A GENETIC APPROACH
TO HEAT RESISTANCE
IN *SALMONELLA* SPECIES

STEPHEN JON MOORE

A THESIS PRESENTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

MARCH 1992

DECLARATION

I hereby declare that this thesis has been composed by myself and that all work reported is my own.

ABSTRACT

The aim of this project was to develop the genetics of heat tolerance in the mesophilic food-poisoning bacterium *Salmonella typhimurium* strain SA2009. Since this required the isolation of mutants having a reduced or increased resistance to heat it was necessary to establish the heat resistance of strain SA2009 in liquid growth medium.

Survival curves initially showed a great deal of variability when exponentially-growing bacteria were heated but by careful control of a number of important parameters such as the precise stage of growth of the bacteria and the volume of preheated medium into which they were dispersed the variability was removed.

Three methods were used in an attempt to generate mutants. Two involved a search for heat-sensitive mutants by the induction of mutagenesis, viz treatment of exponentially-growing bacteria with the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and the use of transposons which by insertion into genes disrupt their function. The third method selected for spontaneous (or heat-induced) heat-resistant mutants of *S. typhimurium* by repeated cycles of heating cultures to reduce substantially the number of viable bacteria and then allowing the survivors to give rise to a culture which was heat treated etc.

None of these methods provided mutants with altered heat tolerance. During these experiments an anomaly between the survival of putative mutants in liquid media and on the surface of agar plates was observed. Apparently-sensitive organisms detected on the surfaces of agar plates gave heat survival curves identical to that of the wild type.

An alternative strategy was to attempt the transfer the gene(s) responsible for the extreme heat resistance of *Salmonella senftenberg* strain 775W into a wild type strain of *S. typhimurium* and *Escherichia coli*.

A gene library of *S. senftenberg* 775W DNA was constructed in the positive selection plasmid pUN121. A culture of the restriction/modification deficient *E. coli*, strain MM294 was transformed with this plasmid library and cycled through several heat treatments. Two clones of *E. coli* were isolated that had greatly increased heat resistance similar to that of *S. senftenberg* 775W.

The heat resistance of *S. senftenberg* was compared with a normal heat resistant *S. senftenberg*, JT577 and *S. typhimurium* SA2009. *S. senftenberg* 775W had the same maximum growth temperature and inability to recover after thermal stress in the presence of cell wall and protein synthesis inhibitors as the others. However, it did have a slightly higher resistance to ^{60}Co γ-irradiation and was, after an equal reduction in viability due to thermal stress, able to recover salt tolerance sooner and recover in the presence of an RNA synthesis inhibitor. All three strains developed increased heat resistance when pre-incubated at 42°C compared with 37°C, and the protein that hybridized to *E. coli* GroEL-derived antibody, appeared to be heat inducible.

CONTENTS

Page No.

Title...i

Declaration...ii

Abstract..iii

List of Table...ix

List of Figures...x

CHAPTER ONE. INTRODUCTION

1.1 Theory and measurement of heat resistance.......1

1.2 Factors affecting heat resistance...............5

1.2.1 Factors affecting survival prior to thermal stress.....................................5

1.2.1.1 Growth Temperature..........................5

1.2.1.2 Growth Phase................................6

1.2.2 Factors affecting survival after thermal stress.....................................7

1.2.2.1 Recovery media..............................7

1.3 Thermal injury and recovery.....................8

1.3.1 Energy requirements for the recovery of thermally injured cells.........................9

1.3.2 Cell wall damage and repair during thermal injury and recovery............................10

1.3.3 Cell membrane damage and repair during thermal injury and recovery............11

1.3.4 Structural protein damage and repair during thermal injury and repair......................14

1.3.5 Enzyme damage and repair during thermal injury and recovery............................15

1.3.6 Ribosome and rRNA degradation and re-synthesis during thermal injury and recovery..18

1.3.7 DNA damage and repair during thermal injury and recovery.................................22

1.3.8 Summary of introduction.......................24

1.4 Heat shock response.............................25

1.4.1 Proteins......................................27

1.4.2 Regulation of the response....................28

1.4.2.1 Inducers, targets and signal(s)...........28

1.4.2.2 Effector..................................28

1.4.2.3 Modulators................................30

1.4.3 Conservation of the heat shock response......31
1.4.4 Heat induced thermotolerance..................31

CHAPTER TWO. MATERIALS AND METHODS
2.1 Bacterial strains.............................33
2.2 Plasmids......................................33
2.3 Maintenance of cultures.......................33
2.4 Media...33
2.5 Solutions and buffers.........................38
2.5.1 DNA manipulation............................38
2.5.2 Polyacrylamide electrophoresis..............39
2.5.3 Western blotting............................40
2.5.4 Phosphate buffer............................40
2.5.5 STEP buffer.................................40
2.5.6 Lowry protein determination, solutions......40
2.6 Chemicals.....................................41
2.7 Antibiotics and nutrient supplements..........41
2.8 Enzymes.......................................41
2.9 Antibodies....................................42
2.10 Measurement of bacterial growth..............42
2.11 Heat treatment...............................42
2.11.1 Heat inactivation...........................42
2.11.2 Standard heat inactivation.................43
2.11.3 Replica-plate heat inactivation.............43
2.11.4 Replica-plate heat inactivation (using tooth picks)..........................44
2.11.5 Heat induced thermotolerance...............44
2.11.6 Thermal conditioning.......................45
2.12 Gamma irradiation............................45
2.13 Transposon mutagenesis.......................46
2.13.1 Making a P22 bacteriophage lysate of *S.typhimurium* TT10423........................46
2.13.2 Transposon mutagenesis of *S.typhimurium* TT10427.....................................46
2.13.3 Transduction of *Tn*10 from *S.typhimurium* TT10427 in to *S.typhimurium* SA2009............47
2.13.4 Producing a P1 lysate......................47
2.14 Isolation of plasmid DNA from strains of *S.typhimurium* and *E.coli*......................48

2.14.1 Small scale preparation of plasmid DNA (mini-preps)....48
2.14.2 Large scale preparation of plasmid DNA....49
2.15 Isolation of chromosomal DNA from strains of *E.coli*, *S.typhimurium* and *S.senftenberg*....50
2.16 Phenol/chloroform extraction of DNA....51
2.17 Recombinant DNA manipulation....51
2.17.1 Restriction digestion of DNA....52
2.17.2 Electrophoresis of DNA....52
2.17.3 Ligation of DNA fragments....54
2.17.4 Determination of DNA concentration....54
2.17.5 Sizing DNA molecules and fragments from agarose gels....55
2.17.6 Sucrose gradient size fractionation of *Sau*3A chromosomal partial digest....55
2.18 Transformation of *S.typhimurium* and *E.coli*....58
2.18.1 Preparation of competent cells....58
2.18.2 Transformation procedure....58
2.19 Separation of proteins by SDS-polyacrylamide gel electrophoresis....59
2.19.1 Preparation of separating gel (12.5%)....59
2.19.2 Preparation of stacking gel (4.8%)....59
2.19.3 Running the gel....59
2.20 Western blotting....60
2.20.1 Antibody detection....61
2.21 Determination of protein concentration....61

CHAPTER THREE. RESULTS

3.1 Introductory Work....64
3.2 Alteration in heat resistance by means of genetic mutation....69
3.3 Genetic mutation using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine....71
3.3.1 Measuring changes in heat resistance of MNNG treated *S. typhimurium* SA2009....72
3.3.2 Genetic mutation using transposon mutagenesis.74
3.4 Thermal conditioning....76
3.5 Genetic work on heat resistance....82
3.5.1 P22 Transduction....84
3.5.2 P1 Transduction....90

3.5.2.1 Production of a P1 lysate from *S. senftenberg* SM002....93
3.5.2.2 Removal of P1 phage....95
3.6 Gene library....97
3.6.1 Making a plasmid library....97
3.6.1.1 Preparation of plasmid DNA....97
3.6.1.2 Preparation of chromosomal DNA....97
3.6.1.3 Ligation of DNA fragments....99
3.6.2 Transformation of host bacteria....99
3.6.3 Characterisation of host bacteria....101
3.6.4 Multiplication of the heat resistant transformants....103
3.6.5 Search for heat resistant transformants....104
3.7 Physiological traits....108
3.7.1 Growth temperature....110
3.7.2 Additional genetic material....110
3.7.3 DNA repair, γ-irradiation resistance....113
3.7.4 Recovery after heat treatment....113
3.7.4.1 Requirements for the recovery from thermal injury....115
3.7.4.2 Effects of protein synthesis inhibition on the recovery of thermally damaged cells....117
3.7.4.3 Effects of cell wall synthesis inhibition on the recovery of thermally damaged cells....118
3.7.4.4 Effect of RNA synthesis inhibition on the recovery of thermally damaged cells....119
3.7.5 Heat induced thermotolerance....124
3.7.5.1 Effect of cell wall, protein and RNA synthesis inhibition on the recovery of thermally damaged, thermally induced *S. typhimurium* SA2009....124
3.7.5.2 Effect of cell wall, protein and RNA synthesis inhibition on the recovery of thermally damaged, thermally induced *S. senftenberg* JT577....125
3.7.5.3 Effects of cell wall, protein and RNA synthesis inhibition on the recovery of thermally induced, thermally damaged *S. senftenberg* 775W....125
3.7.6 Heat inducible proteins....135

CHAPTER FOUR. DISCUSSION
4.1 Variability of thermal survival curves....138

4.2 Anomaly of survival capabilities..............139
4.3 Evidence for *S. senftenberg* 775W elevated heat resistance..........................144
4.3.1 Cell membrane repair..........................144
4.3.2 Ribosome and rRNA repair......................147
4.3.3 Protein synthesis.............................150
4.4 Transfer of heat characteristics..............151

Acknowledgements......................................154

Bibliography..155

LIST OF TABLES

1.1 Known heat inducible proteins and their genes 26
2.1 Strains of *Salmonella typhimurium* used........34
2.2 Strains of *Salmonella senftenberg* used........35
2.3 Strains of *Escherichia coli* used..............35
2.4 Plasmids used and their original hosts........36
3.1 Mutation frequencies of *S. typhimurium* SA2009 after exposure to MNNG.................72
3.2 Presence or absence of growth of strains *S. typhimurium* and *S. senftenberg* after exposure to 55°C on the surface of an LB agar plate.................................80
3.3 Survival of single and mixed cultures of SM001 and *S. typhimurium* SA2009 after exposure to 55°C on an agar plate.............83
3.4 The quantitative ability of P22, derived from *S. typhimurium* LT2, to plaque on *S. senftenberg* 775W and *S. typhimurium* LT2....85
3.5 The quantitative ability of P22, derived from *S. typhimurium* CH1305 and containing *Tn10* elements, to plaque on *S. senftenberg* 775W and *S. typhimurium* LT2 and give rise to tetracycline resistant transductants..........86
3.6 Sensitivity/resistance of *S. senftenberg* 775W transductants to tetracycline, P22 and heat...87
3.7 Number of P1 particles, derived from *S. typhimurium* CH1026, that would plaque on *S. typhimurium* CH223...........................91

3.8 Number of *S. typhimurium* CH223 and *S. senftenberg* 775W chloramphenicol resistant colonies after transduction with P1 phage derived from *S. typhimurium* CH1026...92

3.9 Sensitivity/resistance of *S. senftenberg* 775W transduced with P1 phage derived from *S. typhimurium* CH1026; to chloramphenicol, heat and growth temperature....................93

3.10 The quantitative ability of P1 phage derived from *S. senftenberg* SM002 to plaque on *S. senftenberg* 775W and *S. typhimurium* CH223..94

3.11 The quantitative ability of phage P1 derived from *S. senftenberg* SM002 to plaque on *S. senftenberg* 775W and *S. typhimurium* CH223 suspended in 10 mM $MgSO_4$ and $CaCl_2$............95

3.12 The quantitative ability of phage P1 derived from *S. senftenberg* SM002 to plaque on *S. typhimurium* CH223 and *S. senftenberg* 775W, SM031 which had been cured of the phage P1....96

3.13 Presence or absence of growth of strains of *S. typhimurium, S. senftenberg* & *E. coli* after exposure to 55°C on an agar plate. 103

3.14 Presence or absence of growth of strains of *S. typhimurium, S. senftenberg* & *E. coli* after exposure to 55°C on an agar plate.......106

LIST OF FIGURES

1.1 Survival curves of bacteria thermally inactivation in liquid broth..................3

1.2 Models for induction of the heat shock response....................................29

2.1 Agarose gel electrophoresis of the products of a partial digest of *S. senftenberg* 775W DNA with *Sau*3A.................................53

2.2 Apparatus for loading a sucrose gradientin to centrifuge tubes..........................56

2.3 Agarose gel electrophoresis of the products of a partial digest of *S. senftenberg* 775W DNA with *Sau*3A taken from fractions of a sucrose gradient.............................57

2.4 Visualisation of proteins on Western blots....62

3.1 Survival of *S. typhimurium* SA2009 in 20 ml of LB at 55°C.....................................65

3.2 Survival of *S. typhimurium* SA2009 in 180 ml of LB at 55°C.....................................66

3.3 Survival of *S. typhimurium* SA2009 at 55°C through the growth cycle at 37°C following exposure to 55°C for 5 minutes...............68

3.4	Survival of *S. typhimurium* SA2009 at 50°, 52°, 55°, 57° and 59°C in 180 ml of LB........70
3.5	Survival of thermally cycled *S.typhimurium* SA2009 at 55°C.................................78
3.6	Survival of thermally cycled strains of *S. typhimurium* strain 1, *S. typhimurium* SA2009 and *S. senftenberg* 775W at 55°C........79
3.7	Restriction map and genetic markers in the positive selection plasmid pUN121.............98
3.8	*Bcl*I digests of vector:fragment constructs from transformed *E. coli* MM294................100
3.9	Survival of *S. senftenberg* JT577, 775W, *S. typhimurium* SA2009 and *E. coli* MM294 in LB at 55°C......................................102
3.10	Survival of *S. senftenberg* 775W, *E. coli* MM294 and *E. coli* MM294 transformants in LB at 55°C......................................107
3.11	Survival of *S. senftenberg* 775W, *E. coli* MM294 and *E. coli* MM294 transformants in LB at 55°C......................................109
3.12	Growth of *S. senftenberg* 775W, JT557 and *S. typhimurium* SA2009 in LB at 37°C...........111
3.13	Growth of *S. senftenberg* 775W, JT557 and *S. typhimurium* SA2009 in LB at 39°C, 42°C 46°C and 48°C (from top left to bottom right)......112
3.14	Survival of *S. senftenberg* 775W, JT577 and *S. typhimurium* SA2009 after exposure to a cobalt-60 ν-irradiation source................114
3.15	Growth of *S. senftenberg* 775W, JT577 and *S. typhimurium* SA2009 during recovery from a three log reduction in survival at 55°C.....116
3.16	Recovery of heat-injured *S. typhimurium* SA2009 grown at 37°C in the presence of metabolic inhibitors...........................120
3.17	Recovery of heat-injured *S. senftenberg* JT577 grown at 37°C in the presence of metabolic inhibitors...........................121
3.18	Recovery of heat-injured *S. senftenberg* 775W grown at 37°C in the presence of metabolic inhibitors...........................122
3.19	Recovery of heat-injured *S. typhimurium* SA2009 grown at 42°C in the presence of metabolic inhibitors...........................126
3.20	Recovery of heat-injured *S. senftenberg* JT577 grown at 42°C in the presence of metabolic inhibitors...........................127

3.21 Recovery of heat-injured *S. senftenberg* 775W grown at 42°C in the presence of metabolic inhibitors....128

3.22 Change in survival at 55°C *S. typhimurium* SA2009 grown at 37°C and 42°C....132

3.23 Change in survival at 55°C *S. senftenberg* JT577 grown at 37°C and 42°C....133

3.24 Change in survival at 55°C *S. senftenberg* 775W grown at 37°C and 42°C....134

3.25 Western blot of total cell extracts from *S. senftenberg* 775W, *S. senftenberg* JT577 and *S. typhimurium* SA2009 grown at different incubation temperatures using antibody to GroEL....137

CHAPTER ONE

INTRODUCTION

1.1 Theory and measurement of heat resistance

The effects of mild heating (50°-60°C) and of pasteurization on microbial, and particularly bacterial viability, have been known since the discoveries of Pasteur between 1860 and 1864. Despite this long history, a basic understanding of the mechanisms involved in thermal death has not yet been obtained. However, a picture is beginning to emerge of what happens when vegetative bacteria are subjected to mild thermal stress.

The measurement of bacterial resistance to mild thermal stress is usually achieved by exposing cells to an elevated temperature and determining the number of survivors after various exposure times, using viable plate counts. The results are then plotted on a semilogarithmic scale against time on a linear scale, with the resulting 'survival curve' illustrating the progressive death of the population. Most microbiology text books suggest that curves of thermal inactivation of bacteria are straight lines; indeed Hansen & Rieman (1963) stated "many people take logarithmic survivor curves almost for granted". In practice though, heat inactivation curves are not all exponential and usually fall into four general types (*Figure* 1.1). The biological significance of the curves, in terms of mechanisms involved, has not yet been ascertained; nevertheless, several hypotheses have been proposed which describe inactivation events occurring in a random or a progressive manner within the cell.

The simplest form of the first hypothesis assumes that each organism is killed by a single lethal event occurring at random. An exponential curve therefore implies that the culture is uniformly susceptible to the mild thermal stress, and can be expressed thus;

$$n/n^{o} = \ln(-k^{1}t) \qquad (1)$$

where k^1 is the slope of the survival curve, n^o is the initial number of viable bacteria and n the number of bacteria surviving exposure time t. Most of the early workers considered all bacterial thermal inactivation to be logarithmic, being the result of inactivation of a single site within the cell. Moats (1971) argued that if thermal inactivation was a first order reaction, i.e., only requiring the inactivation of a single site, then surely the rate should be highest at the start of the heating process when the number of bacteria is highest, and thus could not explain the initial lag in death which frequently occurs. Non-exponential curves like A, C, & D are accounted for by this hypothesis by introducing extra assumptions while retaining the central postulate. This theory explains curve A, in that a cell or any other site can be altered by the joint action of two or more hits. This occurs where each site is composite and each of its n parts must be affected by at least one effective unit before a response is produced. An obvious example is killing a clump of bacteria, each bacterium has to be 'hit' to prevent colony formation. Hansen & Rieman (1963) showed that *Streptococcus faecalis* gave an initial lag when heated in skimmed milk and concluded it was because more than one essential molecule had to be destroyed before death of the clump took place. However, the site could just as easily have been an area within the cells. such as the ribosomes or cell membrane. Curve C is similar to curve A but has a tail, which Wood (1956) suggested was due to a very small fraction of the population being extraordinarily resistant to inactivation. Wood also stated that, if it did not appear high on the survival curve (about 1% survival), it had little influence on the initial shape of the curve, and therefore (one must presume) was ignored. Curve D, a concave survivor curve, indicates a population heterogeneous in heat resistance. In the simplest example, suppose there are cells with two distinct heat

Figure 1.1 <u>Survival curves of bacteria thermally inactivated in liquid broth</u>

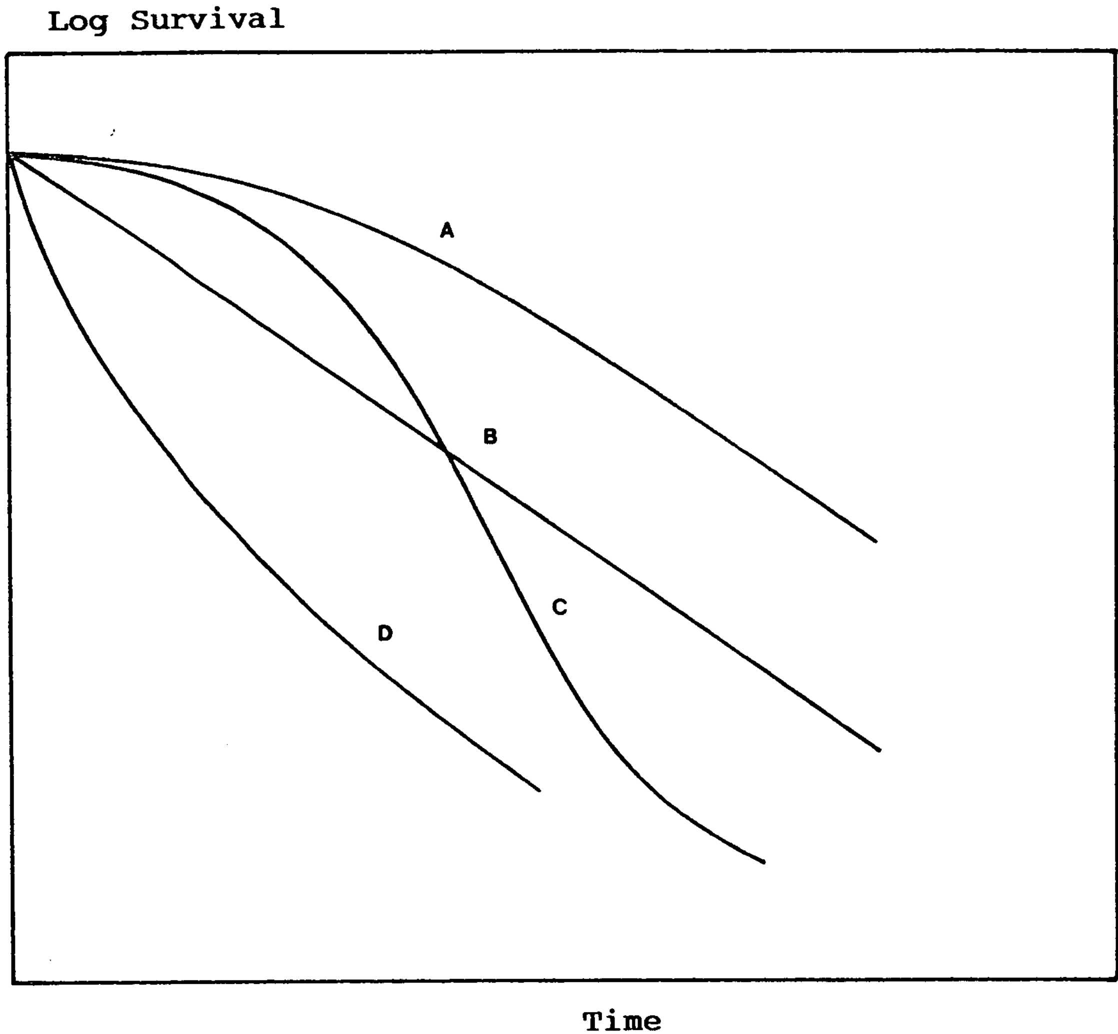


Figure 1.1 Non-exponential survival curves. Curve A, convex survival curve; initial lag in death rate followed by an approximately logarithmic death rate, commonly observed; curve B, logarithmic death rate as described in textbooks; curve C, similar to curve A but has a tail; curve D, commonly observed with cells in logarithmic growth phase and considered to indicate a heterogeneous population. From Moats (1971).

resistances, and, having different rates of inactivation, the survival curve formed will be a composite of the two cell types inactivations and will invariably resemble curve D.

A second hypothesis has been put forward to account for heat inactivation, in which cell death is assumed to occur by the thermal interaction of a large number of molecules. This postulates that each organism is inactivated by the joint action of a very large number of 'events' each of which occurs progressively. If the organisms were all equally susceptible, the survival curve would remain at 100% as long as the number of events per cell *x*, was less than the number required to kill the cell, and would then fall away rapidly to zero when *x* had been exceeded. However, no curves of this form have been described and therefore the shape of the curves are held to be determined solely by the variation of individual susceptibilities. Such variation could be due to age differences of the individual cells of the population, or to inherent non-uniformity in the heat resistance of cells.

In describing the thermal reactions of bacterial cells, there are two parameters which can be obtained from the logarithmic portion of the survival curve. These are the D value and Z value. The D value or decimal reduction time represents the time required, at a particular temperature, to reduce the surviving fraction of organisms 10-fold or by 1 log. The Z value is the number of degrees required to alter the D value 10-fold. These values, although not generally thought to be the best way of comparing bacterial heat resistances, as they are misleading when cultures give non-logarithmic death rates (Moats, Dabbah & Edwards, 1971), are convenient in assessing survival during the pasteurizing heat treatment given to foodstuffs. It may prove to be ill-advised to rely too heavily on survival curves to predict bacterial

numbers after pasteurization in view of the misleading nature of survival curves.

1.2 Factors affecting heat resistance

The survival curve is a graphical representation, at a specific temperature, of bacterial heat resistance. However other parameters can influence bacterial survival on exposure to lethal temperatures. The important parameters which are known to affect bacterial heat resistance have been reviewed by Hansen & Rieman (1963) and by Tomlins & Ordal (1976). I therefore intend to discuss only a few of the parameters here.

1.2.1 Factors affecting survival prior to thermal stress.

1.2.1.1 Growth Temperature

It has been well documented that increasing the growth temperature of a bacterium can increase the heat resistances to levels above those seen for growth at the optimum temperature. *Salmonella typhimurium* (Mackey & Derrick, 1986) and *Salmonella senftenberg* 775W (Mohamed & Davies, 1983) have both been reported to show increased thermal resistances when pre-incubated at temperatures well above their optimum; 42°, 45° and 48°C for *S. typhimurium* and 44°C for *S. senftenberg* 775W. This phenomenon has recently become an area of intense research and is usually referred to as the heat-shock response. This area will be dealt with later in section 1.4, but the effects of elevated growth temperatures on the ratio of saturated:unsaturated fatty acids in the bacterial cell membrane are discussed below.

The bacterial cell membrane is essentially composed of phospholipids and proteins. Bacteria are able to control the relative amounts of saturated and unsaturated fatty acids incorporated into membrane phospholipids. In

this way they are able to alter the phase transition temperature of the membrane, permitting sufficient fluidity for movement of integral proteins, and yet solid enough to maintain its structural role (McGarrity & Armstrong, 1975).

Psychrophilic bacteria which require a relatively liquid cell membrane are typically characterized by having relatively high proportions of unsaturated or anteisobranched fatty acyl groups compared with related mesophiles. Thermophilic bacteria on the other hand, which require a more gel-like membrane, have a higher level of saturated straight-chain fatty acids (McElhaney, 1976). Therefore, in order to maintain membrane fluidity at higher or lower growth temperatures, a mesophile will need to effect a corresponding rise or fall in the saturated:unsaturated fatty acid ratio. Hansen & Skadhauge (1970) observed an increase in the proportion of saturated fatty acids, together with an elevated heat resistance in *Escherichia coli* cells grown at 43°C compared with 30°C. Katsui *et al.* (1981) found that decreasing the growth temperature of *E. coli* to 15°C led to an increase in the proportion of unsaturated fatty acids and a lowering of the heat resistance compared with cells grown at 37°C. It was also suggested that changes in membrane fluidity with pre-incubation temperature, influences the heat stability of certain membrane-associated structures or functions which are critical in thermal death. Katsui *et al* (1981) went on to demonstrate this increase in heat resistance in various kinds of micro-organisms and indicated that these phenomena were general, at least for mesophiles grown at optimal temperature (Katsui, 1982).

1.2.1.2 Growth Phase

It has been known for many years that exponential phase cells are more susceptible to environmental changes

than stationary phase cells. For example *S. typhimurium* and *S. senftenberg* 775W show a marked decrease in their thermal resistances during the mid-log phase of growth (*Figure* 3.1.3). Kito *et al.* (1972) has reported that the proportion of saturated fatty acids in membrane phospholipids of *E.coli* is higher in stationary phase than in exponential phase cells. This would suggest that the phase transition temperature of stationary phase membranes is higher than exponential phase membranes, and this may account for increased thermostability and heat resistance.

1.2.2 Factors affecting survival after thermal stress

1.2.2.1 Recovery media

The recovery medium in itself does not affect the heat resistance of an organism exposed to the lethal effects of heat, as damage to the cell has already occurred. However, the nature of the recovery medium has a crucial effect upon the number of survivors and therefore the perceived heat resistance. After heating, the cell is stressed and without the correct recovery conditions will lose viability. Therefore, various recovery media have been developed, particularly by the food industry, which provide a suitable environment for the recovery of salmonellae from their thermally-stressed state.

The different recovery requirements of sub-lethally damaged cells has been used to good effect in investigations into bacterial thermal damage. Busta & Jezeski (1963) noted a difference between the plate counts of a thermally injured population of cells, on media with and without sodium chloride (NaCl). They suggested that those cells unable to form colonies on the media containing the high concentration of NaCl, had incurred damage to the cell membrane resulting in loss of

selective permeability. This loss of permeability is characteristic and can be used for the quantitative enumeration of thermal injury, since injured cells show this increased sensitivity to NaCl and other compounds.

1.3 Thermal injury and recovery

When a vegetative, mesophilic bacterium is exposed to a stress such as mild heating, the damage incurred may result in a loss of viability in a proportion of the population. This loss of viability is thought to be associated with: (i) the loss of cell membrane integrity, demonstrated by altered transport characteristics (Pierson & Ordal, 1971) and by the leakage of amino acids, potassium ions and 260nm-absorbing material, although these are only short lived, as the internal pools are reformed prior to salt tolerance returning (Iandolo and Ordal, 1966); (ii) the degradation of 16S ribosomal RNA and 30S ribosomal subunits (Rosenthal & Iandolo, 1970; Tomlins & Ordal, 1971*a*; and Sogin & Ordal, 1967); and (iii) some, although not extensive metabolic damage (Bluhm & Ordal, 1969 and Tomlins, Pierson & Ordal, 1971).

When heat-damaged cells are returned to nutrient broth, they exhibit what is called an extended lag (Jackson and Woodbine, 1963; & Kaufmann *et al.*, 1959), or recovery period. However, if the heat-damaged cells are placed in minimal media, recovery does not occur (Iandolo & Ordal, 1966), indicating a dependence upon external sources of nutrients for recovery. Heinmet, Taylor & Lehman (1954), observed that revival in minimal media could be attained if the damaged cells were pre-incubated in 0.2% solutions of various metabolites. Garvie (1955) refuted this claim, suggesting that recovery was merely due to the multiplication of surviving cells. This latter statement is now commonly thought to be incorrect, with recovery, generally measured by the return of salt

tolerance, occurring prior to cell division (Iandolo & Ordal, 1966; Stiles & Witter, 1965; and Tomlins & Ordal, 1971). Iandolo & Ordal (1966) found that the minimal requirements for the recovery of injured *Staphylococcus aureus* are: an energy source, e.g., glucose; a complex nitrogen source, e.g., a minimum of four amino acids; and phosphate. It should therefore be possible, using radio-labelled compounds, to discover which components of the cell are damaged and which are essential for the recovery of thermally-injured cells.

1.3.1 Energy requirements for the recovery of thermally injured cells

It has been well documented that 2,4-dinitrophenol (2,4-DNP) is able to uncouple oxidative phosphorylation along the electron transport chain (Racker, 1965). Therefore, it should be possible by plating a population of thermally-injured cells on a medium containing 2,4-DNP and with citrate as the sole energy source, (citrate having to be oxidatively phosphorylated in order to generate the energy units of adenosine triphosphate [ATP]), to indicate whether recovery is energy dependent. Tomlins & Ordal (1971) showed that *S. typhimurium* incubated on citrate medium after heat treatment, was more susceptible to 2,4-DNP than uninjured cells incubated on the medium. Tomlins & Ordal (1971) also noted in *S. typhimurium,* that which Iandolo & Ordal (1966) had described for *S. aureus*, viz., that if the recovery medium contained a compound from which substantial quantities of ATP could be synthesized, e.g., glucose, the effect of 2,4-DNP was not observed. They suggested that some energy-dependent metabolic processes are essential for the recovery of thermally-injured *S. typhimurium* and *S. aureus*.

1.3.2 Cell wall damage and repair during thermal injury and recovery

The function of the cell wall is to maintain the rigidity of the organism and to protect the delicate underlying structures. There are, generally speaking, two types of cell wall: Gram-positive; and Gram-negative, which differ in their chemical composition. The cell walls of Gram-positive organisms, for example staphylococci, are comprised of peptidoglycan linked to teichoic acid, whereas the walls of Gram-negative bacteria, for example, *S. typhimurium* are more complex, three-layered structures (Costerton, Ingram & Cheng, 1974). Peptidoglycan comprises the inner layer while the outer layer is made up of interpenetrating lipopolysaccharide (LPS) and lipoprotein.

The lipid component of the outer envelope of Gram-negative bacteria, a non-specific permeability barrier, would appear to be particularly vulnerable to thermal attack. Lysozyme, an enzyme capable of hydrolysing the N-acetylmuramyl-(1-4)-B-linkages in peptidoglycan and which is inactive against normal Gram-negatives, produces lethal effects against cells previously thermally stressed. Indeed, Hitchener & Egan (1977) have shown that *E. coli* subjected to mild thermal stress, 48°C for 60 min, released approximately 20% of the outer envelope's LPS. Katsui *et al.* (1982a) has indicated that this release of LPS from thermally injured *E. coli*, 55°C for 30 min, is by "blebbing" of the outer membrane, particularly at the septa of dividing cells. Hitchener & Egan (1977) went on to point out that when cellular LPS was depleted by 30% by washing in solutions containing EDTA, the cells showed no increase in the rate of thermal death or structural injury produced by heating at 48°C. They suggested that cells are capable of tolerating considerable outer membrane damage without increased sensitivity to heating at 48°C and this suggests that

outer membrane damage is not the direct cause of thermal death. Katsui *et al.* (1982*a*) agreed with Hitchener & Egan in that outer membrane damage is not the direct cause of thermal death, since although surface blebs were seen in 11% of cells heated, no substantial death was observed after the first 15 seconds of heating.

It appears unlikely that repair of the cell wall is important in the recovery of injured microbes, *S. aureus* being able to recover in the presence of penicillin (Hurst *et al.*, 1973; and Iandolo & Ordal, 1966). No data as yet has been published on the recovery of Gram-negative bacteria in the presence of an inhibitor of cell wall synthesis, and it would therefore be premature to make any assumptions about its importance in recovery.

1.3.3 Cell membrane damage and repair during thermal injury and recovery

Lying beneath the cell wall is the cell membrane, a semi-permeable structure mainly composed of lipoprotein. Its function is to control the passage of solutes into, and waste and other products out of, the cell. A means of detecting damage to the cell membrane is therefore the measurement of various substances released from the cell or accumulation of substances normally excluded by an undamaged membrane.

During thermal injury, the protein, polypeptide and nucleic acid components of the cell are broken down into smaller fractions, and are then able to leak out of the cell through the cell membrane. The question arises, is such leakage responsible for death, or is it the result of some other primary changes induced elsewhere in the cells? It seems reasonable to infer that leakage is a secondary effect, as it occurs as a result of cellular degradation and/or membrane damage. Bryne and Chapman (1964) studied the physiochemical structure of lipid

membranes and suggested that the cytoplasmic membrane would be expected to be very sensitive to temperature changes, since such membranes existed on the borderline of a temperature-sensitive phase transition. If the cell membrane is such a fragile structure, what sort of membrane damage could arise, from which would ensue intracellular penetration or extracellular leakage? There are two possibilities, first, there is a melting of the lipid component of the lipoprotein membrane, and second, there is physical damage of another kind, viz., that leading to small "holes" in the membrane (Marquis and Corner, 1967).

In order to determine the physical state of the membrane, the extent of damage incurred must be investigated. Pierson, Tomlins & Ordal (1971), reported that the recovery of *S. typhimurium* from thermal injury, involved lipid synthesis. It is therefore likely that these lipids are used in the repair of heat damaged membrane and are important in recovery. Tomlins, Vaaler & Ordal (1972), using ^{14}C labelled glucose, showed that 26.6% of lipid synthesis occurred during recovery from thermal damage. As the heating menstruum yielded almost no lipid at all, disruption of the membrane during injury must have occurred without significant release of lipid. However, Hurst *et al.* (1973), reported a one-third loss of membrane lipid by *S. aureus* during thermal injury.

The repair of thermally-damaged membrane probably occurs early in recovery, being necessary for the accumulation of essential intracellular pools required for other biosynthetic pathways (Tomlins *et al.*, 1972; and Pierson *et al.*, 1971). Hurst *et al.* (1973) noted that salt tolerance was regained when other membrane functions were still impaired. A possible explanation for this was that numerous sites on the membrane were damaged during heat treatment but they were repaired at different rates, the return of salt tolerance being an

indication of the repair of an early one. Based on both experimental and calculated values for *S. faecalis*, Moats (1971) postulated a kinetic explanation for thermal injury and death, suggesting there were > 100 possible sites for thermal inactivation, a concept in agreement with multiple injury to the membrane.

Tomlins *et al.* (1972) measured the phospholipid and neutral lipid levels of normal *S. typhimurium* as 92% and 8% respectively, these correlating well with previously published values (Ames, 1968; Macfarlane, 1962; and Olsen & Ballou, 1971). They noted that during the recovery of *S. typhimurium* from thermal injury, the nature and extent of lipid and phospholipid species synthesis was similar to that of normal cells. The only difference was in the higher levels of cardiolipin. Cardiolipin has been observed to accumulate in the stationary phase of *Clostridium butyricum* (Bauman, Hagan & Goldfine, 1965) and in S. *aureus* (Houtsmuller & Van Deenan, 1965). The authors of these papers suggested that recovering cells may synthesize phospholipid characteristic of late stationary phase cells. The synthesis of fatty acids during the recovery of heat-injured *S. typhimurium*, unlike the phospholipids, varies greatly from that found in uninjured cells. The most striking difference was the large increase in unsaturated fatty acids ($C_{16:1}$, $C_{18:1}$) with reciprocal decrease in both short chain saturated fatty acids up to and including $C_{14:1}$, and cyclopropane fatty acids ($C_{17:0}$ cyclo and $C_{19:0}$ cyclo).

Growing evidence suggests that the loss of membrane integrity during thermal injury is partially responsible for the loss of cell viability through leakage of intracellular components. It is perhaps also true to say that the repair of membrane lesions, by lipid synthesis, is essential for recovery, lest the cells starve due to an inability to accumulate the components necessary for growth.

1.3.4 Structural protein damage and repair during thermal injury and repair

The coagulation or denaturation of a protein by heat is the result of partial or total unfolding of the three-dimensional arrangement of polypeptides which make up the protein. This three dimensional structure is held together by a delicate balance of different noncovalent forces; hydrogen bonds, hydrophobic, ionic and Van der Waals interactions, etc. (Schulz & Schirmer, 1979). Upon increasing the temperature, all these forces diminish and the protein macromolecule unfolds, i.e., acquires a less ordered confirmation. Thermal unfolding occurs at temperatures which vary greatly according to the protein. The temperature range in which a protein is stable depends on the balance between hydrogen bonds and hydrophobic interactions, which stabilize the native structure. Other factors, for example pH, can affect thermal unfolding; at low pH, thermal unfolding occurs at lower temperatures than at neutral pH because the noncovalent interactions which are dependent upon surface charge are reduced, and the energy required to disrupt them lowered.

Proteins do not necessarily undergo complete conformational change during thermal denaturation. Aune *et al.* (1967) estimated that about 25% of native-like structure remains in thermally denatured proteins. However, losing 75% of original conformation is obviously enough to render useless the structure and therefore function, in the case of enzymes, of most proteins. The conformational changes associated with thermal denaturation need not be permanent and renaturation can and does occur. Irreversibility is frequently attributable to the formation of aggregates, presumably resulting from intermolecular disulphide cross-links, or intermolecular non-covalent associations.

Because of the deleterious affects of heat upon protein structure, it is possible that protein coagulation may be the main cause of cell death. However, work has shown that storage of cells in concentrated sucrose solutions at 60°C, reduced the rate of protein denaturation of cellular proteins, without influencing the rate of loss of viability of the suspensions (Brandt, 1967). This could therefore indicate that protein denaturation is not necessarily the prime cause of thermal injury in vegetative bacteria. This does not rule out the possibility that some highly essential proteins are always inactivated by heat, irrespective of the conditions used. It has been reported that chloramphenicol, a potent inhibitor of protein synthesis, has no effect upon the recovery of heat treated *S. aureus* (Bluhm & Ordal, 1969, Iandolo & Ordal, 1966 & Sogin & Ordal, 1967). Tomlins & Ordal (1971) on the other hand reported that both chloramphenicol and chlorotetracycline, another inhibitor of protein synthesis, both prevented recovery of thermally stressed *S. typhimurium*. As *S. typhimurium* is reported not to suffer extensive metabolic damage during thermal injury (Tomlins *et al.*, 1971), essential structural protein damage can be assumed to occur, repair of which requires protein synthesis. Section 1.3.6 on repair of ribosomes indicates that 30S ribosome maturation is dependent upon new protein synthesis.

1.3.5 Enzyme damage and repair during thermal injury and recovery

Enzymes are proteins, and like all proteins heat affects them to different degrees. However, unlike most structural proteins even a very small conformational change of an enzyme's three-dimensional shape could render it inactive. The active site of enzymes always consists of several amino acid residues brought together

only in the native three-dimensional structure (Anfinsen & Scheraga, 1975). Therefore, a slight thermally-induced unfolding may result in a disassembling of the active centre and, hence, enzyme inactivation. While unfolding seems to be a universal and general stage in enzyme thermoinactivation, the subsequent events can be divided into two groups: covalent and noncovalent, and they usually result in the permanent inactivation of the enzyme as they prevent reversion to the native state.

