Starvation-survival in *Escherichia coli*

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DECLARATION

The data contained in this thesis are the result of original research by myself under the supervision of Dr C. S. Dow, unless otherwise stated. All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree.

Fiona E. Robertson

September 1995

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ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPS	Ammonium persulphate
ATP	Adenosine triphosphate
cfu	Colony-forming units
cm	Centimetre
DAPI	4, 6-diamidino-2-pheylindole
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dpm	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Disodium ethylenediamine tetra-acetic acid
EM	Electron microscopy
g	Gramme
HEPES	(N-[2-hydroxyethyl] piperazine)-N'-[2-ethane] sulphonic acid
hr	Hour
kDa	KiloDalton
LPS	Lipopolysaccharide
M	Molar
M	Molar
mA	Milliamperes
mCi	MilliCurie
mg	Milligramme
min	Minute
ml	Millilitre
mm	Millimetre

mM	Millimolar
mmol	Millimole
mRNA	Messenger RNA
nm	Nanometre
NP-40	Nonidet-40
OD	Optical density
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PVA	Polyvinyl alchohol
RGB	Resolving gel buffer
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGB	Stacking gel buffer
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
Tris	Tris (hydroxymethyl) methylamine
UV	Ultraviolet
V	Volts
v/v	Volume per volume
VH	Volt hours
w/v	Weight per volume
°C	Degree Celsius
μCi	MicroCurie
μg	Microgramme

µl	Microlitre
µm	Micrometre
µmol	Micromole
µM	Micromolar
%	Percentage

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"When in a state of hunger, one ought not to undertake labour"

(Aphorism 16, Hippocrates, 400BC)

SUMMARY

The population dynamics of carbon-starved *E. coli* K12 cultures was investigated. It was found that less cell lysis occurred when cells were previously grown in low glucose concentrations. Exponential-phase cells grown previously in 0.05% (w/v) glucose had survival rates comparable with their stationary-phase counterparts, suggesting that the rate of growth is more important in determining the outcome of starvation than the phase of batch culture growth. Long-term-starved cells (18-24 months) showed very little protein, DNA and RNA synthesis. Methionine was shown to alter the *de novo* synthesis protein profiles of long-term-starved cells and growth was seen to occur in the presence of methionine. This suggests that radio-labelling of proteins with ^{35}S-methionine in these cells should be interpreted with care as the cells have been subjected to a nutrient upshift. Radio-labelling of proteins with ^{3}H-leucine did not have the same effect. The ATP content of cells during prolonged incubation was shown to decrease in the first 48 hours incubation, increase until 5-7 days incubation then decrease after 7-8 days. After 13 days a slow, steady increase occurred. The ATP content of cells incubated for 16 days was higher than that of 48 hour-incubated cells. The physiology of long-term-starved cells was investigated with respect to their permeability to routine bacteriological stains (e.g. DAPI, saffranin, Geimsa) and it was found that very few of these dyes were able to penetrate the cells, indicating that a decrease in cell permeability may be an important factor in survival as is seen in endospores of *Bacillus* species and swarmer cells of *Rhodomicrobium vannielii* and *Caulobacter crescentus*. Resistance of long-term-starved cells to heat and biocide challenge was increased in comparison with exponential- and short-term (48 hour) stationary-phase cells and the resistance to biocides was shown to be retained through subsequent generations. Examination of the nucleoids of long-term-starved cells revealed that a more condensed form was present in cultures incubated for over 14 days, suggesting that dehydration of the DNA had occurred, similar to the situation found in endospores of *Bacillus* species and suggestive of dormancy. Analysis of outer-membrane proteins and lipopolysaccharide of long-term-starved cells showed that alterations occurred to the surface of the cells and it was demonstrated that hydrophobicity changes occurred. Hydrophobicity reached a maximum after 48 hours incubation then subsequently declined between days 2 and 3 which corresponded with an increase in cell numbers. Cell surface hydrophobicity was shown to be a potential method for separating heterogeneous, carbon-starved populations into homogeneous sub-populations. The data suggest that *E. coli* produces a dormant survival cell type which is morphologically and physiologically distinct from the parent cell.

Chapter 1. Introduction

1.1. The natural environment

Compared with bacteria grown under laboratory conditions or residing within a host such as the human body, bacteria in the natural environment have a more stressful existence. The first problem they must overcome, if they are to survive, is the fluctuation in the level of nutrient availability. Even when they find adequate levels of nutrients they then have to be able to cope with fluctuations in temperature, pH, osmolarity and UV irradiation. In addition, they will have to compete with other micro-organisms for the available nutrients. Since one way of overcoming competition is to remove competitors, many micro-organisms have evolved mechanisms by which they can produce substances toxic to competing micro-organisms, e.g. pyocins of *Pseudomonas* species, colicins of *Escherichia coli* species, klebicins of *Klebsiella* species and a vast array of antibiotics produced by bacteria and fungi. Resistance to these substances, in addition to excreting their own will enhance the survival of bacteria.

Nutrient limitation is probably the most common stress encountered by bacteria. Without adequate levels of the basic building blocks, phosphate, nitrogen and carbon, bacteria cannot carry out their basic metabolic pathways. Consequently they have evolved mechanisms which allow them to scavenge for nutrients even when these nutrient levels are very low and certain bacteria have evolved mechanisms whereby they can either escape the adverse conditions or become quiescent until more favourable conditions return. These mechanisms are induced when the substrates become limiting and it has become apparent that they are controlled by very complex mechanisms.

1.2. Differentiating bacteria

1.2.1. Endospore production

Bacillus and *Clostridium* species, amongst others, produce the most resistant cell type of the prokaryotes. This form of survival mechanism allows the

genetic material of the cell to withstand extremes of heat, desiccation, nutrient deprivation, radiation and strong acid and alkaline conditions.

Initiation of sporulation is controlled by the phosphorelay, a signal transduction pathway which responds to cell cycle, environmental and metabolic signals, the nature of which have still to be determined (Burbulys *et al.*, 1991). The phosphorelay system activates SpoOA (Trach *et al.*, 1991), a transcription factor which recognises the OA box (TGNCGAA) in the promoters it controls (Strauch *et al.*, 1990), initiating transcription from *spoIIA*, *spoIIE* and *spoIIG* operons required for the transition from stage II to stage III of the sporulation process (Trach *et al.*, 1991; Satola *et al.*, 1992; York *et al.*, 1992). Figure 1.1 summarises the mechanism by which the phosphorelay is thought to function.

There is evidence to suggest that a low intracellular level of guanine nucleotides is of importance in initiation of sporulation (Freese *et al.*, 1979). In addition, extracellular oligopeptides have been suggested to play a role in signalling the cell density of a culture, since strains which are deficient in oligopeptide permease, and therefore fail to transport oligopeptides into the cell, are impaired in sporulation. (Grossman & Losick, 1988). There are a number of oligopeptides which are excreted by cells and it has yet to be determined which one(s) are important in initiation of sporulation.

The structure of the spore is responsible for its extraordinary resistance. Endospores are extremely dehydrated and the core contains high levels of the calcium chelate of dipicolinic acid; this may be the factor which confers heat resistance. Around the outside of the spore is a keratin-like protein rich in disulphide bonds which cannot be solubilised unless these bonds are reduced, thus the spore coat is impervious to chemical and organic solvent assault. Inside this layer is the cortex which contains multiple layers of extremely loosely cross-linked peptidoglycan, specific to the spore and responsible for dehydration and development of heat resistance (Warth, 1978). A separate layer of peptidoglycan is found beneath this and will eventually form the cell wall on germination.

Three small acid soluble proteins (SASPS), α, β and γ, are found in the spore core and are used as a source of amino acids on resumption of vegetative growth (Setlow, 1988). When overexpressed in *E. coli*, the α and β SASPS cause the chromosome to become condensed and transcriptionally inactive (Setlow, *et al.*, 1991). It is thought that they play a role in the axial filament formation seen during the initiation of sporulation and have been shown to protect the DNA of spores from freeze-drying damage (Fairhead *et al.*, 1994).

The complexity of the spore structure and the differentiation process leading to endospore formation indicates the sophistication of the genetic systems which control sporulation. It has been shown that multiple, alternative σ factors are produced and these, in concert with regulatory proteins, initiate and control differential gene expression in the forespore and mother cell compartments. The cascade of σ factors has been reviewed (Strangier & Losick, 1990). Differential gene expression involves different σ factors operating in the two compartments - σ^E in the mother cell and σ^F in the forespore. The whole process from initiation of sporulation to a fully resistant endospore being released from the mother cell takes 8-10 hours and the regulation of gene expression and control of morphogenesis throughout this period has been comprehensively reviewed in Errington (1993)

The germination of endospores is no less complex. On the addition of nutrients, fresh spores fail to germinate without prior activation such as mild heat-treatment (Keyan, 1978). On germination, the spore breaks down the loosely cross-linked peptidoglycan and uses it to provide an amino acid source (Foster & Johnstone, 1990). Phosphogyceric acid, supplied by the mother cell during the sporulation process is swiftly converted to ATP. This provides the energy required by pre-formed mRNA to utilise the amino acids in protein synthesis and vegetative growth resumes (For a comprehensive review of the genetic events involved in sporulation see (Losick *et al.*, 1986).

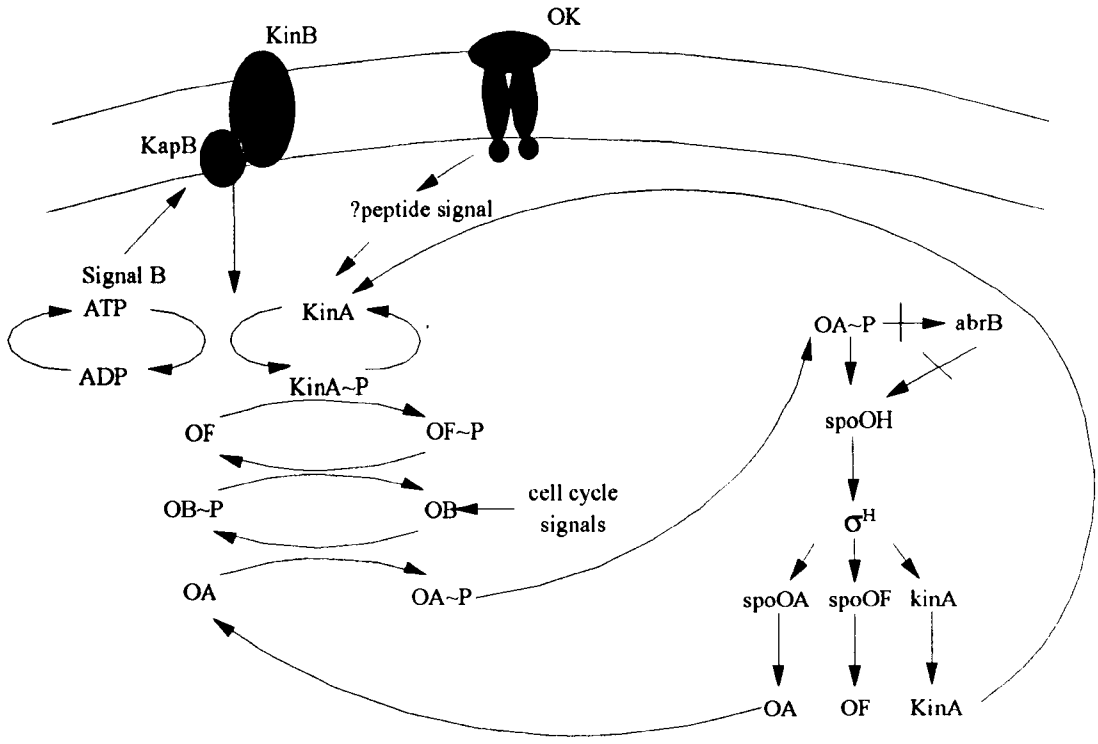


Figure 1.1. Simplified, schematic representation of the events which initiate sporulation in *Bacillus subtilis* (adapted from Hoch, 1993) showing the autoregulation of the phosphorelay system. σ^H, as well as initiating transcription from the promoters of the phosphorelay genes, also initiates transcription of further *spo* genes required for sporulation. Abbreviations; OF - *spoOF* gene product; OB - *spoOB* gene product; OA - *spoOA* gene product; KapB - KinB activator protein (?); KinA - kinase A, KinB - kinase B, OK - oligopeptide permease, ~P - phosphorylation; scored arrow - suppression of transcription.

1.2.2. Prosthecate bacteria

Bacteria such as *Rhodomicrobium* and *Caulobacter* produce motile swarmer cells as part of their life-cycle. All show obligate polar growth with asymmetric division. The study of the metabolism of swarmer cells has been facilitated by the ease with which homogeneous populations can be obtained (Whittenbury & Dow, 1977; Swoboda & Dow, 1979).

Caulobacter crescentus reproduces by obligate swarmer cell production (Poindexter, 1964). The swarmer cell, motile by a single, polar flagellum, is unable to reproduce until it has differentiated into the reproductive form. The swarmer cell sheds it's flagellum and, at the former site of flagellum attachment, a prostheca, or stalk, is formed by which the cell can raise its cell body clear of the substratum to which it has attached, possibly isolating the cell from competitors present on the surface of the substratum or attach to other *Caulobacter* cells within the culture, forming rosettes. The prostheca has also been suggested to be involved in flotation in aquatic environments since prosthecae retard sedimentation characteristics of the cells (Morgan & Dow, 1985). In addition it has been demonstrated that prostheca length is inversely proportional to phosphate availability suggesting that elongation of the prostheca enhances phosphate uptake by increasing the surface area:volume ratio and may function as a specialist uptake organelle (Schmidt & Stanier, 1966; Poindexter, 1981). The cell then elongates and a daughter swarmer cell is formed at the pole distal to the prostheca (Figure1.2). Protein synthesis occurring in swarmer cells is much reduced compared to that seen in vegetative cells and the pattern is unique (Milhausen & Agabian, 1981). In addition it has been shown that the nucleoids of swarmer cells are unable to sustain ribosomal RNA synthesis (Swoboda *et al.*, 1982a). This may be due to the highly condensed nature of swarmer cell nucleoids (Swoboda *et al.*, 1982b).

Rhodomicrobium vannielii has a more complex life cycle. In favourable conditions, ovoid cells are linked by branching cellular filaments, giving rise to

multicellular arrays and growth occurs by budding at the tip of actively growing filaments. Swarmer cells are produced which, in favourable conditions, immediately differentiate into the vegetative form. These swarmer cells are formed and released from the parent cell by binary fission (Whittenbury & Dow, 1977). When conditions are not favourable, swarmer cell differentiation does not occur and their numbers increase in batch culture. In addition, a simplified vegetative cell cycle occurs whereby swarmer cells shed their flagella and develop a prostheca at the end of which a daughter swarmer cell develops. In this cycle, no multicellular arrays are formed (Dow & France, 1980).

During phototrophic growth in batch culture swarmer cells differentiate into the vegetative form during exponential growth. As stationary phase approaches, swarmer cell differentiation ceases and this is thought to be due to a reduction in light intensity caused by cell shading in dense populations. When synchronised swarmer cells are kept anaerobically in the dark, no differentiation occurs. Since *R. vannielii* is photosynthetic, this deprives cells of an exogenous energy source, but not carbon. It has been suggested that the swarmer cell represents a growth-precursor cell which enables survival in adverse conditions (Dow *et al.*, 1983). No DNA synthesis occurs in these cells although, unlike endospores, mRNA and protein synthesis continues, but at a much reduced rate and no ribosomal RNA synthesis occurs (Potts & Dow, 1979; Scott & Dow, 1986). When swarmer cells are returned to light conditions, differentiation occurs. RNA and protein synthesis rates increase and ribosomal RNA synthesis resumes, but the pattern of protein synthesis is different from that seen in inhibited swarmer cells (Dow *et al.*, 1983). One protein in particular is present in swarmer cells and rapidly disappears on induction (Porter & Dow, 1987). Synchronised swarmer cells synthesise an 11.5kDa protein under dark conditions, the presence of which correlates with the inhibition of swarmer cell differentiation . When synchronised swarmer cells are exposed to light for 30 minutes, very little difference is seen in the level of this protein present in the cell and the cells fail to

differentiate. After 1 hour exposure to light, there is an obvious reduction in the amount of this protein and the cells shed their flagellae, becoming non-motile. Prostheca development follows after exposure to light for 2 hours (Porter & Dow, 1987). This suggests that the 11.5kDa protein may function to inhibit swarmer cell differentiation, ensuring that differentiation does not occur until sufficient energy is available, in the form of light, to allow vegetative growth to be initiated and sustained successfully. In addition, swarmer cells have been shown to be more resistant to rifampicin than vegetative cells although the relative resistance of RNA polymerase from both vegetative and swarmer cells to the antibiotic are the same, indicating that a different mechanism is responsible for the enhanced resistance of the swarmer cells (Scott *et al.*, 1987). The same authors showed that resistance to sodium deoxycholate was enhanced in swarmer cells and postulated that the resistance to this and rifampicin was due to changes in the permeability properties of the cells.

The nucleoids of *R. vannielii* and *C. crescentus* have been examined and it was found that, in both organisms, the swarmer cell nucleoid was in a more highly condensed state than that of the vegetative cells, as determined by sucrose density gradient centrifugation (Evinger & Agabian, 1979; Dow *et al.*, 1982, 1983; Swoboda *et al.*, 1982b). The nucleoid of *E. coli* has been shown to condense when protein synthesis is arrested by the addition of chloramphenicol (Zusman *et al.*, 1973). It has been suggested that active genes are normally "pulled" from the nucleoid to the edge, transcribed, then returned (Drlica, 1987). If protein synthesis is inhibited, this process would not occur and the nucleoid would remain condensed. This mechanism may account for the condensation of the nucleoid seen in swarmer cells, but, since it is known that protein synthesis occurs in these cells, it seems unlikely.

The swarmer cells of *R. vannielii* and *C. crescentus* appear to be a survival and dispersal mechanism ensuring that the more resistant progeny do not differentiate into the environmentally sensitive vegetative cell type in unfavourable

conditions and are provided with a mechanism by which they can escape, i.e. motility.

In addition to these cell types, *R. vannielii* also produces angular exospores (Gorlenko, 1969). Once sporulation has been initiated vegetative cell growth ceases and up to four exospores are formed by one vegetative cell at the filament tip (Whittenbury & Dow, 1977). These authors demonstrated that exospores are more resistant to desiccation, heat, UV irradiation and lysozyme treatments and show a decreased ability to take up routine bacteriological stains, suggesting that their permeability is much reduced.

An interesting phenomenon seen during the study of *R. vannielii* swarmer cells suggested that "tiny", non-motile cells were produced by binary fission of cells developing at the tips of growing filaments, without any increase in biomass (Dow & France, 1980). The implication is that a third survival cell type may be produced although the "tiny" cells were not more resistant than vegetative cells. The physiology of these cells has not been investigated and so it is not known if they are metabolically similar to normal swarmer cells. The significance of these cells is unknown, but they may represent a mechanism by which cells can increase their surface area:volume ratio and/or increase the number of copies of genetic material to enhance the probability of at least one copy surviving the conditions. This is reminiscent of reductive division seen in marine *Vibrio* species (Novitsky & Morita, 1976; Amy & Morita, 1983; Oliver *et al.*, 1991).

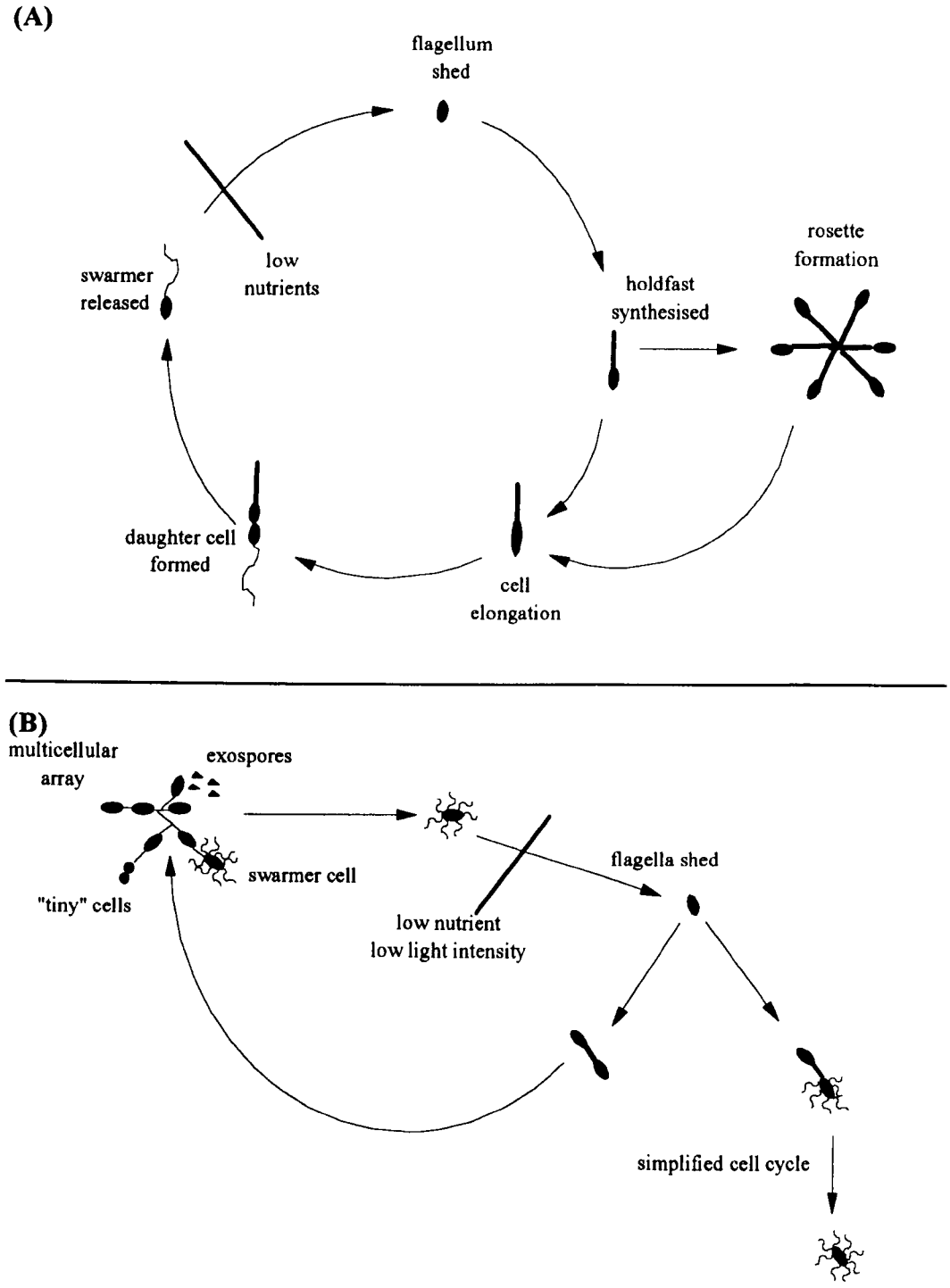


Figure 1.2. The cell cycles of *Caulobacter crescentus*, (A) and *Rhodomicrobium vannielii*, (B). Differentiation of swarmer cells is blocked when environmental conditions are unfavourable, represented by the heavy line. Cell cycle differentiation is obligate in *Caulobacter*, while *Rhodomicrobium* can grow as a multicellular array without the production of swarmer cells.

1.2.3. Other differentiating bacteria

The above information is obviously not exhaustive and many other survival cell types are known. Myxobacteria produce cyst-like exospores surrounded by a thick layer of polysaccharide which is resistant to heat and desiccation. Exospores are produced when cells within a culture aggregate and form a fruiting body within which the exospores form. During exospore formation temporal synthesis of new proteins is seen.

Streptomycetes are prokaryotic organisms which resemble fungi in their growth cycle. They grow as a mycelium mat which spreads over the agar surface. Eventually aerial hyphae form and, at their tips, a chain of spores develops. The spores released then form a new mycelial mat. Spores of streptomycetes are not much more resistant to stress than the vegetative cell mass and it is thought that their formation is primarily a dispersal mechanism.

Arthrobacter species, a Gram positive soil coryneform forms non-motile coccoid forms when subjected to starvation. In nutrient-rich conditions, the cocci elongate to rods and certain species become motile. As stationary-phase approaches, the cells again form non-motile cocci. It has been shown that the coccoid form is more resistant to starvation conditions than the rod-shaped form (Boylen & Ensign, 1970).

More recently surface-induced alterations in motility have been described in *Vibrio parahaemolyticus*. In liquid environments cells are motile by a polar flagellum while growth on solid surfaces induces differentiation to swarmer cells, motile by peritrichous flagellae and a unique σ factor seems to be involved (McCarter & Wright, 1993). A similar differentiation has been described in *E. coli* and *Salmonella typhimurium* although the authors reported that the source of medium which supported swarming was critical (Eiken agar (Eiken, Tokyo) supported swarmer formation while Difco agar did not; Harshey & Matsuyama, 1994).

Filamentous cyanobacteria possess the ability to fix nitrogen, which they carry out in specialised cells called heterocysts. Cyanobacteria produce oxygen to which nitrogenase is extremely sensitive. By localising nitrogen fixation within heterocysts which lack the ability to photosynthesise and thus produce oxygen they are able to circumvent this problem. The walls of the heterocyst are extremely thick, presumably for protection against oxygen. Heterocyst formation occurs in conditions of nitrogen starvation. Heterocysts supply adjoining cells with fixed nitrogen and receive carbon compounds from their neighbours to supply the energy required for nitrogen fixation. Unlike previously described mechanisms, heterocyst formation is a terminal differentiation as reversion to the vegetative cell type does not occur.

1.3. Non-differentiating bacteria

Non-differentiating bacteria such as *Escherichia coli*, are defined as those bacteria not exhibiting a visibly distinct cell type either obligately or in response to environmental conditions. They do, however, possess mechanisms which allow them to alter their metabolism to take account of the availability of specific nutrients and to enhance the cell's ability to remain viable when the nutrient levels are very low.

1.3.1. Phosphate limitation

Phosphate is relatively abundant in nature, but much of it occurs in the form of insoluble salts which cannot be utilised by bacteria. Consequently it is a growth-limiting nutrient and many diverse bacteria have evolved efficient assimilation systems.

In *E. coli*, phosphate limitation induces the synthesis of alkaline phosphatase which allows the utilisation of phosphate sources previously unavailable to the cell, e.g. organic phosphate esters. Active alkaline phosphatase is located in the periplasm allowing utilisation of even those phosphates which cannot penetrate the cell. The alkaline phosphatase gene is only one of a number which are induced when phosphate is limited. These genes, arranged in a number of scattered operons constitute the *pho* regulon. The *pho* regulon is controlled by a two-component sensory system and when phosphate levels are low, a high affinity uptake system is induced, of which alkaline phosphatase is only a small part. Figure 1.3 shows a diagrammatic representation of the system.

