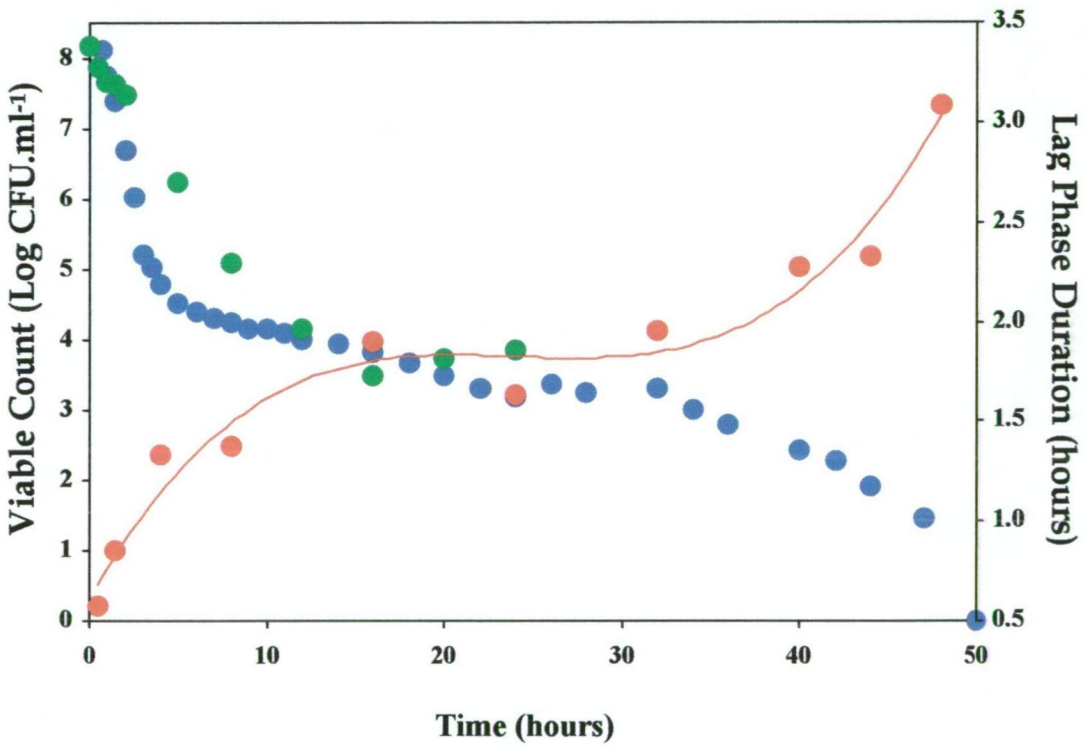


Figure 4.1 Low pH-induced [pH 3.5, 25°C] loss of viability of exponential phase populations of *E. coli* M23, A(●) and B(●), and the lag phase duration (●) of a series of cultures derived from population B by dilution in TSB (pH 7)[2].

[1] Inoculation lowered the pH of the fresh medium by no more than 0.2 pH units.

[2] This dataset represents the collaborative efforts of the author and her colleagues (L. A. Mellefont, C. T. Shadbolt, and T. Ross), both in terms of experimental design and conduct. The experiment performed was exceptionally labour intensive, and consequently its repetition was not possible during the course of this study.

manner designed to yield a series of cultures with similar initial viable counts. The lag phase duration of those secondary cultures, estimated from viable count datasets obtained at 25°C, is also illustrated in Figure 4.1. While the viable counts of population A declined in a triphasic manner, as expected, population B exhibited atypical inactivation kinetics (the reader is referred to section 3.5). Bacterial lawns covered all of the plates prepared for the enumeration of survivors in samples withdrawn from population B twenty-five hours or more after the onset of the low pH treatment. As a result no information concerning the viable count of population B, other than that it exceeded 10^4 cfu.ml^{-1}, could be determined beyond that time point. The lag phase duration of cultures derived from population B increased from 34 minutes for the culture prepared half an hour after the low pH treatment commenced to 185 minutes for the culture prepared 48 hours after the low pH treatment commenced. The lag phase duration of the secondary cultures increased rapidly amongst populations prepared during the first fifteen hours of the low pH treatment of population B, negligibly amongst populations prepared during the subsequent twenty hours, then rapidly beyond that time.

4.4.2 Investigating Inherent Differential Resistance as a Basis for Non-Linear Inactivation Kinetics

4.4.2.1 *By the Dilution of Stationary Phase Populations*

The time-dependent low pH-induced [pH 3.5, 25°C] loss of viability of three dilute stationary phase populations (diluted 1:1000 in fresh TSB, spent TSB, or heat-treated spent TSB), and of the stationary phase population employed in their preparation, is illustrated in Figure 4.2. The inactivation kinetics of the stationary phase population were, as expected, triphasic. After declining by three orders of magnitude in the first sixteen hours, the viable count of the stationary phase population stabilized just below 1×10^7 cfu.ml^{-1}, declining by less than 0.3 log units

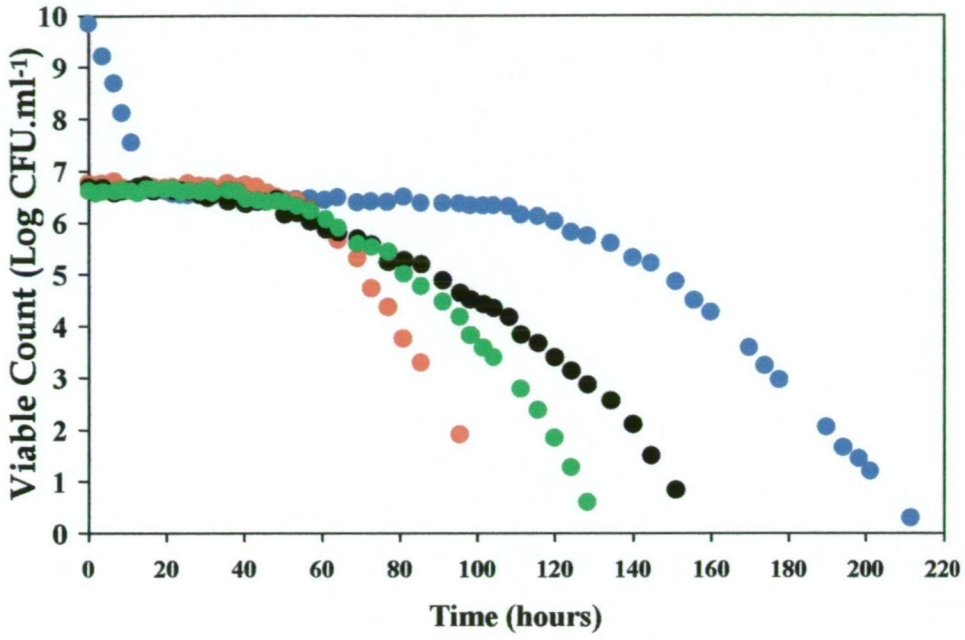


Figure 4.2 Time-dependent low pH-induced [pH 3.5, 35°C] loss of viability of three dilute stationary phase populations of *E. coli* M23 [dilute 1:1000 in fresh TSB (●), spent TSB (●), and heat-treated spent TSB (●)], and of the stationary phase population employed in their preparation (●).

before a second rapid phase of inactivation began one hundred hours later. By contrast the dilute stationary phase populations exhibited only two phases of inactivation, the first being characterised by a negligible decline in viability and the second by the rapid and complete loss of population viability. While corresponding to the second and third phases of inactivation exhibited by the stationary phase population, the onset of the phase of rapid and complete inactivation occurred much earlier in the dilute stationary phase populations than in the non-dilute population. In addition, Figure 4.2 illustrates that during the second phase of inactivation exhibited by the dilute stationary phase populations the viability of the population prepared in fresh TSB declined more rapidly than that of the population prepared in heat-treated spent TSB which, in turn, declined more rapidly than that of the population prepared in spent TSB.

4.4.2.2 *Further Experiments with Dilute Stationary Phase Populations*

Figure 4.3 illustrates the time-dependent loss of viability of six dilute stationary phase populations of *E. coli* M23 exposed to an inimical low pH treatment [pH 3.5, 25°C]. Prepared from the same stationary phase culture populations A, B, and D exhibited biphasic inactivation kinetics with an initial period of negligible decline in viability being followed by a period of rapid decline. The kinetics of inactivation exhibited by population E are analogous. On the basis of the viable count datasets obtained for populations B and D it is evident that the onset of the second phase of inactivation occurred more rapidly in the presence of nalidixic acid than in its

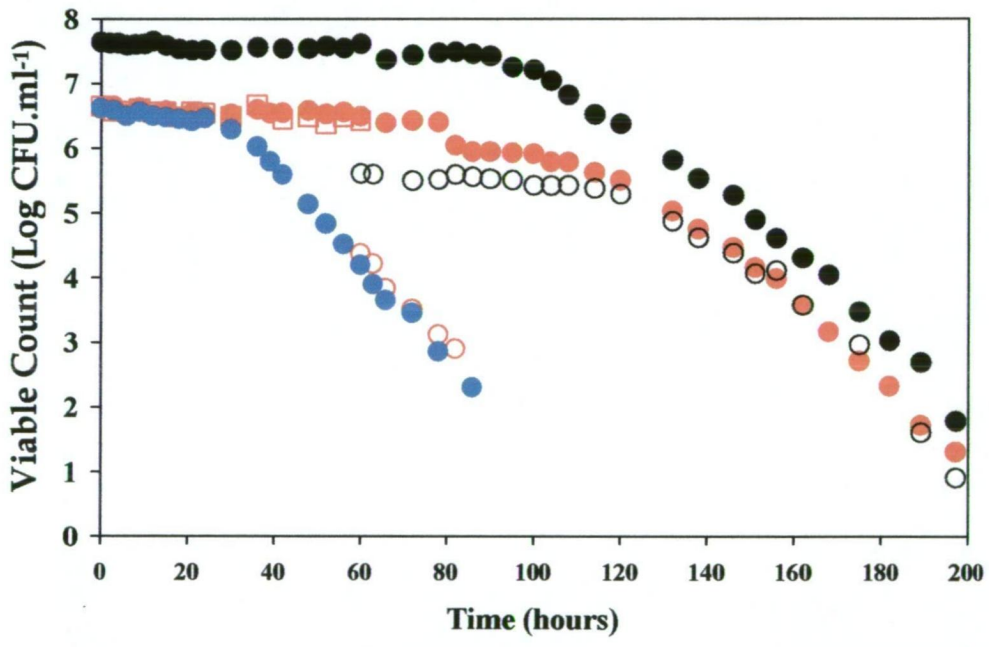


Figure 4.3 Time-dependent loss of viability of dilute stationary phase populations of *E. coli* M23 exposed to an inimical low pH treatment [pH 3.5, 35°C]. Populations A-D were prepared by diluting a stationary phase population 1:100 in fresh TSB pH 7 (A/●), 1:1000 in fresh TSB pH 7 (B/●), 1:1000 in fresh TSB pH 3.5 (C/□), and 1:1000 in fresh TSB pH 7 containing 15 μg.ml^{-1} nalidixic acid (D/●). Populations E (O) and F (○) were prepared by diluting the low pH-treated populations A and B, respectively, 1:100 in fresh TSB pH 7.

absence. By comparing the survival curves of populations A, B and D it becomes apparent that while the magnitude of the dilution did not influence the rates of loss of viability, it may have altered the duration of the first phase of inactivation. Although the data are not entirely clear, the duration of the first phase observed for population B does appear somewhat longer than that observed for population A. That the onset of the second phase of inactivation occurred later in population E than in population A also supports that notion. Counter, however, is the fact that population D exhibited only a single rapid phase of inactivation, reflecting the behaviour of its parental population (i.e. population B) only in terms of the final rate of inactivation. The viability of population C, prepared and challenged as a means of assessing the influence of the pH of the dilution medium on the subsequent viability of a population, was monitored over a relatively short time frame. The data obtained, however, clearly illustrate that the pH of the dilution medium has no influence on the initial rate of inactivation of the population prepared (compare population B with population C).

4.4.3 Investigating Phenotypically Acquired Resistance as a Basis for Non-Linear Inactivation Kinetics

The time-dependent low pH-induced [pH 3.0, 35°C] loss of viability of exponential phase *E. coli* M23 challenged in the presence and absence of chloramphenicol is illustrated in Figure 4.4. No consistent difference is apparent in the kinetics of inactivation exhibited by the two populations.

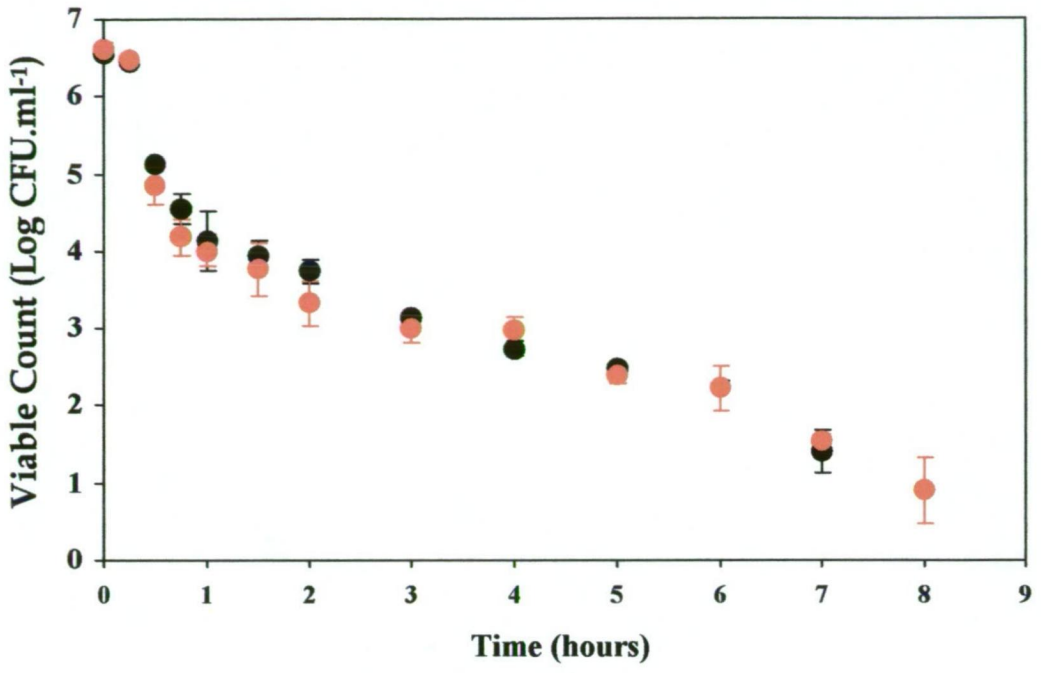


Figure 4.4 Time-dependent low pH-induced [pH 3.0, 35°C] loss of viability of *E. coli* M23 challenged in the presence (●) and absence (●) of chloramphenicol (25 μg/ml).

4.4.4 Investigating the Potential for pH Amelioration in *E. coli* Populations Exposed to an Inimical Low pH Treatment

Figure 4.5 illustrates the temporal changes that occurred in the pH of an 18-hour[1] culture of *E. coli* M23 whose pH was initially set at a value of 3.5. Similar datasets were obtained from twenty-three cultures that ranged from purely exponential to late stationary phase. Where an increase in the pH of a culture was observed, the pH rose rapidly before reaching a plateau. This trend is exemplified by the dataset presented in Figure 4.5.

[1] The population was prepared by incubating a dilute [1:1000 in TSB pH 7] 2^0Exp phase culture at 25°C for 18 hours.

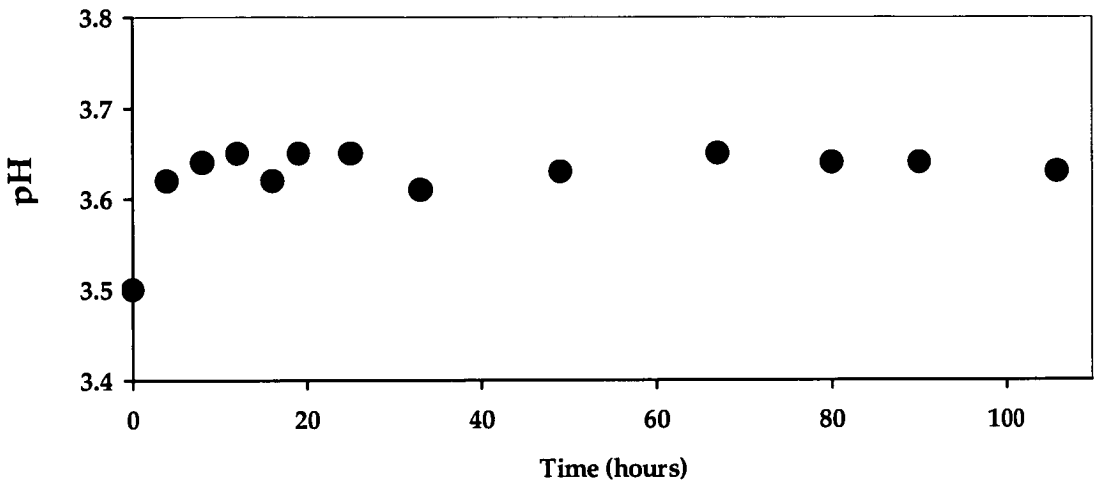


Figure 4.5 Temporal changes in the pH of an 18 hour culture of *E. coli* M23 whose pH was initially set at a value of 3.5.

Figure 4.6 illustrates the average value of the pH 'plateau' as a function of the physiological age of a culture. While the pH of purely exponential phase cultures rose above its initial value marginally, if at all [≤ 0.06 pH units], the pH of cultures whose physiological age was between 10 and 30 hours rose by 0.1 pH unit or more. The largest increase observed (0.26 pH units) occurred in the 11-hour culture, with the magnitude of the increase observed declining gradually amongst older cultures.

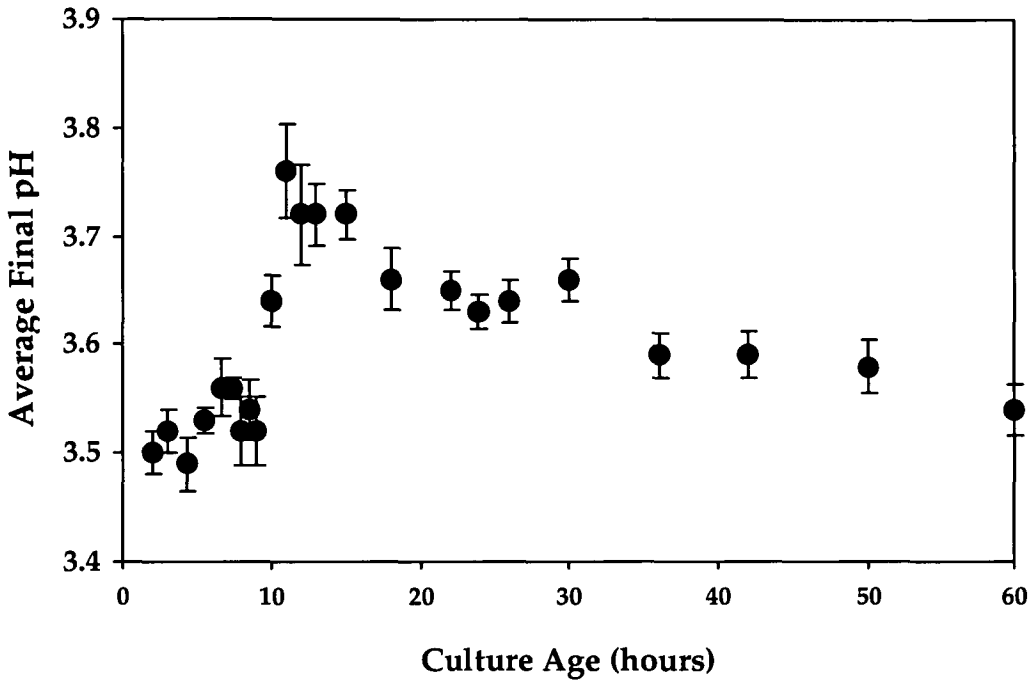


Figure 4.6 Influence of physiological age on the average value of the pH 'plateau' of *E. coli* cultures whose pH was initially set at a value of 3.5.

4.5 DISCUSSION

4.5.1 Non-Linearity - Experimental Artefact or Genuine Feature of Survivor Curves?

Hypotheses concerned with the basis of the shape of non-linear log survivor-time plots can be grouped into two main classes: those that explain non-linearity as an experimental artefact and those that account for it as a genuine phenomenon (Figure 4.7). Proponents of the first class of hypothesis have identified four potential causes of non-linearity:

1) genetic inhomogeneity (Ball and Olson, 1957; Prokop and Humphrey, 1970; Cerf, 1973; Stumbo, 1973);

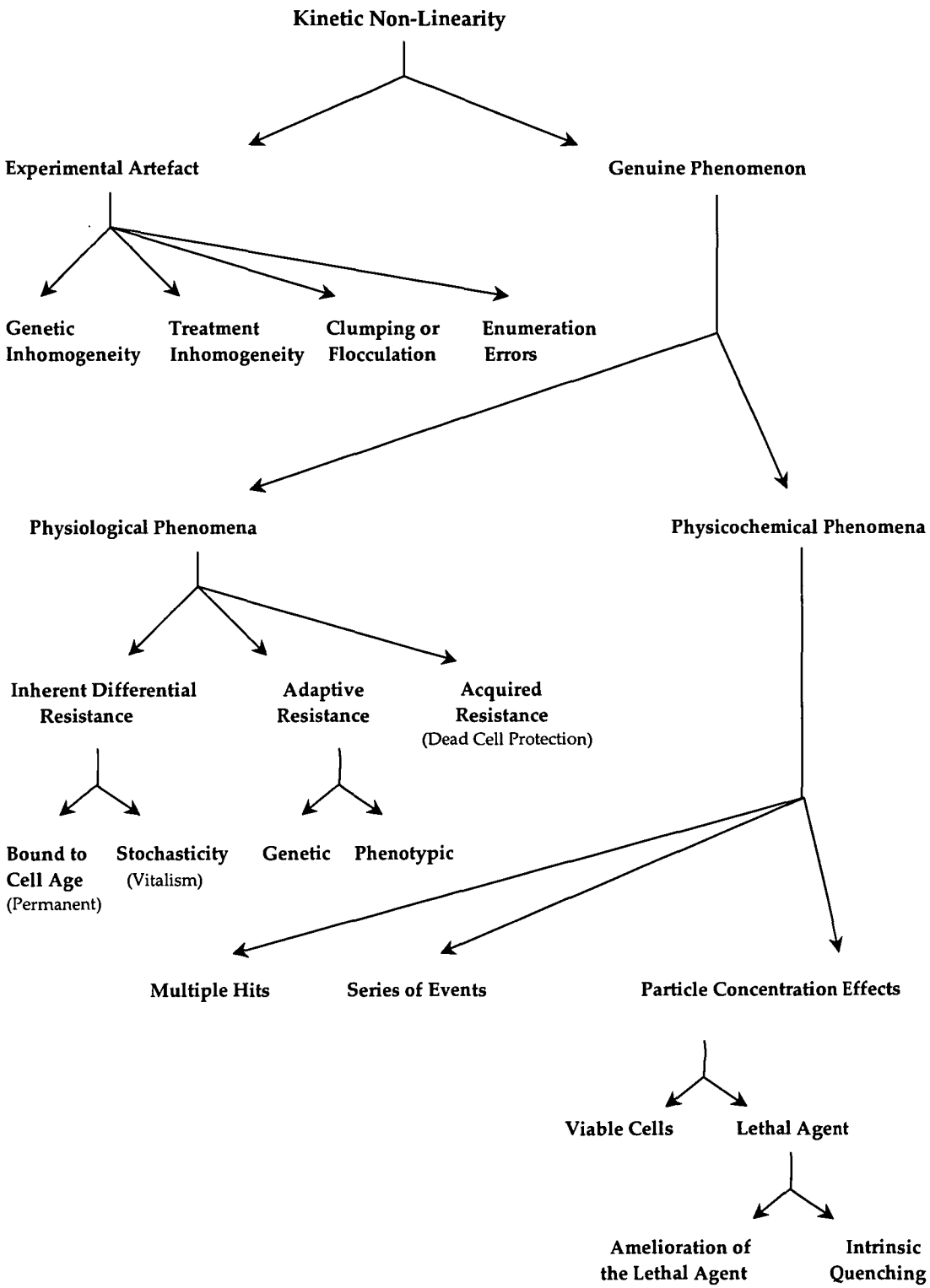


Figure 4.7 Classification of hypotheses concerned with the basis of the shape of non-linear log survivor-time plots used in the present discussion.

2) lack of 'treatment' uniformity [e.g. by way of local variations in temperature or concentration of the lethal agent] (Roberts and Ingram, 1965; Stumbo, 1973; Teo et al., 1996);
3) clumping or flocculation (Rahn, 1945; Stumbo, 1973; Stringer et al., 2000); and
4) errors in the methods employed for the enumeration of survivors (Chick, 1910; Roberts and Hitchins, 1969; Prokop and Humphrey, 1970; Stumbo, 1973).

While experimental artefacts undoubtedly account for a proportion of the non-linear survival curves present in the literature experiments can be, and have been, designed to eliminate kinetic artefacts. In the present study precautions were taken to ensure that experimental populations were genetically homogeneous and that each population as a whole experienced a uniform 'treatment'. On the basis of the microscopic inspection of samples withdrawn during different phases of inactivation, clumping and flocculation were also discounted as potential causes of the non-linearity observed.