Enzyme molecules are built up from the 20 different amino acids and often also contain other molecules such as nucleotides, sugars, lipids, metal ions etc. These structures can undergo numerous chemical reactions but these reactions are usually slower at ambient temperatures. At higher temperatures the rate of such reactions are greatly enhanced and spatially separated parts of the protein molecule are brought together enabling them to react. Reactions involving sulphur-containing amino acid residues are often responsible for thermoinactivation of enzymes. For example, in an enzyme containing both cysteine and cystine, the formation of new intermolecular and/or intramolecular cross-links by disulphide exchange can take place, especially at alkaline pH's (Warner & Levy, 1959; Steinrauf & Dandliker, 1958). Hydrolytic scission of disulphides in protein to form one residue of thiocysteine and one of dehydroalanine has been well documented (Cecil & McPhee, 1959).

ϵ-Amino groups of lysine and terminal α-amino groups are excellent nucleophiles. Since the former are abundant in enzymes, they can participate in many deteriorative reactions. The most common among these are; (i) formation of new cross-links by attachment to dehydroalanine, produced as a result of hydrolytic cleavage of S-S groups (Bohak, 1964; Ziegler, Melchert & Lurken, 1967); (ii) heat-induced isopeptide formation

with the carboxyl groups of aspartic and glutamic acid or their amides and; (iii) reactions with reducing sugars if the latter are present in the system. Enzymes unfolded by heat are susceptible to attack by proteases present in solution (McLendon & Radany, 1978) and this results in thermal inactivation by proteolysis.

Thermally-unfolded enzyme molecules can undergo two types of non-covalent transformations: polymolecular (aggregations) and monomolecular (formation of incorrect structures).

Upon unfolding, hydrophobic regions of the enzyme (which had previously been hidden in the centre of the structure) become exposed to the solution. This is thermodynamically unfavourable and in order to reduce the free energy of the system, the unfolded molecules must interact with each other, the hydrophobic spots being the areas of contact. This kind of interaction will result in protein aggregation and is commonly seen when an egg is poached.

In very dilute solutions of enzymes, aggregation is highly unlikely but thermal inactivation still takes place. A model for this was proposed by Klibanov & Mozhaev (1978) in which the unfolded molecule can intramolecularly re-fold into new structures, different from native enzyme conformations and form kinetically or thermodynamically stable structures, which are catalytically inactive. Even after cooling, these incorrect structures remain because a high kinetic barrier prevents spontaneous refolding to the native structure. In a real system, noncovalent thermoinactivation should always include both poly- and monomolecular processes.

Tomlins *et al.* (1971) carried out enzyme assays on heat-injured *S. typhimurium* and found that it had all the

enzymes necessary for a functional TCA cycle and many other important enzymes. They therefore suggested that metabolic damage was not an important mechanism contributing to the thermal injury of this bacterium. *S. aureus* on the other hand loses the function of a number of enzymes present in the TCA cycle and other pathways. It was concluded that the irreversible denaturation of these enzymes may be responsible for increased nutritional requirement for recovery in *S. aureus* (Iandolo & Ordal, 1966).

1.3.6 Ribosome and rRNA degradation and resynthesis during thermal injury and recovery

Degradation of RNA by thermal injury was first recognized when investigators found that bacterial cells e.g., *E. coli and S. aureus* (Califano, 1952) and *S. aureus* (Iandolo & Ordal, 1966), lost 260nm-absorbing material when they were subjected to temperatures slightly higher than their maximum growth temperature. Allwood & Russell (1967 & 1968) investigated the leakage of 260nm-absorbing material and RNA from thermally injured *S. aureus* and found that, at a temperature of up to 50°C, there was a direct correlation between the loss of RNA and loss of viability. This situation is hardly surprising given that thermally-injured cells lose selective permeability of the membrane and the leakage of soluble pools of low molecular weight precursors into the extracellular environment takes place (Iandolo & Ordal, 1966).

Degradation of rRNA and ribosomes *in vivo* during thermal injury has been studied in *S. aureus* (Sogin & Ordal, 1967; Rosenthal & Iandolo, 1970; Rosenthal *et al.*, 1972); and *S. typhimurium* (Tomlins & Ordal, 1971*a*). The sedimentation rates of ribosomes, from heated and unheated cultures of *S. typhimurium*, were compared on sucrose gradients. The thermally-injured cells contained

one type of ribosomal particle with a sedimentation coefficient of 47S but no 30S. This effect has also been observed in thermally injured *S. aureus* (Sogin & Ordal, 1967; Rosenthal & Iandolo, 1970; and Rosenthal *et al.*, 1972).

Polyacrylamide gel electrophoresis of rRNA extracted from heated and unheated cells of *S. typhimurium* showed complete degradation of the 16S RNA species and partial degradation of the 23S RNA species (Tomlins & Ordal, 1971*a*).

Work on mesophilic and thermophilic bacteria has been performed in order to understand the ability of thermophilic bacteria to grow at high temperatures and to relate this stability to ribosomes (Stenesh & Yang, 1967; Saunders & Campbell, 1966; and Pace & Campbell, 1967) Based on these and other studies, it was proposed the degradation of ribosomes, measured by the increase in absorbency at 260nm of rRNA by thermal-injury involved more than nonenzymatic hydrolysis of RNA. Natori, Nozawa & Mizuno (1966) found that when *E. coli* ribosomes were incubated *in vitro* in various concentrations of phosphate, they became unfolded and lost ribosomal proteins and were more sensitive to ribonuclease attack than were untreated ribosomes. Ethylenediaminetetra-acetic acid (EDTA) has also been shown to cause destabilization of isolated *S. aureus* ribosomes, similar to the effects seen in phosphate buffer (Haight & Ordal, 1969). The authors postulated that intracellular degradation of ribosomes during heat injury was due to the action of polynucleotide phosphorylase and a ribonuclease. Strange & Shon (1964) suggested that ribosome damage following sublethal heating of *Enterobacter aerogenes* was due to the loss of magnesium. They proposed that magnesium was required for ribosome integrity, and that a ribonuclease was responsible for ribosomal damage. Hurst & Hughes (1978) demonstrated the

importance of magnesium in ribosome stability in *S. aureus* during thermal action. They sublethally heated *S. aureus* in phosphate buffer or phosphate buffer containing EDTA and in both cases the 30S subunit was destroyed, its destruction increasing with time. However, if the heating menstruum was changed to Tris buffer containing $MgCl_2$, ribosome destruction did not take place. What was also interesting, was that these cells became as salt sensitive as the cells heated in phosphate buffer but were able to regain tolerance in the presence of actinomycin D, a potent inhibitor of RNA synthesis. This therefore suggests that ribosomal damage is not a primary cause of cell death.

Evidence indicates that heat-injured *S. typhimurium* (Tomlins & Ordal, 1971*a;* and Gomez *et al.*, 1973) and *S. aureus* (Iandolo & Ordal, 1966) need to resynthesize RNA as an obligatory requirement for recovery. Allwood & Russell (1969) reported that RNA synthesis occurred during the early stages of recovery of heat-injured *S. aureus* but at a slower rate than that of unheated exponentially-growing cells. Tomlins & Ordal (1971) performed incorporation experiments using a selective inhibitor of RNA synthesis and showed that RNA synthesis did occur during the recovery of *S. typhimurium* from thermal injury. They also found that RNA synthesis ceased before recovery was complete and concluded that it was not the rate limiting step in recovery. Also, protein synthesis occurred during the latter stages of recovery, and it is possible that these proteins attach to rRNA to form the ribosomal subunits. In fact, Flowers & Martin (1980) noted that ribosomes were assembled from newly synthesized RNA with at least some of the protein which existed before injury.

When the rRNA profiles of the co-electrophoresis in polyacrylamide gels, of control *S. typhimurium* cells and cells recovering from thermal injury were compared, the

rRNA synthesized during recovery was fractionated into four peaks; 23S and 16S and their respective precursor molecules 24S and 17S RNA. When injured cells were similarly incubated, but in the presence of chloramphenicol, the maturation of 17S and 16S RNA stopped (Tomlins & Ordal, 1971*a*). This might suggest that newly synthesized protein is required for maturation of 16S RNA, whereas 17S RNA can be built up using pre-existing protein.

The ribosomal particles generated during partial recovery of *S. typhimurium* from thermal injury, can be divided into two broad peaks by co-sedimentation with control uninjured cells. The 50S and 48S regions correspond to the 50S region, while the 31S, 28S, 26S, and 22S ally to the control 30S region, the majority of the peak being in the 22S range. When injured cells were allowed to recover for a longer period, there was a shift in the relative amounts of 30S precursor particles towards the 30S region and away from the 22S region. The addition of chloramphenicol to the recovery medium altered the ribonucleoprotein profile. The 50S and 48S particles were still present in the 50S region, while in the 30S region, 22S particles formed the major peak but there was a shoulder present in the curve at 32S. This suggests that inhibition of protein synthesis resulted in the inhibition of 30S ribosomal particle maturation, without affecting the formation of mature or precursors of 50S particles (Tomlins & Ordal, 1971a).

Evidence clearly suggests that rRNA and ribosome degradation does occur during thermal injury and that the extent of degradation depends upon the severity of heating and composition of the heating menstruum. It also seems likely that rRNA and ribosomes are not the primary cause of cell death, neither are they the rate limiting step in recovery. The 50S ribosomal particle and its constituent RNA and proteins seem slightly more

heat stable than the 30S ribosomal particle. Also, the ribosomal proteins from degraded ribosomes are re-used during recovery but the synthesis of some 30S ribosomal proteins are necessary for maturation (Tomlins & Ordal, 1971*a*).

1.3.7 DNA damage and repair during thermal injury and recovery

At optimum temperatures for mesophilic bacteria (30°-37°C), the lowest energy, and therefore most stable state DNA can attain is the Watson & Crick double helix. The forces holding the two complementary strands together are: (i) hydrogen bonding between bases; (ii) base stacking and related hydrophobic interactions; and (iii) electrostatic forces of phosphate groups. The strands will only usually separate during transcription, to allow RNA polymerase to act etc., and then only in localised regions.

Raising the ambient temperature will break the hydrogen bonds maintaining the main association between the bases and resulting in separation of the two strands. As the temperature is raised still further, the proportion of single-stranded to double-stranded DNA will increase until all the DNA is single-stranded. This phenomenon is referred to as thermal melting (*Tm*) and is determined by following the absorbence at 260nm as a function of the temperature of the DNA solution, and determining the mid-point of the hyperchromic increase resulting from the disruption of base stacking (Marmur & Doty, 1962). The *Tm* values of the majority of mesophilic bacteria, under physiological conditions, lie in the range 85°-95°C. The variability in values corresponds to the variability in base pair composition, as thermal melting of the secondary structure of DNA is dependent upon the guanine:cytosine (GC) base pair content. The number of hydrogen bonds holding these pairs together is

greater than for the adenosine-thymine (AT) base pairs, and therefore the energy required to break them is greater. Obviously if DNA damage occurs at sub-lethal temperatures (50°-60°C) then it must be something other than strand separation.

Single-strand breaks can be induced by thermally stressing DNA, and are caused by spontaneous hydrolysis in the temperature range 80°-100°C (Eigner, Boedtker & Michaels, 1961). Depurination also occurs at these elevated temperatures (Greer & Zamenhof, 1962). Again these temperatures are well above the sub-lethal range and would not explain DNA damage at the lower temperatures. Bridges, Ashwood-Smith & Munson (1969 & 1969*a*), reported DNA single-strand breaks in *E. coli* at sub-lethal temperatures, e.g., 52°C, similar to those induced by γ-radiation. In further studies, Sedgwick & Bridges (1972) concluded that DNA strand degradation observed in several *E. coli* strains, differing in their DNA polymerase I activity, were consistent with attack by native nucleases which were released or activated by mild heating. Later, Woodcock & Grigg (1972) reported not only single- but double-strand breaks; c.100 double-strand breaks in the *E. coli* genomic DNA by heating at 52°C.

Most bacterial cells are able to repair single- and double-strand lesions upon post-heating incubation at physiological temperatures. Woodcock & Grigg (1972) noted that *E. coli* previously heated at 52°C, and subsequently incubated at 37°C in phosphate buffer plus thymidine, gave an increase in DNA molecular weight returning it to its former value in 30 min. During this period there was recovery of cell viability. However, the inhibitor of DNA repair by pyronin Y, reduced both viability and DNA molecular weight. They also noted that little or no loss of DNA was observed during injury and recovery and that heating the DNA *in vitro* at 52°C did

not result in strand breakage. They concluded that repair of the DNA strand breaks (rather than replication) occurred during the recovery period and that DNA breakage was due to enzymic action and not as a direct effect of temperature.

Genetic evidence implicating DNA in heat damage and repair, comes from studies showing that a variety of DNA repair-deficient mutants are more sensitive to heat than their wild type parents. Pauling & Beck (1975) measured the viabilities after 20 min at 52°C, of a wild type and a ligase-deficient mutant of *E. coli*, as 31% and 0.1% respectively. They concluded that the mutant was unable to repair the DNA breaks. Bridges *et al.* (1969*a*) found that *rec*$^-$ and *uvr*$^-$ DNA repair mutants of *E. coli* were also more heat sensitive than their wild type parents.

Despite the wide ranging experimental evidence suggesting possible mechanisms by which DNA breaks are produced *in vivo*, the system is still not very well understood. However, a number of observations point to enzymatic attack as the cause of these breaks after heat damage: e.g., Eigner *et al.*, 1961; Grecz & Bhatarakamol, 1977; Gomez & Sinskey, 1973; and Woodcock & Grigg, 1972.

1.3.8 Summary of introduction

Thermal injury has clearly been shown to cause numerous biochemical lesions in Gram-negative and Gram-positive bacteria. In *S. typhimurium* these lesions include the loss of ribosomal RNA, a decrease in the internal K^+/Na^+ ratio, a loss of salt tolerance for growth, a loss of membrane lipids, a creation of an extended lag period for growth onset, but no real enzyme damage (glycolysis and TCA cycle) such as occurs in thermally damaged *S. aureus*. Recovery from sublethal thermal injury occurred during the extended lag period for injured cells, and this was parallelled by recovery

of growth tolerance to 7.5% NaCl. Use of inhibitors has shown that salt tolerance recovery did not require cell division, cell wall synthesis but did require protein synthesis. Membrane lipid resynthesis was shown not to be necessary for salt tolerance recovery, while ribosomal RNA synthesis has been clearly shown to parallel the return of salt tolerance and that RNA synthesis inhibition prevents the recovery of salt tolerance by injured cells.

1.4 Heat shock response

A comparison of the total protein composition of a mesophilic bacterial culture grown at 23°C and at 37°C would reveal very little difference. Contrasting growth at steady state, between 13°C and 46°C would show marked differences in many proteins, but this would mainly be a reflection of the higher energy demands required of cells grown at extremes of temperature. However, shifts up and down in growth temperature, even of a minor nature will result in large changes in the proteins synthesised. These changes occur within several minutes and involve up to 100-fold increases in protein levels (Yamamori *et al.*, 1978 and Yamamori & Yura, 1982). Most other cellular proteins are switched off and those that are induced belong to a small group of highly conserved proteins, the heat shock proteins (hsps). Once the cell is returned to a normal growth temperature, the hsps are repressed.

There are at least 17 hsps in *E. coli*; the organism on which the majority of the research work on hsps has been focused. All 17 are shown in *Table* 1.1. The functions of many remain unknown, while many of those with known functions are associated with the growth of bacteriophages. Although their function within an un-infected cell and role in heat shock remains unclear, many suggestions have been made.

Table 1.1 Known heat inducible proteins of *E. coli* and their corresponding genes

Protein No.	Mol Wt.	Protein Name	Gene
1	25,300	GrpE	*grpE*
2	62,883	GroEl	*mopA/ groEL*
3	69,121	DnaK	*dnaK*
4	70,263	Sigma	*rpoD*
5	14,700		*htpE*
6	10,670	GroES	*mopB/ groES*
7	71,000		*htpG*
8	33,400		*htpH*
9	48,500		*htpI*
10	60,500	lysyl-tRNA synthetase form II	*lsyU*
11	10,100		*htpK*
12	21,500		*htpL*
13	84,100		*htpM*
14	13,500		*htpN*
15	21,000		*htpO*
16	94,000		*lon*
17	40,975	Lon, DnaJ	*dnaJ*

1.4.1 Proteins

The GroE proteins are known to be essential for the morphogenesis of many (perhaps all) coliphages. GroEL and GroES are derived from the linked genes *groEL* and *groES*, but their function within the heat shock response remains unclear. Bochkareva *et al*, (1988) has demonstrated that GroEL has an affinity for unfolding or misfolding proteins and it impedes aggregation (or promotes disaggregation) of the latter by using the energy of ATP hydrolysis. This would suggest that the synthesis of this protein would slow or prevent the aggregation of proteins during exposure to high temperatures and speed the return of normal structural and function of any proteins unfolded by heat.

The DnaK protein in *E. coli* belongs to a highly conserved group. The human *hsp70* gene product is 73% identical to that in *Drosophila* and 50% identical to DnaK in *E. coli* (Lindquist, 1986). Many hypotheses have been put forward for the function of these proteins in a heat injured cell. One possibility is that DnaK is capable of recognizing and disaggregating clumps of precipitated and denatured proteins. Also it is potentially capable of unfolding soluble abnormally folded protein chains Ketter & Simon (1988). Further, DnaK may be involved in the dispersal of these granular deposits. The action of dispersal may expose the interior of the deposits to the action of proteolytic enzymes thereby facilitating their break down. The disaggregation function may also unfold the abnormal proteins and perhaps give then a second chance to unfold in to their proper conformation. Pelham (1986) has suggested an ATP-dependant activity capable of dissociating hydrophobic aggregates of denatured protein, for a 70 KD family of stress proteins which includes DnaK.

1.4.2 Regulation of the response

1.4.2.1 Inducers, targets and signal(s)

Induction of the heat shock response is defined on the basis of the synthesis of half a dozen or so of the major shock heat proteins. However, induction is not solely as a result of temperature shifts; there are in fact many other inducers. The immediate target of these inducers remains unclear but likely candidates include DNA and its replication protein structure and synthesis, and the cytoplasmic membrane.

In view of the range of inducers and the variation in target sites, are these targets independent of each other or are they part of a sequential activation cascade?

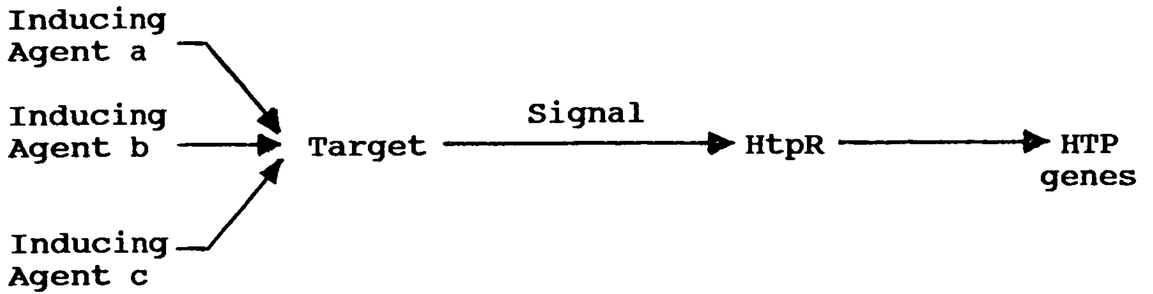
In *Figure* 1.2: Model A illustrates the view that there is one common target (protein denaturation); Model B suggests that the response is somehow triggered by multiple, equivalent signals; and Model C implies that, whatever the inducer, a single signal (such as a low-molecular weight metabolite, or alarmone) is ultimately generated to activate the HtpR-dependant response. However, because of the large number of inducing agents the total cellular picture may be a combination of all of these possibilities.

1.4.2.2 Effector

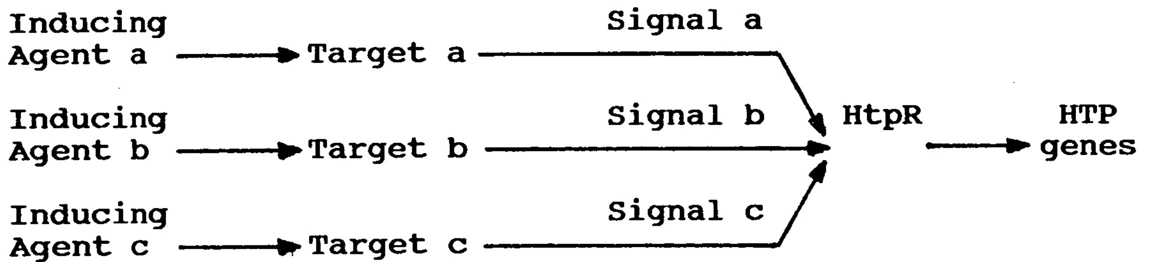
The only so far discovered effector of the heat shock response is the product of the *htpR* gene. The structural and functional similarity of HtpR and the heat-inducible RNA polymerase sigma factor (sigma-70) have given rise to HtpR being called sigma-32 (Grossman *et al*, 1984).

Figure 1.2 Models for induction of the heat shock response

Model A



Model B



Model C

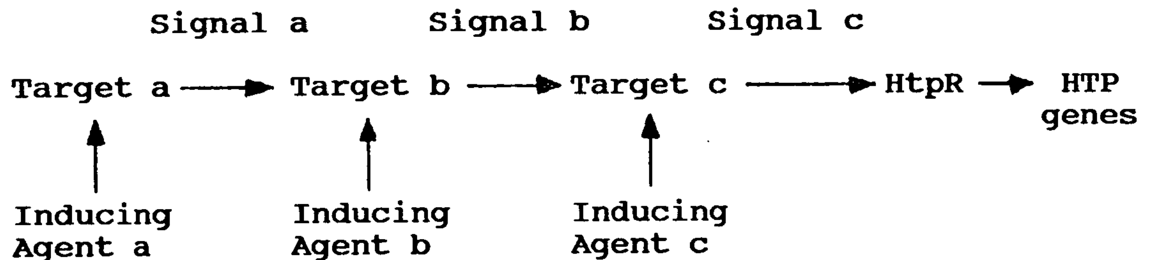


Figure 1.2 Alternative models for induction of the heat shock response in *E. coli* and *S. typhimurium*. Three inducing agents, representing the large environmental conditions provoking the response, are depicted. In Model A, there is assumed to be a single target for all inducers. In Model B, the inducer interacts with separate targets, and separate signals are thereby generated, but each signal directly turns on the HTP regulon. In Model C, there is only one directly effective signal, the others act indirectly by giving rise to the effective one. From (Neidhardt and VanBogelen, 1987).

Regulation of the heat shock response is under transcriptional control, this being evident from the rifampicin sensitivity of the process (Yamamori & Yura, 1980). The mRNA's transcribed during a shift up in temperature appear to have normal half-lives, are initiated within 15 seconds (30° to 42°C) and reach a maximum functional concentration within approximately 3.5 minutes (Yamamori & Yura, 1980).

Given that activation of the heat shock genes is under transcriptional control, how can HtpR initiate such rapid activation. Firstly by a rapid increase in HtpR, secondly an equally rapid activation of pre-existing but inactive HtpR, or thirdly, new synthesis combined with activation. Over-production of HtpR gives an exaggerated heat shock response, (Grossman *et al*, 1984), while over-production of sigma-70 suppresses the heat shock response (Yamamori *et al.*, 1982). Competition must therefore occur between the two sigma subunits with variations in concentration having an influence on protein synthesis and their concentration being influenced by environmental conditions. This may explain the heat inducible nature of sigma-70 as it is necessary to assure the possibility of the cell escaping from the potentially lethal exclusive synthesis of heat shock protein.

1.4.2.3 Modulators

The product of the *dnaK* gene has been implicated as a modulator of the heat shock response (Tilley *et al.*, 1983). The *dnaK*756 mutant allele results in cells unable to turn off the heat shock response at 43°C, while synthesizing higher than normal levels of HTP at 30°C. Cells with the *htpR*165 and *dnaK*756 double mutation, lack a heat shock response suggesting DnaK is not a direct suppressor but that HtpR counteracts the repression.

Theoretically sigma-70 can be regarded as a modulator, but this may have more to do with the molecular mechanism of the response than with its functional modulation.

1.4.3 Conservation of the heat shock response

Genetic conservation of the heat shock response is unusual in that it not only involves some of the genes and proteins but also the controlling mechanism (positive-acting transcription factors) and general behaviour (transient hyperactivity of heat shock genes leading to rapid elevation of the concentration of their products, after a temperature shift). If this is considered alongside the presence of the response in procaryotic and eucaryotic and even in archaebacteria, it must have arisen in the earliest common form of cellular life, perhaps 2 to 2.5 billion years ago, and must be under continual positive selection in all current living systems.

1.4.4 Heat induced thermotolerance

The overall function of the hsps in bacteria is not yet understood, but it does appear that they confer thermal resistance on those organisms in which they are induced. Cells transferred to a lethal temperature of 55°C have a greater resistance if they have been pre-exposed to an elevated growth temperature, e.g., 42°C. Mackey & Derrick (1986) reported that *S. typhimurium* grown at 42°, 45° or 48°C had an increased thermal resistance, at 55°C, which reached a maximum after 2 h and was maintained as long as the cells were held at the elevated growth temperature, in this case 10 h. Yamamori & Yura (1982) reported that *E. coli* previously grown at 42°C, only had a transient increase in resistance which reached a maximum at 30 min and dasappeared by 60 min. However, they did report that cells of some strains in

steady-state growth at 45° to 46°C have a permanently increased thermal resistance compared with cells grown at 30°C (unpublished observation, Yamamori *et al.*, 1982).

It would appear that the heat-shock response acts as an aid to growth and survival at high temperatures. Though why it should have been retained in an inducible state by all forms of life, even those able to grow at very high temperatures, 70°-80°C, remains unclear. It may be that it is a system designed to deal with shifts in temperature rather than high temperatures per se.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacterial strains

Strains of *Salmonella typhimurium* are listed in *Table* 2.1, strains of *Salmonella senftenberg* in *Table* 2.2 and strains of *Escherichia coli* in *Table* 2.3.

2.2 Plasmids

Plasmids used during the course of this study are listed in *Table* 2.4. Some of them were transferred to hosts other than those in which they were received.

2.3 Maintenance of cultures

Strains of *S. typhimurium*, *S. senftenberg* and *E. coli* were maintained on LB agar. Genetically-marked strains and those carrying plasmids were routinely subcultured on to supplemented media for characterisation of the phenotype. Permanent stocks were kept at -70°C in 7% dimethyl sulphoxide (DMSO). The culture to be stored was grown overnight in an appropriate medium and 1 ml was added to 70 μl of DMSO and flash frozen in ethanol at -70°C before storage at -70°C. Liquid cultures were grown in 5 ml volumes in 20 ml Universal bottles, 50 ml volumes in 250 ml Erlenmeyer flasks or 1 l volumes in 2 l flasks on an orbital shaker incubator at 180 rpm and 37°C unless otherwise stated.

2.4 Media

The following media formulations were used:

(1) Luria Broth (LB)

	gl^{-1}
Bactotryptone (Difco)	10
Yeast Extract	5
NaCl	10

Table 2.1 Strains of *Salmonella typhimurium* used

STRAIN	GENOTYPE	SOURCE
SA2009 LT2	*aroA*$^-$, *tyr*$^-$, *phe*$^-$, *trp*$^-$, *paba*$^-$	K.E. Sanderson[1]
LT2	wild type	C.F. Higgins[2]
CH223 LT2	*bio*$^-$	C.F. Higgins[2]
CH1026 LT2	P1*cml*	C.F. Higgins[2]
CH1305	*Tn*10 Tc^R	C.F. Higgins[2]
TT10423 LT7	*proAB* 47/F'128 *pro*$^+$ *lac*$^+$ zzf1231::*Tn*10 16 17 Tc^R	C.F. Higgins[2]
TT10427 LT2	pNK972 Amp^R	C.F. Higgins[2]
MR18	wild type	T.A. Roberts[3]
MR19		T.A. Roberts[3]
MR20		T.A. Roberts[3]
MR21		T.A. Roberts[3]

Tc^R - Tetracycline resistance
Amp^R - Ampicillin resistance
P1*cml* P1 phage carrying chloramphenicol resistance

(1) Salmonella Genetic Stock Centre, Dept. of Biology, University of Calgary, Alberta, Canada.
(2) Dept. of Biochemistry, University of Dundee, Scotland, U.K.
(3) A.F.R.C. Institute of Food Research, Bristol Laboratory, Langford, Bristol, U.K.
(4) Dept. of Molecular Biology, University of Edinburgh, Scotland, U.K.

Table 2.2 Strains of *Salmonella senftenberg* used

STRAIN	GENOTYPE	SOURCE
775W	wild type	NCTC[1]
JT577	wild type	NCTC[1]
SM001	St^R	775W
SM002	P1*cml*	775W
SM031		SM002

St^R - Streptomycin resistance.
P1*cml* P1 phage carrying chloramphenicol resistance

(1) National Collection of Type Cultures, Colindale London, U.K.

TABLE 2.3 Strains of *Escherichia coli* used

STRAIN	GENOTYPE	SOURCE
MM294	*pro*⁻, *usdR*⁻, *thi*⁻ *endoA*⁻, *hsdM*-	D. Lilley[1]
GM161	Amp	G.A. Reid[2]

Tc^R - Tetracycline resistance
Amp^R - Ampicillin resistance

(1) Dept. of Biochemistry, University of Dundee, Scotland, U.K.
(2) Dept of Microbiology. University of Edinburgh, U.K. Scotland, U.K.

Table 2.4 Plasmids used and their original hosts

PLASMID	HOST	SELECTABLE MARKER	SOURCE
pNK972	*S. typhimurium* TT10427	Tc^R	C.F. Higgins[1]
pUN121	*E. coli* GM161	Amp^R	G.A. Reid[2]

Tc^R - Tetracycline resistance

Amp^R - Ampicillin resistance

(1) Dept. of Biochemistry, University of Dundee.

(2) Dept. of Microbiology, University of Edinburgh.

(2) LC Medium

As LB medium but after autoclaving the following sterile solutions were added per litre:

Calcium Chloride (0.5M)	4.0 ml
Glucose (40%)	2.5 ml
Thymine (0.25%)	4.0 ml

(3) LC top agar

As LC medium except $6gl^{-1}$ of agar was added prior to autoclaving

(4) 50 x VB salt solution

Distilled Water	670 ml
$MgSO_4.7H_2O$	10 g
Citric acid.H_2O	100 g
K_2HPO_4	500 g
$NaHNH_4PO_4.H_2O$	175 g

Each salt was added in the order indicated and completely dissolved before adding the next.

(5) VB minimal media

50 x VB salts	20 ml
Water	980 ml

This solution was autoclaved and 10 ml of 40% sterile glucose added.

Media were solidified as required with 15 gl^{-1} Agar No. 1 (Oxoid). Sterilisation of media was by autoclaving at 15 psi for 15 minutes.

2.5 Solutions and buffers

2.5.1 DNA manipulation

(1) TE buffer, pH 7.4

Tris.HCl	(10 mM)
EDTA	(1 mM)

(2) TEG buffer, pH 8.0

Tris.HCl	(25 mM)
EDTA	(10 mM)
Glucose	(50 mM)

(3) 10 x DNA ligase buffer

Tris.HCl, pH 7.6	(660 mM)
$MgCl_2$	(50 mM)
Dithiothreitol	(1 mM)
ATP	(10 mM)

(4) Low-salt buffer

Tris. HCl, pH 7.5	(10 mM)
$MgCl_2$	(10 mM)
Dithiothreitol	(1 mM)

(5) Medium-salt buffer

Tris. HCl, pH 7.5	(10 mM)
$MgCl_2$	(10 mM)
Dithiothreitol	(1 mM)
NaCl	(50 mM)

(6) High-salt buffer

Tris. HCl, pH 7.5	(50 mM)
$MgCl_2$	(10 mM)
Dithiothreitol	(1 mM)
NaCl	(100 mM)

(7) 10 x TBE running buffer, pH 8.0

Tris base	(0.89 M)
Boric acid	(0.89 M)
EDTA	(20 mM)

(8) 10 x STOP loading buffer

Ficoll	(25%)
Bromophenol blue	(25%)

2.5.2 Polyacrylamide electrophoresis

(1) Acrylamide stock solution

Acrylamide	(30% w/v)
Bisacrylamide	(0.8% w/v)

(2) 4 x Stacking gel buffer

Tris.HCl, pH 8.8	(1.5 M)
SDS	(0.4% w/v)

pH was adjusted to 6.8 with approximately 30 ml of concentrated HCl

(3) 4 x Resolving buffer

Tris.HCl, pH 6.8	(0.5 M)
SDS	(0.4% w/v)

(4) 10 x Running buffer

Tris base	(0.25 M)
Glycine	(2.0 M)
SDS	(1% w/v)

(5) 2 x Sample buffer

4 x Stacking buffer	(25% v/v)
Glycerol	(20% v/v)
SDS	(3% v/v)
2-Mercaptoethanol	(3% v/v)
Bromophenol blue	(0.01% w/v)

(6) Staining solution

Methanol	(20% v/v)
Acetic acid	(7.5% v/v)
PAGE blue 83	(0.1% w/v)

(7) Destaining solution

As above but without the PAGE blue 83.

2.5.3 Western blotting

(1) Transfer buffer

Tris.HCl pH 8.3	(250 mM)
Glycine	(1.5 M)

(2) TBS

Tris.HCl, pH 7.5	(10 mM)
NaCl	(150 mM)

2.5.4 Phosphate buffer, 0.067 M, pH 7.0

	gl^{-1}
KH_2PO_4	4.56
$NH_2HPO_4.2H_2O$	4.73

2.5.5 STEP buffer

SDS	(0.5% w/v)
Tris.HCl, pH 7.5	(50 mM)
EDTA	(0.4 M)

2.5.6 Lowry protein determination, solutions

(1) Solution A

Na_2CO_3	(2% w/v)
Sodium tartrate	(1.6% w/v)
NaOH	(0.4% w/v)
SDS	(1% w/v)

(2) Solution B

$CuSO_4$	4 g
Solution A	100 ml

2.6 Chemicals

Sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), Tris(hydroxymethyl)aminomethane (Tris base), acrylamide and bisacrylamide (electrophoretic grade) and routinely-used chemicals were purchased from BDH Chemicals Ltd., England. Ficoll, agarose (type 1-low EEO), ethidium bromide, bovine serum albumin (BSA) were purchased from Sigma Chemical Co., London. Dithiothreitol (DTT), ultra pure phenol and N,N,N'-tetramethylethylenediamine (Temed) were purchased from BRL Bethesda Research Laboratories (UK) Ltd., Cambridge, England. Dimethyl sulphoxide was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England. *Hin*d III restricted lambda DNA was purchased from New England Biolabs, Bishop Stratford, England.

2.7 Antibiotics and nutrient supplements

All antibiotics and nutrient supplements used in this work were purchased from Sigma. Sterilisation was by filters, pore size 0.45 μm, if necessary.

2.8 Enzymes

T_4 DNA ligase was purchased from Gibco BRL Bethesda Research Laboratories. Pancreatic RNase, lysozyme and Proteinase K (fungal) were purchased from Sigma. Restriction endonucleases were purchased from several sources:- New England Biolabs, Bishop Stratford, England; Boehringer Mannheim, Miles Laboratories, Stoke Poges, England; Gibco BRL Bethesda Research Laboratories; NBL Enzymes Ltd., Cramlington, England. All restriction

endonucleases were used in accordance with the manufacturers' instructions and buffers where supplied.

2.9 Antibodies

Antisera to the GroEL heat shock protein was a gift from Dr Ian Marsh, University of Edinburgh. It was stored at -20°C until required.

2.10 Measurement of bacterial growth

Growth rates were determined by following changes in the turbidity of the cultures, measured in a spectrophotometer at an optical density of 600 nm (OD_{600}). For the measurement of cell density prior to heat inactivation a nephalometer (Evans Electroselenium Ltd., Halstead) with orange filter was used.

2.11 Heat treatment

2.11.1 Heat inactivation

A loopful of surface growth, of the strain under investigation on an LB agar plate, was added to 5 ml of LB in a 20 ml Universal bottle and incubated for 18 h at 37°C in a shaking incubator. A volume of the stationary phase cells was inoculated in to 50 ml of fresh LB in a 250 ml conical sidearm flask so as to give a nephalometer reading of 5. The cells were incubated at 37°C in a shaking incubator and the cell density measured at intervals until a nephalometer reading of 25 was obtained. Twenty ml of the cells was then aseptically transferred to a 1 l conical flask containing 180 ml of fresh LB. This had been submerged up to the neck in a shaking water bath, which had been allowed to equilibrate to the temperature of the water bath. Aliquots were then removed at set time intervals, starting at time zero, and serial dilutions performed in fresh LB prior to viable

counting. Viable counts were made on LB agar plates after incubation for 18-24 h at 37°C.

2.11.2 Standard heat inactivation

A loopful of surface growth, of the strain under investigation on an LB agar plate, was added to 5 ml of LB in a 20 ml Universal bottle and incubated for 18 h at 37°C in a shaking incubator. A volume of the stationary phase cells was inoculated in to 50 ml of fresh LB in a 250 ml conical flask so as to give an (OD_{600}) of 0.003. The cells were incubated at 37°C in a shaking incubator and the cell density measured at intervals until an OD_{600} reading of 0.1 was obtained. Twenty ml of the cells was then aseptically transferred to a 1 l conical flask containing 180 ml of fresh LB. This had been submerged up to the neck in a shaking water bath, which had been allowed to equilibrate to the temperature of the water bath. The cells were then thermally inactivated so as to give a three logarithmic reduction in the survival.

2.11.3 Replica-plate heat inactivation

A stationary phase culture of the cells under investigation was diluted so as to give approximately 100 colonies when 0.1 ml was spread on an LB agar plate after incubation at 37°C for 18 h. The plates were then replica-plated using sterile velveteen cloths and replica-plating block on to fresh LB agar plates. The original plate was kept as a reference while the replicate was placed in a high temperature incubator at 55°C with the door closed. Five minutes was allowed for re-heating and stabilization before a watch was started. Once the correct period of time had elapsed the plates were removed and placed on a wooden bench to cool to room temperature. These plates were incubated at 37°C for 18 h to determine the number of survivors. The incubator

was allowed to equilibrate for 30 min before it was used again.

2.11.4 Replica-plate heat inactivation (using tooth picks)

Two LB agar plates were placed over grids which divided each plate up in to 100 numbered squares. A sterile tooth pick was used to transfer part of a colony on to each of the plates in the same numbered square. This was repeated until all the squares were filled.

Both sets of plates were incubated at 37°C for 18-24 h. One plate was kept as the reference, while the other was replica-plated using a sterile velveteen block and replica-plating block on to a fresh LB agar plate. The replicate was placed in a high temperature incubator at 55°C and the door closed. Five minutes was allowed for re-heating and stabilization before a watch was started. Once the correct period of time had elapsed the plate was removed and placed on a wooden bench to cool to room temperature. These plates were incubated at 37°C for 18 h to determine the number of survivors. The incubator was allowed to equilibrate for 30 min before it was used again.

2.11.5 Heat induced thermotolerance

The strain to be heat induced was grown to a cell density, as measured by a spectrophotometer, of 0.1 as described in section 2.11.2. Three ml of the culture was inoculated in to 27 ml of LB in a 250 ml conical flask which was in a water bath, and had been set at either 42°, 45°, or 48°C. The cells were incubated at the elevated temperature for 10 h and aliquots were taken at 0, 15, 30, 45, 60 min intervals and at every hour after that up to 10 h. The cell density of these aliquots was adjusted to a spectrophotometer reading of 0.1, prior to

heat inactivation at 55°C, (2.11.1). Viable counts were performed at time zero and after 5 min on LB agar plates. Counts were made after incubation for 18-24 h at 37°C.

2.11.6 Thermal conditioning

A loopful of surface growth, of the strain under investigation on an LB agar plate, was added to 5 ml of LB in a 20 ml Universal bottle and incubated for 18 h at 37°C in a shaking incubator. A volume of the stationary phase cells was inoculated in to 50 ml of fresh LB in a 250 ml conical flask so as to give an OD_{600} of 0.003. The cells were incubated at 37°C in a shaking incubator and the cell density measured at intervals until an OD_{600} reading of 0.1 was obtained. Twenty ml of the cells was then aseptically transferred to a 1 l conical flask containing 180 ml of fresh LB. This was submerged up to the neck in a shaking water bath, which had been allowed to equilibrated to the temperature of the water bath. The cells were then thermally inactivated so as to give a three logarithmic reduction in survival.

From the surviving cells, 0.1 ml was transferred in to 5 ml of fresh LB and incubated for 18 h. The method was repeated using the cultured cells for as many cycles as was required. After each cycle, 1 ml of the survivors was taken and 20% (v/v) sterile glycerol added and frozen down at -70°C to be kept as a permanent.