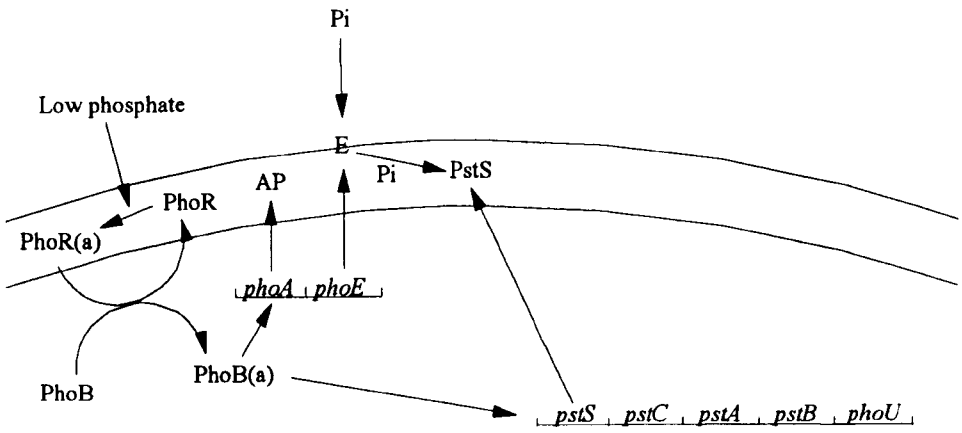


Figure 1.3. Simplified, schematic diagram indicating the regulation of the *pho* regulon. Low phosphate levels activate PhoR, a membrane histidine kinase, by phosphorylation which in turn activates PhoB, the response regulator in the same manner. PhoB then stimulates transcription of a number of genes involved in enhanced phosphate uptake. AP - alkaline phosphatase; E - product of *phoE* gene, an outer-membrane porin; PstS - high affinity periplasmic phosphate binding protein; pstC - encodes a peripheral membrane protein, *pstA* and *B* - encode integral membrane proteins; *phoU* - product required for phosphate repression of the *pho* regulon.

1.3.2. Nitrate limitation

Enteric bacteria preferentially utilise ammonia as a nitrogen source. When ammonia levels drop below 1mM, *E. coli* responds by increasing the expression of a number of operons which constitute the nitrogen starvation regulon. The expression of these genes firstly facilitates ammonia uptake at low concentrations by a high-affinity uptake system and then enables the utilisation of alternative nitrogen sources by the production of the necessary membrane uptake proteins and biosynthetic enzymes.

The induction of the regulon appears to involve a two-component sensory system, much like the phosphate starvation regulon, leading to the production of an alternative σfactor, σ^{60}, the product of the *rpoN* gene (*glnF*, *ntrA*) which transcribes the genes of the regulon. Figure 1.4 illustrates the mechanism by which the regulon is induced.

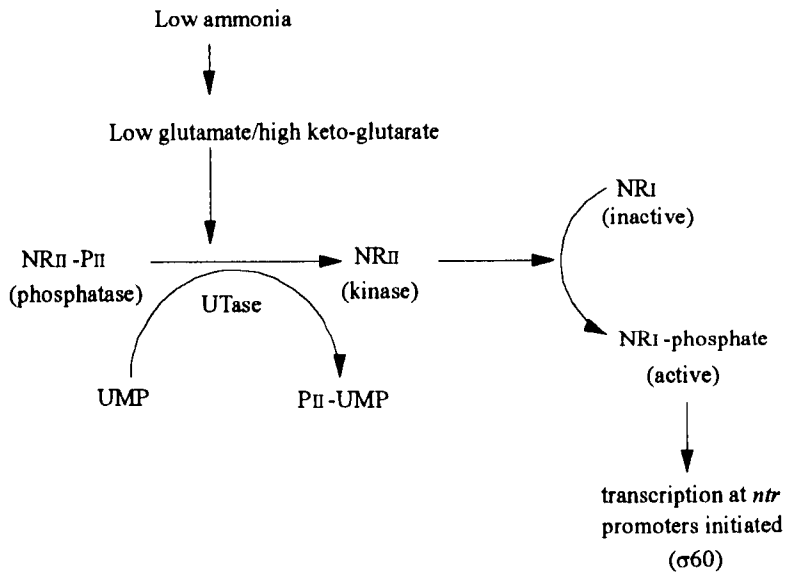


Figure 1.4. Simplified, schematic diagram of the two-component sensory system which controls the nitrogen starvation regulon. The removal of PII from NRII converts NRII to a kinase which then phosphorylates NRI. NRI activates transcription at Ntr promoters. UTase - urididdyltransferase, NRII - NRI kinase/NRI-phosphate phosphatase; NRI-*glnG* gene product; PII - *glnB* gene product.

1.3.3. Carbon limitation

It has been determined that, in oceanic systems which constitute the bulk of the biosphere, carbon is the most likely nutrient to be limiting with respect to bacterial growth since levels of organic carbon can be as low as 30μg l^{-1} (Kirchman, 1990; Velimirov & Walenta, 1992). In addition much of this may be in forms which bacteria are not able to utilise.

The carbon starvation response is not understood to the same extent as the responses to nitrogen and phosphorous starvation. This is because carbon starvation induces a multiplicity of seemingly disparate responses. It has been determined that, like phosphorous and nitrogen, a regulon system is induced, but this regulon is on a much larger scale and many of the genes involved seem to have nothing to do with the search for and enhanced uptake of carbon sources. In addition there is induction of genes which were previously thought to be induced in response to other stresses exclusively, for example, carbon starvation induces heat-, osmotic-, oxidative and acid-shock proteins in addition to proteins involved in the nitrogen and phosphorous starvation regulons and proteins which play a role in cell wall and membrane synthesis. The carbon starvation regulon is dealt with in more detail in Section 1.6.

1.4. Studies on marine *Vibrio* species

Starvation survival studies on non-differentiating bacteria began in earnest in marine *Vibrio* species as it was thought that bacteria which existed in a low-nutrient environment would be more likely to exhibit specific starvation responses. Previously it had been reported that various organisms did not survive longer than 5 days (Boylen & Ensign, 1970). It was found that, on starvation, *Vibrio* species ANT300, a psychrophilic marine vibroid, underwent a reductive division, giving rise to a large increase in cell numbers with no prior increase in biomass when starvation was encountered (Novitsky & Morita, 1976, 1977, 1978). It was found that the magnitude of the cell number increase was related to

the age of the cells in batch culture when starvation was encountered and this appeared to be due to an increase in chromosome copies per cell as stationary phase continued (Novitsky & Morita, 1977). This explains why DNA synthesis is not required for reductive division to occur.

Reductive division may enhance starvation survival in two ways; firstly the increase in cell numbers increases the probability that at least one copy of the genome will survive the adverse conditions and secondly the increase in surface area:volume ratio may increase the likelihood of substrate being encountered while reducing the volume of cytoplasm requiring maintenance. It was found that, as starvation continued, the DNA content of the cells decreased in a biphasic manner, possibly representing the degradation of incomplete copies of the chromosome and extrachromosomal DNA (Novitsky & Morita, 1977).

During starvation the DNA, RNA and protein content of cells decreased rapidly and the number of ribosomes also decreased (Amy *et al.*, 1983). The DNA and protein content then rose slightly before reaching a steady state. The RNA was seen to continue rising throughout the experiment as was ATP content although the authors suggested that this may be due to a decrease in viable counts affecting the calculations.

It has subsequently been shown that marine *Vibrio* species undergo significant changes in response to nutrient starvation. A high-affinity glucose-binding protein with a K_m value of 0.55 is produced in place of the normal glucose-binding protein with a K_m value of 4.6 (Albertson *et al.*, 1990b). Alterations in the protein composition of the outer-membrane (Albertson *et al.*, 1987), cytoplasmic-membrane and periplasm (Nystrom *et al.*, 1988) were detected and a concomitant rise in resistance to autolysis was demonstrated (Nystrom & Kjelleberg, 1989). During starvation the functional half-life of mRNA molecules produced during growth and starvation showed a 2-3-fold increase while mRNA molecules specific to starvation had half-lives of up to 70 minutes (Albertson *et al.*, 1990a). This stabilisation of mRNA during starvation

may be a mechanism by which the demand for starvation protein synthesis can be met in a more economical way than during active growth.

Marine *Vibrio* species alter their protein synthesis profiles on encountering nutrient deprivation. When cells are starved for carbon, nitrogen and phosphate singly or in various combinations, distinct alterations in profile are obtained. Some proteins are produced only under one regime while others are found to be produced under all regimes (Nystrom *et al.*, 1992). These authors demonstrated that starvation for carbon seemed to induce a stress resistance which starvation for the other two nutrients did not and suggested that starvation for carbon in particular was the trigger which allowed for long-term survival in starvation conditions.

A parallel system has been found and extensively studied in *E. coli* and is dealt with in more detail in Section 1.6.

1.5. Viable but nonculturable bacteria

It has long been recognised that aquatic and soil environments contain a greater number of bacterial cells than can be cultured. These cells were observed by microscopy and tended to be very small. It was thought that these viable but non-culturable cells represented a survival mechanism which allowed them to colonise nutrient poor environments.

Since the 1980s, a viable but non-culturable state has been described for organisms which are not known for their colonisation of nutrient-poor conditions. This state has been described as the ability to respond in a limited manner to nutrients, but the inability to form colonies on plating. In a classic experiment, *Vibrio cholerae* was starved until viable plate counts reached zero and the culture was then inoculated into the ligated ileal loop of a rabbit. Not only was the typical cholera response of fluid accumulation in the lumen observed, but, when cultured, the fluid gave rise to typical *V. cholerae* colonies (Colwell *et al.*, 1985). The same phenomenon has been seen in human volunteers (Colwell *et al.*, 1990).

The viable but non-culturable state has now been described in *E. coli* (Xu *et al.*, 1982), *Salmonella* species (Roszak *et al.*, 1984; Turpin *et al.*, 1993), *Campylobacter jejuni* (Rollins & Colwell, 1986; Jones *et al.*, 1991), *Klebsiella pneumoniae, Enterobacter aerogenes, Streptococcus faecalis, Micrococcus flavus* (Byrd *et al.*, 1991), *Vibrio vulnificus* (Oliver *et al.*, 1991), *Shigella* species (Colwell *et al.*, 1985; Rahman *et al.*, 1994), *Aeromonas salmonicida* (Allen-Austin *et al.*, 1984; Morgan *et al.*, 1991, 1993) and *Pasteurella piscicida* (Magarinos *et al.*, 1994)

Aeromonas salmonicida and *Pasteurella piscicida* are fish pathogens and thus are associated with aquatic environments. The remainder all tend to be associated with the human body either as commensals or pathogens. This demonstrates that the viable but non-culturable state is not solely associated with bacteria which are considered to be adapted for natural environmental survival.

The mechanisms which operate to prevent cells from dividing and forming colonies is not understood, but there are many possibilities. Firstly, young *Bacillus* endospores fail to germinate when supplied with the appropriate nutrients unless they have been subjected to a prior mild stress such as radiation, heat, acid or reducing agents (Keyan, 1978). This suggests that the presence of nutrients may not be detected by the spore due to the impermeability of the spore coat and the acid treatment increases permeability. The immediate germination of old spores may be due to cumulative damage to the spore coat which would allow the detection of nutrients. A mechanism of this type may explain the viable but non-culturable state. Resuscitation has been shown to be effected by pre-incubation with weak acids (Heinmets *et al.*, 1953). Secondly, a pathogenic bacterium, when removed from a nutrient-poor environment to a nutrient-rich one, i.e. the human body, may develop requirements for growth factors which it did not previously require. *Legionella* species are ubiquitous in the natural environment in nutrient-poor conditions, but require very complex media and specific conditions to allow growth under laboratory conditions, suggesting that

the failure of viable but non-culturable bacteria to form colonies may be a function of unsuitable culture conditions, even in bacteria where optimum growth conditions for the organism within the laboratory are known, e.g. *E. coli*. Thirdly, the viable cells may be killed by the growth conditions supplied resulting in the failure to form colonies. For example, recovery of sub-lethally acid-shocked *Staphylococcus aureus* was enhanced using solid medium repair on tryptone soya peptone yeast extract egg-yolk pyruvate medium at 23°C as opposed to enumeration on Baird-Parker medium at 37°C, but this was not true for heat-shocked cells (VanNetten *et al.*, 1990). The same authors demonstrated that heat-shocked *Micrococcus* species incubated at 42°C were suppressed in comparison with incubation at 37°C. It is possible that incubation at 42°C represents more of a stress to the commensal *Micrococcus* species than it does to the pathogenic *Staphylococcus aureus* and the prior heat-shock had damaged *Micrococcus* cells to such an extent that they are unable to elicit the heat-shock response. Incubation at 37°C results in slower growth which may allow the cells to repair the damage in more favourable conditions and thus recover. It is possible that the rapid increase in substrates experienced by starving cells causes substrate-accelerated death to occur in much the same manner.

It has been shown that the efficiency of culture of stressed bacteria can depend on the recovery conditions used. In some cases it has been found that the use of dilute, non-selective media and acclimation temperatures will increase the viable counts obtained (Roszak *et al.*, 1984; Magarinos *et al.*, 1994; Votyakova *et al.*, 1994). This suggests that the higher concentration of nutrients found in many routine laboratory media may cause substrate-accelerated death and therefore account for the observation of viable but non-culturable cells.

1.6. Carbon starvation survival

When bacteria experience carbon-starvation rather than carbon-limitation of growth rate, they undergo a significant molecular re-alignment which results in

cells possessing an enhanced ability to withstand a number of stresses. The same is not true of phosphate or nitrate starvation.

In the last fifteen years, much research has been carried out on non-differentiating bacteria such as *E coli* and *Salmonella typhimurium*.

1.6.1. Protein synthesis and carbon-starvation survival

It has been shown that carbon-starvation survival depends on the ability of the cells to carry out protein degradation and synthesis at the onset of starvation (Reeve *et al.*, 1984a, b). Novel proteases are produced which degrade non-essential preformed proteins such as enzymes involved in energy-wasting metabolic pathways. Shutting them down saves valuable energy and provides an endogenous amino acid source for the synthesis of other proteins necessary for starvation survival. At the onset of carbon-starvation, *E. coli* has been shown to undergo a temporally ordered programme of starvation-specific protein synthesis involving the induction of 40-80 genes (Groat *et al.*, 1986; Spector *et al.*, 1986). The whole process takes 9-10 hours and results in the formation of cells with increased resistance to a variety of stresses (Grossman *et al.*, 1985; Jenkins *et al.*, 1988, 1991; Matin *et al.*, 1989).

From a study of 30 of these proteins it has been demonstrated that the production of 19 of them is dependent on cyclic AMP levels and the production of the remaining 11 is cyclic AMP independent (Schultz *et al.*, 1988). The latter class have been named Pex proteins (post exponential). Cyclic AMP-deficient mutants, unable to synthesise cyclic AMP-dependent proteins show no decrease in the ability to survive starvation; thus it is inferred that the production of the Pex proteins confers enhanced survival rates under starvation conditions. Almost all of the Pex proteins belong to a common class of proteins which are induced under various stress regimes. Interestingly, three of the proteins which are cyclic AMP-dependent under carbon starvation conditions are cyclic AMP-independent when

the cells are starved for nitrogen, suggesting that two different regulatory processes are involved in their induction, (Schultz *et al.*, 1988).

The functions of all the proteins produced by starving, non-differentiating cells have not been elucidated, but it has been shown that preventing protein synthesis at the onset of starvation leads to a dramatic reduction in survival rates, (Groat *et al.*, 1986). In peptidase-deficient mutants, starvation survival is also much reduced, thus demonstrating that protein degradation is as important in starvation survival as protein synthesis, (Reeve *et al.*, 1984b). It has been demonstrated that the survival of *E. coli* and *S. typhimurium* is enhanced when starved in the presence of a multiply peptidase-deficient mutant of *S. typhimurium*, strain TN852, (Bockman *et al.*, 1986). Peptidase-deficient mutants excrete oligopeptides which have been shown to be taken up by wild-type cells. Only a small amount (about 11%) of the available amino acids were shown to be used as a carbon source. The remainder were incorporated into newly synthesised proteins. From this it can be seen that an endogenous supply of amino acids for starvation-protein synthesis is crucial to starvation survival. In addition, it has been shown that inhibition of protein synthesis by chloramphenicol during starvation greatly reduces the survival rate, the effect being greater the earlier the chloramphenicol is added (Groat *et al.*, 1986). Addition of chloramphenicol after nine hours of starvation has no effect on starvation survival. This suggests that a form of differentiation into a more resistant cell type is occurring and takes a defined length of time after which maximum resistance is attained.

It has been shown that several of the Pex proteins are known heat-shock proteins whose production is controlled by alternative σ factors. DnaK, GroEL and HtpG are heat-shock proteins produced when *E. coli* is starved for carbon. The production of these three proteins is known to be under the control of the *rpoS* (*katF*) gene product. This protein has been identified as a minor σ factor, σ^S (σ^{38}) (Jenkins *et al.*, 1991)

It has been shown that σ^S is a major regulator of the starvation response (Bohannon *et al.*, 1991). Several of the growth-phase regulated genes which seem to require σ^S for their induction include *katE* which encodes a stationary phase-induced catalase, *xthA* encoding exonuclease III and *appA* encoding acid phosphatase.

1.6.2. The role of σ^S in starvation survival

The *rpoS* gene product was initially identified in *E. coli* as being required for the synthesis of catalase II, encoded by *katE* (Loewen & Triggs, 1984). It was subsequently shown that the transcription of a number of seemingly unrelated genes is affected by mutations in *rpoS*. These genes include *xthA* (Sak *et al.*, 1989), *bolA* (Bohannon *et al.*, 1991; Lange & Hengge-Aronis, 1991a), *appCBA* (Touati *et al.*, 1991), *mcbA* (Bohannon *et al.*, 1991), *otsBA* (Kaasen *et al.*, 1992) and genes encoding over 30 carbon-starvation-specific proteins (Lange & Hengge-Aronis, 1991b; McCann *et al.*, 1991). Although seemingly unrelated, one common feature of all of these genes is that they are expressed as cells either enter stationary phase, are subjected to a nutrient down-shift or are subjected to starvation conditions with the exception of *xthA*, transcription from which is down-regulated in these conditions.

The nucleotide sequence of *rpoS* showed a 181 base pair region having 65% similarity with the corresponding region of *rpoD* which encodes σ^{70} and 85% similarity in a second 38 base pair segment. In addition, a 62 amino acid region of the predicted RpoS protein sequence exhibited 85% similarity with the corresponding σ^{70} sequence including a segment implicated in core polymerase binding (Mulvey & Loewen, 1989). This suggests that RpoS is a σ factor (σ^S) induced by stress which is then involved in the induction of a number of genes whose products are involved in stress survival. A similar gene has been identified in *S typhimurium* and its expression is essential for the survival of the organism in macrophages (Fang *et al.*, 1992) and in *Shigella flexnerii* the gene is essential for

acid resistance (Small & Falkow, 1992). In both cases the virulence of the organisms is enhanced. Several loci have been identified in *S. typhimurium* which are essential for carbon-starvation survival - *stiA*, *stiB* and *stiC* - and it has been suggested that σ^S is involved in regulating their expression in conjunction with ppGpp. For a review of the genetics of starvation-survival in *S. typhimurium* see Spector & Foster (1993).

It has been demonstrated that, of the 32 carbon-starvation proteins which require σ^S for their expression, 6 have been identified as Pex proteins, whose induction at the onset of carbon-starvation is cyclic AMP independent. These 6 proteins belong to the core set that is synthesised in response to starvation for several nutrients. It was also demonstrated that *rpoS* mutants failed to develop cross-protection against osmotic, oxidative and heat stresses (McCann *et al.*, 1991). In vitro, *rpoS* functions as a σ factor (Tanaka *et al.*, 1993) and many of the genes which require σ^S for their induction contain a unique sequence at the -10 region of the promoter, CGGC(T/A)AGTA. These have been described as "gearbox" promoters since transcription from them is inversely proportional to the growth rate of the cell. However, it has been demonstrated that the gearbox is not a sequence universally recognised by σ^S. It fails to initiate transcription from the gearbox of *mcbA* (Lange & Hengge-Aronis, 1991a), the first gene in a complex operon essential for the microcin Mcc B17 production and immunity which inhibits DNA synthesis (Genilloud *et al.*, 1989), but has been shown to transcribe several typical σ^{70} promoters. It does not, however, transcribe nine from genes involved in the synthesis of components of the translational apparatus (Tanaka *et al.*, 1993). However, the promoter of the *fic* gene has been shown to work only with σ^S. This promoter has the common -10 TATAAT-like region, but lacks a recognisable σ^{70} promoter -35 sequence (Tanaka *et al.*, 1993). This arrangement has also been found in the *poxB* gene which encodes pyruvate oxidase, whose activity has been shown to increase as cells approach stationary phase and reaches a maximum in early stationary phase and whose transcription

requires a functional σ^s (Chang *et al.*, 1994). It has been reported that the product of *poxB*, acetate, which is found in the supernatant of stationary phase cultures, is able to induce *rpoS* transcription (Schellhorn & Stones, 1992).

Two genes regulated positively by σ^s have been shown to contain two promoters. CFA synthase which converts lipids into cyclopropane fatty acids (CFAs) is encoded by the *cfa* gene in *E. coli*. Throughout the growth curve, *cfa* is transcribed constitutively from a typical σ^{70} promoter. The second promoter is active only during the transition from exponential- to stationary-phase and is functional only in cells containing a functional *rpoS* gene product (Wang & Cronan, 1994). CFAs are major membrane phospoholipid components found in many bacteria and it has been suggested that the increased levels of CFA synthase at entry to stationary-phase allows the ever-decreasing lipid substrate pool to be efficiently converted to CFAs, possibly ensuring membrane integrity as stationary-phase and possible starvation are encountered.

A second gene containing two promoters, one of which is specifically induced during the transition to stationary-phase is *bolA*, a morphogene of *E. coli*. This promoter contains the gearbox sequence CGGC(T/A)AGTA at the -10 position (Aldea *et al.*, 1989). Overproduction of *bolA* results in a spherical morphology (Aldea *et al.*, 1988) and it has been shown that σ^s is required for its expression as stationary-phase is approached (Lange & Hengge-Aronis, 1991a). For further details of the mechanism by which *bolA* causes a cell size reduction, see Section 1.6.3).

The regulation of σ^s itself is very poorly understood. Acetate has already been mentioned as being able to induce σ^s production. Cyclic AMP appears to play a role in stimulating transcription, although *Δcya* strains retain the ability to induce *rpoS*-dependent *pex* genes (Schultz *et al.*, 1988). There is evidence that post-transcriptional regulation can compensate for the lack of *rpoS* transcription in the absence of cyclic AMP (McCann *et al.*, 1993). This type of multiple regulation has been seen in other stress responses such as the regulation of σ^{32}

(*rpoH*), a minor alternative σ factor which regulates the heat shock response (Nagai *et al.*, 1990). It has been suggested that post-transcriptional regulation may involve differential mRNA stabilities, mRNA interaction with ribosomes, translation initiation or ribosome movement (Loewen *et al.*, 1993) although evidence for the mechanisms remains to be discovered.

Overall, the evidence to date shows that *rpoS* encodes an alternative σ factor which is a major regulator of the starvation response. How σ^S achieves this is unknown. Gearbox promoters are not universally recognised by σ^S, suggesting a much more complex mechanism than recognition of unique promoter regions. A second, unknown factor may be involved, possibly a DNA-binding protein which binds to the unique promoter site preventing binding of σ^{70} and enhancing the binding of σ^S.

1.6.3. Starvation-induced cell division

Cell division at the onset of carbon-starvation was first observed in non-differentiating bacteria during studies of the antarctic marine *Vibrio* species ANT300. Starvation behaviour in marine bacteria was studied because their existence in such low-nutrient environments suggested that starvation-survival might be enhanced as compared with enteric organisms. ANT300 showed the ability to increase dramatically cell numbers with no parallel increase in cell biomass (Novitsky & Morita, 1976, 1977). This reductive division resulted in small cells which, after 3 weeks starvation, could pass through a 0.4μm pore size filter. A similar mechanism was not seen in a marine *Pseudomonas* species (Kurath & Morita, 1983), but was demonstrated in *Vibrio cholerae* during nutrient starvation (Baker *et al.*, 1983).

When *E. coli* is grown in batch culture, cell size is seen to decrease as stationary-phase is approached. This was seen as a function of the constant time required to replicate the chromosome in a background where other metabolic reactions have slowed. It has now been demonstrated that this cell size reduction

is very carefully regulated. In rich medium, two cell size reductions are seen. The first occurs at optical densities between 1 and 2.5 and is independent of the *rpoS* gene product. However the second, which occurs at optical densities above 2.5, does not occur in *rpoS* mutants (Lange & Hengge-Aronis, 1991a) and it has been demonstrated that induction of *rpoS* stimulates transcription of *bolA*. BolA, in turn, controls the expression of PBP6 (Aldea *et al.*, 1989). PBP6 catalyses the removal of D-alanine from the pentapeptide side-chain of peptidoglycan. This increase in the amount of tetrapeptide probably leads to the increase in tripeptide which is the preferred substrate for PBP3. Although PBP3 levels within the cell remain constant, the increase in substrate increases the activity of PBP3. This enzyme catalyses transpeptidation in cross-wall peptidoglycan synthesis, leading to a cell division (Begg *et al.*, 1990). Figure 1.4 illustrates the probable pathway which leads to septation during growth and at entry to the stationary-phase.

The unknown factor which causes the switch from RodA control to σ^S control has yet to be determined. Suitable candidates include acetate which accumulates in the growth medium as stationary phase is approached and oligopeptides which have been shown to be involved in the initiation of sporulation in *Bacillus subtilis* (see Section 1.2.1). This mechanism may represent a method by which increasing cell density is detected by cells in a batch culture. A similar system of positive feed-back regulation is seen in the *lux* operon of *Photobacterium fischeri* whereby N-acyl-L-homoserine lactone is excreted into the medium. When homoserine lactone levels in the medium go above a certain threshold, it is taken up by the cells and is involved in the induction of the *lux* operon and bioluminescence of the cells results. Homoserine lactone production has been detected in a number of diverse bacteria such as *Enterobacter*, *Citrobacter* and *Serratia*, (reviewed in Williams *et al.* (1992)), and has been shown to regulate the production of exoenzymes which confer infectivity on *Erwinia carotovora*, a potato pathogen (Jones *et al.*, 1993). The idea of a small diffusable molecule being involved in *rpoS* induction is an attractive idea

since it may also help to explain the synchronisation of *E. coli* cells as they enter the stationary phase (Cutler & Evans, 1966). Prior to synchronisation, the cells in the population are heterogeneous with respect to size and the phase of the cell division cycle the cells are in. For the culture to become synchronous, cells further advanced in the cell cycle must be halted in their development while those which lag behind catch up. This suggests that a control mechanism exists which may prevent initiation of further rounds of chromosome replication, but allows the completion of those already begun. Alternatively cells could be halted after chromosome replication has occurred, but before septum formation has begun.

In actively growing cells initiation of chromosome replication takes place at every copy of the origin of replication, *oriC*, at each doubling of cell volume (Helmstetter & Leonard, 1987). Chromosome replication takes 40 minutes thus cells with a doubling time of under 40 minutes carry out multiple rounds of chromosome replication at once to ensure that each daughter cell has one copy. The control of initiation is not fully understood, but it is thought to involve activation of DnaA, the only protein known to be required for initiation. Activation of DnaA occurs when it is complexed with *oriC* and is catalysed by phospholipids (Crooke *et al.*, 1992). Once activated, DnaA is prevented from initiating replication on the newly-formed double strands by a delay in methylation of adenine groups at the origin (Campbell & Kleckner, 1990). This delay is 30-40% of the cell cycle and is independent of growth rate. If a further round of replication is to take place, initiation then occurs. The decision as to whether a further round of replication is to take place may be dependent on the concentration of a regulator molecule within the cytoplasm of the cell and the breakdown or synthesis of this molecule may prevent DnaA complexing with further double strands. The delay in initiation due to methylation of adenine may allow cellular concentrations of this molecule to increase or decrease to levels which do not allow initiation to take place. When methylation of adenine is completed the initiation window has now closed. In rapidly growing cells, cellular

concentrations of the regulator molecule would increase or decrease rapidly and initiation of the new double strands would take place as soon as methylation of adenine has taken place.