The fourth potential source of artefactual non-linearity noted above is less readily eliminated and therefore warrants particular consideration. Prokop and Humphrey (1970) noted that kinetic artefacts may result from counting techniques that yield inherently variable results at low cell densities. In the present study, specific measures were taken to enhance the reliability of counts obtained when population viabilities were low (for further details the reader is referred to section 3.3.2.1). In addition, the fact that similar non-linear patterns of inactivation were observed at both low and high cell densities (see Figure 3.8) implies that the non-linearity observed at low cell densities is genuine. Roberts and Hitchins (1969) and Stumbo (1973) noted that the enumeration protocol employed may also affect the

observed D ('death rate') value. Ross (pers. comm., 1999), seeking to explain the occurrence of non-linear inactivation kinetics developed that idea suggesting that if the true surviving population is not recovered by the enumeration method employed, an injury-recovery phenomenon may give rise to triphasic inactivation kinetics such as those described in this study. That concept is illustrated in Figure 4.8. While significant efforts were made to optimise the enumeration protocol employed in the present study (see Chapter 2) it is not possible, at present, to demonstrate unequivocally that a given method facilitates recovery of all the viable cells in a sample. If failure to recover the true surviving population was to account for some or all of the non-linearity observed, however, it should be possible to demonstrate the occurrence of an injury-recovery phenomenon. To investigate the

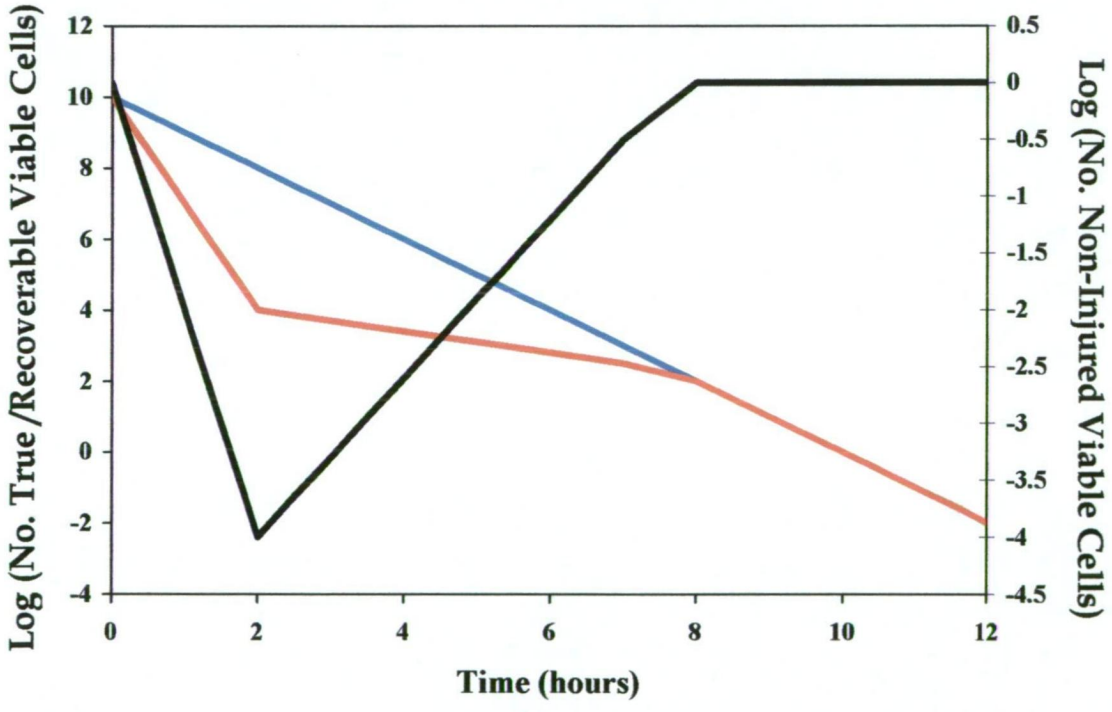


Figure 4.8 Diagrammatic representation of an injury-recovery phenomenon that could account for the occurrence of triphasic inactivation kinetics. Illustrated are time-dependent changes in the true number of viable cells [both recoverable and non-recoverable] (_), in the number of recoverable viable cells (_), and in the number of non-injured viable cells (_). Figure constructed by Ross (pers. comm., 1999).

possible occurrence of such a phenomenon in *E. coli* populations exposed to inimical low pH constraints two methods were employed. The first relies on the differential sensitivity to bile salts exhibited by injured and non-injured cells and thus yields an estimate of the number of cells sustaining membrane-associated injury. The second method employs lag phase duration as an indicator of injury and relies on the assumption that the larger the proportion of injured cells in an inoculum (or alternatively the more extensive the injury sustained by a similar number of injured cells), the greater the time required to repair injury and initiate growth. No indication of the nature of the injury sustained, or of the 'distribution' of injury within a population is provided by that method. While the data obtained with both methods (Figure 3.5 and Figure 4.1 illustrate the data obtained with the first and second method respectively) demonstrate the occurrence of injury in populations of *E. coli* exposed to inimical low pH constraints, neither dataset illustrates a recovery phenomenon that could account for the triphasic nature of the inactivation kinetics observed. On the basis of that observation, and the foregoing remarks, it is concluded that the triphasic inactivation kinetics observed in the present study are not due to methodological limitations or errors.

4.5.2 Explaining Kinetic Non-Linearity as a Genuine Phenomenon

As noted in the introduction, the vitalistic and mechanistic hypotheses were founded on alternate assumptions – the former on the assumption that the individuals of an isogenic population are not identical but that they exhibit inherent differences in resistance to inimical agents, and the latter on the assumption that the individuals of an isogenic population[1] are identical in their

[1] Note that Chick (1908, 1910) also stipulated that the population must be uniform with respect to cell age.

resistance to inimical constraints. In attempting to justify their views, proponents of the vitalistic hypothesis have argued that the mechanistic assumption is unreasonable, pointing out that "variation is a fundamental law of living matter, and to postulate a uniform cell population is to assume a condition that has never been demonstrated" (Withell, 1942). Their opponents have generally responded by directing attention to " .... the fact that biological characteristics are distributed as a rule in a manner quite different from that which they [the vitalists] ... assumed in formulating their theory" (Lee and Gilbert, 1918). While theoretical objections to both assumptions have been raised, experimental evidence clearly negating one or the other has not been presented.

Although conceived to explain log linear inactivation kinetics, the vitalistic hypothesis has been employed, and the mechanistic hypothesis modified, to explain kinetic non-linearity as a genuine phenomenon. Several alternative hypotheses, formulated without regard for the mechanistic and vitalistic schools of thought, have also been presented. In the discussion that follows hypotheses advanced to explain kinetic non-linearity as a genuine phenomenon are grouped into two main classes: those that explain the shape of survivor curves in terms of physiological (resistance) phenomena and those that explain it in terms of physicochemical phenomena (Figure 4.7).

4.5.2.1 *Hypotheses that Explain the Shape of Survivor Curves in terms of Physiological (Resistance) Phenomena*

Inherent Differential Resistance (The Vitalistic Hypothesis)

As noted earlier, the vitalistic hypothesis states that the individuals of a clonal population exhibit inherent differences in resistance to inimical agents and that the shape of survivor curves is determined by the distribution of individual resistances.

In attempting to evaluate the vitalistic hypothesis, its advocates and opponents have encountered the same difficulty – an inability to quantify the resistance of individual cells and thereby demonstrate that the individuals of a population are, or are not, differentially resistant. Falk and Winslow (1926) alluded to that quandary observing [as noted in Chapter 3] that the "... resistance of a cell ... is not an independently measurable factor. Resistance is known only as a function of time .... it represents, therefore, a state whose existence is *assumed* but not *proved*." That statement remains true today and, therefore, a different approach must therefore be employed to resolve the issue at hand.

Consider the resistance of a bacterial population. Like that of an individual cell, the resistance of a bacterial population is (at present) known only as a function of time. While it is impossible to determine experimentally whether the time an individual survives is determined by its resistance, by chance (i.e. stochastically), or by both, it is possible to demonstrate that the time a (large) population survives is not determined stochastically. Consequently it is possible to quantify the *average* resistance of a bacterial population. Consider two bacterial populations (A and B) who, exposed to the same inimical treatment, exhibit the inactivation kinetics depicted in Figure 4.8. Population A is more resistant to the treatment imposed than population B, but it can only be so for one of two reasons. Either the mechanism of inactivation is different in the two populations or the constituent cells of population A are *on average* more resistant than those of population B. While the former argument may apply when the two populations are genetically different, it cannot apply if the populations are genetically indistinguishable. In such a case one must conclude that the cells of the two populations *are* differentially resistant. Since the resistance of a population as a whole reflects the resistance of its constituent cells (uniform or not), it is also evident that the survivor curves of two

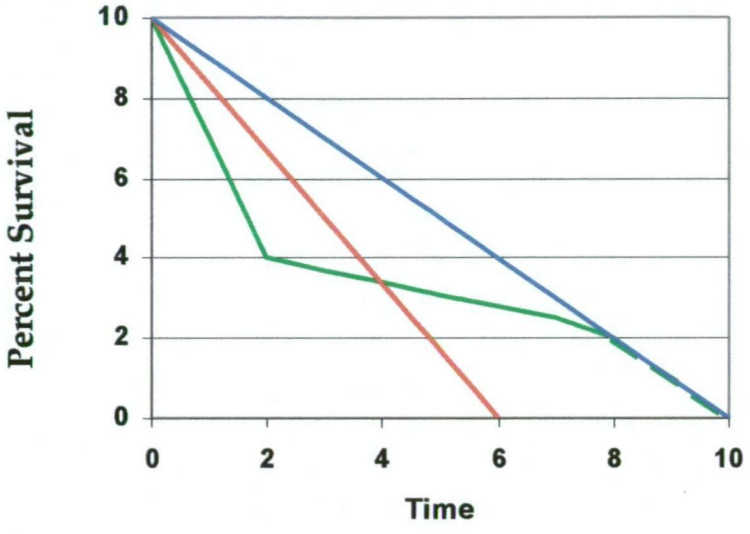


Figure 4.8 Diagrammatic representation of the loss of viability of three bacterial populations (A _ , B _ and C _) exposed to the same inimical treatment. Note that the initial viable count of these three theoretical populations is the same.

genetically identical populations of equal resistance[1] must assume the same form unless the resistance of the individuals of at least one of the populations is not uniform. If two genetically identical populations of equal resistance exhibit different inactivation kinetics when exposed to the same inimical treatment (exemplified by the inactivation kinetics of populations A and C in Figure 4.8), one must conclude that the individuals of at least one of the populations are differentially resistant.

While such a situation is theoretically possible one must ask whether or not it can be demonstrated experimentally, and if so, what the nature of the differential resistance is. Consider again the data presented in Figure 3.7. That data depicts

[1] Note the importance of defining population resistance in terms of the time taken for 'complete' loss of population viability.

time-dependent loss of viability of four genetically identical populations exposed to the same low pH treatment. It clearly illustrates that the low pH resistance of two genetically identical populations can differ (compare the 1^0Exp population with the 3^0Exp population) and that two genetically identical populations of equal resistance can exhibit different inactivation kinetics when exposed to the same inimical treatment (compare the 2^0Exp population with the 4^0Exp population). One is led to conclude, therefore, that the low pH resistance of a population reflects the resistance of its constituent cells and that the resistance of individual cells within a genetically homogeneous population is not uniform. Since the populations whose inactivation is depicted in Figure 3.7 differed only in the (predicted maximum) number of residual stationary phase cells (i.e. in the potential number of individuals that had not begun to divide), it is also concluded that the resistance of a population is bound, to some extent, to the physiological age of its constituent cells. Chick (1908, 1910) reached the same conclusion but maintained her allegiance to the mechanistic school of thought, contending that while kinetic non-linearity (and specifically tailing) can be attributed to 'permanent' differences (i.e. those bound to cell age) in the resistance of individuals within a population, in an ideal case (i.e. in the case of a population whose individuals are of a uniform age), the resistance of individual cells will be identical and inactivation will proceed in accordance with the logarithmic law of decline. Srivastava and Thompson (1965) provided data that clearly negates the mechanistic assumption, however. Using a synchronous culture, those authors demonstrated that the resistance of *E. coli* (in their case to phenol) varies with the stage of the cell cycle, resistance being a minimum when cells are dividing. Since even the cells of a purely exponential phase population exhibit a distribution with regard to stages of the cell cycle, it follows that there is a distribution of resistance within such populations (i.e. that the individuals are differentially resistant).

Not only do the individuals of exponentially growing clonal populations exhibit differences with regard to stages of the cell cycle, they exhibit compositional, and consequently physiological, differences. Such differences stem from stochasticity, or noise, in gene expression (Elowitz et al., 2002) and are realized as variations in the transcriptomes, proteomes, and metabolomes of individual cells. Elowitz et al. (2002) have suggested that these subtle cell-to-cell variations may play crucial roles in determining the fate of individual cells. With the identification of molecular markers[1] or distinct expression profiles that lead the cells of a 'homogeneous' population to respond differentially to the same inimical constraint it may be possible, in the future, to confirm that notion, to 'measure' the resistance of individual cells, and to characterize the distribution of individual resistances within a population.

Having concluded that there will be a distribution of resistance states within a clonal population, and that the resistance of a population as a whole reflects a weighted average of the individual resistances, it is necessary to ask whether or not the shape of inactivation curves can be described fully by the distribution of resistance states within a population. Several proponents of the vitalistic hypothesis (Withell, 1942; Jordan and Jacobs, 1944; Jordan et al., 1947; Augustin et al., 1998; Peleg and Cole, 1998; Peleg, 2000) have attempted to illustrate agreement between a particular type of distribution (e.g. the normal, log-normal, Weibull, or Fermi distribution) and the "distribution of resistances of a population" derived from experimental datasets. Because the resistance of individual cells cannot (yet) be measured, however, the accuracy with which a theoretical distribution reflects the true distribution of resistances within a population cannot be verified. Consequently inactivation models developed on the basis of theoretical resistance

[1] A potential molecular resistance marker is evaluated in Chapter 5.

distributions cannot be used to reliably predict responses outside the range of conditions tested.

In the present study, the vitalistic hypothesis was evaluated in a manner that rendered the determination of the true distribution of individual resistances within a population unnecessary. It was argued that if the shape of log survivor-time plots is determined only by the inherent distribution of individual resistances within a population that physiologically identical populations would yield survivor curves that were identical in shape irrespective of their initial cell densities. To test that assumption the inactivation kinetics of dilute stationary phase populations were investigated. The data obtained (Figure 4.2 and Figure 4.3) clearly illustrate that non-dilute stationary phase populations exhibit three phases of inactivation when exposed to an inimical low pH treatment, while dilute stationary phase populations exhibit only two phases of inactivation (corresponding to the second and third phase of inactivation exhibited by non-dilute stationary phase populations). While the individuals of a clonal population will exhibit inherent differences in terms of their resistance to inimical constraints, this finding demonstrates that the shape of survivor curves depicting the low pH-induced loss of viability of *E. coli* is not determined by the distribution of resistances alone.

Having demonstrated that the inherent differential resistance of individuals within a population cannot fully account for the shape of log-survivor time plots depicting the low pH-induced loss of viability of *E. coli* the discussion turns now to the evaluation of hypotheses that have been advanced to explain the two main types of deviation from linearity observed (i.e. shoulders and tails).

Adaptive Resistance (Genetic or Phenotypic)

A number of investigators studying the loss of viability of microbial populations have suggested that tailing may result from the prolonged survival of a small number of cells that develop resistance *during* the inimical treatment. Gage and Stoughton (1906) entertained the possibility that the 'resistant survivors' of heat-treated populations of *B. coli* had acquired adaptive mutations and attempted, as did Vas and Proszt (1957), Moats et al. (1971), and Humpheson et al. (1998), to propagate a population that exhibited enhanced resistance to thermal stress by successively sub-culturing cells from the resistant tails of heat-treated populations. Those attempts to develop a population 'superior' in terms of its thermal tolerance failed, leading the authors to conclude that those cells surviving into the tail were not genetic mutants or, that if they were, the mutations were very unstable. Booth (pers. comm., 1998) working with low pH-treated populations of *E. coli* arrived at the same conclusion.

By contrast phenotypic adaptation has been endorsed as the basis of several tailing phenomena. Humpheson et al. (1998) suggested that the tailing they observed in *Salmonella* populations exposed to inimical thermal constraints resulted from the *de novo* synthesis of protective proteins in a proportion of the population. They presented experimental evidence supporting that hypothesis by illustrating that the tailing observed was substantially reduced by the addition of chloramphenicol (an inhibitor of protein synthesis). Shadbolt et al. (1999) subsequently illustrated that the occurrence of tailing in populations of *E. coli* exposed to inimical water activity constraints is substantially reduced by the addition of chloramphenicol. That said, data obtained from analogous experiments performed in the present study (Figure 4.4) illustrate that the kinetics of inactivation exhibited by populations of *E. coli* subjected to inimical low pH constraints are not influenced by the presence of chloramphenicol. Studies published recently by Booth et al. (1999) and Jordan et al.

(1999b) corroborate that observation. Consequently it is concluded that enhanced resistance, acquired by means of *de novo* protein synthesis, does not explain the non-linearity of survivor curves depicting the low pH-induced loss of viability of *E. coli*. In addition, data presented in Figure 3.4D illustrate that the duration of Phase 1 decreased as the stringency of the inimical treatment employed increased. If the occurrence of unusually slow rates of inactivation (Phase 2 in the present study) were attributable to phenotypic adaptation, the reverse scenario would be expected.

Acquired Resistance - Dead Cell Protection

The possibility that cells surviving for extended periods (i.e. into the tail) are resistant to the inimical constraints imposed because they acquire compounds released into the medium by cells killed during the initial stages of inactivation was first entertained by Lange (1922, cited by Rahn, 1945). Rahn (1945) indicates that Lange demonstrated that the rate of death of *B. coli* and *Staphylococcus aureus* exposed to inimical thermal constraints decreased two- to three-fold when dead cells of the same species were added to challenge medium. By contrast, Moats et al. (1971) and Humpheson et al. (1998) subsequently illustrated that dead bacteria in the heating menstrum provided no protective effect. In contemplating these conflicting reports, it is pertinent to note that the survival data obtained when dead cells are added to a viable population (and visa versa) are likely to be confounded by several factors. The most obvious of these is a reduction in the oxygen tension of cultures at high cell densities. Several observations made during the course of the present study provide clear evidence that the reduced rate of inactivation observed in *E. coli* populations subjected to inimical low pH constraints (i.e. Phase 2) cannot be attributed to the survival of cells that acquire resistance via the uptake of dead cell material, however. The first observation that leads to that conclusion was drawn from the data presented in Figures 3.7 and 3.8. That data illustrates that in purely exponential phase populations the fraction of cells surviving into Phase 2 is

not constant but that it decreases as the initial viable count increases. That dilute stationary phase populations exhibit a phase of negligible decline, analogous to the second phase of inactivation observed in non-dilute stationary phase populations, at the onset of a low pH challenge also demonstrates that Phase 2 is not attributable to a state of cellular resistance afforded by dead cell material.

4.5.2.2 *Hypotheses that Explain the Shape of Survivor Curves in terms of Physicochemical Phenomena*

The discussion turns now to the evaluation of those hypotheses that originated from the mechanistic school of thought and which explain the shape of survivor curves in terms of physicochemical, rather than physiological, phenomena. In its purest form the mechanistic hypothesis (described also as the 'monomolecular theory' or the 'quantum theory of cell destruction') demands that the individuals of a population are identical and that cell death is the result of a single event. That event has often been described as a random encounter (*hit*) between the lethal agent (*bullet*) and a critical molecule (*target*) within the cell. Accordingly, the process of inactivation is considered analogous to a monomolecular reaction of the first order or a bimolecular reaction with one reagent (the lethal agent) being present in vast excess, and in that instance the reaction kinetics are log linear. The mechanistic hypothesis has been modified to account for the two main types of deviation from linearity observed amongst survivor curves, that is for the occurrence of unusually slow rates of inactivation during the initial stages of inactivation (i.e. a 'shoulder' or convex curve) and for the occurrence of unusually slow rates of inactivation during the final stages of inactivation (i.e. a 'tail' or concave curve).

Multiple hit hypotheses

Modifications of the mechanistic hypothesis advanced to account for the occurrence of unusually slow rates of inactivation during the initial stages of inactivation can

be described as the 'multiple hit' hypotheses. The simplest 'multiple hit' hypothesis was presented by Atwood and Norman (1949) who illustrated that if $n > 1$ hits are required to kill an organism the survivor curve will be rectilinear, the breadth of the shoulder increasing with the number of hits required to cause death. An alternate hypothesis advanced by Rahn (1929) accounts for the same phenomenon by assuming not only that $n > 1$ hits are required to cause the death of a microorganism, but also that $n > 1$ targets must be hit at least once. A mathematical treatment of the two models illustrates that they describe curves of the same general form and that under special circumstances (e.g. for high values of n) they describe identical curves (Atwood and Norman, 1949).

In their 1966 treatise on the death of bacterial populations Dean and Hinshelwood contended that to describe the loss of viability of a microorganism in terms of targets and bullets is unnecessarily crude. They suggested that instead the second tenet of the mechanistic hypothesis be met by assuming that the death of a cell results from an "appropriate conjunction of random events". Hinshelwood (1951) had previously described the cell as "the seat of a complex pattern of chemical reactions". He postulated that when growth is precluded intracellular reactions continue for some time but in a non-integrated manner (since the equilibrium between anabolic and catabolic reactions is disturbed) and that, consequently, a cell loses viability not at the moment its growth is precluded but when a certain number of independent cellular processes, that occur with quasi-periodicity (since they are governed by a complex of non-integrated reactions), occur simultaneously. Hinshelwood noted that in so far as the periodic phenomena discussed are superimposed on a general decline the occurrence of an unusually slow rate of inactivation during the initial stages of inactivation is not improbable, stating that one needs only to assume "... that until a certain stage in the general decay of the cell economy has been reached even an adverse combination of the quasi-periodic

factors does not necessarily preclude recovery". Hinshelwood's hypothesis is thus a 'sophisticated form' of the multi-hit hypotheses presented by Rahn (1929) and Atwood and Norman (1949).

A Series of Related Events

The first hypothesis that explains unusually slow rates of inactivation that occur during the final stages of inactivation as the result of a purely physicochemical phenomenon was presented by Winslow and Falk (1920). They postulated that cell death results not from a single event, nor from multiple independent events, but from a series of related events. That idea was subsequently advocated by Komemushi and Terui (1967), and Brannen (1968) who developed a mathematical model that yielded convex curves by assuming that death results from several reactions of the first order.

Particle Concentration Effects

Several hypotheses have been presented that attribute declining rates of microbial inactivation to 'the particle concentration effect' – that is, to a decline in the concentration of reacting species. Such hypotheses have been formulated in terms of the declining probability of a lethal event (collision) between a viable cell (target) and the lethal agent (bullet), determined by:

i) the *concentration of viable cells*, and

ii) the [effective] *concentration of the lethal agent.*

i) *The Probability of a Lethal Event is Determined by the Concentration of Viable Cells*

Casolari (1981) invoked the particle concentration effect to explain the shape of thermal inactivation curves. He postulated that cellular targets are inactivated when cells are struck by water molecules having a level of kinetic energy sufficient to cause light intracellular molecules to collide with the less motile cellular targets

(macromolecules) in a disorderly manner and with sufficient energy to disrupt intermolecular bonds. That the probability of such events would decline as the number of living cells declined was, he suggested, the cause of the 'tailing off' of survivor curves. Casolari (1981) also presented an analogous model for chemical inactivation kinetics.

ii) *The Probability of a Lethal Event is Determined by the Effective Concentration of the Lethal Agent*

Often it is assumed that lethal chemical agents are present in such quantities that their concentration can be considered constant throughout the duration of an experiment (e.g. Chick, 1908; Lee and Gilbert, 1918). Johnston et al. (2000) and Lambert and Johnston (2000) challenged that assumption, postulating that the concentration of the lethal agent declines during the course of an experiment and that such a decline results in a decline in the death rate coefficient. Those authors presented a mechanistic model in which the lethal agent is 'quenched'/inactivated in a process that follows the kinetics of a first order chemical reaction and suggested that the membranes of cells ruptured by the lethal agent may effect the quenching process. Along similar lines, Watkins and Winslow (1932) suggested that a reduction in the rate of inactivation of a population might be caused by the elimination of products that neutralize or inhibit the action of the disinfecting agent. Their hypothesis stemmed from the observation that *B. coli* liberates acidic substances when suspended in alkaline solutions, and alkaline substances when suspended in acidic solutions (Winslow and Falk, 1923; Shaughessy and Falk, 1924; Shaughessy and Winslow, 1927). To test the possibility that the reduced rates of inactivation observed in the present study (Phase 2) may be attributable to alkalinization of the medium the pH of twenty-three cultures of varying physiological age was monitored during the course of a low pH challenge. Adjusted initially to a value of 3.5, the pH of many of the cultures rose by a

measurable amount (Figure 4.5 and Figure 4.6). Only low density purely exponential phase populations and a very late stationary phase population (cultivated 60 hours from the time of inoculation) effect no substantial change in the pH of the culture medium. The pH of few of the cultures rose above the growth/no growth boundary of *E. coli* M23 (i.e. pH 3.7), however. That all of the populations examined exhibited a reduced rate of inactivation without all effecting a measurable change in the pH of the culture medium, that the pH increases observed occurred much faster than the onset of the reduced rate of inactivation, and that dilute stationary phase populations exhibit 'Phase 2-type' kinetics without increasing the pH of the culture medium, lead one to conclude that the pH increases observed were not responsible for the reduced rate of inactivation observed. Jordan et al. (1999b) also investigated such a possibility. Observing no increase in the pH of a cell-broth mixture adjusted to pH 3.0, they also concluded that alkalinization of the medium as an explanation of the 'resistant tail' could be ruled out.

During the course of the foregoing discussion it has been concluded that:

a) the individuals of bacterial populations are inherently differentially resistant to inimical agents;
b) the distribution of individual resistance states is reflected in the shape of survivor curves but the shape of survivor curves is not determined by the distribution of individual resistance states alone; and
c) hypotheses that explain the shape of survivor curves purely in terms of physicochemical phenomena are not consistent with the kinetics of inactivation exhibited by low pH-treated populations of *E. coli*.

The shape of the survivor curve must, therefore, reflect both physiological and physicochemical phenomena, that is it must reflect both the distribution of

individual resistance states and the mechanism of inactivation. In the discussion that follows, a novel hypothesis is advanced to explain the triphasic inactivation kinetics exhibited by low pH-treated populations of *E. coli*.

4.5.2.3 *Inactivation Curves Reflect both Physiological and Physicochemical Phenomena – A Novel Hypothesis*

With regard to the kinetics of inactivation exhibited by low pH-treated populations of *E. coli* it is here proposed that the mechanism underpinning the loss of viability observed during Phase 1 differs from that underpinning the loss of viability observed during Phase 2 and 3. This notion derives essentially from two observations, the first being that Phase 1 and Phase2/3 are influenced differentially by the stringency of the low pH treatment employed (Figure 3.4), and the second that viable injured cells were detected during the second phase of inactivation but not during the first phase of inactivation (Figure 3.5). Specifically it is postulated:

I) that the loss of viability observed during Phase 2/3 is mediated by low pH *per se* [i.e. directly by a high concentration of hydrogen ions]

II) that the loss of viability observed during Phase 1 is mediated by a substance produced by *E. coli* during its growth (described here as an Alternative Lethal Agent [ALA]) which is not toxic to the cell at growth-permissive pH values but that is 'potentiated' and unconditionally toxic to the cell when the pH is growth non-permissive;

III) that the ALA is labile in the absence of viable cells;

IV) that the ALA is secreted by cells in a manner sensitive to the physiological age/state of a population (i.e. it is not secreted by actively dividing cells in 'purely exponential phase' populations but it is secreted as the cell density/physiological age of a population rises above some critical value); and

V) that the ALA is not produced in exceptionally large quantities (i.e. the number of ALA molecules produced does not exceed the number of viable cells in a population substantially) and that it is therefore quenched during the first phase of inactivation.

With regard to the first and second postulates presented it is noted that the magnitude of the loss of viability observed during Phase 1 is independent of the stringency of the treatment employed except at high temperatures [i.e. 45-50°C] (Figure 3.4), that the duration of Phase 2 decreases with the stringency of the inimical treatment employed (Figure 3.3 and other data not shown), and that non-dilute stationary phase populations exhibit three phases of inactivation [Phase 1, 2 and 3] while dilute stationary phase populations exhibit only two phase of inactivation [Phase 2 and 3] (Figure 4.2 and 4.3). On the basis of data describing the low pH-induced loss of viability of a non-dilute stationary phase population and a dilute stationary phase population prepared in spent broth [i.e. the first experiment performed with dilute stationary phase populations] (Figure 4.2) it was initially tempting to speculate that the loss of viability observed during Phase 1 is dependent on the viable cell density of a population. That no Phase 1-type inactivation was observed in stationary phase populations diluted 1000-fold or 100-fold (Figure 4.3) and that the viable cell density at which Phase 2 was observed is not fixed (Figure 3.8) indicates, however, that the viable cell density *per se* is not responsible for the occurrence of Phase 1-type inactivation kinetics. That the onset of the second phase of inactivation exhibited by dilute stationary phase populations occurs much sooner in the presence of nalidixic acid than in its absence (Figure 4.3) indicates that an inability to repair damaged DNA hastens the onset of the third phase of inactivation. This observation may imply that the loss of viability observed during Phase 3 is the result of 'accumulated injury'.