2.12 Gamma irradiation

Overnight cultures were diluted, 1 ml of cells to 20 ml of LB and grown to a turbidity of 30 to 40 (approximately $1x10^{8}$ viable units ml^{-1} in exponential phase). Samples of the cultures were centrifuged at 4000 rpm for 10 min and then washed and resuspended in the same volume of phosphate buffer. The cells were then irradiated (using

a cobalt-60 source at 10.6 Gray min^{-1}) in 3 ml aliquots with oxygen bubbling through.

2.13 Transposon mutagenesis

2.13.1 Making a P22 bacteriophage lysate of *S.typhimurium* TT10423

A loopful of surface growth, from *S.typhimurium* strain TT10423 on an LB agar plate, was added to 5 ml of LB in a 20 ml Universal bottle and incubated for 1-2 h at 37°C in a shaking incubator. To this was added 3 l of a P22 phage suspension (8.5 x 10^9 pfu ml^{-1}) and the culture re-incubated at 37°C for 3-4 h. The culture took on a stringy quality and was spun down in a MSE bench centrifuge at 3000 rpm for 15 min. The supernatant was taken off with a sterile glass pasteur pipette and stored over 0.5 ml of chloroform at 4°C.

2.13.2 Transposon mutagenesis of *S.typhimurium* TT10427

A loopful of surface growth, from *S.typhimurium* strain TT10427 on an LB ampicillin (20 μg ml^{-1}) agar plate, was added to 5 ml of LB ampicillin (20 μg ml^{-1}) in a 20 ml Universal bottle and incubated for 1-2 h at 37°C in a shaking incubator. On to five LB tetracycline (12.5μg ml^{-1}) agar plates, 0.1 ml of the 18 h culture of *S.typhimurium* TT10427 was spread. One hundred microlitres of the P22 lysate from 2.13.1 was spread on to these five plates and on to a sixth LB tetracycline (12.5 μg ml^{-1}) agar plate was spread 0.1 ml of the P22 lysate, as a control. The plates were incubated at 37°C for 24 h and the colonies that grew up were pooled and centrifuged in a MSE bench centrifuge at 3000 rpm for 15 min. The supernatant was poured off and the pellet washed several times in fresh LB before storage at 4°C.

2.13.3 Transduction of *Tn*10 from *S.typhimurium* TT10427 in to *S.typhimurium* SA2009

Using the method described in section 2.13.1, a P22 lysate of the pooled *S.typhimurium* TT10427 from section 2.13.2 was made. One hundred microlitres of an 18 h culture of *S.typhimurium* SA2009 was spread on to each of five LB tetracycline (12.5 μg ml^{-1}) agar plates. One hundred microlitres of the P22 lysate of the pooled *S.typhimurium* TT10427 was spread on to these five plates and on to a sixth LB tetracycline (12.5μg ml^{-1}) agar plate was spread 0.1 ml of the P22 lysate as a control. The plates were incubated at 37°C for 24 h and the colonies that grew up were pooled and centrifuged in a MSE bench centrifuge at 3000 rpm for 15 min. The supernatant was poured off and the pellet washed several times in fresh LB before storage at 4°C.

2.13.4 Producing a P1 lysate

A loopful of surface growth, from a P1 lysogenic bacterium on a LB agar plate was added to 5 ml of LC in a 20 ml Universal bottle and incubated for 18 h at 30°C in a shaking incubator. From this culture 50μl was added to 10 ml of fresh LC in a Universal bottle and incubated until the cells had entered early logarithmic phase, this corresponded to an OD_{600} of 0.02 and took 1-2 h. The culture was then transferred to a water bath at 45°C for 2-3 min, before vigourous shaking for 1-2 h at 37°C in an orbital shaker. When lysis was complete, 0.1 ml of chloroform was added and the Universal bottle vortexed for 15-20 sec before centrifugation at 4500 rpm for 10 min. The supernatant was then carefully transferred to a sterile screw capped bottle and 0.1 ml of chloroform added before storage at 4°C.

It was important to ensure that the culture was not overgrown before induction, otherwise a poor phage yield

was obtained. If the cells did not lyse after 3-4 h, chloroform was added to lyse the cells, and the supernatant taken off as useable lysate could be obtained.

2.14 Isolation of plasmid DNA from strains of *S.typhimurium* and *E.coli*

Small and large scale isolation of DNA from transformed *S. typhimurium* and *E. coli* were performed essentially as described by Birnboim & Doly (1979). CsCl/ethidium bromide buoyant density gradients were carried out according to Maniatis *et al.* (1982).

2.14.1 Small scale preparation of plasmid DNA (mini-preps)

Cells were grown overnight in 5 ml of LB containing an appropriate selective antibiotic. Cells were harvested by centrifugation at 8000 rpm for 5 min in a Sorvall SS34 rotor at room temperature and allowed to drain upside down for 2 min. The pellet was resuspended in 0.1 ml TEG and transferred to a 1.5 ml microcentrifuge tube. Cells were lysed by the addition of 0.2 ml of (0.2 M NaOH, 1% (w/v) SDS), vortexed and left on ice for 5 min. Chromosomal DNA and the majority of the cellular protein was precipitated by the addition of 0.15 ml of 3 M sodium acetate, pH 5.0. This was left on ice for 5 min prior to centrifugation at 4°C for 10 min in a MSE microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube ensuring that none of the white precipitate was transferred. The plasmid DNA was extracted with 0.5 ml of phenol:chloroform (1:1), followed by extraction with 0.5 ml of chloroform, see 2.16. The DNA was precipitated by 0.9 ml ethanol and left for 5 min at room temperature. The DNA was pelleted by centrifugation at 4°C for 10 min, the supernatant was removed and the tube inverted to drain for 5 min. The

pellet was dried under vacuum before resuspending in 50μl TE.

2.14.2 Large scale preparation of plasmid DNA

Cells were grown in 1 l of LB plus the relevant selective antibiotic. Chloramphenicol amplification was optional and if used, the cells were grown to an OD_{600} of 0.1. Chloramphenicol, 150 μg ml^{-1} final concentration, was added and the culture shaken overnight at 37°C. Cells were harvested at 8000 rpm in a Sorvall SA600 rotor at room temperature and resuspended in 9 ml TEG. The cell suspension was transferred to two 50 ml Sorvall tubes and 0.5 ml of fresh lysozyme 15 mg ml^{-1} (in TEG) was added to each tube. Tubes were vortexed and left to stand on ice for 15 min. To each tube 15 ml (0.2 M NaOH, 1% (w/v) SDS) was added and vortexed. After a further 5 min incubation on ice, 11.25 ml 3 M sodium acetate was added, mixed by inversion 2-3 times and left on ice for 45 min before centrifugation at 16000 rpm for 40 min in a Sorvall SS34 rotor at 4°C. The supernatant was transferred to a 300 ml Sorvall tube and the DNA precipitated with 45 ml isopropanol for 5 min at room temperature and spun at 1000 rpm in a Sorvall SA600 rotor at room temperature for 20 min.

The supernatant was discarded and the tube inverted to drain for 5 min before the pellet was resuspended in 5.4 ml TE and transferred to a sterile 15 ml corex tube. To this was added 5.85 g CsCl which was dissolved and left at 4°C for 1 h to precipitate RNA. This precipitate was removed by centrifugation at 9000 rpm in a Sorvall SS34 rotor at 4°C for 30 min. The supernatant was transferred to a 10 ml polyallomer ultracentrifuge tube containing 0.6 ml ethidium bromide (10 mg ml^{-1}) and mixed. The density of the final solution was adjusted to 1.58-1.60 g ml^{-1} and a CsCl/ethidium bromide buoyant density gradient was formed by centrifuging at 35000 rpm in a Sorvall

OTD65 ultracentrifuge (r89.5 rotor) for 24-48 h at 20°C. After centrifuging, plasmid DNA was visualised on the gradient using U.V. light and carefully removed, using a 2 ml syringe with a 19 gauge needle to puncture the tube wall, into a clean silanized 30 ml corex tube. The ethidium bromide was extracted by shaking 3-4 times with an equal volume of isopropanol saturated with NaCl and water, until the top (aqueous) layer was clear. The DNA was then diluted with 8 ml TE and precipitated with 8 ml isopropanol for 1 hour at -20°C before being spun down at 8000 rpm in a Sorvall SS34 rotor at 4°C for 20 min. The pellet was dried and resuspended in 0.4 ml TE and phenol/chloroform extracted once before the DNA was ethanol precipitated. Finally the DNA pellet was vacuum dried and resuspended in 0.2 ml TE before storage at 4°C

2.15 Isolation of chromosomal DNA from strains of *E.coli*, *S.typhimurium* and *S.senftenberg*

Cells were grown in 10 ml of LB overnight and harvested at 8000 rpm for 5 min at room temperature in a Sorvall SS34 rotor. The pellet was resuspended in 0.5 ml (50 mM Tris.HCl, pH 8.0, 50mM EDTA) and frozen at -20°C. Fresh lysozyme was added, 50μl of a 10 mg ml^{-1} solution made up in 0.25 M Tris.HCl pH, 8.0, to the frozen cells and thawed in a room temperature water bath. When just thawed the cells were put on ice for 45 min and then freshly made proteinase K, at a final concentration of 1 mg ml^{-1} in STEP, was added and mixed well before incubating in a 50°C water bath for 1 h with occasional mixing. After cooling, 0.6 ml Tris.HCl buffered phenol was added and mixed gently for 5 min to form an emulsion before centrifugation at 2000 rpm at room temperature for 15 min in a Sorvall SS34 rotor. The upper aqueous layer was transferred to a clean 5 ml corex tube and the phenol step repeated until the aqueous layer was uncontaminated. This layer was then transferred to a clean tube containing 0.1 volume 3 M sodium acetate and mixed by

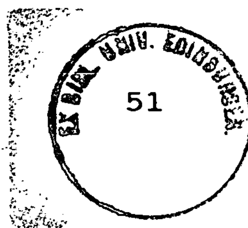
inversion before the addition of 2 volumes ethanol. The DNA was precipitated with gentle mixing, spooled off using a glass rod and dissolved in 0.2 ml TE. Once dissolved, 200 $\mu g\ ml^{-1}$ final concentration of RNase, was added and incubated at 37°C for 30 min. An equal volume of chloroform was added and mixed by inversion to form an emulsion and centrifuged at 2000 rpm at room temperature for 15 min in a Sorvall SS34 rotor. The upper aqueous phase was transferred to a 1.5 ml microcentrifuge tube containing 0.1 volume 3 M sodium acetate and mixed by inversion before adding 2 volumes ethanol. The DNA precipitated with inversion was spooled off and dissolved in 0.2 ml (50 mM Tris, 1 mM EDTA) and stored at 4°C

2.16 Phenol/chloroform extraction of DNA

Extractions were performed routinely on DNA samples both during isolation of plasmid DNA from *E. coli* and *S. typhimurium* and other DNA manipulation procedures. The DNA sample was diluted to at least 0.2 ml as required and the salt concentration adjusted to 0.3 M with 3 M sodium acetate. An equal volume of phenol:chloroform (1:1) was added and vortexed for 10 seconds to give an emulsion before centrifugation in a MSE microcentrifuge for 5 min. The upper aqueous layer was transferred to a 1.5 ml microcentrifuge tube and an equal volume of chloroform added before vortexing for 10 sec. Microcentrifugation was repeated for a few seconds and the aqueous phase transferred to a 1.5 ml microcentrifuge tube containing 2 volumes of ethanol (-20°C), and the DNA precipitated with inversion. The DNA was pelleted in a microcentrifuge at 4°C for 15 min. The pellet was then drained, dried under vacuum and resuspended in an appropriate volume of TE.

2.17 Recombinant DNA manipulation

Procedures were followed essentially as laid down in Maniatis *et al.* (1982).


EX BIBL. UNIV. EDINBURGH

2.17.1 Restriction digestion of DNA

Restriction endonucleases were used according to manufacturers instructions and with manufacturers buffer (where provided). Digestions for analytical purposes, typically contained 0.5-2μg of DNA in a total volume of 20μl; 1-3 units of enzyme; 0.1 volume of enzyme buffer; 2μg BSA and 4μg RNase (for mini-prep digests) and were carried out at the appropriate temperature for 1-24 h. Checking for complete digestion was carried out by agarose gel electrophoresis, (linear DNA moving slower than covalently-closed-circular [ccc] plasmid DNA on 0.8% gels).

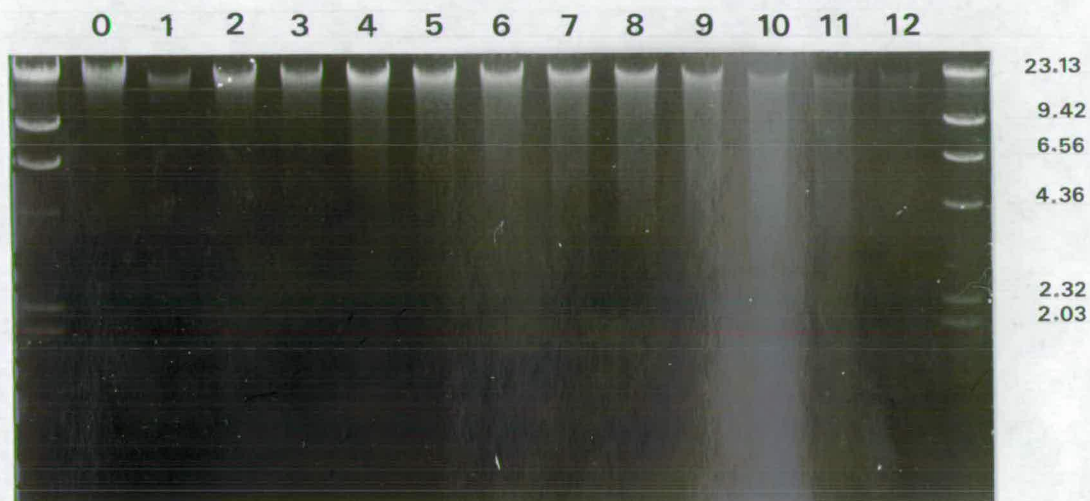
Partial chromosomal digests were carried out using a much higher DNA concentration of approximately 50μg per 100μl reaction mixture, and samples were taken at increasing time intervals from commencement, stopping the reaction with 0.1 volume of STOP loading buffer and leaving the samples on ice until they had all been collected. Samples were run on a gel (*Figure* 2.1) and the time exposure was chosen that gave the desired size range.

2.17.2 Electrophoresis of DNA

Restriction digests were assessed by electrophoresis on agarose gels (0.7-1.0% w/v) run in TBE at a constant current of 50 mA. DNA was visualised under ultra violet illumination using a transilluminator (Fotodyne Inc, New Berlin, Wisconsin, USA) in the presence of ethidium bromide. Electroelution of DNA fragments from agarose gels was achieved by excising the desired band of DNA with a scalpel and eluting the fragment using a Biotrap (Schliecher Schuell Ltd).

Figure 2.1 Agarose gel electrophoresis of the products of a partial digest of *S. senftenberg* 775W DNA with *Sau*3A

Photograph showing partial digestion products on an 0.8% agarose gel, with *Hin*dIII molecular weight markers on the left and right (sizes shown in kb). Samples were taken after, left to right, 0 to 12 minutes from a reaction mixture containing 1.25 units of *Sau*3A per 100μl per 50 μg of DNA



2.17.3 Ligation of DNA fragments

Ligations were performed in volumes of between 10-50μl, depending on fragment concentration, and in 0.1 volume of 10 x ligation buffer. 1 unit of T_4 ligase was used for each reaction and ligations were carried out for 5-12 h at 15°C. A 3:1 molar ratio of fragment:vector was observed.

2.17.4 Determination of DNA concentration

Two methods were employed to determine DNA concentrations, the Pye Unicam 596-500 U.V. spectrophotometer or by comparison with a known concentration of lambda standard on an agarose gel.

The U.V. spectrophotometric method involved adding a known volume of the DNA sample (2 to 15 μl) to 1 ml of 0.2 M NaOH in a silica cuvette and taking absorbance readings at 260 nm, (the assumption that 50μg ml^{-1} of DNA gave a reading of 1 was made). More often the absorbance was measured at 260 and 280 nm, and using a nomograph, the DNA concentration could be calculated more accurately and it also gave an indication of the protein content.

The second method involved running several wells of known volumes of the sample (usually 1 to 5 μl) along side a known concentration of lambda *Hin*d III digest (2 to 5 μl, depending on the gel, at 0.2 $\mu g \, \mu l^{-1}$). After ethidium bromide staining and visualisation under U.V., the DNA concentration was estimated by direct comparison with the lambda 23 kb fragment, which is equivalent to approximately half of the total lambda fragments. Of the two methods, the spectrophotometric method is the most accurate but it requires large amounts of DNA.

2.17.5 Sizing DNA molecules and fragments from agarose gels

Sizing of DNA molecules and fragments was done by comparison with the restriction patterns produced by *Hind* III restricted bacteriophage lambda standard. This produced fragments of 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, and 0.56 kb in size. Although not as accurate as the equation described by Southern (1979), the graphical method, where the migration rates of DNA molecules is inversely proportional to the logarithm of the molecular weight, it was faster and easier where accuracy was not so important.

2.17.6 Sucrose gradient size fractionation of *Sau*3A chromosomal partial digest

Two sucrose solutions, 10% and 40% were made up in (1 M NaCl, 20 mM Tris.HCl pH 8.0, 5 mM EDTA) and autoclaved to remove nuclease activity. A linear gradient of the two solutions was constructed in two 10 ml ultra centrifuge tubes, using a gradient maker and peristaltic pump (flow rate 2 ml/min). The gradient was added in a smooth flow using a sealed glass Pasteur pipette (*Figure* 2.2). To each tube was added 150μg of digested chromosomal DNA in 0.2 ml. The tubes were balanced and spun at 26000 rpm in a MSE superspin (swingout rotor) at 15°C for 24 h with the vacuum on and the break off. The gradient was then taken off in 0.5 ml fractions into numbered microcentrifuge tubes.

A range of samples were visualised on a 0.8% agarose gel to determine the fragment size (*Figure* 2.3). Those samples that were required had the sucrose removed. Two volumes of distilled water were added to the required fractions. To this, 0.1 times the new volume of 3 M sodium acetate pH 5.6 and two times the new volume of ethanol was added to precipitate the DNA. This was mixed

Figure 2.2 Apparatus for loading a sucrose gradient in to centrifuge tubes

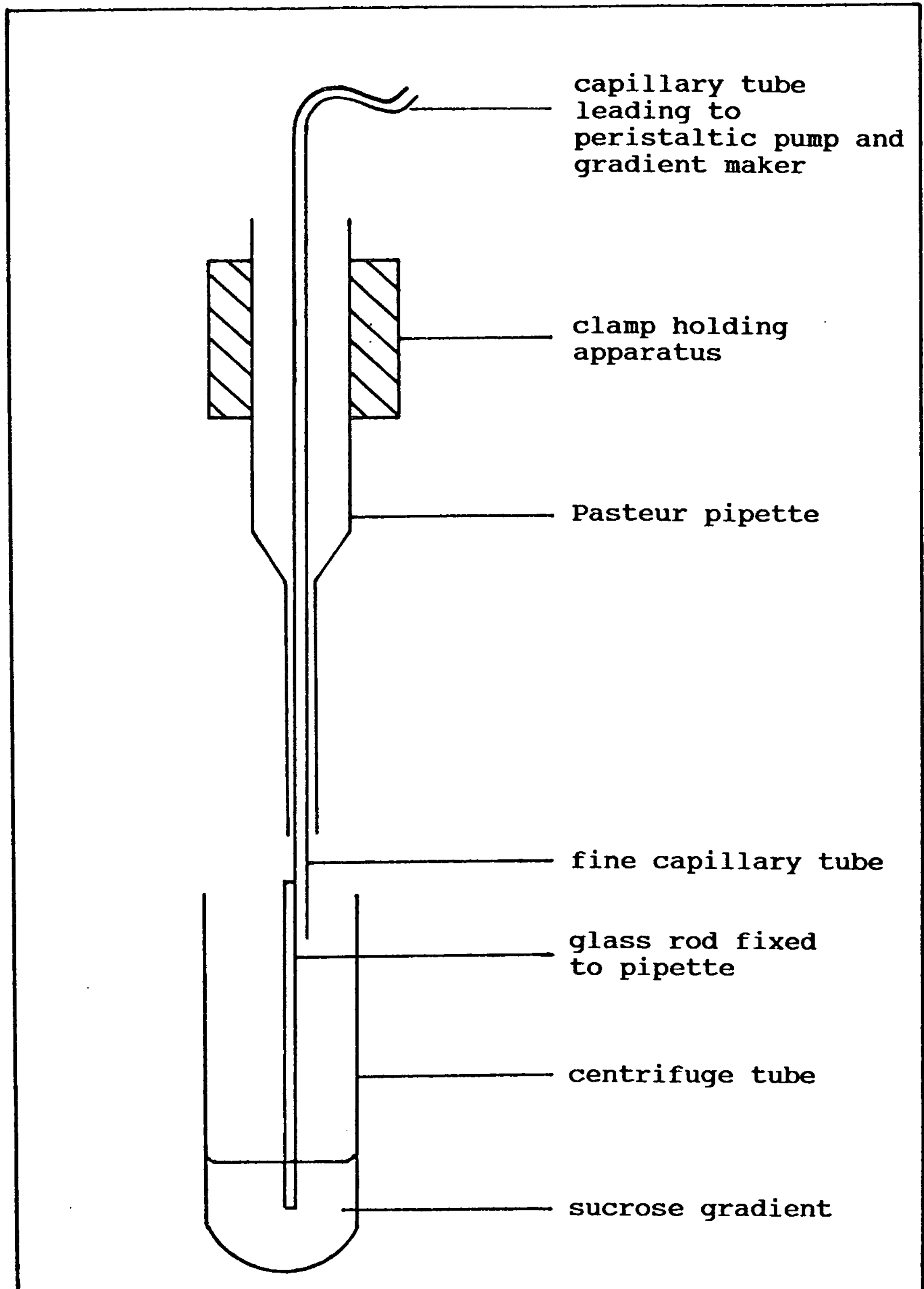
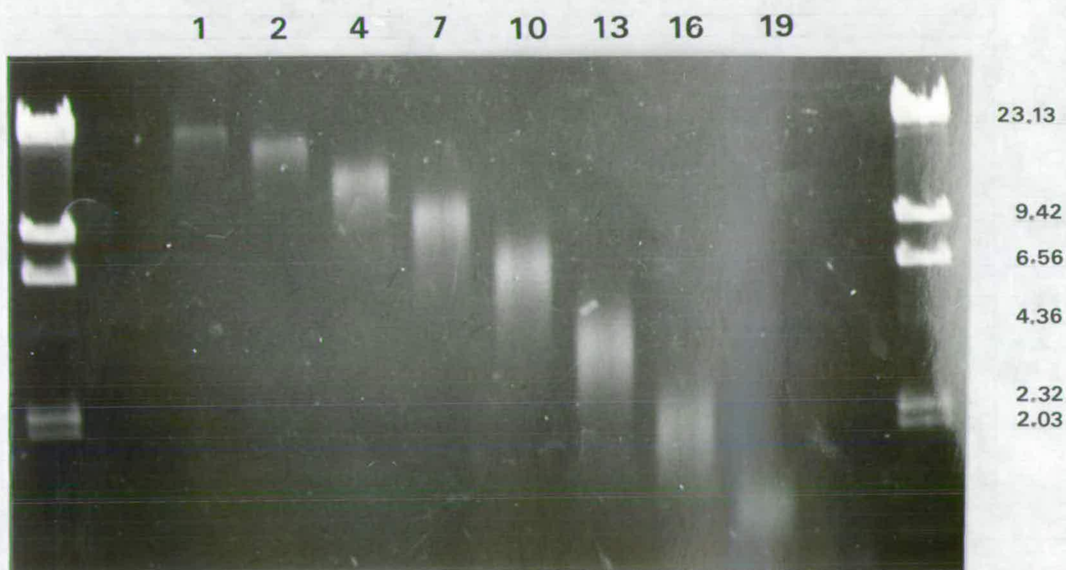


Figure 2.3 Agarose gel electrophoresis of the products of a partial digest of *S. senftenberg* 775W DNA with *Sau*3A taken from fractions of a sucrose gradient

Photograph showing products of sucrose gradient size fractionated *Sau*3A partially digested *S. senftenberg* 775W DNA on an 0.8% agarose gel, with *Hin*dIII wolecular weight markers on left and right (sizes in kb). Samples run were taken from fractions 1,2,4, 7, 10, 13, 16, 19 (left to right), with fraction 1 being taken from the bottom of the ultra centrifuge tube and fraction 19 from the top.



thoroughly and put in a dry ice/ethanol bath for 5 min. They were then centrifuged at 18000 rpm, 4°C for 30 min, the supernatant poured off and any excess dabbed off the rim with a tissue. The DNA was washed with 70% (v/v) ethanol and respun at 18000 rpm at 4°C. The ethanol was discarded and the pellet dried under vacuum prior to resuspending in an appropriate volume of TE.

2.18 Transformation of *S.typhimurium* and *E.coli*

Performed essentially as described by Mandel & Higa (1970).

2.18.1 Preparation of competent cells

A culture of the appropriate strain was grown to an OD_{600} of 0.3 in LB. Cells were then harvested at 8000 rpm at 4°C for 5 min in a SS34 rotor and resuspended in 0.5 volumes of ice-cold 0.1 M $CaCl_2$. After 30 min on ice, the cells were spun down and resuspended in 0.1 volume of ice-cold 0.1 M $CaCl_2$, the cells were now competent and could be stored for future use at -70°C by the addition of 20% (v/v) sterile glycerol.

2.18.2 Transformation procedure

Plasmid DNA or ligation mixture (1-100 ng) was suspended in 0.1 ml of TMC (10 mM Tris.HCl pH 7.0, 10 mM $MgCl_2$, 10 mM $CaCl_2$) and added to 0.2 ml of competent cells and incubated on ice for 30 min. The mixture was then heat shocked at 42°C for 2 min and 1 ml of LB added before incubating at 37°C for 1-3 hours. The cells were then pelleted in a microcentrifuge and resuspended in 0.1 ml of LB before plating out on LB agar plates containing an appropriate antibiotic and incubated overnight at 37°C.

2.19 Separation of proteins by SDS-polyacrylamide gel electrophoresis

The procedure used was essentially that described by Laemmli (1970), with some modifications.

All glass plates (10 cm x 10 cm) and spacers were wiped with ethanol prior to assembly, and once clamped together were sealed with Vaseline.

2.19.1 Preparation of separating gel (12.5%)

Acrylamide stock solution 12.5 ml, distilled water 10 ml and 4 x resolving buffer were mixed in a 100 ml beaker. To this was added 0.2 ml APS (10% w/v) and 40μl TEMED. The mixture was quickly mixed and poured between the glass plates to within 3-4 cm of the top of the plates. The gel was then overlaid with 2 ml of water-saturated n-butanol and allowed to polymerise. After polymerisation the n-butanol was poured off and the gel surface rinsed several times with water and allowed to drain.

2.19.2 Preparation of stacking gel (4.8%)

Acrylamide stock solution 2.6 ml, distilled water 9 ml and 4 x stacking buffer 4 ml were mixed in a 50 ml beaker. To this was added 0.1 ml APS (10% w/v) and 80μl TEMED and quickly mixed and poured between the plates on top of the resolving gel to the lip of the plates. A perspex comb was inserted in to the stacking gel, ensuring no bubbles were trapped in the teeth of the comb.

2.19.3 Running the gel

When the stacking gel had polymerised, the comb and the bottom spacer were removed. The gel was then clamped to a slab gel electrophoresis tank and 1 x running buffer

added to both reservoirs ensuring the wells and bottom of the plates were submerged. Any residual acrylamide was removed from the wells and any air bubbles occupying the space between the bottom plates ejected using a syringe. Samples to be loaded were first suspended in an appropriate volume of 2 x sample buffer and boiled for 3 min. Electrophoresis was carried out at a constant current of 30 mA until the bromophenol blue had just reached the bottom of the gel. Gels were stained in PAGE blue staining solution overnight and then transferred to destaining solution, which was changed frequently, until necessary.

2.20 Western blotting

SDS-polyacrylamide gels were run as described in section 2.19.3 but after electrophoresis, gels were not stained. A Hybond-Nylon membrane was cut to fit the size of gel and two sheets of Whatman 3 MM filter paper similarly cut. The nylon membrane and filter paper were soaked in 1 x transfer buffer for at least 5 min along with two Scotchbrite pads and a sandwich assembled as follows, ensuring no air bubbles formed between the layers;

Plastic holder (top +ve)
Scotchbrite pad
Filter paper
Hybond-Nylon membrane
Gel
Filter paper
Scotchbrite pad
Plastic holder (bottom -ve)

The sandwich was placed in an LKB 2005 TRANSPHOR electroblotting unit filled with 5 litres of 1 x transfer buffer ensuring all wires were covered and the gel was nearest to the -ve terminal. Proteins were transferred for 1-2 hours at 0.6-2.0 Amps or overnight in the cold

room at 0.3-0.5 Amps. After transfer, the sandwich was removed from the tank and the nylon membrane placed in 100 ml 20% (w/v) skimmed milk powder made up in TBS and gently shaken overnight to block unbound sites

2.20.1 Antibody detection

Proteins were detected essentially as described by Glass *et al.*, (1981) modified by Johnson *et al.*, (1984). The blocked membrane was placed in 50 ml 5% milk solution made up in TBS. To this was added 10-30μl of antisera and the membrane was gently shaken at room temperature for 5 hours. The milk solution was tipped off and the membrane washed for 4 x 5 min in 100 ml TBS. A 50 ml 5% milk solution containing 10μl of Horse Radish Peroxidase-conjugated antirabbit IgG (Biorad) was added and the membrane incubated as before for a further 2 hours. The membrane was washed as before and the following solution made up and added to the membrane;

0.5 ml dianisidine (5 mg ml^{-1})
1.0 ml imidazole (0.1 M, pH 7.4)
0.1 ml 30% H_2O_2
8.4 ml distilled H_2O

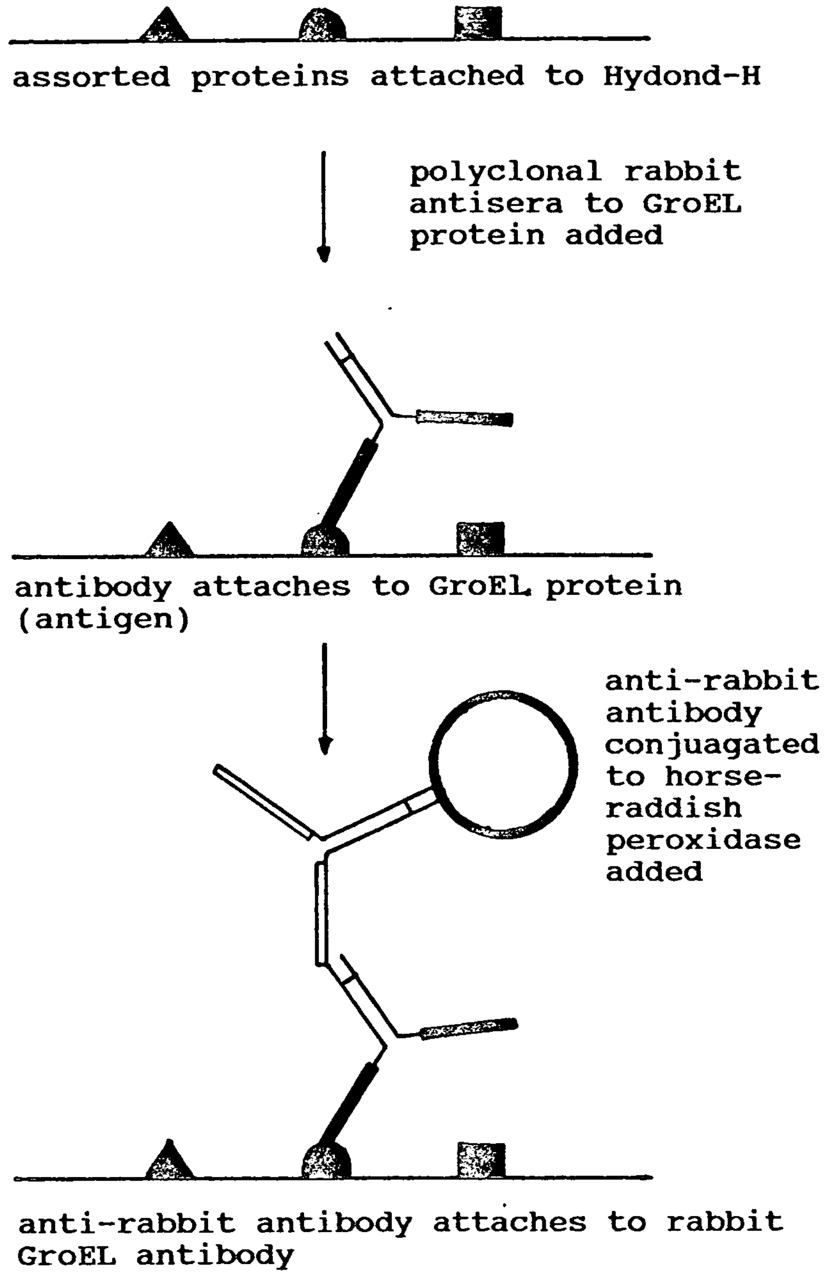
The membrane was gently shaken until a brown colour developed and the reaction stopped by washing the membrane in distilled water (*Figure* 2.4).

2.21 Determination of protein concentration

The method of Lowry (1951), was used.

Protein samples were diluted to a volume of 1 ml in H_2O as necessary, mixed with 3 ml solution B and left for 30 min. To this was added 1ml of a 1:1 dilution of Folin reagent made up in H_2O, mixed and left for a further 30 min. The absorbance at 660 nm was measured for each

Figure 2.4 Visualisation of proteins on Western blots



sample against a blank of the above mixture but without the protein sample.

A series of standard concentrations in the range 0-150μg ml^{-1} of BSA was prepared each time an assay was performed and a standard curve of absorbance at 660 nm versus protein concentration prepared. The protein concentration in each unknown sample could then be determined from this curve.

CHAPTER THREE

RESULTS

3.1 Introductory Work

The survival of *S. typhimurium* strain SA2009 at elevated temperatures in liquid media was assayed. Cells were grown to mid-logarithmic phase at 37°C and then heat inactivated as described in section 2.11.1. Initially experiments were carried out in conditions where the bacteria under test were added to small volumes of pre-heated Luria Broth (9 ml) and *Figure* 3.1 shows the survival curves from three separate thermal inactivation experiments at 55°C. Although all three thermal survival experiments were performed on cells at the same concentration, determined by nephalometer readings, the survival curves were different. One curve shows an initial shoulder and a tail, another has a reduced shoulder and no tail, while the third has two shoulders. Similar experiments were carried out at lower and higher inactivation temperatures but they also did not give reproducible survival curves (data not shown here). It was assumed that these variations and non-linear curves were due to fluctuations in the temperature of the cell suspension during thermal inactivation. Indeed Gould (personal communication, 1987) suggested that adiabatic changes resulting from using small inactivation volumes was sufficient to cause shoulders and large variations in survival curves. It was therefore decided to increase the volume of pre-heated Luria Broth to 180 ml with a corresponding increase in the volume of mid-logarithmic phase cells. *Figure* 3.2 shows the thermal survival curves of two different sets of mid-logarithmic phase cells at 55°C using 180 ml of LB. Conditions were closely monitored each time the thermal inactivations were performed, but *Figure* 3.2 still shows some variation between the two curves. For further work to be productive it was important to resolve the problem of variation in thermal survival curves.

Figure 3.1 Survival of *S. typhimurium* SA2009 in 20 ml of LB at 55°C

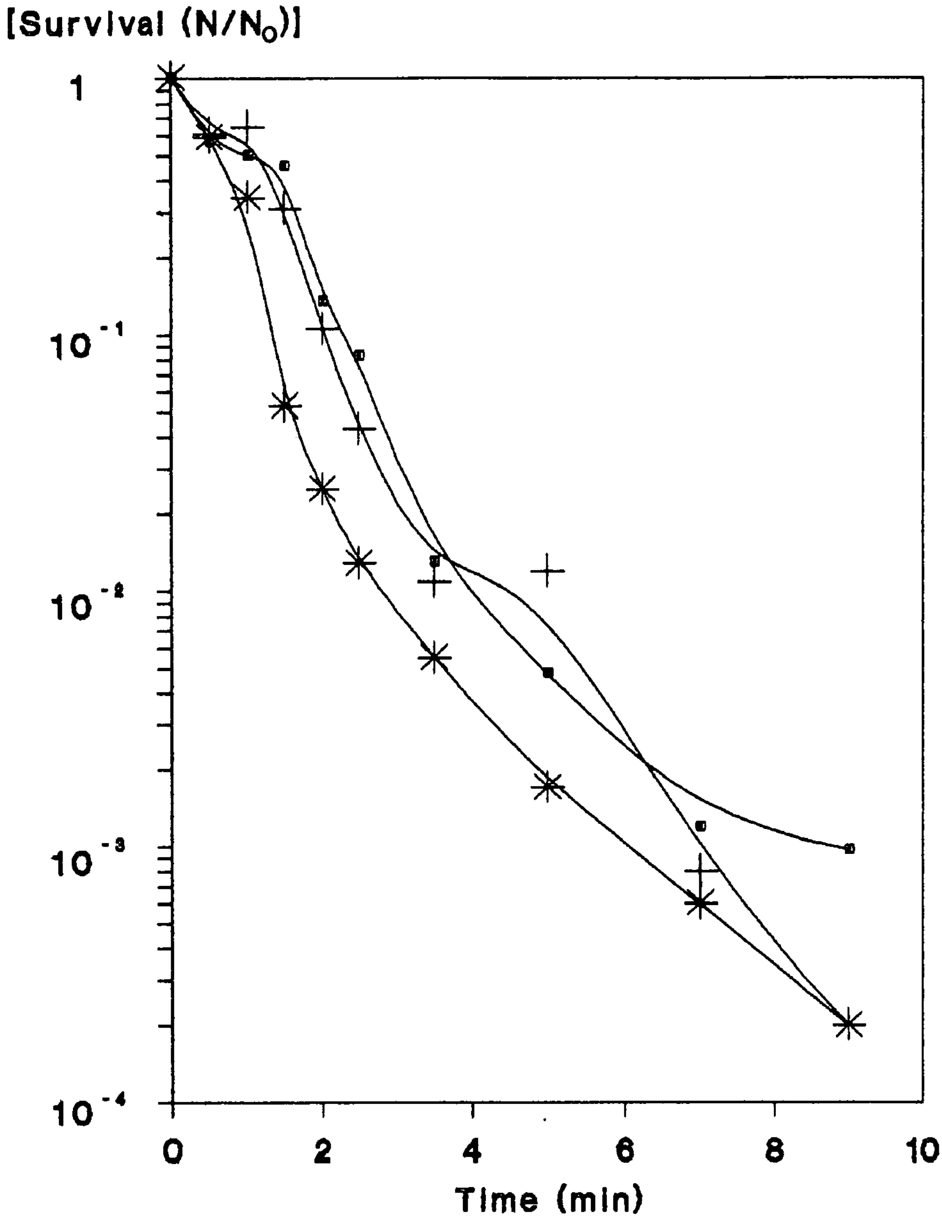


Figure 3.1. Survival (N/N$_o$ where N$_o$ is the initial number of viable bacteria and N the number of bacteria surviving exposure time t, in min) in 9 ml of Luria Broth at 55°C, of 1 ml of mid-logarithmic phase *S. typhimurium* SA2009 measured on three separate occasions, represented as(+), (*), and (▪).