There is some evidence that the regulation of DNA replication involves cyclic AMP concentration, which increases with decreasing growth rate and may act as a negative regulator of the process (Hughes *et al.*, 1988). Transcription of the promoters of *mioC*, a gene adjacent to the origin may influence initiation. *mioC* transcription is stringently controlled and its expression is negatively regulated by growth rate (Chiaramello & Zyskind, 1989, 1990). It is possible that control of these factors may allow cells to be halted prior to initiation of chromosome replication, resulting in the observed synchrony. For a recent review of cell cycle events see Donachie (1993).

The cell size reduction seen as cells approach stationary-phase indicates that the relationship between initiation of chromosome replication and cell volume is altered. The involvement of σ^S in this process has already been discussed and it is possible that σ^S or a σ^S-controlled gene is involved in allowing initiation of chromosome replication to occur at lower cell volumes.

It has recently been demonstrated that mutants blocked in the production of homoserine in the threonine biosynthetic pathway fail to induce σ^S production and that this could be reversed by the addition of exogenous homoserine lactone, suggesting that homoserine lactone, or a metabolic derivative of it, was involved in the regulation of *rpoS* expression (Huisman & Kolter, 1994) and it was suggested that this molecule may act both as an intracellular and intercellular signalling mechanism in induction of the carbon-starvation regulon. Further details are given in Chapter 4.

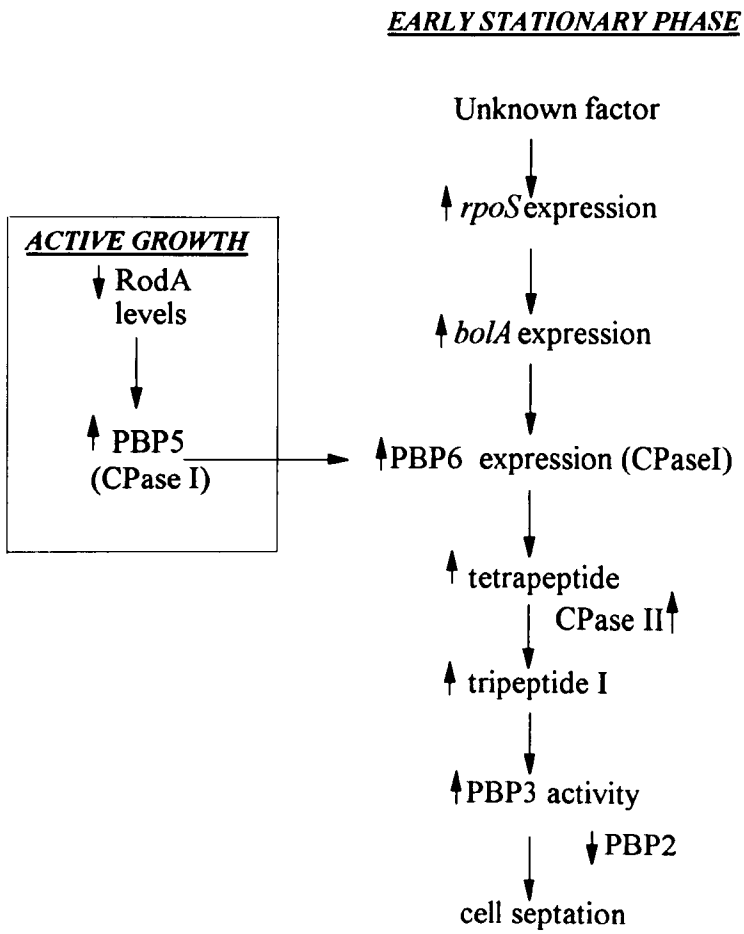


Figure 1.5. Sequence of events which leads to the formation of a septum and, subsequently cell division. Abbreviations; CPase I - carboxypeptidase I, CPase II - carboxypeptidase II, PBP - penicillin-binding protein. The unknown factor which causes the switch from RodA control to σ^S control is probably an extracellular signalling substance.

1.7. Other stationary phase regulators

Although the *rpoS* gene product has been shown to be a major regulator of the starvation response, certain anomalies have indicated that it is not the sole regulator. A second regulatory protein has been found in *E. coli* which is the product of a starvation-induced gene *sspA* (Williams *et al.*, 1994). SspA was found to co-purify with RNA polymerase (Ishihama & Saitoh, 1979), but confusion over its identity meant that it was subsequently listed as OmpA. Williams *et al.*, (1994) demonstrated that *sspA* is part of an operon which includes the *sspB* gene and the operon is involved in the positive and negative regulation of several proteins during exponential- and stationary-phase growth. They suggested that SspA and SspB are part of a regulatory cascade which involves RNA polymerase and starvation-induced promoters Co-purification of SspA with RNA polymerase, confirmation that it was not OmpA and the effect of mutations in the gene on stationary-phase protein synthesis suggested that the interaction of SspA with RNA polymerase may be part of the mechanism by which altered gene expression is achieved in the transition from exponential- to stationary-phase growth. Induction of the *sspAB* operon is not dependent on σ^s or the related heat-shock sigma factor, σ^{32}.

One important protein whose expression is affected by Δ*sspA* is H1 (Hns), a DNA-binding histone-like protein, whose expression increased in the absence of SspA, suggesting that SspA may play a regulatory role in Hns production. Hns accumulates in stationary phase and condenses DNA (Spassky *et al.*, 1984) and has been implicated in DNA supercoiling alterations in response to environmental stimuli (Hulton *et al.*, 1990).

A universal stress protein, UspA, has been implicated in modulations of the flow of carbon in central metabolic pathways in *E. coli* and is induced in response to a number of stress regimes. It is thought that the induction of UspA restricts the synthesis of acetate or increases the utilisation of acetate and a *uspA*

mutant has demonstrated elevated levels of acetate in the growth medium (Nystrom & Neidhardt, 1993)

Overall, the responses of non-differentiating bacteria to carbon-starvation suggests that, the vast majority of alterations are not involved in the scavenging for alternative carbon sources as is the case in the *pho* and *ntr* regulons, but is more concerned with the survival of the organism during carbon-starvation until carbon is again available. This is analogous to the sporulation process in *Bacillus* species and suggests that the production of a more resistant cell type may occur in non-differentiating bacteria.

1.8. Aims

The aims of this experimental work were to examine the physiology of long-term-starved cultures of a laboratory strain of *E. coli* K12 and to attempt to determine the mechanism by which resistant cell types arise within cultures.

Chapter 2. Materials and Methods

2.1. Media and growth conditions

2.1.1. Minimal medium

E. coli K12 was grown in M9 mineral salts medium, (Lark *et al*, 1963). This contained, per litre of double distilled water;

$Na_2HPO_4.2H_2O$	7g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g
$MgSO_4.7H_2O$	0.2g
$CaCl_2$	20mg

The pH was adjusted to 7.0 with 1M NaOH and the medium sterilised by autoclaving at 121°C for 15 minutes. Glucose, which had been filter-sterilised through a 0.22μm cellulose acetate filter (Acrodisc, 32mm, Gelman Sciences) was added after sterilisation as a carbon and energy source. The glucose concentrations used are discussed in the appropriate Results sections. Cultures were incubated aerobically at 37°C with shaking at 200 rpm.

2.1.2. Complex media

Cultures were streaked to purity on nutrient agar (Oxoid) and MacConkey agar (Oxoid), made up according to the manufacturer's instructions and incubated aerobically at 37°C.

2.1.3. Maintenance of cultures

Stock cultures of all organisms were stored as follows;

a) nutrient broth with 10% (v/v) glycerol in liquid nitrogen

b) nutrient agar slopes at 4°C

c) nutrient agar plates at 4°C

2.1.4. Assessment of growth in liquid culture

Absorbance measurements were carried out at 600nm using a Shimadzu UV-150-02 Double Beam spectrophotometer.

2.2. Carbon starvation protocols

2.2.1. Strict starvation

Cells were harvested, during growth in batch culture, by centrifugation at 1, 500*g* for 20 minutes in an MSE Mistral 1000 benchtop centrifuge, washed in pre-warmed M9 medium with no added glucose and placed in the same volume of pre-warmed M9 with no added glucose in a Quick-Fit flask capped with a Suba-Seal. Cells were then incubated aerobically at 37°C.

2.2.2. Prolonged incubation of stationary phase cells

For small volumes, cells were grown to stationary phase in M9 medium in a Quick-Fit flask capped with a Suba-Seal, with a limiting concentration of glucose. The incubation was continued at the same temperature as before for the required time.

Larger volumes of cells were subjected to prolonged incubation in vessels containing 2, 5 or 10 litre volumes. As the surface area to volume ratio in the culture vessels was not optimum, blended air (BOC) was constantly bubbled through the culture medium at 100ml min^{-1} and a magnetic stirrer was used to aid aeration. Figure 2.1 shows a diagrammatic representation of the arrangement of the culture vessels and the sampling method.

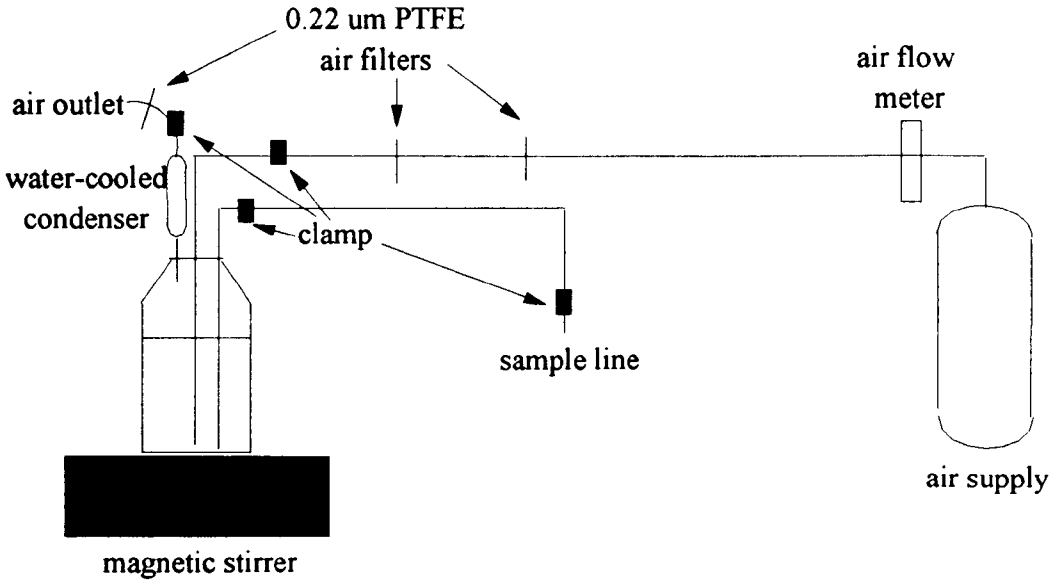


Figure 2.1. Diagram of apparatus for obtaining large volumes long-term starved cultures. More than one vessel could be connected in parallel to the air supply. Cultures were sampled by clamping the air outlet, allowing the pressure within the vessel to rise. Culture was forced into the sampling line and prevented from entering the air inlet line using a clamp. After sampling, the air oultet was opened and the sample line was clamped. The sample line was then washed out with 70% ethanol and clamped at the end and close to the vessel. Water-cooled condensers were used when the incubation time was greater than 1 week to minimise evaporation.

2.2.3. Synchronisation of *Escherichia coli*

E. coli K12 was synchronised using a stationary phase method, (Cutler & Evans, 1966). 5ml of an overnight culture was added to 100ml of prewarmed M9 medium containing 0.5% (w/v) glucose in a 1 litre Quick-Fit flask and the culture incubated at 37°C with shaking at 200 rpm. Half a doubling time after stationary phase was reached, determined by absorbance measurements at 600nm, fresh medium was added to the culture, the amount being equivalent to a 1:7 dilution. The culture was incubated as previously and, at the same time point as before, another 1:7 dilution was carried out. Synchrony was demonstrated, as measured by viable counts and total particle counts.

2.2.4. Starvation of synchronised cells

Cultures were synchronised as described in Section 2.2.3. Once synchrony was established, cells were harvested rapidly at 15 minute intervals by centrifugation at 1, 500*g* for 20 minutes in an MSE Mistral 1000 benchtop centrifuge and inoculated into M9 medium with no added glucose in Quick-Fit flasks capped with a Suba-Seal.

2.3. Assessment of starvation survival

2.3.1. Viable counts

Viable counts were measured by spreading dilutions of culture onto M9 agar containing 0.02% (w/v) glucose, i.e. starvation medium. This minimised the occurrence of substrate-accelerated death. All batches of medium were tested by plating dilutions of an overnight culture of *E. coli* K12 grown in M9 with 0.1% (w/v) glucose and compared with counts obtained on nutrient agar plates. Plates were incubated at 30 or 37°C for 24 and 48 hours. Prolonged incubation up to 10 days in a moist chamber demonstrated that no additional colonies arose after this time.

2.3.2. Total particle counts

Total particle counts were carried out using two different methods. Initially, particles were enumerated using a Coulter Counter ZM (Coulter Euro Diagnostics GMBH) with a 30μm-orifice probe. Data from the Coulter Counter were sent to a Coulter Channelyzer 256 to obtain size-distribution profiles of the cells being analysed. In later work, a CellFacts particle counter (Microbial Systems Limited) was used in conjunction with CellFacts Industrial software. As with the Coulter Counter, size-distribution profiles were obtained. Samples for both methods of analysis were prepared in the same way. The samples were diluted in the appropriate electrolyte solution to which 0.3% (v/v) glutaraldehyde (Agar Scientific) had been added to fix the cells. Experiments showed that total particle counts and size distributions remained stable over 14 days in electrolyte with 0.3% glutaraldehyde at 4°C, allowing samples to be stored and counted together.

2.4. Separation of cell types from starving cultures

A number of methods were used in an attempt to separate cell types from starving cultures.

2.4.1. Filtration

Cells were filtered through 0.45μm and 0.22μm cellulose acetate filters, (Acrodisc, 32mm, Gelman Sciences) in an attempt to isolate small cells. Viable counts and total particle counts were carried out on the filtrates using nutrient agar and M9 starvation plates as described in Sections 2.3.1. and 2.3.2. respectively.

2.4.2. Percoll density gradient centrifugation

Percoll (Pharmacia) was used according to the manufacturer's instructions, the solution made isotonic using NaCl. The samples were layered onto the top of the Percoll and centrifuged at 23, 700g for 45 minutes in a JA20 fixed-angle rotor in a Beckman centrifuge for 45 minutes. Calibration beads with known densities were centrifuged in the same manner as a control.

2.4.3. Binding to phenyl-sepharose

Separation of cells based on cell-surface hydrophobicity differences was carried out on a 10 ml phenyl-sepharose CL-4B (Sigma) column. A 25ml glass graduated pipette was plugged at the bottom with glass wool. 10ml of phenyl-sepharose was diluted 1:1 with 1M NaCl and added to the pipette. The column was saturated with a 7-day starved culture. When the optical density of the eluate equalled that of the original culture the column was deemed saturated and excess culture was eluted with 1M NaCl. Cells were removed from the phenyl-sepharose by washing with discrete, decreasing NaCl concentrations. The eluate obtained with each NaCl concentration was collected and the cells harvested by centrifugation at 4, 000*g* in an MSE Mistral benchtop centrifuge for 30 minutes.

2.5. Protein profile analysis

2.5.1. Preparation of protein samples

2.5.1.1. Total cellular protein

Crude lysates were produced by adding one volume of 6xSDS sample buffer to five volumes of sample. The sample buffer contained 0.375M Tris-HCl, pH 6.8, 60mM DTT, 12% (w/v) SDS and 10% (v/v) glycerol. Samples were incubated at 100°C for five minutes and then stored at -20°C until required.

Protein concentrations were determined using the method of Lowry *et al.*, (1951).

2.5.1.2. Soluble proteins

To prepare soluble proteins, cells were sonicated for 15 seconds min^{-1} for 5 minutes using a Jencons ultrasonicator fitted with a 4mm probe at an amplitude of 6 microns peak to peak. Whole cells and membrane fragments were removed by centrifugation at 48, 400g for 60 minutes at 4°C. A Bio-Rad assay, based on Bradford (1976), was used to estimate the protein concentration in each sample according to the manufacturer's instructions. For SDS-PAGE one volume of 6x SDS sample buffer was added to five volumes of sample. For iso-electric focusing 0.27g of solid urea was added to every 0.5 ml of sample, giving a final concentration of 9M. One volume of lysis buffer was added and the sample loaded onto the gel (See Section 2.5.3.1.)

2.5.1.3. Cytoplasmic-membrane and outer-membrane proteins

To obtain samples of outer-membrane proteins, cells were harvested, washed and resuspended in 1ml 10mM Tris-HCl pH 8.0. The cells were ruptured by sonication, as described in Section 2.5.1.2. Whole cells were removed by centrifugation at 1, 500*g* for 20 minutes at 4°C. The resulting supernatant was centrifuged at 48, 400*g* in a JA20 fixed-angle rotor of a Beckman centrifuge for 60 minutes at 4°C to harvest the membranes. The pellet was resuspended in 150 µl of distilled H_2O and stored at -20°C. When required, the membrane suspension was thawed, split into three 50µl volumes and extracted with 8 volumes of membrane extraction detergent which contained 0.075g N-lauryl sarcosine (Sigma), 4.45ml distilled H_2O and 50µl 1M Tris-HCl pH 7.6. This reagent was made up immediately before use. The sample was incubated at room temperature for 20 minutes to allow the cytoplasmic portion of the sample to solubilise. The sample was then centrifuged at 48, 400*g* as before for 90

minutes at 20°C to pellet the insoluble outer-membranes. The resulting pellet was resuspended in the appropriate buffer, 50µl of SDS sample buffer, if one-dimensional SDS-PAGE was to be carried out, or 50µl of lysis buffer if two-dimensional electrophoresis was to be carried out. Samples in SDS sample buffer were boiled and loaded onto an SDS-PAGE slab gel. Samples in lysis buffer could not be boiled as cyanate ions are formed in concentrated urea solutions which would interfere with iso-electric focusing. These samples were mixed thoroughly for at least 10 minutes using a vortex mixer and frequent pipetting. This was necessary because some membrane proteins are less soluble in urea buffers than in SDS buffers. It has been suggested that boiling membrane preparations in SDS buffer followed by the addition of two volumes of lysis buffer will result in complete solubilisation of the membranes with the subsequent replacement of protein-attached SDS with urea. In practice, it was found that the SDS was not fully removed and, being an ionic detergent, it interfered with the iso-electric focusing, preventing proper separation of proteins in the samples. Prior to electrophoresis, the amount of protein in each sample was estimated (Lowrey *et al.*, 1953).

2.5.1.4. Trichloroacetic acid precipitation of proteins

Precipitation of proteins in very dilute samples was accomplished using trichloroacetic acid, (Groat e*t al.*, 1986). An equal volume of 20% (v/v) ice-cold TCA was added to the protein sample and incubated on ice for 15 minutes. The precipitate was collected by microcentrifugation in a Microcentaur at 13, 000 rpm, washed in 5% (w/v) TCA and then washed in acetone. The acetone was discarded and the precipitate dried in a stream of nitrogen before the protein was dissolved in water or the appropriate buffer.

2.5.1.5. Determination of protein concentration

Routinely, the protein concentration of samples was estimated using Bio-Rad reagent according to the manufacturer's instructions. However, in some cases, the buffer in which the protein sample was prepared contained substances which interfered with the assay. In these cases the Lowry method was used, (Lowrey *et. al.*, 1953).

2.5.2. One-dimensional SDS-PAGE

The following solutions were used to cast uniform acrylamide gels;

2x resolving gel buffer - 0.2% (w/v) SDS in 0.75M Tris-HCl pH 8.8 (RGB)

2x stacking gel buffer - 0.2% (w/v) SDS in 0.25M Tris-HCl pH 6.8 (SGB)

Acrylamide stock : acrylamide 44g
bis-acrylamide 0.8g

The acrylamide and bis-acrylamide were dissolved in 100ml of distilled water with gentle stirring and then filtered through a 0.22μm cellulose acetate filter (Acrodisc, 32mm, Gelman Sciences).

The resolving gel was made up as described in Table 2.1. All figures are in millilitres unless otherwise stated.

Table 2.1. Composition of linear polyacrylamide gels

Gel strength (w/v)	15%	12%	10%	8.5%
2x RGB	27	27	27	27
Acrylamide stock	18.4	14.8	13.6	10.6
1% (w/v) AMPS	1.9	1.9	1.9	1.9
TEMED	100µl	100µl	100µl	100µl
H_20	7.2	10.8	12.0	15.0

The stacking gel was made up as described in Table 2.2. All figures refer to millilitres unless otherwise stated.

Table 2.2. Composition of polyacrylamide stacking gels

Gel strength (w/v)	7.5%	4.5%	2.5%
2x SGB	10	10	10
Acrylamide stock	3.3	3.0	2
1% (w/v) AMPS	0.5	0.5	0.5
TEMED	40µl	40µl	40µl
H_20	6.7	7	8.8

The running buffer contained, per litre

0.025M Tris-HCl pH 8.8	
Glycine	14.4g
SDS	0.1% (w/v)

The samples were loaded onto the gel and the gel run at 40mA for 3-4 hours until the bromophenol blue indicator dye reached the bottom of the gel. On occasion, gels were run at 8mA overnight.

2.5.3. Two-dimensional polyacrylamide gel electrophoresis

2.5.3.1. Iso-electric focusing

The following solutions were used;

Sonication buffer

0.01M Tris-HCl pH 7.4	
H_2O	100ml

0.102g of $MgCl_2$ was added. To 10 ml of this, RNase (Sigma) was added to a final concentration of 50 $\mu g\ ml^{-1}$.

DNase solution

0.01M Tris-HCl pH 7.4	
H_2O	100ml

20.4mg of $MgCl_2$ was added. To 10ml of this 10 mg of DNAase (Sigma) was added. This solution was stored at -20°C in 50µl aliquotes.

Lysis buffer

Urea	5.7g
NP-40 (10% w/v)	2.0ml
Ampholines 5-7 pH	0.4ml, (Sigma)
Ampholines 3.5-10 pH	0.1ml, (Sigma)
(DTT)	15.4mg, (Sigma)

The solution was made up to 10ml with H_2O and stored at -20°C in 0.5ml aliquotes

Acrylamide stock solution

Acrylamide	28.38g
Bis-acrylamide	1.62g
Distilled H_2O to 100ml.	

The solution was stored at room temperature in the dark.

Gel overlay solution

Urea	4.8g
H_2O to 10 ml.	

This solution was stored in 0.5ml aliquotes at -20°C.

Sample overlay solution

Urea	5.4g
Ampholines 5-7 pH	0.2ml
Ampholines 3.5-10 pH	0.05ml
H_2O to 10ml.	

This solution was stored in 0.5ml aliquotes at -20°C.

Sample extraction buffer

Tris-HCl pH 6.8	0.0625M
Glycerol	10% (v/v)
DTT	10mM
SDS	2.3% (w/v)

Rod gel preparation

Urea	5.5g
Acrylamide stock	1.33ml
NP-40 (10% w/v)	2ml
Ampholines 5-7 pH	0.4ml
Ampholines 3.5-10 pH	0.1ml
H_2O	1.97ml
TEMED	7µl

10µl of 10% (w/v) ammonium persulphate was added immediately before casting.

The gels were cast to a length of 10cm in 3mm diameter glass tubes and overlaid with gel overlay solution. They were allowed to polymerise for 1-2 hours. The gel overlay solution was removed and 20µl of lysis buffer was placed on top of the gel and overlaid with distilled H_2O. The gel was then allowed to polymerise for a further 1-2 hours. The lysis buffer and distilled H_2O were removed and the gels placed in the gel tank. 20µl of fresh lysis buffer was placed on the gels and the tubes were filled with 0.02M NaOH. The upper buffer reservoir was filled with 0.02M NaOH and the lower buffer reservoir with 0.01M H_3PO_4. The gels were pre-run at 200V for 15 minutes, 300V for 30 minutes and 400V for 30 minutes. The power was turned off and the upper buffer reservoir emptied. The NaOH and lysis buffer were removed from the gel tubes and the samples were placed on the gels and overlaid with 20µl of sample overlay

solution and then 0.02M NaOH. The upper buffer reservoir was filled with fresh 0.02M NaOH. The gels were focused at an average of 700V for a total of 10, 000VH. After focusing, the gels were extruded from the glass tubes using water pressure from a syringe and equilibrated at room temperature for two hours in sample extraction buffer. The rod gels were then stored at -20°C until required or immediately loaded onto the second dimension resolving gel.

2.5.3.2. Second dimension SDS-PAGE

The second dimension was run on an SDS-PAGE system using a uniform 12% (w/v) polyacrylamide gel as described in Section 2.5.2.1. To 5ml of sample extraction buffer, 0.05% (w/v) bromophenol blue and 1.5% (w/v) agarose were added. This was boiled and allowed to cool to 50°C. The rod gel was drained of sample extraction buffer. Sufficient agarose mixture was placed on top of the stacking gel to allow contact between the rod gel and the slab gel then the rod was placed directly on top of the molten agarose. More agarose mixture was used to seal any gaps and fill any air bubbles between the stacking gel and the rod gel. Running buffer was placed in the upper and lower buffer reservoirs. The gel was electrophoresed, with cooling, until the bromophenol blue reached the bottom of the resolving gel. The gel was removed from the glass plate sandwich and fixed in 40% (v/v) methanol in a polythene sandwich box overnight, with two changes of methanol.

2.5.4. Silver staining of polyacrylamide gels

Fixed polyacrylamide gels were stained with silver, (Wray *et al.*, 1981) to allow the visualisation of protein bands. 1.6g of $AgNO_3$ (Sigma) was dissolved in 8ml of H_2O to give a 20% (w/v) stock solution. 42ml of 0.36% (w/v) NaOH was placed in a 200ml measuring cylinder and 2.5ml of concentrated (29.4% (w/v)) ammonium hydroxide added. The stock silver solution was added dropwise with shaking to ensure that the solution cleared after each drop was added. The

solution was made up to 200ml with H_2O. The fixed gel was drained of 40% (v/v) methanol and the silver stain was added. The gel was gently shaken in stain for 15 minutes after which the stain was poured off, the gel removed and the sandwich box thoroughly rinsed, first with tap water then with distilled H_2O. The gel was returned to the sandwich box and washed for ten minutes in distilled water with one change. The developing solution, 0.4ml formaldehyde, 2.5ml 1% (w/v) citric acid, 497.1ml H_2O, was added and the gel shaken gently until the protein bands were visible. To stop the reaction the developer was poured off and a solution of 40% (v/v) methanol and 30% (v/v) acetic acid was added. The gels were stored in this solution until required.

Gels were photographed on a light box using a Praktica MTL 3 camera and Tmax 100 professional black and white film which was developed according to the manufacturer's instructions and printed onto photographic paper, (Kodak Kodabrome II RC F2 Professional black and white).

2.5.5. Destaining of silver-stained polyacrylamide gels

In some cases it was necessary to destain gels to lighten the background or to allow washing and restaining of contaminated gels. The destain contained 50 ml Kodak Unifix photographic fixer, 225 ml methanol and 200 ml H_20. Before re-staining a destained gel, it was necessary to wash the gel in three changes of 40% (v/v) methanol, one wash being carried out overnight.