Data indicating that all of the cells of a population that are injured during the first phase of inactivation are also dead (Figure 3.5) is consistent with the suggestion that the ALA is unconditionally toxic (i.e. death mediated by the ALA occurs in an all-or none fashion). The lability of the hypothetical ALA in the absence of viable cells is suggested to account for the fact that Phase 1-type inactivation was not observed in dilute stationary phase populations prepared in spent broth. In this regard it is important to note that the spent broth was prepared 'in advance' (i.e. 24 hours before the dilute stationary phase population was prepared and challenged).

The fourth point of the hypothesis presented stems from the observation that Phase 1-type inactivation is more extensive (i.e. it is responsible for the death of a greater proportion of the population) in exponential phase populations than stationary phase populations, that dilute exponential phase populations exhibit Phase 1-type inactivation kinetics, and that the 'tail' of exponential phase populations is eliminated by dilution (Shadbolt, pers. comm., 2001).

That Phase 1-type inactivation is not responsible for the death of entire populations implies that a hypothetical ALA molecule would be found in limiting quantities and that it would be quenched as inactivation progressed. That the magnitude of decline observed during the first phase of inactivation decreases with the cell density of non-exponential phase populations (Figure 3.8) lends support to that notion.

If correct, testable corollaries of the hypothesis presented are:

a) that stationary and exponential phase populations diluted in freshly prepared spent broth would exhibit Phase 1-type inactivation; and

b) that stationary phase populations harvested and resuspended in fresh broth at their original concentration would not exhibit Phase 1-type inactivation.

With regard to the hypothesis presented it is interesting to note that Datta and Benjamin (1999) observed that the acid sensitivity of stationary phase populations of *E. coli* is cell density dependent (although they present no kinetic data). They too speculate that stationary phase cultures may produce a diffusible substance that makes them more sensitive to low pH. While evidence has been presented which indicates that *E. coli* produces small diffusible molecule that mediate acid resistance (Rowbury and Goodson 1999; Rowbury and Goodson, 2001), no such compound has been implicated in enhancing acid sensitivity. In studying the effects of the addition of spent medium on the regrowth of starved *E. coli* cells, however, Weichart and Kell (2001) observed that the addition of raw supernatants at high concentrations led to an inhibition of growth (in terms of both growth rate and yield). They reported that a significant fraction of the inhibitory material could be removed by ion-exchange treatment and concluded that the inhibitory effects observed were not a consequence of the lowered substrate concentration (effected by the addition of supernatants), but a consequence of inhibitory products. Weichart and Kell (2001) postulated that such compound(s) might constitute an inhibitory 'signal' excreted by cells in stationary phase. It is tempting to speculate that such a molecule, inhibitory under otherwise growth permissive conditions, might act synergistically with other inhibitory constraints to effect cell death.

In summary, it has been demonstrated that the three phase inactivation kinetics exhibited by low pH-treated populations of *E. coli* are not artefactual and that they are not adequately explained by existing hypotheses advanced to account for non-linear survivor curves. A novel and testable hypothesis consistent with the kinetics of inactivation observed has thus been presented.

CHAPTER 5 EVALUATING THE ROLE OF CYCLOPROPANE FATTY ACIDS AS MOLECULAR MEDIATORS OF LOW PH TOLERANCE IN *E. COLI*

5.1 ABSTRACT

The role of cyclopropane fatty acids (CFAs) as molecular mediators of the low pH tolerance of *E. coli* was evaluated. Initially, the *cfa* mutant *E. coli* YYC1106 and the isogenic wild type strain, FT1, were employed for this purpose. Survival curves depicting the time-dependent low pH-induced loss of viability of exponential phase populations of these strains demonstrated that the low pH tolerance (intrinsic and inducible) of *E. coli* FT1 exceeds that of *E. coli* YYC1106. While this finding lends support to the notion that CFAs play some part in mediating the low pH tolerance of *E. coli*, results demonstrating that *E. coli* YYC1106 exhibits an adaptive response to low pH, and that the magnitude of this adaptive response is similar to the magnitude of the adaptive response exhibited by *E. coli* FT1, prompted further investigations. A comparative analysis of the fatty acid composition and the low pH tolerance of *E. coli* FT1 with that of other wild type strains indicated that *E. coli* FT1 is atypical of wild type strains in several respects that render it, and consequently *E. coli* YYC1106, inappropriate for the purposes of this study. For this reason a new mutant (*E. coli* JBM1) containing an in-frame deletion in *cfa* was constructed from *E. coli* Frag1, a strain shown to be typical of wild type *E. coli* in those respects that *E. coli* FT1 was atypical. No difference in the intrinsic or inducible low pH tolerance of *E. coli* JBM1 and its parental strain was observed and consequently the notion that CFAs mediate, in part, the intrinsic and/or inducible low pH tolerance of *E. coli* was rejected. The findings of this study are discussed in

relation to those of Chang and Cronan (1999) who also sought to evaluate the role that CFAs play in mediating the low pH tolerance of *E. coli*.

5.2 INTRODUCTION

A marked shift in the fatty acid profile of *E. coli*, effected by the post-synthetic conversion of monounsaturated fatty acids (MUFAs) to their cyclopropane derivatives, occurs as cultures enter stationary phase and upon exposure to certain mild physical and chemical stresses (Knivett and Cullen, 1965; Knivett and Cullen, 1967; Cronan, 1968; McGarrity and Armstrong, 1975; McGarrity and Armstrong, 1981; Arneborg et al., 1993; Brown et al., 1997). Esterified into membrane localised phospholipid molecules a significant proportion of the primary MUFAs of *E. coli*, *cis*-9-hexadecenoic acid (16:1ω7c) and *cis*-11-octadecenoic acid (18:1ω7c), are converted *in situ* to their cyclopropane derivatives, *cis*-9,10-methylene hexadecenoic acid (cy17:0) and *cis*-11,12-methylene octadecenoic acid (cy19:0), by the addition of a methylene group at the site of unsaturation. S-adenosyl-L-methionine (S-AdoMet) serves as the methylene donor and cyclopropane fatty acid (CFA) synthase, a 43kDa cytoplasmic enzyme encoded by *cfa*, catalyses this membrane modification (Law et al., 1963; Thomas and Law, 1966; Cronan et al., 1979). While much is known regarding the synthesis of CFAs, the enzyme responsible for their formation, and the temporal and environmental regulation of its cellular abundance, the physiological significance of CFA formation has for many years puzzled investigators.

The conversion of MUFAs to CFAs is both energetically expensive and carefully regulated, and many have argued that it may be an adaptive strategy designed to protect the cell from unfavourable environments by altering the physical and/or chemical properties of the membrane in a biologically relevant manner (Law et al.,

1963; Cronan, 1968; Law, 1971; McGarrity and Armstrong, 1975; Dunkley et al., 1991; Grogan and Cronan, 1997). Studies illustrating that *E. coli* mutants defective in the synthesis of CFA synthase, and hence of CFAs, are not impaired in their ability to withstand a wide variety of inimical conditions (Taylor and Cronan, 1976; Calcott et al., 1984; Grogan and Cronan, 1986) have, however, failed to substantiate this notion.

In a recent study of the acid habituation of *E. coli*, Brown et al. (1997) illustrated a strong correlation between the CFA content and the survival of acid habituated and non-habituated populations of five strains exposed to inimical low pH constraints. This led them to suggest that CFAs may decrease the proton permeability of the membrane and thereby act as molecular mediators of the low pH tolerance (intrinsic and/or inducible) of *E. coli*. This chapter evaluates that hypothesis.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial Strains, Bacteriological Media, Chemical Reagents & Equipment

Details of the bacterial strains, bacteriological media, chemical reagents and equipment employed throughout this study, together with the methods employed for bacterial maintenance and recovery, are presented in Appendix A.

5.3.2 General Methods

5.3.2.1 *Preparation of Acid Habituated and Non-Habituated Populations*

60 ml volumes of TSB were inoculated with individual colonies of *E. coli* and incubated overnight at 37°C with shaking. Parallel exponential phase cultures were prepared subsequently by diluting the overnight cultures 1:1000 in fresh TSB then incubating under the conditions described above until an optical density [OD_{450}] of 0.1 (i.e. a viable count of *ca.* 5×10^7 cfu.ml^{-1}) was attained. At this time the pH of

the growth medium of those cultures destined for habituation was rapidly (within 1 minute) adjusted to pH 5.0 with 10M HCl, while the pH of the growth medium of the non-habituated cultures remained unaltered. Incubation of both the pH adjusted and non-adjusted cultures was continued, as described above, until an optical density [OD_{450}] of 0.26, corresponding to a viable count of *ca.* 1 x 10^8 cfu.ml^{-1}, was attained.

5.3.2.2 *Fatty Acid Extraction, Methylation, and Analysis*

200 ml volumes of acid habituated and non-habituated exponential phase populations were prepared in duplicate (3.3.2.1) and harvested by centrifugation (12 400 g for 10 minutes at 4°C). After being washed twice in 200 ml volumes of sterile saline (0.85% NaCl, w/w) the cell pellets were resuspended in 2 ml volumes of the same. Lipid extraction, by the modified one-phase $CHCl_3$-MeOH-H_2O Bligh and Dyer method (Bligh and Dyer, 1959; White et al., 1979), was performed immediately. An aliquot (50%) of the total solvent extract was saponified with 5% KOH (w/v) in 80:20 MeOH-milli-Q water (v/v) at 60°C. The non-saponifiable neutral lipid fraction was recovered, after addition of milli-Q water, and by extraction with C_6H_{14}-$CHCl_3$ (4:1, v/v). Acidification of the remaining aqueous layer with an excess of mineral acid liberated the free fatty acids which were collected by extraction with C_6H_{14}-$CHCl_3$ (4:1, v/v). Methylation of the fatty acids to yield their fatty acid methyl esters (FAME) was achieved by reaction with MeOH-HCl-$CHCl_3$ (10:1:1, v/v/v) for 1 hour at 100°C.

FAME samples were made up to a known concentration with chloroform containing a C_{23} FAME internal injection standard and analysed by gas chromatography (GC). GC was performed on a Hewlett Packard 5890 gas chromatograph equipped with a 50 m x 0.32 mm internal diameter cross-linked

methyl silicone (HP1) fused-silica capillary column and flame ionisation detector (FID). By means of a Hewlett Packard 7673A automatic sampler, samples were injected at 50°C in the splitless mode with a 2 minute venting time. Temperature-programmed after one minute, the oven temperature rose from 50°C to 150°C at a rate of 30°C min^{-1}, then at a rate of 2°C min^{-1} to 250°C. A final temperature of 300°C was then attained at 5°C min^{-1}, this final temperature being maintained for fifteen minutes. Hydrogen was used as the carrier gas, and the injector and detector were maintained at 290°C and 310°C respectively. Peak areas were quantified using chromatography software.

Selected FAME samples were also analysed by gas chromatography-mass spectroscopy (GC-MS). GC-MS analyses were performed on a Hewlett Packard 5890 gas chromatograph and 5970 mass selective detector (MSD) fitted with a direct capillary inlet and a split/splitless injector. Mass spectral data were acquired and processed on a Hewlett Packard 59970C Workstation operated in scan acquisition mode. Identification of fatty acids (as FAME) was achieved using retention time and mass spectral data. The location and position of the double bonds in monounsaturated FAME were determined by GC-MS analysis of their dimethyl disulphide (DMDS) adducts as described by Nichols et al. (1986). This procedure also served to confirm the identification of cyclopropane fatty acids.

5.3.3 Evaluating the Role of CFAs as Mediators of Low pH Tolerance - Part I

The fatty acid profiles of acid habituated and non-habituated populations (5.3.2.1) of the *cfa* mutant *E. coli* YYC1106, and of its parental strain FT1, were analysed (5.3.2.2). Survival curves depicting the time-dependent loss of viability of habituated and non-habituated populations of *E. coli* FT1 and YYC1106 exposed to growth non-permissive low pH constraints [pH 3.0, 35°C] were also prepared

(3.3.2.1). These survival curves were prepared as a means of evaluating the intrinsic and inducible acid tolerance of the two strains, and consequently, of evaluating the hypothesis that CFAs act as molecular mediators of low pH tolerance in *E. coli*.

5.3.4 Construction of a New E. coli cfa Mutant

5.3.4.1 *Primers, Plasmids, and Bacterial Growth Conditions*

Table 5.1 lists the primers, and Table 5.2 the plasmids, employed in the construction and analysis of a new *E. coli cfa* mutant. The location of the primer binding sites is illustrated in Figure 5.1. For DNA purification and for the selection of specific genetic constructs *E. coli* were grown at 37°C in Luria-Bertani broth (LB) or on the surface of Luria-Bertani Agar (LBA). Selective agents were filter sterilised and added where necessary to LB at room temperature or to molten LBA cooled to

Table 5.1 Primers used in construction and analysis of a new *E. coli cfa* mutant[1].

Primer	Sequence
CFA1F	5′-TAGATGTCTAGAGTGTGATGGCGATTGCC-3′ (*Xba*I)
CFA1R	5′-GCCACTCGGTTGGCGATACGGTACC-3′
CFA2F	5′-TCGCCAACCGAGTGGCTCGCTAAAG-3′
CFA2R	5′-TAGATGGCATGCCATGTGGTGGAGTTACC-3′ (*Sph*I)
CFA3F	5′-AAAAGCACCGCCGGAC-3′
CFA3R	5′-GCAGAAAAAACCGCGAG-3′
CFA4F	5′-TACGGGGACAGGATCG-3′
CFA4R	5′-GCATCAGCGGTATTAGC-3′
CFA5F	5′-TCCCAAGAGTCGTGCG-3′
CFA5R	5′-TGTGGAATTGTGAGCGG-3′
CFAOF	5′-ACCGTGGTTGCTGGTG-3′
CFAOR	5′-CAGTTTGGGCGATTTGC-3′

[1]The underscored bases indicate complementary regions incorporated to facilitate the fusion of two DNA fragments covering the *cfa* termini.

Table 5.2 Plasmids employed in the construction of a new *E. coli cfa* mutant.

Plasmid	Description/Relevant Phenotype	Reference
pUC18	Ap^R	NBL Gene Sciences
pJB1	pUC18 containing a DNA fusion fragment covering the *cfa* termini; Ap^R	This study
pST4	Cm^R, Suc^S	Levina et al., 1999
pJB3	pDM4 (recovered from pST4) containing a DNA fusion fragment covering the *cfa* termini; Ap^R	This study

55°C. Ampicillin was employed at a final concentration of 25 $\mu g.ml^{-1}$, chloramphenicol at 12.5 $\mu g.ml^{-1}$, and both isopropyl-1-thio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) at 0.2 $\mu M.ml^{-1}$. Sucrose (5% w/v) was employed as a selective agent in LBA that contained no NaCl (LBA-S) and an incubation temperature of 30°C was used to assess sucrose resistance.

5.3.4.2 *DNA Manipulation and Sequence Analysis*

Polymerase Chain Reaction (PCR)

Prepared on ice, each PCR reaction (100 µl) contained PCR buffer (10 µl of 10x stock with $MgCl_2$), dNTPs (16 µl of 12.5mM stock), Taq polymerase (0.5 µl), primers (1.25 µl/primer of 20 µM stock), mQ-dH_2O (added to give a final volume of 100 µl) and a DNA template. Generally whole cells picked from a colony with a sterile needle provided the DNA template although in some instances a genomic preparation or plasmid DNA was used. In the latter instances approximately 0.1 µg of DNA was added to the reaction and the volume was taken into account by adjusting the amount of mQ-dH_2O added. The PCR cycle conditions varied with the primers

used and the size of the expected product but in general the cycle parameters fell within the following limits:

94°C for 1 minute (denaturation)

50-56°C for 1 minute (annealing)

72°C for 2-3 minutes (extension)

An initial lysis step of 94°C for 4 minutes was included when whole cells were used as the source of the DNA template. After checking that the correct product had been obtained by running 10 µl of the reaction on an agarose gel, the PCR products were cleaned using Promega's Wizard™ PCR Preps DNA Purification system.

DNA Separation by Agarose Gel Electrophoresis

DNA separation by agarose gel electrophoresis was carried out according to the protocol described by Maniatis et al. (1982).

Klenow Treatment

Klenow was used to blunt-end sticky-ended PCR products used for cloning purposes. Klenow, concentrated dNTPs, and 1 x buffer were added to the cleaned PCR product and the volume made up to 20 µl with mQ-dH_20. The reactions were incubated at room temperature for 30 minutes then at 37°C for 30 minutes. A heat treatment (65°C for 10 minutes) was then employed to inactivate the Klenow and a Hybaid Recovery™ DNA Purification Kit was used to purify the blunt-ended product.

Preparation of Plasmid DNA

The QIAprep Spin Miniprep Kit (Quiagen) was employed for the preparation of plasmid DNA.

Restriction Endonuclease Digestions

Restriction endonuclease digestions were carried out according to the protocol described by Maniatis et al. (1982).

Purification of Vectorial DNA

Plasmid DNA digests were separated by agarose gel electrophoresis. Following excision of the desired band the vectorial DNA was purified by means of the Hybaid Recovery™ DNA purification Kit II and eluted into mQ-dH_2O.

Calf Intestinal Phosphatase (CIP) Treatment

Plasmid DNA that had been restricted to yield compatible ends was treated with calf intestinal phosphatase in order to prevent self-ligation of the plasmid termini. After inactivating the restriction endonucleases used to digest the plasmid DNA with a heat treatment (65°C for 10 minutes) the reactions were cooled to room temperature. 1 x CIP buffer and CIP were added and the reactions were then incubated at 37°C for 60 minutes. A Hybaid Recovery™ DNA Purification Kit was employed to purify plasmid DNA following CIP treatment.

Ligations

DNA fragments to be ligated (vector and insert) were quantified and the volume of each fragment used for ligation was calculated according to the following equation:

$$\left\{ \frac{\text{Concentration of vector x size of insert (Kbp)}}{\text{Concentration of insert x size of vector (Kbp)}} \right\} \times \text{insert:vector ratio}$$

Insert:vector ratios of 0:1, 2:1 and 3:1 were typically employed and a total of 50 ng of DNA was added to each ligation reaction. Ligation reactions were carried out in 15 µl volumes of 1 x T4 DNA ligase buffer (by adjusting with mQ-dH_20). The insert, vector, and mQ-dH_2O were mixed first, heated to 65°C for 2 minutes, then cooled to room temperature before the ligase buffer and ligase (1 unit) were added. For sticky-ended and blunt-ended ligations the reactions were held at 4°C and 15°C, respectively, overnight.

Transformation

A modification of the calcium chloride procedure described by Maniatis et al. (1982) was employed for transformation. Briefly, LB (20 ml) was inoculated with 1 ml of an overnight culture of the strain to be transformed and incubated with shaking at 37°C until an optical density [OD_{650nm}] of 0.4 was attained. Aliquots (1.5 ml) of the culture thus prepared were transferred to sterile eppendorfs and the cells harvested by centrifugation (1 min at 14,000 rpm). The supernatant was removed, the cell pellet was resuspended in 0.5 ml ice-cold $CaCl_2$ (50mM), and the eppendorf tubes placed on ice for 10 minutes. Harvested a second time, the cells were resuspended in 300 µl ice-cold $CaCl_2$ (50mM) and placed on ice for 90 minutes. 1 µl of plasmid or 10 µl of ligation mix was added to the competent cells and mixed by inversion. After incubating on ice for a further 60 minutes, the cells were heat shocked at 42°C for 2 minutes in a waterbath. 1 ml of LB was immediately added to the cells, and the eppendorfs placed at 37°C for 60 minutes. After incubation the cells were harvested by centrifugation (as described above) resuspended in 100 µl LB, and surface plated onto the appropriate antibiotic-containing LB plate. Competent cells with no DNA added were used as a negative control and a plasmid with the same antibiotic resistance as the other transformations was used as a positive control. The plates were incubated overnight at 37°C and transformants purified on fresh antibiotic-containing LB plates.

DNA Sequencing

The ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit was employed for DNA sequencing. Sequencing reactions were performed according to the manufacturer's directions and the extension products obtained were purified by ethanol precipitation. An automated sequencer (ABI A377) was employed for sequence analysis and each sequence was obtained in duplicate from both strands.

5.3.4.3 *Construction of a* cfa *Null Mutant*

A chromosomal deletion in *cfa* was generated by overlap extension PCR (Ho et al., 1989) and allelic exchange using the counter-selectable suicide vector, pDM4 (Milton et al., 1996). The primers used are listed in Table 5.1 and the location of the primer binding sites, and the procedure employed for construction of the *cfa* mutant, are illustrated in Figure 5.1. Designed to yield two ~ 600 bp DNA fragments that cover the *cfa* termini and have complementary 3′ ends, the primer pairs CFA1F/CFA1R and CFA2F/CFA2R were initially employed in separate PCR reactions. The DNA fragments amplified in these reactions were purified and combined 1:1 to generate a fusion fragment containing the desired deletion. Amplified by PCR with the two external primers (CFA1F and CFA2R), the fusion fragment was purified, end filled with Klenow, and ligated into pUC18 that had been digested with *Sma*I and treated with calf intestinal phosphatase to prevent self-ligation. The ligation products were transformed into *E. coli* JM109 and those clones containing the recombinant plasmid (pJB1) were identified as white colonies from the chloramphenicol-resistant sucrose-sensitive colonies obtained. The chromosomal integration of pJB3 was verified by PCR analysis with a primer complementary to the plasmid (CFA5R) and another complementary to the chromosome but outside the chromosomal regions amplified by the primer pairs CFA1F/CFA1R and CFA2F/CFA2R (CFAOF). Following the growth of *E. coli* JBI1 in LB, chloramphenicol-sensitive sucrose-resistant colonies were isolated and screened for plasmid excision and for the desired chromosomal deletion in *cfa* by PCR with the chromosomal primers CFAOF and CFAOR. The Frag1,Δ*cfa* strain obtained in this manner was designated *E. coli* JBM1.

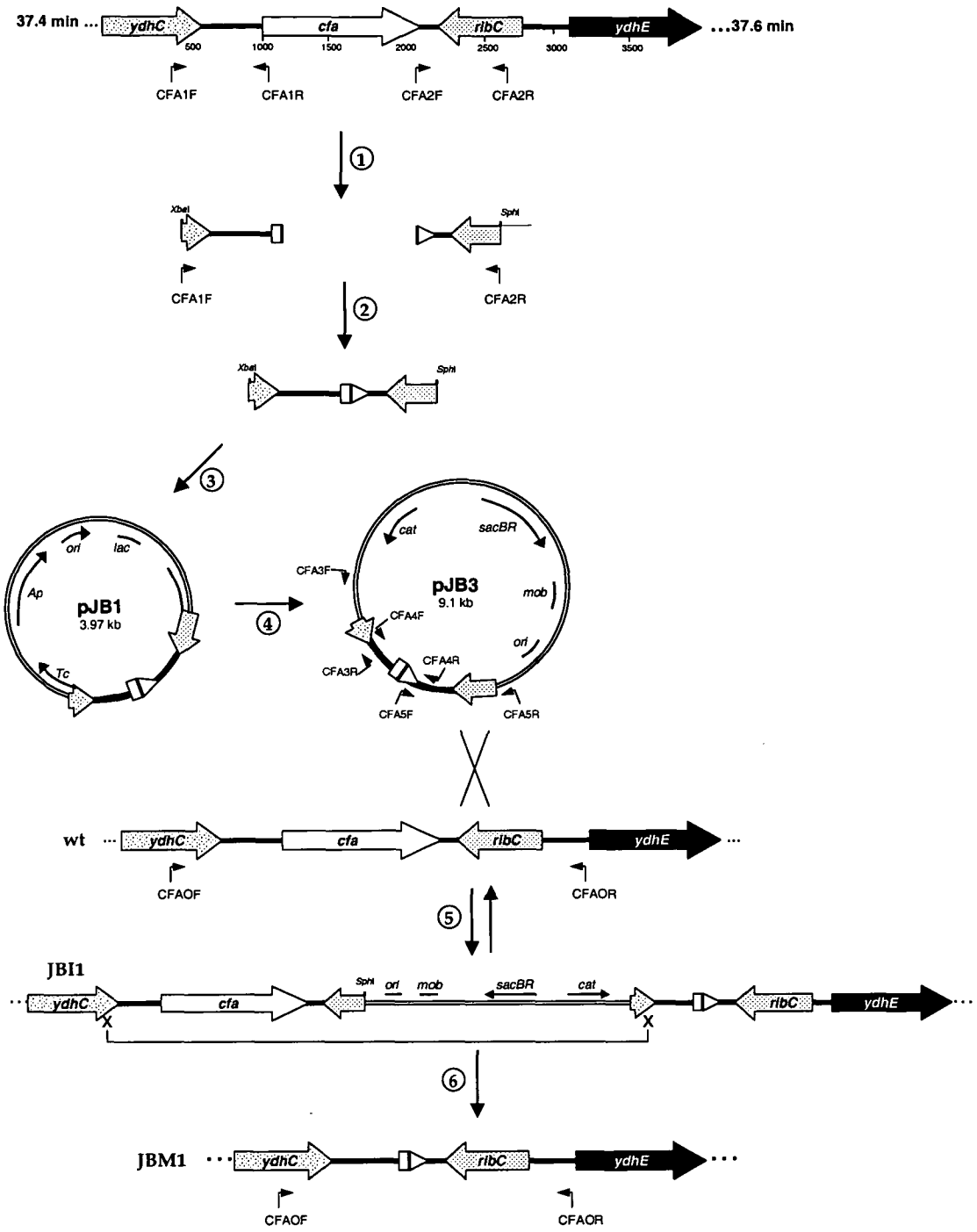


Figure 5.1 Schematic of the procedure employed in the construction of a *cfa* null mutant. ①, PCR yielding two DNA fragments covering the *cfa* termini with complementary 3′ ends; ②, PCR to amplify the fusion fragment containing the desired deletion; ③, ligation of the fusion fragment into pUC18 to yield pJB1; ④, excision of the fusion fragment from pJB1 and ligation into the suicide vector pDM4 to yield pJB3; ⑤, integration of the plasmid into the chromosome by homologous recombination yielding pJBI1; ⑥, allelic replacement resulting from a second recombination event.

5.3.5 Evaluating the Role of CFAs as Mediators of Low pH Tolerance – Part II

The fatty acid profiles of acid habituated and non-habituated populations (5.3.2.1) of the new *cfa* mutant *E. coli* JBM1, and of the isogenic wild-type strain Frag1, were analysed (5.3.2.2). To evaluate the hypothesis that the CFA content of the cell membrane plays a role in determining the tolerance of *E. coli* to acidic yet *growth permissive* low pH constraints, the influence of pH on the cell yield and growth rate of the two strains grown under neutral and sub-optimal pH conditions was determined. *E. coli* Frag1 and *E. coli* JBM1 were inoculated to 60 ml volumes of TSB and incubated overnight in an oscillating waterbath maintained to 37°C. Cultures thus prepared were employed as experimental inocula in growth rate and cell yield determinations. For each strain a series of pH adjusted broths (TSB) with pH values ranging from 4.5 to 7.0, and whose temperature was equilibrated to 37°C, was inoculated to ~ 2×10^6 CFU.ml^{-1} for growth rate determinations. An aliquot from each culture was removed aseptically immediately after inoculation for pH analysis, and the growth of each culture was monitored turbidimetrically (450nm). Generation times were estimated by linear regression analysis of log OD-time plots. A second series of pH adjusted broths (1/5 strength TSB) with pH values ranging from 3.6 to 6.9, and whose temperature was equilibrated to 25 °C, was inoculated to ~ 2×10^7 CFU.ml^{-1} for cell yield determinations. The change in optical density (ΔOD) of each culture, from its starting value to the value at the optical density plateau, was recorded as a measure of cell yield.