Figure 3.2 <u>Survival of *S. typhimurium* SA2009 in 180 ml of LB at 55°C</u>

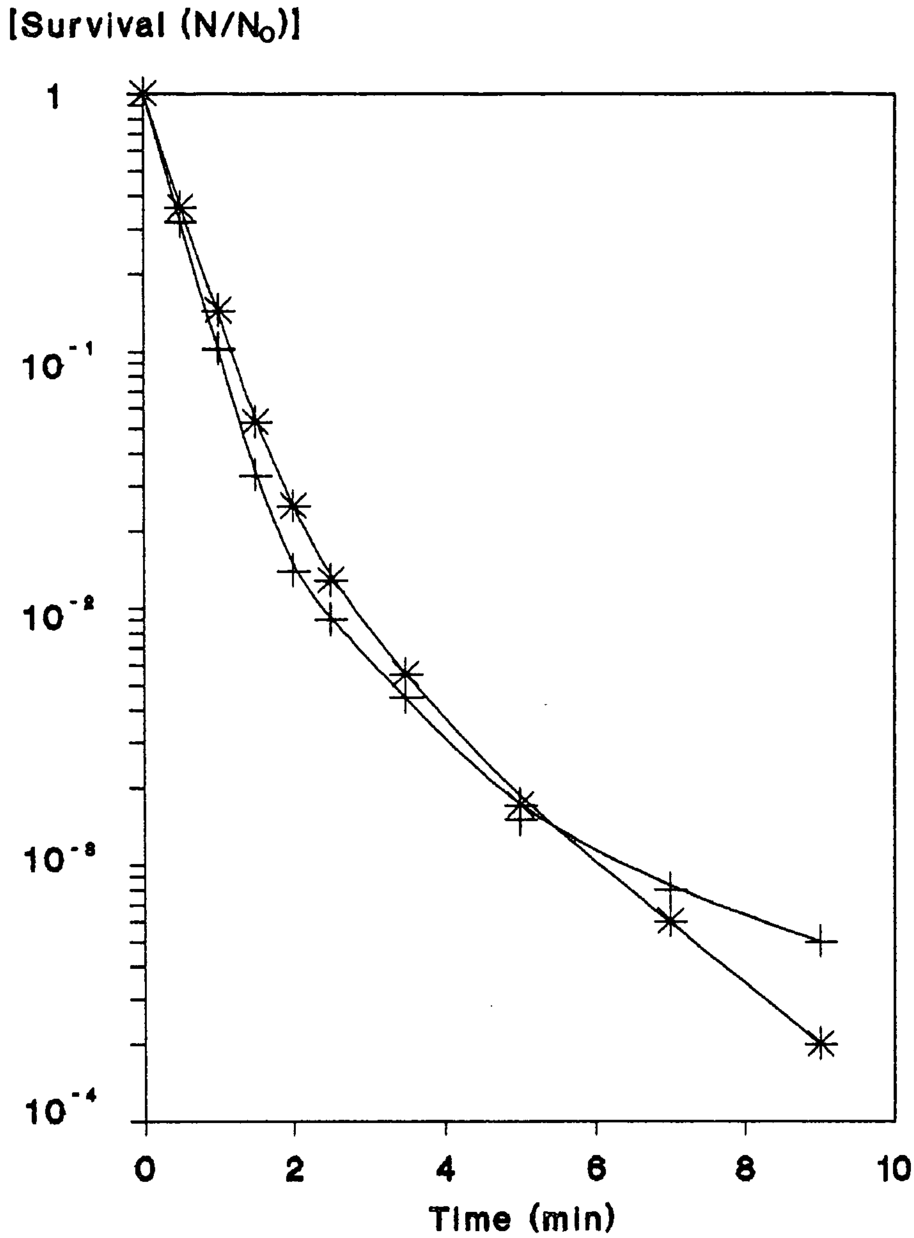


Figure 3.2. Survival (N/N_o) in 180 ml of LB at 55°C, of 20 ml of mid-logarithmic phase *S. typhimurium* SA2009 measured on two separate occasions represented as (+) and (*).

The variables that could possibly have had an effect on the thermal survival curves were therefore investigated. The obvious variables were: (i) wall thickness of inactivation vessel; (ii) phase of growth prior to thermal inactivation; (iii) length of incubation of thermally damaged cells and (iv) batch of inactivation and recovery media used. Variables that could be assumed to be constant throughout the experiments were: (i) speed of the rotary shaker; (ii) water bath temperature; (iii) pipette volumes; (iv) accuracy of serial dilutions and (v) spreading of cells onto recovery media. Of the four variables investigated, the phase of growth prior to thermal inactivation proved to be the only one that gave any significant alteration in the survival curves. When bacteria in the same phase of growth were heat inactivated, reproducible survival curves were only obtained if great care was taken while measuring cell density when predicting growth phase. Also great care was needed when preparing media as this could, if a poor batch of medium was used, give measurable variations in survival curves. The importance of comparing results from cells in the same phase of growth cannot be emphasised enough. *Figure* 3.3 shows how the survival at 55°C in LB of *S. typhimurium* SA2009 to a set thermal exposure, varied during the course of the growth cycle at 37°C. Survival is at its greatest during the lag phase and stationary phase, but the rate of change between these two phases is great. This would mean that a delay in using cells obtained for a thermal inactivation experiment would result in changes in their thermal survival and would make comparison impossible. This also explained why the initial use of a nephalometer as the means of measuring optical density did not give reproducible survival curves. Phase of growth was initially measured by comparing nephalometer readings against a standard curve, thereby determining the mid-logarithmic phase of growth. The nephalometer proved to be very temperamental and even after calibration,

Figure 3.3 <u>Survival of *S. typhimurium* SA2009 at 55°C through the growth cycle at 37°C following exposure to 55°C for 5 minutes</u>

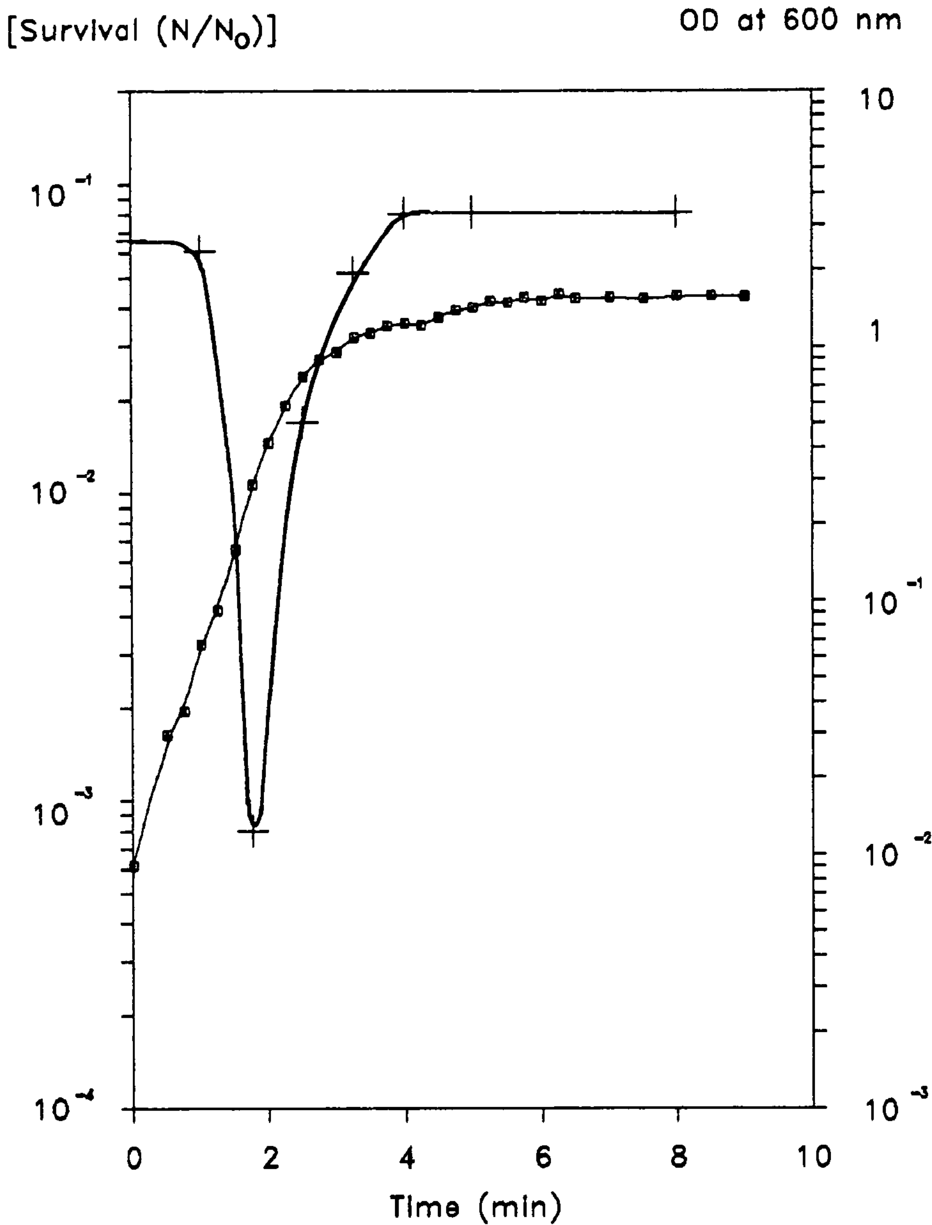


Figure 3.3. The effect of growth phase at 37°C, measured by OD at 600 nm (•), on the survival (+) of 20 ml of *S. typhimurium* SA2009 in 180 ml of LB to 5 min in 180 ml at 55°C.

readings could to be extremely variable even on the same sample.

To counteract this, a spectrophotometer was used as a more accurate measure of cell density/phase of growth. By accurately predicting the phase of growth it was possible to remove much of the variability in the thermal survival curves that results from growth phase differences. Once all the variables had been eliminated, it was possible to produce a series of survival curves at different lethal temperatures for mid-logarithmic phase *S. typhimurium* strain SA2009. *Figure* 3.4 shows survival curves at 50°, 52°, 55°, 57° and 59°C. All these curves were produced on the same day using the same batch of Luria Broth, Luria Broth agar plates and cells at the same optical density started from the same stationary phase culture. The experiments were repeated and survival curves were comparable and within the bounds of experimental error. These curves were then regarded as the standard against which other survival curves could be compared and variations in heat resistance measured.

3.2 Alterations in heat resistance by means of genetic mutation

Once a standard and reliable set of survival curves had been obtained for *S. typhimurium* SA2009, changes in the organism's ability to survive at elevated temperatures, caused by mutation, could be measured. It was initially decided to produce changes in *S. typhimurium* SA2009's ability to withstand elevated temperatures by means of random disruptional mutations in the DNA. This would prevent normal transcription of the particular gene(s) affected, and result in loss of some normal protein function. If the resultant protein was in some way involved in survival at elevated temperatures or recovery from damage caused by elevated temperatures, then this organism should be more sensitive to heat.

Figure 3.4 <u>Survival of *S. typhimurium* SA2009 at 50°, 52°, 55°, 57° and 59°C in 180 ml of LB</u>

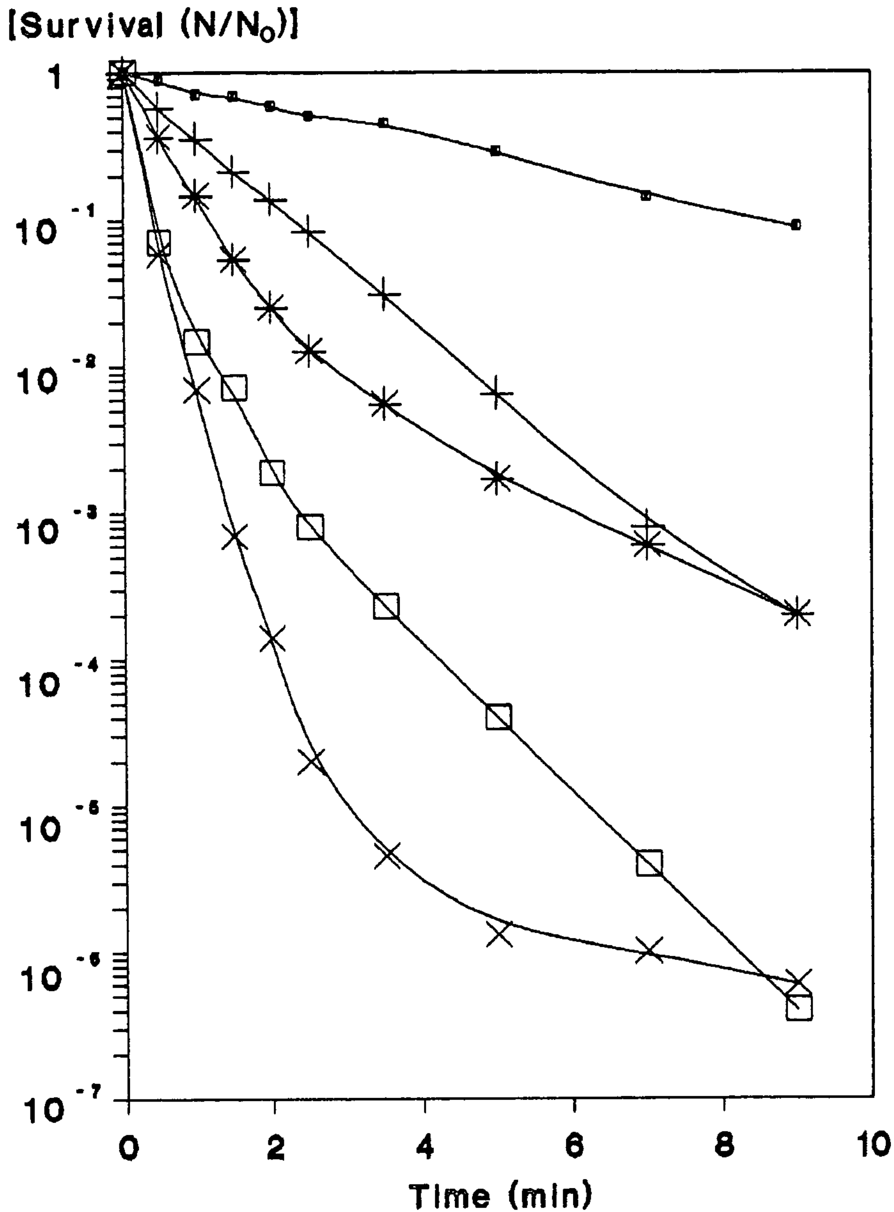


Figure 3.4. Survival (N/N_o), in 180 ml of LB at 55°C of 20 ml of mid-logarithmic phase cells of *S. typhimurium* SA2009 at 50°C (▪), 52°C (+), 55°C (*), 57°C (□) and 59°C (×).

Once mutated organisms with a reduced heat resistance had been isolated, it would be possible to determine the position and function of the affected gene and therefore determine what its function was in thermal survival/recovery.

3.3 Genetic mutation using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is a very powerful alkylating agent and will spontaneously transfer alkyl groups to ring nitrogen atoms of bases in DNA. The most frequently observed reaction is the alkylation of the N-7 position of guanine, but a variety of other ring nitrogen alkylations occur at low frequency. This alkylation of the N-7 of guanine causes ionization of the molecule which readily alters its base-pairing specificity. Alkylation at certain other positions blocks base pairing completely. When alkylation of purines occurs it labilizes their bonds to deoxyribose and causes extensive depurination of the DNA. All of these changes occur when cells are exposed to MNNG and contributes to both transition (purine:purine or pyrimidine:pyrimidine replacement) and transversion (purine:pyrimidine or pyrimidine:purine replacement) mutations (Stanier, 1981).

Treatment of a population of exponential phase S. typhimurium SA2009 with 10 μg ml^{-1} of MNNG for 20 min, resulted in a population which showed an induced mutation frequency of 1.4 x 10^{-5}, as calculated by the increased proportion of the population being resistant to 10μg ml^{-1} of the antibiotic rifampicin. *Table* 3.1 shows the mutation frequency after 0, 5, 10 and 20 minutes.

Table 3.1 <u>Mutation frequencies of *S. typhimurium* SA2009 after varying exposure times to MNNG, measured by resistance to 10μg ml^{-1} rifampicin.</u>

Exposure to MNNG (min)	TVC ml^{-1}	No. rif^R colonies ml^{-1}	Mutation Frequency
0	2.0×10^{10}	0	0
5	9.5×10^{9}	1.39×10^{6}	1.46×10^{-4}
10	1.0×10^{10}	1.37×10^{6}	1.37×10^{-4}
20	7.8×10^{9}	1.1×10^{5}	1.4×10^{-5}

TVC - Total Viable Count
rif^R - rifampicin resistance

As a control, an untreated sample of the culture was plated out after 0, 5, 10 and 20 minutes intervals on to LB rifampicin (10μg ml^{-1}) agar plates and incubated with the MNNG treated cells to measure the degree of spontaneous mutation. No colonies grew on any of the control plates, indicating that all the rifampicin-resistant colonies present on the plates were MNNG-induced mutants.

Those cultures derived from the 10 min exposure were retained for further study as they showed the highest mutation frequency. After 10 minutes exposure, the mutation frequency began to fall, probably because the number of mutations per cell began to rise, as would the probability of at least one being lethal.

3.3.1 Measuring changes in heat resistance of MNNG treated *S. typhimurium* SA2009

The survivors of the 10 min MNNG exposure experiment were screened to determine whether any of them had become heat sensitive. Survivors were plated out on to LB agar

plates so as to give approximately 100 colonies per plate. These were then exposed to a temperature of 55°C in a high temperature incubator according to the procedure laid out in section 2.11.3.

Not surprisingly colony size appeared to play an important part in thermal survival. Larger colonies obviously had a greater number of cells and therefore viable units to survive the exposure. Also the degree of shielding for those bacteria in the centre of a large colony would be greater. A standard colony size was therefore required in order to make any thermal inactivation times comparable. Colonies were therefore transferred from the original plate to a gridded plate using sterile tooth picks as described in section 2.11.4. Once this technique had been introduced it quickly became apparent that the plates were not receiving an even distribution of heat throughout the time span of the experiment, and a control was set up to ensure even distribution. One hundred wild type colonies were tooth picked on to an LB agar plate and replica-plated, and the replicates placed in the high temperature incubator for a period that should just kill all the bacteria. After incubation at 37°C for 18 hours, areas within each plate showed colonies giving strong or medium growth, while others had no growth. This problem was easily remedied. The shelf on which the plates were placed happened to be drilled with a regular pattern of $\simeq$ 2 cm diameter holes. On replacing this shelf with a solid one and repeating the experiment, no growth was seen on any of the plates wherever they had been placed. The experiment was repeated once again, but this time reducing the exposure time to ensure that no areas were receiving more heat than any other. After incubation, no colonies were absent and it was assumed an even distribution of heat within the incubator took place. Several thousand colonies were screened using this technique and although some 11 colonies were, on the first screening thought to

have a reduced heat resistance at 55°C, none of them were found to have an altered heat resistance when screened further. In fact second and third screenings showed them to have exactly the same heat resistance as the wild type.

3.3.2 Genetic mutation using transposon mutagenesis

The apparent inability to isolate any *S. typhimurium* SA2009 organisms with altered heat resistances, as compared to the wild type, using the surface plate method posed a dilemma. Although a population of mutated cells had been created using MNNG, the mutant organisms being screened were indistinguishable from wild type organisms. The mutation frequency indicated that at least 7000 organisms had to be screened before a mutated organism would necessarily be screened (providing heat resistance was due to a single gene) As the plate screen method was quite involved, it was necessary to ensure that only mutated cells were screened. A method was therefore employed which allowed the identification of those cells that had a disruption in the DNA sequence. To this end the technique of transposon mutagenesis was employed. The transposable element, *Tn*10, was introduced into *S. typhimurium* SA2009 by the action of the bacteriophage P22 as described in (section 2.13). Once inside, the transposable element randomly inserts itself in the bacterial genome producing insertional mutations, disrupting the normal function of the gene and therefore the protein for which it codes. The *Tn*10 carries a tetracycline-resistance gene which can be used to positively screen all those bacteria containing an insertional element. By plating out a population of treated cells on to LB agar containing (12.5 $\mu g\ ml^{-1}$) tetracycline, a discernible mutant population was available for screening, to identify if the insertion was in a gene that conferred thermal resistance. These tetracycline-resistant colonies were pooled and plated

out to give individual colonies, which were then tooth picked on to LB agar plates containing tetracycline (12.5 μg ml^{-1}), replica plated, and exposed in a high temperature incubator to 55°C,(section 2.11.4).

Every plate that was tested for heat resistance contained 99 tetracycline resistant transduced strains of *S. typhimurium* SA2009, and one tetracycline-resistant strain of *S. typhimurium* SA2009 that had the same heat resistance, on both LB agar plates and in LB at 50°C, 52°C, 55°C, 57°C and 59°C as the wild type *S. typhimurium* SA2009. In all, 7920 tetracycline-resistant transductants of *S. typhimurium* SA2009 were screened using this method, 17 of which showed reduced heat resistances. All 17 transductants were tested for their ability to survive in liquid culture as compared with the standard curves of *S. typhimurium* SA2009 at elevated temperatures. Cells were heat inactivated using the standard method and recovered on LB and LB tetracycline (20 μg ml^{-1}) agar plates; this was to ensure that the presence of tetracycline in the agar did not in any way inhibit or enhance the recovery of the cells after heat treatment. When all the recovery curves were compared against the standard, there was no statistically-measurable difference, and none that would have suggested any difference in resistance by the plate method.

The plate method was therefore refined to make it more sensitive. It was assumed that the tooth pick replica-plating method, although a good primary screen, did not give reproducible or sensitive enough results, due to the variations in inocula size and density of colony growth. An alternative method was devised in which a very small inoculum of cells from the colony under investigation was transferred in to a small volume (0.2 ml) of LB and mixed using a vortex mixer. This was placed at 37°C for 2-3 hours and spotted in 3 μl volumes on to the surface of an LB tetracycline (20μg ml^{-1}) agar

plate and allowed to dry. Plates were then placed in the high temperature incubator set at 55°C for a predetermined length of time, before incubation at 37°C for 18 hours. This method requires more time in setting up the screen but produces reproducible results with a higher sensitivity. Nevertheless, no isolates of *S. typhimurium* SA2009 were found to have altered thermal survival capabilities even though this method was the most sensitive and reproducible plate method that could be devised.

3.4 Thermal conditioning

The work so far undertaken had endeavoured to isolate organisms with increased sensitivity to heat, as compared to the wild type. As neither mutational method had succeeded, an attempt was made to isolate organisms with elevated heat resistances. A method was employed which was capable of generating organisms with increased resistance to heat.

It had been reported that by repeated heat treatment and growth of survivors, the heat resistance of the cycled cultures is increased in comparison with non-cycled cells (Corry and Roberts, 1970). This cycling or conditioning, involves thermal inactivation down to 0.1% survival and then re-growth of the survivors prior to the next round of thermal inactivation. Corry and Roberts (1970) were able to show a doubling in the D^{55} value for those cells cycled 13 times and a 3 times higher D^{55} value for those cells cycled 26 times.

S. typhimurium SA2009 was cycled using the method described in section 2.11.6. After each fifth cycle the survivors were heat inactivated and the percentage survival measured using the viable count method described in section 2.11.1, but using the spectrophotometer to measure cell density. The strain was cycled altogether

30 times and six survival curves produced for comparison against the original survival curve of the non-cycled cells. *Figure* 3.5 shows an increase in the thermal survival of the cycled strains. The increase occurred after 10 cycles, and although small was consistently greater than the wild type strain. No further increase was observed until 20 cycles, when the increase was again only small but consistent. All the curves of cycled strains were repeated several times to ensure that any increase was not due to experimental variations. The 30th cycled strain showed the greatest increase, but this was only small, and nowhere near the increase shown by the works of Corry and Roberts (1970). They reported D^{55} values of their 39th cycled strain to be close to that of *S. senftenberg* 775W, described as the most heat resistant *Salmonella* strain.

Cultures of the cycled strains from the Corry and Roberts experiments and of *S. senftenberg* 775W were obtained. *Figure* 3.6 shows the thermal survival curves of the strains of *S. typhimurium*; SA2009, MR18, MR19, MR20, MR21 and *S. senftenberg* 775W. The curves of *S. typhimurium* SA2009 and MR18, the non-cycled strain from Corry and Roberts, are very similar up to three and a half minutes at 55°C, but *S. typhimurium* MR18 has a steeper reduction in survival. There was a progressive increase in the heat resistance at 55°C of *S. typhimurium* MR19, MR20 and MR21, when compared with that of *S. typhimurium* MR18. Corry and Roberts (1970) described *S. typhimurium* MR21 as having a D^{55} value close to that of *S. senftenberg* 775W. These results confirm the similarity between the heat resistance of *S. typhimurium* MR21 and *S. senftenberg* 775W.

D^{55} values of 11.0 for *S. senftenberg* 775W and 4.7 for *S. typhimurium* MR21 were obtained. The value for *S. senftenberg* 775W is close to that of 13.0 obtained by Corry and Roberts (1970) but the value for *S. typhimurium*

Figure 3.5 <u>Survival of thermally cycled *S.typhimurium* SA2009 at 55°C</u>

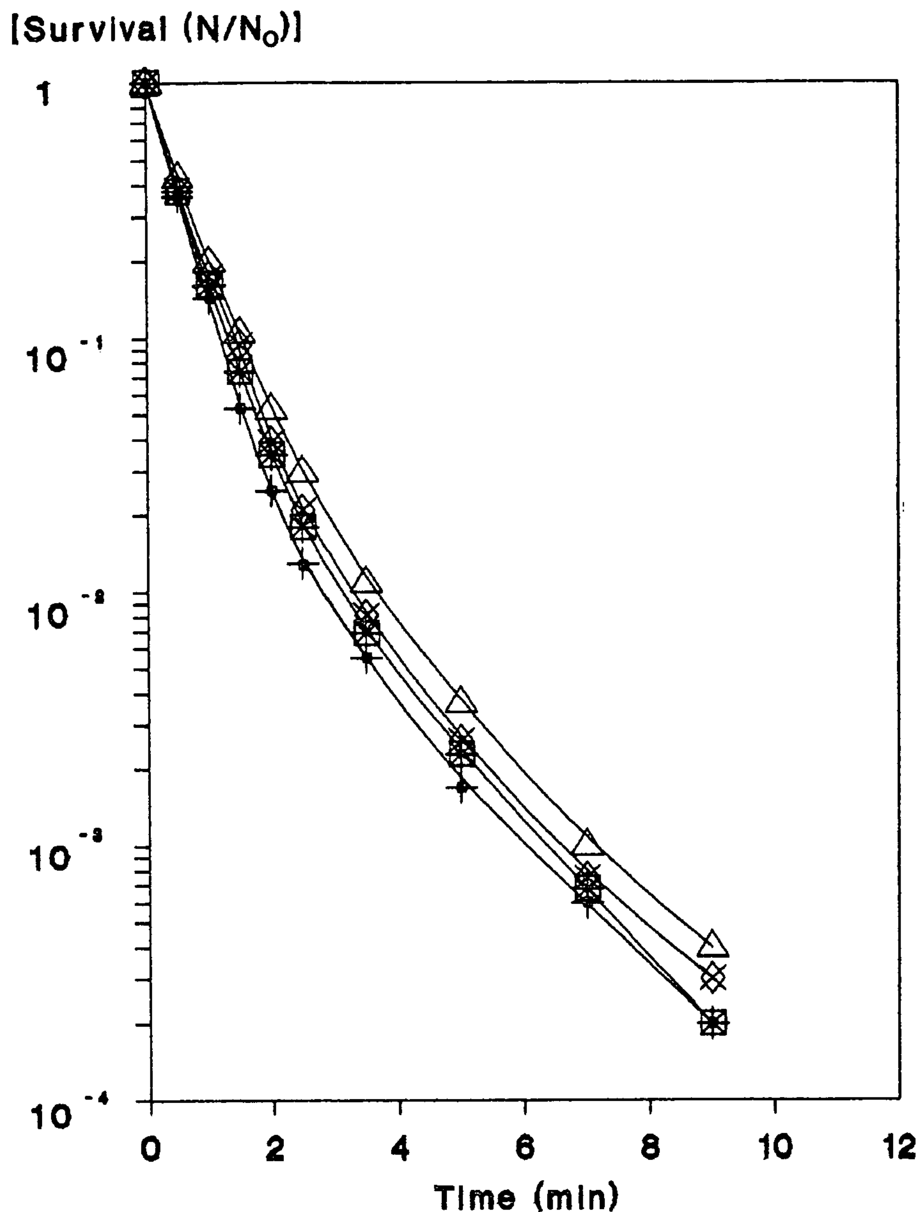


Figure 3.5. Survival (N/N$_o$), in 180 ml of LB at 55°C of 20 ml of mid-logarithmic phase cells of *S. typhimurium* SA2009 and heat cycled derivatives. Number of thermal cycles, 0 (•), 5 (+), 10 (□), 15 (*), 20 (×), 25 (◇), and 30 (△).

Figure 3.6 <u>Survival of thermally cycled strains of *S. typhimurium* strain 1, *S. typhimurium* SA2009 and *S. senftenberg* 775W at 55°C</u>

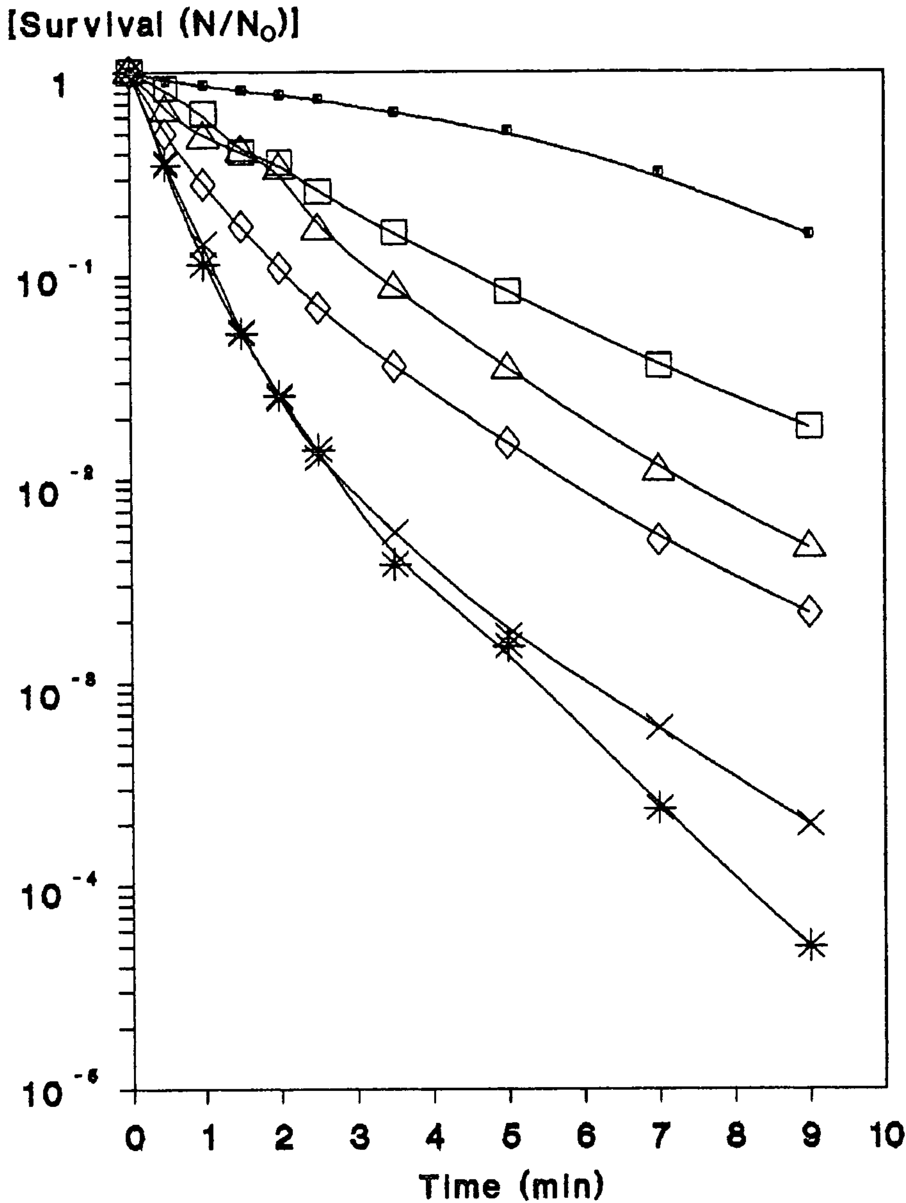


Figure 3.6. Survival in 180 ml of LB at 55°C, of 20 ml of mid-logarithmic phase wild type *S. typhimurium* strain 1 (MR18) (*), 13th cycle (MR19) (◇), 26th cycle (MR20) (△) & 39th cycle (MR21) (□), and *S. senftenberg* 775W (■) and *S. typhimurium* SA2009 (×).

MR21 is almost one third their value of 11.7. The explanation for this may lie in the nature of the inactivation and recovery media which was discussed in section 1.2.2. Nevertheless, the cycled strains obtained from Corry and Roberts (1970) had survival capabilities far greater than those of the cycled strains of *S. typhimurium* SA2009.

All the *Salmonella* strains so far tested for heat resistance in liquid media were spotted out using the 3µl method described in (section 3.3.2), and placed in a 55°C incubator before being taken out and incubated at 37°C for 18-24 h. Those producing visible growth were marked as positive, while those showing no growth were marked negative.

Table 3.2 <u>Presence or absence of growth of strains *S. typhimurium* and *S. senftenberg* after exposure to 55°C on the surface of an LB agar plate</u>

	Duration of exposure to 55°C in min / Growth after exposure to 55°C						
Strain	0	45	46	47	48	49	50
SA2009 wild type	+	+	+	-	-	-	-
SA2009 30th cycle	+	+	+	-	-	-	-
MR18	+	+	+	-	-	-	-
MR19	+	+	+	-	-	-	-
MR20	+	+	+	-	-	-	-
MR21	+	+	+	-	-	-	-
775W	+	+	+	+	+	+	+

All the strains were capable of surviving for 46 min but only *S. senftenberg* 775W was able to survive beyond 46 min. *S. senftenberg* 775W in fact survived for 57 min, 12 min longer than any of the other strains tested. This

ability to survive in both broth and on plates, but particularly on plates, made it an ideal subject for the study of heat resistance in Salmonellae.

At this point it was decided to switch the emphasis of study away from *S. typhimurium* SA2009 to *S. senftenberg* 775W, but still using the information obtained from the work on *S. typhimurium* SA2009 as a comparative *Salmonella* having a normal heat resistance.

The initial work done had shown *S. senftenberg* 775W to be more heat resistant at 55°C than *S. typhimurium* SA2009, which could be described as having an average heat resistance for the Salmonellae species. Winter *et al.*, (1946) first reported a strain of *S. senftenberg* that had a greater heat resistance than that of 16 other isolates of *Salmonella* they tested. The strain was designated as *S. senftenberg* 775W by Solowey *et al.*, (1948). Ng *et al.*, (1969) reported *S. senftenberg* 775W had a heat resistance which was 30 times greater than that of their reference strain of *S. typhimurium*, Tm-1. Ng *et al.* (1969) also reported that of 19 strains of *S. senftenberg* tested for heat resistance, *S. senftenberg* 775W proved to be the only one that had such an elevated heat resistance. If *S. senftenberg* 775W was a *senftenberg* strain, which serology suggested, then perhaps it had gained this increased heat resistance by the acquisition or development of a physiological trait, possibly in the same way as Corry and Roberts (1970) had induced heat resistance in their strain of *S. typhimurium*. It was therefore decided to examine the heat resistance of *S. senftenberg* 775W and determine whether the trait could be transferred to a recipient organism. Initial work was centred on the transfer of genes from *S. senftenberg* 775W to *S. typhimurium* SA2009.

3.5 Genetic work on heat resistance

Genetic studies would involve looking for low numbers of organisms having elevated heat resistances in large populations of organisms with wild type heat resistances. An experiment was therefore constructed to ensure that, if a genetically altered organism with an elevated thermal survival capability was produced, it could be separated from wild type organisms using a simple method. The method involved the production of an antibiotic resistant strain of *S. senftenberg* 775W.

A streptomycin resistant strain of *S. senftenberg* 775W was generated by spreading a dense culture of stationary phase *S. senftenberg* 775W onto the surface of 5 LB streptomycin (150μg ml^{-1}) agar plates and incubating them for 18 hr at 37°C. Only one colony was isolated which was resistant to streptomycin, up to 2 mg ml^{-1}, and it had the same heat resistance properties as the wild type *S. senftenberg* 775W (results not shown), and was termed SM001.

Both SM001 and *S. typhimurium* SA2009 were grown to mid-logarithmic phase and serially diluted so as to give cultures containing 10 SM001 and 10^6 *S. typhimurium* SA2009 organisms per 0.1 ml. The bacteria were mixed, again so as to give cultures that contained 10 SM001 and 10^6 *S. typhimurium* SA2009 per 0.1 ml. One hundred microlitres of each suspension was spread on to an LB agar plate and an LB streptomycin (250 μg ml^{-1}) agar plate, and allowed to dry. This series of six plates was then placed in a high temperature incubator set at 55°C, for a pre-set period of time, before being removed and incubated for 18-24 hr at 37°C. This was repeated for a series of time exposures.

Table 3.3 shows the low number of SM001 having a higher heat resistance than the vast majority, *S.*

typhimurium SA2009, and greatly increasing its percentage presence. The percentage of each of the two organisms present in the mixed culture was altered after 47 min exposure. SM001 had 10% survival, while *S. typhimurium* SA2009 had 0.0074% survival. This would greatly reduce the amount of work needed to isolate heat resistant variants in a population of wild type heat resistant organisms. However, the lower number of survivors in a mixed culture would suggest that a less stringent exposure time should be used so as to ensure that the low numbers of heat resistant organisms could be isolated.

Table 3.3 Survival of single and mixed cultures of SM001 and *S. typhimurium* SA2009 after varying exposure times to 55°C in a high temperature incubator using a surface plate method.

	Duration of exposure to 55°C in min Growth media							
	45 min		46 min		47 min		48 min	
Strain	LB	LBS	LB	LBS	LB	LBS	LB	LBS
SM001	9	8	7	7	1	1	0	0
SA2009	TNTC	0	188	0	0	0	0	0
SM001/ SA2009	TNTC	9	743	1	64	1	0	0

TNTC - Too Numerous To Count
LB - Luria Broth Agar Plates
LBS - Luria Broth Streptomycin (250μg ml^{-1}) Agar Plates

The mixed culture of SM001 and *S. typhimurium* SA2009 had a higher number of *S. typhimurium* SA2009 survivors than did the *S. typhimurium* SA2009 culture alone, while

the SM001 isolation rate was lower in the mixed culture than for SM001 alone. This suggests that SM001 may in some way be able to induce heat resistance in *S. typhimurium* SA2009, and by the same token, *S. typhimurium* SA2009 may in some way reduce the heat resistance of SM001.

3.5.1 P22 Transduction

P22 is a generalised temperate transducing phage which infects *S. typhimurium*. When the phage passes into the lytic phase, along with packaging its own DNA in the phage head it will, at a rate of about 2% in the wild type phage, package host bacterial DNA (Ebel-Tsipis *et al.*, 1972). The P22 DNA is a single linear duplex of $2.6x10^7$ to $2.8x10^7$ daltons and can be replaced by up to 1 % of the bacterial genome (Tye *et al.*, 1974).

As work had already been carried out using the bacteriophage P22, it was used again as a vector for transducing DNA from *S. senftenberg* 775W into *S. typhimurium* SA2009. First *S. senftenberg* 775W was tested for its ability to plaque P22, i.e., whether it was sensitive to P22 derived from *S. typhimurium* LT2. A P22 lysate grown on *S. typhimurium* LT2, as described in (section 2.13.1), was mixed with *S. senftenberg* 775W and with *S. typhimurium* LT2. One hundred microlitres of an 18 hr culture of *S. senftenberg* 775W and *S. typhimurium* LT2 were mixed with 0.1 ml of the phage suspension at dilutions of 10^0, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}. These were then incubated at 30°C for 30 min prior to mixing with 3 ml of LB top agar. These were poured on to LB agar plates and allowed to set before incubating at 37°C for 24 h. The plates were then examined for plaques and the numbers are recorded *Table* 3.4.

Table 3.4 <u>The quantitative ability of bacteriophage P22, derived from *S. typhimurium* LT2, to plaque on *S. senftenberg* 775W and *S. typhimurium* LT2</u>

	Phage dilution Number of plaques per plate				
Strains	10^{0}	10^{-2}	10^{-4}	10^{-6}	10^{-8}
S. senftenberg 775W	0	0	0	0	0
S. typhimurium LT2	TNTC	TNTC	TNTC	TNTC	TNTC

TNTC - Too Numerous To Count

The plaque suspension, which contained approximately 8.6×10^{10} plaque forming units per ml (pfu ml^{-1}), plaqued on *S. typhimurium* LT2 but would not plaque on *S. senftenberg* 775W. There are several explanations as to why P22 grown on *S. typhimurium* LT2 would not plaque on *S. senftenberg* 775W.

(i) The phage particles are unable to recognize an attachment site on the surface of *S. senftenberg* 775W and are therefore unable to introduce their DNA into *S. senftenberg* 775W.

(ii) The DNA has been modified by the restriction/modification system of *S. typhimurium* LT2, and once inside *S. senftenberg* 775W is recognized as foreign and is restricted, again giving rise to no plaques.

It was decided to 'force' P22 in to *S. senftenberg* 775W by the use of a P22 lysate containing a *Tn*10 tetracycline resistance transposable element. This would hopefully show if phage was able to attach to the surface

and insert DNA in to *S. senftenberg* 775W. *Table* 3.5 shows the number of transductants produced.