2.5.6. SDS-PAGE of silver-stained proteins

When two-dimensional electrophoresis was used to separate complex protein mixtures it was necessary to use silver-staining to obtain maximum sensitivity of protein detection. When the detected protein was to be used for polyclonal antibody production it was necessary to ascertain whether the silver-staining procedure had caused cleavage or degradation of the protein. To determine if this was the case, outer-membrane proteins were prepared as

described in Section 2.5.1.3. and electrophoresed on a 12% (w/v) SDS polyacrylamide gel with a 4.5% (w/v) stacking gel as described in Section 2.5.2. The gel was silver stained as described in Section 2.5.4., but the stain stop solution used was 50% (v/v) methanol without acetic acid. This minimised acid cleavage of proline-asparagine bonds in the proteins. A track was cut from the gel and destained as described in Section 2.5.5. for 48 hours or until all traces of stain were removed. The destain solution was removed by washing the gel strip in three changes of 40% (v/v) methanol overnight. The gel strip was equilibrated in 1xSGB for 1-2 hours to allow SDS to attach to the proteins in the gel. The gel strip was gently pushed between a glass sandwich which contained a 12% (w/v) SDS polyacrylamide gel with a 4.5% (w/v) stacking gel. The gel was sealed in place with 1% (w/v) agarose, (Sigma), made up in 1x SGB and electrophoresed for 3 hours at 40 mA.

2.5.7. Radio-labelling of proteins

Cells were pulse-labelled with ^{35}S-methionine (15mCi ml^{-1}, 1000 Ci mmol^{-1}, Amersham International) or L-[4,5-^{3}H] leucine (153Ci mmol^{-1}, Amersham International) by sampling the culture vessel at appropriate times and adding the appropriate amount of radio-labelled amino acid for the desired time. After incubation at 37°C, unlabelled methionine (Sigma) or leucine (Sigma) was added to a final concentration of 1mM to stop isotope incorporation or the sample was denatured immediately. Details of individual radio-labellings are given in the appropriate Results Chapters since in certain circumstances longer labelling times or higher concentrations of radio-labelled amino acid were necessary. Long-term-starved cells were labelled with up to 300μCi ml^{-1} of ^{35}S-methionine or ^{3}H-leucine.

2.5.8. Autoradiography of polyacrylamide gels

Linear polyacrylamide gels were fixed with 3 changes of 40% (v/v) methanol over 4 hours. The gels were placed on 3MM Chr chromatography paper (Whatman), covered with Freshcling (European Plastic Films Manufacturers' Association) and dried under vacuum on a heated gel drier at 60° C for approximately 2 hours. The Freshcling was removed and the dried gels were placed in autoradiography cassettes in direct contact with Hyperfilm-βmax, (Amersham International) for ^{35}S-methionine-labelled proteins and Hyperfilm-^{3}H (Amersham International) for ^{3}H-leucine-labelled proteins.

^{14}C-labelled proteins were detected by soaking the gel in Amplify, (Amersham International) after fixing, but before drying. The dried gels were then placed in direct contact with Hyperfilm MP (Amersham International) for the desired time.

The autoradiographs were developed and fixed according to the manufacturer's instructions and contact-printed directly onto photographic paper (Kodak Kodabrome II RC F2 Professional black and white).

2.6. N-terminal amino acid sequencing of proteins

Three major outer membrane proteins were chosen for sequencing. Outer membranes were prepared as described in Section 2.5.1.3. and two-dimensional electrophoresis was carried out. The gel was soaked in low-glycine transfer buffer for 15 minutes. Hydrophobic polyvinylidene difluoride (PVDF) membrane, pore size 0.2μ, (Bio-Rad) was wetted in 100% (v/v) methanol then soaked in low- glycine transfer buffer for 15 minutes. Low-glycine transfer buffer contained, per litre, 100ml methanol, Tris 5.81g (48mM), glycine 2.93g (39mM), SDS 0.3g (0.03% w/v). Proteins were blotted onto the membrane as described in Section 2.16.2., except that low-glycine buffer was used. After transfer, the proteins were stained using Coomassie blue (0.025% Coomassie Blue R 250 (Sigma) in 40% (v/v) methanol) for 5 minutes then the membrane

was destained in 50% (v/v) methanol until the protein spots became visible (about 2 minutes). The spots were cut out and sent to Leicester University for sequencing.

2.7. Penicillin-binding protein analysis

Fluctuations in the levels of the various penicillin-binding proteins present within cells during starvation were investigated by ^{14}C-penicillin binding, (Buchanan & Sowell, 1982).

Cells were harvested and resuspended in ice-cold 10mM sodium phosphate buffer. The cells were ruptured by sonication, as described in Section 2.5.1.2. Unbroken cells were removed by centrifugation at 1, 500*g* for 20 minutes at 4°C. The supernatant was centrifuged at 48, 000g using a JA20 fixed angle rotor in a Beckman centrifuge for 60 minutes at 4°C to harvest the membranes. The membranes were washed twice in ice-cold, 10mM sodium phosphate buffer then resuspended in the same buffer. 10µl of 5M sodium phosphate buffer was added per 1ml of sample. 200µl of the sample was removed and incubated with 20µl of benzyl [^{14}C] penicillin potassium, (50-60mCi mmol^{-1}, Amersham International) for 10 minutes at 30°C. The reaction was stopped by adding 5µl of 120mg ml^{-1} penicillin G (Sigma) and 10µl of 20% (w/v) N-lauryl sarcosine (Sigma), to solubilise the cytoplasmic membrane fraction. The sample was incubated at room temperature for 20 minutes. Insoluble outer-membranes were removed by centrifugation for 90 minutes at 48, 000g as before at 20°C. To every 100µl of supernatant, 50µl of sample buffer (0.3% (w/v) SDS, 30% (v/v) glycerol, 0.1M DTT, 0.2M Tris-HCl pH 6.8) and 2µ l of 1M DTT solution was added and the sample boiled for 3 minutes. The sample was then loaded onto a 12% (w/v) linear acrylamide gel with a 4.5% (w/v) acrylamide stacking gel and electrophoresed as described in Section 2.5.2.

2.8. Long-term starvation of *E. coli* K12

2.8.1. Apparatus

Small volume cultures of *E. coli* K12 were incubated in carbon-limited conditions in Suba-Sealed flasks as described in Section 2.2.2. Volumes of 2, 5 10 or 20 litres were set up in Quickfit vessels in a 30°C warm room. Blended air (BOC) was supplied from a cylinder through two 0.22 μm pore size polytetrafluoroethylene (PTFE) Midisart 2000 air filters (Sartorius) connected in series. The air outlet of each vessel was connected to one 0.22 μm PTFE filter. To minimise the effects of water evaporation from the cultures, the air outlet was placed at the top of a water-cooled condenser, (see Figure 2.1).

2.8.2. Growth conditions

A seed culture was prepared by growing *E. coli* K12 in 100ml M9 containing 0.1% (w/v) glucose at 37°C overnight. The culture was inoculated aseptically into a large-volume vessel by injection through one Suba-seal and glucose was added to 0.05% (w/v) through a second Suba-seal. Both punctured Suba-seals were washed with 70% (v/v) ethanol and sealed with ethanol-washed parafilm. Blended air was supplied constantly at 100ml $litre^{-1}$.

2.9. Macromolecular synthesis rates

2.9.1. Determination of the rate of protein synthesis

A 0.5 ml sample of cells was labelled with ^{35}S-methionine or ^{3}H-leucine (Amersham International) at 1μCi ml^{-1} for 10 minutes at 30°C. The sample was then added to 0.5 ml of ice-cold 10% (w/v) TCA and incubated at 4°C for 30 minutes. The sample was filtered onto Whatman glass fibre filter discs (GF/F), washed with 5% (w/v) TCA, then absolute ethanol and finally a mixture of equal parts ethanol and ether. The filters were transferred to scintillation vials and

dried overnight at 60°C. 3 ml of Optiphase 'Safe' scintillation fluid (LKB) was added to each vial and the samples were counted in an LKB Wallac 1219 Rackbeta liquid scintillation counter. Unlabelled filters in scintillation fluid and scintillation fluid alone served as blanks. Total particle and viable counts were carried out as described in Sections 2.3.1 and 2.3.2.

2.9.2. Determination of the rate of DNA synthesis

0.5ml of culture was labelled with [methyl-^{3}H] thymidine (79Ci mmol^{-1}, Amersham International) at 1 μCi ml^{-1} . The culture was incubated at 30°C for 2 minutes then 0.5ml of ice-cold 10% (w/v) TCA was added. The samples were incubated on ice for 15 minutes then the TCA precipitable material was collected on GF/F filter discs. The filters were washed with 5% (w/v) TCA, then absolute ethanol before being placed in scintillation vials and incubated at 60°C until dry. 3ml of Optiphase 'Safe' scintillation fluid (LKB) was added to each vial and the samples counted on an LKB liquid scintillation counter as before. Total particle and viable counts were carried out as described in Sections 2.3.1. and 2.3.1.

2.9.3. Determination of the rate of RNA synthesis

0.5ml of culture was labelled with [5-^{3}H] uridine (27Ci mmol^{-1}, Amersham International) at 1μCi ml^{-1} for 30 seconds at 30°C, then treated as for DNA synthesis. Total particle and viable counts were carried out as described in Sections 2.3.1 and 2.3.2.

2.10. Light, phase-contrast and fluorescence microscopy

All light and phase-contrast microscopy was carried out on an Olympus microscope at a magnification of x1000. Using a trinocular headpiece an Olympus PM-6 camera and an Olympus light meter were attached. Black and white photography and printing was carried out using Tmax 100 Professional black and white film which was developed according to the manufacturer's

instructions and printed onto Kodak photographic paper (Kodabrome II RC Professional black and white). Colour photography was carried out using 35mm Kodak Gold ISO 100 and developed and printed by Colab (Coventry, England).

2.10.1. Gram stain

The method of Kopeloff and Beerman (1922) was used except crystal violet was used in place of methyl violet (Mackie & McCartney, 1989).

2.10.2. Giemsa stain

Cells were spread onto an acid-washed glass slide and air dried. The slides were immersed in 1M HCl at 60°C for five minutes then RNase (50μg ml^{-1}) was spotted onto the cells and the slides incubated in a humid environment at 37°C for 1 hour. The slides were then washed in H_2O and immersed in 10% (v/v) Giemsa (BDH, supplied as a 0.68% (v/v) in ethanol/glycerol) in 0.1M sodium phosphate buffer for 1 hour. The stain was washed off with water, the slides allowed to dry in air and the cells were examined as wet mounts using phase-contrast microscopy.

2.10.3. β-polyhydroxybutyrate stain

Samples were heat-fixed onto glass slides then the slide was immersed in 0.3% (w/v) sudan black in ethylene glycol for 5-15 minutes. The slides were drained and air-dried, dipped in xylene several times and then blotted dry. The cells were counterstained in 0.5% (w/v) aqueous saffranin for 5-10 seconds which stains the cytoplasm pink. The slides were rinsed in water, blotted and allowed to air-dry. In some cases, modifications of this method were used. These are described in the appropriate Results section. The slides were then observed at a magnification of x1000 using light microscopy.

2.10.4. Modified spore stain

Cells were heat-fixed onto a clean glass slide and the slide was flooded with carbol fuchsin (Sigma). The slide was heated until the steam rose and the heat source was removed to prevent boiling. This was repeated several times for a total of 5 minutes. The cells were observed at a magnification of x1000 using light microscopy.

2.10.5. Rhodamine 123 stain

Cells were harvested and washed three times in 50mM Tris-HCl, 5mM EDTA, pH 8. This permeabilised the outer-membrane of the Gram negative cell allowing the dye to make contact with the cytoplasmic-membrane and so be taken up. The cells were resuspended in their original supernatant and rhodamine 123 (Sigma) was added to a final concentration of 0.26μM, (Kaprelyants & Kell, 1992). The cells were incubated at room temperature for 10 minutes before being observed using an Nikon Optiphot UFX-II UV microscope fitted with a rhodamine filter (490nm).

2.10.6. DAPI stain (Hiraga *et al*, 1989; Eliasson *et al*, 1992)

Cells were spread onto an ethanol-washed slide and allowed to dry at room temperature. The cells were fixed with methanol at -20°C for ten minutes and washed in six changes of tap water. 10μl of poly-L-lysine (5μg ml^{-1}, Sigma) was spread on top of the cells with a plastic pipette tip and allowed to dry at room temperature. 10μl of 0.2μg ml^{-1} DAPI (Sigma) in 30% (v/v) glycerol and 10% (v/v) Mowiol, (Harlow Chemical Co., Essex, UK) was spotted onto the slide and an ethanol-washed coverslip was placed on top. The coverslip was sealed in place using soft white paraffin and the slides stored in the dark and viewed the same day using oil immersion phase-contrast with UV illumination using a Nikon Optiphot UFX-II microscope fitted with a UV filter. This allowed visualisation of the whole cell with phase contrast while also allowing the blue

fluorescence of the nucleoid to be seen. Photography was carried out using 35mm Kodak Gold ISO 100 colour film with a Nikon FX-35A camera

2.10.7. Mithramycin/ethidium bromide (MEB) staining

This stain was employed to stain DNA within cells, (Boye *et al.*, 1983) . Cells were fixed in 70% (v/v) cold aqueous ethanol for a minimum of 10 minutes. They were then washed twice in 0.1M Tris-HCl, pH 7.4 and resuspended in MEB.

MEB stain contained;

1mg mithramycin A, (Sigma Chemicals)

0.5mg ethidium bromide, (Sigma Chemicals)

0.102 g $MgCl_2$

0.117 g NaCl

20 ml 0.1M Tris-HCl buffer, pH 7.4.

2.11 Transmission electron microscopy

2.11.1. Negative staining of whole, fixed cells

Copper grids (300 mesh, Agar Scientific Ltd.) were coated with formvar and 10µl of a suspension of cells was spotted onto each grid. After 5 minutes, excess fluid was removed using filter paper and the sample was allowed to air-dry. 25% (v/v) EM grade gluteraldehyde (Agar Scientific Ltd.) was placed on the grid for 1 minute then washed off gently by placing the grid face-down in 3 successive drops of sterile distilled water to remove salt crystals from the sample. 10µl of filtered 0.1% (w/v) phosphotungstic acid was placed on the grid and the excess immediately blotted off. The grid was allowed to air-dry then observed using a Jeol JEM-100S transmission electron microscope at 80, 000 V.

2.11.2. Ultrathin sectioning of cells

2.11.2.1. Epoxy resin embedding

Cells were harvested by centrifugation at 1, 500*g* for 20 minutes in a Mistral 1000 benchtop centrifuge. The pellet was resuspended in 50mM HEPES buffer containing 2.5% (v/v) EM grade gluteraldehyde (Agar Scientific Ltd) and allowed to fix for 2-4 hours at room temperature. The cells were washed twice in 50mM HEPES buffer (Sigma) and then harvested. The cell pellet was resuspended in 1% (w/v) osmium tetroxide (Agar Scientific Ltd) for 2-4 hours at room temperature after which the cells were washed twice in 50mM HEPES buffer then harvested. The cell pellet was resuspended in 2-3 drops of molten 2% (w/v) Noble agar (Agar Scientific Ltd) at 45°C. The suspension was spread onto an alcohol-cleaned glass microscope slide and allowed to solidify at room temperature after which the agar was diced into 1mm cubes. The cubes were incubated in deionised water for 15 minutes at room temperature, then in 2% (w/v) filtered uranyl acetate for 2 hours. The cubes were washed in 2-5 changes of deionised water then twice in 2-3ml of 70% (v/v) ethanol for 15 minutes each time. They were then transferred to 80% (v/v) and 95% (v/v) ethanol, successively, for 15 minutes each then finally to 100% ethanol for 30 minutes. The cubes were placed separately in 1ml of fresh 100% ethanol and 1ml of complete plastic medium (Agar Scientific Ltd., made up according to the manufacturer's instructions) was added and mixed gently. The vial was incubated at room temperature with occassional mixing until the cube sank to the bottom, when another 1ml of plastic medium was added. After the cube had sunk, the plastic medium/ethanol mixture was removed and 2ml of plastic medium was added. Again the cube was left to sink after which it was transferred to a size 00 Beem capsule (Agar Scientific Ltd) containing 0.55ml plastic medium. The Beem capsules were left at room temperature until the cube had sunk to the bottom, then, with the caps off, the capsules were placed in a 60°

C oven overnight to allow the plastic medium to harden, usually overnight. The plastic medium with the cube was removed from the capsule, trimmed with a razor blade and fixed to the chuck of an ultramicrotome, (Leica), and sections were cut.

2.11.2.2. Cryosectioning

Cells were harvested, fixed and washed as described in Section 2.10.1. The cells were suspended in 50 µl of 2.3M sucrose. Approximately 20 µl of suspension was placed on the head of a roughened cryosectioning stub and plunged upright into liquid nitrogen. The stub was held under the surface of the liquid nitrogen until boiling ceased and was then transferred immediately to a pre-cooled cryosectioning chamber (Leica). The stub was fixed to the chuck using pre-cooled forceps and the sample was trimmed before sections were cut using a glass knife. The cut sections were picked up using a 2.3M sucrose drop on a 300µl platinum wire loop (Agar Scientific Ltd) and transferred to a formvar-coated copper EM grid (Agar Scientific Ltd). The grid was picked up with the loop and placed section-side down on a drop of deionised water to wash off the sucrose. To stain the sections, the grid was transferred, section-side down to three consecutive drops of 1% (w/v) osmium tetroxide, three consecutive drops of deionised water, then three consecutive drops of a mixture containing one part 2% (w/v) uranyl acetate and nine parts 2% (v/v) polyvinylalcohol. Excess uranyl acetate/PVA mixture was removed by blotting with filter paper and the sections were allowed to air dry. Unstained sections could be kept for long periods by placing them face down on 1% (w/v) gelatin plates which had a thin layer of PBS on the surface. When required they were floated off the surface of the gelatin with a small volume of PBS and stained as described.

2.12. Lipopolysaccharide analysis

2.12.1 Extraction of lipopolysaccharide

Outer membrane proteins were prepared as described in Section 2.5.1.2. The protein concentration was adjusted to 2mg ml^{-1} and an equal volume of 2x sample buffer was added. This contained 0.25M Tris-HCl, pH 8, 40%(v/v) glycerol, 8%(w/v) SDS, 20mM DTT, 0.005%(w/v) bromophenol blue. The sample was boiled for 5 minutes. 25 μg Proteinase K (Sigma) made up at 2.5mg ml^{-1} in 1x sample buffer, was added to each 100μg protein. The sample was incubated at 60°C for 1 hour and stored at -20°C until required.

2.12.2. Electrophoresis of lipopolysaccharide

LPS was electrophoresed in a 15% (w/v) polyacrylamide gel as described in Section 2.5.2.1. except that 4M urea was included in the resolving gel. Smooth LPS from *E. coli* 0111:B4 (Sigma) was used as a control, 20μl of a 10mg ml^{-1} solution in 1x sample buffer being loaded onto the gel after Proteinase K digestion.

2.12.3. Silver staining of lipopolysaccharide

The method of Hitchcock and Brown was used, (Hitchcock & Brown, 1983). Briefly, gels were soaked overnight in 200ml 25%(v/v) isopropanol in 7% (v/v) acetic acid then oxidised for 5 minutes in 150ml distilled water with 1.05g periodic acid (Sigma) and 4ml 25% (v/v) isopropanol in 7% (v/v) acetic acid, made up immediately before use. The gel was washed in 200ml distilled water for 30 minutes eight times then stained for 10 minutes in a solution containing 28ml 0.1M NaOH, 1ml concentrated (29.4%, w/v) ammonium hydroxide, 5ml 20% (w/v) silver nitrate (Sigma) and 115ml distilled water, made up just before use with constant stirring. The gel was washed for 10 minutes in 200ml distilled water four times. The gel was soaked for 10-20 minutes in

developer which contained 50mg citric acid and 0.5ml formaldehyde in 1 litre of distilled water, made up just before use. The developer was made up at 25°C and kept at this temperature during development to prevent the staining of proteins. Once developed, the gel was soaked in stain stop solution which contained 200ml distilled water and 10% (v/v) acetic acid for 1 hour after which it was washed in 200ml distilled water and stored in an airtight container with a little distilled water to prevent desiccation.

2.13. Nucleoid sedimentation characteristics

Cells were harvested from 3-5 ml of culture as described previously and resuspended in 0.4ml 1M NaCl, in 0.1M Tris-HCl, pH 8.1. 0.08ml of the same solution, but containing lysozyme to a final concentration of 10mg ml^{-1} and 0.05M EDTA, was added to the cell suspension and the sample was incubated at 4°C for 45 seconds. 0.5ml of a solution containing 1M NaCl, 1% (w/v) Brij 58 (Sigma), 0.01M EDTA, 1% (w/v) N-lauryl sarcosine (Sigma), 0.1M $MgCl_2$ and 5mg ml^{-1} sodium deoxycholate was added. To obtain membrane-associated nucleoids, the sample was incubated at 4°C for 30 minutes. The samples were centrifuged at 23, 000g for 5 minutes (excluding acceleration and deceleration times) at 4°C in a SW28 swing-out rotor in a Beckman ultracentrifuge on a 10-30% (w/v) sucrose gradient containing 1M NaCl, 0.01M Tris-HCl, pH 8.1, 0.001M EDTA, 1mM DTT (Sigma) and 0.1M $MgCl_2$.

The position of DNA in the sucrose gradient was determined by collecting the gradient from above using a Buchler Auto Densi-Flow II^C connected to a Pharmacia peristaltic pump. The collected gradient was passed through a Pharmacia Dual Path Monitor UV-2. The optical density of the gradient was measured by the monitor on the 1mm path at 280nm with the maximum deflection set at 2 units. A Parmacia RC2 two-channel recorder was used to record changes in optical density. The delay in optical density changes due to the length of tubing was calculated by the addition of herring sperm DNA

(Sigma) to distilled water while the apparatus was operating and the time lag marked on the chart recording.

The sedimentation coefficient of bacterial nucleoid DNA was calculated by comparison with a calibration curve obtained using T4 phage. Phage lysate was prepared by overlaying nutrient agar plates with 3ml of top agar to which had been added 200µl of an overnight culture of *E. coli* K12 and 10 µl of a phage dilution (10^0 to 10^{-6}). Top agar contained 0.3% (w/v) agarose in nutrient broth. After overnight incubation, the top agar was aseptically scraped off plates showing semi-confluent plaque formation into a glass universal container. 1ml of chloroform was added and the preparation was vortexed for 15 minutes. Debris was removed by centrifugation at 1, 500*g* for 20 minutes in an MSE Mistral 1000 benchtop centrifuge and the supernatant collected. 1ml of chloroform was added and the supernatant was stored at 4°C until required.

To obtain a calibration curve, phage was layered onto a 10-30% (w/v) sucrose gradient and centrifuged at 23, 000g as described above. The distance travelled by the phage at 5 minute intervals was determined and plotted against time.

2.14. Hydrophobicity assessment

2.14.1. Bacterial adherence to hexadecane (BATH)

Cells were harvested by centrifugation at 1, 500*g* for 30 minutes then washed twice in PUM buffer which contained, per litre, 22.2g $K_2HPO_4.3H_2O$, 7.26g KH_2PO_4, 1.8g urea, 0.2g $MgSO_4.7H_2O$. The cells were resuspended in PUM buffer to an optical density of approximately 1.5 at 400nm. 1ml of n-hexadecane (Sigma) was added to 1.2ml of cell suspension and incubated at 30 °C for 10 minutes. The suspension was vortexed for 2 minutes and the two phases were allowed to separate by standing at room temperature for 15 minutes, after which the optical density of the aqueous phase was measured as before.

The percentage bacterial adherence to hexadecane was calculated as shown below;

% adherence to hexadecane = (Starting OD - Final OD) x 100 / Starting OD

2.14.2. Hydrophobic interaction chromatography (HIC)

Glass Pasteur pipettes were plugged at the bottom with glass wool then the glass wool was washed twice with 95% (v/v) ethanol followed by 1M NaCl. Phenyl sepharose CL4B (Sigma) was diluted 1:1 with 1M NaCl and 0.6ml was added to each pipette. Cells were harvested and washed twice in 1M NaCl and resuspended in 1M NaCl and the absorbance at 600mn was measured. The cell suspension was passed through the phenyl sepharose column and the absorbance of the eluate measured as before. The percentage retention by phenyl sepharose was calculated. Unsubstituted sepharose was used as a control to take into account any physical trapping of the cells in the column.

2.15. Measurement of cellular ATP levels

The ATP content of cells was estimated using a commercially available kit (Boerringer-Mannheim) according to the manufacturer's instructions. All solutions used were provided in the kit. Samples and standard solutions were prepared in a black microtitre plate kept on ice throughout, except during cell lysis. Briefly, cells were lysed by incubating with an equal volume of cell lysis buffer at room temperature. An equal volume of luciferase was added and the relative light units emitted by each sample was determined using a Luminoscan RT luminometer.

A standard curve was prepared by diluting a stock ATP solution to 10^{-6} - 10^{-12} in dilution buffer. The standards were then treated in the same manner as the samples. The amount of ATP in each sample was determined using the regression data from the standard curve.

2.16 Surface antigen detection

2.16.1. Polyclonal antibody production

Outer-membrane proteins were prepared as described in Section 2.5.1.3 separated by two-dimensional electrophoresis (Section 2.5.3) and detected by silver staining as described in Section 2.5.4. The required spot was cut out from 4 gels for each injection and destained as described in Section 2.5.5. The destain solution was removed by washing the pieces of acrylamide in 40% (v/v) methanol for 24 hours and the sample stored at -20°C in 40% (v/v) methanol. When required, the acrylamide pieces were washed twice in sterile PBS for 1 minute each time then crushed between two ethanol-flamed glass microscope slides in a small amount of sterile PBS. The crushed acrylamide was washed into an Eppendorf tube with a small volume of PBS and an equal volume of sterile PBS was added. The sample was placed in a 2ml glass syringe, connected to a second by a Luer lock and an equal volume of Freund's complete adjuvant (Sigma) was added. The sample and adjuvant were homogenised by passage. A half-lop rabbit was injected subcutaneously with the protein sample. All subsequent injections given consisted of the same protein in Freund's incomplete adjuvant (Sigma). A second, booster dose was given four weeks later. 10 days after the second injection, the rabbit was bled by nicking the marginal ear vein and blood was collected in a glass universal and stored at 4°C overnight. The serum was then separated from the clot and centrifuged at 1, 500*g* for 5 minutes to remove residual red blood cells and stored at -20°C. The serum was checked for the presence of antibody by Western blotting, (Section 2.16.2.) A second boost was given four weeks after the first and the rabbit bled 10 days later. Again the serum was checked for the presence of antibody by Western blotting.

2.16.2. Western blotting

Outer-membrane proteins were separated by SDS-PAGE or two-dimensional electrophoresis in 12% (w/v) polyacrylamide with a 4.5% (w/v) stacking gel, as described in Section 2.5.2 and The gel was soaked for 10-15 minutes in western transfer buffer (WTB; 25mM Tris-HCl, pH 8.3, 192mM glycine and 20% (v/v) methanol). A nitro-cellulose filter (Hybond C, Bio-Rad) was cut to the same size as the gel, soaked in WTB and placed on top of the gel. The gel and filter were placed between six sheets of filter paper, three on each side, which had been soaked in WTB. A porous sponge was placed on either side of filter paper sandwich and placed in the cassette of a Transblot tank containing WTB. The proteins were transferred to the nitro-cellulose filter at 65 V for 3 hours at 4°C. The filter was stained with 0.1% (w/v) Ponceau S (Sigma) in 5% (w/v) TCA. The positions of molecular weight standards were marked and the membrane was destained in PBS for 10 minutes. The membrane was soaked in blocking buffer which contained 2% (w/v) dried milk (Marvel) and 0.1% (v/v) Tween 20 (Sigma) in PBS for 1 hour to allow blocking to take place. The filter was transferred to blocking buffer containing an appropriate dilution of antibody and incubated for one hour at room temperature then overnight at 4°C. The filter was then washed 3 times in 10 ml PBS containing 0.1% (v/v) Tween 20 for 5 minutes each, then twice for 1 minute each time.