To evaluate the hypothesis that CFA content of the cell membrane plays a role in determining the tolerance of *E. coli* to *growth non-permissive* low pH constraints, survival curves depicting the time-dependent low pH-induced [pH 3.0, 35°C] loss of viability of habituated and non-habituated populations (5.3.2.1) of *E. coli* JBM1 and Frag1 were prepared (3.3.2.1).

5.4 RESULTS

5.4.1 Evaluating the Role of CFAs as Mediators of Low pH Tolerance – Part I

5.4.1.1 *Fatty Acid Profiles of the* cfa *mutant* E. coli *YYC1106 & its Parental Strain*

The fatty acid composition of acid habituated and non-habituated populations of the *cfa* mutant, *E. coli* YYC1106, and of its parental strain, FT1, is presented in Table 5.3. As expected, no CFAs were detected in populations of *E. coli* YYC1106 (habituated or non-habituated). While CFAs were present in populations of *E. coli* FT1, only cy17:0 was detected in non-habituated populations. A conspicuous decrease in the proportion of MUFAs (from 46.5% to 12.8%), and a concomitant increase in the proportion of cyclopropane and saturated fatty acids (from 0.5% to 5.5%, and from 53.1% to 81.6%, respectively) of *E. coli* FT1 occurred during acid

Table 5.3 Fatty acid composition (% of total FAs[1]) of non-habituated (N) and habituated (H) populations of the *cfa* mutant, *E. coli* YYC1106, and of its parental strain, FT1.

Fatty Acid	Strain/Treatment			
	FT1/N	**YYC1106/N**	**FT1/H**	**YYC1106/H**
14:0	3.87	4.21	5.19	3.46
16:0	46.77	43.43	71.53	50.15
18:0	2.42	5.72	4.92	3.75
Σ SATFAs	**53.06**	**53.36**	**81.64**	**57.36**
14:1ω7c	ND	ND	ND	ND
16:1ω7c	24.62	26.78	3.69	22.29
18:1ω7c	21.84	19.86	9.14	20.35
Σ MUFAs	**46.46**	**46.64**	**12.83**	**42.64**
cy17:0	0.48	ND	3.87	ND
cy19:0	ND	ND	1.66	ND
Σ CFAs	**0.48**	**ND**	**5.53**	**ND**

[1] Only fatty acids comprising ≥ 0.1% of the total are included in this table.

ND indicates that a particular fatty acid was not detected in that instance.

habituation. In populations of *E. coli* YYC1106 a much less pronounced low pH-induced shift in the fatty acid profile was observed, the MUFA component decreasing slightly (from 46.6% to 42.6%) and the saturated fatty acid (SATFA) component increasing by a corresponding amount (from 53.4 % to 57.4%). Interestingly, the fatty acid profiles of the two strains were dominated by SATFAs both before and after acid habituation, this class of fatty acids comprising between 53.0 and 81.6 percent of the total fatty acids in each case.

5.4.1.2 *The Low pH Tolerance of* E. coli *FT1 and* E. coli *YYC1106*

Figure 5.2 illustrates the time-dependent loss of viability of acid habituated and non-habituated populations of *E. coli* FT1 and *E. coli* YYC1106 during exposure to inimical low pH constraints [pH 3.0, 35°C]. Both *E. coli* FT1 and *E. coli* YYC1106

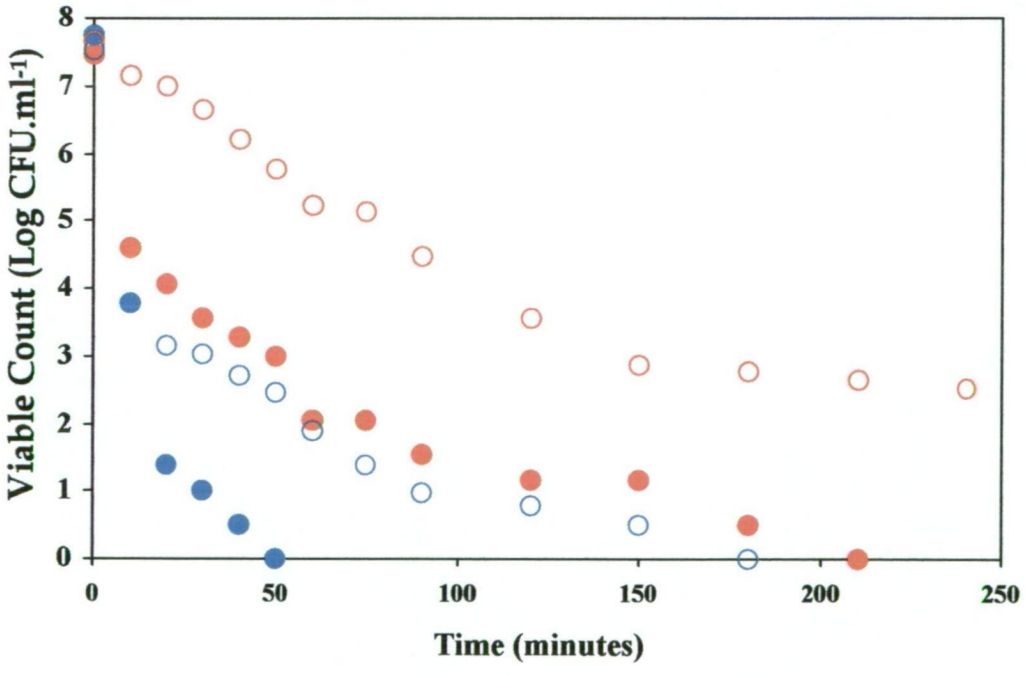


Figure 5.2 Loss of viability in acid habituated (open circles) and non-habituated (closed circles) populations of the *cfa* mutant *E. coli* YYC1106 (●○), and of its parental strain, FT1 (●○), exposed to growth non-permissive low pH constraints [pH 3.0, 35°C].

exhibited an adaptive response to low pH, the survival of populations exposed to mildly acidic conditions for a cell doubling during growth (acid habituated) being greater than that of their neutral-grown (non-habituated) counterparts. The low pH tolerance of both acid habituated and non-habituated populations of *E. coli* FT1, however, exceeded that of *E. coli* YYC1106.

5.4.2 Construction of a New *E. coli cfa* Mutant

A new mutant (*E. coli* JBM1) containing an in-frame deletion in *cfa*, the gene encoding CFA synthase, was constructed by overlap extension PCR and allelic exchange (5.3.4). With the aid of a suicide vector that replicates only in bacterial hosts supplying in *trans* the π protein encoded by *pir* (Kaniga et al., 1991), the wild-type allele was replaced with a DNA fusion fragment covering the *cfa* termini and containing the desired 1071 bp deletion. Integration of the suicide vector carrying the mutated allele into the host (*E. coli* Frag1) genome was the result of a single homologous recombination event and the plasmid integrant, *E. coli* JBI1 (Frag1, *cfa*::pJB3), was obtained by selecting for the vector-borne antibiotic resistance marker, *cat*. A second counter selectable marker derived from *Bacillus subtilis*, *sacB*, was also carried on the suicide plasmid. Regulated in *cis* by the sequence *sacR*, *sacB* encodes levansucrase, a 50 kDa enzyme that catalyses the hydrolysis of sucrose and the synthesis of levans (Gay et al., 1983). The production of levansucrase in *E. coli* is lethal in the presence of 5% sucrose (Gay et al., 1985; Simon et al., 1991). Double recombinants containing either the truncated allele (resulting from successful allelic exchange) or the wild-type allele (resulting from abortive allelic exchange) were thus obtained by screening for sucrose resistance and for loss of the antibiotic resistance marker. Double recombinants containing the desired deletion were then identified by screening the isolates obtained by PCR with primers pairs external to, and encompassing, *cfa*. With the primer pairs CFAOF/CFAOR and CFA1F/CFA1R the PCR reactions yielded DNA fragments of ~ 2.59 kbp and ~ 2.35 kbp,

respectively, in the case of the wild type, and ~1.52 kbp and 1.28 kbp in the case of the *cfa* mutant. The DNA fragments obtained in PCR reactions with the primer pair CFA1F/CFA2R, and with DNA templates derived from the wild type and the *cfa* mutant, are shown in Figure 5.3.

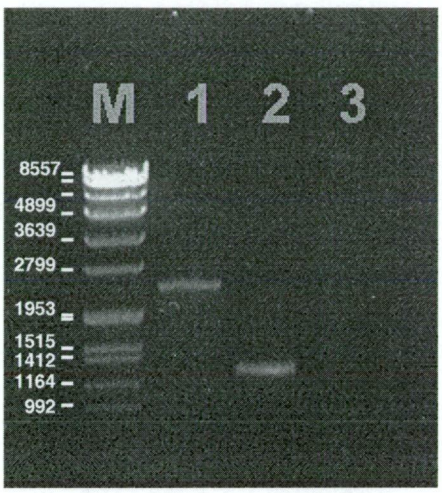


Figure 5.3 DNA fragments obtained in PCR reactions with the primer pair CFA1F/CFA2R when the DNA template employed was genomic DNA derived from Frag1 [wild-type] (1), genomic DNA derived from JBM1 [Δ*cfa*] (2), and when no DNA template was added [negative control] (3). The molecular weight marker (M) is an *Eco*RI-digest of the DNA of the bacteriophage SPP1.

It should be noted that the subcloning procedure used to clone the DNA fragment, created by overlap extension PCR, into the suicide vector was employed to overcome a difficulty encountered in cloning the fragment directly into the suicide vector. Cloning the fragment into pUC18 then removing it by digestion with *Xba*I and *Sac*I made cloning the fragment into pDM4 a much easier task. Also, the sucrose sensitivity of strains containing the *sacB* gene was highly dependent on incubation temperature and on the sodium chloride concentration of the medium employed. This phenomenon was also noted by Blomfield et al. (1991) who suggested that sucrose (5% w/v) be employed as a selective agent in LBA that

contained no NaCl and that an incubation temperature of 30°C be used to assess sucrose resistance.

5.4.3 Evaluating the Role of CFAs as Mediators of Low pH Tolerance – Part II

5.4.3.1 *Influence of Low pH on the Growth Characteristics of* E. coli *JBM1 and* E. coli *Frag1*

The influence of pH on the cell yield of *E. coli* Frag1 and *E. coli* JBM1 cultured under substrate-limited sub-optimal pH conditions is illustrated in Figure 5.4. The cell yield of the *cfa* mutant mirrored that of the wild type strain, declining gradually as the pH of the growth medium fell below a value of 6.0, then abruptly as the lower pH limit for growth was approached. The generation time of *E. coli* JBM1, which was influenced little by the pH of the growth medium at pH values greater than 5.5, was also found to be comparable to that of *E. coli* Frag1 (Table 5.4). Below pH 5.5 the generation times of the two strains increased markedly.

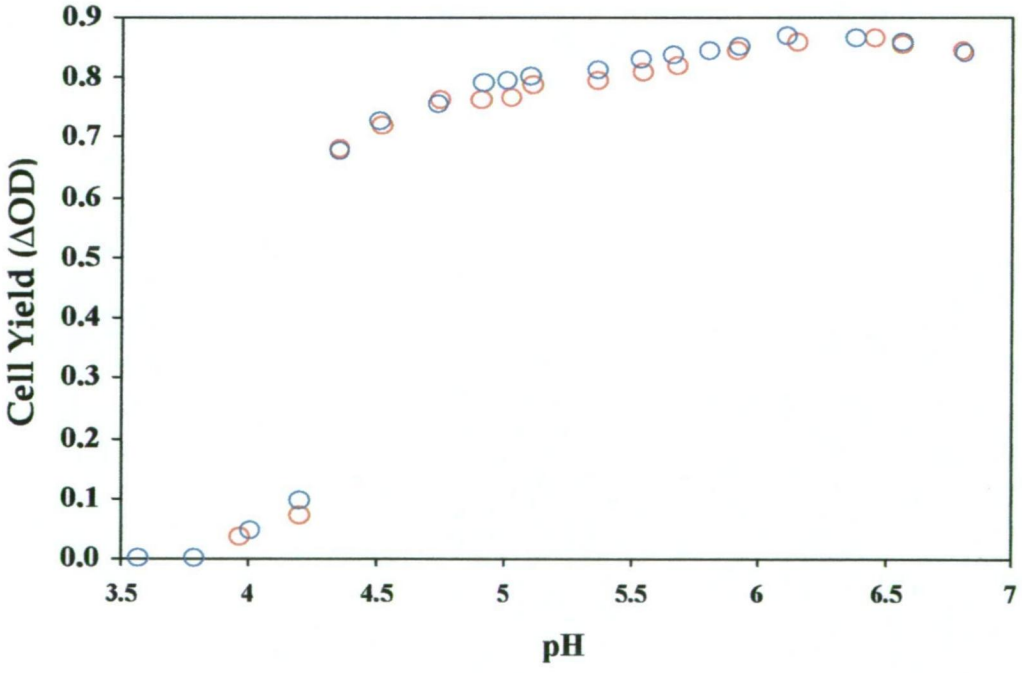


Figure 5.4 Influence of pH (sub-optimal) on the cell yield of substrate limited batch cultures of the *cfa* mutant *E. coli* JBM1 (○) and the isogenic wild type strain, Frag1 (○).

Table 5.4 Generation times of the *cfa* mutant, *E. coli* JBM1, and of the isogenic wild-type strain, Frag1, cultured in TSB at 37°C under neutral and sub-optimal pH conditions.

	Generation Time (minutes)	
pH	***E. coli* Frag1**	***E. coli* JBM1**
7.0	26.6	26.0
6.5	26.4	26.3
6.0	26.2	25.9
5.5	27.3	28.3
5.0	31.7	31.0
4.75	32.0	32.0
4.5	43.9	43.6

5.4.3.2 *Fatty Acid Profiles of* E. coli *JBM1 & its Parental Strain, Frag1*

The fatty acid composition of acid habituated and non-habituated populations of the *cfa* mutant, *E. coli* JBM1, and of the isogenic wild type strain, Frag1, is presented in Table 5.5. Consistent with the truncation of *cfa* in *E. coli* JBM1, no CFAs were detected in acid habituated or non-habituated populations of this strain. CFAs were present in both acid habituated and non-habituated populations of *E. coli* Frag1, however, where they were found to comprise 2.1 and 19.9 percent of the total fatty acids derived from non-habituated and habituated populations, respectively. SATFAs and MUFAs comprised almost equal proportions of the total fatty acids derived from non-habituated populations of the *cfa* mutant and its parental strain, the proportion of MUFAs being in slight excess to the proportion of SATFAs, and the ratio of MUFAs:SATFAs being slightly lower in populations of *E. coli* Frag1 than in populations of *E. coli* JBM1 (1.03 compared with 1.06) because of the absence of CFAs in the latter. In acid habituated populations, SATFAs clearly dominated the fatty acid profiles of the two strains comprising 69.7 and 55.1

percent of the total fatty acids extracted from populations of *E. coli* Frag1 and JBM1, respectively. Non-habituated populations of *E. coli* Frag1 contained a substantially (39.3 percent) higher proportion of MUFAs than did acid habituated populations, the MUFAs present in non-habituated populations being replaced by almost equal proportions of CFAs (17.8 percent) and SATFAs (21.5 percent) during acid habituation. By contrast, a much smaller (6.6 percent) decrease in the MUFA component of the fatty acid profile of *E. coli* JBM1 was induced by acid habituation, and in this case the MUFAs present in non-habituated populations were replaced completely by SATFAs.

Table 5.5 Fatty acid composition (% of total FAs[1]) of non-habituated (N) and habituated (H) populations of the *cfa* mutant, *E. coli* JBM1, and of its parental strain, Frag1.

Fatty Acid	Strain/Treatment			
	Frag1/N	**JBM1/N**	**Frag1/H**	**JBM1/H**
14:0	6.57	5.70	8.86	5.35
16:0	39.29	38.60	58.82	46.32
18:0	2.34	4.13	2.00	3.39
Σ SATFAs	**48.20**	**48.43**	**69.68**	**55.06**
14:1ω7c	0.37	0.31	0.25	0.18
16:1ω7c	32.37	33.92	4.18	29.71
18:1ω7c	16.99	17.34	5.98	15.05
Σ MUFAs	**49.73**	**51.57**	**10.41**	**44.94**
cy17:0	2.07	ND	16.02	ND
cy19:0	ND	ND	3.89	ND
Σ CFAs	**2.07**	**ND**	**19.91**	**ND**

[1] Only fatty acids comprising • 0.1% of the total are included in this table.

ND indicates that a particular fatty acid was not detected in that instance.

5.4.3.3 *The Intrinsic and Inducible Acid Tolerance of* E. coli *Frag1 and JBM1*

Figure 5.5 illustrates the time-dependent loss of viability observed in acid habituated and non-habituated populations of *E. coli* JBM1 and Frag1 exposed to growth non-permissive low pH constraints [pH 3.0, 35°C]. While the inactivation kinetics of non-habituated populations of the two strains were biphasic with an initial period of rapid decline (resulting in a five log reduction in the viable count) being followed by a protracted period of much slower decline, no initial period of rapid decline was apparent in the acid habituated populations. The survival of populations of both *E. coli* Frag1 and JBM1 was thus enhanced by exposure to mild acidity (pH 5 for a cell doubling), the difference in the viable counts of acid habituated and non-habituated populations being in excess of four log after a four

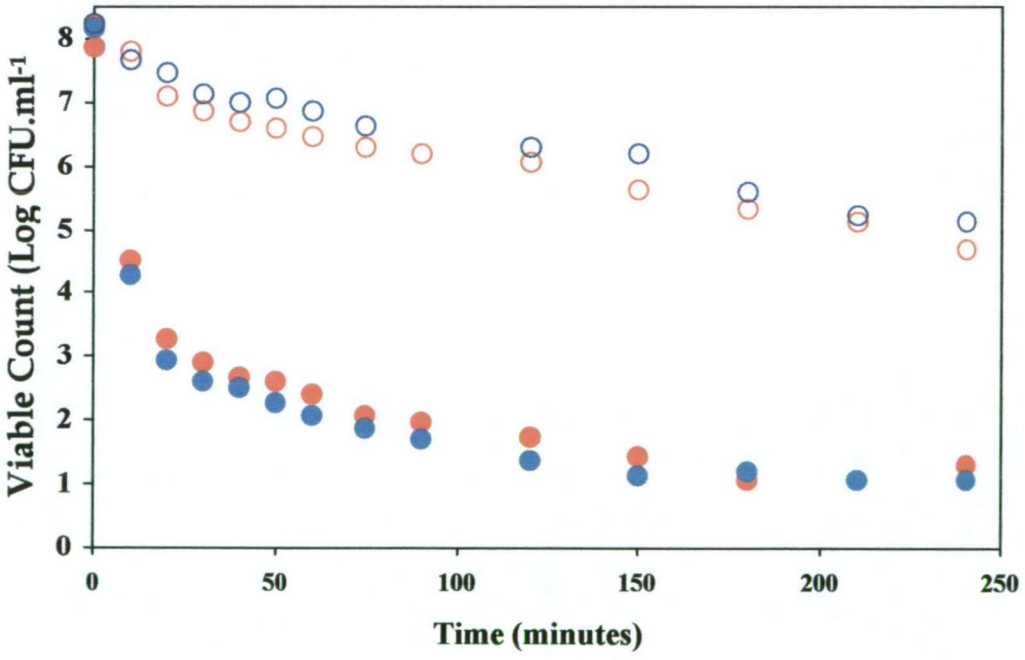


Figure 5.5 Loss of viability in acid habituated (open circles) and non-habituated (closed circles) populations of the *cfa* mutant *E. coli* JBM1 (●○), and of the isogenic wild type strain, Frag1 (●○), exposed to growth non-permissive low pH constraints [pH 3.0, 35°C].

hour low pH treatment. No consistent difference in the survival of the *cfa* mutant and the isogenic wild type strain (acid habituated or not) was apparent.

5.5 DISCUSSION

That *E. coli* mutants defective in the synthesis of CFAs were constructed prior to the conception of the present study (Taylor and Cronan, 1976; Grogan and Cronan, 1984; Grogan and Cronan, 1986; Chang and Cronan, 1999) was fortuitous. Constructed by John Cronan and his colleagues at the University of Illinois, these mutants were generated by three different procedures and hence carry *cfa* lesions of three distinct types. Derived from the methionine auxotroph *E. coli* FT1, and comprising the first class of *cfa* mutants generated, *E. coli* FT16 and FT17 (*cfa*-1) were isolated by means of a tritium suicide procedure in which wild type cells were killed as the result of the incorporation of tritiated methyl groups into CFAs (Taylor and Cronan, 1976). Not null mutants, but mutants in which the specific activity of CFA synthase was reported to be less than ten percent of that of the wild type (Taylor and Cronan, 1976; Cronan et al., 1979), *E. coli* FT16 and FT17 were employed by Cronan et al. (1979), and later by Calcott et al. (1984), who sought to reveal the biological role of CFAs in *E. coli*.

E. coli G176-13 (*cfa*-2), G176-14 (*cfa*-3), and G176-16 (*cfa*-4) comprise the second class of *cfa* mutants, are derived from *E. coli* FS173, and carry *cfa* lesions that are the result of Tn*10*-mediated chromosomal rearrangements (Grogan and Cronan, 1984). Grogan and Cronan (1986) have illustrated that these rearrangements are inversions with endpoints within *cfa* and a Tn*10*-element (*zdg-299*::Tn*10*) located some 10-13 kbp downstream of *cfa*. Although all three mutants retain the *cfa* sequence they are completely devoid of CFA synthase anctivity and hence of CFAs. In this respect the mutations they carry are superior to those carried by *E. coli* FT16

and FT17. That the mutations carried by *E. coli* G176-13, G176-14 and G176-16 are discrete, however, has not been illustrated.

The third and final class of *cfa* mutants carry a kanamycin-resistance gene (*kan*) in *cfa* that blocks its transcription after codon 17 (of the 382). This mutation (*cfa*::*kan*) is without polar effects on downstream genes because *cfa* is transcribed as a monocistronic mRNA. Derived from *E. coli* FT1, ZK126, and MG1655, the strains harbouring this mutation make no CFAs detectable by gas chromatography or collision induced dissociation electrospray mass spectroscopy, and have been designated *E. coli* YYC1106, YYC1272, and YYC1273 (Chang and Cronan, 1999). Because the mutation carried by these strains was known to be both absolute and discrete, *E. coli* YYC1106[1] was regarded as the *cfa* mutant of choice for this study and was the strain initially employed together with its parental strain, *E. coli* FT1, to evaluate the role that CFAs play as molecular mediators of low pH tolerance in *E. coli*.

The results obtained in the analysis of fatty acids derived from acid habituated and non-habituated populations of *E. coli* YYC1106 and FT1 (Table 5.3) clearly demonstrate, as do the results of Chang and Cronan (1999), that the *cfa*::*kan* mutation carried by *E. coli* YYC1106 prevents CFA biosynthesis. These results also indicate that a significant proportion of the MUFAs of *E. coli* FT1 are either converted to CFAs or replaced by SATFAs during acid habituation. A substantial (eleven-fold) increase in the CFA content of *E. coli* FT1 was induced by acid habituation and in this respect, *E. coli* FT1 is representative of the five wild type

[1] While personal communication with Y.Y. Chang and J.E. Cronan alerted me to the availability of *E. coli* YYC1106 before this study commenced, I was unaware of the existence of *E. coli* YYC1272 and YYC1273 until after *E. coli* JBM1 had been constructed.

strains examined by Brown et al. (1997). Confirmation of this fact and of the null phenotype of *E. coli* YYC1106 was necessary to ensure the validity of conclusions, concerning the role of CFAs in low pH tolerance, drawn from survival curves depicting the time-dependent loss of viability that occurred in populations of *E. coli* YYC1106 and FT1 exposed to inimical low pH constraints. Illustrated in Figure 5.2, these survival curves demonstrate that exponential phase populations of the wild type strain *E. coli* FT1, habituated or not, are more acid tolerant than exponential phase populations of the *cfa* mutant *E. coli* YYC1106. While this pairwise analysis of the survival curves leads naturally to the conclusion that CFAs do play a role in determining the low pH tolerance of *E. coli*, an integrated analysis of the four survival curves indicates that CFAs cannot be regarded as sole mediators of the adaptive acid tolerance response of *E. coli* and, in fact, prompts one to question the validity of the conclusion drawn.

That *E. coli* FT1 and YYC1106 both exhibit an adaptive response to low pH (Figure 5.2) clearly indicates that CFAs play, at most, a partial role in mediating the adaptive acid tolerance response of *E. coli*. That the magnitude of the adaptive response exhibited by *E. coli* YYC1106 is not very dissimilar to the magnitude of the adaptive response of *E. coli* FT1 (Figure 5.2) in turn suggests that CFAs play only a minor role, if any, in mediating the adaptive acid tolerance response of *E. coli*. Should the CFA content of acid habituated cultures be shown to have little bearing on their low pH tolerance, the possibility that CFAs play a role in determining the intrinsic acid tolerance of *E. coli* would appear remote given the fact that CFA component of acid habituated cultures is between five and thirty times higher than that of non-habituated cultures (Brown et al., 1997; Table 5.3 and Table 5.5).

Use of the *cfa* mutant *E. coli* YYC1106, and of its parental strain, FT1, to evaluate the role that CFAs play as molecular mediators of low pH tolerance in *E. coli* thus

renders only one thing certain – that CFAs are not sole mediators of the adaptive acid tolerance response of *E. coli*. Whether or not CFAs play a partial role in mediating the intrinsic acid tolerance of *E. coli*, or the adaptive acid tolerance response of this organism, cannot be ascertained conclusively from an analysis of survival curves depicting the time-dependent low pH-induced loss of viability of these strains. A comparative analysis of the fatty acid composition and the low pH tolerance of *E. coli* FT1 with that of other wild type strains, and of the growth rate of *E. coli* FT1 and YYC1106, sheds some light on why this might be so.

Consider again the results obtained in the analysis of fatty acids derived from acid habituated and non-habituated populations of *E. coli* FT1 (Table 5.3). Although the magnitude of the relative increase in the CFA content of *E. coli* FT1 observed during acid habituation is representative of that observed in other wild type strains, the magnitude of the absolute increase in the CFA content of this strain is, in fact, much lower (Table 5.6). Brown et al. (1997) observed that the magnitude of the absolute increase in the CFA content of five wild type strains induced by acid habituation ranged from 11.5 to 22.0 percent (mean 17.1 ± 4.0). By contrast, only a 5 percent increase (from 0.5 to 5.5) in the CFA content of *E. coli* FT1 was induced by acid habituation. This finding indicates that *E. coli* FT1 may be impaired in its ability to upregulate *cfa* during growth at sub-optimal pH values.