Table 3.5 The quantitative ability of bacteriophage P22, derived from *S. typhimurium* CH1305 and containing *Tn*10 elements, to plaque on *S. senftenberg* 775W and *S. typhimurium* LT2 and give rise to tetracycline resistant transductants

	Phage dilution Number of plaques per plate				
Strains	10^{0}	10^{-2}	10^{-4}	10^{-6}	10^{-8}
S. senftenberg 775W	60	0	0	0	0
S. typhimurium LT2	TNTC	TNTC	1690	16	0

Most of the *S. senftenberg* 775W colonies that grew up on the LB tetracycline (12.5 g ml^{-1}) agar plates were very small and gave rise to very small colonies when re-streaked on to LB tetracycline (12.5 g ml^{-1}) agar plates. A few were normal sized colonies that gave rise to normal sized colonies when re-streaked. The re-streaking was done as quickly as possible to reduce the risk of phage re-entering the bacteria and rendering them P22 resistant again. Sixteen were picked from single colonies from the re-streaked LB tetracycline (12.5 μg ml^{-1}) agar plates, and put through two sets of green plates. These are pH indicator plates that go dark green under acid conditions. Phage present in a bacterium will alter the amount of glucose metabolized and hence increase acid production giving rise to very dark green colonies. Streaking through two sets of green plates and only re-streaking the pale green colonies should give rise to phage free cells. On re-streaking the colonies back on to LB tetracycline plates, many were not tetracycline

resistant, suggesting they had lost their *Tn*10 transposable elements. All sixteen colonies were also tested for sensitivity to the phage and heat resistance. *Table* 3.6 shows *S. senftenberg* 775W transductants' sensitivity/resistance to tetracycline, P22 derived from *S. typhimurium* LT2 and heat.

Table 3.6 Sensitivity/resistance of *S. senftenberg* 775W transductants to tetracycline, P22 and heat.

Colony	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Tet^R	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-
$P22^S$	-	-	-	-	-	-	-	-	*	-	*	-	*	-	-	-
$Heat^R$	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+

Note * thin lawn of cells

The colonies 9, 11 and 13 when tested for P22 sensitivity gave very thin lawns of cells suggesting a 'sick' cell. These cells also had heat resistances, using the surface plate method, equivalent to *S. typhimurium*, far less than *S. senftenberg* 775W.

Although all 60 of the transductants proved to be tetracycline resistant when re-streaked directly on to LB tetracycline agar plates, those that gave rise to very small colonies lost their resistance when passed through green plates. Only those transductants that gave normal sized colonies i.e., 9, 11 and 13 retained their tetracycline resistance when passed through green plates.

Why do many of the transductants lose their tetracycline resistance? When bacterial DNA is transduced with P22 it can either become associated with the DNA of the recipient bacteria or persist in the

recipient bacteria as intact, non-integrated, non-replicating double stranded molecules (Ebel-Tsipis *et al.*, 1972*a*). It would appear that the very small colonies on the LB tetracycline agar plates, have part of the *S. typhimurium* LT2 CH1305 genome that contains the *Tn*10 element, and therefore allows growth on tetracycline. The colonies are small because the element of DNA does not replicate and the colony is the multiplication of 1 cell. When these colonies are streaked on to green plates, the presence of the *Tn*10 element has no influence on the ability of the cells to grow, and so passage through two sets of green plates will essentially remove the *Tn*10 element from the population. The normal sized colonies have presumably integrated the *Tn*10 element in to their genome. However, although the element is able to be maintained in the cell, its presence affects the normal cell function, making it unusable for further work.

Despite the loss of the *Tn*10 elements in the small colonies and the sick nature of the normal sized colonies, *S. senftenberg* 775W was infected by phage, and providing they had not been re-infected by new phage, should have been P22 sensitive. However none of them were P22 sensitive. Why? The phage they were propositioned with had been derived from *S. typhimurium* LT2. Although they may have taken up phage DNA once before, it would still be recognized as foreign and be restricted. The only way phage DNA would successfully re-infect *S. senftenberg* 775W, is if it had had one passage through *S. senftenberg* 775W. In doing this, phage DNA could become modified by the *S. senftenberg* 775W restriction/modification enzyme system.

In an effort to maximise the possibility of producing *S. senftenberg* 775W that would liberate attenuated P22 phage DNA, a method was derived to optimise the use of the *Tn*10 element. It was therefore

decided to utilize the hyper-helper-hopper plasmid, pNK972 in an effort to produce a stably-integrated *Tn*10 element that would liberate P22 phage and possess all the physiological attributes of *S. senftenberg* 775W. An attempt was made to transform pNK972 into *S. senftenberg* 775W, and the method chosen was electroporation.

Electroporation is the use of high-voltage, high-current exponential electric pulses across a cuvette which contains a cell suspension and DNA, generated by a Gene Pulser Transfection apparatus (Bio-Rad Laboratories, Richmond, California CA94804). Methods were provided by Bio-Rad technical publications (1987) along with the Gene pulser transfection apparatus operating instructions and applications guide (Bio-Rad Laboratories, Richmond California CA94804).

The plasmid was obtained from *S. typhimurium* TT10427 by performing a mini-prep as described in 2.14.1. *S. senftenberg* 775W was grown in LB at 37°C to the required turbidity, spun down, washed twice in electroporation buffer (272 mM sucrose, 7 mM sodium phosphate pH 7.4 and 1 mM $CaCl_2$), and resuspended in ice cold buffer at approximately $1x10^8$ cfu ml^{-1}. Four sets of four 1 ml replicates of the cell suspensions were transferred to Gene Pulser cuvettes and left on ice for 10 min, before adding 15μg, 20μg, 25μg and 50μg of pNK972 to one cuvette in each set respectively, and leaving on ice for a further 10 min. One cuvette from each of the four series was electroporated at a constant 25 μFD (microfarad) and at either 1.0, 1.5, 2.0 or 2.5 KV. After treatment, each cuvette was kept on ice for 10 min prior to cells and DNA being transferred to 9 ml of LB and incubated for 18 hr for expression of the ampicillin marker on the plasmid. Cells were then diluted on to LB ampicillin (20μg ml^{-1}) agar plates and incubated for 24-72 hr. As a control, *S. typhimurium* TT10423 was also electroporated using 15 g of pNK972 at 2.5 KV and 25 μFD.

No transfected *S. senftenberg* 775W cells were produced using this method, while 1970 transfected *S. typhimurium* TT10423 cells grew up after 72 hr. This number could be considered low compared with the probable number of plasmid molecules present in the 15μg of DNA. This could possibly be explained by the nature of the conditions used for electroporation, as they were not designed for Salmonellae. However, no more efficient electroporation methods were known at that time.

Since the production of a *S. senftenberg* 775W attenuated P22 phage lysate proved to be unattainable, it was decided to use an alternative phage vector. The phage P1 was chosen because viable phage, carrying an antibiotic marker, was available. Use of P1*cml* allowed those cells containing viable phage DNA to be identified and isolated due to chloramphenicol resistance.

3.5.2 P1 Transduction

P1 is a generalised temperate transducing phage. P1 will transduce host bacterial DNA as 0.3% of the phage lysate. Its DNA is a single linear duplex of $6x10^7$ to $6.6x10^7$ daltons and it can package 2% of the host genome (Ikeda & Tomizawa, 1965).

The *S. typhimurium* strain CH1026 contains a lysogenic P1 phage which carries a chloramphenicol resistance marker. The phage is temperature sensitive and will enter the lytic cycle if the bacterium is grown over 37°C. It was therefore decided to try and produce a P1 lysogenic *S. senftenberg* 775W.

A P1 lysate of *S. typhimurium* CH1026 was produced according to section 2.13.4. This phage preparation was then titred on *S. typhimurium* CH223. One hundred microlitres of an 18 hr culture of *S. typhimurium* CH223,

grown at 37°C, was mixed with 0.1 ml of phage at dilutions of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}. This was incubated at 30°C for 30 min prior to mixing with 5 ml of LC top agar and pouring on LC agar plates. The plates were incubated at 37°C for 18 h or until plaques began to appear, but before they were overgrown. *Table* 3.7 shows the number of P1 bacteriophage particles present in the lysate produced from *S. typhimurium* CH1026

Table 3.7 Number of P1 bacteriophage particles, derived from *S. typhimurium* CH1026, that would plaque on *S. typhimurium* CH223.

Phage Dilution	pfu	pfu ml^{-1}
10^{-2}	TNTC	-
10^{-4}	TNTC	-
10^{-6}	808	$8.1x10^{9}$
10^{-8}	9	$9.0x10^{9}$
No cells	0	0
No phage	0	0

TNTC - Too Numerous To Count

pfu - Plaque Forming Units

The phage titre was quite low at $8.5x10^{9}$ pfu ml^{-1}. However it could be used to transduce *S. senftenberg* 775W to chloramphenicol resistance. One hundred microlitres of 18 hr cultures of *S. senftenberg* 775W and *S. typhimurium* CH223 grown at 37°C and mixed with 0.1 ml of P1 phage at dilutions of 10^{0}, 10^{-2}, 10^{-4}, and 10^{-6}. These were incubated at 30°C for 30 min to allow phage attachment, prior to mixing with 5 ml of LC top chloramphenicol (50 μg ml-1) agar and poured on LC chloramphenicol (50μg ml-1) agar plates. The plates were incubated at 30°C for 24 h. Table 3.8 shows the number of transductants of *S. typhimurium* CH223 and *S. senftenberg* 775W to chloramphenicol resistance.

Table 3.8 Number of *S. typhimurium* CH223 and *S. senftenberg* 775W chloramphenicol resistant colonies after transduction with P1 bacteriophage derived from *S. typhimurium* CH1026

Phage Dilution	CH223 cfu	775W cfu
10^{0}	TNTC	49
10^{-2}	TNTC	3
10^{-4}	31	0
10^{-6}	0	0

TNTC - Too Numerous To Count
cfu - Colony Forming Units

The number of transduced *S. typhimurium* CH223 was 3.1×10^{6} ml^{-1} while there were only 490 transduced *S. senftenberg* 775W's per ml. The *S. senftenberg* 775W transduced colonies were very different sizes, and except for the very small colonies, gave rise to normal sized colonies when streaked on to LB chloramphenicol (50 μg ml^{-1}) agar plates. These transductants were streaked through two sets of green plates and re-streaked on to chloramphenicol (50 μg ml^{-1}) agar plates. As with the tetracycline resistant *S. senftenberg* 775W's transduced by P22, they were not antibiotic resistant. It must therefore be assumed that without the environmental pressure of chloramphenicol, *S. senftenberg* 775W will lose the P1 phage DNA. The colonies from the LB chloramphenicol (50 μg ml^{-1}) agar plates remained chloramphenicol resistant when re-streaked on to fresh LB chloramphenicol (50 μg ml^{-1}) agar plates. These transductants were tested for heat resistance in the high temperature incubator and also for temperature sensitivity, which would indicate the presence of the P1 phage, (*Table* 3.9).

Table 3.9 Sensitivity/resistance of *S. senftenberg* 775W transduced with P1 bacteriophage derived from *S. typhimurium* CH1026; to chloramphenicol, heat and growth temperature.

Colony	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Cml^R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$Heat^R$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth @ 30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth @ 42°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.5.2.1 Production of a P1 lysate from *S. senftenberg* SM002

The P1 phage should lysogenise when *S. senftenberg* 775W is grown at 42°C and give rise to a P1 phage population with modified DNA that would not be restricted in *S. senftenberg* 775W. A P1 lysate was therefore prepared from a lysogenic strain of *S. senftenberg* 775W, SM002, using the method described in section 2.13.4. The lysate was then used to plaque *S. typhimurium* CH223 and *S. senftenberg* 775W. (method as before). *Table* 3.10 shows the number of plaques formed on each strain.

Table 3.10 The quantitative ability of bacteriophage P1 derived from *S. senftenberg* SM002 to plaque on *S. senftenberg* 775W and *S. typhimurium* CH223.

	Phage dilution Number of plaques per plate			
Strain	10^0	10^{-2}	10^{-4}	10^{-6}
S. typhimurium CH223	0	0	0	0
S. senftenberg 775W	150	0	0	0

The number of plaques seen on each of the strains is not easily explained. With no plaques on *S. typhimurium* CH223 it would suggest that the DNA of P1 has indeed been modified and that restriction has taken place on entering *S. typhimurium* CH223. However the low number of plaques on *S. senftenberg* 775W might well suggest that the DNA has not been modified and is restricted once inside *S. senftenberg* 775W. It was therefore decided to try to improve attachment by using varying levels of $MgSO_4$ and $CaCl_2$. Seven hundred microlitres of each cell type was spun down and resuspended in the following concentrations of $MgSO_4$ and $CaCl_2$ prior to mixing with the 0.1 ml of the phage preparation: 10 mM $MgSO_4$ + 5 mM $CaCl_2$; 10 mM $MgSO_4$ + 10 mM $CaCl_2$; 10 mM $MgSO_4$ + 50 mM $CaCl_2$; 10 mM $MgSO_4$ + 100 mM $CaCl_2$; 0 mM $MgSO_4$ + 50 mM $CaCl_2$; 0 mM $MgSO_4$ + 100 mM $CaCl_2$.

The concentrations of $MgSO_4$ and $CaCl_2$ that gave the highest number of plaques on *S. senftenberg* 775W was 10 mM $MgSO_4$ + 10 mM $CaCl_2$, and the results in *Table* 3.11 indicate the number of plaques formed by P1 on *S. senftenberg* 775W and *S. typhimurium* CH223 using these concentrations of $MgSO_4$ and $CaCl_2$ in the LC top agar.

Table 3.11 <u>The quantitative ability of bacteriophage P1 derived from *S. senftenberg* SM002 to plaque on *S. senftenberg* 775W and *S. typhimurium* CH223 suspended in 10 mM $MgSO_4$ and $CaCl_2$.</u>

Strain	Phage dilution / Number of plaques per plate			
	10^0	10^{-2}	10^{-4}	10^{-6}
S. typhimurium CH223	0	0	0	0
S. senftenberg 775W	180	1	0	0

Table 3.11 indicates that there was no real increased in the number of plaques observed. It was therefore decided to try and plaque the P1 lysate from *S. senftenberg* 775W on a *S. senftenberg* 775W that had already plaqued P1. First of all the P1 lysogenic strain of *S. senftenberg* 775W, SM002, had to be cured of the P1 bacteriophage.

3.5.2.2 Removal of P1 phage

An 18 hr culture of the lysogenic *S. senftenberg* 775W, SM002, was grown at 30°C and 0.1 ml was plated out on to LB agar plates at 1/100th and 1/1000th dilutions, and incubated at 37°C and 42°C for 24 h. Two hundred and forty colonies grew up on the 1/1000th dilution incubated at 42°C and 16 were picked and restreaked on to fresh LB agar plates and incubated at 30°C. These were then streaked on to LB agar and LB chloramphenicol (50μg ml^{-1}) agar plates and each set incubated at 30°C for 18 hr. All colonies grew on the LB agar plate while some did not grow on the LB chloramphenicol (50μg ml^{-1}) agar plate. It was assumed that those that did not grow on the LB chloramphenicol (50μg ml^{-1}) agar plate had lost the P1

phage, but this was double-checked by regrowing them on LB plates at 42°C. One of these colonies was then used to plaque the lysate from the lysogenic *S. senftenberg* 775W, SM031.

Table 3.12 <u>The quantitative ability of bacteriophage P1 derived from *S. senftenberg* SM002 to plaque on *S. typhimurium* CH223 and *S. senftenberg* 775W, SM031 which had been cured of the bacteriophage P1.</u>

	Phage dilution Number of plaques per plate			
Strain	10^0	10^{-2}	10^{-4}	10^{-6}
S. typhimurium CH223	0	0	0	0
S. senftenberg SM031	TNTC	TNTC	TNTC	TNTC

Table 3.12 shows that *S. senftenberg* 775W, SM031, that had been infected by and cured of P1, plaqued P1 derived from *S. senftenberg* 775W, SM002, at a frequency of $>3.0\times10^9$ pfu ml^{-1}. This would suggest that the attachment site for P1 was present in this strain and that is why it has a much higher plaquing frequency than the wild type *S. senftenberg* 775W.

Although P1 was now able to plaque on *S. senftenberg* 775W and lysates were available, no recipient strain of *S. typhimurium* was available to transduce genetic material into. The *S. senftenberg* 775W lysates could have presumably been transduced in to *S. senftenberg* JT577, but the genetic background of this organism is unknown and any further work would prove difficult. It was therefore decided to produce a genomic library from *S. senftenberg* 775W chromosomal DNA.

3.6 Gene library

Although the bacteriophages P22 and P1 are ideal genetic vectors they did not prove useful in the transfer of genetic material between *S. senftenberg* 775W and *S. typhimurium*. It was decided to construct a library of the *S. senftenberg* 775W genome using an alternative vector, i.e., a plasmid. By joining regions of the *S. senftenberg* 775W chromosome to a plasmid, the phenotypic nature of the *S. senftenberg* 775W genes could be expressed and the effects on the host bacterium measured.

3.6.1 Making a plasmid library

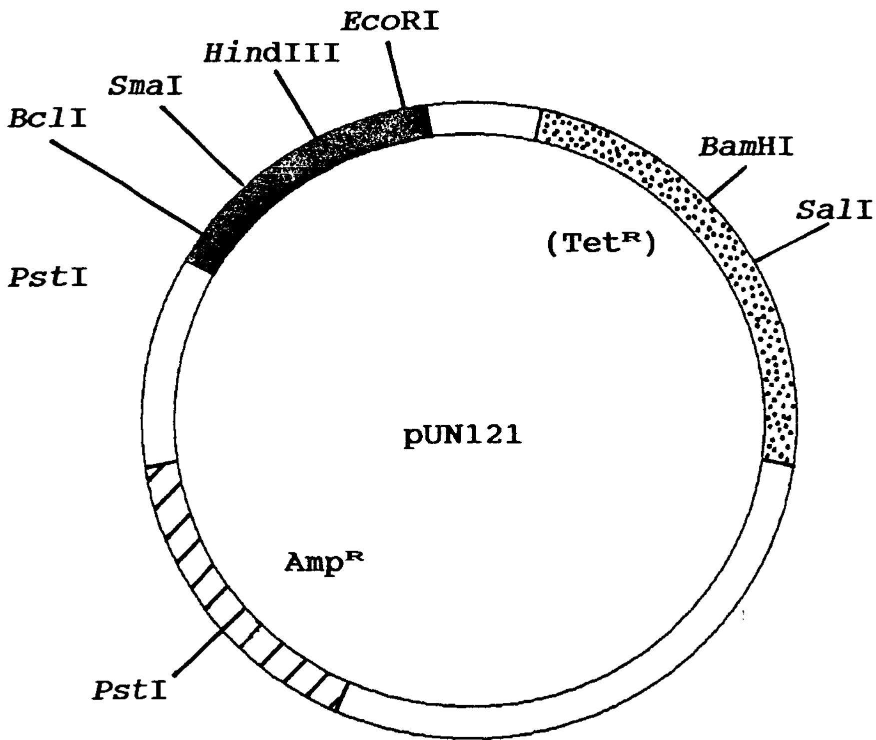
3.6.1.1 Preparation of plasmid DNA

The plasmid used was pUN121, a positive selection plasmid, (*Figure* 3.7). Under circumstances where the plasmid does not contain cloned DNA, the lambda *CI* gene present expresses lambda repressers which binds to the operator and prevents the promotor from functioning; therefore the plasmid does not confer tetracycline resistance. When foreign DNA fragments are inserted in to the *CI* gene, no lambda repressors is produced enabling the promotor to function and consequently tetracycline resistance is expressed. The cloning site used was the *Bcl*I site which produced ends compatible with *Sau*3A-cut chromosomal DNA. A plasmid preparation of pUN121 was carried out, see (section 2.14.2), and the resulting plasmid DNA concentration was determined using the spectrophotometric method, described in section 2.17.4. The plasmid DNA was then cut with *Bcl*I, (2.17.1).

3.6.1.2 Preparation of chromosomal DNA

S. senftenberg 775W chromosomal DNA was obtained using the method described in section 2.15, and the DNA

Figure 3.7 Restriction map and genetic markers in the positive selection plasmid pUN121



concentration was measured, (2.17.4). Once a clean sample of chromosomal DNA was obtained, a partial digest using *Sau*3A was performed, (2.17.1).

3.6.1.3 Ligation of DNA fragments

So as to get the most efficient ligation mixture of vector and partial *S. senftenberg* 775W DNA digest, a specific size of *S. senftenberg* 775W DNA fragment had to be obtained. The size range 4-6 kb would give the optimum return of the vector-fragment ligation and so the *S. senftenberg* 775W DNA partial digest was size fractionated using a sucrose gradient, (2.17.6), and the concentration of the 4-6 kb fragments was measured using the method in 2.17.4.

To 3.5 μg of *Bcl*I-cut pUN121 and 3.5 g of 4-6 kb *Sau*3A-cut *S. senftenberg* 775W DNA was added; 10 μl of 10 x ligation buffer, 5 μl T_4 DNA ligase and made up to 0.1 ml with 5 mM Tris-HCl pH 7.6. This mixture was left at room temperature overnight.

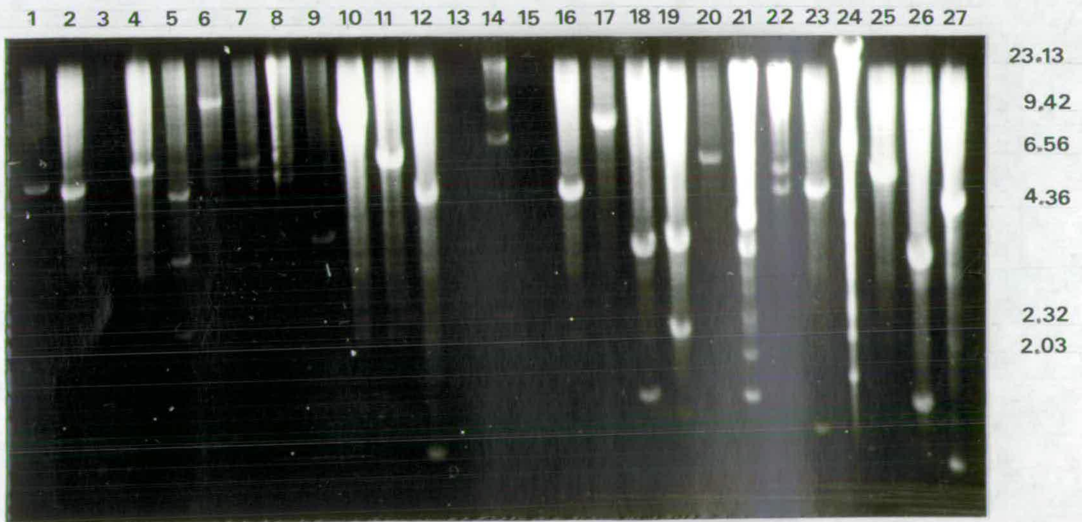
3.6.2 Transformation of host bacteria

The bacteria used as the recipient strain for the vector-fragment construct was *E. coli* MM294. The ligation mix from 3.6.1.3 was transformed in to competent *E. coli* MM294, (2.18.2). The resulting tetracycline and ampicillin resistant transformants were pooled and stored in 7% DMSO. Mini-preps, (2.14.1), were performed on some of the transformants and five microlitres of the resulting plasmid DNA was digested with *Bcl*I, (2.17.1). *Figure* 3.8 shows the digest of each of the transformants after separation on a 0.8% agarose gel. The average size of the inserts was 4-6 kb.

Figure 3.8 <u>*BclI* digests of vector:fragment constructs from transformed *E. coli* MM294</u>

Photograph showing *BclI* cut, plasmid (pUN121)/ fragment (*S. senftenberg* 775W chromosomal DNA) constructs and lambda standard (*Hind*III digested) DNA run on an 0.8% agarose gel.

Lane 1 - 12	pUN121/*S. senftenberg* 775W DNA constructs
Lane 13	*BclI* cut pUN121
Lane 14	Lambda standard (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 0.56 kb)
Lane 15	Uncut pUN121
Lane 16 - 27	pUN121/*S. senftenberg* 775W DNA constructs



3.6.3 Characterisation of host bacteria

Before the gene bank could be screened for genes conferring heat resistance, the heat resistance of the strain to be the host, *E. coli* MM294, had to be characterised and compared with the three organisms under study. By doing this, any alterations in the heat resistance of *E. coli* MM294 could be measured and conclusions drawn about the possible cause. A thermal survival curve of mid-logarithmic phase *E. coli* MM294, at 55°C in LB, was performed as described in section 2.11.1, but using the spectrophotometer to measure cell density. *Figure* 3.9 shows the survival curves of mid-logarithmic phase *S. senftenberg* 775W, JT577, *S. typhimurium* SA2009 and *E. coli* MM294 when exposed at 55°C in LB. *E. coli* MM294 had only a slightly lower heat resistance than *S. typhimurium* SA2009 for the first 3.5 min at 55°C. After 3.5 min, survival of *E. coli* MM294's fell rapidly for the remainder of the experiment, 8 min, when survival was almost 1.5 logs lower than *S. typhimurium* SA2009. This shape of curve was consistent, and allowed scope for an increase in survival due to the presence of genetic material from *S. senftenberg* 775W, to levels between *E. coli* MM294's survival and that of *S. senftenberg* 775W. The resistance of *E. coli* MM294, compared with the other strains studied, was measured using the modified surface plate method, (3.3.2). *Table* 3.13 shows the presence or absence of growth after exposure in a high temperature incubator at 55°C followed by incubation afterwards at 37°C for 18-24 hr.

From *Table* 3.13, wild type *E. coli* MM294 can be seen to have the same ability to survive at 55°C on the surface of an agar plate as all the other Salmonellae investigated, except *S. senftenberg* 775W. Again this difference would allow for any increase in resistance of transformed *E. coli* MM294 to be picked up. It was hoped that the transformants with increased resistances, would

Figure 3.9 <u>Survival of *S. senftenberg* JT577, 775W, *S. typhimurium* SA2009 and *E. coli* MM294 in LB at 55°C</u>

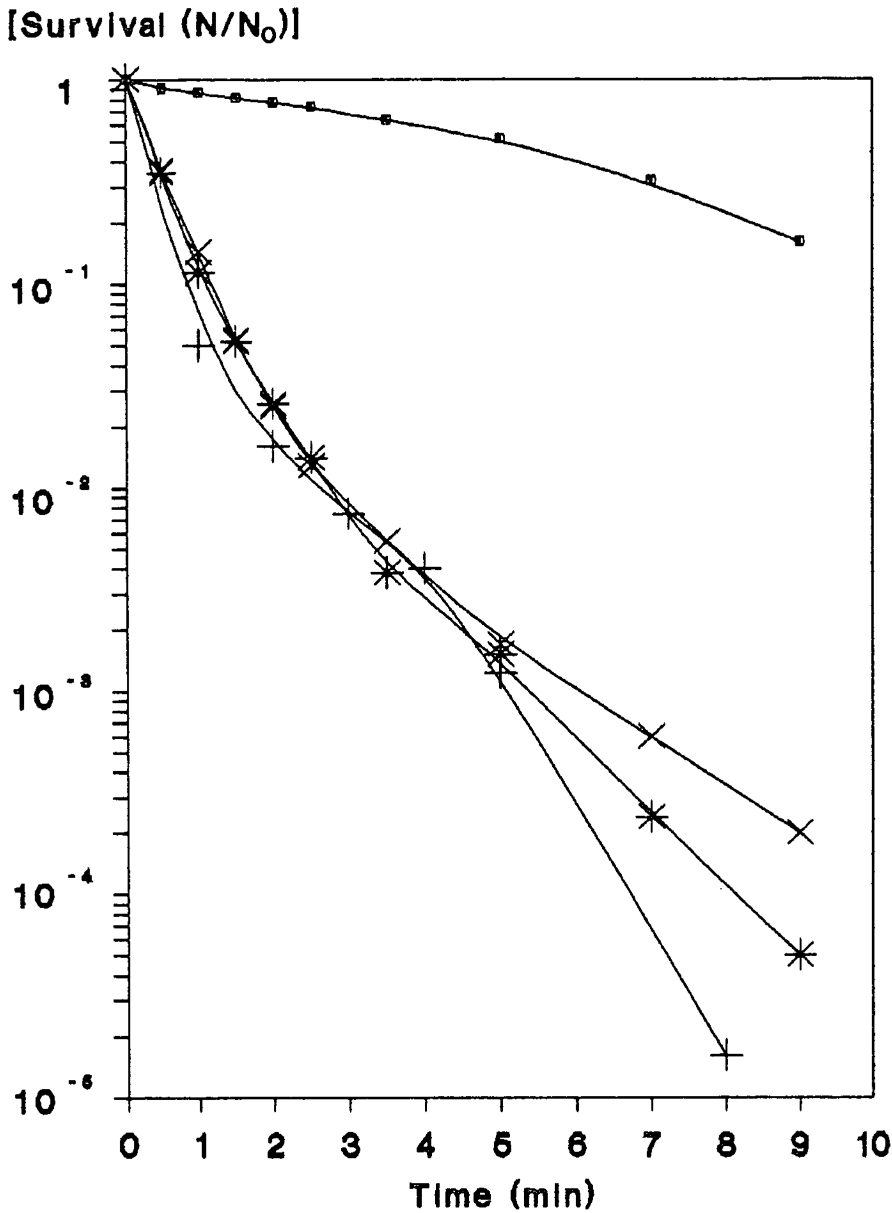


Figure 3.9. Survival (N/N_o), in 180 ml of LB at 55°C of 20 ml of mid-logarithmic phase cells of *S. typhimurium* SA2009 (×), *S. senftenberg* JT577 (*), *S. senftenberg* 775W (▪) and *E. coli* MM294 (+)

first be picked up using the plate method and then the heat resistance could be measured more accurately using the method described in section 2.11.1, but using the spectrophotometer to measure cell density.

Table 3.13 Presence or absence of growth of strains of *S. typhimurium S. senftenberg, E. coli* after exposure to 55°C on the surface of an LB agar plate

	Duration of exposure to 55°C in min						
	Growth after exposure to 55°C						
Strain	0	45	46	47	48	49	50
SA2009	+	+	+	-	-	-	-
MM294	+	+	+	-	-	-	-
MR18	+	+	+	-	-	-	-
MR19	+	+	+	-	-	-	-
MR20	+	+	+	-	-	-	-
MR21	+	+	+	-	-	-	-
775W	+	+	+	+	+	+	+
JT577	+	+	+	-	-	-	-

3.6.4 Multiplication of the heat resistant transformants

If heat resistant transformants were produced, it was assumed that their presence could be identified and multiplied by heat cycling using a method similar to that described in section 2.11.6.

From the pooled *E. coli* MM294 transformants, 10 μl was inoculated in to 50 ml of LB in a 250 ml conical flask and incubated at 37°C in a shaking incubator. The OD_{600} was measured until it reached 0.1, and at this point 20 ml was transferred into 180 ml of LB in a 1 l flask which was submerged up to the neck in a shaking

water bath at 55°C. After 10 min, 1 ml was diluted into 9 ml of LB containing $12.5\mu g\ ml^{-1}$ of tetracycline and $20\mu g\ ml^{-1}$ of ampicillin and incubated at 37°C for 18 hr in a shaking incubator, and 0.1 ml was plated on to five LB ($12.5\mu g\ ml^{-1}$ tetracycline and $20\mu g\ ml^{-1}$ ampicillin) agar plates and incubated at 37°C overnight. This procedure was repeated using the cells from the previous heat inactivation, but this time the initial OD_{600} in the 50 ml of LB was adjusted to 0.003. After each cycle the average percentage survival from the five plates was calculated, using the viable count from the unheated cells to give the initial value. The percentage survival was 0, $1.2x10^{-4}$, $2.6x10^{-4}$ and $4.3x10^{-3}$ for cycles 0, 1, 2 and 3 respectively. This percentage survival change was equivalent to the average number of survivors per plate increasing from <1, to 6, 13 and 215. A control of untransformed *E. coli* MM294 was also cycled, however the number of survivors remained at <1. The absence of increasing cell numbers in the control, suggests that amplification of the transformed survivors was in part or in whole due to the presence of a small number of heat resistant transformants.

3.6.5 Search for heat resistant transformants

The pooled *E. coli* MM294 transformants were diluted in LB so that when 0.1 ml was spread on an LB (12.5 g ml^{-1} tetracycline and $20\mu g\ ml^{-1}$ ampicillin) agar plate and incubated at 37°C for 18 hr, it would give approximately 100 colonies. Cells from these colonies were then heat inactivated for increasing time intervals on the surface of an LB ($12.5\mu g\ ml^{-1}$ tetracycline and $20\ \mu g\ ml^{-1}$ ampicillin) agar plate using the modified surface plate method described in section 3.3.2. After exposure at 55°C, each plate was incubated at 37°C for 18-24 hr. The presence or absence of growth at 24 hr was noted and the exposure times compared with those obtained for wild type *E. coli* MM294 and *S. senftenberg* 775W.

Apart from variations in heat resistance, the transformants fell in to two categories, those producing smooth colonies and those producing rough colonies. The rough colonies were very sticky and posed problems when trying to take a portion of the colony for the modified surface plate heat inactivation method. However, the nature of the colony did not seem to bear any relation to the resistance or otherwise of the transformants. Both rough and smooth colonied transformants had heat resistances that were equal to or greater than that of the parental strain, and also no transformant studied showed a resistance lower than wild type *E. coli* MM294.

Only 630 transformants were screened for altered heat resistances using the modified surface plate method, but of those, several were discovered to have appreciably higher heat resistances than that of wild type *E. coli* MM294. *Table* 3.14 shows the presence or absence of growth of those cells found to have the highest heat resistances using the modified surface plate method.

All four transformants were found to have heat resistances, on the surface of an LB agar plate at 55°C, that exceeded those of the wild type. The transformants R6 and R22 were found to have the highest resistances, with resistance lasting at least 4 min longer than the wild type. Transformants S6 and R101 has resistances of 2 and 1 minutes respectively, greater than the wild type.

Table 3.14 Presence or absence of growth of strains of *S. typhimurium* and *S. senftenberg* and *E. coli* after exposure to 55°C on the surface of an LB agar plate

	Duration of exposure to 55°C in min						
	Growth after exposure to 55°C						
Strain	0	45	46	47	48	49	50
SA2009	+	+	+	-	-	-	-
MM294	+	+	+	-	-	-	-
R6	+	+	+	+	+	+	+
S6	+	+	+	+	+	-	-
R22	+	+	+	+	+	+	+
R101	+	+	+	+	-	-	-
775W	+	+	+	+	+	+	+

Although the resistances of S6 and R101 were not significantly greater, the values were consistent. The next step was to compare the resistances of the transformants at 55°C in liquid broth and *Figure* 3.10 compares the survival curves of *E. coli* MM294, *S. senftenberg* 775W and the four transformants. The survival curves of the transformants fell in to two categories, of which R22 and R6 were in one and R101 and S6 were in the other. All four transformants had survival curves that were greater than wild type. The curve of R22 followed the survival of *S. senftenberg* 775W for the first 2.5 min and then fell to approximately 1 log lower after 9 min but was 3 logs higher than that of wild type *E. coli* MM294. Survival of R6 was initially lower than R22 but the change in resistance mirrored closely that of R22. After 5 min the survival curve of R6 followed exactly that of R22. The survival of S6 and R101 followed each other closely too, except here the final survival was very close to that of wild type *E. coli* MM294, but the initial survival was over 1 log

Figure 3.10 <u>Survival of *S. senftenberg* 775W, *E. coli* MM294 and *E. coli* MM294 transformants in LB at 55°C</u>

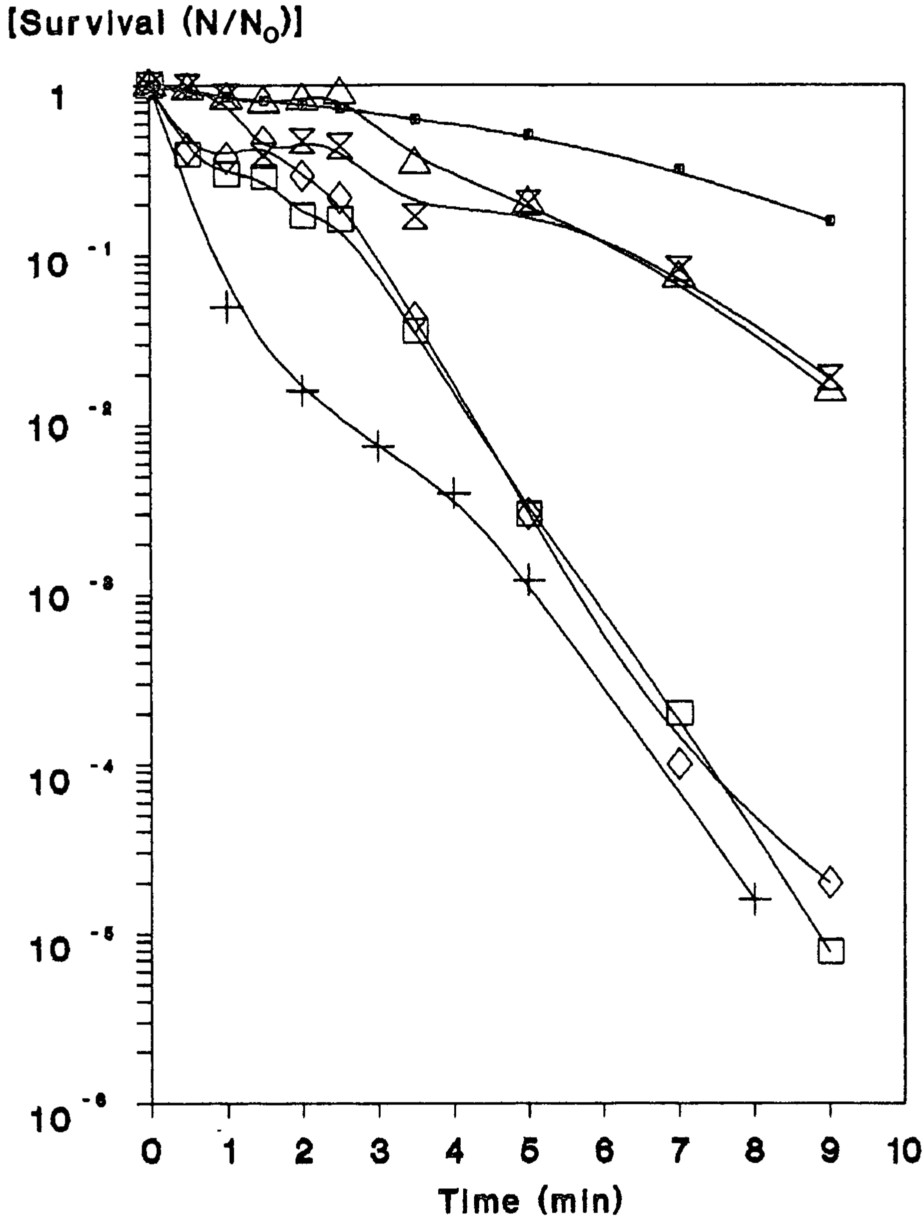


Figure 3.10. Survival (N/N_o), in 180 ml of LB at 55°C of 20 ml of mid-logarithmic phase cells of *S. senftenberg* 775W (•), *E. coli* MM294 (+) and *E. coli* MM294 transformants R6 (⧖), S6 (◇), R101 (□) and R22 (△).

higher. It was decided to see if the increasing survival of the transformed *E. coli* MM294 cells was due to the presence of the vector, pUN121, or the vector:fragment ligation. A mini-prep, (2.14.1), was performed on each of the four transformants and the individual plasmids transformed back in to *E. coli* MM294. Along with the vector:ligation transformants, *E. coli* MM294 cells were transformed with the vector alone to see if this affected heat resistance. Thermal survival curves were obtained for the five transformants and compared against those of wild type *E. coli* MM294 and *S. senftenberg* 775W. *Figure* 3.11 shows the survival of each of the strains and survival did not vary appreciably between that seen in *Figure* 3.10. The survival of the *E. coli* MM294 transformed with pUN121 was almost identical to that of the wild type. Although further work, for which sufficient time was not available, was necessary to provide conclusive evidence that the elevated resistance seen in the transformed *E. coli* MM294, was attributable to the DNA fragments from *S. senftenberg* 775W, the balance of argument was in its favour.

3.7 Physiological traits

It was decided to examine physiological traits to see if they could go some way to explain *S. senftenberg* 775W's elevated heat resistance. As much work had already been carried out on *S. typhimurium* SA2009 in this project, it was used as a reference against which *S. senftenberg* 775W could be measured. To make sure that any differences observed between *S. typhimurium* SA2009 and *S. senftenberg* 775W were not just difference between the species, a non-heat-resistant strain of *S. senftenberg* was obtained; *S. senftenberg* JT577.