2.16.3. Detection of antigen-antibody complex on filters

The filter was placed in PBS containing 0.1% (v/v) Tween 20 and a 1:300 dilution of goat anti-rabbit peroxidase-conjugated IgG (Sigma) and incubated at room temperature for 1-2 hours with shaking. The membrane was washed twice in 10 ml PBS containing 0.1% (v/v) Tween 20 for 10 minutes each, then twice in 10 ml PBS for 10 minutes each wash. The membrane was transferred to the staining solution which contained 0.1% (v/v) hydrogen peroxide in PBS. Immediately before use, 30mg of 4-chloro-1-naphthol (Sigma) was dissolved in

20ml methanol and added to the hydrogen peroxide solution. Before the reaction was completed, the membrane was washed in 3 changes of water over 30 minutes and then air-dried. After photography, the membrane was stored in the dark.

2.17. Assessment of cross-protection

2.17.1. Heat challenge

Samples were removed from cultures at the required times and incubated in a 60°C waterbath in a glass universal container. At two minute intervals samples were removed for total particle and viable counts, carried out as described in Section 2.3.

2.17.2. Biocide challenge

Minimum inhibitory concentrations of each biocide were determined by exposing cells to a dilution series of biocide. The biocides were diluted in M9 containing 0.05% (w/v) glucose in microtitre plates and a 1 in 10 dilution of cells was added to each dilution. The plates were incubated at 30°C and examined for growth at 24 and 48 hours. Those wells which showed no growth were subcultured onto M9 starvation plates and incubated at 30°C for 48 hours to determine the minimum bactericidal concentration.

To compare biocide resistance, cells were exposed to the minimum bactericidal concentration of biocide and samples were removed at 30 minute intervals. Viable counts were carried out to determine the death rates of the cells.

Chapter 3. Kinetics of carbon-starved populations

3.1. Introduction

When cells growing in batch culture experience depletion of one or more nutrients and/or the build-up of toxic metabolites, growth is seen to slow down and/or stop, i.e. cell numbers, as determined by viable counts, no longer increase.

By carefully balancing the constituents of minimal medium, cells can be starved for specific nutrients either separately or in various combinations without the added complication of toxic metabolite build-up.

Numerous published articles on the subject of starvation survival have suggested that starvation for carbon triggers a survival response which protects against many forms of stress (Grossman *et al.*, 1985; Morgan *et al.*, 1986; Jenkins *et al.*, 1988; Hartke *et al.*, 1994), whereas starvation for other nutrients such as phosphate or nitrate elicit only a response specific for that particular starvation regime.

The accepted view that cells entered the stationary phase when nutrients are depleted and then began to die off during the decline phase was questioned when investigations into the viable, but non-culturable state began. It had been thought that only bacteria specifically adapted for life in oligotrophic environments were able to survive long periods without nutrients, particularly a carbon-source (Boylen & Ensign, 1970), but it has become apparent that bacteria previously considered to be adapted to high-nutrient environments are able to survive carbon-starvation for surprisingly long periods (Xu *et al.*, *1982;* Allen-Austin *et al.*, 1984; Roszak *et al.*, 1984; Colwell *et al.*, 1985; Grimes *et al.*, 1986; Rollins & Colwell, 1986; Roszak & Colwell, 1987; Byrd *et al.*, 1991; Jones *et al.*, 1991; Oliver *et al.*, 1991; McKay, 1992; Morgan *et al.*, 1993; Duncan *et al.*, 1994; Rahman *et al.*, 1994; Stern *et al.*, 1994).

Work on stationary phase populations has suggested that survival during starvation may depend on the growth conditions previously experienced by cells, (Kolter, 1992; Zambrano *et al.*, *1993*). This work also posed the interesting

question of why some cells survive and others within the same population, and apparently experiencing the same conditions, do not.

It has been shown that to survive carbon starvation, *E. coli* must synthesise starvation-specific proteins (Reeve *et al.*, 1984a). It is possible that the rate of growth of a batch culture when starvation is encountered may have some bearing on the outcome. It has been shown that exponential-phase cells are unable to survive stress conditions as well as stationary-phase cells (Gauthier *et al.*, 1992; Nystrom *et al.*, 1992). At a rapid rate of cell division, with multiple chromosome replication initiation sites, a cell encountering sudden starvation would need to finish all rounds of chromosome replication initiated before ceasing growth. It is possible that this would deplete the energy reserves of the cell, thus leaving it unable to synthesise starvation-survival proteins.

It has been demonstrated that *E. coli* exhibits natural synchrony as it enters the stationary-phase, (Cutler & Evans, 1966). This suggests that a signal alerts the cells within a culture to the imminent shortage of nutrients. During exponential growth the population of a batch culture is heterogeneous with respect to the phase of cell division that the cells are in. To become synchronous, some cells must be "stopped" or slowed to allow others to catch up. This suggests a mechanism which stops vegetative growth in preparation for stationary-phase. This slow, natural entry to stationary-phase, compared with the sudden entry experienced by exponential-phase cells when they are placed in starvation medium allows the cells to finish all rounds of chromosome replication before the nutrients are fully depleted. Thus less of their stored energy needs to be used for this purpose and can be utilised in the synthesis of starvation-specific proteins.

This hypothesis also suggests that exponential-phase cells growing in batch culture in very low glucose concentrations may survive sudden carbon starvation better than those cells in rich medium and as well as stationary-phase cells.

In order to investigate the above questions, the kinetics of carbon-starved populations of *E. coli* were examined.

Aims

The aims of the following experiments were to determine the optimal medium composition to use in subsequent experiments and to determine which aspects of growth conditions played a role in starvation-survival. The conditions examined were growth rate and growth phase.

3.2. Results and discussion

3.2.1. The relationship between growth rate and starvation survival

In order to determine the optimum glucose concentration for use in prolonged incubation experiments an *E. coli* K12 was inoculated into 3 flasks containing M9 minimal medium with 0.5%, 0.05% and 0.02% (w/v) glucose. It was important to ensure that stationary-phase cell density was kept to a minimum to prevent the build up of toxic metabolic waste products and to prevent cryptic growth while ensuring that the normal growth kinetics of batch culture were retained. The growth in the flasks was monitored by absorbance measurements at 600nm as described in Section 2.1.4.

Figure 3.1 shows the absorbance at 600nm of the three cultures, monitored over 11 hours. The doubling time of each culture was calculated to be 1.8, 3.3 and 17.3 hours for 0.5%, 0.05% and 0.02% (w/v) glucose respectively. As expected they differed in final absorbance at stationary-phase. It has been shown that *E. coli* exhibits unidirectional polar growth at generation times exceeding 60 minutes (Donachie & Begg, 1970; Begg & Donachie, 1977). It can be seen from these results that cultures grown in 0.02% (w/v) glucose did not exhibit normal batch culture kinetics, while cells grown in 0.5% (w/v) glucose resulted in a dense population carrying the dual risks of cryptic growth and the

build-up of toxic metabolites occurring. Cultures grown in 0.05% (w/v) glucose, however, exhibited normal batch culture kinetics with distinct lag-, exponential- and stationary-phases, while the lower cell density reduced the probability of crypyic growth and toxic metabolite build-up occurring. For these reasons it was decided that 0.05% (w/v) glucose wouls be used subsequently. Since glucose levels above 0.05% (w/v) resulted in a greater number of cells in a culture, it was assumed that at 0.05% (w/v) the glucose was the limiting factor which caused entry to the stationary phase and not the exhaustion of any other component of the medium. It was decided to use 0.02% (w/v) glucose in M9 medium as the solid medium for subsequent viable counts as this would minimise the effects of substrate-accelerated death.

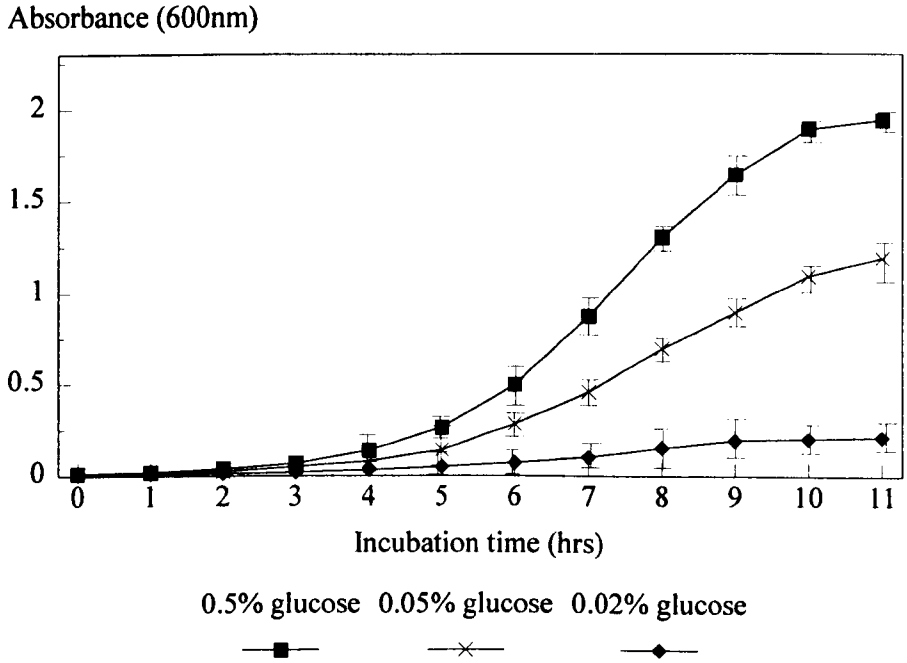


Figure 3.1. *E. coli* K12 was grown in minimal medium with 0.5, 0.05 and 0.02% (w/v) glucose provided as a carbon source. Growth was assessed by absorbance measurements at 600nm. Each glucose concentration was tested in triplicate flasks and triplicate samples were taken from each. The data represent the mean absorbance measurement of nine readings. Error bars represent ± one standard deviation.

To investigate the hypothesis that growth rate and not only growth phase affects starvation survival and is the cause of exponential-phase cells being less able to survive than stationary-phase cells (Gauthier *et al.*, 1992; Nystrom *et al.*, 1992), an *E. coli* K12 was inoculated into two flasks containing 0.05% and 0.5% (w/v) glucose and incubated aerobically at 37°C with shaking at 200rpm. The absorbance at 600nm in each flask was monitored hourly and when exponential growth had begun, half of each culture was removed and the cells were harvested by centrifugation, washed in M9 without glucose and resuspended in M9 medium without glucose, as described in Section 2.2.1. The second half of each culture was allowed to reach stationary phase and incubation was continued. Viable and total particle counts were carried out on the four populations of starving cells over a period of 14 days, as described in Sections 2.3.1 and 2.3.2. Figures 3.2 and 3.3 show the viable and total particle counts obtained.

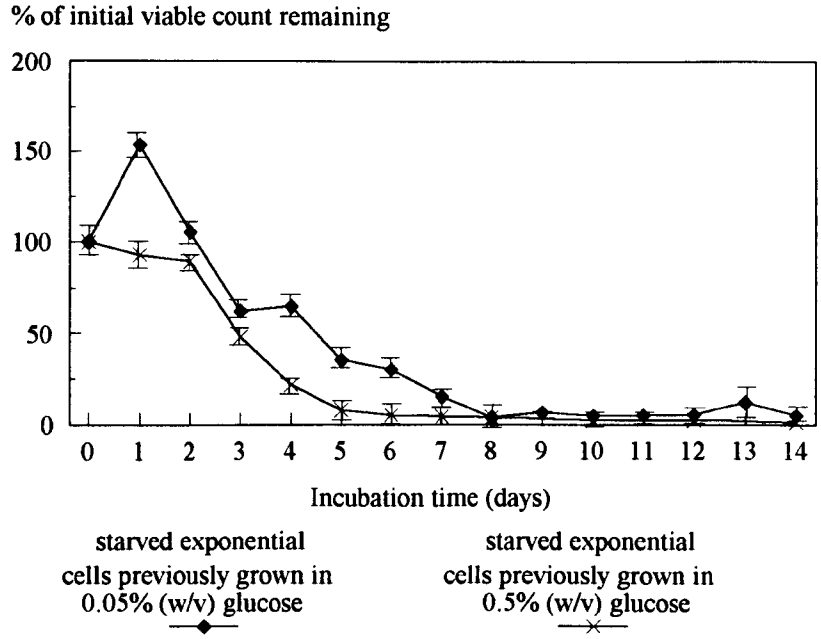
It can be seen from Figure 3.2 (A) that the viable counts of exponential-phase cells subjected to strict starvation were affected by the concentration of glucose which the cells had previously been grown in. The viable counts of cells previously grown in 0.05% (w/v) showed an increase in the first 24 hours which was not seen in cells which had been previously grown in 0.5% (w/v) glucose and remained higher until 8 days incubation. When stationary-phase cells were examined it was seen that, although the cells previously grown in 0.05% (w/v) glucose did not exhibit the same increase in viable count in the first 24 hours as the exponential-phase cells, it remained significantly higher than the viable count of the cells previously grown in 0.5% (w/v) glucose until 7 days incubation (Figure 3.2 (B)). The cells which had previously been grown in 0.5% (w/v) glucose exhibited a dramatic decrease in viable count in the first 24 hours, having decreased to only 0.1% of the initial viable count. This suggests that a build-up of toxic metabolites had played a role or that another component of the medium had become limiting. The carbon-starvation response has been tentatively identified as one which confers cross-protection against many other types of

stress. Other stress responses appear to be very specific in that they confer protection only against the specific stress experienced. It seems reasonable to assume that the large decrease in viable counts is due to an alternative growth-limiting factor with a specific stress-response being elicited. For example, the marine *Vibrio* species S14 is markedly more tolerant to a variety of stresses after starvation for carbon than after starvation for nitrogen or phosphorous (Nystrom *et al.*, 1992). In the present experiment, the phosphorous:nitrogen ratio in the medium was 2:1. Chemical analysis of *E. coli* shows that nitrogen represents 14% and phosphorous represents 3% (Neidhardt *et al.*, 1990). This suggests that in the present experiments nitrogen will become growth-limiting before phosphorous in media containing 0.5% (w/v) glucose. In addition, nitrogen starvation does not result in protection against acid, heat or peroxide shock, which carbon starvation has been shown to confer. It is therefore plausible that carbon-starvation protects cells against toxic metabolites. This has not been investigated in the present study, but could easily be accomplished by repeating the above experiment with an increased concentration of salts so that, even at 0.5% (w/v) glucose, glucose would be the limiting factor. In this case, the amount of growth which would be achieved would result in metabolite build-up in the medium. By reducing the concentration of each salt in turn, the effect of each limitation on metabolite resistance could be investigated. Finally, the role played by cell density-sensing mechanisms may be important in induction of the carbon-starvation response, as discussed in Chapter 1.

When total particle counts were examined it was seen that they behaved in a different manner (Figure 3.3 (A)). The total particle counts of exponential cells previously grown in 0.05% (w/v) glucose increased as the incubation period continued, while those of cells previously grown in 0.5% (w/v) glucose showed a 50% decrease in the first 24 hours. Since the viable count of these cells did not show a similar decrease in the same period (Figure3.2 (A)) this suggests that the decrease in total particle count represents the lysis of dead cells and indicates

that, when starvation is encountered, at least 50% of exponential cells die and lyse. The total particle counts of stationary phase cells behaved differently. Cells grown in both concentrations of glucose initially showed an increase in total particle count; between days 1 and 2 for cells previously grown in 0.05% (w/v) glucose and between days 2 and 3 for cells previously grown in 0.5% (w/v) glucose (Figure 3.3 (B)). The latter cells subsequently showed a steep decline after day 3 before beginning a slow, gradual decline throughout the time of the experiment. The cells previously grown in 0.05% (w/v) glucose, while showing a decline between days 3 and 5, subsequently increased and by day 14 were significantly higher than they had been at stationary phase.

(A)



(B)

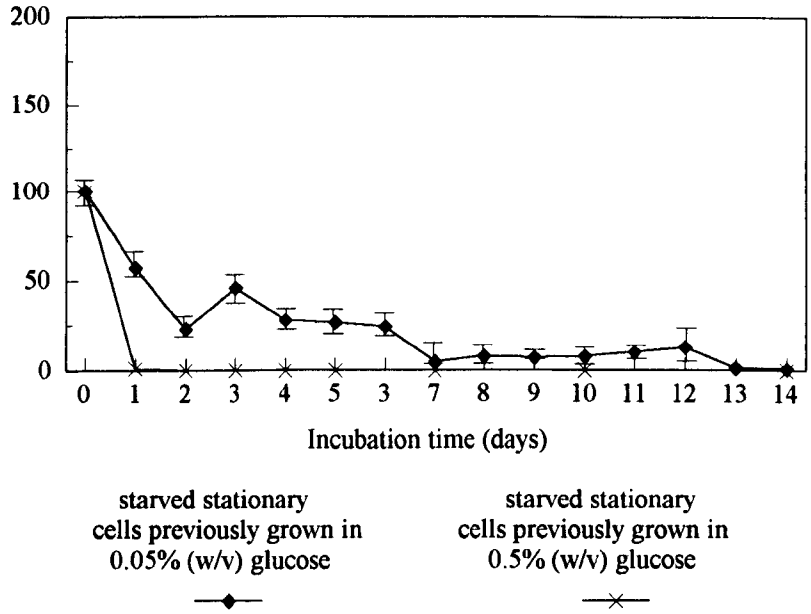
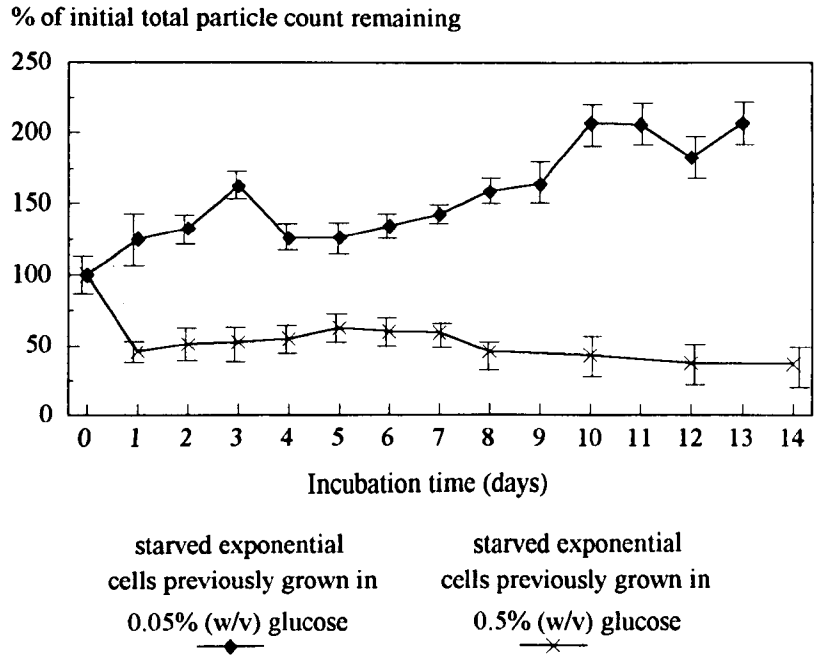


Figure 3.2. Cells were grown in 0.05% and 0.5% (w/v) glucose in triplicate. One half of each culture was harvested in exponential phase and inoculated into minimal medium without a carbon source. The other half was allowed to reach stationary phase and all four cultures were incubated for 14 days. Viable counts were carried out on a daily basis on starvation medium in triplicate. The data represents the mean value of nine counts at each time point. Error bars represent ± one standard deviation.

(A)



(B)

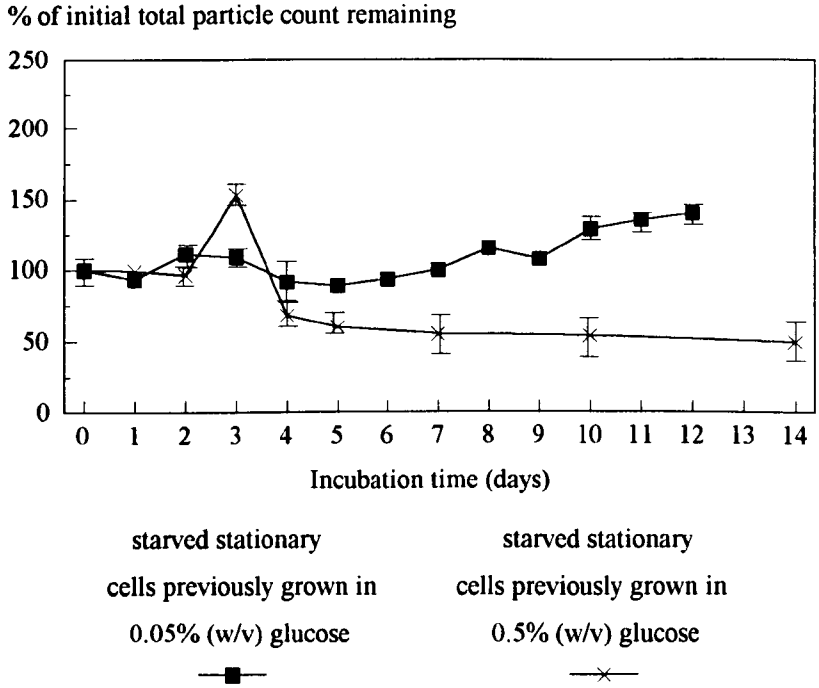
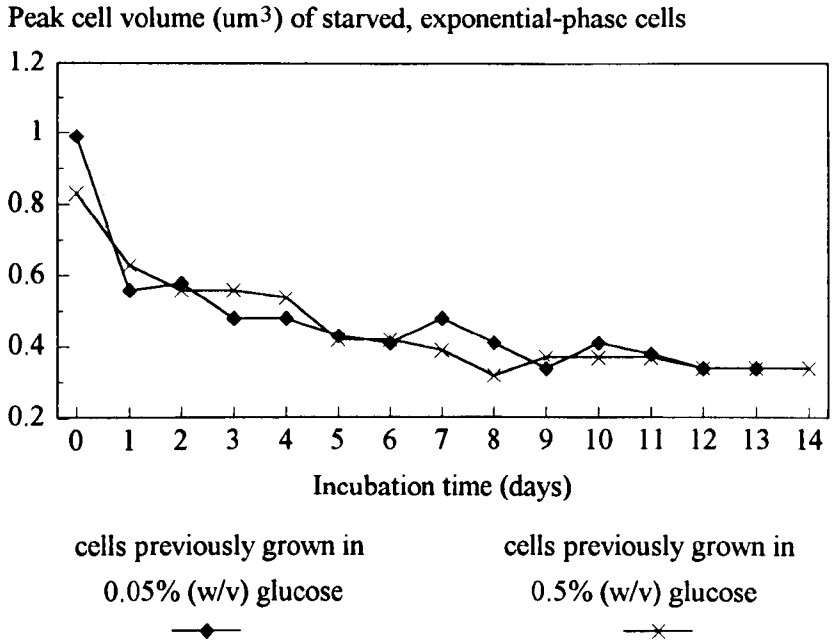


Figure 3.3. Cells were grown in 0.05% and 0.5% (w/v) glucose in triplicate. One half of each culture was harvested in exponential phase and inoculated into minimal medium without a carbon source. The other half was allowed to reach stationary phase and all four cultures were incubated for 14 days. Total particle counts were carried out on a daily basis using a CellFacts particle analyser on triplicate samples. The data represents the mean value of nine counts at each time point. Error bars represent ± one standard deviation.

When the peak cell volumes of the four cultures were measured over the course of the experiment, it was found that all decreased to 0.4μm^3. The usual size for actively growing *E. coli* is 2-4 x 0.6μm in Luria-Bertani broth (Donachie, 1993). In the minimal media used the difference in peak cell volumes of exponential-phase cells at the onset of starvation was negligible; 1μm^3 for cells grown in 0.05% (w/v) glucose and 0.82μm^3 for cells grown in 0.5% (w/v) glucose. However, cells previously grown in 0.05% glucose showed a larger decrease in peak cell volume between exponential- and stationary-phase than cells previously grown in 0.5% glucose.

It was also noted that exponential-phase cells previously grown in 0.5% (w/v) glucose exhibited a second peak in their cell volume profile which tended to distort the major peak and overlapped with the interference channel (data not shown). The extremely small size of the particles represented by this peak tends to suggest that it has been caused by the presence of cell lysis debris.

(A)



(B)

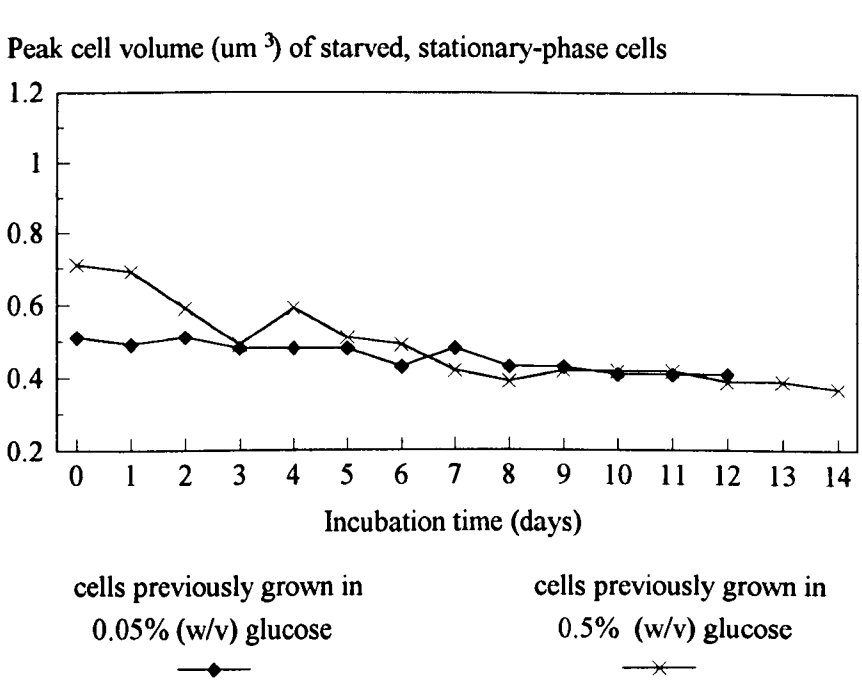
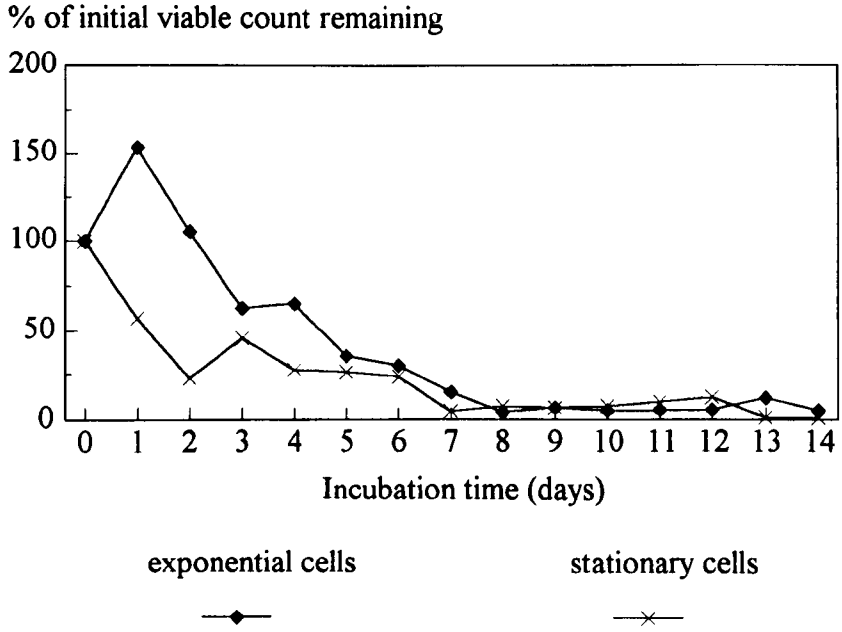


Figure 3.4. Cells were grown in 0.05% and 0.5% (w/v) glucose. One half of each culture was harvested in exponential phase and inoculated into minimal medium without a carbon source. The other half was allowed to reach stationary phase and all four cultures were incubated for 14 days without a carbon source. Cell size profiles were measured on a daily basis using a CellFacts particle analyser.