Early studies published by Cronan and his colleagues indicate that more than thirty percent of the phospholipid-associated acyl chains of stationary phase populations of *E. coli* FT1 may be CFAs (Taylor and Cronan, 1976; Cronan et al., 1979). That the CFA content of acid habituated populations of *E. coli* FT1 examined in this study did not exceed 5.5 percent of the total fatty acids (Table 5.3) is, therefore, somewhat surprising. This result is consistent with the findings of Calcott et al. (1984),

however, who reported that the CFA content of stationary phase populations of *E. coli* FT1 never exceeded 5.1 percent of the total fatty acids of that strain.

Table 5.6 CFA content (as a percentage of the total fatty acids) of acid habituated and non-habituated populations of wild type *E. coli* examined in this study[A] and by Brown et al. (1997)[B], and the increase in the CFA content of these strains that was induced by acid habituation.

	Stain						
CFA Content	**FT1[A]**	**R91[B]**	**R172[B]**	**M23[B]**	**O157:H-[B]**	**MJR[B]**	**Frag1[A]**
Non-habituated Population[1]	0.5	0.5	1.3	2.4	4.1	0.9	2.1
Acid habituated Population[2]	5.5	15.7	20.4	24.4	22.0	12.4	19.9
Increase in CFA Content							
Relative % Increase (= 2÷1)	11	31.4	15.7	10.2	5.4	13.8	9.5
Absolute % Increase (= 2-1)	5.0	15.2	19.1	22.0	17.9	11.5	17.8

Figure 5.6 facilitates a comparison of the time-dependent loss of viability observed in an acid habituated population of *E. coli* FT1 exposed to growth non-permissive low pH constraints with that observed in populations of other wild type strains exposed to the same inimical treatment. By comparison with these wild type strains *E. coli* FT1 exhibits a low level of acid tolerance, post habituation, a finding that would not be unexpected if the acid tolerance of acid habituated populations of *E. coli* was known to be linked to the proportion of CFAs in the membrane. Interestingly, Chang and Cronan (1999) also commented on the unusual acid sensitivity of *E. coli* FT1, a characteristic of this strain which they attribute to a deficiency in RpoS function. Such a deficiency might explain the fact that *E. coli* FT1 converts an unusually low proportion of the MUFA component of its phospholipids to CFAs when exposed to stress (Calcott et al., 1984; Table 5.5).

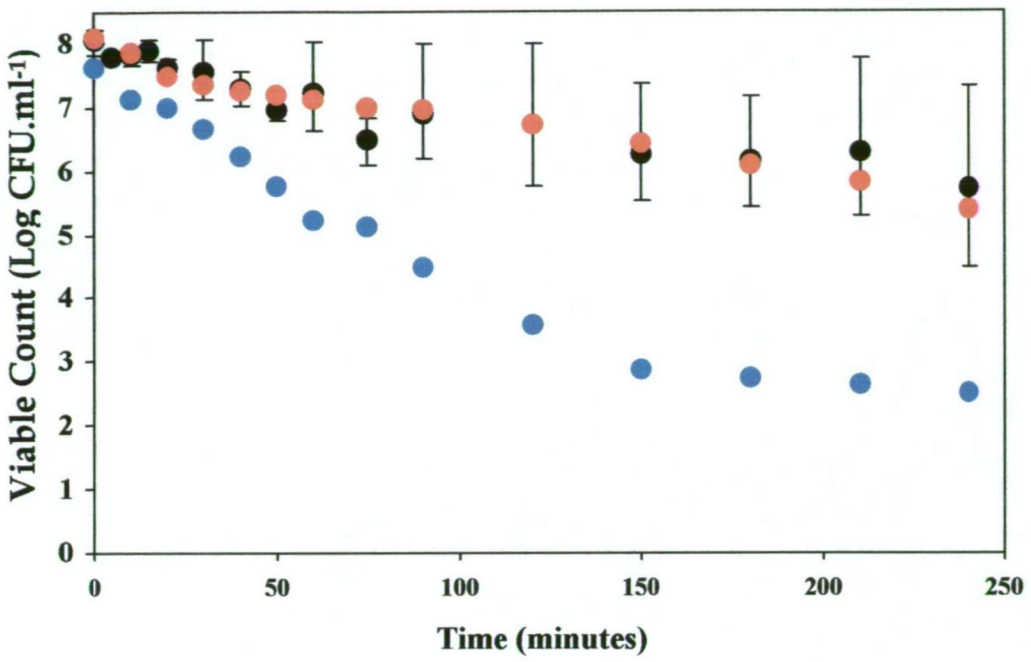


Figure 5.6 Loss of viability in acid habituated populations of *E. coli* FT1 (●) and *E. coli* Frag1 (●) exposed to inimical low pH constraints [pH 3.0, 35°C], and the mean of the viable counts Brown et al. (1997) reported for five wild type strains exposed to the same inimical treatment (●). Y-error bars indicate the minimum and maximum viable counts observed.

Stress-induced increases in the CFA content of *E. coli* appear to mediated by an increase in the rate of RpoS-directed *cfa* transcription (Chang and Cronan, 1999). That strains with a deficiency in RpoS will be impaired in their ability to upregulate the proportion of MUFAs converted to CFAs when exposed to stress is thus to be expected.

In addition to the data already presented which illustrates that *E. coli* FT1 is atypical of wild type *E. coli* in several respects that render it (and hence *E. coli* YYC1106) inappropriate for the purposes of this study, data made available by Presser (pers. comm., 1998) illustrates that *E. coli* FT1 is atypical of wild type *E. coli* in terms of its growth rate. At pH values between 4.5 and 7.5 the growth rate of *E. coli* FT1 is substantially lower than that of *E. coli* Frag1 and JBM1 (Figure 5.7).

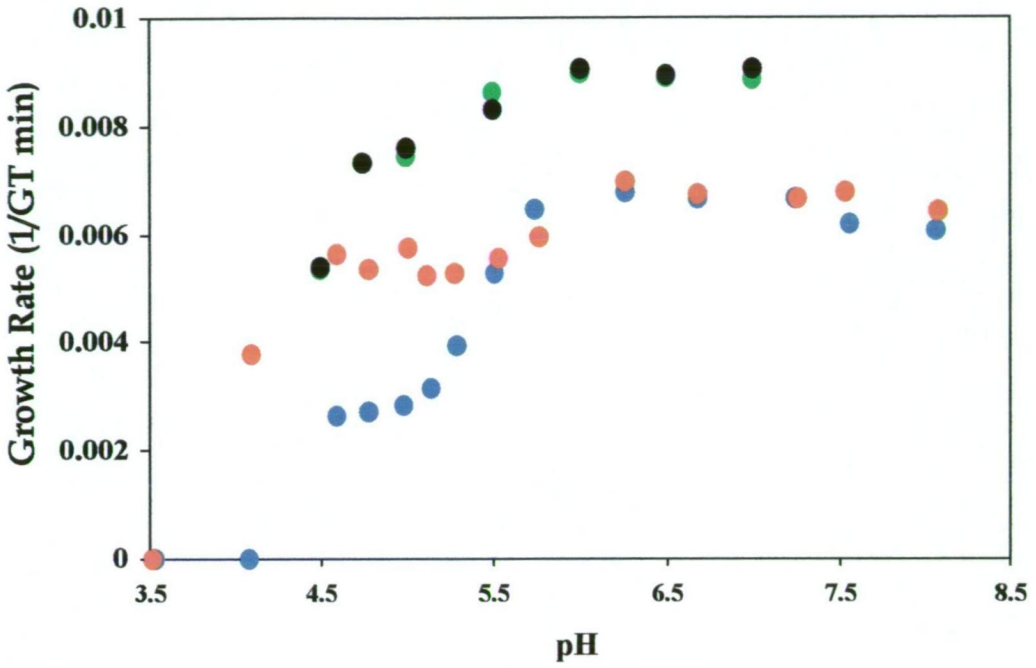


Figure 5.7 Influence of pH on the growth rate of *E. coli* FT1 (●), *E. coli* YYC1106 (●), *E. coli* Frag1 (●), and *E. coli* JBM1 (●). Data in this figure pertaining to *E. coli* FT1 and *E. coli* YYC1106 were provided by K.A. Presser, 1998.

Perhaps of more concern, however, is the fact that the growth rate of *E. coli* YYC1106 is considerably lower than that of its parental strain at pH values less than 5.5 (Figure 5.7). While this finding may imply that CFAs play a role in determining the sensitivity of *E. coli* to environments whose pH is moderately acidic yet growth permissive, it may also indicate that the *kan* gene impairs the growth of *E. coli* under moderately acidic conditions. Dufourc et al. (1984) reported that cyclopropanation of phospholipid-associated MUFAs increases the stability of biomembranes, and Yatvin et al. (1986) that the presence of a cyclopropane ring within membrane fatty acids acts to increase membrane viscosity. Should such a CFA-mediated increase in the stability and viscosity of the cell membrane decrease its proton permeability, a difference in the growth characteristics of strains competent and defective in the synthesis of CFAs might be expected at pH values

below which their synthesis is induced. Rowbury (1995) indicates that the low pH inducible molecular systems of *E. coli* are fully activated when the pH of the growth medium falls to 5.5 – that pH value below which a difference in the growth rate of *E. coli* FT1 and YYC1106 is observed. That the growth characteristics of *E. coli* will be affected by the CFA content of the membrane under moderately acidic conditions goes hand in hand with the notion that the low pH tolerance of *E. coli* exposed to growth non-permissive low pH constraints is linked to their CFA content. The possibility that the *kan* gene harboured by *E. coli* YYC1106 reduces its potential for growth under moderately acidic conditions cannot be ruled out, however. To evaluate this possibility, and the original hypothesis, a new *cfa* mutant was constructed.

Derived from *E. coli* Frag1 the new *cfa* mutant, *E. coli* JBM1, carries an in-frame deletion in *cfa* (between codon 21 and 378). This mutation, like the *cfa*::*kan* mutation harboured by *E. coli* YYC1106, is both discrete and absolute. It has the added advantage of being unmarked, however, which ensures that any difference in the behaviour of *E. coli* JBM1 and the isogenic wild type strain can be ascribed only to a difference in their abilities to convert MUFAs to CFAs. While the fact that the mutation carried by *E. coli* JBM1 is absolute was verified by the results obtained in the analysis of fatty acids derived from populations of this strain (Table 5.5), the discrete and unmarked nature of the lesion was ensured by the method employed in the construction of *E. coli* JBM1.

The results obtained in the analysis of fatty acids derived from acid habituated and non-habituated populations of *E. coli* Frag1 (Table 5.5) demonstrate that both the relative and absolute low pH-induced increase in the CFA content of this strain is typical of that observed in other wild type strains (Table 5.6). Moreover, an analysis of the time-dependent low pH-induced loss of viability of *E. coli* Frag1

indicates that the low pH tolerance of this strain, post habituation, is representative of that of other wild type strains (Figure 5.6). In those respects that *E. coli* FT1 is atypical of other wild type strains, *E. coli* Frag1 is typical. The new *cfa* mutant, *E. coli* JBM1, and the isogenic wild type strain, Frag1, were therefore deemed appropriate for the purposes of this study.

Before examining the low pH tolerance of *E. coli* JBM1 and Frag1 under growth non-permissive low pH constraints, the influence of pH (sub-optimal) on the cell yield and growth rate of these strains was examined. Krist et al. (1998) indicate that the cell yield of substrate-limited batch cultures can be regarded as a measure of the efficiency with which substrate is converted to biomass, and therefore, of the relative energetic burdens that different environmental conditions pose to microbial growth. As previously noted (1.5), the regulation of cytoplasmic pH is generally regarded to be an energy requiring process. Should the CFA content of a population have a bearing on its sensitivity to moderately acidic yet growth-permissive low pH constraints, strains defective in the synthesis of CFAs would be expected to divert a greater amount of energy from growth into maintenance, and hence exhibit a lower cell yield when grown at sub-optimal pH values, than strains capable of synthesising CFAs. Figure 5.4 portrays the influence of pH on the cell yield of the *cfa* mutant *E. coli* JBM1 and the isogenic wild type strain, Frag1. That the cell yield of *E. coli* JBM1 mirrors that of Frag1 under sub-optimal pH conditions indicates that CFAs, in fact, do not play a role in determining the sensitivity of *E. coli* to moderately acidic yet growth permissive low pH constraints. This conclusion is also supported by data which illustrate that the growth rates of *E. coli* Frag1 and JBM1 are comparable across the growth permissive low pH range (Table 5.4 and Figure 5.7). These data, in turn, indicate that the difference in the growth rate of *E. coli* FT1 and YYC1106 may be attributed to the presence of the *kan* gene in the *cfa* mutant. Strains harbouring the *cfa*::*kan* mutation are, therefore, likely to be

inappropriate for studies that seek to assess the role of CFAs as mediators of low pH tolerance.

Survival curves depicting the time-dependent low pH-induced loss of viability of *E. coli* Frag1 and *E. coli* JBM1, generated as a means of evaluating the hypothesis that CFAs mediate, in part, the tolerance of *E. coli* to growth non-permissive low pH constraints are presented in Figure 5.5. These survival curves illustrate that the low pH tolerance (intrinsic and inducible) of the *cfa* mutant, *E. coli* JBM1, is identical to that of its parental strain. The hypothesis presented must, on the basis of this finding, be rejected – the conclusion being that CFAs play no role in mediating the intrinsic low pH tolerance of *E. coli*, or in mediating the adaptive acid tolerance response of this organism.

Interestingly, Chang and Cronan (1999) have also sought recently to evaluate the hypothesis presented by Brown et al. (1997). After comparing the low pH tolerance of *E. coli* YYC1272, YYC1273 and YYC1106 (three strains harbouring the *cfa::kan* mutation) with that of their parental strains, *E. coli* ZK126, MG1655 and FT1, Chang and Cronan concluded that the synthesis of CFAs is an important factor in the protection of cells exposed to growth non-permissive low pH constraints. While they point out that "... this lipid modification is clearly not the only mechanism that gives efficient survival ....", they assert that "...." *CFA formation probably provides the greatest protection against acid shock of any single structural* molecule."

Utilising three different protocols (A, B and a hybrid [A/B])[1], they illustrate that at least two low pH protective mechanisms are found in *E. coli* – one dependent on the presence of amino acids in the medium before and during the low pH challenge (amino acid decarboxylases) and the other, they suggest, dependent on the formation of CFAs. They go on to indicate that when protocol A is employed, the effect of CFAs on the acid tolerance of *E. coli* may be masked by the activity of the inducible amino acid decarboxylases. Having employed a type A protocol in the present study, the possibility that the influence of CFAs on the acid tolerance of *E. coli* could be masked in the results presented in Figure 5.5 needed to be addressed. Acid habituated and non-habituated populations of *E. coli* Frag1 and JBM1 (5.3.2.1) were thus washed in a minimal broth (Minimal Broth Davis, pH 7), then resuspended in the same before being exposed to a low pH treatment (3.3.2.3). This protocol, which mirrors[2] the hybrid protocol described by Chang and Cronan (1999), should have rendered the amino acid decarboxylases of *E. coli* Frag1 and JBM1 inoperative during the course of the low pH challenge. Any affect of CFAs on the low pH tolerance of these strains should thus have been 'unmasked'. Data obtained using this new protocol (not shown) provided no cause for the withdrawal of the original conclusion reached in the present study.

As does the present study, Chang and Cronan's study includes a comparison of the low pH tolerance of exponential phase populations of a *cfa* mutant (*E. coli* YYC1272)

---

[1] Populations of *E. coli* were both grown and exposed to a low pH treatment in a complex broth (Protocol A) or in a minimal broth (Protocol B). For the hybrid protocol (A/B), populations of *E. coli* were grown in a complex broth then washed and resuspended in a low pH adjusted minimal broth.

[2] Note that the hybrid protocol employed by Chang and Cronan (1999) involved washing and resuspension in a minimal broth that was *already* adjusted to a pH of 3.

and its parental strain (ZK126) post habituation. Their data indicate that the magnitude of the adaptive response of the *cfa* mutant exceeds that of the wild type strain. While this finding implies that the induced expression of *cfa* and the subsequent synthesis of CFAs has little to do with the adaptive acid tolerance response of *E. coli*, Chang and Cronan maintain otherwise. Unfortunately, methodological inconsistencies[1] in that paper and those authors' decision to omit data does not permit rigorous evaluation of their conclusions presented. That those authors draw some of their conclusions from invalid comparisons[2] is, likewise, unfortunate. In light of these facts, and an awareness that strains harbouring the *cfa*::*kan* mutation are likely to be inappropriate for studies that seek to assess the role of CFAs as mediators of low pH tolerance, it is here proposed that CFAs play no role in mediating the low pH tolerance of *E. coli* and that the physiological significance of CFA formation remains an intriguing question.

---

[1] A careful examination of the methods employed by Chang and Cronan (1999) reveals multiple inconsistencies in the preparation/challenge of cultures under different protocols and in different experiments.

[2] By way of example, Chang and Cronan (1999) report that "when tested by protocol A, there was little or no difference in survival of YYC1272 and ZK126, whereas when tested by protocol B the *cfa* strain survived at a 100-fold lower rate." In so doing they compare the survival of the two strains after a low pH treatment of 40 minutes (Protocol A) with their survival after a low pH treatment of 120 minutes (Protocol B). There was, in fact, little difference in the survival of the two strains evaluated under protocol B after a 40 minute low pH treatment.

SUMMARY AND CONCLUSIONS

At the close of this dissertation the reader's attention is drawn back to the objectives of the study. The primary objective of this study was to expand knowledge and understanding of the process(es) of the low pH-induced inactivation of *E. coli*. To meet that objective several approaches were employed. Initially [Chapter 1] literature concerned with *E. coli*, its responses to low pH, and the molecular basis of those responses was reviewed. While substantial amounts of quantitative information relating the growth response characteristics of *E. coli* to the primary physicochemical parameters controlling bacterial growth, including pH, has been collected and modelled, relatively little effort has been made to systematically describe the response characteristics of populations of *E. coli* exposed to analogous lethal constraints. It was noted that a lack of methodological consistency between studies concerned with the process of low pH-induced inactivation, the inherent sensitivity of survival responses to environmental and physiological parameters, and the fragmentary/sparse nature of many of the kinetic datasets published, make it exceptionally difficult to draw conclusions concerning the mechanism(s) underpinning low pH-induced inactivation, or indeed the process of low pH-induced inactivation itself, from the literature.

In the second chapter uncertainties associated with quantifying microbial viability and the loss thereof were addressed. Moreover, experimental methods that permitted the loss of viability of *E. coli* populations subjected to inimical low pH constraints to be characterised without ambiguity, and without the introduction of kinetic artefacts, were developed. Specifically, precautions were taken to ensure that experimental populations were genetically homogeneous and that each

population as a whole experienced a uniform 'treatment'. Culturability was endorsed as the (currently) most appropriate means of assessing microbial viability and substantial efforts were made to optimize the recovery protocol employed. The composition of the diluent, the physical structure and the composition of the outgrowth medium, and the incubation temperature employed for recovery were considered in the selection of the recovery protocol.

Employing the protocols developed, the process of low pH-induced inactivation of *E. coli* was characterized [Chapter 3]. Initial viability datasets generated illustrated that, when subjected to inimical low pH constraints, *E. coli* populations exhibit two distinct phases of inactivation – an initial phase of rapid inactivation and a second protracted phase of much slower inactivation. The influence of the stringency of the inimical treatment employed, and of temperature, on the rates of inactivation that occur during the first and second phases of inactivation, and on the magnitude and duration of the first phase of inactivation was investigated. Viability datasets obtained during the course of that investigation demonstrated the occurrence of a third phase of inactivation in low pH-treated populations of *E. coli.* That phase of inactivation is marked by a dramatic increase in the rate of inactivation and persists until the viable count falls below the limit of detection.

While knowledge of the fraction of a population surviving into the tail is often regarded as being 'industrially significant (since it is the survivors that will cause spoilage and/or safety issues), the present study demonstrates that 'tail cells' do not survive indefinitely. On the basis of the kinetics observed it is inferred that the stability/safety of acidic products will increase rapidly after a fixed period of time. Knowledge of the influence of physiological and physicochemical parameters on

the duration of the second and third phases of inactivation will, therefore, also be of industrial significance. The present study illustrates that the kinetics of inactivation exhibited by *E. coli* populations subjected to inimical low pH constraints are extremely sensitive to small differences in the physiological state of populations, that the rate, magnitude and duration of the three phases of low pH-induced inactivation may be influenced by environmental parameters in a non-continuous and differential manner, and consequently that extrapolation in the absence of a mechanistic understanding of the phenomena observed is foolhardy. That stationary phase populations incubated for extended periods of time exhibit an increasing sensitivity to low pH constraints provides a particularly striking example of the non-intuitive nature of the responses observed.

That the three main types of curves observed in inactivation studies (i.e. linear, shouldered or tailing) can be superimposed to yield a triphasic inactivation curve of the form observed in the present study leads the author to suggest that the apparent lack of comparability of published datasets stems from differences in the experimental 'systems' employed by different investigators (shouldered curves are generally observed when the responses of dilute stationary phase populations are studied while linear or tailing curves are generally observed when non-dilute populations are employed) and from a common failure among investigators to characterise inactivation curves in their entirety (linear and tailing curves). While systematic kinetic studies concerned with the loss of viability of microbial populations are labour intensive, a significant amount of information relating to the mechanism(s) of the process of inactivation can be gleaned from the data obtained.

In Chapter 4 hypotheses concerned with the basis of the shape of non-linear survivor curves were introduced and evaluated. Although many of the hypotheses introduced were conceived during the early 1900s, few have been systematically evaluated. That investigation revealed that no single hypothesis could adequately account for the kinetics of inactivation exhibited by *E. coli* populations subjected to inimical low pH constraints. A case for the inherent differential sensitivity of individuals within a population was presented and it was concluded that the shape of the survivor curve reflects both the distribution of individual resistance states within a population (a physiological phenomenon) and the mechanism of inactivation (a physicochemical phenomenon). A novel and testable hypothesis based on the notion that the mechanism underpinning the loss of viability observed during the first phase of inactivation differs from that underpinning the loss of viability observed during the second and third phases of inactivation was presented.

In the final chapter the construction of an *E. coli* mutant defective in the synthesis of cyclopropane fatty acid synthase and hence cyclopropane fatty acids was described. That mutant, together with its parental strain, was employed to evaluate the author's hypothesis that cyclopropane fatty acids mediate the intrinsic and/or inducible low pH tolerance of *E. coli*. Experiments illustrating that the mutant and parental strain exhibited no differences in their growth and survival responses at low pH clearly negated that hypothesis. That conclusion has not been endorsed by all investigators, however.

In conclusion, the present study has refined considerably our understanding of both the process and mechanisms of the low pH-induced inactivation of *E. coli*.

Moreover, it provides a framework for quantitative investigations concerned with inactivation of *E. coli* by non-thermal environmental constraints.

REFERENCE LIST

Abdul-Raouf, U.M., Beuchat, L.R. and Ammar, M.S., 1993. Survival and growth of *Escherichia coli* O157:H7 in ground, roasted beef as affected by pH, acidulants, and temperature. *Appl. Environ. Microbiol.* 59: 2364-2368.

Abee, T. and Wouters, J.A., 1999. Microbial stress response in minimal processing. *Int. J. Food Microbiol.* 50: 65-91.

Abshire, R.L., Bain, B. and Williams, T., 1980. Resistance and recovery studies on ultraviolet irradiated spores of *Bacillus pumilus*. *Appl. Environ. Microbiol.* 39: 695-701.

Ahamad, N. and Marth, E.H., 1999. Behaviour of *Listeria monocytogenes* at 7, 13, 21, and 35°C in Tryptose Broth Acidified with Acetic, Citric or Lactic Acid. *J. Food Prot.* 52: 688-695.

Alterkruse, S.F. and Swerdlow, D.L., 1996. The changing epidemiology of foodborne diseases. *Am. J. Med. Sci.* 311: 23-29.

Alterkruse, S.F., Cohen, M.L. and Swerdlow, D.L., 1997. Emerging foodborne diseases. *Emerg. Infect. Dis.* 3: 285-294.

ANZFA (Australia New Zealand Food Authority), 1999. Food safety standards costs and benefits – An analysis of the regulatory impact of the proposed national food safety reforms. Commonwealth of Australia.

Archer, D.L., 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. *Trends Food Sci. Tech.* 7: 91-95.

Arneborg, N., Salskov-Iversen, A.S. and Mathiasen, T.E., 1993. The effect of growth rate and other growth conditions on the lipid composition of *Escherichia coli*. *Appl. Microbiol. Biot.* 39: 353-357.

Arnold, K.W. and Kaspar, C.W., 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 61: 2037-2039.

Atwood, K.C. and Norman, A., 1949. On the interpretation of multi-hit survival curves. *Proc. Natl. Acad. Sci. USA* 35: 696-709.

Auger, E.A., Redding, K.E., Plumb, T., Childs, L.C., Meng, S.Y. and Bennett, G.N., 1989. Construction of *lac* fusions to the inducible arginine- and lysine decarboxylase genes of *Escherichia coli* K12. *Molec. Microbiol.* 3: 609-620.

Augustin, J.C., Carlier, V. and Rozier, J., 1998. Mathematical modeling of the heat resistance of *Listeria monocytogenes*. *J. Appl. Microbiol.* 84: 185-191.

Bacus, J., 1997. Processing procedures to control *Salmonella* and *E. coli* in fermented sausage products. AIFST 1997 Convention Paper. *Food Aust.* 49: 543-547.

Ball, C.O. and Olson, F.W.C., 1955. Foundation of food process calculation methods. *Food Res.* 20: 666-686.

Baranyi, J. and Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. *Int. J. Food Microbiol.* 23: 277-294.

Barer, M.R., 1997. Viable but non-culturable and dormant bacteria: time to resolve an oxymoron and a misnomer? *J. Med. Microbiol.* 46: 629-631.

Bearson, S., Bearson, B. and Foster, J.W., 1997. Acid stress responses in enterobacteria. *FEMS Microbiol. Lett.* 147: 173-180.

Bearson, S.M.D., Benjamin, W.H., Swords, W.E. and Foster, J.W., 1996. Acid shock induction of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. *J. Bacteriol.* 178: 2572-2579.

Benito, A., Ventoura, G., Casadei, M., Robinson, T. and Mackey, B., 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. *Appl. Environ. Microbiol.* 1564-1569.

Benjamin, M.M. and Datta, A.R., 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. *Appl. Environ. Microbiol.* 61: 1669-1672.

Besser, R.E., Lett, S.M., Weber, J.T., Doyle, M.P., Barrett, T.J., Wells, J.G. and Griffin, P.M., 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *JAMA* 269: 2217-2220.

Bettelheim, K.A., 1996a. Enterohemorrhagic *Escherichia coli*: a new problem, an old group of organisms. *Aust. Vet. J.* 73: 20-26.

Bettelheim, K.A., 1996b. Enterohemorrhagic *E. coli* – A review. *International Food Hygeine* : 5-9.

Bligh, E.G. and Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911-917.

Blomfield, I.C., Vaughn, V., Rest, R.F. and Eisenstein, B.I., 1991. Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature sensitive pSC101 replicon. *Molec. Microbiol.* 5: 1447-1457.

Bogosian, G., Morris, P.J.L. and O'Neil, J.P., 1998. A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. *Appl. Environ. Microbiol.* 64: 1736-1742.

Booth, I.R., 1985. Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* 49: 359-378.

Booth, I.R., 1998. Personal communication.

Booth, I.R., 1999. The regulation of intracellular pH in bacteria, In: D.J. Chadwick and G. Cardew (Eds), *Bacterial Responses to pH* Novartis Foundation Symposium 221. John Wiley and Sons, Chichester, pp 19-37.