Figure 3.11 <u>Survival of *S. senftenberg* 775W, *E. coli* MM294 and *E. coli* MM294 transformants in LB at 55°C</u>

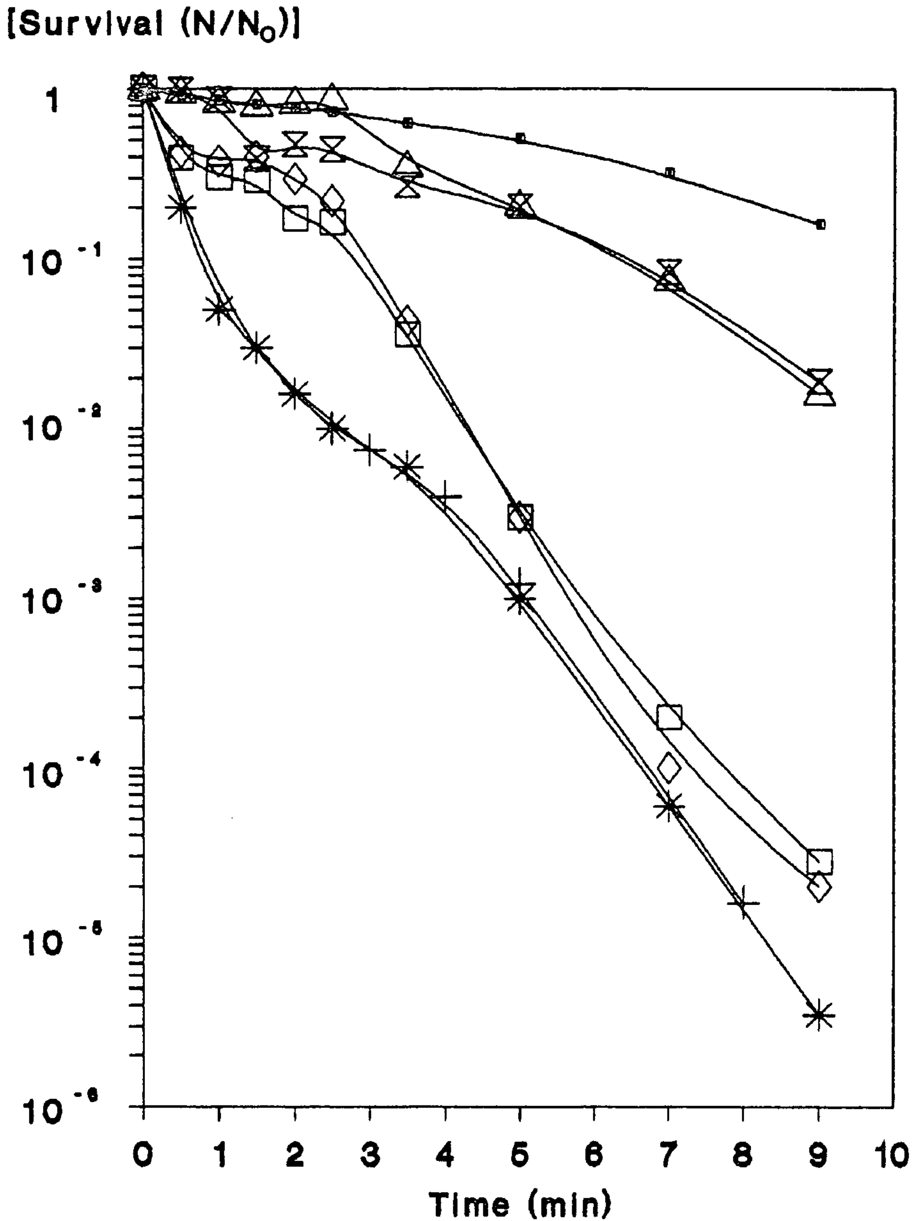


Figure 3.11. Survival (N/N_o), in 180 ml of LB at 55°C of 20 ml of mid-logarithmic phase cells of *S. senftenberg* 775W (▪), *E. coli* MM294 (+), *E. coli* MM294+pUN121 (*) and *E. coli* MM294 transformants using plasmids from the transformants R6 (⧖), S6 (◇), R101 (□) and R22 (△).

3.7.1 Growth temperatures

S. senftenberg 775W's growth temperature parameters had never been studied as far as could be determined from the literature. It was therefore quite feasible to consider that *S. senftenberg* 775W's ability to survive at these elevated temperatures was merely a physical effect of having an elevated maximum growth temperature. This maximum growth temperature could be close to those used for the heat inactivation experiments and would therefore not have the same lethal effect as it had on *S. typhimurium* SA2009. The maximum growth temperature of each organism was measured.

An aliquot of an 18 h culture grown at 37°C was transferred to 100 ml of fresh LB in a conical flask, pre-conditioned in a shaking water bath set at the required temperature. Samples were taken at set time intervals and measured against an LB blank in a spectrophotometer. *Figures* 3.12 and 3.13 show the growth curves of *S. senftenberg* 775W, *S. senftenberg* JT577 and *S. typhimurium* SA2009 at 37°, 39°, 42°, 46° and 48°C. These figures show all three organisms had the same growth rates and maximum growth temperatures.

3.7.2 Additional genetic material

The presence or absence of plasmid DNA in *S. senftenberg* 775W was investigated, since plasmids are often the site of genes conferring additional physiological characteristics. Both strains of *S. senftenberg* were treated as described in section 2.14.2, and their DNA run on an agarose gel that would reveal both high and low molecular weight plasmids. After staining the gel with ethidium bromide, no bands were observed in those lanes containing *S. senftenberg* 775W or *S. senftenberg* JT577, suggesting that neither contained plasmids. However the possibility that a plasmid had

Figure 3.12 <u>Growth of *S. senftenberg* 775W, JT557 and *S. typhimurium* SA2009 in LB at 37°C</u>

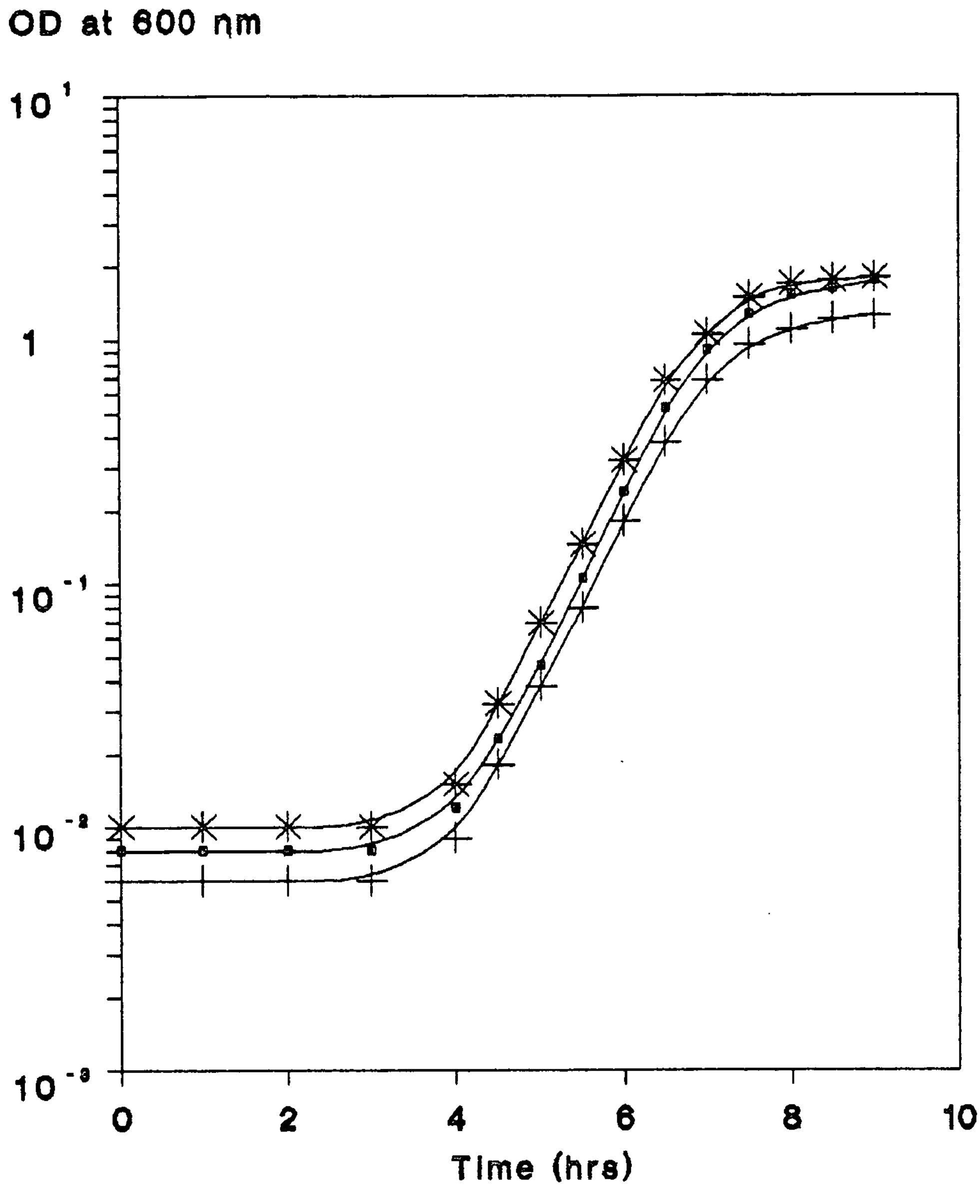


Figure 3.12. Growth curve of cultures incubated in 100 ml of LB in a shaking water bath at 37°C. Growth was assayed at 600 nm. *S. senftenberg* 775W (*), *S. senftenberg* JT577 (▪) and *S. typhimurium* SA2009 (+).

Figure 3.13 Growth of *S. senftenberg* 775W, JT557 and *S. typhimurium* SA2009 in LB at 39°C, 42°C 46°C and 48°C (from top left to bottom right)

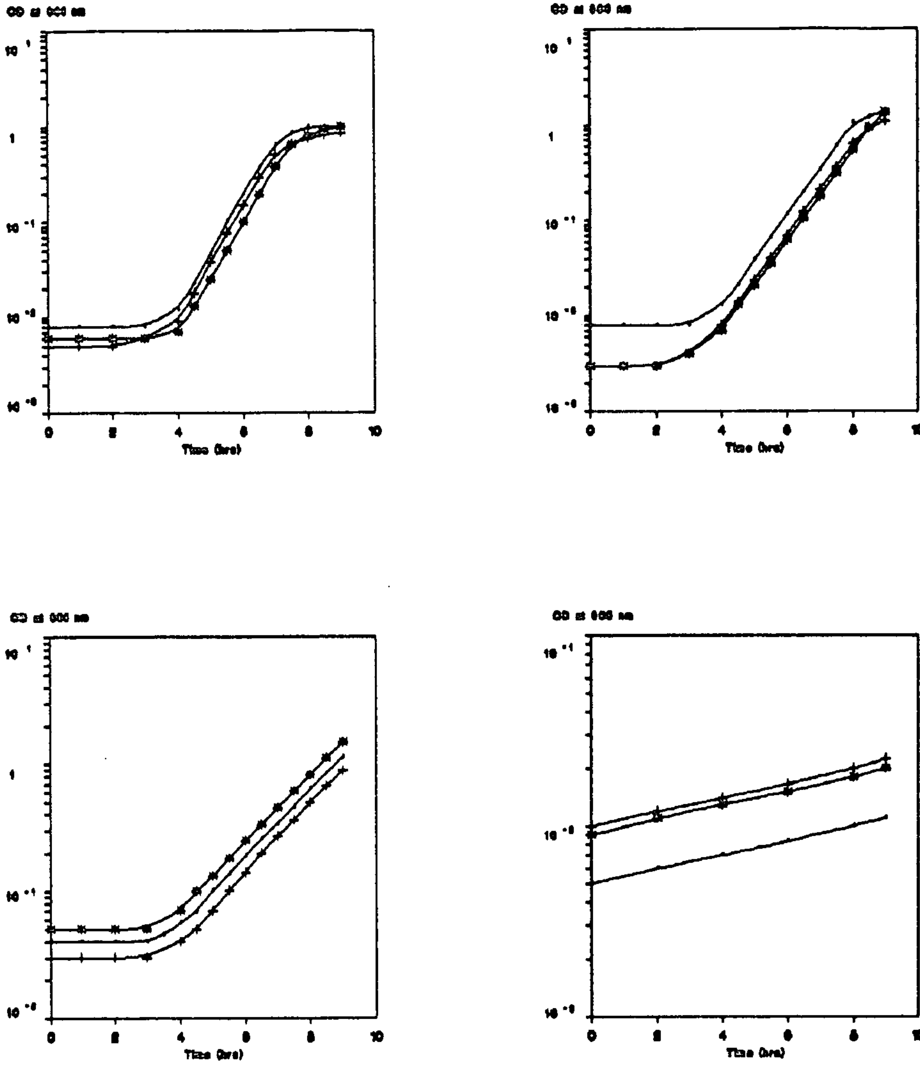


Figure 3.13. Growth curve of cultures incubated in 100 ml of LB in a shaking water bath at 39°C, 42°C 46°C and 48°C (from top left to bottom right). Growth was assayed at 600 nm. *S. senftenberg* 775W (*), *S. senftenberg* JT577 (▪) and *S. typhimurium* SA2009 (+).

recombind with the chromosome could not be ruled out nor that the plasmids were extremely large and fragmented along with the chromosome.

3.7.3 DNA repair, γ-irradiation resistance

The single- and double-strand DNA breaks induced at mild-heating temperatures 52°-55°C, are similar to those that occur in cells exposed to ionizing irradiation. If DNA repair is essential as part of total cell recovery in heat-treated cells, as it is in γ-irradiated cells, then an organism containing an efficient DNA repair system may well recover faster after heat treatment. If *S. senftenberg* 775W were able to repair DNA damage caused by heat treatment more efficiently than other strains of *Salmonella*, it might have been expected to have been able to repair γ-irradiated induced damage more efficiently too. *S. senftenberg* 775W, JT577 and *S. typhimurium* SA2009 were exposed to gamma rays in a ^{60}Co source (see section 2.12) for 75 min, and survival measured through the course of exposure. *Figure* 3.14 shows *S. senftenberg* 775W to have a slightly greater ability to survive exposure to ^{60}Co γ-irradiation than *S. senftenberg* JT577 or *S. typhimurium* SA2009. However the magnitude of this increased survival was quite small and certainly not sufficient in itself to explain *S. senftenberg* 775W's increased thermal survival capabilities.

3.7.4 Recovery after heat treatment

After thermal injury, bacteria go through what is generally termed an extended lag prior to cell division restarting. During this lag period cellular damage induced by thermal exposure is repaired, and the length of this lag period can be varied by altering the recovery media (Iandolo & Ordal, 1966). It was decided to compare the recovery capabilities of *S. senftenberg* 775W, JT577 and *S. typhimurium* SA2009 when recovered in the same rich

Figure 3.14 <u>Survival of *S. senftenberg* 775W, JT577 and *S. typhimurium* SA2009 after exposure to a cobalt-60 γ-irradiation source</u>

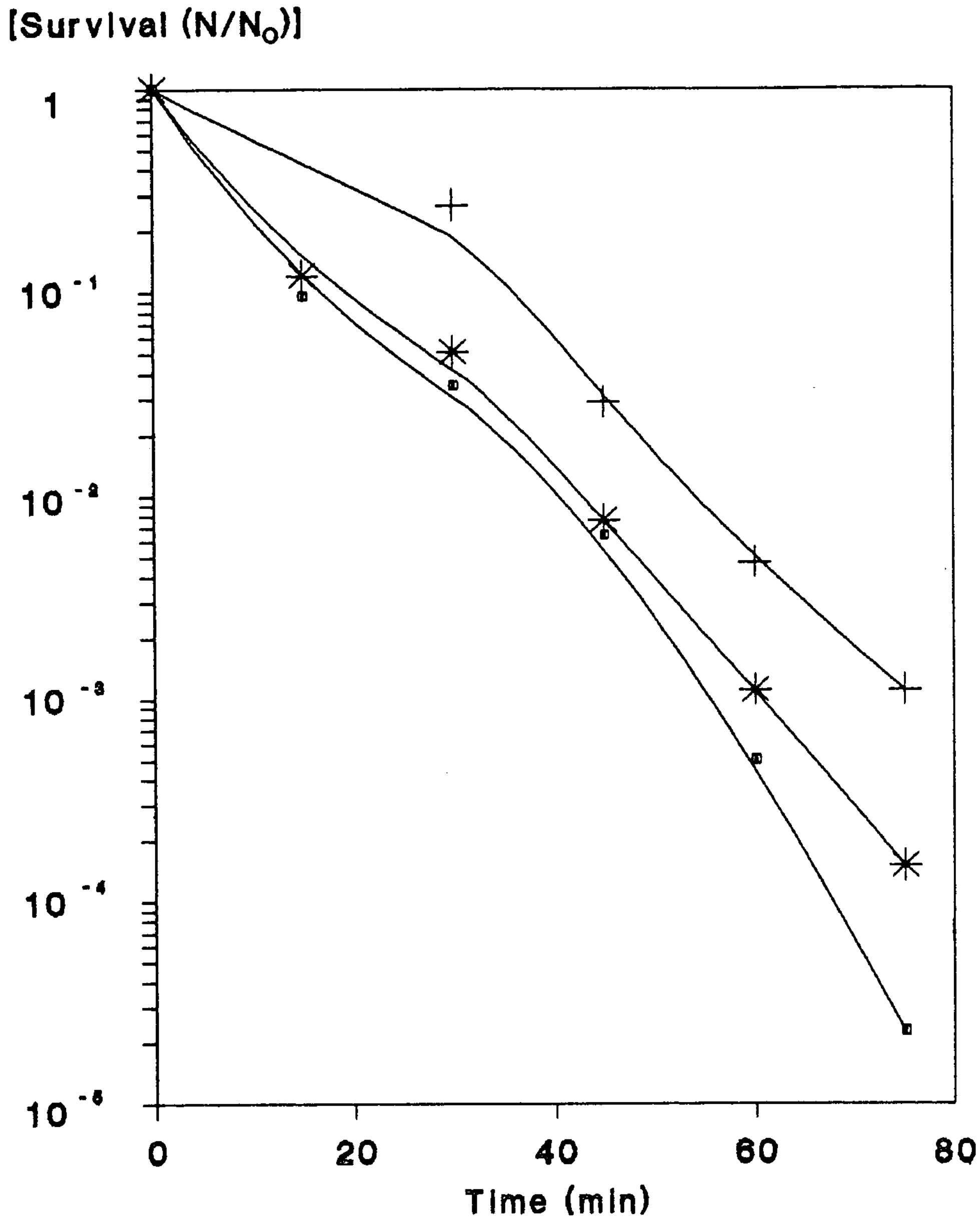


Figure 3.14. Survival of *S. senftenberg* 775W (+), *S. typhimurium* SA2009 (▪) and *S. senftenberg* JT577 (*) when exposed to a ^{60}Co γ-irradiation source and recovered on LB agar at 37°C.

medium, Luria Broth, after exposure to the same degree of thermal injury.

All three organisms were thermally treated according to section 2.11.2 and recovered at 37°C in a shaking water bath. As a control, a 200 ml cell suspension in LB of each organism, at the same stage in the growth cycle and at the same initial OD_{600}, was placed in a 37°C shaking water bath. Recovery was measured by monitoring the change in OD_{600}.

All three organisms exhibited an extended lag post thermal injury, (*Figure* 3.15). The lag period of *S. senftenberg* JT577 and *S. typhimurium* SA2009 were very similar at about 3.25 hr, after which time cell division restarted. *S. senftenberg* 775W had a 2.25 hr lag period, prior to cell division recommencing. After the lag period all three organisms showed increases in OD_{600} equivalent to that of the control. The control curve in *Figure* 3.15 represents all three organisms, as they all had almost identical growth curves. All three recovering organisms reached terminal OD_{600} values very similar to that of the control. The next step was to see if *S. senftenberg* 775W's elevated heat resistance was due to more efficient repair systems for repairing damaged cellular sites, post thermal injury.

3.7.4.1 Requirements for the recovery from thermal injury.

The single- and double-strand breaks induced in DNA by ^{60}Co γ-irradiation have already been discussed in section 3.7.3. This section will look at the use of metabolic inhibitors post thermal injury, and their effect on recovery. This method has been well documented in *S. typhimurium* (Tomlins & Ordal, 1971).

Figure 3.15 <u>Growth of *S. senftenberg* 775W, JT577 and *S. typhimurium* SA2009 during recovery from a three log reduction in survival at 55°C</u>

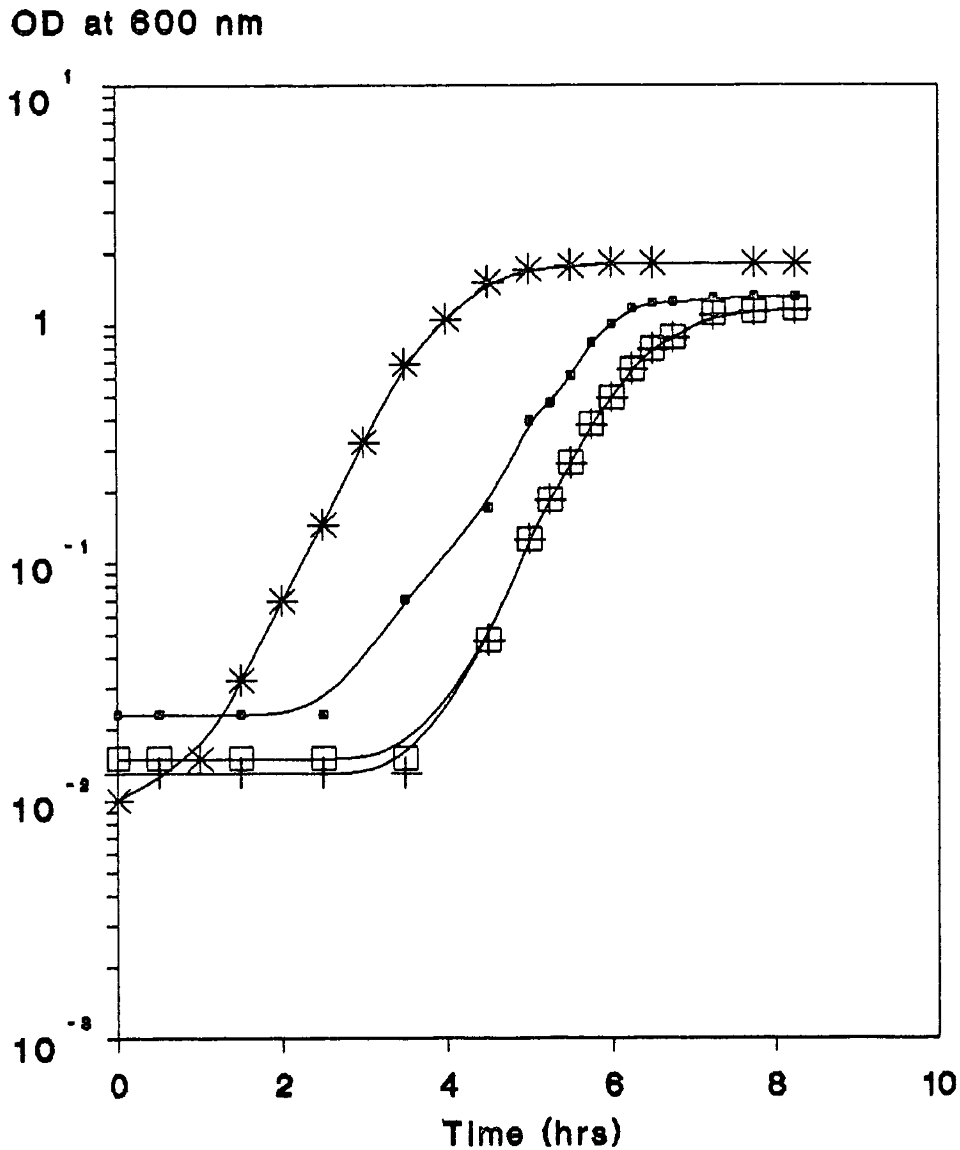


Figure 3.15. Recovery and growth at 37°C in LB after a standard thermal exposure (2.11.2) in LB at 55°C, measured by the change in OD at 600 nm of *S. senftenberg* 775W (■), *S. typhimurium* SA2009 (□) and *S. senftenberg* JT577 (+). A control (*) represents the growth of all three organisms at 37°C when not exposed to a standard thermal exposure.

Thermally damaged cells were recovered in the presence of metabolic inhibitors at the highest concentration that allowed growth. The concentrations were worked out for each organism by inoculating 0.1 ml of an 18 hr culture of the organism, into 100 ml of LB at 37°C which contained a known concentration of the metabolic inhibitor. The OD_{600} was then measured every 30 min. The concentration chosen was the highest at which cell division still took place.

The method used for thermal inactivation was described in section 2.11.2. Two 20 ml samples of the heat inactivated cells were transferred into two 500 ml conical flasks, one containing 120 ml of fresh LB, the other 120 ml of fresh LB and an appropriate concentration of antibiotic. These were then incubated at 37°C in a shaking water bath from which aliquots were taken for enumeration of survivors on LB agar and LB + (7%) NaCl agar plates. The number of surviving cells could be obtained from the LB agar plate count, while the LB + (7%) NaCl agar plate gave the number of un-injured cells. The number of injured cells could therefore be calculated, it being the difference between the two counts.

3.7.4.2 Effects of protein synthesis inhibition on the recovery of thermally damaged cells

Chlorotetracycline inhibits protein synthesis on 70S and 80S ribosomes, although 70S ribosomes are more sensitive. Specifically it inhibits the binding of aminoacyl-tRNA to the ribosomal acceptor site.

Using the method for determining the maximum non-inhibitory level of metabolic inhibitor described in section 3.7.4.1, the concentration of chlorotetracycline used was calculated to be 2 μg ml^{-1} for all three organisms.

Examination of *Figures* 3.16, 3.17 and 3.18 show that recovery of all three organisms was inhibited in the presence of chlorotetracycline. This dependence upon the synthesis of new protein in *S. typhimurium* has been reported by Tomlins & Ordal (1971). Section 1.3.5 points out that: *S. typhimurium* has all the enzymes for a functional TCA cycle and many other important enzymes after thermal exposure and that protein denaturation may not necessarily be the prime cause of thermal injury. However it does not eliminate the possibility that some highly essential proteins are always inactivated.

The control experiments showed that return of salt tolerance, or recovery, took almost two hours for S. senftenberg JT577 and S. typhimurium SA2009 but was almost immediate for S. senftenberg 775W. Cell division returned after the same period after post thermal inactivation as it had done in Figure 3.15.

3.7.4.3 Effects of cell wall synthesis inhibition on the recovery of thermally damaged cells

Penicillin interferes with the terminal step in the cross-linking of peptidoglycan chains. This is the transpeptidation of the N-terminus of the amino acid or peptide bridge on to the penultimate D-alanine of the pentapeptide chain with the release of the D-alanine.

Using the method described in section 3.7.4.1, the concentrations of penicillin were calculated to be 6 μg ml^{-1} for *S. typhimurium* SA2009, 2 μg ml^{-1} for *S. senftenberg* 775W and 4 μg ml^{-1} for *S. senftenberg* JT577.

Recovery of all three organisms was inhibited in the presence of penicillin. No literature could be found on the dependence of new cell wall synthesis for the recovery of Gram-negative bacteria, although the Gram-

positive organism *S. aureus* is able to recover in the presence of penicillin. However the inability of these three Gram-negative organisms to recover in the presence of penicillin suggests that partial or total repair of the cell wall is necessary for recovery and cell division to begin.

3.7.4.4 Effect of RNA synthesis inhibition on the recovery of thermally damaged cells

Rifampicin specifically inhibits bacterial DNA-dependant RNA polymerases by blocking RNA chain initiation. It binds very tightly to the β-subunit of the polymerase in a molar ratio of 1:1.

Using the method described in section 3.5.4.1, the concentrations of rifampicin used were calculated to be 2 $\mu g\ ml^{-1}$ for *S. senftenberg* 775W, 8 $\mu g\ ml^{-1}$ for *S. typhimurium* SA2009 and 4$\mu g\ ml^{-1}$ for *S. senftenberg* JT577.

Recovery of *S. senftenberg* JT577 and *S. typhimurium* SA2009 was dependent upon the synthesis of new RNA as recovery did not occur in the presence of rifampicin. *S. senftenberg* 775W on the other hand was able to recover in the presence of the inhibitor, recovery taking around 6 hr. Once recovery was complete, growth was at the same rate as the control and they both reached similar terminal OD_{600} values.

S. senftenberg 775W has been shown to have a greater ability to survive at lethal temperatures (50°-60°C) than the other two organisms studied. The work in section 3.7.4 showed that *S. senftenberg* 775W was able to begin cell division after exposure to thermal damage, faster than *S. typhimurium* SA2009 or *S. senftenberg* JT577. As cell division can only recommence when the internal pools, lost during thermal damage have reaccumulated, cell membrane integrity must also have returned. *Figures*

Figure 3.16 Recovery of heat-injured *S. typhimurium* SA2009 grown at 37°C in the presence of metabolic inhibitors

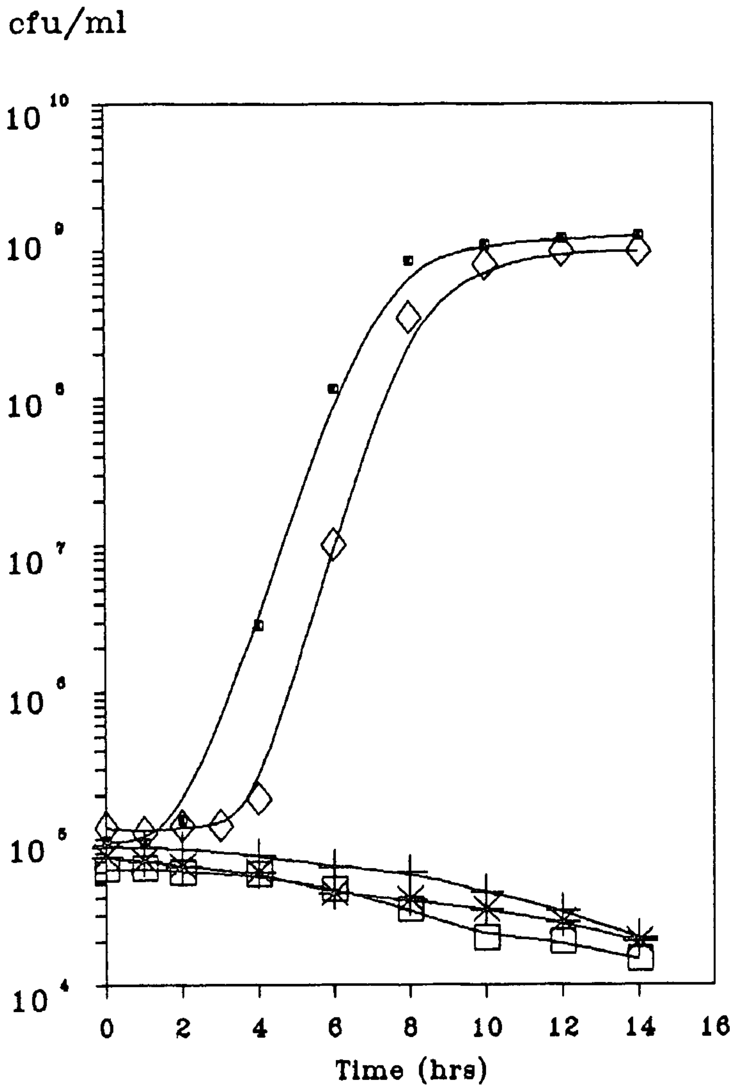


Figure 3.16. Recovery and growth of heat-injured *S. typhimurium* SA2009 incubated in LB plus 8 μg ml^{-1} of rifampicin, 6 μg ml^{-1} of penicillin and 2 μg ml^{-1} of chlorotetracycline. The cells were heat injured in LB by a standard thermal exposure (2.11.2). Cells incubated in LB and recovered on LB agar (◇) and LB + 7% NaCl agar (■). Cells incubated in LB plus 8 μg ml^{-1} of rifampicin and recovered on LB + 7% NaCl agar (+), 6 μg ml^{-1} of penicillin and recovered on LB + 7% NaCl agar (*) and 2μg ml^{-1} of chlorotetracycline and recovered on LB + 7% NaCl agar (□)

Figure 3.17 <u>Recovery of heat-injured *S. senftenberg* JT577 grown at 37°C in the presence of metabolic inhibitors</u>

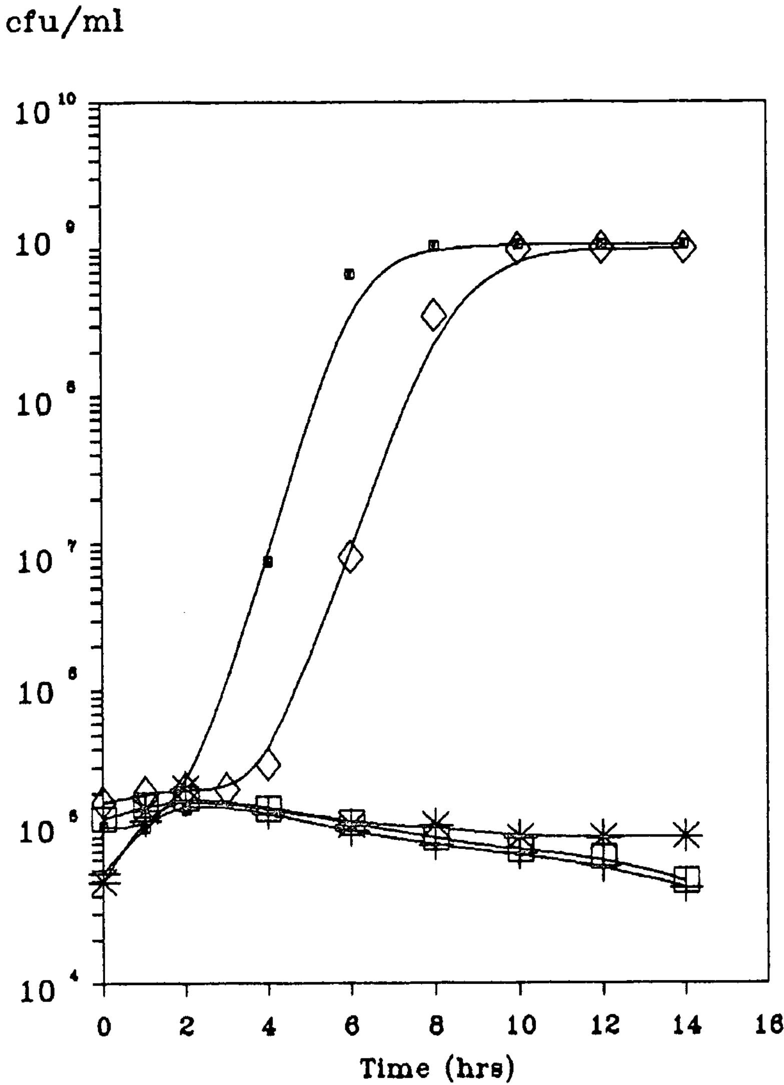


Figure 3.17. Recovery and growth of heat-injured *S. senftenberg* JT577 incubated in LB plus 4 μg ml^{-1} of rifampicin, 4 μg ml^{-1} of penicillin and 2 μg ml^{-1} of chlorotetracycline. The cells were heat injured in LB by a standard thermal exposure (2.11.2). Cells incubated in LB and recovered on LB agar (◇) and LB + 7% NaCl agar (■). Cells incubated in LB plus 8 μg ml^{-1} of rifampicin and recovered on LB + 7% NaCl agar (+), 6 μg ml^{-1} of penicillin and recovered on LB + 7% NaCl agar (*) and 2μg ml^{-1} of chlorotetracycline and recovered on LB + 7% NaCl agar (□)

Figure 3.18 <u>Recovery of heat-injured *S. senftenberg* 775W grown at 37°C in the presence of metabolic inhibitors</u>

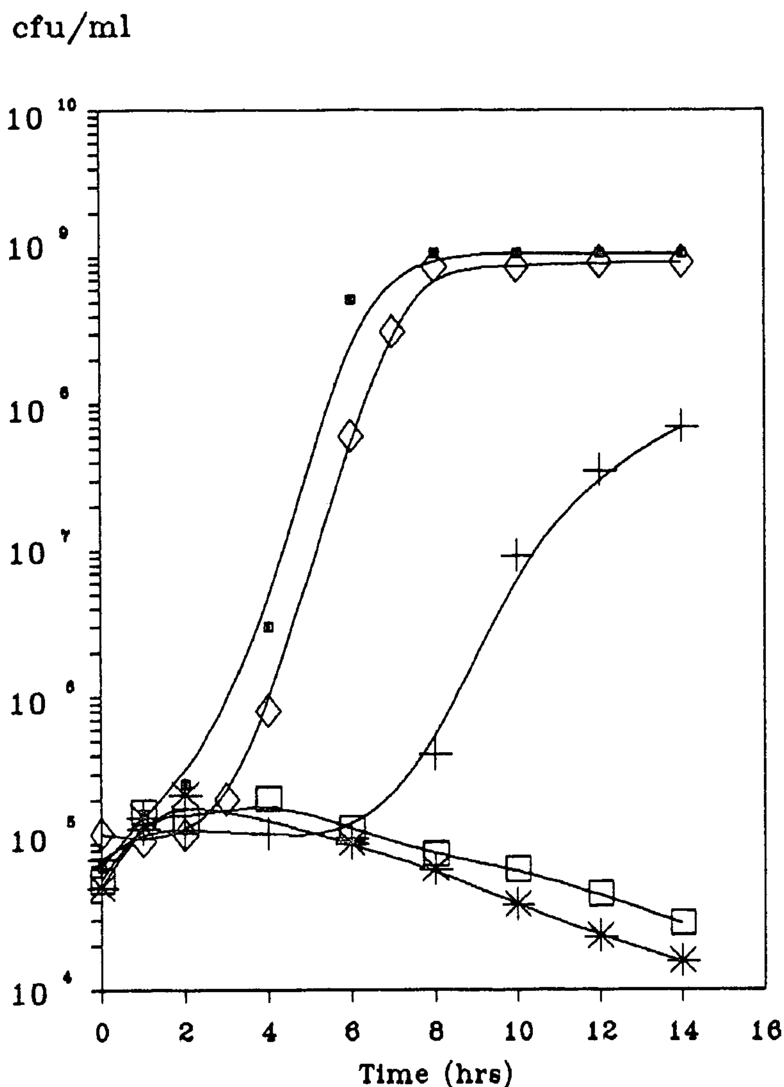


Figure 3.18. Recovery and growth of heat-injured *S. senftenberg* 775W incubated in LB plus 2 μg ml^{-1} of rifampicin, 2 μg ml^{-1} of penicillin and 2 μg ml^{-1} of chlorotetracycline. The cells were heat injured in LB by a standard thermal exposure (2.11.2). Cells incubated in LB and recovered on LB agar (◇) and LB + 7% NaCl agar (▪). Cells incubated in LB plus 8 μg ml^{-1} of rifampicin and recovered on LB + 7% NaCl agar (+), 6 μg ml^{-1} of penicillin and recovered on LB + 7% NaCl agar (*) and 2μg ml^{-1} of chlorotetracycline and recovered on LB + 7% NaCl agar (□)

3.16, 3.17 and 3.18 show the return of salt tolerance, which is dependent upon the return of cell membrane integrity (Busta & Jezeski, 1963), in the three organisms studied. *S. senftenberg* 775W regains salt tolerance immediately after transfer to the recovery media, while *S. senftenberg* JT577 and *S. typhimurium* SA2009 require approximately 1 hr to regain salt tolerance. It could therefore be assumed that as the return of salt tolerance follows cell membrane repair, that *S. senftenberg* 775W must have either incurred only slight membrane damage or has facilitated rapid repair. If this is the case, then the loss of essential internal pools necessary for biosynthesis and presumably repair and recovery would be minimised. But if *S. senftenberg* 775W does only undergo slight cell membrane damage, why is there not a bigger difference between the strains in the time taken for cell division to begin again? It may be that repair of other sites damaged in all three organisms must begin before cell division can recommence. Possible sites are; the cell wall as all three organisms are unable to recover in the presence of a cell wall synthesis inhibitor, and specific proteins may also need repairing.

The conditions in which thermally injured organisms are recovered is critical. Injured organisms are unable to recover when placed in minimal media due to the loss of internal pools, and unless they are transferred to a nutritionally richer medium will lose viability. Therefore, if *S. senftenberg* 775W has lost little of its internal pools because of only slight cell membrane damage, repair can begin quickly. However if the time taken to return cell membrane integrity is great, organisms on the borderline between maintaining and losing viability may well die.

3.7.5 Heat induced thermotolerance

When mesophilic bacteria are exposed to a shift up in the growth temperature to one slightly above their optimum, they exhibit an increased heat resistance when subsequently exposed to lethal temperatures, 50°-60°C. This induced, elevated heat resistance has been described as transient in *E.coli*, reaching a maximum after 30 min and disappearing by 60 min (Yamamuri & Yura, 1982); while in *S. typhimurium* the elevated resistance continued as long as incubation was at the shifted temperature (Mackey & Derrick, 1986)

If a shift up in growth temperature induces an increase in heat resistance, it may be reflected in the start of, or improvement in, the ability to recover in the presence of a metabolic inhibitor. All three organisms were thermally induced according to section 2.11.5. They were then recovered in LB with and without metabolic inhibitors as described in 3.7.4.1, and the concentrations of inhibitor used were as previously calculated in 3.7.4.1.

3.7.5.1 Effect of cell wall, protein and RNA synthesis inhibition on the recovery of thermally damaged, thermally induced *S. typhimurium* SA2009

Recovery of thermally induced, thermally injured *S. typhimurium* SA2009 was inhibited in the presence of chlorotetracycline, penicillin and rifampicin, (*Figure* 3.19). Induction at 42°C did not alter dependence upon the synthesis of new protein, cell wall or RNA in *S. typhimurium* SA2009. However, recovery of the control was altered by heat induction. Incubation at 42°C resulted in the complete absence of any lag or recovery period, which had been 3.5 hr for cells grown at 37°C. The rate of cell division was less in the heat induced control, suggesting a reduced efficiency, perhaps as a result of

incomplete recovery but sufficient to allow cell division.