If the data for exponential- and stationary-phase cells previously grown in 0.05% (w/v) glucose are compared (Figure 3.5) it can be seen that the differences between the two cell types are not as marked as the differences seen between exponential- and stationary-phase cells grown in 0.5% (w/v) glucose (Figure 3.6). These results suggest that although the phase of growth the cells are in when starvation is encountered has some bearing on their subsequent ability to survive, the main factor influencing survival is the rate of growth prior to starvation. Since exponential-phase cells experience more rapid growth than cells entering stationary-phase, results obtained from these experiments will always tend to suggest that exponential-phase cells survive starvation less successfully than stationary-phase cells. However, Figure 3.5 indicates that when the exponential-phase growth rate is reduced sufficiently, in this case by providing a much lower level of carbon and energy source, the exponential-phase cells will survive almost as well as the stationary-phase cells and the kinetics of the population will mimic that of the stationary-phase cells.

Of more importance, however is the observation that total particle counts of exponential-phase cells previously grown in 0.05% glucose increased during carbon-starvation (Figure 3.5 B) and the peak cell volume decreased (Figure 3.4 A). This suggests that a reductive division has taken place. Reductive division has been demonstrated to occur in marine *Vibrio* species and it has been suggested that this is a mechanism by which cells increase their chances of surviving starvation conditions until nutrients are again available (See Chapter 1, Section 1.4). This mechanism has not been described previously for *E. coli*, but it is suggested that, given the appropriate conditions, *E. coli* uses this strategy to enhance survival during nutrient starvation.

(A)



(B)

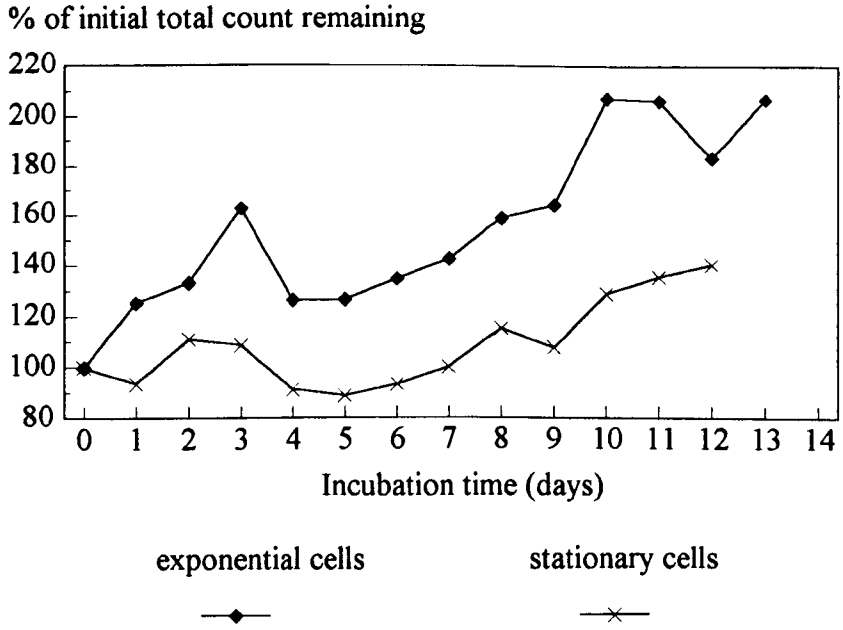
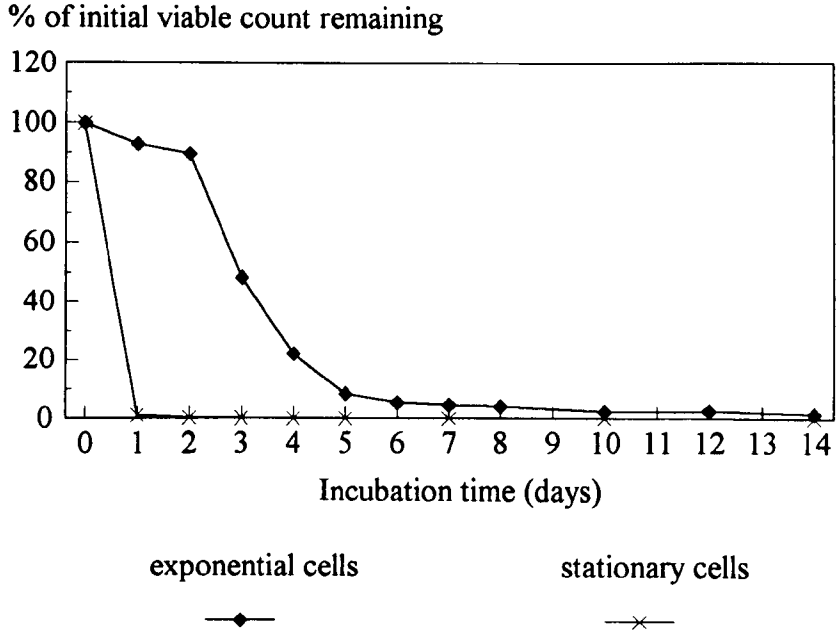


Figure 3.5. Comparison of survival rates of exponential and stationary phase cells, as viable and total counts, grown in 0.05% (w/v) glucose. The data presented here are the same as those presented in Figures 3.2 and 3.3, but in a different combination.

(A)



(B)

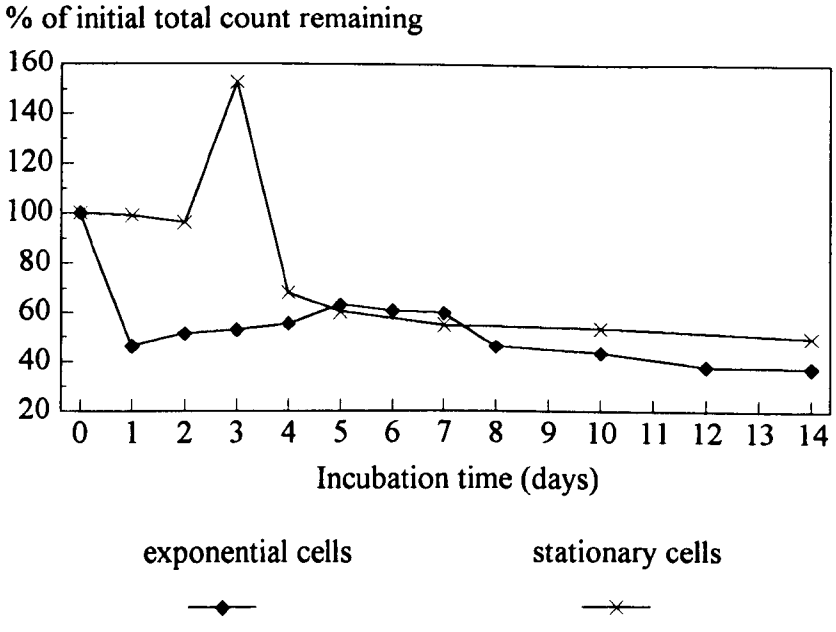


Figure 3.6. Comparison of survival rates of exponential and stationary phase cells, as viable and total counts, grown in 0.5% (w/v) glucose. The data presented here are the same as those presented in Figures 3.2 and 3.3, but in a different combination.

Although the death rate for starved exponential-phase cells previously grown in 0.5% (w/v) glucose can be explained as a function of the rate of growth it does not explain why a death curve is obtained, i.e. why some cells die and others do not. Figure 3.6 (B) shows that after 14 days starvation at least 50% of the initial cells had died and lysed because the total particle count had decreased by this amount. However, some cells still grew on plating. The stationary phase cells did not show a death curve when assessed by viable count, but showed a sudden decrease to almost zero.

3.2.2. Natural synchrony and starvation survival

One of the major differences between exponential-and stationary-phase cells is the relative heterogeneity of the populations. As has already been mentioned, as cells enter stationary-phase, they seem to exhibit natural synchrony. Exponential-phase cells do not exhibit synchrony unless they have been specially prepared. It was thought that this difference may be responsible for the different population starvation kinetics seen and that the phase of the cell division cycle a cell is in when starvation is encountered may determine the outcome. To investigate this, a culture of *E. coli* K12 was synchronised using the method of Cutler and Evans, (1966; Section 2.2.3). Many methods of obtaining synchronised cultures involves disruption of normal cell cycle events and it is possible that this method may do the same. It is thought that this mechanism may involve cell-cell interaction. Figure 3.7 shows the total particle count of a synchronised culture, the arrows indicating the sampling points. At these points, cells were harvested from the culture, washed in M9 medium without glucose and resuspended in M9 medium without glucose. The total particle counts were observed for 17 days and the total particle counts and peak cell volumes measured (Section 2.3.2). These results are shown in Figure 3.8. If the phase of the cell division cycle plays no role in starvation survival it was expected that all the cultures would behave in the same fashion. This was not the case.

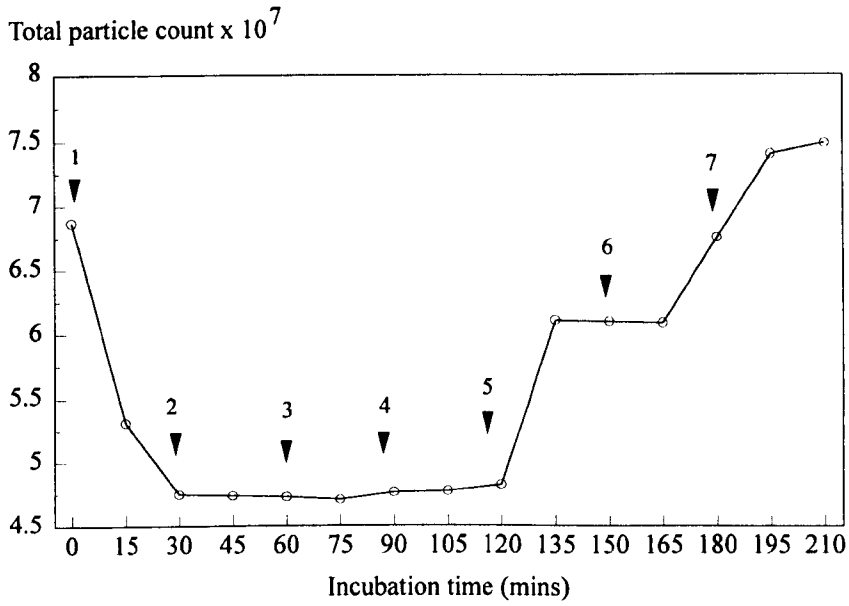
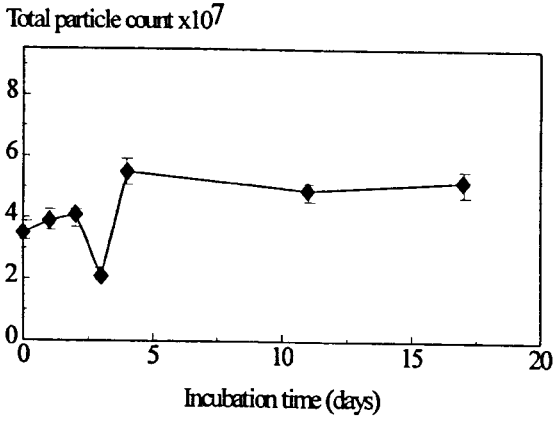
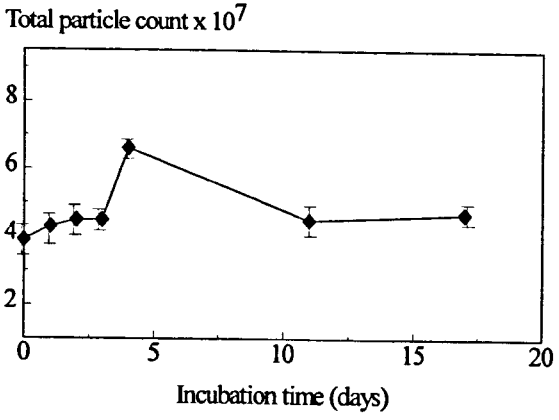


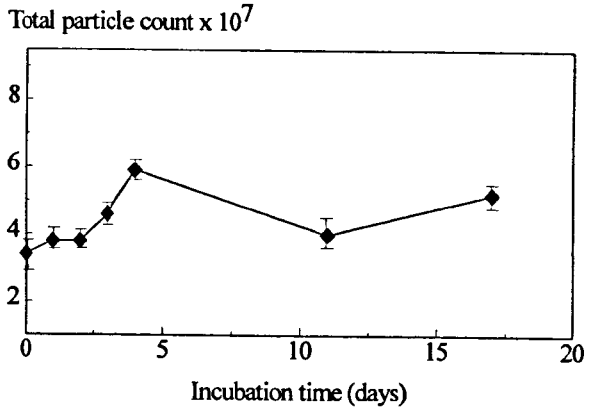
Figure 3.7. Cells were synchronised using the stationary phase method of Cutler and Evans (1966) and at the arrowed time points, cells were harvested and subjected to strict carbon-starvation.



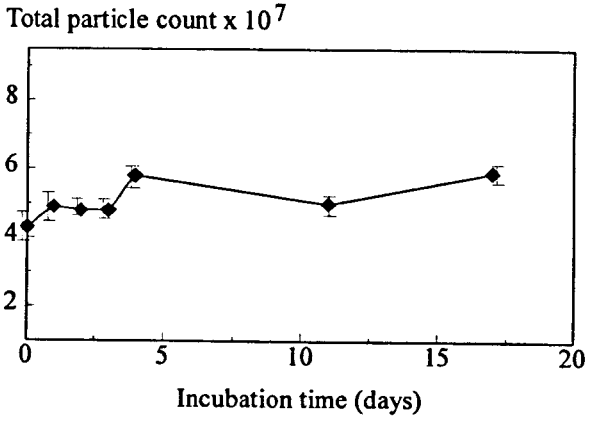
Flask 1



Flask 3



Flask 2



Flask 4

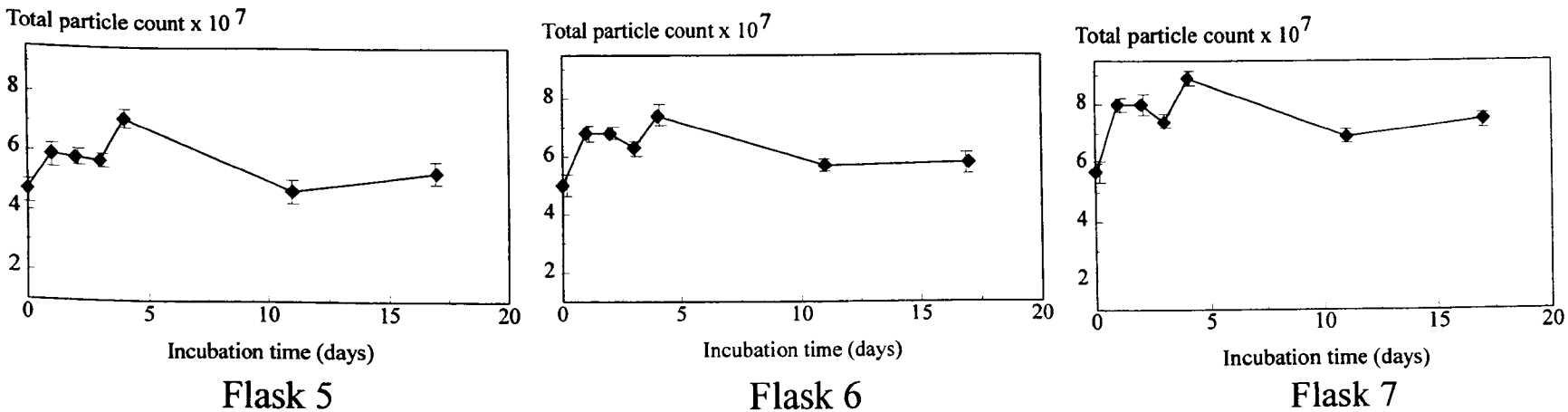


Figure 3.8. Survival of cells harvested from a synchronised culture, as determined by total particle count. Synchronised cells were inoculated into M9 medium with 0.5% (w/v) glucose . At 30 minute intervals (marked with arrows on Figure 3.7) cells were harvested and subjected to strict starvation for a period of 17 days. Synchronised cultures were set up in triplicate and three samples of cells from each were sampled at each time point. Values represent the mean value of nine samples. The error bars represent ± one standard deviation.

Although differences were apparent in the starvation-survival characteristics of cells sampled from a synchronised culture, no discernible pattern could be seen which related to the phase of the cell division cycle the cell was in when starvation was encountered. The differences seen in the behaviour of the starved populations (Figure 3.8) are more likely to be due to the effect of lag-phase on the cells. The increase in cell numbers seen by day 1 of starvation was noticeable in Flasks 5, 6 and 7 and suggests that the cells completed the cell cycle which they had embarked upon before responding to starvation conditions.

A major problem in this experiment was that partial synchrony and not full synchrony was achieved. The step-wise increases in cell numbers seen in Figure 3.7 do not represent a douling of cell numbers and the time between the step-wise increases is shorter than the doubling time expected in this medium. For this reason the results must be interpreted with care. The differences noted between the cells from each sampling point may due to the emergence of the cells from stationary- and lag-phase, rather than any differences in the point of the cell division cycle the cells had reached.

A second problem with this experiment was that. because only partial synchrony was achieved, synchronicity was lost very quickly, before rapid cell growth had begun. It could therefore be argued that true exponential growth was not occurring when the cells were subjected to strict starvation. This problem is inherent in most methods of obtaining synchronised cultures - synchronicity is lost after 2-3 cell division cycles. The method of Cutler and Evans (1966) supposedly gives good synchrony over 10 or more division cycles, as does the method of Kepes (1980) which relies on limitation of sulphur throughout numerous doublings before the addition of excess sulphur. In the present work sulphur limitation would have presented an additional stress, the avoidance of which was felt to be important. The success of the method of Cutler and Evans, however, depends greatly on the time of harvest of the cells. There is only a short period of time where cells will exhibit synchronicity in fresh medium. This suggests that

population synchrony is not vital in starvation-survival, but may be a function of it. In addition it suggests that, although a well-synchronised population can be obtained, in most cases the best which can be achieved is partial synchrony.

Overall it can be said that no evidence was found that the point of the cell division cycle the cell is in has any bearing on starvation survival. However the present experimental conditions dictated that, at most, two chromosome replication forks were present, since the doubling time of the culture was around 45 minutes. It is possible that this did not represent rapid enough growth to show any effect and that using cultures with much lower doubling times and, consequently a greater number of replication forks, would show a cell cycle-dependent survival pattern. In addition, inhibition of initiation in rapidly growing cells may be a useful method for investigating this hypothesis.

3.2.4. Conclusions

The results presented in this chapter demonstrate that although exponential cells are indeed more resistant to starvation for carbon and energy, this owes more to the rate of growth experienced by the cells than to the phase of growth the cells are in when starvation is encountered. However, even when the culture kinetics of starving exponential cells approximates that of starving stationary-phase cells, their survival rate is still reduced. These results suggest that the changes which take place at entry to stationary-phase and which enhance starvation-survival may take place to a greater extent when cells are growing extremely slowly, even if the cells are in exponential-phase.

In the natural environment, bacteria are more likely to encounter conditions which allow only very slow growth or mimic entry to the stationary-phase than they are to encounter sudden starvation after a surplus of nutrients. It is possible that a reduction in the growth rate triggers a response in the cell which results in a more resistant cell type. It has been shown that starvation-survival depends on the ability of the bacterium to carry out protein degradation and synthesis at the onset of starvation (Reeve *et al.*, 1984a, b). If cells encounter sudden starvation they may be unable to carry out the degradation of superfluous proteins and the synthesis of starvation-survival proteins before cellular carbon and energy reserves are exhausted. In rapidly growing cells, the chromosome can have multiple replication initiation sites and multiple replication forks. Before cell division ceases, the cell must complete all the rounds of chromosome replication which have been initiated. This will utilise a portion of the cell's energy resources, a commodity in short supply in carbon-starved cells. In addition, proteins involved in chromosome replication and cell division will still be required and will not be available for use as an amino acid source. The supply of amino acids at the onset of carbon-starvation has been shown to be extremely important in survival (Reeve *et al.*, 1984b; Bockman *et al.*, 1986). Another factor which may have a bearing on the increased sensitivity of exponential cells to starvation may be the

delay which would be experienced in initiation of transcription of the *rpoS* gene. It has been demonstrated that the *rpoS* gene product, σ^S, is required for the transcription of a large number of genes involved in resistance to many forms of stress (Loewen & Hengge-Aronis, 1994). σ^S is required for a cell size reduction which occurs in cultures with high cell density approaching stationary-phase (Lange & Hengge-Aronis, 1991a), suggesting that the changes which take place resulting in enhanced resistance may begin before stationary-phase is reached. Exponential-phase cells subjected to sudden, strict starvation will not have begun preparation for starvation conditions and will, in effect, lag behind stationary-phase cells during starvation-induced molecular realignment. In addition, the cell size reduction indicates that initiation of chromosome replication occurs at a lower cell mass than in exponential-phase cells, suggesting a modulation of the control mechanism which enables initiation to take place.

It is evident that cell death occurring in stationary-phase starved cells, is much less than that seen in exponential-phase starved cells. The natural synchrony seen as cells enter the stationary-phase suggests a cell-density-sensing mechanism by which cells within a culture receive the same signal at the same time. This type of regulatory system is seen in the *lux* operon of *Photobacterium fischeri* (Eberhard *et al.*, 1981). Homoserine lactone is excreted by the cells and when its concentration in the growth medium reaches a certain level it is taken up by the cells and acts as a positive regulator of the *lux* operon. This allows cells to detect the density of the population. There is evidence that homoserine lactone plays a role in the induction of the *rpoS* gene (Huisman & Kolter, 1994). The addition of exogenous homoserine lactone to *E. coli* cells deficient in threonine biosynthesis was shown to restore normal levels of σ^S expression. However, the authors also showed that σ^S expression occurred at normal levels in *E. coli* cultures which had a low cell density and suggested that two signalling pathways involving homoserine lactone were involved; one pathway which involves intracellular regulation by non-acylated homoserine lactone and a second which

involves acylation of the molecule to allow it to cross the cell membrane and become an intercellular signalling molecule. The intracellular signalling of starvation conditions is thought to involve the stringent response, stimulating the expression of the threonine biosynthetic pathway. Increased threonine levels in addition to slow translation would cause feedback inhibition and the accumulation of homoserine, an intermediate in the threonine biosynthetic pathway. For a review see Salmond *et al.* (1995). Further details of this proposed mechanism are discussed in Chapter 4, Section 4.2.1.

Chapter 4. Rates of macromolecular synthesis during starvation and on nutrient upshift.

4.1. Introduction

During vegetative growth bacteria continually synthesise macromolecules such as DNA, RNA and proteins. The replication of the bacterial chromosome is solely for the purpose of cell proliferation. RNA and protein synthesis, whilst being essential to supply the progeny with the necessary macromolecules, is also required for the parent cell to grow and remain a viable, vegetative cell. Messenger RNA molecules are degraded within the cell, the half-life depending on the specific messenger. The same can be said for many proteins, thus there is a constant turnover of these molecules. This allows the cell to control its metabolism by switching off the production of messenger RNA and thus protein synthesis. Preformed, active molecules already present will be degraded and will not continue to function. In addition, many enzymes are necessary only at specific times within the cell replication cycle and therefore will be synthesised or activated at certain stages and not at others. The degradation or inactivation of these molecules prevents inappropriate activity within the cell.

As cells enter the stationary-phase in batch culture, bulk protein synthesis is reduced (Reeve *et al.*, 1984b). However, novel proteins are synthesised which are not seen during vegetative growth. The synthesis of these proteins takes place over a period of 9 hours, after which no further novel protein synthesis is thought to occur. In the "non-differentiating" bacteria it has long been accepted that a turnover of macromolecules must take place during periods of non-growth. It has been reported that, using a labelling time of 2 days instead of five minutes, proteins can be labelled during starvation, suggesting that starving, "non-differentiating" cells do indeed continue to synthesise macromolecules, but at a much reduced rate (Rahman *et al.*, 1994). However, this may be a function of the labelling itself, rather that continuing protein synthesis.

During starvation, cells can be recovered from the medium on conventional plate culture, suggesting that, in "non-differentiating" organisms, there must be a turnover of macromolecules to maintain the energy balance of the

cell, the essential macromolecular components and functions, e.g. proton motive force and DNA supercoiling. This view is based on the belief that "non-differentiating" bacteria do not have a specific form of survival cell. In the spore-forming bacteria such as *Bacillus* species, no metabolic activity can be detected in the endospore and it is, in effect, in limbo. However, the *Bacillus* endospore contains mRNA species not found in vegetative cells. This mRNA is used for protein synthesis once germination has begun, relieving the awakening cell of the additional energy and substrate burden of producing its own messenger.

Protein synthesis in swarmer cells of *R. vannielii* and *C. crescentus* continues, but at a much reduced rate and the pattern of proteins synthesised is different from that seen in vegetative cells (Milhausen & Agabian, 1981; Dow *et al.*, 1983) . DNA synthesis does not occur in swarmer cells and it has been shown that no ribosomal RNA synthesis occurs (Swoboda *et al.*, 1982a). The same is true for non-differentiating marine *Vibrio* species (see Section 1.4). Similar macromolecular synthesis pattern are seen in survival cells of other differentiating bacteria (see Sections 1.2.2 and 1.2.3).

Aims

The aims of the following experiments were to compare the rates of synthesis of the macromolecules during active growth and long-term starvation in *E. coli* K12 in order to determine if macromolecular synthesis does indeed take place during long-term starvation and to compare the findings with the known macromolecular synthesis patterns of differentiating organisms.

4.2. Results and discussion

4.2.1. Protein synthesis

The rate of protein synthesis was determined for batch cultures from lag phase to stationary phase. Cells grown in 0.05% (w/v) glucose were labelled with ^{35}S-methionine for 5 minutes then the TCA-precipitable material was collected and isotope incorporation assessed using liquid scintillation counting, as described in Section 2.9.1. Experiments with longer labelling times demonstrated that further incorporation took place suggesting that the amount of radio-label was not exhausted within 5 minutes and therefore any differences found would be due to differences the rate of isotope incorporation (data not shown). Figure 4.1 shows the amount of isotope incorporation per cell during the growth curve and the total cell count at each sampling time.