Booth, I.R. and Kroll, R.G., 1989. The preservation of foods by low pH, In: G.W. Gould (Ed.), *Mechanisms of Action of Food Preservation Procedures*. Elsevier Applied Science, London, pp 119-151.

Booth, I.R., Thomson-Carter, F., Carter, P., Jordan, S., Park, S., Malcolm, L. and Glover, J., 1999. Acid tolerance of *Escherichia coli* - the sting in the tail? Rowett Research Symposium pp. 27-38.

Brannen, J.P., 1968. A rational model for thermal sterilization of microorganisms. *Math. Biosci.* 2: 165-179.

Breeuwer, P. and Abee, T., 2000. Assessment of viability of microorganisms employing fluorescence techniques. *Int. J. Food Microbiol.* 55: 193-200.

Brock, T.D. and Madigan, M.T., 1991. *Biology of microorganisms* (6th Ed.). Prentice Hall, Englewood Cliffs, New Jersey.

Brooks, S.C., 1918. A theory of the mechanism of inactivation, hemolysis and similar processes. *J. Gen. Physiol.* 1: 61-80.

Brown, J.L., 1996. The acid habituation of *Escherichia coli*. Honours Thesis, University of Tasmania.

Brown, J.L., Ross, T., McMeekin, T.A. and Nichols, P.D., 1997. Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *Int. J. Food Microbiol.* 37: 163-173.

Brown, M.H. and Booth, I.R., 1991. Acidulants and low pH, In: N.J. Russell and G.W. Gould (Eds), *Food Preservatives*. Blackie, Glasgow, pp 22-43.

Buchanan, R.L. and Bagi, L.K., 1994. Expansion of response surface models for the growth of *Escherichia coli* O157:H7 to include sodium nitrite as a variable. *Int. J. Food Microbiol.* 23: 317-332.

Buchanan, R.L., Bagi, L.K., Goins, R.V. and Phillips, J.G., 1993. Response surface models for the growth kinetics of *Escherichia coli* O157:H7. *Food Microbiol.* 10: 303-315.

Buchanan, R.L. and Cygnarowicz, M.L., 1990. A mathematical approach to defining and calculating the duration of the lag phase. *Food Microbiol.* 7: 237-240.

Buchanan, R.L. and Doyle, M.P., 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*. *Food Technol.* 10: 69-76.

Buchanan, R.L. and Edelson, S.G., 1996. Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. *Appl. Environ. Microbiol.* 62: 4009-4013.

Buchanan, R.L. and Edelson, S.G., 1999. pH-dependent stationary-phase acid resistance response of enterohemorrhagic *Escherichia coli* in the presence of various acidulants. *J. Food Prot.* 62: 211-218.

Buchanan, R.L. and Klawitter, L.A., 1992. The effect of incubation temperature, initial pH, and sodium chloride on the growth kinetics of *Escherichia coli* O157:H7. *Food Microbiol.* 9: 185-196.

Buzby, J.C. and Roberts, T., 1997. Economic costs and trade impacts of microbial foodborne illness. *World Health Statistics Quarterly* 50: 57-66.

Cairns, J., Overbaugh, J. and Miller, S., 1988. The origin of mutants. *Nature* 335: 142-145.

Calabrese, J.P. and Bissonnette, G.K., 1990. Improved membrane filtration method incorporating catalase and sodium pyruvate for detection of chlorine-stressed coliform bacteria. *Appl. Environ. Microbiol.* 56: 3558-3564.

Calcott, P.H., Oliver, J.D., Dickey, K. and Calcott, K., 1984. Cryosensitivity of *Escherichia coli* and the involvement of cyclopropane fatty acids. *J. Appl. Bacteriol.* 56: 165-172.

Campbell, N.A., 1990. *Biology*. Benjamin/Cummings Publishing Co., Redwood City, California.

Casolari, A., 1981. A model describing microbial inactivation and growth kinetics. *J. Theor. Biol.* 88: 1-34.

Castanie-Cornet, M.P., Penfound, T.A., Smith, D., Elliott, J.F. and Foster, J.F., 1999. Contol of acid resistance in *Escherichia coli*. *J. Bacteriol.* 181: 3525-3535.

CDC, 1995. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami – Washington and California, 1994. *Morbidity and Mortality Weekly Report* 44: 157-160.

CDC, 1997. Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider – Connecticut and New York, October 1996. *JAMA* 277: 781-783.

CDNANZ, 1997. Foodborne disease: Towards reducing foodborne illness in Autralia. Technical report series No. 2.

Cerf, O., 1977. Tailing of survival curves of bacterial spores. *J. Appl. Bacteriol.* 42: 1-19.

Charm, S.E., 1958. The kinetics of bacterial inactivation by heat. *Food Technology* 12: 4-8.

Chang, Y.Y. and Cronan, J.E., 1999. Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. *Molec. Microbiol.* 33: 249-259.

Cheng, C.M., and Kaspar, C.W., 1998. Growth and processing conditions affecting acid tolerance in *Escherichia coli* O157:H7. *Food Microbiol.* 15: 157-166.

Cheville, A.M., Arnold, K.W., Buchrieser, C., Cheng, C.M. and Kaspar, C.W., 1996. *RpoS* regulation of acid, heat and salt tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 62: 1822-1824.

Chick, H., 1908. An investigation of the laws of disinfection. *J. Hyg. Camb.* 8: 92-158.

Chick, H., 1910. The process of disinfection by chemical agencies and hot water. *J. Hyg. Camb.* 10: 237-286.

Chivell, W.C., 1995. State Coroners Report – Finding of Inquest into the death of Nikki Dearne Robinson.

Clark, W.M. and Lubs, H.A., 1917. Hydrogen electrode potentials of phthalate, phosphate and borate buffer mixtures. *J. Biol. Chem.* 25: 479.

Cohen, B., 1922. Disinfection studies – the effects of temperature and hydrogen ion concentration upon the viability of Bact. coli and Bact. typhosum in water. *J. Bacteriol.* 7: 183-230.

Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A. and Levine, M.M., 1996. Viable but nonculturable *Vibrio cholerae*-O1 revert to a cultivable state in the human intestine. *World J. Microb. Biot.* 12: 28-31.

Conner, D.E. and Kotrola, J.S., 1995. Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. *Appl. Environ. Microbiol.* 61: 382-385.

Cook, A.M., Roberts, T.A. and Widdowson, J.P., 1964. Gamma irradiation of *Bacillus subtilis* spores in the presence of sugars. *J. Gen. Microbiol.* 34: 185-193.

Corlett, D.A. and Brown, M.H., 1980. pH and acidity, In: J.H. Silliker and R.P. Elliot (Eds), *Microbial ecology of foods* (Volume 1) - *Factors affecting life and death of microorganisms*. Academic Press, New York. 92-111.

Cotter, P.A., Chepuri, V., Gennis, R.B. and Gunsalus, R.P., 1990. Cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the *fnr* gene product. *J. Bacteriol.* 172: 6333-6338.

Cronan, J.E., 1968. Phospholipid alterations during growth of *Escherichia coli. J. Bacteriol.* 95: 2054-2061.

Cronan, J.E., Reed, R., Taylor, F.R. and Jackson, M.B., 1979. Properties and biosynthesis of cyclopropane fatty acids in *Escherichia coli. J. Bacteriol.* 138: 118-121.

Cutter, C.N and Siragusa, G.R., 1994. Efficacy of organic acids against *Escherichia coli* O157:H7 attached to beef carcass tissue using a pilot scale model carcass washer. *J. Food Prot.* 57: 97-103.

Czechowicz, S.M., Santos, O. and Zottola, E.A., 1996. Recovery of thermally-stressed *Escherichia coli* O157:H7 by media supplemented with pyruvate. *Int. J. Food Microbiol.* 33: 275-284.

Dabbah, R., Moats, W.A. and Mattick, J.F., 1969. Factors affecting resistance to heat and recovery of heat-injured bacteria. *J. Dairy Sci.* 52: 608-614.

Datta, A.R. and Benjamin, M.M., 1999. Cell density dependent acid sensitivity in stationary phase cultures of enterohemorrhagic *Escherichia coli* O157:H7. *FEMS Microbiol. Lett.* 181: 289-295.

Dean, A.C.R. and Hinshelwood, Sir C., 1966. *Growth, function and regulation in bacterial cells.* Oxford University Press, London.

Desmarchelier, P.M., 1996. Foodborne disease: emerging problems and solutions. *MJA* 165: 668-671.

Desmarchelier, P.M. and Grau, F.H., 1997. *Escherichia coli,* In: A.D. Hocking, G. Arnold, I. Jenson, K. Newton, and P. Sutherland (Eds), *Foodborne Microorganisms of Public Health Significance* (Fifth Edition). AIFST (NSW Branch) Food Microbiology Group, Australia, pp 231-264.

Diez-Gonzalez, F. and Russell, J.B., 1999. Factors affecting the extreme acid resistance of *Escherichia coli* O157:H7. *Food Microbiol.* 16: 367-374.

Dorsa, W.J., 1997. New and established carcass decontamination procedures commonly used in the beef-processing industry. *J. Food Prot.* 60: 1146-1151.

Doyle, M.P., 1994. The emergence of new agents of foodborne disease in the 1980s. *Food Res. Int.* 27: 219-226.

Doyle, M.P. and Padhye, V.V., 1989. *Escherichia coli*, In: M.P. Doyle (Ed.), *Foodborne Bacterial Pathogens*. Marcel Dekker, New York, pp 235-281.

Dufourc, E.J., Smith, I.C.P. and Jarrell, H.C., 1984. Role of cyclopropane moieties in the lipid properties of biological membranes: a ^{2}H NMR structural and dynamical approach. *Biochemistry* 23: 2300-2309.

Dunkley, E.A., Guffanti, A.A., Clejan, S. and Krulwich, T.A., 1991. Facultative alkaliphiles lack fatty acid desaturase activity and lose the ability to grow at near-neutral pH when supplemented with an unsaturated fatty acid. *J. Bacteriol.* 173: 1331-1334.

Eijkman, 1908. Die ueberlebungskurve bei abtötung der bakterien durch hitze. *Biochem. Zeitschr.* 11: 12-20.

Ellison, A., Anderson, W., Cole, M.B. and Stewart, G.S.A.B., 1994. Modelling the thermal inactivation of *Salmonella typhimurium* using bioluminescence data. *Int. J. Food Microbiol.* 23: 467-477.

Elowitz, M.B., Levine, A.J., Siggia, E.D. and Swain, P.S., 2002. Stochastic gene expression in a single cell. *Science* 297: 1183-1186.

Falk, C.R., Bucca, H.B. and Simmons, M.P., 1939. A comparative study of the use of varying concentrations of agar in the test medium used to detect contaminants in biologic products. *J. Bacteriol.* 37: 121-131.

Falk, I.S. and Winslow, C.E.A., 1926. A contribution to the dynamics of toxicity and the theory of disinfection. *J. Bacteriol.* 11: 1-25.

Fillingame, R.H. and Divall, S., 1999. Proton ATPases in bacteria: comparison to *Escherichia coli* F_1F_0 as the prototype, In: D.J. Chadwick and G. Cardew (Eds), *Bacterial Responses to pH* Novartis Foundation Symposium 221. John Wiley and Sons, Chichester, pp 218-234.

Finkel, S.E. and Kolter, R., 1999. Evolution of microbial diversity during prolonged starvation. *Proc. Natl. Acad. Sci. USA* 96: 4023-4027.

Finkel, S.E., Zinser, E.R. and Kolter, R., 2000. Long-term survival and evolution in stationary phase, In: G. Storz and R. Hengge-Aronis (Eds), *Bacterial Stress Responses*. ASM Press, Washington, D.C., pp 231-238.

Foster, J.W., 1999. Microbial response to acid stress, In: G. Storz and R. Hengge-Aronis (Eds), *Bacterial Stress Responses*. ASM Press, Washington, D.C., pp 99-115.

Foster, J.W., and Hall, H.K., 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. *J. Bacteriol.* 172: 771-778.

Foster, J.W. and Moreno, M., 1999. Inducible acid tolerance mechanisms in enteric bacteria, In: D.J. Chadwick and G. Cardew (Eds), *Bacterial Responses to pH* Novartis Foundation Symposium 221. John Wiley and Sons, Chichester, pp 55-74.

Foster, P.L., 1992. Directed mutation: between unicorns and goats. *J. Bacteriol.* 174: 1711-1716.

Fulmer, E.I. and Buchanan, R.E., 1923. Studies on toxicity. *J. Gen Physiol.* 6: 77-89.

Gage, S.D. and Stoughton, G.V.E., 1906. A study of the laws governing the resistance of *B. coli* to heat. *Science* 23: 216-217.

Gale, E.F., 1940. Production of amines by bacteria – the decarboxylation of amino-acids by strains of *Bacterium Coli*. *Biochem. J.* 34: 392-413.

Gale, E.F., 1946. The bacterial amino acid decarboxylases. *Adv. Enzymol.* 6: 1-32.

Gale, E.F. and Epps, H.M.R., 1942. The effect of pH of the medium during growth on the enzymic activities of bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the biological significance of the changes produced. *Biochem. J.* 36: 600-618.

Gatenby, A.A., 1992. Protein folding and chaperonins. *Plant Mol. Biol.* 19: 677-687.

Gay, P., Le Coq, D., Steinmetz, M., Berkelman, T. and Kado, C.I., 1985. Positive selection procedure for entrapment of insertion sequence elements in Gram-negative bacteria. *J. Bacteriol.* 164: 918-921.

Gay, P., Le Coq, D., Steinmetz, M., Ferrari, E. and Hoch, J.A., 1983. Cloning structural gene *sacB,* which codes for exoenzyme levansucrase of *Bacillus subtilis*: expression of the gene in *E. coli. J. Bacteriol.* 153: 1424-1431.

Gerba, C.P., Rose, J.B. and Haas, C.N., 1996. Sensitive populations: who is at the greatest risk? *Int. J. Food Microbiol.* 30: 113-123.

Giannella, R.A., Broitman, S.A. and Zamcheck, N., 1972. Gastric acid barrier to ingested microorganisms in man: studies *in vivo* and *in vitro. Gut* 13: 251-256.

Glass, K.A., Loeffelhoz, J.M., Ford, J.P. and Doyle, M.P., 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. *Appl. Environ. Microbiol.* 58: 2513-2516.

Glover, J. and Malcolm, L., 1998. Personal communication.

Glover, J., Malcolm, L., Thomson-Carter, F.M., Carter, P., Jordan, S., Park, S.J. and Booth, I.R., 1998. Unpublished observations (Grant application draft).

Goodson, M. and Rowbury, R.J., 1989. Habituation to normal lethal acidity by prior growth of *Escherichia coli* at a sublethal acid pH value. *Lett. Appl. Microbiol.* 8: 77-79.

Gorden, J. and Small, P.L.C., 1993. Acid resistance in enteric bacteria. *Infect. Immun.* 61: 364-367.

Griffin, P.M. and Tauxe, R.V., 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli,* and the associated hemolytic uremic syndrome. *Epidemiol. Rev.* 13: 60-97.

Grogan, D.W. and Cronan, J.E., 1984. Genetic characterisation of the *Escherichia coli* cyclopropane fatty acid (*cfa*) locus and neighbouring loci. *Mol. Gen. Genet.* 196: 367-372.

Grogan, D.W. and Cronan, J.E., 1986. Characterisation of *Escherichia coli* mutants completely defective in synthesis of cyclopropane fatty acids. *J. Bacteriol.* 166: 872-877.

Grogan, D.W. and Cronan, J.E., 1997. Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol. Mol. Biol. R.* 61: 429-441.

Gross, R., Aricò, B. and Rappuoli, R., 1989. Families of bacterial signal-transducing proteins. *Mol. Microbiol.* 3: 1661-1667.

Hall, B.G., 1988. Adaptive evolution that requiresmultiple spontaneous mutations. I. Mutations involving an insertion sequence. *Genetics* 120: 887-897.

Hall, B.G., 1990. Spontaneous point mutations that occur more often when they are advantageous than when they are neutral. *Genetics* 126: 5-16.

Hall, R.L., 1999. The IUFoST Founders Lecture – Food safety: elusive goal and essential quest. *Food Aust.* 51: 601-606.

Hall, H.K., Karem, K.L. and Foster, J.W., 1995. Molecular responses of microbes to environmental stress, In: R.K. Poole (Ed.), *Advances in Microbial Physiology* Vol 37. Academic Press, San Deigo, pp 229-272.

Harold, F.M. and van Brunt, J., 1977. Circulation of H+ and K+ across the plasma membrane is not obligatory for bacterial growth. *Science* 197: 372-373.

Henderson-Smith, J., 1921. The killing of *Botrytis* spores by phenol. *Ann. Appl. Biol.* 8: 27.

Hengge-Aronis, R., 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. *Cell* 72: 165-168.

Hengge-Aronis, R., 1999. Interplay of global regulators in the general stress response of *Escherichia coli*. *Curr. Opin. Microbiol.* 2: 148-152.

Hengge-Aronis, R., 2000. The general stress response in *Escherichia coli*, In: G. Storz and R. Hengge-Aronis (Eds), *Bacterial Stress Responses*. ASM Press, Washington, D.C., pp 99-115.

Hershey, A.D., 1939. Factor limiting bacterial growth VII. Respiration and growth properties of *Escherichia coli* surviving sublethal temperatures. *J. Bacteriol.* 38: 563-578.

Hewlett, R.T., 1909. Milroy Lectures on Disinfection. *Lancet* 1: 741.

Hewlett, R.T. and Hall, G.N., 1911. Influence of culture media on germination of anthrax spores with special reference to disinfection experiments. *J. Hyg. Camb.* 11: 473-480.

Heyde, M. and Portalier, R.C., 1987. Regulation of the outer membrane porin proteins of *Escherichia coli* by pH. *Mol. Gen. Genet.* 208: 511-517.

Heyde, M. and Portalier, R., 1990. Acid shock proteins of *Escherichia coli*. *FEMS Microbiol. Lett.* 69: 19-26.

Hiatt, C.W., 1964. Kinetics of the inactivation of viruses. *Bacteriol. Rev.* 28: 150-163.

Hickey, E.W. and Hirshfield, I.N., 1990. Low-pH-induced effects on patterns of protein synthesis and on internal pH in *Escherichia coli* and *Salmonella typhimurium*. *Appl. Environ. Microbiol.* 56: 1038-1045.

Hill, C., O'Driscoll, B. and Booth, I., 1995. Acid adaptation and food poisoning microorganisms. *Int. J. Food Microbiol.* 28: 245-254.

Hinshelwood, Sir C., 1951. Decline and death of bacterial populations. *Nature* 167: 666-669.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R., 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51-59.

Hollaender, A., Stapleton, G.E. and Martin, F.L., 1951. X-Ray sensitivity of *E. coli* as modified by oxygen tension. *Nature* 167: 103-104.

Humpheson, L., Adams, M.R., Anderson, W.A. and Cole, M.B., 1998. Biphasic thermal inactivation kinetics in *Salmonella enteritidis* PT4. *Appl. Environ. Microbiol.* 64: 459-464.

Ingraham, J.L. and Marr, A.G., 1996. Effect of Temperature, Pressure, pH, and Osmotic Stress on Growth, In: F.C. Neidhardt (Ed.), Escherichia coli *and* Salmonella *Cellular and Molecular Biology* (Volume 2, 2nd Edition). ASM Press, Washington, D.C., pp1570-1578.

Jacobs, S.E., 1960. Some aspects of the dynamics of disinfection. *Report of Proceedings of the British Pharmaceutical Conference* Newcastle upon Tyne. 9T-18T.

Jarvis, B., 1989. Statistical Aspects of the Microbiological Analysis of Foods. *Progress in Industrial Microbiology* Vol. 21. Elsevier, Amsterdam.

Jawetz, E., melnick, J.L. and Adelberg, E.A., 1958. *Review of medical microbiology* (3rd Ed.). Lange Medical Publications, Los Altos.

Jay, J.M., 2000. Modern Food Microbiology (6th Ed.). Aspen Publishers, Inc., Maryland.

Jensen, M.S. and Bainton, D.F., 1973. Temporal changes in pH within the phagocytic vacuole of the polymorphonuclear neutrophilic leukocyte. *J. Cell Biol.* 56: 379-388.

Johnston, M.D., Simons, E.A. and Lambert, R.J.W., 2000. One explanation for the variability of the bacterial suspension test. *J. Appl. Microbiol.* 88: 237-242.

Jones, D.M., Sutcliffe, E.M. and Curry, A., 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. *J. Gen. Microbiol.* 137: 2477-2482.

Jordan, S.L., Glover, J., Malcolm, L., Thomson-Carter, F.M., Booth, I.R. and Park, S.F., 1999a. Augmentation of killing of *Escherichia coli* O157 by combinations of lactate, ethanol and low-pH conditions. *Appl. Environ. Microbiol.* 65: 1308-1311.

Jordan, K.N., Oxford, L. and O'Byrne, C.P., 1999b. Survival of low-pH stress by *Escherichia coli* O157:H7 – Correlation between alterations in the cell envelope and increased acid tolerance. *Appl. Environ. Microbiol.* 65: 3048-3055.

Jordan, K.N., Hall, S. and McClure, P.J., 1999c. Osmotic stress on dilution of acid injured *Escherichia coli*. *Lett. Appl. Microbiol.* 28: 389-393.

Jordan, R.C. and Jacobs, S.E., 1944. Studies in the dynamics of disinfection. I. New data on the reaction between phenol and *Bact. Coli* using an improved technique, together with an analysis of the distribution of resistance amongst cells of the bacterial populations studied. *J. Hyg. Camb.* 43: 275-289.

Jordan, R.C., Jacobs, S.E. and Davies, H.E.F., 1947. Studies in the dynamics of disinfection. XI. The effect of lethal temperatures on standard cultures of *Bact. Coli*. IV. An investigation of that portion of the population which survives prolonged exposure at pH 7.0. *J. Hyg. Camb.* 47: 342-353.

Jordan, K.N., Oxford, L. and O'Byrne, C.P., 1999b. Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alterations in the cell envelope and increased acid tolerance. *Appl. Environ. Microbiol.* 65: 3048-3055.

Käferstein, F.K., 1997. Food safety: a commonly underestimated public health issue. *World Health Statistics Quaterly* 50: 3-4.

Käferstein, F.K., Motarjemi, Y. and Bettcher, 1997. Foodborne disease control: a transnational challenge. *Emerg. Infect. Dis.* 3: 503-515.

Kaniga, K., Delor, I. And Cornelis, G.R., 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* 109: 137-141.

Kaprelyants, A.S., Gottschal, J.C. and Kell, D.B., 1993. Dormancy in non-sporulating bacteria. *FEMS Microbiol. Rev.* 104: 271-286.

Kasimoglu, E., Park, S.J., Malek, J., tseng, C.P. and Gunsalus, R.P., 1996. Transcriptional regulation of the proton-translocating ATPase (*atpIBEFHAGDC*) operon of *Escherichia coli*: Control by cell growth rate. *J. Bacteriol.* 178: 5563-5567.

Kaur, J., Ledward, D.A., Park, R.W.A. and Robson, R.L., 1998. Factors affecting the heat resistance of *Escherichia coli* O157:H7. *Lett. Appl. Micro.* 26: 325-330.

Kell, D.B., 2000. Personal communication.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R. and Barer, M.R., 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Ant. van Leeu.* 73: 169-187.

Kihara, M and Macnab, R.M., 1981. Cytoplasmic pH mediates pH taxis and weak-acid repellent taxis of bacteria. *J. Bacteriol.* 145: 1209-1221.

Knivett, V.A. and Cullen, J., 1965. Some factors affecting cyclopropane acid formation in *Escherichia coli*. *Biochem. J.* 96: 771-776.

Knivett, V.A. and Cullen, J., 1967. Fatty acid synthesis in *Escherichia coli*. *Biochem. J.* 103: 299-306.

Knøchel, S. and Gould, G., 1995. Preservation microbiology and safety: Quo vadis? *Trends Food Sci. Tech.* 6: 127-131.

Kobayashi, H., Murakami, N. and Unemoto, T., 1982. Regulation of the cytoplasmic pH in *Streptococcus faecalis*. *J. Biol. Chem.* 257: 13246-13252.

Kobayashi, H., Saito, H. and Kakegawa, T., 2000. Bacterial strategies to inhabit acidic environments. *J. Gen. Appl. Microbiol.* 46: 235-243.

Kolter, R., Siegele, D.A. and Tormo, A., 1993. The stationary phase of the bacterial life cycle. *Annu. Rev. Microbiol.* 47: 855-874.

Komemushi, S. and Terui, G., 1967. On the change of death rate constant of bacterial spores in the course of heat sterilization. *J. Ferment. Technol.* 45: 764-768.

Krist, K.A., 1997. Description and mechanisms of bacterial growth responses to water activity and compatible solutes. PhD Thesis, University of Tasmania.

Krist, K.A., Ross, T. and McMeekin, T.A., 1998. Final optical density and growth rate; effects of temperature and NaCl differ from acidity. *Int. J. Food Microbiol.* 43: 195-203.

Kroll, R.G. and Booth, I.R., 1981. The role of potassium transport in the generation of a pH gradient in *Escherichia coli. Biochem. J.* 198: 691-698.

Kroll, R.G. and Booth, I.R., 1983. The relationship between intracellular pH, the pH gradient and potassium transport in Escherichia coli. *Biochem. J.* 216: 709-716.

Kroll, R.G. and Patchett, R.A., 1992. Induced acid tolerance in *Listeria monocytogenes. Lett. Appl. Microbiol.* 14: 224-227.

Krönig, B. and Paul, T., 1897. Die chemische grundlage der lehre von der giftwirkung und desinfektion. *Ztschr. F. Hyg.* 25: 1-112.

Lahti, E., Johansson, T., Honkanen-Buzalski, T., Hill, P. and Nurmi, E., 2001. Survival and detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* during the manufacture of dry sausage using two different starter cultures. *Food Microbiol.* 18: 75-85.

Lamanna, C. and Mallette, M.F., 1959. *Basic Bacteriology – Its Biological and Chemical Background.* The Williams and Wilkins Company, Baltimore.

Lambert, R.J.W. and Johnston, M.D., 2000. Disinfection kinetics: a new hypothesis and model for the tailing of log-survivor/time curves. *J. Appl. Microbiol.* 88: 907-913.

Lange, R. and Hengge-Aronis, R., 1991a. Identification of a central regulator of stationary –phase gene expression in *Escherichia coli*. *Mol. Microbiol.* 5: 49-59.

Lange, R. and Hengge-Aronis, R., 1991b. Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor σ^S. *J. Bacteriol.* 173: 4474-4481.

Law, J.H., 1971. Biosynthesis of cyclopropane rings. *Acc. Chem. Res.* 4: 199-203.

Law, J.H., Zalkin, H. and Kaneshiro, T., 1963. Transmethylation reactions in bacterial lipids. *Biochim. Biophys. Acta* 70: 143-151.

Lee, R.E. and Gilbert, C.A., 1918. On the application of the mass law to the process of disinfection – being a contribution to the "mechanistic theory" as opposed to the "vitalistic theory". *J. Phys. Chem.* 22: 348-372.

Lee, R.M. and Hartman, P.A., 1989. Optimal pyruvate concentration for the recovery of coliforms from food and water. *J. Food Prot.* 52: 119-121.

Lee, I.S., Slonczewski, J.L. and Foster, J.W., 1994. A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. *J. Bacteriol.* 176: 1422-1426.

Leistner, L., 2000. Basic aspects of food preservation by hurdle technology. *Int. J. Food Microbiol.* 55: 181-186.