3.7.5.2 Effect of cell wall, protein and RNA synthesis inhibition on the recovery of thermally damaged, thermally induced *S. senftenberg* JT577

The results are similar to those for *S. typhimurium* SA2009 seen in section 3.5.5.1. Incubation at 42°C prior to thermal inactivation did not alter *S. senftenberg* JT577's dependence upon cell wall, protein and RNA synthesis (*Figure* 3.20). The control, as with *S. typhimurium* SA2009, was affected by heat induction. There was no lag present but cell division was at a slower rate than cells grown at 37°C prior to thermal injury.

3.7.5.3 Effects of cell wall, protein and RNA synthesis inhibition on the recovery of thermally induced, thermally damaged *S. senftenberg* 775W

S. senftenberg 775W remained unable to recover in the presence of protein and cell wall synthesis inhibitors, but the recovery time in the presence of a RNA synthesis inhibitor was reduced from 6 h to less than 1 hr, (*Figure* 3.21). Cell division for *S. senftenberg* 775W, recovered in the presence of rifampicin and appeared to be in two phases: a slow rate beginning almost immediately after transfer to the recovery medium which lasted 4 h; and a second faster rate, equivalent to that of the control, from 4 h to the end of the exponential phase at 8 to 9 h. This two stage growth curve suggested that recovery was not complete until 4 h but sufficient repair had taken place for cell division to begin. The control, as with *S. typhimurium* SA2009 and *S. senftenberg* JT577, has no lag period and the rate of cell division is greater, but not as fast as that seen in *Figure* 3.18.

Figure 3.19 <u>Recovery of heat-injured *S. typhimurium* SA2009 grown at 42°C in the presence of metabolic inhibitors</u>

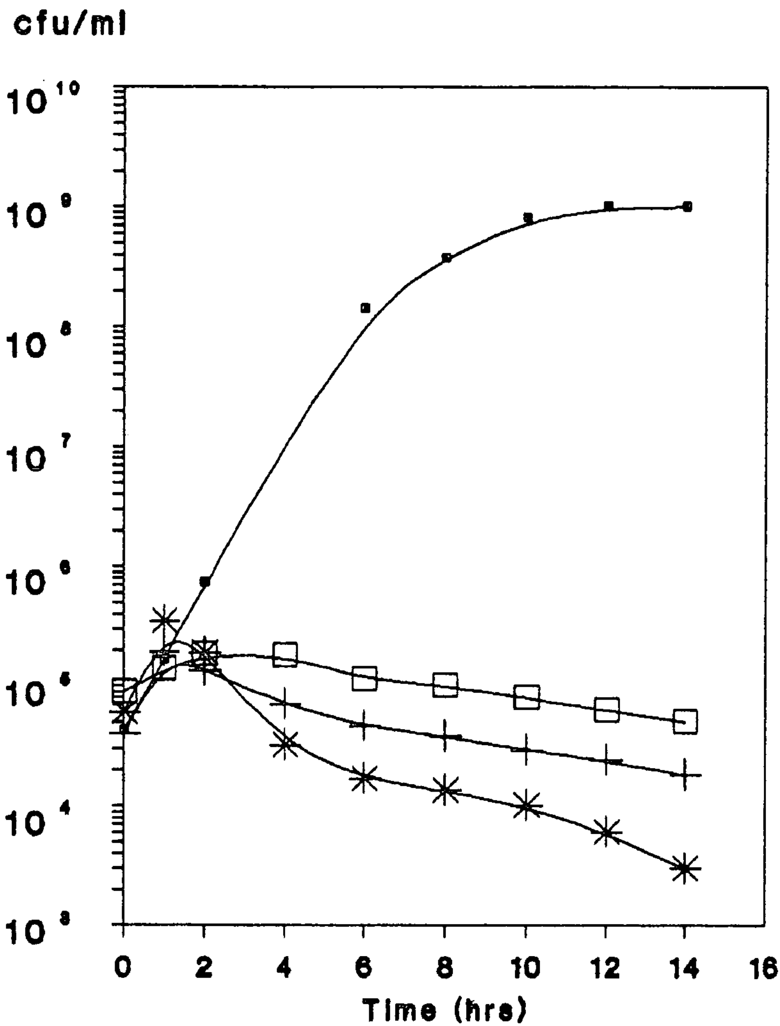


Figure 3.19. Recovery and growth of, 42°C heat induced *S. typhimurium* SA2009, after heat injury when incubated in LB plus 8μg ml^{-1} of rifampicin, 6 μg ml^{-1} of penicillin and 2μgml^{-1} of chlorotetracycline. The cells were heat injured in LB by a standard thermal exposure (2.11.2). Cells incubated in LB and recovered on LB agar (▪) and LB + 7% NaCl agar (▪). Cells incubated in LB plus 8μgml^{-1} of rifampicin and recovered on LB + 7% NaCl agar (+), 6μg ml^{-1} of penicillin and recovered on LB + 7% NaCl agar (*) and 2 μg ml^{-1} of chlorotetracycline and recovered on LB + 7% NaCl agar (□)

Figure 3.20 <u>Recovery of heat-injured *S. senftenberg* JT577 grown at 42°C in the presence of metabolic inhibitors</u>

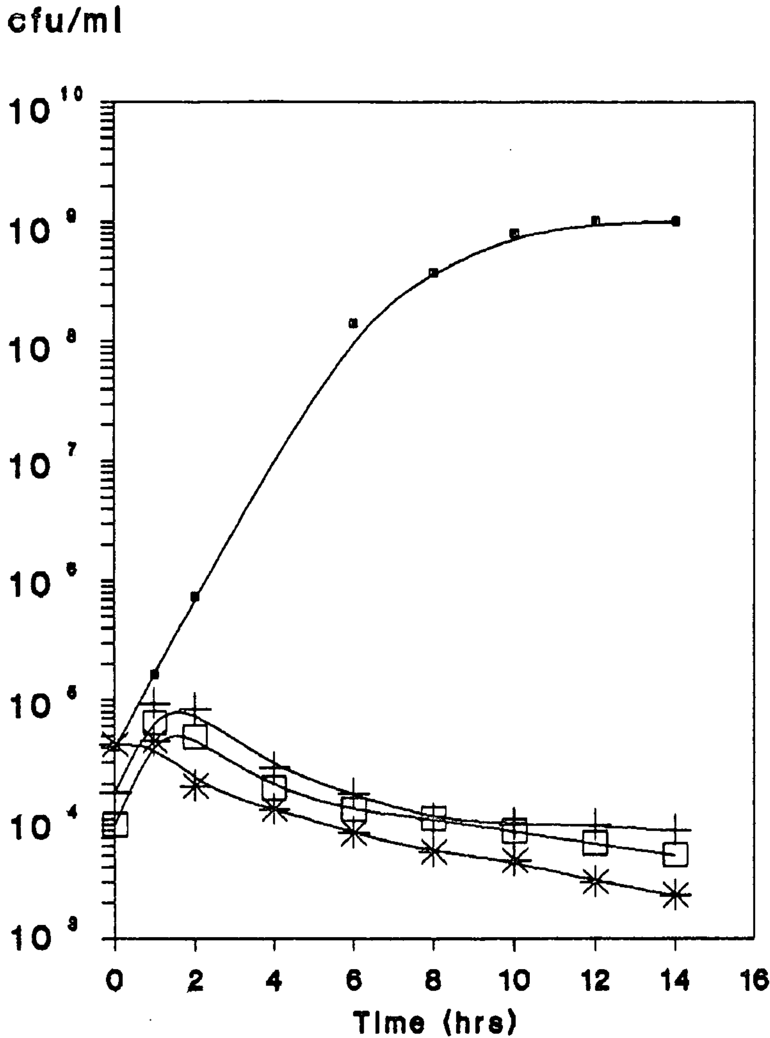


Figure 3.20. Recovery and growth of, 42°C heat induced *S.senftenberg* JT577, after heat injury when incubated in LB plus 4μg ml^{-1} of rifampicin, 4μg ml^{-1} of penicillin and 2 μg ml^{-1} of chlorotetracycline. The cells were heat injured in LB by a standard thermal exposure (2.11.2). Cells incubated in LB and recovered on LB agar (▪) and LB + 7% NaCl agar (▪). Cells incubated in LB plus 8μg ml^{-1} of rifampicin and recovered on LB + 7% NaCl agar (+), 6μg ml^{-1} of penicillin and recovered on LB + 7% NaCl agar (*) and 2 μg ml^{-1} of chlorotetracycline and recovered on LB + 7% NaCl agar (□)

Figure 3.21 <u>Recovery of heat-injured *S. senftenberg* 775W grown at 42°C in the presence of metabolic inhibitors</u>

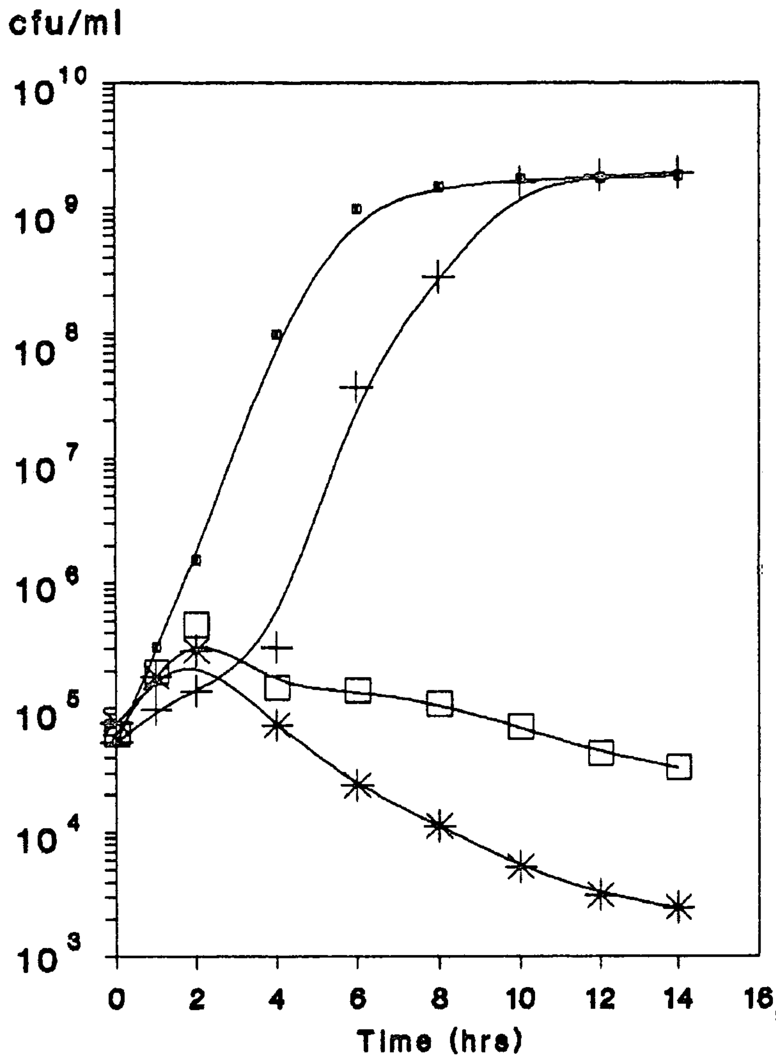


Figure 3.21. Recovery and growth of, 42°C heat induced *S.senftenberg* 775W, after heat injury when incubated in LB plus 2 μg ml^{-1} of rifampicin, 2μg ml^{-1} of penicillin and 2μgml^{-1} of chlorotetracycline. The cells were heat injured in LB by a standard thermal exposure (2.11.2). Cells incubated in LB and recovered on LB agar (■) and LB + 7% NaCl agar (•). Cells incubated in LB plus 8μgml^{-1} of rifampicin and recovered on LB + 7% NaCl agar (+), 6μg ml^{-1} of penicillin and recovered on LB + 7% NaCl agar (*) and 2 μg ml^{-1} of chlorotetracycline and recovered on LB + 7% NaCl agar (□)

All three organisms were still unable to recover in the presence of protein and cell wall synthesis inhibitors. *S. senftenberg* JT577 and *S. typhimurium* SA2009 were also unable to recover in the presence of a RNA synthesis inhibitor, suggesting some damage must have occurred to these areas for which repair was necessary. All three organisms had no lag period prior to cell division recommencing, and this ability of all three organisms to regain salt tolerance suggests they had been able to either limit the damage induced or improve the repair of damage induced or, more likely, a combination of the two. This evidence suggests that return of cell membrane integrity, indicated by the return of salt tolerance, is an important factor in maintaining viability. In cells incubated at 37°C, return of salt tolerance occurs before cell division restarts. However in cells incubated at 42°C, salt tolerance and the return of cell division are indistinguishable. Nevertheless it is difficult to draw conclusions as to how cell membrane integrity increases viability, except to say that the loss of internal pools would be limited and it would be a reflection of the cells overall status.

Incubation at 42°C prior to thermal injury appeared to induce an elevated heat resistance in all three organisms. However the magnitude of the increase had not yet been qualified. *Figure* 3.3 showed that the heat resistance of *S. typhimurium* SA2009 varied through the course of the growth cycle. The experiment described in section 3.1 was repeated for *S. senftenberg* 775W, and it was found that the change in resistance followed that for *S. typhimurium* SA2009 (work not shown). It was decided to see if the altered heat resistance seen in heat induced cultures followed a similar pattern for cells grown at 37°C, or whether they showed the changes described by Mackey & Derrick (1986) for cells grown at 42°C.

Each organism was grown up to an OD_{600} of 0.1 as described in section 2.11.3. However 1 l rather than 50 ml of LB culture media was used to grow up the cells. After the first 20 ml aliquot of cells was taken for a 5 min heat inactivation at 55°C, each culture was split into 2 x 490 ml volumes. One was incubated at 42°C while the other continued to be incubated at 37°C. All cultures were incubated for 10 hr and 20 ml aliquots were removed at set time intervals for thermal inactivation.

Figures 3.22, 3.23 and 3.24 show how the resistance of the three organisms changed throughout the course of growth at 37°C and 42°C. The survival of cells grown at 37°C varied very little for the first 1.5 hr for all three organisms. This was than followed by another 1.5 hr in which survival increased exponentially. *S. senftenberg* 775W showed the greatest rise in survival with a 1.5 x logarithmic increase, while *S. senftenberg* JT577 and *S. typhimurium* SA2009 showed an approximate 1 x logarithmic rise. Maximum survival for all three was reached after approximately 4 hr and from there survival remained at the same level. These curves follow closely those in *Figure* 3.3. The first 1.5 hr corresponds to the period around the mid-logarithmic phase of growth (which all the cells were in), in which cells are at their most sensitive to heat. The next 1.5 hr corresponds to cells entering the early stages of stationary phase growth. The remaining period, until the end of the experiment, corresponds to the cells fully entered into stationary phase and at their most resistant.

For cells grown at 42°C, the increase in thermal survival was similar for all three organisms. There was no initial lag period after transfer to the elevated growth temperature, there was in fact an exponential rise in resistance. Maximum survival was reached 2 hr after transfer to 42°C. Resistance of *S. senftenberg* JT577 and

S. typhimurium SA2009 peaked at around 1.75-2 hr, after the shift up in temperature, and then declined for the remainder of the experiment. The resistance of *S. senftenberg* 775W showed the same exponential rise for 1.75-2 hr as *S. senftenberg* JT577 and *S. typhimurium* SA2009, but resistance remained at the higher level throughout the course of the experiment. In all three cases, survival of the cells grown at 42°C exceeded 1, suggesting there were less viable organisms prior to heating than after 5 min at 55°C. What is more likely is that the counts seen prior to the 55°C exposure were artificially low, probably due to the presence of hydrogen peroxide in the rich recovery media, and in some way exposure to 55°C induced the cells catalase/peroxidase systems. The induction of these systems would have the affect of reducing hydrogen peroxide potency, and cell viability would appear to increase. Mackey & Seymour (1987) reported that the addition of catalase or peroxidase to rich recovery media restored viable counts of heat-injured *recA*, *recB* and *polA* strains to wild type levels. These three mutations when present singly in *E. coli* K12 increased its sensitivity to mild heat. Mackey & Seymour (1987) also noted that the catalase activity of a strain of *E. coli* heated at 52°C, increased to a maximum level after approximately 3 minutes at this increased temperature. It may be that this kind of increase in catalase activity during heating could explain *S. senftenberg* 775W, JT577 and *S. typhimurium* SA2009 increase in survival. Indeed Mackey (personal communication) suggested that the counts obtained on LB agar plates, were lower than the OD_{600} would have suggested.

The resistance seen in cells grown at 37°C can be associated with changes that occur in the cell through the course of the growth cycle. The changes in resistance seen for cells grown at 42°C, must involve the

Figure 3.22 <u>Change in survival at 55°C *S. typhimurium* SA2009 grown at 37°C and 42°C</u>

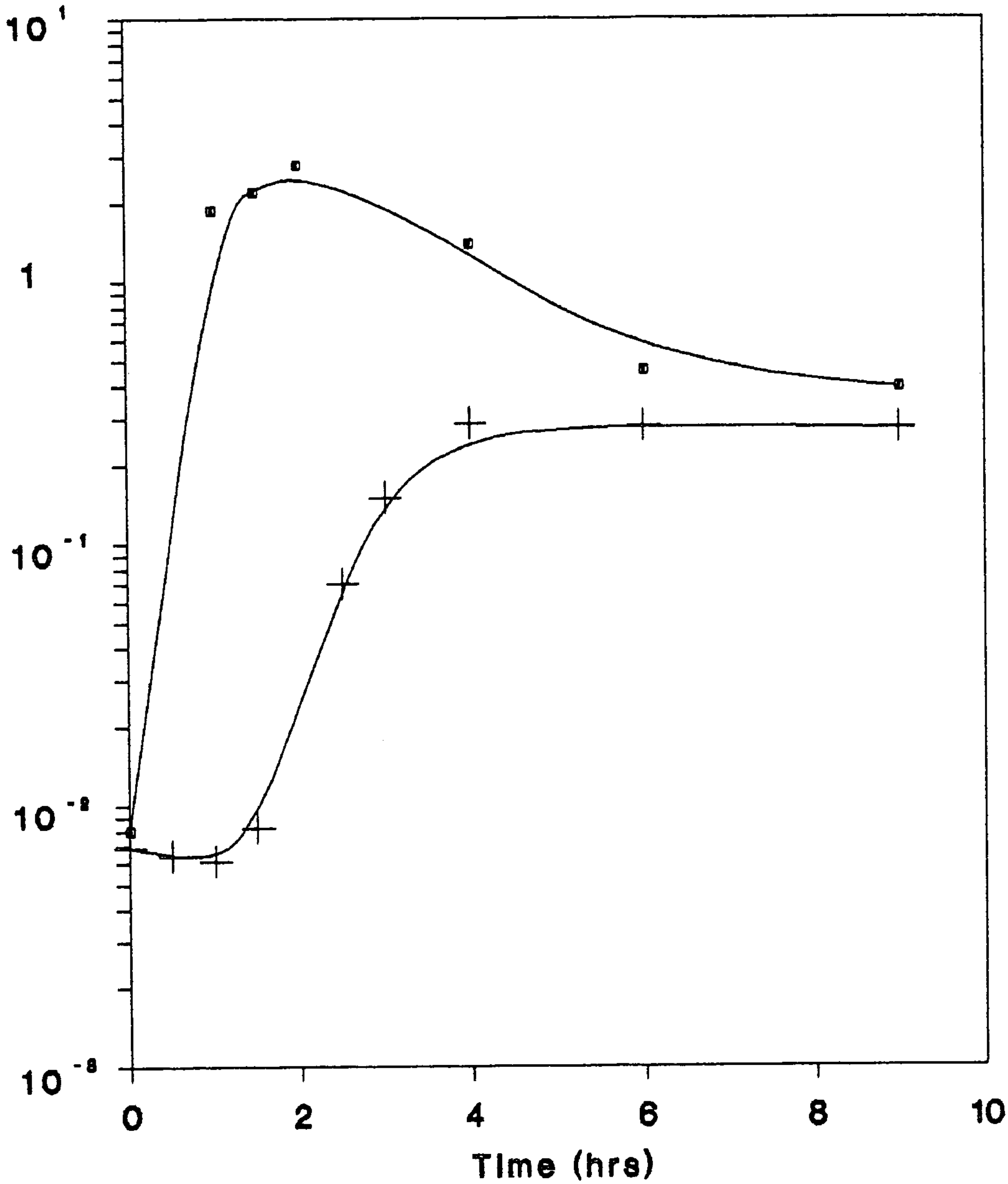


Figure 3.22. The effect of growth temperature, 37°C (+) and 42°C (▪) during incubation, on the survival of 20 ml of mid-logarithmic *S. typhimurium* SA2009 to 5 min in 180 ml of LB at 55°C

Figure 3.23 <u>Change in survival at 55°C *S. senftenberg* JT577 grown at 37°C and 42°C</u>

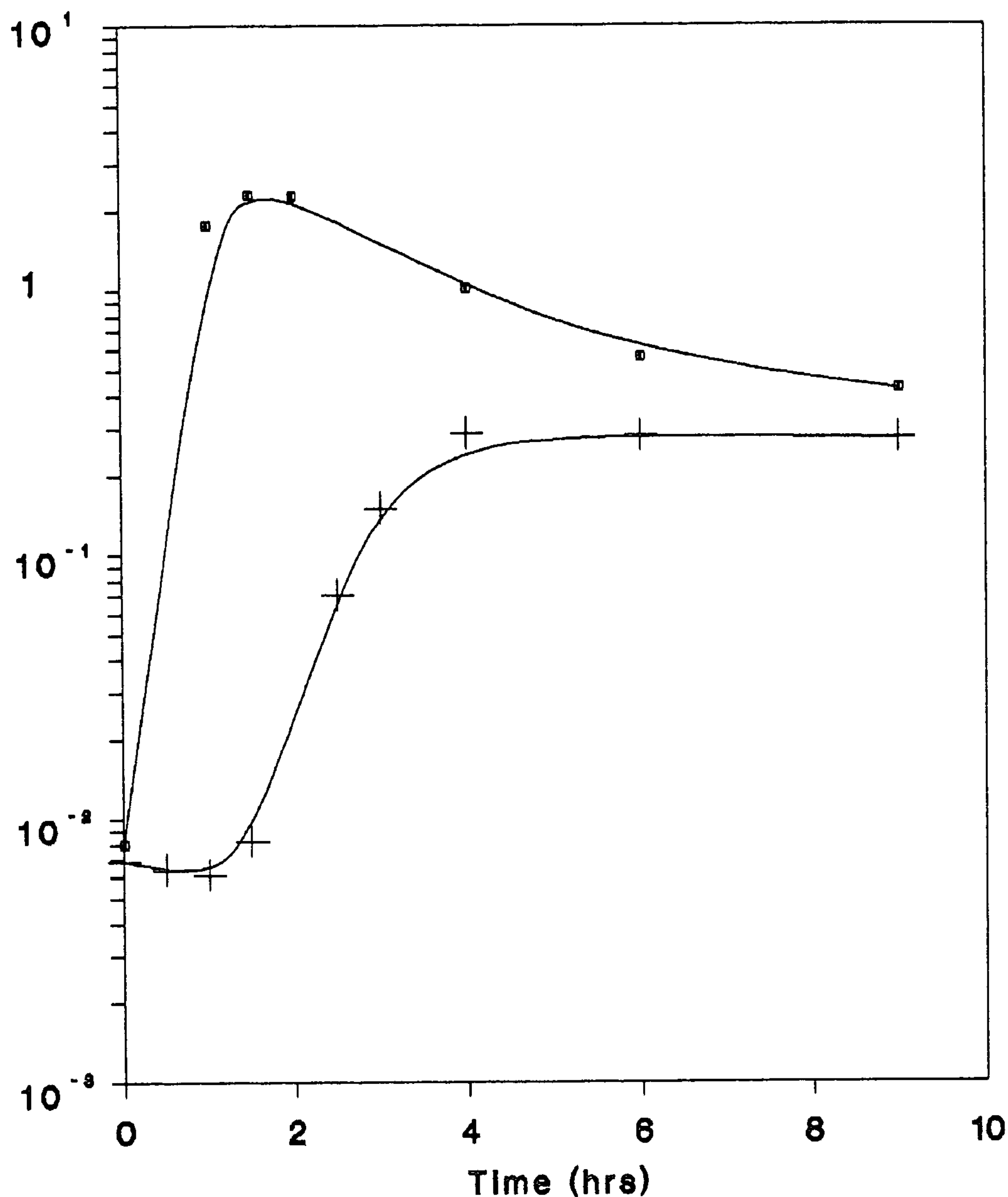


Figure 3.23. The effect of growth temperature, 37°C (+) and 42°C (▪) during incubation, on the survival of 20 ml of mid-logarithmic *S. senftenberg* JT577 to 5 min in 180 ml of LB at 55°C

Figure 3.24 <u>Change in survival at 55°C *S. senftenberg* 775W grown at 37°C and 42°C</u>

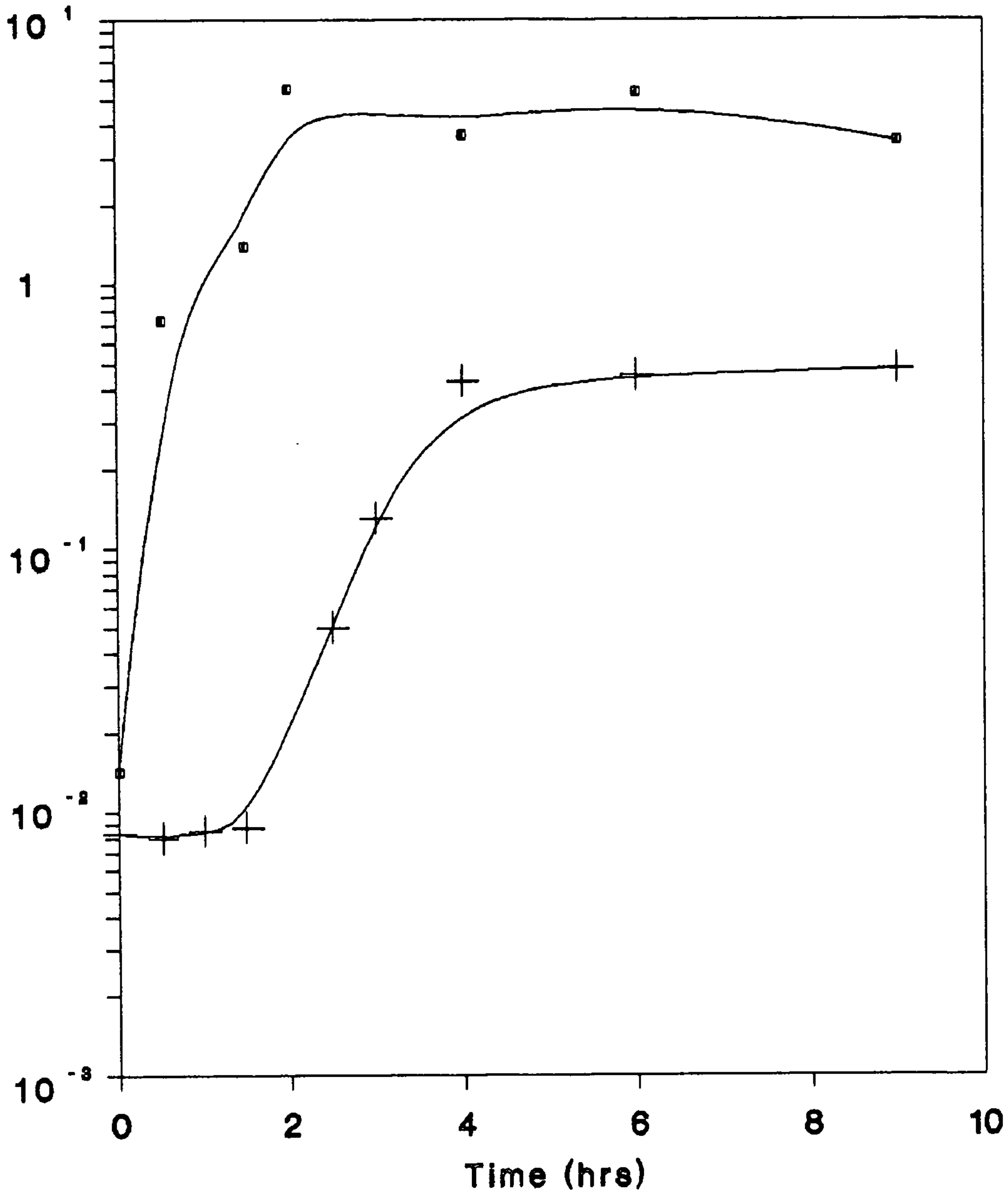


Figure 3.24. The effect of growth temperature, 37°C (+) and 42°C (▪) during incubation, on the survival of 20 ml of mid-logarithmic *S. senftenberg* 775W to 5 min in 180 ml of LB at 55°C

induction of systems that can rapidly alter the ability to survive at elevated temperatures.

The ability of *S. senftenberg* 775W to survive to a greater extent than *S. senftenberg* JT577 or *S. typhimurium* SA2009, with or without heat induction, could perhaps be explained by the level of synthesis of heat shock proteins. Although no heat shock protein has been shown to directly increase heat resistance, their induction does enable cells to survive longer when exposed to lethal temperatures. It was decided to see if *S. senftenberg* 775W had in fact higher basal levels of any heat inducible protein, or whether induction gave rise to higher levels of these proteins.

3.7.6 Heat inducible proteins

Although not shown directly from the work in section 3.7.5, it could be assumed that *S. senftenberg* 775W when heat induced, synthesised heat shock proteins (hsp) similar to those found in *E. coli*. Mutants that synthesise induced levels of the hsp's at 37°C have elevated heat resistances. It was therefore decided to see if *S. senftenberg* 775W synthesised higher than normal levels of hsp's at 37°C and also at a temperature that would induce hsp's, e.g., 42°C.

Total protein SDS-polyacrylamide page gels were prepared, (see section 2.19), to compare the levels of protein present at optimal and up-shifted growth temperatures (37° & 42°C, respectively) in *S. senftenberg* JT577, 775W and *S. typhimurium* SA2009. Comparisons of total protein, at the same concentration for all three organisms (see section 2.21), revealed no measurable difference between them at the two incubation temperatures (gels not shown). As the numbers of bands present on a total protein gel made it difficult to compare specific protein bands, a specific heat-inducible

protein, GroEL, was identified for further investigation. A polyclonal rabbit antisera to *E. coli* GroEL was obtained which could be used to quantify the levels of GroEL in each of the bacteria under study and determine how it varied at different growth temperatures.

Figure 3.25 shows strong homology between the GroEL antibody of *E. coli* and the total cell extracts of the three organisms. The amount of antibody, determined by the intensity of the bands, varied with growth temperature but not between strains. The intensity of the bands increased when the bacteria were up-shifted in growth temperature and increased over the period of incubation examined. However, *S. senftenberg* 775W did not have a higher basal level of GroEL at 37°C or after induction at 42°C than *S. senftenberg* JT577 or *S. typhimurium* SA2009. There was no positive or negative control for this experiment due to time constraints.

Although there are other heat inducible proteins, and higher than average levels of these proteins can not be ruled out in *S. senftenberg* 775W grown at 37°C, GroEL does not appear to be at levels higher than in *S. senftenberg* JT577 and *S. typhimurium* SA2009. Although these proteins are induced by an up-shift in growth temperature and this up-shift results in elevated resistance, a direct relationship between the proteins and elevated heat resistance cannot be drawn.

Figure 3.25 Western blot of total cell extracts from *S. senftenberg* 775W, *S. senftenberg* JT577 and *S. typhimurium* SA2009 grown at different incubation temperatures using antibody to GroEL

Lane 1 - *S. senftenberg* 775W in mid-logarithmic phase of growth at 37°C

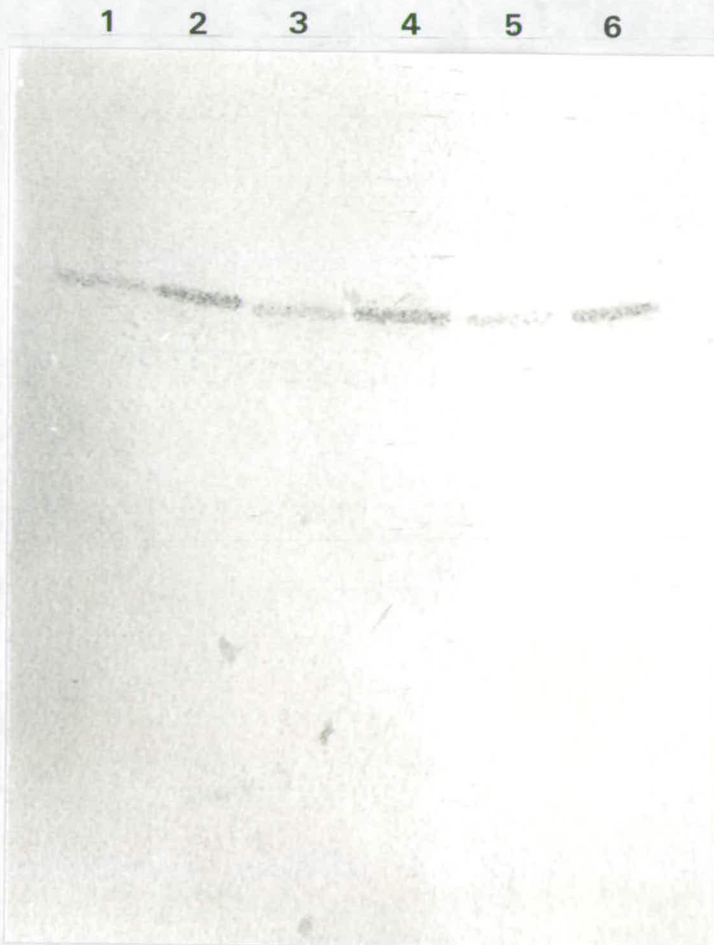
Lane 2 - *S. senftenberg* 775W transferred to 42°C for 1 hour from mid-logarithmic phase cells at 37°C

Lane 3 - *S. senftenberg* JT577 in mid-logarithmic phase of growth at 37°C

Lane 4 - *S. senftenberg* JT577 transferred to 42°C for 1 hour from mid-logarithmic phase cells at 37°C

Lane 5 - *S. typhimurium* SA2009 in mid-logarithmic phase of growth at 37°C

Lane 6 - *S. typhimurium* SA2009 transferred to 42°C for 1 hour from mid-logarithmic phase cells at 37°C



CHAPTER FOUR

DISCUSSION

4.1 Variability of thermal survival curves

The variability of the thermal survival curves of *S. typhimurium* SA2009 in LB at 55°C was very frustrating. Without a reproducible set of these curves, comparisons could not be made between the wild type and mutant populations.

Much has been written about the variability of survival curves of vegetative bacterial cells, most of it about the shape of the curves. As stated in section 1.1, many microbiological text books show thermal inactivation curves as straight lines. This work has shown that heat inactivation of *S. typhimurium* SA2009 at 55°C in LB does not give straight line survival curves, but concave curves. The obvious danger of assuming straight line survival occurs when attempting to predict numbers of viable organisms after a set thermal exposure during food processing. Moats *et al.*, (1971) pointed out that, of the two assumptions used as the theoretical basis for survival curves being logarithmic, i.e., populations of single strains of bacteria being homogeneous with regard to heat resistance; and thermal death of bacteria being unimoloecular, i.e., a single site lethal event; neither were valid. He suggested experimental evidence indicated that the first was seldom true and that the second is incompatible with the evidence of sublethal injury. He therefore suggested that there was no theoretical basis for assuming that survival curves should be logarithmic or that any deviation was due to experimental error.

The model that best describes the concave curves obtained during this study is based on the assumption that survival reflects inherent phenotypic variations in the susceptibility of the individual members of a population (Allwood & Russell, 1970). Susceptibility to heat within the population is uniform, and the shape of the curve is determined by the non-uniform distribution

of heat resistance within the population. Using this model, explanations can be given for variations from straight line survival, for example, tails on survival curves can be explained by a section of the population having a higher level of resistance compared with the rest of the population.

Although many other factors are known to affect the shape of survival curves, e.g., growth medium, recovery medium, growth temperature, these variables were kept constant throughout this study to try to maintain reproducibility. *Figure* 3.3 highlighted the significance of keeping all variables constant so as to give credibility to a standard thermal survival curve. Stationary and exponential phase cells are known to have appreciably different thermal survival capabilities. *Figure* 3.3 indicated that the rate of change of resistance during the two phases was great. Cells taken at any time well into stationary phase had no measurable difference in resistance, while exponentially dividing cells greatly varied in their ability to survive at 55°C. Although the reasons behind this difference in terms of providing an explanation for thermal survival are important, they are not crucial in ensuring reproducible survival curves. What is important is that reproducibility can only come from removing or reducing all possible variables.

4.2 Anomaly of survival capabilities

The inability to isolate *S. typhimurium* SA2009 with either increased or decreased thermal survival capabilities, as compared to the wild type, was disappointing and an explanation is not obvious. This failure initially hampered progress. One might assume from the number of putative mutants screened, and providing the surface plate method worked, a colony having an altered heat resistance would have been found.

For example, inactivating the function of the *htpR* gene would give rise to cells that grew normally at 37°C but could not be induced to synthesise hsp's when there was a shift-up in the surrounding temperature. Therefore the gradual rise in temperature, to which cells on the surface of an agar plate when placed in a high temperature incubator would be exposed to, should induce the heat shock response. Cells lacking the *htpR* gene would therefore not be as heat resistant as wild type cells and should be distinguished from cells with a functional *htpR*. However none with lower heat resistance were isolated.

The inability to separate *Salmonella* strains, which could be shown to have different heat resistances in liquid broth, by means of the surface plate method, did not become apparent until the heat cycled strains of Corry & Roberts (1970) were obtained. These *Salmonella* strains had D^{55} values of up to 4.7 min for the 39th cycled strain (*S. typhimurium* MR21) as compared to 1.7 min for *S. typhimurium* SA2009. However they were inseparable in terms of their ability to survive on the surface of an agar plate when exposed to 55°C in a high temperature incubator. This may suggest that whatever the cause of *S. typhimurium* MR21's ability to survive in broth, it did not enable it to survive on the surface of a plate.

It has been shown that the accumulation of hydrogen peroxide (H_2O_2) in agar reduced the colony-forming ability of injured microorganisms (Flowers *et al.*, 1977 and Martin *et al.*, 1976). This and other peroxides have been shown to increase when cells are grown aerobically due to aerobic respiration. Martin *et al.*, (1976), has suggested that this accumulation in or around the cells may be a universal phenomenon in injured cells due to decreased catalase activity. Therefore when cells are heat inactivated on the surface of a plate, or recovered

on plates for viable counting after heat inactivation in liquid broth, the combination of reduced catalase activity and H_2O_2 accumulation could render some of the already injured cells non-viable.

Why should *S. senftenberg* 775W be able to survive for longer than *S. typhimurium* MR21 on the surface of an agar plate when incubated at 55°C? The reason may lay in the thermal stability of *S. senftenberg* 775W's catalase and other peroxidase enzymes. Alternatively it may be the expression of the genes that code for these enzymes that is more heat stable, or it may be a combination of the two. Whatever the cause, *S. senftenberg* 775W would have active peroxidases, allowing injured organisms to retain or regain viability. Rayman *et al.*, (1978) reported that the addition of Fe^{2+} (Iron Sulphate $FeSO_4.7H_2O$), MnO_4^- (Potassium Permanganate $KMnO_4$) and catalase, which are all capable of inactivating H_2O_2, increased the survival of a heat-injured strain of *S. senftenberg*. The survival increased by 102, 170 and 151 times respectively, as compared to recovery on Tryptone Soya Agar alone. If *S. senftenberg* 775W does have active peroxidase, after thermal stress, then not only could it protect itself from the effects of accumulated H_2O_2 but also other surrounding cells. The results in *Table* 3.3 show an increase in the survival of *S. typhimurium* SA2009 on the surface of an agar plate when inactivated with *S. senftenberg* SM001, the streptomycin resistant form of *S. senftenberg* 775W, as compared to inactivation alone. After 46 min at 55°C, the number of *S. typhimurium* SA2009 survivors on the mixed culture plate was almost 4 times that of the *S. typhimurium* SA2009 plate alone. After 47 min there were no survivors on the *S. typhimurium* SA2009 plate, but 64 on the *S. typhimurium* SA2009/*S. senftenberg* SM001 plate. At the same time as *S. typhimurium* SA2009 was increasing in percentage survival on the mixed culture plate, *S. senftenberg* SM001 reduced in percentage survival. After 46 min at 55°C, 7 of the 10 *S.*

senftenberg SM001 organisms remained viable when inactivated alone, while only 1 of the 10 was viable on the mixed culture plate. One minute earlier at 45 min the same number of *S. senftenberg* SM001 cells were viable on both *S. senftenberg* SM001 and *S. typhimurium* SA2009/*S. senftenberg* SM001 plates. Why should there be a difference? After 45 min many of the *S. typhimurium* SA2009 will presumably have been injured and some may not have active peroxidases, which includes catalase. Hydrogen peroxide will accumulate due to aerobic respiration as cells begin to repair cellular damage. However sufficient *S. typhimurium* SA2009 cells will have functional peroxidase systems to reduce hydrogen peroxide to non-inhibitory levels. As a result, *S. senftenberg* SM001 will not be affected whether inactivated alone or with *S. typhimurium* SA2009. After 46 min the number of injured *S. typhimurium* SA2009 cells has risen, more are beginning to repair damage and so producing H_2O_2, but fewer have efficient peroxidase systems. Although all the *S. senftenberg* SM001 will presumably have active catalase and this could allow some of the injured *S. typhimurium* SA2009 to recover, the extremely high levels of H_2O_2 will cause most of the *S. senftenberg* SM001 to lose viability.