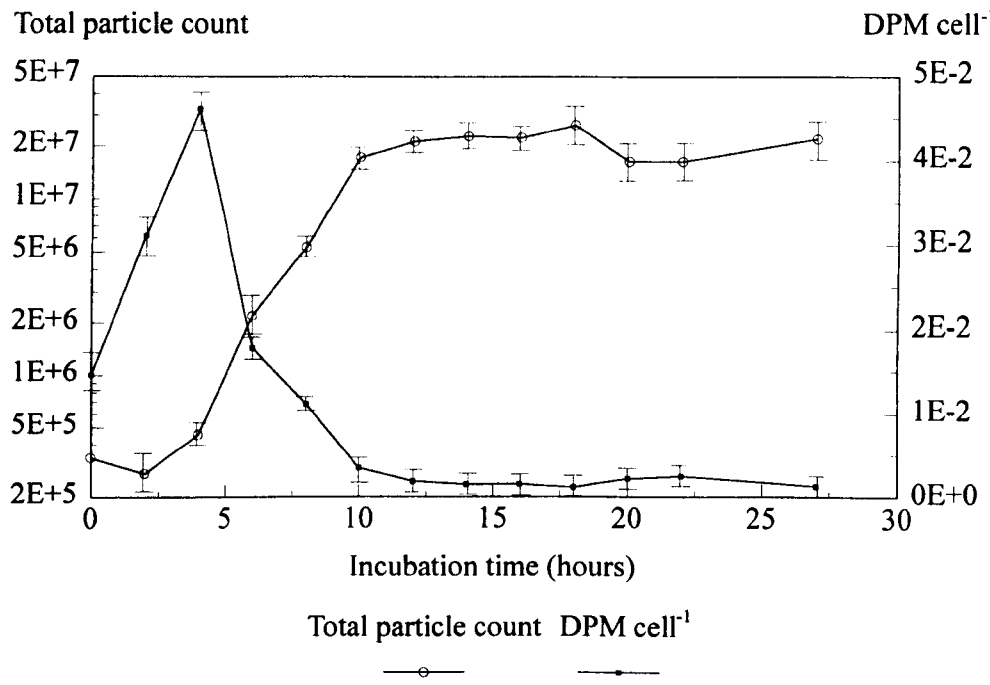


Figure 4.1. The relationship between the growth curve and ^{35}S-methionine incorporation cell^{-1}. Three separate batch cultures were grown in M9 minimal medium with 0.05% (w/v) glucose. Triplicate samples were removed during growth and radio-labelled with 10μCi ^{35}S-methionine ml^{-1}. At the same time total particle counts were carried out using a Coulter Counter and the rate of isotope incorporation cell^{-1} was calculated and expressed as disintegrations per minute (DPM). The values plotted represent the mean of nine values and the error bars represent ± one standard deviation.

This indicates that the maximum rate of isotope incorporation and thus protein synthesis occurs in the lag phase, just before exponential growth begins. This can be explained by considering that cells in the lag phase have to adjust to the new conditions in which they find themselves. In the present experiments all batch cultures were kept as similar as possible, bearing in mind the individuality of batch cultures. All inocula for the flasks in these experiments were 24 hour-incubated cultures of *E. coli* K12 inoculated into the same batch of pre-warmed M9 medium, thus cells were in stationary-phase when inoculated into the flasks and would have shut down much of their metabolic activity, with the subsequent degradation and inactivation of enzymes required for growth. Previous experiments had shown that, under these conditions, cultures reached the stationary phase in 9 hours. To allow vegetative growth to resume, degraded enzymes must be resynthesised. Once adequate concentrations of enzymes have been achieved during the lag-phase, the cell cycle resumes and cells must synthesise adequate proteins to replace the enzymes which are degraded as part of the cellular control mechanisms and to supply the progeny with adequate molecules to ensure continued metabolism until the progeny are able to synthesise adequate concentrations themselves.

The rate of isotope incorporation in cells incubated over 61 days and a long-term-starved cultures (24 months) were determined as described in Section 2.9.1. Three samples were labelled in triplicate and the results obtained are shown in Table 4.1. Boiled, 2 day-incubated cells were used as a control. It can be seen that, when calculated using total particle counts, the rate of isotope incorporation $cell^{-1}$ is very low, but is greater than that of the control cells. When calculated using viable counts, the rate of isotope incorporation shows an increase as the incubation continues. The 24 month-starved culture has an isotope incorporation rate lower than that of the control cells when determined by total particle counts, but when determined by viable counts, the rate of isotope incorporation is very high.

Table 4.1. Rate of protein synthesis as determined by the rate of isotope incorporation

Incubation time	Total particle count	dpm cell^{-1} (determined by total particle count)	Viable count	dpm cell^{-1} (determined by viable count)
2 days	$8.7x10^{8}$	$5.20x10^{-4}$ (1.2)	$8.7x10^{8}$	$3.98x10^{-4}$ (2.1)
8 days	$7.1x10^{8}$	$2.36x10^{-5}$ (0.9)	$6.3x10^{9}$	$2.64x10^{-6}$ (2.4)
13 days	$6.4x10^{8}$	$6.80x10^{-5}$ (1.8)	$5.3x10^{8}$	$8.24x10^{-5}$ (1.9)
29 days	$8.7x10^{8}$	$2.24x10^{-5}$ (1.7)	$2.5x10^{8}$	$7.72x10^{-5}$ (3.7)
47 days	$4.4x10^{8}$	$4.00x10^{-5}$ (2.4)	$3.8x10^{6}$	$4.60x10^{-3}$ (2.6)
61 days	$4.5x10^{8}$	$5.56x10^{-5}$ (1.3)	$9.6x10^{5}$	$2.60x10^{-2}$ (1.6)
24 months	$1.05x^{9}$	$1.17x10^{-5}$ (3.5)	$3.4x10^{5}$	$3.60x10^{-2}$ (4.1)
boiled cells		$1.41x10^{-5}$ (2.0)		

^{35}S-methionine incorporation during prolonged incubation. Triplicate samples from three cultures were radio-labelled with ^{35}S-methionine, (10μCi ml^{-1}) for 5 minutes. TCA-insoluble material was collected on Whatman GF/F glass fibre filter discs and the amount of isotope incorporation determined using a Beckman scintillation counter, as described in Section 2.9.1. Total particle and viable counts were determined as described in Section 2.3.1 and 2.3.2. The data represent the mean of nine samples and standard deviations, given in brackets, are expressed as a percentage of the mean.

The results seem to indicate that, when calculated using viable cell count ml^{-1}, the rate of isotope incorporation increased as starvation continued, but when calculated using total particle counts ml^{-1}, the rate of isotope incorporation decreased. The results obtained for long-term-starved cells suggest that, when calculated using viable cell count ml^{-1} the rate of isotope incorporation, and consequently protein synthesis, was higher than in early stationary phase. To resolve this difference it was necessary to investigate the isotope-labelling pattern of proteins in long-term-starved cells.

In order to examine the pattern of protein synthesis during long-term starvation it was necessary to determine the optimum radio-labelling time which would allow visualisation of the proteins by SDS-PAGE and autoradiography. In batch cultures, radio-labelling of proteins currently being synthesised can be carried out using a 5 minute labelling time with ^{35}S-methionine. However, in starving cells metabolism is much slower. It was thought that if protein synthesis in starving cells was carried out at a very low level, it may prove very difficult to incorporate enough radio-isotope to be able to visualise bands on SDS-PAGE or spots on two-dimensional electrophoresis gels. It has been reported that viable but non-culturable cells of *Shigella dysenteriae* were shown to incorporate methionine after 2 days incubation with 1μCi ml^{-1} (Rahman *et al.*, 1994). However, concerns about the effect of the radio-labelling time itself on the pattern of protein synthesis led to the investigation of the optimum labelling time in long-term-starved *E. coli* cells.

A. 18 month-starved *E. coli* K12 culture was harvested and resuspended in 1/10 of the supernatant from the centrifugation. ^{35}S-methionine was added at 280μCi ml^{-1} and at various time intervals a 0.5ml sample was removed and prepared for SDS PAGE, as described in Section 2.5.1.1. It was found that proteins from non-starving cells were sufficiently radio-labelled using 15μCi ^{35}S-methionine for 5-10 minutes to allow visualisation of proteins separated by one- and two-dimensional PAGE (data not shown). In long-term-starved cells,

however, it was found that this labelling regime resulted in no visible bands or spots on one-dimensional SDS-PAGE and two-dimensional electrophoresis respectively (data not shown). By experimenting with varying concentrations of ^{35}S-methionine it was found that protein labelling could be achieved at 280μCi ml^{-1}. The samples were analysed using SDS-PAGE on 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels as described in Section 2.5.2. Each track was loaded with the same volume of sample, 20μl, so that comparisons could be made of the amount of radio-label in each band and thus the rate of protein synthesis could be compared for each band. The gel was subjected to autoradiography as described in Section 2.5.8. Faint bands were visible at 30 minutes radio-labelling time (Figure 4.2) indicating that the rate of protein synthesis in long-term-starved cells is much slower. In addition, however, starvation-induced alterations in the composition of the outer membrane may cause a much reduced rate of methionine uptake (See Chapter 5). Incorporation of ^{35}S-methionine into proteins of viable but non-culturable *Sh. dysenteriae* was found to take 2 days when isotope was provided at 1μCi ml^{-1} (Rahman *et al.*, 1994). In the present experiments the incorporation was much more rapid. This apparent contradiction may be explained by comparing the conditions the experiments utilised. Rahman *et al* (1994) generated viable non-culturable *Sh. dysenteriae* by inoculation of stationary-phase cells grown in a rich medium (trypticase soy broth) into water which constitutes an osmotic shock and may have caused disruption of the normal function of the membrane transport systems. In the present experiments cultures were starved by continuous incubation in glucose-limited M9 medium and did not become wholly non-culturable - after 18 months starvation 1 in 10 cells showed the ability to form colonies on starvation media. The finding of Rahman *et al* (1994) that stationary-phase cells freshly inoculated into water were less able to incorporate ^{35}S-methionine than cells which had been incubated in water for 4-8 weeks supports the theory that the rapid transfer to water may have caused damage to

the cells. If this is the case, then leakage of cell contents, whether from the periplasm or cytoplasm, may have allowed less damaged cells within the population to repair gradually some of the damage done and this may explain the increase in isotope incorporation seen as incubation in water continued. In addition the amount of ^{35}S-methionine used in the present experiments was much higher than that used by Rahman *et al* (1994).

As the radio-labelling time increased, the bands seen on autoradiography became more numerous and more intense, as had been expected. This can be explained by two mechanisms; the very low rate of protein synthesis in long-term-starved cells means that isotope incorporation is very slow; alternatively, the presence of methionine may have affected the rate of protein synthesis, increasing it by, in effect, serving as a nutrient source for the cells. On closer examination, it was seen that two protein bands showed a different pattern of isotope labelling and protein synthesis (arrowed in Figure 4.2). These proteins, with molecular weights of 47.5 and 82.5kDa, could be seen in those samples labelled for 30 minutes to 1 hour. At 2 hours radio-labelling time, these proteins could no longer be seen suggesting that they were no longer being synthesised. Indeed, since this experiment employed continuous labelling, the proteins must have been degraded, suggesting a breakdown mechanism operating to remove them from the cells. This suggests that the labelling time alone is not responsible for the heavier labelling of the proteins, but that the presence of the methionine is altering the metabolism of the cells and switching on previously shutdown protein synthesis. This suggests that both mechanisms suggested above are operating.

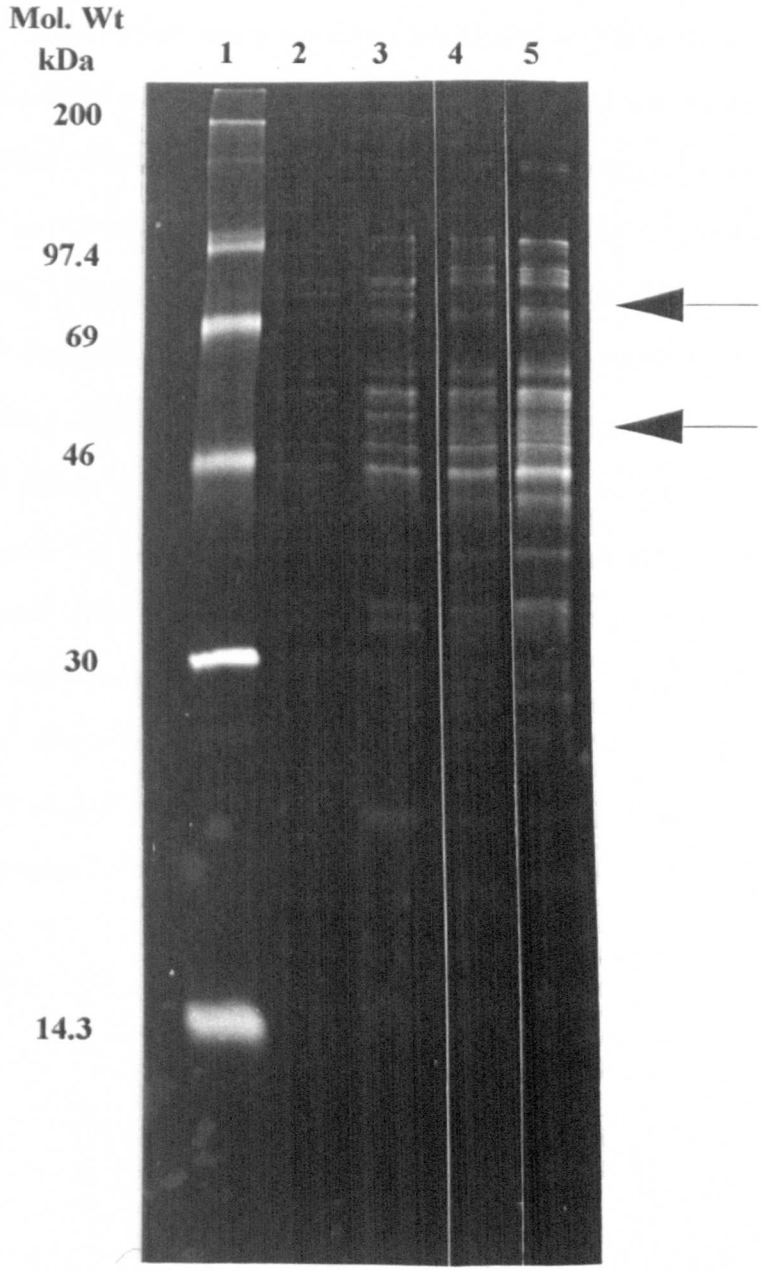


Figure 4.2. Autoradiograph showing the effect of radio-labelling time on the efficiency of radio-labelling of proteins using ^{35}S-methionine and the effect on the protein synthesis profile in 18 month-starved *E. coli*. Cells were continuously radio-labelled for 3 hours with ^{35}S-methionine, (280μCi ml^{-1}). Samples were removed at various time points, incubated at 100°C for 5 minutes in 6x sample buffer, as described in Section 2.5.1.1 and the proteins separated by SDS-PAGE using a 12% (w/v) resolving gel and 4.5% (w/v) stacking gel. 70μl of sample were loaded per track. Track 1 - molecular weight markers; track 2 - 30 minutes; track 3 - 1hr; track 4 - 2hr; track 5 - 3 hr.

To determine how soon after the addition of ^{35}S-methionine the synthesis of the 82.5kDa protein began, the previous experiment was repeated, but samples were taken from 5 minutes radio-labelling time onwards. It was found that at 5 and 10 minutes no bands were visible, suggesting that no protein synthesis was occurring (Figure 4.3). The autoradiograph obtained was scanned using a Hewlett Packard monochrome Scanjet and densitometry was carried out using QuantiScan software (Microbial Systems Ltd, marketed by Biosoft). This showed that peaks were present in the 5 and 10 minute-labelled samples which corresponded in position with bands in longer-labelled samples (Figure 4.4). By 15 minutes faint bands could be seen. The peaks found using densitometry corresponded in position to the peaks which were visible in longer-labelled samples. Densitometry also revealed that both the 82.5 and 47.5 kDa proteins were still present, but their levels were much reduced with respect to the levels of other proteins being synthesised.

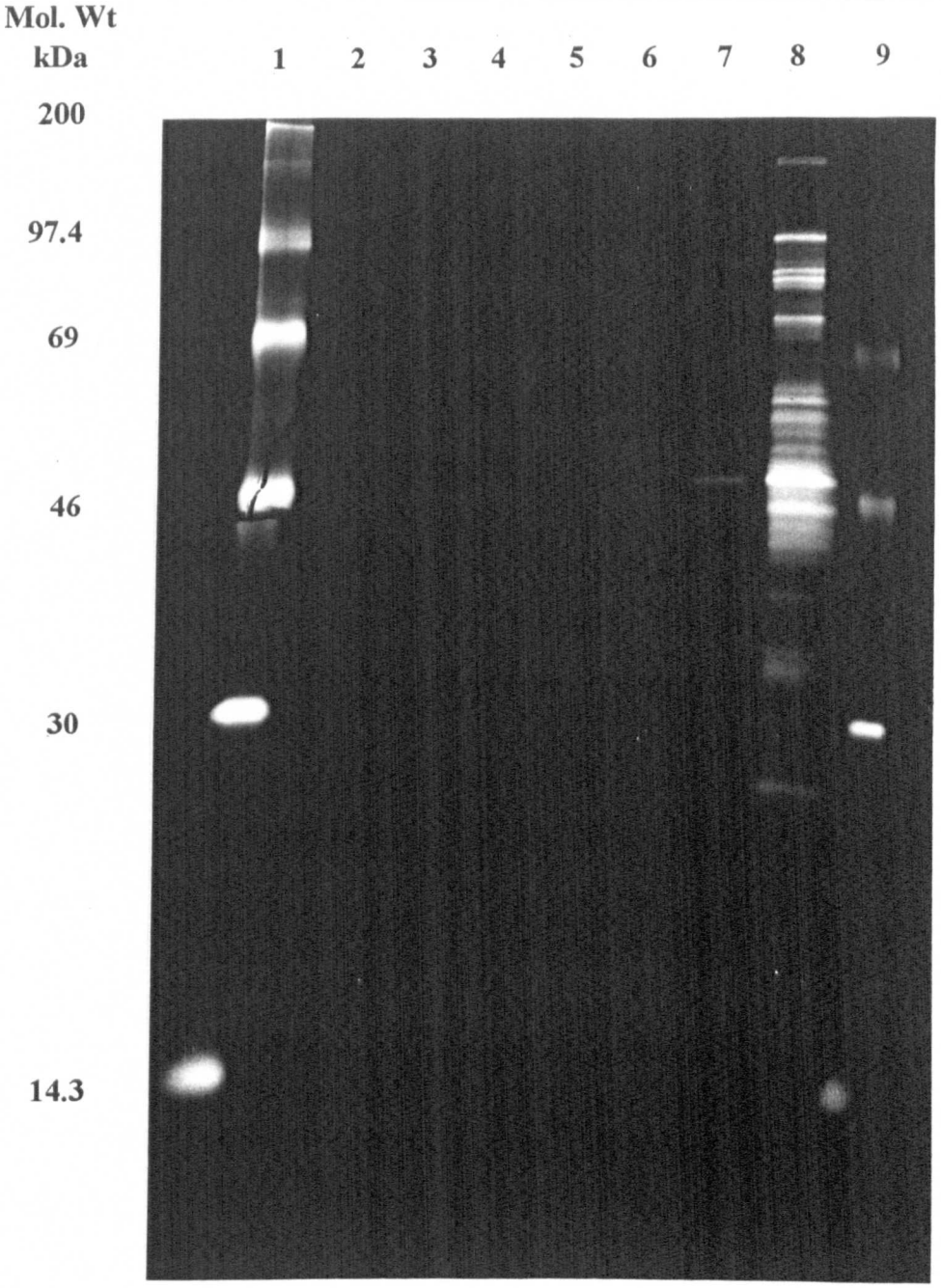
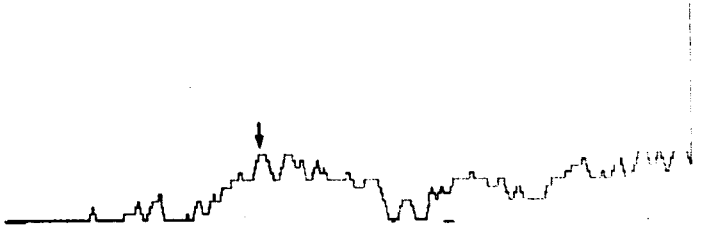


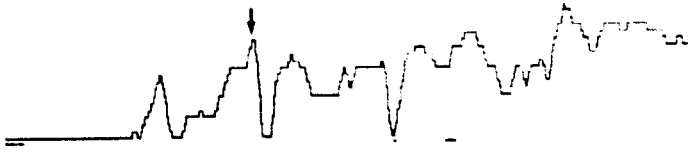
Figure 4.3. 18 month-starved cells were radio-labelled with ^{35}S-methionine (280μCi ml^{-1}). Samples were removed at various times after the addition of radio-label and proteins separated by SDS-PAGE on 12% (w/v) resolving gels with 4.5% stacking gels. Track 1-molecular weight markers; track 2 - 5 min; track 3 - 10 min, track 4 - 15 min; track 5 - 20 min, track 6 - 30 min, track 7 - 1 hr, track 8 - 2 hr, track 9 - molecular weight markers. 20μl were loaded per track. Densitometry was carried out on samples and the resulting profiles are shown in Figure 4.4.

Track 1

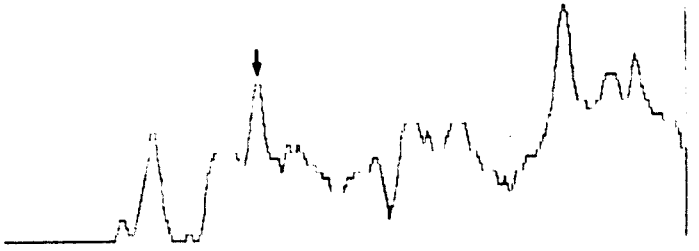
Track 2



Track 3



Track 4



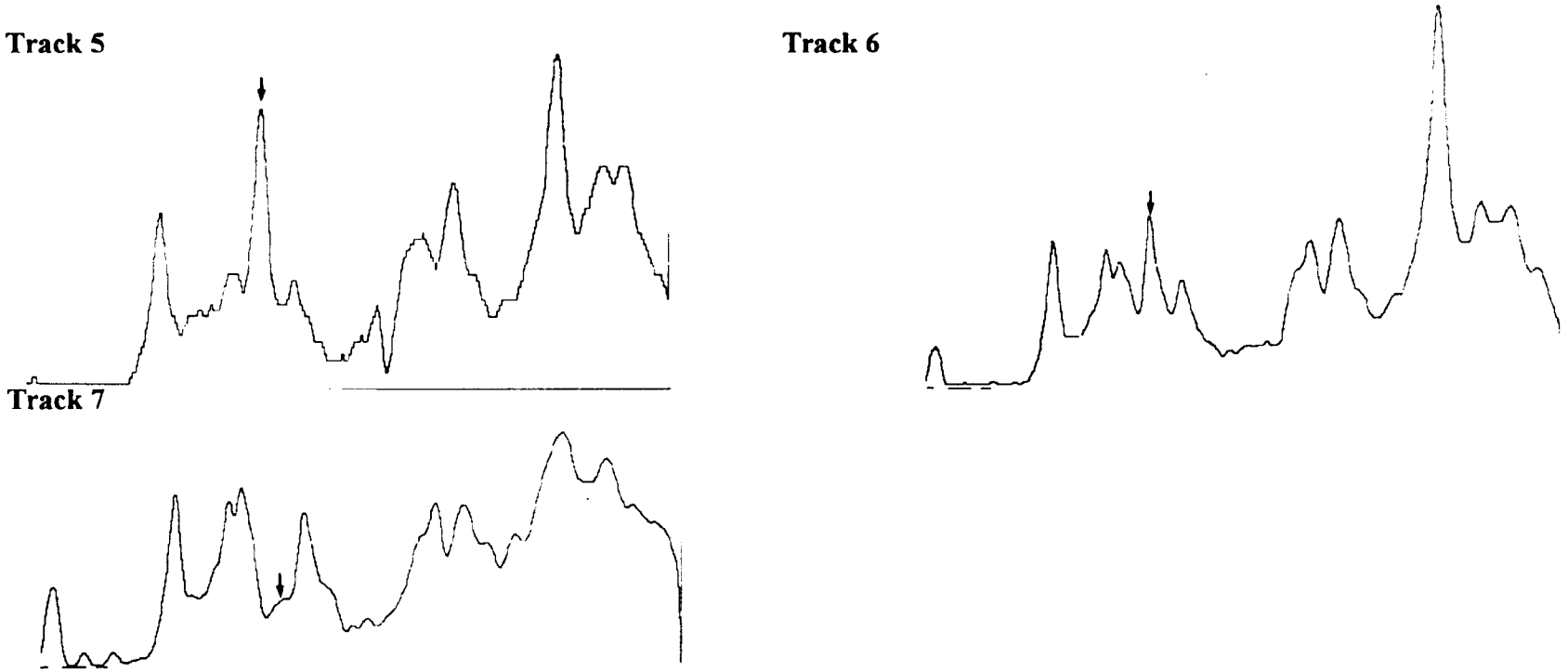


Figure 4.4. Long-term-starved cells were radio-labelled with 35S-methionine (280μCi ml^{-1}) and samples were removed at various times then separated by SDS-PAGE on 12% (w/v) resolving gel with 4.5% (w/v) stacking gels. After autoradiography, the resulting image was scanned and analysed by densitometry. The peak corresponding to the 82.5 kDa protein is marked with an arrow. Track 1 - 5 minute label, track 2 - 10 minutes, track 3 - 15 minutes, track 4 - 30 minutes, track 5 - 1hr, track 6 - 2hrs, track 7 - 3hrs.

These results suggest that the calculation of the rate of isotope incorporation cell^{-1} (Table 4.1) was more accurate when the total particle count was used, particularly when long-term-starved cells were examined. The results in Table 4.1 indicate that the amount of isotope incorporation cell^{-1} decreased during starvation when total particle count was used, but increased when viable count was used. The autoradiograph (Figures 4.2 and 4.3) show that very little isotope was incorporated into protein the first ten minutes of ^{35}S-methionine labelling and was so low as to be unquantifiable.

To determine if vegetative growth resumed in the presence of methionine, viable and total counts were carried out as described in Sections 2.3.1 and 2.3.2 on cells supplied with unlabelled 0.3μM methionine. This demonstrated that methionine did indeed allow vegetative growth to resume (Figure 4.5) and represented a nutrient upshift. During feeding of cells with unlabelled methionine it was not possible to radio-label cells with ^{35}S-methionine. Consequently it was decided to use ^{3}H-leucine to pulse-label methionine-fed long-term-starved cells. It was found that, although vegetative growth resumed, very little isotope incorporation took place, suggesting that the cells synthesised their own leucine in the preference of an exogenous nutrient/amino acid source (Figure 4.6). After 3.5 hours radio-labelling, one band became visible and by 5 hours 2 bands were evident, neither of which were as apparent when cells were radio-labelled with ^{35}S-methionine alone (Figure 4.2). To determine if leucine was unable to enter the cells they were continuously labelled with ^{3}H-leucine without methionine feeding. It was found that, although leucine did enter the cells, the pattern of protein synthesis was different from that found with continuous labelling with ^{35}S-methionine (Figure 4.7). Although there were common proteins synthesised under both regimes, there was no evidence of any alteration in the pattern of protein synthesis during the labelling time. As the labelling time increased, the bands seen on the autoradiograph became more intense, as had been seen with ^{35}S-methionine, but the 47.5kDa protein band was

present throughout the labelling time. The two proteins shown in Figure 4.6 were found to be labelled much sooner than when cells had been supplemented with unlabelled methionine. These proteins had molecular weights of 34 and 39 kDa. This suggests that, although leucine was taken up and incorporated into proteins, it did not elicit the same response as methionine and that in the presence of methionine, ^{3}H-leucine incorporation into protein was much lower than when leucine was the sole amino acid provided.