Lemcke, R.M. and White, H.R., 1959. The heat resistance of *Escherichia coli* cells from cultures of different ages. *J. Appl. Bacteriol.* 122: 193-201.

Levina, N., Tötemeyer, S., Stokes, N.R., Louis, P., Jones, M.A. and Booth, I.R., 1999. Protection of *Escherichia coli* cells against extreme turgor by activation of MscS

and MscL mechanosensitive channels: identification of genes required for MscS activity. *EMBO J.* 18: 1730-1737.

Leyer, G.J. and Johnson, E.A., 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. *Appl. Environ. Microbiol.* 58:2075-2080.

Leyer, G.J. and Johnson, E.A., 1993. Acid adaptation induced cross protection against environmental stresses in *Salmonella typhimurium. Appl. Environ. Microbiol.* 59: 1842-1847.

Leyer, G.J., Wang, L.L. and Johnson, E.A., 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. *Appl. Environ. Microbiol.* 61: 3752-3755.

Lin, J., Lee, I.S., Frey, J., Slonczewski, J.L. and Foster, J.W., 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium, Shigella flexneri,* and *Escherichia coli. J. Bacteriol.* 177: 4097-4104.

Lin, J., Smith, M.P., Chapin, K.C., Baik, H.S., Bennett, G.N. and Foster, J.W., 1996. Mechanism of acid resistance in enterohemorrhagic *Escherichia coli. Appl. Environ. Microbiol.* 62: 3094-3100.

Loewen, P.C. and Hengge-Aronis, R., 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. *Annu. Rev. Microbiol.* 48: 53-80.

Loewen, P.C., Hu, B., Strutinsky, J. and Sparling, R., 1998. Regulation in the *rpoS* regulon of *Escherichia coli. Can. J. Microbiol.* 44: 707-717.

Mackey, B.M., 1999. Injured Bacteria, In: B. Lund (Ed.), *The microbiological safety and quality of food.* Aspen Publishers, Gaithersburg, pp 315-341.

Mackey, B.M. and Derrick, C.M., 1982. A comparison of solid and liquid media for assessing the sensitivity of heat-injured *Salmonella typhimurium* to selenite and tetrathionate media, and the time needed to recover resistance. *J. Appl. Bacteriol.* 53: 233-242.

Mackey, B.M., Miles, C.A., Parsons, S.E. and Seymour, D.A., 1991. Thermal denaturation o whole cells and cell components of *Escherichia coli* examined by differential scanning calorimetry. *J. Gen. Microbiol.* 137: 2361-2374.

Madsen, T. and Nyman, M., 1907. Zur theorie der desinfektion. *Zeitschr. F. Hyg.* 57: 388-404.

Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. *Molecular Cloning: A Laboratory Manual,* Cold Springs Harbour Laboratory, Cold Spring Harbour, NY.

Martin, S.E., Flowers, R.S. and Ordal, Z.J., 1976. Catalase: its effects on microbial enumeration. *Appl. Environ. Microbiol.* 32: 731-734.

Maurice, J., 1994. The rise and rise of food poisoning. *New Sci.* 144: 28-33.

Mayhew, M. and Hartl, F.U., 1996. Molecular-chaperone proteins, In: (F.C. Neidhardt (Ed.), Escherichia coli *and* Salmonella – *Cellular and Molecular Biology* (Volume 1). ASM Press, Washington, D.C., 922-937.

McCann, M.P., Kidwell, J.P. and Matin, A., 1991. The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli. J. Bacteriol.* 173: 4188-4194.

McClure, P.J. and Hall, S., 2000. Survival of *Escherichia coli* in foods. *J. Appl. Micro.* 88: 61S-70S.

McDonald, L.C., Hackney, C.R. and Ray, B., 1983. Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. *Appl. Environ. Microbiol.* 45: 360-365.

McDougald, D., Rice, S.A., Weichart, D. and Kjelleberg, S., 1998. Nonculturability: adaptation or debilitation? *FEMS Microbiol. Ecol.* 25: 1-9.

McGarrity, J.T. and Armstrong, J.B., 1975. The effects of salt on phospholipid fatty acid composition in *Escherichia coli* K-12. *Biochim. Biophys. Acta* 398: 258-264.

McGarrity, J.T. and Armstrong, J.B., 1981. The effect of temperature and other growth conditions on the fatty acid composition of *Escherichia coli*. *Can. J. Microbiol.* 27: 835-840.

McKellar, R.C. and Knight, K.P., 1999. Growth and survival of various strains of enterohemorrhagic *Escherichia coli* in hydrochloric and acetic acid. *J. Food Prot.* 62: 1466-1469.

McMeekin, T.A., Olley, J., Ross, T. and Ratkowsky, D.A., 1993. *Predictive microbiology: theory and application*. Research Studies Press, Taunton, UK.

Mellefont, L.A., 2001. Predictive Model Development and Lag Phase Characterisation for Applications in the Meat Industry. PhD Thesis, University of Tasmania.

Meng, S.Y. and Bennett, G.N., 1992. Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH. *J. Bacteriol.* 174: 2659-2669.

Milbauer, R. and Grossowicz, N., 1959. Reactivation of chlorine-inactivated *Escherichia coli*. *Appl. Microbiol.* 7: 67-70.

Miles, C.A., Mackey, B.M. and Parsons, S.E., 1993. Differential scanning calorimetry of bacteria. *J. Gen. Microbiol.* 132: 939-952.

Miller, R.J. and Davey, C.B., 1965. Influence of the microphysical structure of a system on the growth of bacteria. *Can. J. Microbiol.* 11: 761-763.

Miller, L.G. and Kaspar, C.W., 1994. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. *J. Food Prot.* 57: 460-464.

Miller, A.J., Smith, J.L. and Buchanan, R.L., 1998. Factors affecting the emergence of new pathogens and research strategies leading to their control. *J. Food Safety* 18: 243-263.

Milton, D.L., O'Toole, R., Horstedt, P and Wolf-Watz, H., 1996. Flagellin A is essential for virulence of *Vibrio anguillarum*. *J. Bacteriol.* 178: 1310-1319.

Moats, W.A., Dabbah, R. and Edwards, V.M., 1971. *J. Food Sci.* 36: 523-526.

Montville, T.J., 1997. Principles which influence microbial growth, survival and death in foods, In: M.P. Doyle, L.R. Beuchat and T.J. Montville (Eds), *Food Microbiology – Fundamentals and Fronteirs*. ASM Press, Washington, D.C., pp. 13-29.

Morgan, D., Newman, C.P., Hutchinson, D.N., Walker, A.M., Rowe, B. and Majid, F., 1993. Verotoxin producing *Escherichia coli* O157 infections associated with the consumption of yoghurt. *Epidemiol. Infect.* 111: 181-187.

Morris, J.G. and Potter, M., 1997. Emergence of new pathogens as a function of changes in host susceptibility. *Emerg. Infect. Dis.* 3: 435-441.

Mossel. D.A.A. and Van Netten, P., 1984. Harmful effects of selective media on stressed microorganisms: nature and remedies, In: M.H.E. Andrew and A.D. Russell (Eds), *The revival of injured microbes*. Academic Press, London, pp 329-371.

Moxon, E.R., Rainey, P.B., Nowak, M.A. and Lenski, R.E.. 1994. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Current Biology* 4: 24-33.

National Centre for Disease Control, 2000. National Notifiable Diseases Surveillence System Annual Disease Data. http://www.health.gov.au/pubhlth/cdi/nndss/nndss2.htm (accessed 31/01/01).

Neely, M.N., Dell, C.L. and Olson, E.R., 1994. Roles of LysP and CadC in mediating the lysine requirement for acid induction of the *Escherichia coli cad* operon. *J. Bacteriol.* 176: 3278-3285.

Neidhardt, F.C., 1996. The enteric bacterial cell and the age of bacteria, In: F.C. Neidhardt (Ed.), Escherichia coli *and* Salmonella typhimurium – *Cellular and Molecular Biology 1* (2nd Edition). American Society for Microbiology, Washington, pp 1-3.

Nichols, P.D., Guckert, J.B. and White, D.C., 1986. Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. *J. Microbiol. Meth.* 5: 49-55.

Nilsson, L., Oliver, J.D. and Kjelleberg, S., 1991. Resuscitation of *Vibrio vulnficus* from the viable but nonculturable state. *J. Bacteriol.* 173: 5054-5059.

Niven, G.W. and Mackey, B.M., 2000. Personal communication.

Niven, G.W., Miles, C.A. and Mackey, B.M., 1999. The effects of hydrostatic pressure on ribosome conformation in *Escherichia coli*: and *in vivo* study using differential scanning calorimetry. *Microbiology* 145: 419-425.

O'Hara, G.W. and Glenn, A.R., 1994. The adaptive acid tolerance response in root nodule bacteria and *Escherichia coli*. *Arch. Microbiol.* 161: 286-292.

Oliver, J.D., 1993. Formation of viable but nonculturable cells, In: S. Kjelleberg (Ed.), *Starvation in Bacteria*. Plenum Press, New York, pp 239-272.

Oliver, J.D. and Bockian, R., 1995. *In vivo* resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 61: 2620-2623.

Oliver, J.D., Hite, F., McDougald, D., Andon, N.L. and Simpson, L.M., 1995. Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. *Appl. Environ. Microbiol.* 61: 2624-2630.

Olson, E.R., 1993. Influence of pH on bacterial gene expression. *Mol. Microbiol.* 8: 5-14.

Ørskov, F., 1984. Genus I – *Escherichia coli*, In: N.R. Kreig (Ed.), *Bergey's Manual of Systematic Bacteriology I.* Williams and Wilkins, Baltimore, 420-423.

O'Sullivan, E. and Condon, S., 1997. Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 63: 4210-4215.

Ozaki, M., Wada, A., Fujita, N. and Ishihama, A., 1991. Growth phase-dependent modification of RNA polymerase in *Escherichia coli*. *Mol. Gen. Genet.* 230: 17-23.

Padan, E., Zilberstein, D. and Schuldiner, S., 1981. pH homeostasis in bacteria. *Biochim. Biophys. Acta* 650: 151-166.

Parry , S.M. and Palmer, S.R., 2000. The public health significance of VTEC O157. In: *E. coli* – friend or foe? Supplement to *J. Appl. Microbiol.* Society for Applied Microbiology Symposium Series Number 29. 88: 38S-50S.

Paul. 1909. Der chemisch reactionsverlauf beim absterben trockener bakterien bei niederen temperaturen. *Biochem. Z.* 18: 1-13.

Pelczar, M.J., Chan, E.C.S. and Krieg, N.R., 1993. *Microbiology – Concepts and Applications*. McGraw-Hill, Inc. New York.

Peleg, M., 2000. Microbial survival curves – the reality of flar "shoulders" and absolute thermal death times. *Food Res. Int.* 33: 531-538.

Peleg, M. and Cole, M.B., 1998. Reinterpretation of microbial survival curves. *Crit. Rev. Food Sci.* 38: 353-380.

Peterson, W.L., Mackowiak, P.A., Barnett, C.C., Marling-Carson, M. and Haley, M.L., 1989. The human gastric bactericidal barrier: mechanisms of action, relative antibacterial activity , and dietry influences. *J. Infect. Dis.* 159: 979-983.

Phan-Thanh, L., Mahouin, F. and Aligé, S., 2000. Acid responses of *Listeria monocytogenes*. *Int. J. Food. Microbiol.* 55: 121-126.

Pirt, S.J., 1965. The maintenance energy of bacteria in growing cultures. *Proc. R. Soc. Lond [Biol]* 163: 224-231.

Postgate, J.R., 1967. Viability measurements and the survival of microbes under minimum stress. *Advances in Microbial Physiology* 1: 1-23. Death in Macrobes and Microbes . Symposium.

Postgate, J.R., 1969. Viable Counts and viability, In: J.R. Norris and D.W. Ribbons (Eds), *Methods in Microbiology* (Volume 1). Academic Press, London, pp 611-628.

Postgate, J.R., 1976. Death in macrobes and microbes, In: T. Gray and J.R. Postgate (Eds), *The survival of vegetative microorganisms*. Cambridge University Press, Cambridge, pp 1-18.

Postgate, J.R. and Hunter, J.R., 1962. The survival of starved bacteria. *J. Gen. Microbiol.* 29: 233-267.

Poynter, D., Hicks, S.J. and Rowbury, R.J., 1986. Acid resistance of attached organisms and its implications for the pathogenicity of plasmid-bearing *Escherichia coli*. *Lett. Appl. Microbiol.* 3: 117-121.

Presser, K.A., 1998. Personal communication.

Presser, K.A., 2001. Physiology and modelling of *Escherichia coli* growth inhibition due to pH, organic acids, temperature and water activity. PhD Thesis, University of Tasmania.

Presser, K.A., Ratkowsky, D.A. and Ross, T., 1997. Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. *Appl. Environ. Microbiol.* 63: 2355-2360.

Presser, K.A., Ross, T. and Ratkowsky, D.A., 1998. Modelling the growth limits (growth/no growth interface) of *Escherichia coli* as a function of temperature, pH, lactic acid concentration, and water activity. *Appl. Environ. Microbiol.* 64: 1773-1779.

Prokop, A. and Humphrey, A.E., 1970. Kinetics of disinfection, In: M.A. Bernarde (Ed.), *Disinfection*. Marcel Dekker, New York, pp 61-83.

Przybylski, K.S. and Witter, L.D., 1979. Injury and recovery of *Escherichia coli* after sublethal acidification. *Appl. Environ. Microbiol.* 37: 261-265.

Rahman, I., Shahamat, M., Chowdhury, M.A.R. and Colwell, R.R., 1996. Potential virulence of viable but nonculturable *Shigella dysenteriae* Type I. *Appl. Environ. Microbiol.* 62: 115-120.

Rahn, O., 1929. The size of bacteria as the casue of the logarithmic order of death. *J. Gen. Physiol.* 13: 179-205.

Rahn, O., 1930. The non-logarithmic order of death of some bacteria. *J. Gen. Physiol.* 13: 395-407.

Rahn, O., 1943. *Chemistry of death.* Cold Spring Harbour Symposium of Quantitative Biology. 2: 70-77.

Rahn, O., 1945. Injury and death of bacteria by chemical agents, In: B.J. Luyet (Ed.), *Biodynamica Monographs* (number 3). Biodynamica, Normandy, Missouri.

Raja, N., Goodson, M., Chui, W.C.M., Smith, D.G. and Rowbury, R.J., 1991a. Habituation to acid in *Escherichia coli*: conditions for habituation and its effect on plasmid transfer. *J. Appl. Bacteriol.* 70: 59-65.

Raja, N., Goodson, M., Smith, D.G. and Rowbury, R.J., 1991b. Decreased DNA damage by acid and increased repair of acid-damaged DNA in acid-habituated *Escherichia coli*. *J. Appl. Bacteriol.* 70: 507-511.

Raven, J.A. and Beardall, J., 1981. The intrinsic permeability of biological membranes to H^+: significance for the efficiency of low rates of energy transformation. *FEMS Microbiol. Lett.* 10: 1-5.

Reichenbach, H., 1911. Die absterbeordnung der bakterien und ihre bedeutung für theorie u. Praxis der desinfektion. *Z. Hyg. InfektKr.* 69: 171-222.

Repaske, D.R. and Adler, J., 1981. Change in intracellular pH of *Escherichia coli* mediates the chemotactic response to certain attractants and repellents. *J. Bacteriol.* 145: 1196-1208.

Riley, L.W., 1987. The epidemiologic, clinical, and microbiological features of hemorrhagic colitis. *Ann. Rev. Microbiol.* 41: 383-407.

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A. and Cohen, M.L., 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New Engl. J. Med.* 308: 681-685.

Riordan, D.C.R., Duffy, G., Sheridan, J.J., Eblen, B.S., Whiting, R.C., Blair, I.S. and McDowell, D.A., 1998. Survival of *Escherichia coli* O157:H7 during the manufacture of pepperoni. *J. Food Prot.* 61: 146-151.

Rius, N., Solé, M., Francia, A. and Lorén, J.G., 1995. Buffering capacity and H+ membrane conductance of Gram-negative bacteria. *FEMS Microbiol. Lett.* 130: 103-110.

Roberts, T.A., 1970. Recovering spores damaged by heat, ionizing radiations or ethylene oxide. *J. Appl. Bacteriol.* 33: 74-94.

Roberts, T.A., 1990. Predictive modelling of microbial growth. *Food Technol. Int. Europe*: 231-235. A. Turner Sterling Publications International Limited, London.

Roberts, R.B. and Aldous, E., 1949. Recovery from ultraviolet irradiation in *Escherichia coli. J. Bacteriol.* 57: 363-375.

Roberts, T.A. and Hitchins, A.D., 1969. Resistance of spores, In: G.W. Gould and A. Hurst (Eds), *The bacterial spore.* Academic Press, London.

Roberts, T.A. and Ingram, M., 1956. The resistance of spores of *Clostridium botulinum* type E to heat and radiation. *J. Appl. Bacteriol.* 28: 125-141.

Robinson, T.P., Ocio, M.J., Kaloti, A. and Mackey, B.M., 1998. The effect of the growth environment on the lag phase of *Listeria monocytogenes*. *Int. J. Food Microbiol.* 44: 83-92.

Roering, A.m., Luchansky, J.B., Ihnot, A.M., Ansay, S.E., Kaspar, C.W. and Ingham, S.C., 1999. Comparative survival of *Salmonella typhimurium* DT 104, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in preservative-free apple cider and simulated gastric fluid. *Int. J. Food Microbiol.* 46: 263-269.

Rompf, A. and Jahn, D., 1999. Ecology of bacteria and fungi in foods: Influence of redox potential and pH, In: R.K. Robinson, C.A. Batt and P.D. Patel (Eds), *Encyclopedia of Food Microbiology*. Academic Press, San Diego, pp 556-563.

Rosche, W.A. and Foster, P.L., 2000. Mutation under stress: adaptive mutation in *Escherichia coli*, In: G. Storz and R. Hengge-Aronis (Eds) *Bacterial Stress Responses*. ASM Press, Washington, D.C., pp 239-248.

Rosenberg, S.M., 1994. In pursuit of a molecular mechanism for adaptive mutation. *Genome* 37: 893-899.

Rosenstein, C. and Levin, I., 1935. The bactericidal and antiseptic action of preservatives frequently used in biological products, and the effect of these preservatives on the potencies of the products. *Am. J. Hyg.* 21: 260-279.

Ross, T., 1999. Assessment of a theoretical model for the effects of temperature on bacterial growth rate, In: *Predictive Microbiology Applied to Chilled Food Preservation*, Proc. Int. Inst. Refrigeration, Commission C2, Quimper, France, June 1997, EUR 18816, pp 64-71.

Ross, T., 1999. Personal communication.

Ross, T., 2002. Personal communication.

Ross, T. and McMeekin, T.A., 1994. Predictive microbiology. *Int. J. Food Microbiol.* 23: 241-264.

Ross, T., McMeekin, T.A. and Baranyi, J., 2000. Predictive microbiology and food safety, In: R.K. Robinson, C.A. Batt and P.D. Patel (Eds), *Encyclopedia of Food Microbiology*. Academic Press, London, pp 1699-1710.

Ross, T. and Shadbolt, C.T., 2001. Predicting pathogen inactivation in uncooked comminuted fermented meat products. Meat and Livestock Australia, Sydney, Australia.

Rosso, L., Lobry, J.R., Bajard, S. and Flandrois, J.P., 1995. Convenient model to describe the combined effects of temperature and pH on microbial growth. *Appl. Environ. Microbiol.* 61: 610-616.

Roszak, D.B. and Colwell, R.R., 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* 51: 365-379.

Roszak, D.B., Grimes, D.J. and Colwell, R.R., 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.* 30: 334-338.

Roth, L.A. and Keenan, D., 1971. Acid injury of *Escherichia coli*. *Can. J. Microbiol.* 17: 1005-1008.

Rowan, N.J., 1999. Evidence that inimical food-preservation barriers alter microbial resistance, cell morphology and virulence. *Trends Food Sci. Technol.* 10: 261-270.

Rowbury, R.J., 1995. An assessment of environmental factors influencing acid tolerance and sensitivity in *Escherichia coli, Salmonella* spp. and other enterobacteria. *Lett. Appl. Microbiol.* 20: 333-337.

Rowbury, R.J., 1997. Regulatory components, including integration host factor, CysB and H-NS, that influence pH responses in *Escherichia coli*. *Lett. Appl. Microbiol.* 24: 319-328.

Rowbury, R.J. and Goodson, M., 1993. PhoE porin of *Escherichia coli* and phosphate reversal of acid damage and killing and of acid induction of the CadA gene product. *J. Appl. Bacteriol.* 74: 652-661.

Rowbury, R.J. and Goodson, M., 1998. Induction of acid tolerance at neutral pH in log-phase *Escherichia coli* by medium filtrates from organisms grown at acidic pH. *Lett. Appl. Microbiol.* 26: 447-451.

Rowbury, R.J. and Goodson, M., 1999. An extracellular stress-sensing protein is activated by heat and u.v. irradiation as well as by mild acidity, the activation producing an acid tolerance-inducing protein. *Lett. Appl. Microbiol.* 29: 10-14.

Rowbury, R.J., Goodson, M. and Wallace, A.D., 1992. The PhoP porin and transmission of the chemical stimulus for induction of acid resistance (acid habituation) in *Escherichia coli*. *J. Appl. Bacteriol.* 72: 233-243.

Rowbury, R.J., Goodson, M. and Whiting, G.C., 1989. Habituation of *Escherichia coli* to acid and alkaline pH and its relevance for bacterial survival in chemically-polluted natural waters. *Chem. Ind.* October: 685-686.

Rowe, M.T. and Kirk, R., 1999. An investigation into the phenomenon of cross protection in *Escherichia coli* O157:H7. *Food Microbiol.* 16: 157-164.

Ryan, F.J., 1959. Bacterial mutation in stationary phase and the question of cell turnover. *J. Gen. Microbiol.* 21: 530-549.

Ryu, J.H. and Beuchat, L.R., 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int. J. Food Microbiol.* 45: 185-193.

Salmond, C.V., Kroll, R.G. and Booth, I.R., 1984. The effect of food preservatives on pH homeostasis in *Escherichia coli*. *J. Gen. Microbiol.* 130: 2845-2850.

Salter, M.A., Ratkowsky, D.A., Ross, T. and McMeekin, T.A., 2000. Modelling the combined temperature and salt (NaCl) limits for growth of a pathogenic *Escherichia coli* strain using nonlinear logistic regression. *Int. J. Food Microbiol.* 61: 159-167.

Samelis, J., Sofos, J.N., Kendall, P.A. and Smith, G.C., 2001. Influence of the natural microbial flora on the acid tolerance response of *Listeria monocytogenes* in a model system of fresh meat decontamination fludis. *Appl. Environ. Microbiol.* 67: 2410-2420.

Sartory, D.P., 1995. Improved recovery of chlorine-stressed coliforms with pyruvate supplemented media. *Wat. Sci. Tech.* 31: 255-258.

Schellhorn, H.E. and Stones, V.L., 1992. Regulation of *katF* and *katE* in *Escherichia coli* K-12 by weak acids. *J. Bacteriol.* 174: 4769-4776.

Semancheck, J.J. and Golden, D.A., 1996. Survival of *Escherichia coli* O157:H7 during fermentation of apple cider. *J. Food Prot.* 59: 1256-1259.

Serrano, R., 1984. Plasma membrane ATPase of fungi and plants as a novel type of proton pump. *Curr. Top. Cell. Regul.* 23: 157: 533-544.

Shabala, S., 2000. Personal communication.

Shabala, L., Budde, B., Ross, T., Siegumfeldt, H. and McMeekin, T., 2002. Responses of *Listeria monocytogenes* to acid stress and glucose availability monitored by measurements of intracellular pH and viable counts. *Int. J. Food Microbiol.* **75:** 89-97.

Shadbolt, C.T., 2001. Personal communication.

Shadbolt, C.T., Ross, T. and McMeekin, T.A., 1999. Nonthermal death of *Escherichia coli*. *Int. J. Food Microbiol.* 49: 129-138.

Shadbolt, C.T., Ross, T. and McMeekin, T. A., 2001. Differentiation of the effects of lethal pH and water activity: food safety implications. *Lett. Appl. Microbiol.* 32: 99-102.

Shaughnessy, H.J. and Falk, I.S., 1924. The effects of some electrolytes on the buffering capacity of *Bacterium coli*. *J. Bacteriol.* 9: 559.

Shaughnessy, H.J. and Winslow, C.E.A., 1927. The diffusion products of bacterial cells as influenced by the presence of various electrolytes. *J. Bacteriol.* 14: 69-99.

Silk, T.M. and Donnelly, C.W., 1997. Increased detection of acid-injured *Escherichia coli* O157:H7 in autoclaved apple cider by using nonselective repair on trypticase soy agar. *J. Food Prot.* 60: 1483-1486.

Simon, R., Höte, B., Klauke, B. and Kosier, B., 1991. Isolation and characterisation of insertion sequence elements from Gram-negative bacteria by using new broad-host-range, positive selection vectors. *J. Bacteriol.* 173: 1502-1508.

Skinner, F.A. and Hugo, W.B., 1976. *Inhibition and inactivation of vegetative microbes*. Academic Press, London.

Slonczewski, J.L. and Blankenhorn, D., 1999. Acid and base regulation in the proteome of *Escherichia coli*, In: D.J. Chadwick and G. Cardew (Eds), *Bacterial Responses to pH* Novartis Foundation Symposium 221. John Wiley and Sons, Chichester, pp 75-92.

Slonczewski, J.L. and Foster, J.W., 1996. pH-regulated genes and survival at extreme pH, In: F.C. Neidhardt (Ed.), Escherichia coli *and* Salmonella typhimurium – *Cellular and Molecular Biology 1* (2nd Edition). American Society for Microbiology, Washington, pp 1539-1549.

Slonczewski, J.L., Gonzalez, T.N., Bartholomew, F.M. and Holt, N.J., 1987. Mu d-directed *lacZ* fusions regulated by low pH in *Escherichia coli*. *J. Bacteriol.* 169: 3001-3006.

Slonczewski, J.L., Macnab, R.M., Alger, J.R. and Castle, A.M., 1982. Effects of pH and repellent tactic stimuli on protein methylation levels in *Escherichia coli*. *J. Bacteriol.* 152: 384-399.

Slonczewski, J.L., Rosen, B.P., Alger, J.R. and Macnab, R.M., 1981. pH homeostasis in *Escherichia coli*: measurement by ^{31}P nuclear magnetic resonance of methylphosphonate and phosphate. *Proc. Natl. Acad. Sci. USA* 78: 6271-6275.

Small, P., Blankenhorn, D., Welty, D., Zinser, E. and Slonczeski, J.L., 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. *J. Bacteriol.* 176: 1729-1737.

Smith, J.L. and Fratamico, P.M., 1995. Factors involved in the emergence and persistence of food-borne disease. *J. Food Prot.* 58: 696-708.

Smith, D.K., Kassam, T., Singh, B. and Elliott, J.F., 1992. *Escherichia coli* has two homologous glutamate decarboxylase genes that map to distinct loci. *J. Bacteriol.* 174: 5820-5826.

Srivasta, R.B. and Thompson, R.E.M., 1965. Influence of bacterial cell age on phenol action. *Nature* 206: 216.

Stapleton, G.E., 1955. Variations in the sensitivity of *Escherichia coli* to ionizing radiations during the growth cycle. *J. Bacteriol.* 70: 357-362.