It may be possible to test this theory by recovering heat inactivated *S. typhimurium* SA2009 in a medium that had sustained the growth of *S. senftenberg* 775W for a period. As a control, LB medium in which *S. typhimurium* SA2009 had been grown could be used to recover *S. typhimurium* SA2009. After recovery for a known period, total viable counts on LB agar plates could be compared. If there was enhanced recovery in the medium that had sustained the growth of *S. senftenberg* 775W, this might suggest the release of a substance from *S. senftenberg* 775W capable of increasing survival of thermally stressed cells. The experiment could be repeated and the medium subdivided into aliquots and heated to temperatures

between 50°C and 100°C for known time periods. Recovery could then be compared between the different temperature aliquotes to see if the enhanced recovery was affected by temperature. This scenario would obviously favour the theory that *S. senftenberg* 775W possessed a heat stable peroxidase, in which case recovery would fall with increasing temperature. The other scenario, the lack of any increase in *S. typhimurium* SA2009's survival, would favour the theory that the expression of the peroxidase genes was heat stable.

If H_2O_2 accumulation and reduced catalase activity is the cause of reduced survival, why should *S. typhimurium* MR21 have higher survival in liquid broth than on the surface of an agar plate, compared with *S. typhimurium* SA2009. This is difficult to explain using this theory except to say that, during thermal inactivation in LB and serial dilution prior to viable counting, H_2O_2 would not be produced to any great extent as aerobic respiration would not take place at the level seen on the surface of an agar plate. Also any H_2O_2 produced would be diluted in the LB of the heating menstruum and during serial dilutions. There is also the factor of increased diffusion in liquid media opposed to solid media. Nevertheless if H_2O_2 accumulation and reduced catalase activity is the cause of differences in resistance, it should be possible to reduce the level of H_2O_2 by overlaying the thermally injured cells. This would reduce aerobic respiration and consequently the levels of H_2O_2. If this does occur and *S. typhimurium* MR21 exhibits the same resistance on plates as it does in LB, it would be possible to use *S. typhimurium* MR21 as the source of genetic material. It would then be possible to use bacteriophage P22 and reduce many of the problems encountered with *S. senftenberg* 775W.

The ability of *S. senftenberg* 775W to survive at high temperatures for measurably longer in both broth and

on the surface of agar plates lent itself for further study. The need for an organism that could survive on the surface of a plate allowed large numbers of cells, with potentially different heat resistances, could be screened at once.

4.3 Evidence for *S. senftenberg* 775W elevated heat resistance

4.3.1 Cell membrane repair

S. senftenberg 775W was able to regain salt tolerance immediately after transfer to the recovery medium. The other organisms required about 1 hour before any evidence of salt tolerance was seen. As mentioned in section 3.7.4.4, this rapid return of salt tolerance/cell membrane integrity could be explained in at least two ways. Firstly, rapid repair of the damaged membrane could take place; secondly, only slight membrane damage is incurred during the thermal exposure. There is of course the possibility of a combination of the two taking place.

Lipid synthesis is known to occur during recovery from thermal damage in *S. typhimurium* (Pierson, Tomlins and Ordal, 1971). Pierson *et al.*, stated, "The repair of the heat damaged membrane must be rapid and probably occurs early during recovery so the cell can fully repair other lesions. If this is the case, then lipid synthesis plays an important role in the repair process". It may be that *S. senftenberg* 775W is able to begin synthesising new lipid material immediately after being transferred to the recovery medium. However, any damage to the membrane would allow internal pools to leak out of the cell, requiring time to re-accumulate them, but only after membrane repair. It would therefore appear more likely that viability is maintained by *S. senftenberg* 775W reducing the level of membrane damage.

It has already been mentioned that the cytoplasmic membrane of mesophilic bacteria is very sensitive to heat as it lies on the borderline of a temperature-sensitive phase transition (Bryne and Chapman, 1964). This phase transition which is between the thermotrophic gel and liquid-crystalline phases, is dependent upon the environmental temperature. If this temperature changes too quickly, for example changing from 37°C to 55°C, the membrane is unable to cope with the increase in energy, and structurally and functionally the membrane will break down. Mesophilic bacteria generally have cell membranes that are liquid-crystalline in structure and contain a high proportion of unsaturated fatty acids. They have a high level of fluidity while maintaining cell form. Thermophilic bacteria on the other hand require a more rigid membrane to cope with the high temperatures and do this by lowering the proportion of unsaturated fatty acids, increasing the chain length of their fatty acyl chains and increasing the proportion of methyl branched chains. The first of these changes is also noted when mesophilic bacteria are grown at elevated temperatures, e.g., 42°C. This is a transient phase change to stabilize the membrane, which reverts back when the temperature returns to 37°C. It may be that *S. senftenberg* 775W's membrane has a lipid composition somewhere between that of a mesophile and a thermophile. In this way when exposed to an elevated temperature, *S. senftenberg* 775W would be able to survive for longer than "true" mesophiles but nowhere near as long as thermophiles. However any changes in lipid composition to form a more rigid membrane would presumably reduce the growth rate at optimum growth temperatures and increase the maximum growth temperature, which for *S. senftenberg* 775W is exactly the same as *S. typhimurium* SA2009 and *S. senftenberg* JT577. Nevertheless it may be possible for *S. senftenberg* 775W to have a lipid composition that gives its membrane some level of thermal resistance while

retaining membrane fluidity equivalent to that of other mesophiles. Thermophilic bacteria are able to maintain a level of membrane fluidity by having a high proportion of methyl branched chains. These chains have been shown to be "fluidizers" rather than "stabilizers" (Bean and Perry, 1974) and may allow thermophiles to adjust to growth at lower temperatures.

The conditions in which thermally injured organisms are recovered is critical. Injured organisms are unable to recover when placed in minimal medium due to the loss of internal pools, and unless they are transferred to a nutritionally richer medium will lose viability. Therefore if *S. senftenberg* 775W by incurring less membrane damage, is able to limit the loss of internal pools, it may be able to begin recovery sooner. The time during recovery in which membrane repair and internal pools are re-established is obviously critical. It is likely therefore that the longer the time taken to return cell membrane integrity, the less the chance the cell has of recovering. It may therefore follow that the shorter the time to return membrane integrity, by whatever means, the greater the chance of maintaining viability.

One could use this theory to provide a partial explanation for the elevated heat resistance seen in all three organisms when pre-incubated at 42°C prior to thermal injury. Although hsp's are known to be synthesised during growth at 42°C, and that they are associated with elevated heat resistance, some of the increase in resistance could be due to the change in the saturated:unsaturated fatty acid ratio. There is an increase in the proportion of saturated fatty acids, along with a decrease in the proportion of unsaturated fatty acids when *E. coli* is incubated at 42°C (Katsui, 1981). This alteration would increase the phase-transition temperature of the membrane, making it more resistant to further increases in temperature. This may

partly explain the increased ability of all three organisms to recover after thermal injury when pre-incubated at 42°C opposed to 37°C. It might therefore be interesting to measure the levels of material in the menstruum, known to leak from the cell during heat damage, during thermal injury of each organism when incubated at 37°C and 42°C prior to the thermal exposure.

4.3.2 Ribosome and rRNA repair

It has been shown that loss of 260 nm-absorbing material is associated with the loss of cell viability, that this material is derived from rRNA, and that the resynthesis of rRNA in *S. typhimurium* is an obligatory requirement for recovery (Tomlin & Ordal, 1971). Ribosome damage in *S. aureus* during mild heating is thought to be due to the action of polynucleotide phosphorylase and ribonuclease (Haight & Ordal, 1969). It was suggested that the damage was the result of lost Mg^{2+} ions that normally stabilize the ribosomes, and this then leaves them open to ribonuclease attack (Hurst & Hughes, 1978). One might assume that if *S. senftenberg* 775W only under goes slight cell membrane damage during mild heating (as suggested in 4.3.1), Mg^{2+} ions could remain at high enough levels to maintain ribosome stability, reducing the risk of enzymic attack. This would have the affect of reducing or removing the time taken for ribosomes to repair and consequently begin the resynthesis of new proteins.

As stated previously, the recovery of heat-injured *S. typhimurium* requires the resynthesis of RNA (Tomlins & Ordal, 1971). Nevertheless, *S. senftenberg* 775W is able to recover in the presence of rifampicin, an inhibitor of RNA synthesis, (*Figure* 3.21).

Firstly, *S. senftenberg* 775W may develop an altered resistance to rifampicin after thermal injury.

Rifampicin is a relatively small, slightly hydrophillic molecule that under normal circumstances should be able to penetrate the cell membrane by diffusion through aqueous channels formed by porin molecules which span the membrane. However changes can occur in the membrane of heat damaged Gram-negative bacteria that prevent passage of this type of antibiotic. *E. coli* has been reported to develop an increased resistance to novobiocin after damage induced by drying (Mackey, 1983). Cellular repair could therefore take place without the inhibitory effects of rifampicin.

Secondly, the half life of mRNA is relatively short, measurable in minutes. Therefore, for recovery to take place in the presence of an RNA synthesis inhibitor, RNA synthesis must occur prior to the addition of the rifampicin by either producing a protein that facilitates recovery directly, or by altering the specificity of the DNA-dependent RNA polymerase, which is the site of rifampicin inhibition. Either way the protein produced must be induced directly or indirectly by the increase in temperature, and be rapidly synthesised. These conditions describe a heat inducible protein which were discussed in section 1.4. The mRNA of heat shock proteins are initiated within 15 seconds when transferred from 30°-42°C (Yamamori & Yura, 1980), but it is quite possible that these proteins are initiated when cells are transferred from 37°C to 55°C. If this were the case, then sufficient could be synthesised prior to the transfer to LB (rifampicin) to allow resistance to rifampicin to develop. If this does occur, what form could the heat inducible protein take? The most obvious is the product of the *htpR* gene. This is the only known effector of the heat shock response and is also a sigma factor (sigma-32) (Grossman, *et al.*, 1984). A bacterial RNA polymerase is made up of four major subunits, designated β, β', σ and α. The σ-subunit initiates binding of the RNA polymerase to the DNA and the β-

subunit is the site at which rifampicin binds. Although rifampicin is unable to bind once chain elongation has progressed beyond the second or third phosphodiester bond, insufficient mRNA would be synthesised in the presence of rifampicin to allow protein synthesis to continue for any length of time. However an alteration to the σ-subunit can, because of its topological relationship to the β-subunit, alter rifampicin's ability to bind to the β-subunit (Stender *et al.*, 1975). It may be that the HtpR protein induced in *S. senftenberg* 775W inhibits, partially or completely, the binding of rifampicin to the DNA dependent RNA-polymerase. If this were the case, pre-incubation of *S. senftenberg* 775W at 42°C would give improved recovery, as more of the sigma-32 protein would be synthesised. However the concomitant heat shock proteins simultaneously induced would make interpretation difficult.

When the organisms were pre-incubated at 42°C prior to the exposure to 55°C, *S. typhimurium* SA2009 and *S. senftenberg* JT577 remained unable to recover in the presence of rifampicin, while *S. senftenberg* 775W began to recover almost immediately. What can be said about these observations regarding *S. senftenberg* 775W recovery? Firstly the rapid recovery seen by *S. senftenberg* 775W would require a high number of functional ribosomes when it was returned to the recovery medium. Secondly, the method by which rifampicin was prevented from inhibiting RNA synthesis would have to remain and possibly be enhanced. Therefore what theories can be given to provide explanations for the increased resistance, taking into account those already given?

The cell membrane increases its phase-transition temperature when incubated at a higher temperature, e.g., 42°C (see section 4.3.1), and increases its ability to maintain integrity at subsequent exposures to lethal temperatures, e.g., 55°C. The membrane would therefore

be less likely to sustain any damage and reduce the risk further of enzymic ribosome breakdown due to loss of Mg^{2+} ions.

One might not expect to see any increase in the ability of *S. senftenberg* 775W to recover in the presence of rifampicin if the initial reason was merely an inability of the antibiotic to cross the membrane. However work highlighted in section 4.3.1 has shown that the cell membranes' fatty acid ratio changes during growth at 42°C as compared to that seen at 37°C. This change may affect the ability of the antibiotic to cross the membrane, leaving *S. senftenberg* 775W open to re-synthesise proteins, providing the machinery for protein synthesis is still functional. Other evidence suggests that increased heat resistance after pre-incubation of *S. typhimurium* at an up-shifted temperature is accompanied by the synthesis of heat shock proteins (Mackey and Derrick, 1990). It might therefore be reasonable to assume that the level of *htpR* present in *S. senftenberg* 775W would be greater at the up-shifted temperature. If this protein is able to reduce rifampicins ability to bind to the RNA-polymerase in *S. senftenberg* 775W, then the higher the level of protein, the greater the ability to recover when returned to optimal growth conditions, even in the presence of rifampicin. Again this is dependent upon having a functional protein synthesis system.

4.3.3 Protein synthesis

None of the organisms investigated were able to recover in the presence of a protein synthesis inhibitor. Tomlins *et al.*, (1971) showed that *S. typhimurium* does not suffer extensive metabolic damage during thermal injury. Growth at 42°C prior to heating at 55°C did not allow cells to recover when protein synthesis was prevented. Knowing that heat induction results in the

synthesis of hsp's, and some have been implicated in the reversal of protein denaturation caused by heat (see section 1.4.4), the protein damage induced must be extensive. It may be possible by varying the lethal temperature, and thereby varying the degree of protein damage, to observe improved recovery in the heat induced cultures when recovered in the presence of protein synthesis inhibitors. Alternatively damage to key heat labile proteins at any lethal temperature may be so great, or irreversible, that recovery would never take place. Even so, *S. senftenberg* 775W's elevated heat resistance, compared with *S. senftenberg* JT577 and *S. typhimurium* SA2009 does not appear to be as a result of improved protein protection or repair.

4.4 Transfer of heat characteristics

The acquisition of an elevated ability to survive at 55°C on the surface of a plate and in LB, by two clones of *E. coli* MM294 following transformation with *S. senftenberg* 775W DNA, was taken as evidence for the transfer of genes conferring heat resistance from *S. senftenberg* 775W and *E. coli* MM294. Although the transfer of genetic material between *S. senftenberg* 775W and *S. typhimurium* SA2009 had initially proved unsuccessful, the use of a restriction/modification deficient strain of *E. coli* as a recipient gave a lead into the heat resistance *S. senftenberg* 775W. Although the size of the fragments present in the recombinant plasmid molecules were never measured, their size should have been between 4-6 kb. A cosmid could have given a fragment insert size of up to 45 kb, but the use of the pUN121 plasmid appears to have been sufficient to transfer some or all of the genes responsible for *S. senftenberg* 775W's heat resistance.

The close similarity between the shapes of the survival curves of clones R6 and R22 could suggest that

both plasmids contained overlapping portions of the *S. senftenberg* 775W chromosome. The shapes of these curves could have several explanations. For example, the gene(s) responsible for the heat resistance seen in *S. senftenberg* 775W when transferred into *E. coli* MM294 did not give fully functional protein(s). Perhaps co-factors are required to provide complete protection and after 2 minutes the protein(s) function begins to become impaired without them. Alternatively the DNA fragments had only some of the genes responsible for heat resistance and full resistance is only seen when all are transferred. Another explanation may be that the proteins produced interact with specific sites inside the cells and these are absent in *E. coli* MM294.

The advantage of having used the pUN121 plasmid, is that further work could have quickly shown how large the region of DNA was that encoded the proteins responsible for the heat resistance. By restriction digests of the inserted fragments with other restriction enzymes, a series of smaller fragments could have been generated. By religating these fragments back into the plasmid the region of DNA responsible for the increased resistance could be located. It would also be possible to compare the digestion patterns of the fragments responsible for increased resistance in R6 and R22 and see if they were overlapping sections. If they were not homologous, both could be transformed into a single recipient and the resistance measured again.

Unfortunately because of limited time, only a few of the transformants were screened for elevated heat resistances. With more time other transformants could have been screened and perhaps others with elevated resistances found. Once a population of transformants with elevated resistances was available, the genes could be mapped. If sufficient homology was present between *S. senftenberg* 775W, *S. typhimurium* and *E. coli*,

recombination experiments could have provided evidence as to the nature of the genes and a possible function. Also the physiological work could have been repeated and the ability of wild type and transformed *E. coli* MM294's to recover in the presence of rifampicin and regain salt tolerance could have been measured. The transfer of heat resistance factors has also been reported between thermophilic and non-thermophilic strains of *Bacillus* spp. MacDonald (1969) showed that temperature-sensitive strains of *B. subtilis* could be transformed to allow growth at 55°C. He suggested that the gene responsible for the increase in resistance was closely linked to the streptomycin region. He went on to postulate that due to the position of the gene for temperature-sensitivity, relative to the streptomycin region, that it was associated with ribosomal structure.

ACKNOWLEDGEMENTS

This thesis is dedicated to the memory of my Grandmother, for whom I stayed good and got clever.

I would like to thank Bev Moseley for his advice, and his unstinting optimism during the course of this Ph.D.

Thanks also to all members of the Microbiology Department, particularly, Vicki Manners, Margaret Rogers, Liz Ellis, Lynn Kennedy and Anne Black.

To all the members of the Biochemistry Department in Dundee, particularly, Chris Higgins and Steve Hyde, I would like to say thank you for putting up with me.

I would like to thank M.A.F.F. for the three year funding of this studentship

I am indebted to many other workers else where, especially; T. A. Roberts I.F.R. Reading, for the heat cycled strains of *Salmonella typhimurium*; I. Marsh Edinburgh University, for supplying *E. coli* GroEL and GroES antibody; D. Jamieson for general advice.

BIBLIOGRAPHY

ALLWOOD, M. C. & RUSSELL, A. D. (1967). Mechanism of thermal injury in *Staphylococcus aureus*. 1 Relationship between viability and leakage. *Applied Microbiology* 15: 1266-1269.

ALLWOOD, M. C. & RUSSELL, A. D. (1968). Thermally induced ribonucleic acid degradation and leakage of substances from the metabolic pool of *Staphylococcus aureus*. *Journal of Bacteriology* 95: 345-349.

ALLWOOD, M. C. & RUSSELL, A. D. (1969). Growth and metabolic activity of heat treated *Staphylococcus aureus*. *Journal of Applied Bacteriology* 32: 79-85.

ALLWOOD, M. C. & RUSSELL, A. D. (1970). Mechanism of thermal injury in nonsporulating bacteria. Advances in Applied Microbiology 12: 89-119.

AMES, G. F. (1968). Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. *Journal of Bacteriology* 95: 833-843.

ANFINSEN, C. B. & SCHERAGA, H. A. (1975). Experimental and theoretical aspects of protein folding. *Advances in Protein Chemistry* 29: 205-301.

AUNE, K. C., SALAHUDDIN, A., ZARLENGO, M. H. & TANFORD, C. (1967). Evidence for residual structure in acid- and heat-denatured proteins. *Journal of Biological Chemistry*. 242: 4486-4489.

BAUMAN, N. A., HAGAN, P. Q. & GOLDFINE, H. (1965). Phospholipids of *Clostridium butyricum* on plasmogen composition and biosynthesis. *Journal of Biological Chemistry* 240: 1559-1567.

BEAN, H. W. & PERRY, J. J. (1974). Microbial degradation and assimilation of *n*-Alkyl-substituted cycloparaffins. *Journal of Bacteriology* 118: 394-399.

BIO-RAD technical publication. (1987). Gene pulser transfection apparatus operating instructions and applications guide. Bio-Rad Laboratories, Richmond, California CA 94804.

BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening plasmid DNA. *Nucleic Acid Research* 7: 1513-1523.

BLUHM, L. & ORDAL, Z. J. (1969). Effect of sub-lethal heat on the metabolic activity of *Staphylococcus aureus*. *Journal of Bacteriology* 97: 140-150.

BOCHKAREVA, E. S., LISSIN, N. M. & GIRSHOVICH, A. S. (1988). Transient association of newly synthesized unfolded proteins with the heat-shock GroEL protein. *Nature* 336: 254-257.

BOHAK, Z. (1964). Nε(DL-2-Amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. *Journal of Biological Chemistry* 239: 2878-2887.

BRANDT, J. F. (1967). Heat effects on proteins and enzymes. In Thermobiology. (Ed. Rose, A. H.). Academic Press p. 25-72.

BRIDGES, B. A., ASHWOOD-SMITH, M. J. & MUNSON, R. J. (1969). Susceptibility of mild thermal and ionizing radiation damage to the same recovery mechanism of *Escherichia coli*. *Biochemical and Biophysical Research Communications* 35: 193-196.

BRIDGES, B. A., ASHWOOD-SMITH, M. J. & MUNSON, R. J. (1969*a*). Correlation of bacterial sensitivities to ionizing radiation and mild heating. *Journal of General Microbiology* 58: 115-124.

BUSTA, F. F. & JEZESKI, J. J. (1963) Effect of sodium chloride concentration in an agar medium on growth of heat shocked *Staphylococcus aureus* *Applied Microbiology* 11: 404-407.

BYRNE, P. & CHAPMAN, D. (1964). Liquid crystalline nature of phospholipids. *Nature* 202: 987-988.

CALIFANO, L. (1952). Liberation d'acide nucleique par les cellules bacteriennes sons l'action de la chaleur. *Bulletin of the World Health Organization* 6: 19-34.

CECIL, R. & MCPHEE, J.R. (1959). The sulfur chemistry of proteins. *Advances in Protein Chemistry* 14: 255-289.

CORRY J. E. L. & ROBERTS, T. A. (1970). A note on the development of resistance to heat and gamma radiation in *Salmonella*. *Journal of Applied Bacteriology* 33: 733-737.

COSTERTON, J. W., INGRAM, J. M. & CHENG, K. J. (1974). Structure and function of the cell envelope of gram-negative bacteria. *Bacteriological Reviews* 38: 87-110.

EBEL-TSIPIS, J., BOTSTEIN, D. & FOX, M. S. (1972). Generalised transduction by phage P22 in *Salmonella typhimurium*. I Molecular origin of transducing DNA. *Journal of Molecular Biology* 71: 433-448.

EBEL-TSIPIS, J., FOX, M. S. & BOTSTEIN, D. (1972*a*). Generalised transduction by bacteriophage P22 in *Salmonella typhimurium.* II Mechanism of integration of transducing DNA. *Journal of Molecular Biology* 71: 449-469.

EIGNER, J. BOEDTKER, H. & MICHAELS, G. (1961). The thermal degradation of nucleic acids. *Biochemica et Biophysica Acta* 51: 165-168.

FLOWERS, R. S., MARTIN, S. E., BREWER, D. G. & ORDAL, Z. J. (1977). Catalase and enumeration of stressed *Staphylococcus* aureus cells. *Annals of Environmental Microbiology* 33: 1112-1117.

FLOWERS, R. S. & MARTIN, S. E. (1980). Ribosome assembly during recovery of heat-injured *Staphylococcus aureus* cells. *Journal of Bacteriology* 141: 645-651.

GARVIE, E. I. (1955). The growth of *Escherichia coli* in buffer substrate and distilled water *Journal of Bacteriology* 69: 393-398.

GLASS, W. F., BRIGGS, R. C. & HNILICA, L. S. (1981). Identification of tissue-specific nuclear antigens transferred to nitrocellulose from polyacrlymide gels. *Science* 211: 70-72.

GRECZ, N. & BHATARAKOMOL, S. (1977). Apurinic acid endonuclease implicated in DNA breakage in *Escherichia coli* subjected to mild heat. *Biochemical and Biophysical Research Communications* 77: 1183-1188.

GREER, S. & ZAMENHOF, S. (1962). Studies on depurination of Dna by heat. *Journal of Molecular Biology* 4: 123-141.

GOMEZ, R. F. & SINSKEY, A. J. (1973). Deoxyribonucleic acid breaks in heated *Salmonella typhimurium* LT-2 after exposure to nutritionally complex media. *Journal of Bacteriology* 115: 522-528.

GOMEZ, R. F. SINSKEY, A. J. DAVIES, R. & LABUZA, T. P. (1973). Minimal medium recovery of heated *Salmonella typhimurium* LT2. *Journal of General Microbiology* 74: 267-274.

GROSSMAN, A. D., ERICKSON J. W. & GROSS, C.A. (1984). The *htpR* gene product of *Escherichia coli* is a sigma factor for heat-shock promoters. *Cell* 38: 383-390.

HAIGHT, R. D. & ORDAL, Z. J. (1969). Thermally induced degradation of staphylococcal ribosomes. *Canadian Journal of Microbiology* 15: 15-19.

HANSEN, N. H. & RIEMANN, H. (1963). Factors affecting the heat resistance of nonsporing organisms. *Journal of Applied Bacteriology* 26: 314-333.

HANSEN, E. W. & SKADHAUGE, K. (1970). The influence of growth temperature on the thermal resistance of *Escherichia coli*. *Dansk Tidsskr Farm* 45: 24-28.

HEIMETZ, F., TAYLOR, W. W. & LEHMAN, J. J. (1954). The use of metabolites in the restoration of viability of heat and chemically inactivated *Escherichia coli*. *Journal of Bacteriology* 67: 5-12.

HITCHENER, B. J. & EGAN, A. F. (1977). Outer-membrane damage in sublethally heated *Escherichia coli* K-12. *Canadian Journal of Microbiology* 23: 311-318.

HOUTSMULLER, V. & VAN DEENAN, L. (1965). On the amino acid esters of phosphotidyl glycerol from bacteria. *Biochemical and Biophysical Acta* 65: 134-141.

HURST, A., HUGHES, A., BEARE-ROGERS, J. L. & COLLINS-THOMPSON, D. L. (1973). Physiological studies on the recovery of salt tolerance by *Staphylococcus aureus* after sub-lethal heating. *Journal of Bacteriology* 116: 901-907.

HURST, A. & HUGHES, A. (1978). Stability of ribosomes of *Staphylococcus aureus* S6 sublethally heated in different buffers. *Journal of Bacteriology* 133: 564-568.

IANDOLO, J. J. & ORDAL, Z. J. (1966). Repair of thermal injury of *Staphylococcus aureus*. *Journal of Bacteriology* 91: 134-142.

IKEDA, H. & TOMIZAWA, J. (1965). Transducing fragments in generalised transduction by phage P1. I. Molecular origin of the fragments. *Journal of Molecular Biology* 14: 85-109.

JACKSON, H. & WOODBINE, M. (1963). The effect of sub-lethal heat treatment on the growth of *Staphylococcus aureus*. *Journal of Applied Bacteriology* 26: 152-158.

JOHNSON, D. A., GAUTSCH, J. W., SPORTSMAN, J. R. & ELDER, J. H. (1984). Improved technique utilizing non fat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Analysis Techniques* 1: 3-8.

KATSUI, N., TSUCHIDO, T., TAKANO, M. & SHIBASAKI, I. (1981). Effect of preincubation temperature on

the heat resistance of *Escherichia coli* having different fatty acid compositions. *Journal of General Microbiology* 122: 357-361.

KATSUI, N., TSUCHIDO, T., TAKANO, M. & SHIBASAKI, I. (1982). Viability of heat-stressed cells of micro-organisms as influenced by pre-incubation and post-incubation temperatures. *Journal of Applied Bacteriology* 53: 103-108.

KATSUI, N., TSUCHIDO, T., HIRAMATSU, R., FUJIKAWA, S., TAKANO, M. & SHIBASAKI, I. (1982*a*). Heat-induced blebbing and vesiculation of the outer membrane of *Escherichia coli*. *Journal of Bacteriology* 151: 1523-1531.

KAUFMANN, O. W., HARMON, L. G., PAILTHORP, O. C. & PFLUG. (1959). Effect of heat treatment on the growth of surviving cells. *Journal of Bacteriology* 78: 834-838.

KELLER, J. A. & SIMON, L. D. (1988). Divergent effects of a *dnaK* mutation on abnormal protein degradation in *Escherichia coli*. *Molecular Microbiology* 2: 31-41.

KITO, M., AIBARA, S., KATO, M. & HATA, T. (1972). Differences in fatty acid composition among phosphatidylethanolamine, phosphatidylglycerol and cardiolipin of *Escherichia coli*. *Biochemical and Biophysical Acta* 260: 475-478.

KLIBANOV, A. M. & MOZHAEV, V. V. (1978). On the mechanism of irreversible thermoinactivation of enzymes and possibilities for reactivation of "irreversibly" inactivated enzymes. *Biochemical and Biophysical Research Communications* 83: 1012-1017.

LINDQUIST, S. (1986). The Heat Shock Response. *Annual Review of Biochemistry* 55: 1151-1191.

MACDONALD, W. C. (1969). Linkage of a temperature sensitive loci to the streptomycin region and its use in recombination studies with streptomycin b mutants in *Bacillus subtilis*. *Canadian Journal of Microbiology* 15: 1287-1291.

MACFARLANE, M. G. (1962). Lipid components of *Staphylococcus aureus* and *Salmonella typhimurium*. *Biochemical Journal* 92: 40-41.

MACKEY, B. M. (1983). Changes in antibiotic sensitivity and cell surface hydrophobicity in *Escherichia coli* injured by heating, freezing, drying or gamma radiation. *FEMS Microbiology Letter* 20: 395-399.

MACKEY, B. M. & DERRICK, C. M. (1986). Elevation of the heat resistance of *Salmonella typhimurium* by sublethal heat shock. *Journal of Applied Bacteriology* 61: 389-393.

MACKEY, B. M. & DERRICK, C. M. (1990). Heat shock protein synthesis and thermotolerance in *Salmonella typhimurium*. *Journal of Applied Microbiology* 69: 373-383.

MACKEY, B. M. & SEYMOUR, D. A. (1987). The effects of catalase on recovery of heat-injured DNA-repair mutants of *Escherichia coli*. *Journal of General Microbiology* 133: 1601-1610.

MANDEL, M. & HIGA, A. (1970). Calcium dependant bacteriophage DNA infection. *Journal of Molecular Biology* 53: 159-162.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *Journal of Molecular Biology* 5: 109-118.

MARQUIS, R. E. & CORNER, T. R. (1967). Permeability changes associated with osmotic swelling of bacterial protoplasts. *Journal of Bacteriology* 93: 1177-1178.

MARTIN, S. E., FLOWERS, R. S. & ORDAL, Z. J. (1970). Catalase: its effects on microbial enumeration. *Annals of Environmental Microbiology* 32: 731-734.

MCELHANEY, R. N. (1976). The biological significance of fatty acid composition of microbial membrane lipids in response to changes in environmental temperature. In *Extreme Environment Mechanisms of Microbial Adaption* (Ed Heinrich, M. R.) pp. 255-281. London & New York: Academic Press.

MCGARRITY, J. T. & ARMSTRONG, J. B. (1975) Effect of salt on phospholipid fatty-acid composition in *Escherichia coli* K-12. *Biochemical and Biophysical Acta* 398: 258-264.

MCLENDON, G. & RADANY, E. (1978). Protein turnover thermodynamically controlled. *Journal of Biological Chemistry* 253: 6335-6338.

MOATS, W. A. (1971). Kinetics of thermal death of bacteria. *Journal of Bacteriology* 105: 165-171.

MOATS, W. A., DABBAH, R. & EDWARDS, V. M. (1971). Interpretation of non-logarithmic survivor curves of heated bacteria. *Journal of Food Science* 36: 523-526.

MOHAMED, N. & DAVIES, R. (1983). Heat resistance of *Salmonella senftenberg* as a function of previous growth temperature. In *Food Microbiology*: Society of Applied Bacteriology Symposium Series No. 11 (Eds Roberts, T. A. & Skinner, F. A.) pp. 374. London & New York: Academic Press.

NATORI, S., NOZAWA, R. & MIZUNO, D. (1966). The turnover of ribosomal RNA of *Escherichia coli* in a magnesium deficient stage. *Biochemica et Biophysica Acta* 114: 245-253.

NEIDHARDT, F. C. & VANBOGELEN, R. A. (1987). Heat Shock Response. In *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*. (Eds Neidhardt, F.C., Ingraham, J. L., Brooks Low, K., Magasanik, B., Schaechter, M. & Umbarger, H. E.) American Society for Microbiology. Washington D.C. pp. 1334-1345.

NG, H., BAYNE, H. G. & GARIBALDI, J. A. (1969). Heat resistance of *Salmonella*: the uniqueness of *Salmonella senftenberg* 775W. *Applied Microbiology* 17: 78-82.

OLSEN, R. W. & BALLOU, C. E. (1971). Acyl phosphotidyl glycerol- A new phospholipid from *Salmonella typhimurium*. *Journal of Biological Chemistry* 246: 3305-3313.

PACE, B. & CAMPBELL, L. L. (1967). Correlation of maximal growth temperature and ribosome heat stability.

Proceedings of the National Academy of Science of the United States of America 57: 1110-1116.

PAULING, C. & BECK, L. A. (1975). Role of DNA ligase in the repair of single strand breaks induced in DNA by mild heating of *Escherichia coli*. *Journal of General Microbiology* 87: 181-184.

PELHAM, H. R. B. (1986). Specifications on the functions of the major heat shock and glucose-regulated protein. *Cell* 46: 959-961.

PIERSON, M. D., TOMLINS, R. I. & ORDAL, Z. J. (1971). Biosynthesis during recovery of heat-injured *Salmonella typhimurium*. *Journal of Bacteriology* 105: 1234-1236.

PIERSON, M. D. & ORDAL, Z. J. (1971). The transport of methyl-α-D-gluco-pyranoside by thermally stressed *Salmonella typhimurium*. *Biochemical Biophysical Research Communications* 43: 378-383.

RACKER, E. (1965). Mechanisms in bioenergetics. Academic Press Inc. N.Y.

RAYMAN, M. K., ARIS, B. & EL DEREA, H. B. (1978). The effect of compounds which degrade hydrogen peroxide on the enumeration of heat-stressed cells of *Salmonella senftenberg*. *Canadian Journal of Microbiology* 24: 883-885.

ROSENTHAL, L. J. & IANDOLO, J. J. (1970). Thermally induced intracellular alteration of ribosomal ribonucleic acid. *Journal of Bacteriology* 103: 833-835.

ROSENTHAL, L. J., MARTIN, S. E., PARIZA, W. A. & IANDOLO, J. J. (1972). Ribosome synthesis of thermally

shocked cells of *Staphylococcus aureus*. *Journal of Bacteriology* 109: 245-249.

SAUNDERS, G. F. & CAMPBELL, L. L. (1966). Ribonucleic acid and ribosomes of *Bacillus stearothermophilus*. *Journal of Bacteriology* 91: 332-339.

SCHULZ, G. E. & SCHIRMER, R. H. (1979). Principles of Protein Structure, Chapter 3. Stringer-Verlag, Berlin and New York.

SEDGWICK, S. G. & BRIDGES, B. A. (1972). Evidence for indirect production of DNA strand scissions during mild heating of *Escherichia coli*. *Journal of General Microbiology* 71: 191-193.

SOGIN, S. J. & ORDAL, Z. J. (1967). Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury in *Staphylococcus aureus*. *Journal of Bacteriology* 94: 1082-1087.

SOLOWEY, M., SUTTON, R. R. & CALESNICK, E. J. (1948). Heat resistance of Salmonella organisms isolated from spray-dried whole-egg powder. *Food Technology* 2: 9-14.

SOUTHERN, E. M. (1979). Gel electrophoresis of restriction fragments. In: Methods in Enzymology (Ed. R. Wu. Academic Press) 68: 152-175.

STANIER, R. Y., ADELBERG, E. A. & INGRAHAM, J. L. (1981). In General Microbiology 4th Edition. Macmillan Press

STEINRAUF, L. K. & DANDLIKER, W. B. (1958). A study of the reaction of the disulfide groups of bovine

serum albumin during heat denaturation. *Journal of the American Chemical Society* 80: 3833-3839.

STENESH, J. & YANG, C. (1967). Characterization and stability of ribosomes from mesophilic and thermophilic bacteria. *Journal of Bacteriology* 93: 930-936.

STENDER, W, STUTZ, A. A. & SCHEIT, K. H. (1975). The modification of DNA-dependent RNA polymerase from *Escherichia coli* by an alkylating derivative of rifampicin SV. *European Journal of Biochemistry* 56: 129-136

STILES, M. E. & WITTER, L. D. (1965). Thermal inactivation, heat injury, and recovery of *Staphylococcus aureus*. *Journal of Dairy Science* 48: 677-681.

STRANGE, R. E. & SHON, M. (1964). Effects of thermal stress on viability and ribonucleic acid *Aerobacter aerogenes* in aqueous suspension. *Journal of General Microbiology* 34: 99-114.

TILLY, K., MCKITTRICK, N., ZYLICZ. & GEORGOPOULOS,. C (1983). The *dna*K protein modulates the heat-shock response of *Escherichia coli*. *Cell* 34: 641-646.

TOMLINS, R. I., PIERSON, M. D. ORDAL, Z. J. (1971). Effect of thermal injury on the TCA cycle enzymes of *Staphylococcus aureus* MF31 and *Salmonella typhimurium* 7136. *Canadian Journal of Microbiology* 17: 759-765.

TOMLINS, R. I. & ORDAL, Z. J. (1971). Requirements of *Salmonella typhimurium* for recovery from thermal injury. *Journal of Bacteriology* 105: 512-518.

TOMLINS, R. I. & ORDAL, Z. J. (1971*a*). Precursor ribosomal ribonucleic acid and ribosome accumulation *in vivo* during the recovery of *Salmonella typhimurium* from thermal injury. *Journal of Bacteriology* 107: 134-142.

TOMLINS, R. I. VAALER, G. L. & ORDAL, Z. J. (1972). Lipid biosynthesis during the recovery of *Salmonella typhimurium* from thermal injury. *Canadian Journal of Microbiology* 18: 1015-1021.

TOMLINS, R. I. & ORDAL, Z. J. (1976). Thermal injury and inactivation in vegetative bacteria. In *Inhibition and Inactivation of Vegetative Microbes*: Society of Applied Bacteriology Symposium Series No. 5 (Eds Skinner, F. A. & Hugo, W. B.) pp. 153-190. London & New York: Academic Press.

TYE, B., HUBERMAN, J. A. & BOTSTEIN, D. (1974). Non-random circular permutation of phage P22 DNA. *Journal of Molecular Biology* 85: 501-532.

WARNER, R. C. & LEVY, M. (1959). Denaturation of bovine plasma albumin. 2 Isolation of intermediates and mechanism of the reaction at para-H 7. *Journal of the American Chemical Society* 81: 5735-5744.

WINTER, A. R., STEWART, G. F., MCFARLANE, V. H. & SOLOWEY, M. (1946). Pasteurization of liquid egg products. III. Destruction of Salmonella in liquid whole egg. *American Journal of Public Health* 36: 451-460.

WOOD, T. H. (1956). Lethal effects of high and low temperatures on unicellular organisms. *Advances in Biological and Medical Physics* 4: 119-165.

WOODCOCK, E. & GRIGG, G. W. (1972). Repair of thermally induced DNA breakage in *Escherichia coli*. *Nature New Biology* 237: 76-79.

YAMAMORI, T., ITO, K., NAKAMURA, Y. & YURA, T. (1978). Transient regulation of protein synthesis in *Escherichia coli* upon shift-up of growth temperature. *Journal of Bacteriology* 134: 1133-1140.

YAMAMORI, T. & YURA, T. (1980). Temperature-induced synthesis of specific proteins in *Escherichia coli*: evidence for transcriptional control. *Journal of Bacteriology* 142: 843-851.

YAMAMORI, T. & YURA, T. (1982). Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in *Escherichia coli* K-12. *Proceedings of the National Academy of Science of the United States of America* 79: 760-764.

YAMAMORI, T., OSAWA, T., TOBE, T., ITO, K. & YURA, T. (1982). *Escherichia coli* gene (*hin*) controls transcription of heat-shock operons and cell growth at high temperatures. In *Heat Shock from Bacteria to Man*: Cold Spring Harbor Laboratory (Eds Schlessinger, M, Ashburner, M. & Tissieres, A.) pp. 131-137. Cold Spring Harbor, New York.

ZIEGLER, K. MELCHERT, I. & LURKEN, C. (1967). Nδ-(2-amino-2-carboxyethyl)-ornathine, a new amino-acid from alkali-treated proteins. *Nature* (London) 214: 404-405.