Stapleton, G.E., Billen, D. and Hollaender, A., 1953. Recovery of X-irradiated bacteria at sub-optimal incubation temperature. *J. Cell. Comp. Physiol.* 41: 345-357.

Stim, K.P. and Bennett, G.N., 1993. Nucleotide sequence of the *adi* gene, which encodes the biodegradative acid-induced arginine decarboxylase of *Escherichia coli*. *J. Bacteriol.* 175: 1221-1234.

Stinger, S.C., George, S.M. and Peck, M.W., 2000. Thermal inactivation of *Escherichia coli* O157:H7. *J. Appl. Microbiol. Symposium* 88: 79S-89S.

Stumbo, C.R., 1973. *Thermobacteriology in food processing* (2nd Ed.). Academic Press, New York.

Sumner, J.L., Ross, T. and McMeekin, T.A., 2000. Food poisoning rates in Australia: an alternative view. *Food Aust.* 52: 274-276.

Sutherland, J.P., Bayliss, A.J. and Braxton, D.S., 1995. Predictive modelling of growth of *Escherichia coli* O157:H7: the effects of temperature, pH and sodium chloride. *Int. J. Food Microbiol.* 25: 29-49.

Sykes, G., 1963. The phenomenon of bacterial survival. *J. Appl. Microbiol.* 26: 287-294.

Tarr, P.I.., 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. *Clin. Infect. Dis.* 20: 1-10.

Tauxe, R.V., 1997. Emerging foodborne diseases: an evolving public health challenge. *Emerg. Infect. Dis.* 3: 425-434.

Taylor, F.R. and Cronan, J.E., 1976. Selection and properties of *Escherichia coli* mutants defective in the synthesis of cyclopropane fatty acids. *J. Bacteriol.* 125: 518-523.

Teo, Y.L., Raynor, T.J., Ellajosyula, K.R. and Knabel, S.J., 1996. Synergistic effect of high temperature and high pH on the destruction of *Salmonella enteritidis* and *Escherichia coli* O157:H7. *J. Food Prot.* 59: 1023-1030.

Thomas, A.D. and Booth, I.R., 1992. The regulation of expression of the porin gene *ompC* by acid pH. *J. Gen. Microbiol.* 138: 1829-1835.

Thomas, H.A., 1942. Bacterial densities from fermentation tube tests. *J. Amer. Water Resours. Ass.* 34: 572-576.

Thomas, J. and Law, J., 1966. Biosynthesis of cyclopropane compounds. 9. Structural and stereochemical requirements for the cyclopropane synthetase substrate. *J. Biol. Chem.* 241: 5013-5018.

Tilden, J., Young, W., McNamara, A., Custer, C., Boesel, B., Lambert-Fair, M.A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J. and Morris, J.G., 1996. A new route of transmission for *Escherichia coli*: Infection from dry fermented salami. *Public Health Briefs* 86: 1142-1145.

Tortora, G.J., Funke, B.R. and Case, C.L., 1992. *Microbiology – An Introduction.* The Benjamin/Cummings Publishing Company, California.

Tsai, Y.W. and Ingham, S.C., 1997. Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in acidic condiments. *J. Food Prot.* 60: 751-755.

Tuttle, J., Gomez, T., Doyle, M.P., Wells, J.G., Zhao, T., Tauxe, R.V. and Griffin, P.M., 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol. Infect.* 122: 185-192.

Uckert, J., 2001. Personal communication.

Uyttendaele, M., Taverniers, I. And Debevere, J., 2001. Effect of stress induced sub-optimal growth factors on survival of *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* 66: 31-37.

Vanderlinde, P.D., 1999. *E. coli* in uncooked fermented meat. Meat and Livetsock Australia. Report MSHE.008. Sydney.

Van Netten, 1996. Decontamination of fresh meats: pitfall and opportunities, In: A. Amagar (Ed.), *Food Safety '96 Proceedings of Fourth Asept International Conference*, pp 55-84.

Vas, K. and Proszt, G., 1957. Observations on the heat destruction of spores of *Bacillus cereus. J. Appl. Bacteriol.* 20: 431-441.

Villarino, A., Bouvet, O., Regnault, B., Delautre, S. and Grimont, P.A.D., 2000. Cellular activities in ultra-violet killed *Escherichia coli. Int. J. Food Microbiol.* 55: 245-247.

Wang, G. and Doyle, M.P., 1998. Heat shock response enhances acid tolerance of *Escherichia coli* O157:H7. *Lett. Appl. Microbiol.* 26: 31-34.

Watkins, J.H. and Winslow, C.E.A., 1932. Factors determining the rate of mortality of bacteria exposed to alkalinity and heat. *J. Bacteriol.* 24: 243-265.

Watson, N., Dunyak, D.S., Rosey, E.L., Slonczewski, J.L. and Olson, E.R., 1992. Identification of elements onvolved in transcriptional regulation of the *Escherichia coli cad* operon by external pH. *J. Bacteriol.* 174: 530-540.

Weagant, S.D., Bryant, J.L. and Bark, D.H., 1994. Survival of *Escherichia coli* O157:H7 in mayonnaise and mayonnaise-based sauces at room temperature and refrigerated temperatures. *J. Food Prot.* 57: 629-631.

Winslow, C.E.A. and Falk, I.S., 1920. *Abst. Bact.* 4: 2.

Winslow, C.E.A. and Falk, I.S., 1923. Studies on salt action. VIII. The influence of calcium and sodium salts at various hydrogen ion concentrations upon the viability of *Bacterium coli. J. Bacteriol.* 8: 215.

White, D.C., Davis, W.M., Nickels, J.S., King, J.D. and Bobbie, R.J., 1979. Determination of the sedimentary microbial biomass by extractable lipid phsophate. *Oecologica* 40: 51-62.

White, H.R., 1951. Variation with age in the resistance of bacterial cells. *Nature* 168: 828-829.

Withell, E.R., 1942. The significance of the variation in shape of time-survivor curves. *J. Hyg.* 42: 124-183.

World Health Organisation, 2000. *Food Safety – An Essential Public Health Issue for the New Millenium.* http://www.who.int./fsf/brochure/food safety/index.htm (accessed 28/01/01).

Xu, H.S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J. and Colwell, R.R., 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb. Ecol.* 8: 313-323.

Yatvin, M.B., Gipp, J.J., Klessig, D.R. and Dennis, W.H., 1986. Hyperthermic sensitivity and growth stage in *Escherichia coli. Radiat. Res.* 106: 78-88.

Zhao, T. and Doyle, M.P., 1994. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in commercial mayonnaise. *J. Food Prot.* 57: 780-783.

Zilberstein, D., Agmon, V., Schuldiner, S. and Padan, E., 1982. The sodium/proton antiporter is part of the pH homeostasis mechanism in *Escherichia coli*. *J. Biol. Chem.* 257: 3687-3691.

Zilberstein, D., Agmon, V., Schuldiner, S. and Padan, E., 1984. *Escherichia coli* intracellular pH, membrane potential, and cell growth. *J. Bacteriol.* 158: 246-252.

Zink, D.L., 1997. The impact of consumer demands and trends on food processing. *Emerg. Infect. Dis.* 3: 467-469.

APPENDIX A: MATERIALS & EQUIPMENT

A.1 Materials

A.1.1 Bacterial Strains

Table A.1 lists the bacterial strains employed during the course of this study. Taken from original plate cultures and resuspended in nutrient broth containing 30% glycerol (NB-Gly), each strain was stored in duplicate at -80°C. One culture from each pair was used for the preparation of experimental inocula while the other was held in reserve. To prepare experimental inocula, cells were removed from the surface of frozen stock cultures with a sterile pipette tip, plated to TPA-P, and incubated for 14 hours at 37°C.

Table A.1 Bacterial Strains (*E. coli*) employed during the course of this study.

	Description	**Reference**
Strain		
M23	Laboratory Strain, OR:H-	Brown et al., 1997
FT1	F⁻ *proC32 purE42 lysA23 trpE38 metE70 metB1 thi-1 str*ʳ	Taylor and Cronan, 1976
YYC1106	FT1, *cfa::kan*	Chang and Cronan, 1999
Frag1	F⁻, *gal, lacZ, rha, thi*	Epstein and Kim, 1971
JM109	*recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi,* Δ(*lac-proAB*), F'[*traD36, proAB*⁺, *laqI*ᵠ, *lacZ*ΔM15]	Yanish-Perron et al., 1985
S17-1λpir	(*thi pro hsdR hsdM*⁺ *recA* RP4-2-Tc::Mu-Km::Tn*7*)	Simon et al., 1983
JBM1	Frag1, Δ*cfa*	This study

A.1.2 Bacteriological Media

The bacteriological media employed throughout this study were prepared and stored according to the manufacturer's (Oxoid, www.oxoid.com) specifications, or as described below.

Brain Heart Infusion Agar with 0.1% sodium pyruvate (BHA-P)

Brain Heart Infusion Agar was prepared from Brain Heart Infusion Broth (Oxoid CM225) by the addition of 1.5% bacteriological grade agar. The addition of 0.1% sodium pyruvate prior to sterilisation yielded BHA-P.

Eosin Methylene Blue Agar (EMB)

(Oxoid CM69)

Eosin Methylene Blue Agar with 0.1% sodium pyruvate (EMB-P)

The addition of 0.1% sodium pyruvate to Eosin Methylene Blue Agar (Oxoid CM69) prior to sterilisation yielded EMB-P.

Luria-Bertani Broth (LB)

LB was prepared by dissolving 10g tryptone, 5g yeast extract, and 10g sodium chloride in 1L of distilled water. The broth was sterilised by autoclaving (121°C for 15 minutes).

Luria-Bertani Agar (LBA)

LBA was prepared from LB (above) by the addition of 1.5% bacteriological agar prior to sterilisation.

Luria-Bertani Agar with 0.1% sodium pyruvate (LBA-P)

LBA-P was prepared from LBA by the addition of 0.1% sodium pyruvate prior to sterilisation.

Luria-Bertani Agar with 5% sucrose – No NaCl (LBA-S)

For the preparation of LBA-S the constituents of LBA, excluding NaCl, were dissolved in a 900 ml volume of distilled water. This solution was sterilised by autoclaving (121°C for 15 minutes), cooled to 55°C, and converted to LBA-S by the addition of a filter sterilised 50% (w/w) sucrose solution (100 ml).

Nutrient Broth with 30% Glycerol (NB-Gly)

Nutrient Broth (Oxoid CM1) was amended with 30% glycerol before sterilisation.

NuBile

NuBile was prepared from Tryptose Phosphate Broth (Oxoid CM283) by the addition of 1.5% bacteriological agar and 0.15% Oxoid Bile Salts No. 3 (Oxoid L56) prior to sterilisation.

Tryptose Phosphate Agar with 0.1% sodium pyruvate (TPA-P)

TPA-P was prepared from Tryptose Phosphate Broth (Oxoid CM283) by the addition of 1.5% bacteriological agar and 0.1% sodium pyruvate prior to sterilisation.

Tryptone Soya Agar with 0.1% sodium pyruvate (TSA-P)

TSA-P was prepared from Tryptone Soya Broth (Oxoid CM129) by the addition of 1.5% bacteriological agar and 0.1% sodium pyruvate prior to sterilisation.

Tryptone Soya Broth (TSB)

TSB (Oxoid CM129) was pH adjusted with 10M HCl/5M NaOH prior to sterilisation. Unless otherwise indicated, the pH of TSB was routinely adjusted to 7.0.

Tryptone Soya Broth with 0.1% sodium pyruvate (TSB-P)

TSB-P was prepared by the addition of 0.1% sodium pyruvate to Tryptone Soya Broth (Oxoid CM129) prior to sterilisation.

A.1.3 Chemicals

For lipid extraction and fractionation, and for the methylation of fatty acids, nanograde reagents were obtained exclusively from Mallinckrodt Baker, Inc. (Paris, Kentucky 40361). The remaining chemicals employed throughout this study were obtained from a variety of commercial distributors.

A.1.4 Molecular Biology Reagents and Kits

The *Taq* DNA polymerase, restriction enzymes, Klenow, Calf Intestinal Phosphatase, DNA ligase, and DNA molecular weight markers employed throughout this study were supplied by Boehringer Mannheim (www.biochem.boehringer-mannheim.com). Deoxynucleotides for PCR were supplied by Amersham Pharmacia (Amersham Pharmacia Biotech. Inc., P.O. Box 1327, Piscataway, NJ 08855-1327, USA). Oligonucleotide primers were synthesized either by Geneworks (www.geneworks.com) or by Genosys Biotechnologies (www.genosys.com), and the PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit employed for DNA sequencing was supplied by Applied Biosystems (www.appliedbiosystems.com). Promega (www.promega.com) supplied the Wizard® PCR Preps DNA Purification System, Thermo Hybaid (Action Court, Ashford, Middlesex, TW15 1XB, UK) the Hybaid Recovery™ DNA Purification Kit, and Qiagen (www.quiagen.com) the QIAprep® Miniprep System.

A.2 Equipment

Automated Sequencer

ABI A377, Applied Biosystems, USA.

Centrifuges

1. Hettich Zentrifugen EBA12 from Andreas Hettich (Gartenstr 100 D-7200, Tuttlingen).

2. Beckman J2-21M/E Centrifuge from Beckman Coulter Pty Ltd (P.O. Box 218, Gladesville, NSW, 2111).

Differential Scanning Calorimeter

Perkin-Elmer DSC 6, Beaconsfield, UK.

Gas Chromatography-Mass Spectroscopy

Hewlett Packard gas chromatograph (5890), Hewlett Packard mass selective detector (5970), Hewlett Packard automatic sampler (7673A) and Hewlett Packard workstation (59970C) from the Hewlett-Packard Company (19091 Pruneridge Avenue, Cupertino, CA 95014 USA).

Incubators

1. Ratek SWB20D shaking waterbaths from Ratek Instruments Pty. Ltd. (Unit 1/3 Wadhurst Drive, Boronia, Australia, 3155). These waterbaths were accurate to within ± 0.1°C of the specified temperatures and were set to oscillate 160-180 times min^{-1}.

2. Temperature Gradient Incubator Advantec TN-2148 from Advantec MFS, Inc. (6691 Owens Drive, Pleasanton, CA 94588, USA).

pH meter

Orion pH meter 250A from Orion Research Inc. (Boston, MA 02129, USA) fitted with Activon AEP 433 flat tip probe from Activon Scientific Products Co. Pty. Ltd. (P.O. Box 505, Pennant Hills, NSW, Australia, 2120). This instrument was calibrated with reference buffers (pH 4.0 and 7.0) before use.

Pipettes

1. Gilson pipettes from Gilson Medical Electronics (B.P. 45, F95469 Villiers-le-Bel, France).

2. Finnpipette® pipettes from Labsystems Oy (P.O. Box 8, FIN-00881, Helsinki, Finland).

Volumes were checked by weighing the dispensed volume of distilled water at room temperature and were typically within ± 1% of the stated volume.

Software

Ultrafit 3.0 (©Biosoft, 37 Cambridge Place, Cambridge, UK) was employed for curve fitting, Cricket Graph 1.3.2 (Cricket Graph Software, 40 Valley Stram Parkway, Malvern, PA 19355) and Microsoft Excel 98 (Microsoft Corporation, www.microsoft.com) for data presentation, DAPA Scientific Software (Kalamunda, Western Australia) for gas chromatography-mass

spectroscopy, and CIA-BEN 2.2 (Spiral Biotech, Inc., 7830 Old Georgetown Rd, Bethesda, MD 20814 USA) for colony image analysis.

Spectrophotometers

Spectronic 20+ [analogue] and Spectronic 20D [digital] from Milton Roy Co. (820 Linden Avenue, Rochester, NY 14625, USA).

Spiral Plater

Autoplate 4000 from Spiral Biotech Inc. (7830 Old Georgetown Rd, Bethesda, MD 20814 USA).

Thermocyclers

1. Peltier Thermal Cycler PTC-200 from MJ Research, Inc. (Waltham, Massachusetts).
2. Thermal Sequencer FTS-960 from Corbett Research (1/14 Hilly St., Mortlake, 2137, Sydney, Australia).

APPENDIX B: INACTIVATION MODELS

The 'Death Curve Model' was fitted to datasets describing biphasic inactivation kinetics, and the 'Three Phase Death Model' to datasets describing triphasic inactivation kinetics. The two models are described below and use of the 'Three Phase Death Model' is exemplified

Death Curve Model

This model describes a two phase concave reduction in log numbers with time as the independent variable and takes the form:

$$y = \text{LOG10}(10\text{^}(Ni)*((f*(10\text{^}(-A*t)))+((1-f)*(10\text{^}(-B*t)))))$$

where: y is the logarithm of the number of viable bacteria

t is time

Ni is the logarithm of the initial number of viable bacteria

f is the proportion of cells inactivated during phase 1

A is the negative slope [Δ log CFU/Δt] of phase 1 (R1)

B is the negative slope of phase 2 (R2)

Three Phase Death Model

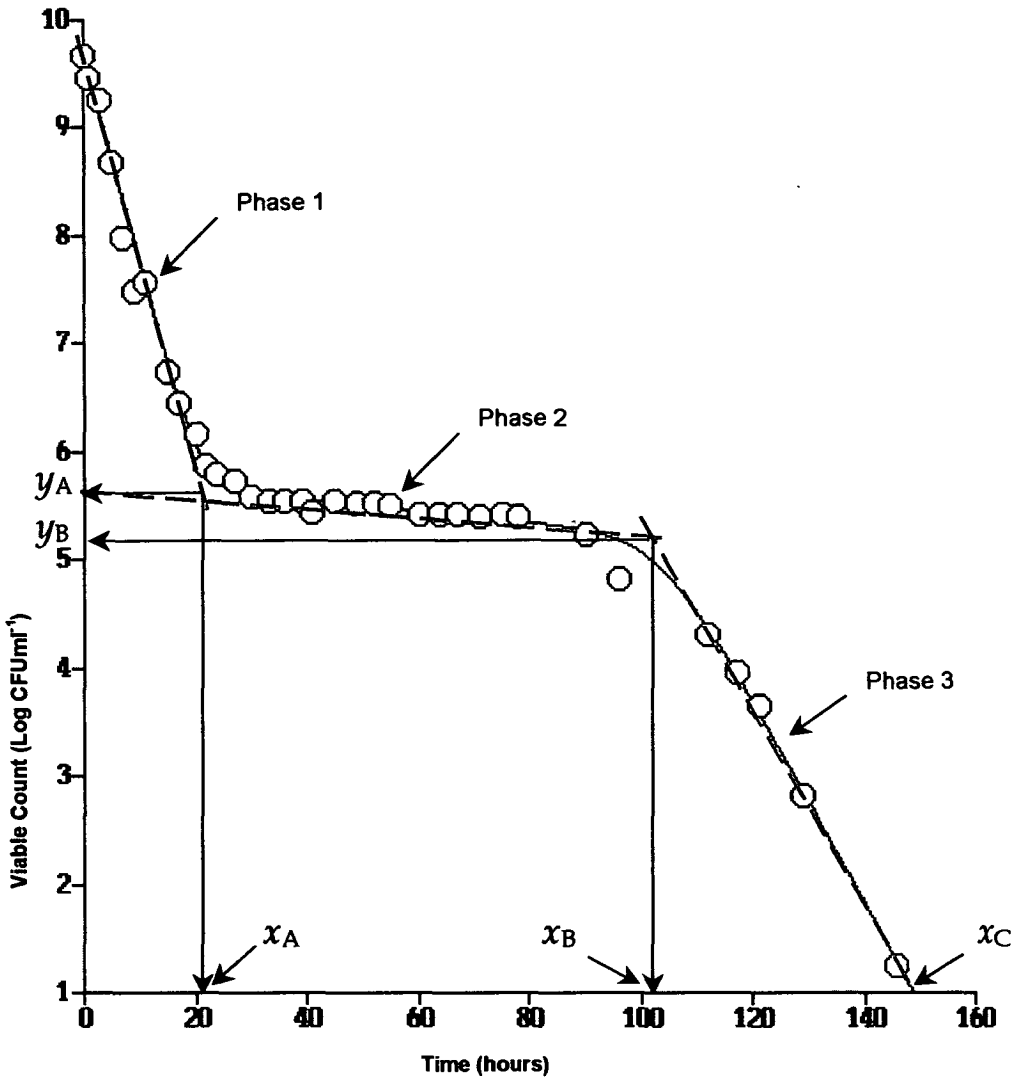
This model describes a three phase reduction in log numbers with time as the independent variable and takes the form:

$$y = \text{LOG10}((10\text{^}(No-Ni-K*t)) + 1 + (1+(10\text{^}Ni/10\text{^}I)) + 2*Ni-$$
$$(\text{LOG10}(10\text{^}(Ni+A*t) + (10\text{^}Ni/10\text{^}I)*10\text{^}(Ni+B*t)))$$

where: y is the logarithm of the number of viable bacteria

t is time

K is the negative slope of phase 1 (R1)



Now the parameters *K, A, B, No, Ni,* and *I* correspond to m_1, m_2, m_3, c_1, c_2, & c_3

x_A and x_B can thus be determined by substituting the values of the fitted parameters into equations 4 and 5

Having done this y_A can be found by substituting x_A for x_1 in equation 1, and y_B can be found by substituting x_B for x_2 in equation 2

Furthermore, x_C can be found by setting y_3 at a value of 0

No is the logarithm of the initial number of viable bacteria

Ni is the point where a line drawn through phase 2 would cross the y-axis

I is the point where a line drawn through phase 3 would cross the y-axis

A is the negative slope of phase 2 (R2)

B is the negative slope of phase 3 (R3)

Example

The 'Three Phase Death Model' was fitted to the viable count dataset of a 15 hour stationary phase culture exposed to low pH [pH 3.5, 25°C] (illustrated diagrammatically over).

Straight line equations representing each of the phases of inactivation can be described thus:

$$y_1 = c_1 + m_1 x_1 \quad \ldots\ldots 1$$

$$y_2 = c_2 + m_2 x_2 \quad \ldots\ldots 2$$

$$y_3 = c_3 + m_3 x_3 \quad \ldots\ldots 3$$

At the intersection of the lines describing Phase 1 and Phase 2 (i.e. x_A, y_A), $y_1 = y_2$ and $x_1 = x_2$

$$\therefore c_1 + m_1 x_A = c_2 + m_2 x_A$$

and solving for x

$$x_A = (c_2 - c_1)/(m_1 - m_2) \quad \ldots\ldots 4$$

At the intersection of the lines describing Phase 2 and Phase (i.e. x_B, y_B), $y_2 = y_3$ and $x_2 = x_3$

By analogy $\quad x_B = (c_3 - c_2)/(m_2 - m_3) \quad \ldots\ldots 5$

In the example provided, the values of the fitted parameters are as follows:

$K = 0.18709$ $No = 9.67463$

$Ni = 5.76203$ $I = 14.83302$

$A = 0.00518$ $B = 0.09296$

Thus:

$$y_1 = 9.67463 - 0.18709x_1 \quad \ldots\ldots 1E$$

$$y_2 = 5.76203 - 0.00518x_2 \quad \ldots\ldots 2E$$

$$y_3 = 14.83302 - 0.09296x_3 \quad \ldots\ldots 3E$$

$x_A = (5.76203 - 9.67463)/((-0.18709) - (-0.00518)) = 21.5084$

$x_B = (14.83302 - 5.76203)/((0.00518) - (-0.09296)) = 103.338$

$x_c = 14.83302/0.09296 = 159.5635$

Also $y_A = 9.67463 - (0.18709 \times 21.5084) = 5.6506$

And $y_B = 5.76203 - (0.00518 \times 103.338) = 5.2267$

Thus:

The duration of Phase 1 (x_A) is 21.51 hours

The duration of Phase 2 ($x_B - x_A$) is 81.83 hours

The duration of Phase 3 ($x_C - x_B$) is 56.23 hours

The size of the kill achieved during Phase 1 ($No - y_A$) is 4.02 log

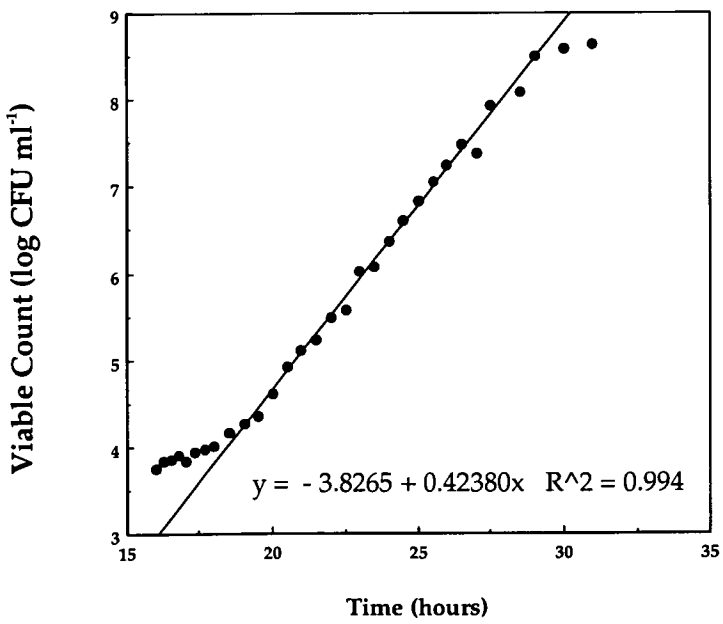
The size of the kill achieved during Phase 2 ($y_A - y_B$) is 0.42 log

The size of the kill achieved during Phase 3 (y_B) is 5.23 log

APPENDIX C: ESTIMATION OF LAG PHASE DURATION

The lag phase duration of bacterial populations was estimated by linear regression as described, by example. The example uses data collected for the growth of a population prepared and monitored as described in Section 2.2.5, presented below in tabular form and as a viable count-time plot.

Time (hours)	Viable Count (Log CFU ml^{-1})	Time (hours)	Viable Count (Log CFU ml^{-1})	Time (hours)	Viable Count (Log CFU ml^{-1})
16.0	3.76	20.0	4.62	25.5	7.05
16.3	3.85	20.5	4.93	26.0	7.25
16.5	3.86	21.0	5.12	26.5	7.49
16.8	3.92	21.5	5.24	27.0	7.39
17.0	3.85	22.0	5.50	27.5	7.93
17.3	3.95	22.5	5.57	28.5	8.09
17.7	3.98	23.0	6.03	29.0	8.50
18.0	4.01	23.5	6.08	30.0	8.58
18.5	4.16	24.0	6.37	31.0	8.64
19.0	4.27	24.5	6.61		
19.5	4.36	25.0	6.84		



Linear regression analysis (Cricket Graph 1.3.2) of the data representing the exponential phase of growth on such a plot (the straight line portion) yields an equation of the form:

$$y = c + mx$$

where: y is the logarithm of the number of viable cells

c is the y-axis intercept

m is the slope

x is the time

which for the example described is:

$$y = -3.8265 + 0.42380x \quad (r^2 = 0.994)$$

Now the lag phase duration is the time that elapses between the time of inoculation and the time at which growth commences. The time of inoculation is known (in this case16 hrs) and the time at which growth commences is the value of x when y is fixed at its initial value. For the example provided then, the lag phase duration is:

$$\text{LPD} = \left[\frac{y(\text{initial}) - c}{m} \right] - \text{time of inoculation}$$

For the example described:

$$\text{LPD} = \left[\frac{3.76 + 3.8265}{0.42380} \right] - 16 = 1.901 \text{ hours}$$