The difference in the response to methionine and leucine may be due to the biosynthetic pathways involved in the synthesis of these amino acids. Methionine is synthesised from oxaloacetate while leucine is synthesised from pyruvate via a completely separate pathway. It is interesting to note that the pathway which gives rise to methionine synthesis also results in the synthesis of threonine and homoserine lactone as shown in Figure 4.8. From homoserine the pathway branches to either threonine or methionine synthesis and is under strict control of methionine. The biosynthesis of methionine is prevented in the presence of exogenous methionine by repression of the synthesis of the *metL* gene product (Cohen & Saint-Girons, 1987). By blocking the pathway homoserine lactone production will be prevented and will result in the repression of σ^{S} (Huisman & Kolter, 1994) which is responsible for the starvation survival response of carbon-starved cells. A reduction in the amount of σ^{S} will prevent transcription from positively regulated genes and may induce transcription from genes required for growth and negatively regulated by σ^{S}. The biosynthesis of leucine occurs by a separate pathway and should have no effect on the synthesis of homoserine lactone and subsequent σ^{S} levels within the cell.

It is possible that the 47.5kDa protein is some form of repressor which prevents the resumption of cell division, since it's disappearance at 2 hours exposure to methionine coincided with an increase in total particle and viable counts. Since methionine is the first amino-acid incorporated into proteins during synthesis it is plausible to hypothesise a repressor system which is de-

repressed by the presence of methionine, but not leucine. This would explain the differences seen in the pattern of protein synthesis when these amino-acids are supplied to starving cells.

The results suggest that great care must be taken when designing and interpreting radio-isotope incorporation studies in long-term-starved cells. In the light of these results, the experiments to determine the rate of protein synthesis during prolonged incubation and in long-term-starved cells were repeated using ^{3}H-leucine. Table 4.2 shows the results obtained.

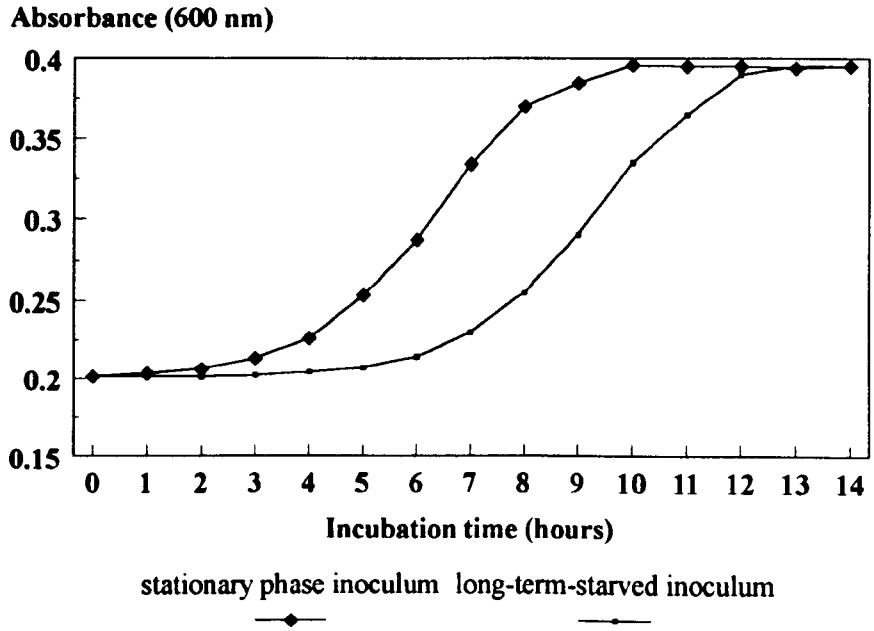


Figure 4.5. The growth curve of 24 hour-incubated and long-term-starved cells when inoculated into fresh M9 medium with 0.3μM methionine. At hourly intervals, samples were removed and the absorbance at 600nm was measured as described in Section 2.1.4.

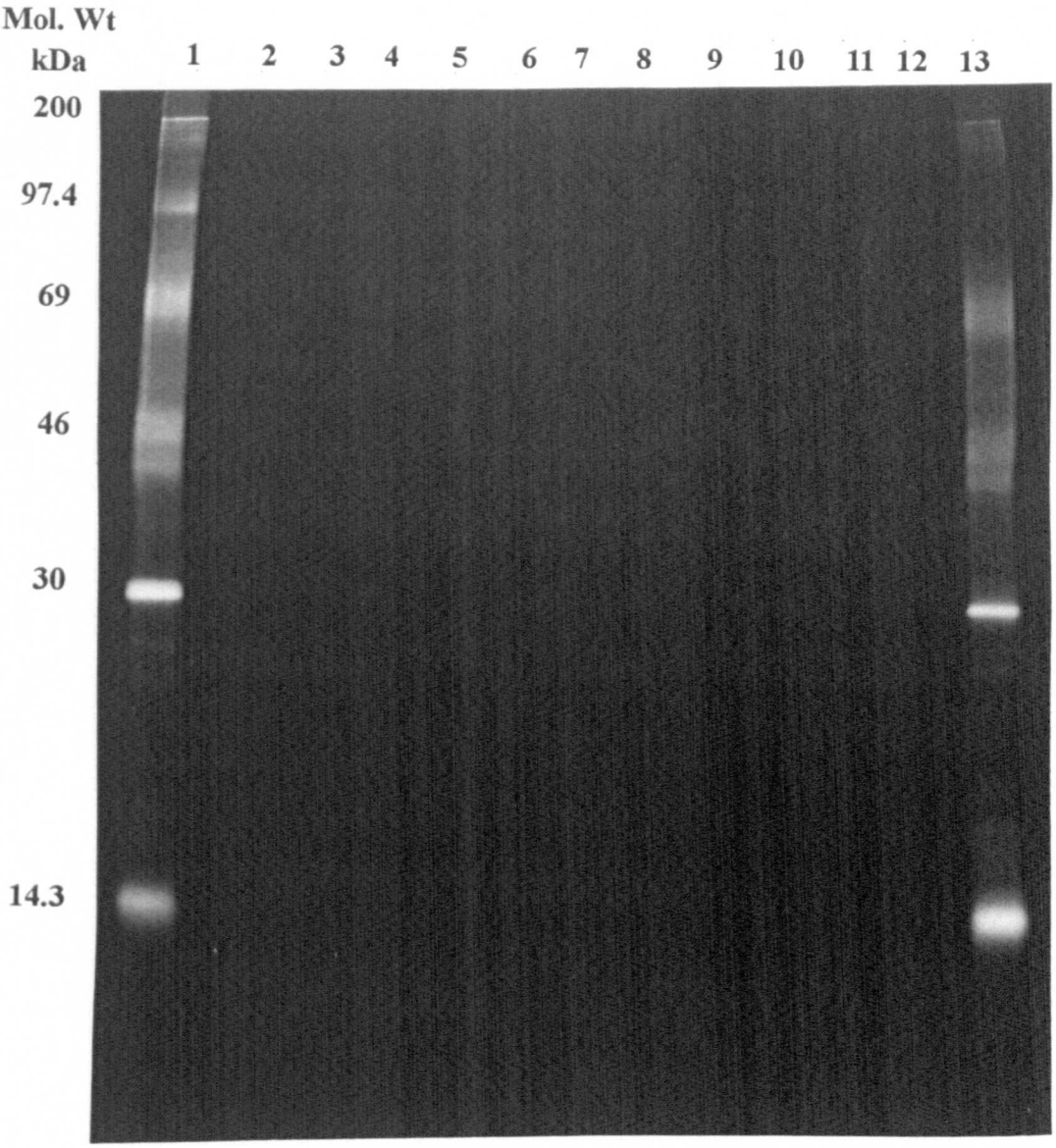


Figure 4.6. 18 month-starved cells were supplied with methionine at a final concentration of 0.3μM and 100μCi ml^{-1} ^{3}H-leucine. Samples were removed at various times and the proteins separated by SDS-PAGE on 12% (w/v) resolving gels with 4.5% (w/v) stacking gelsthen visualised by autoradiography. 70μl of sample were loaded per track. Track 1 and 13 - molecular weight markers, track 2 - 0hrs, track 3 - 0.5 hr, track 4 - 1hr, track 5 - 1.5hrs, track 6 - 2hrs, track 7 - 2.5hrs, track 8 - 3hrs, track 9 - 3.5hrs, track 10 - 4hrs, track 11 - 4.5hrs, track 12 - 5hrs. 20μl of sample were loaded per track.

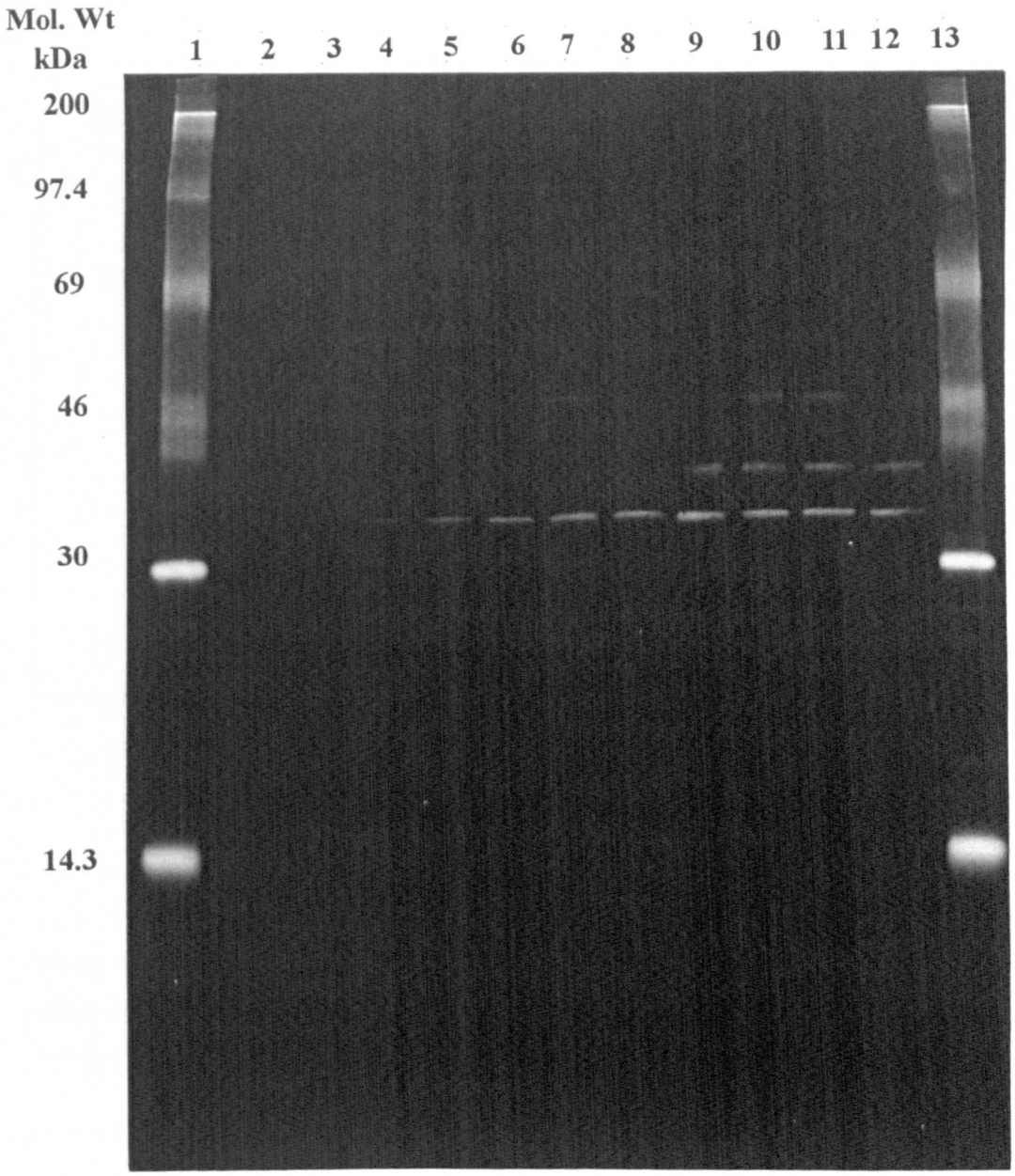


Figure 4.7. 18 month-starved cells were radio-labelled with 100μCi ml^{-1} ^{3}H-leucine. Samples were removed at various time intervals and the proteins separated by SDS-PAGE on 12% (w/v) resolving gels with 4.5% (w/v) stacking gels then visualised by autoradiography. 70μl of sample were loaded per track. Track 1 and 13 - molecular weight markers, track 2 - time 0, track 3 - 0.5hrs, track 4 - 1hr, track 5 - 1.5hrs, track 6 - 2hrs, track 7 - 2.5hrs, track 8 - 3hrs, track 9 - 3.5hrs, track 10 - 4hrs, track 11 - 4.5hrs, track 12 - 5hrs.

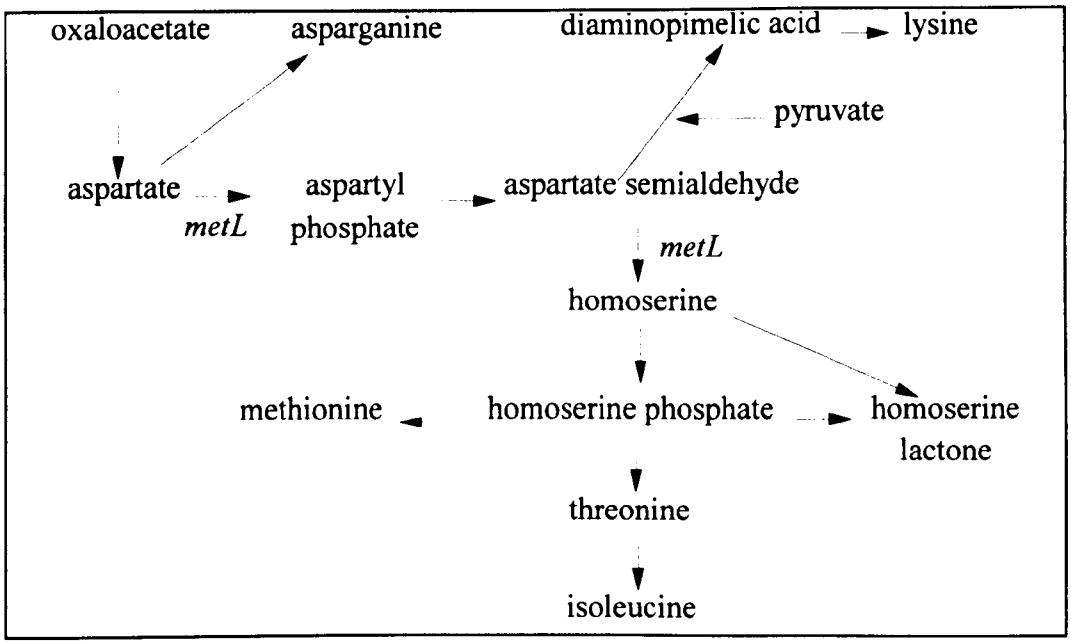


Figure 4.8. Diagrammatic representation of the methionine and threonine biosynthetic pathway indicating the proposed pathway of homoserine lactone production. (Adapted from Huisman & Kolter, 1994; Cohen & Saint-Girons, 1987)

TABLE 4.2. ^{3}H-leucine incorporation during prolonged incubation

Incubation time	dpm cell^{-1} as determined by total particle count	dpm cell^{-1} as determined by viable count
2 days	4.54×10^{-4} (3.1)	4.38×10^{-4} (2.2)
8 days	9.08×10^{-6} (2.6)	1.02×10^{-6} (4.7)
13 days	8.3×10^{-6} (4.5)	1.00×10^{-5} (3.3)
29 days	7.76×10^{-6}(2.3)	2.68×10^{-5} (5.3)
47 days	1.45×10^{-5} (8.6)	1.68×10^{-3} (4.8)
61 days	8.26×10^{-6} (4.4)	3.72×10^{-3} (1.1)
24 months	1.06×10^{-5} (2.8)	4.00×10^{-2} (3.4)
boiled cells	9.35×10^{-6} (1.2)	

^{3}H-leucine incorporation during prolonged incubation. Triplicate samples from each culture were radio-labelled with ^{3}H-leucine (10μCi ml^{-1}) for 5 minutes. TCA-insoluble material was collected on Whatman GF/F glass fibre filter discs and the amount of isotope incorporation determined by liquid scintillation counting. The data represent the mean value and the standard deviation, in brackets, is expressed as a percentage of the mean.

The results for ^{3}H-leucine incorporation followed the same pattern as those for ^{35}S-methionine incorporation. Since the amount of isotope incorporation, as demonstrated by SDS-PAGE, was very low it can again be assumed that the data obtained using total particle counts were more accurate.

In order to compare the protein synthesis pattern of long-term-starved cells during nutrient upshift with the total protein profile of the cells, a long-term-starved culture was radio-labelled with ^{35}S-methionine (280μCi ml^{-1}) and proteins were prepared as described in Section 2.5.1.1. The proteins were separated by SDS-PAGE on 12% (w/v) resolving gels with 4.5% (w/v) stacking gels as described in Section 2.5.2. The gel was silver stained using the method of Wray (Section 2.5.4) then dried and autoradiography was carried out as described in Section 2.5.8.

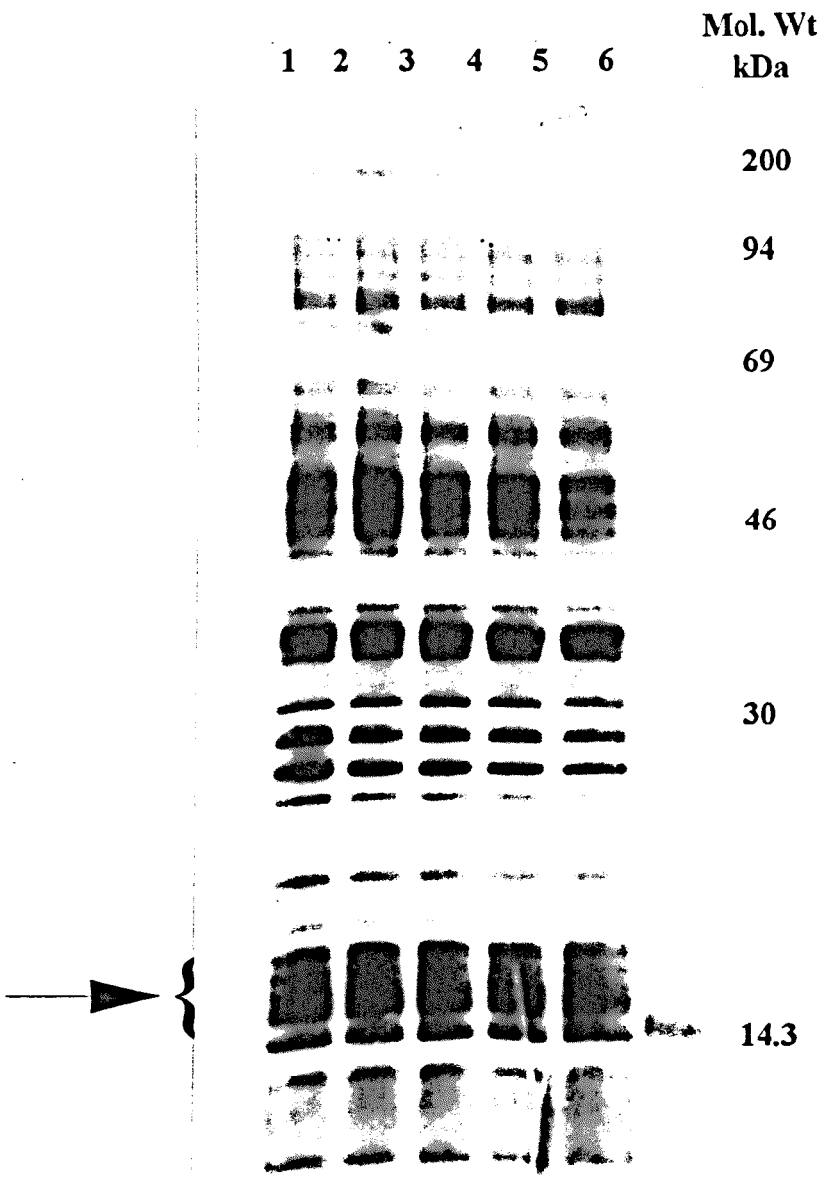


Figure 4.9 (A). Long-term-starved cells were labelled with ^{35}S-methionine (280μCi ml^{-1}). At various time intervals samples were removed and the proteins separated by SDS-PAGE on 12% (w/v) resolving gels with 4.5% (w/v) stacking gels. 20μl of sample were loaded per track. The gel was silver-stained to allow visualisation of the proteins. The gel was then dried and autoradiography was carried out (Figure 4.9 (B)). Track 1 - time 0, track 2 - 1hr, track 3 - 2hr, track 4 - 3hrs, track 5 - 4hrs, track 6 - molecular weight markers.

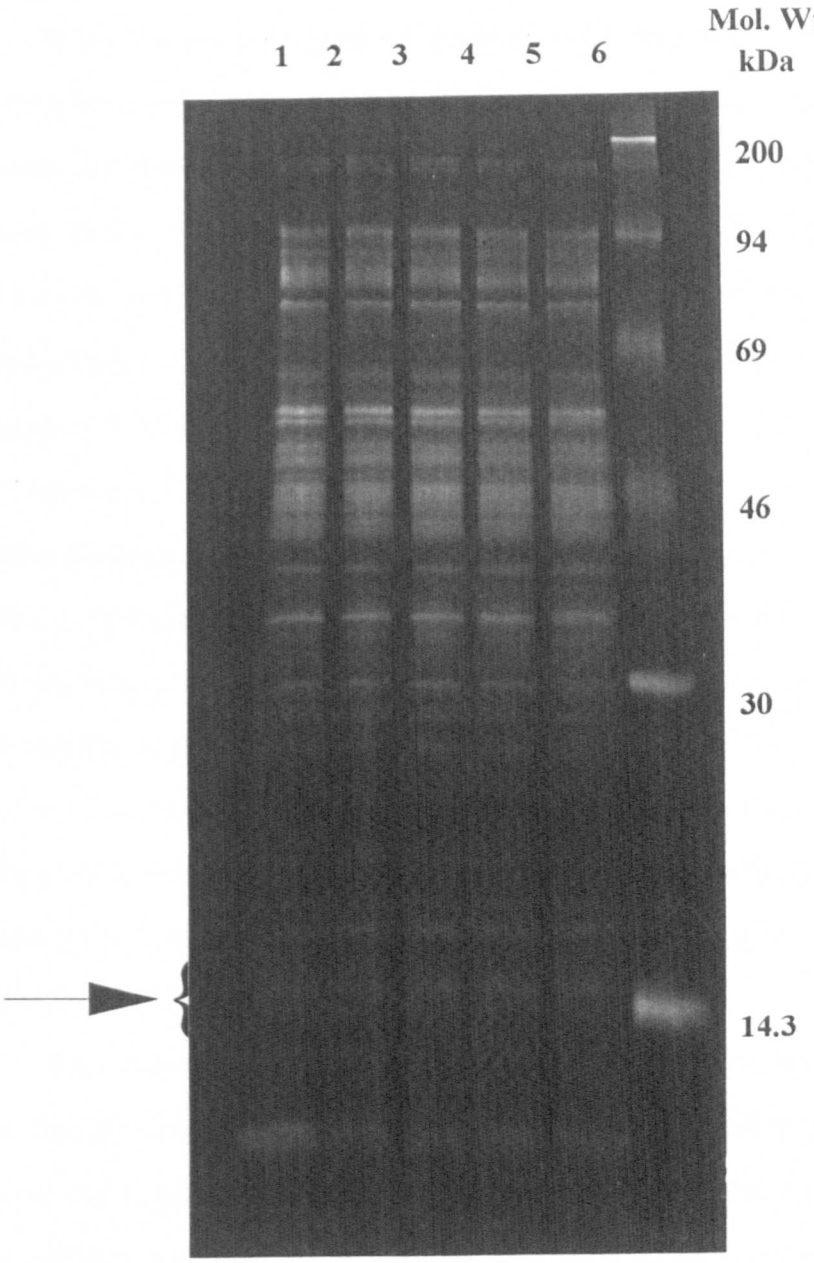


Figure 4.9 (B). Autoradiograph obtained from the silver-stained gel shown in Figure 4.9 (A). Track 1 - time 0, track 2 - 1hr, track 3 - 2hrs, track 4 - 3hrs, track 5 - 4hrs, track 6 - molecular weight markers.

When the autoradiograph of proteins being synthesised (Figure 4.9(B)) was compared with the silver-stained profile of proteins present (Figure 4.9(A)) it was seen that there were many proteins present in long-term-starved cells which were not synthesised until nutrient upshift had begun, (arrowed in Figure 4.9 (A)). This suggests that they are very stable proteins. Although quantification of silver stained proteins is not possible, it does appear that the smaller proteins, (arrowed in Figure 4.9 (A)), were present in relatively high amounts, yet their synthesis levels were low. To determine the location of these proteins - outer-membrane, cytoplasmic-membrane or cytoplasm - samples were prepared as described in Section 2.5.1.2 and 2.5.1.3 and run on 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels as described in Section 2.5.2. Proteins were visualised by silver staining as described in Section 2.5.4 (Figure 4.10). It was found that the major outer membrane proteins were present, but not synthesised. Of the small proteins, most were found in the outer membrane fraction, with trace amounts in the soluble protein and cytoplasmic membrane fractions, suggesting that, during starvation, outer membrane proteins are not synthesised.

Since one-dimensional SDS-PAGE can result in more than one protein being present in one band, two-dimensional electrophoresis was carried out on the radio-labelled samples (Section 2.5.3) to determine if the pattern of synthesis of other proteins was altered. Autoradiography were carried out as described in Section 2.5.8. Figures 4.11 (a)-(d) show the resulting profiles.

One protein showed interesting kinetics in that it could be seen after 30 minutes, 1 hour and 3 hours labelling, but not after 2 hours labelling (arrowed in Figure 4.11). This suggests that the synthesis of this protein is cyclic and that degradation of it occurs between 1 and 2 hours labelling before synthesis begins again. A number of proteins were seen to disappear after 3 hours labelling.

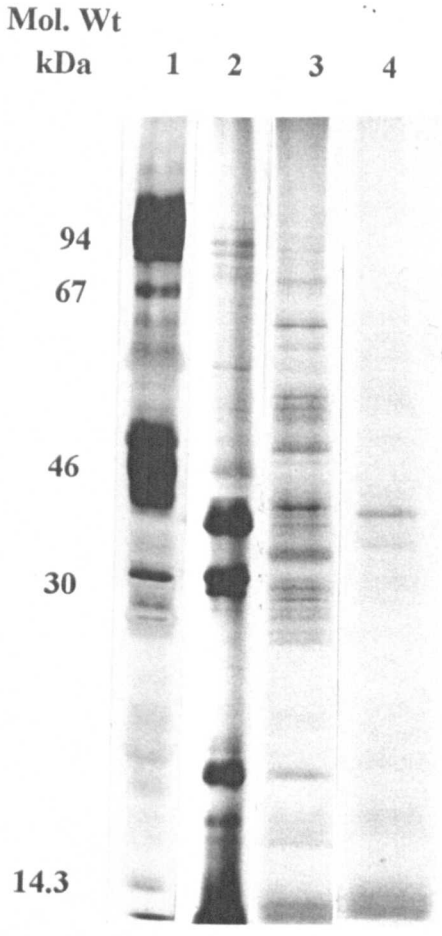
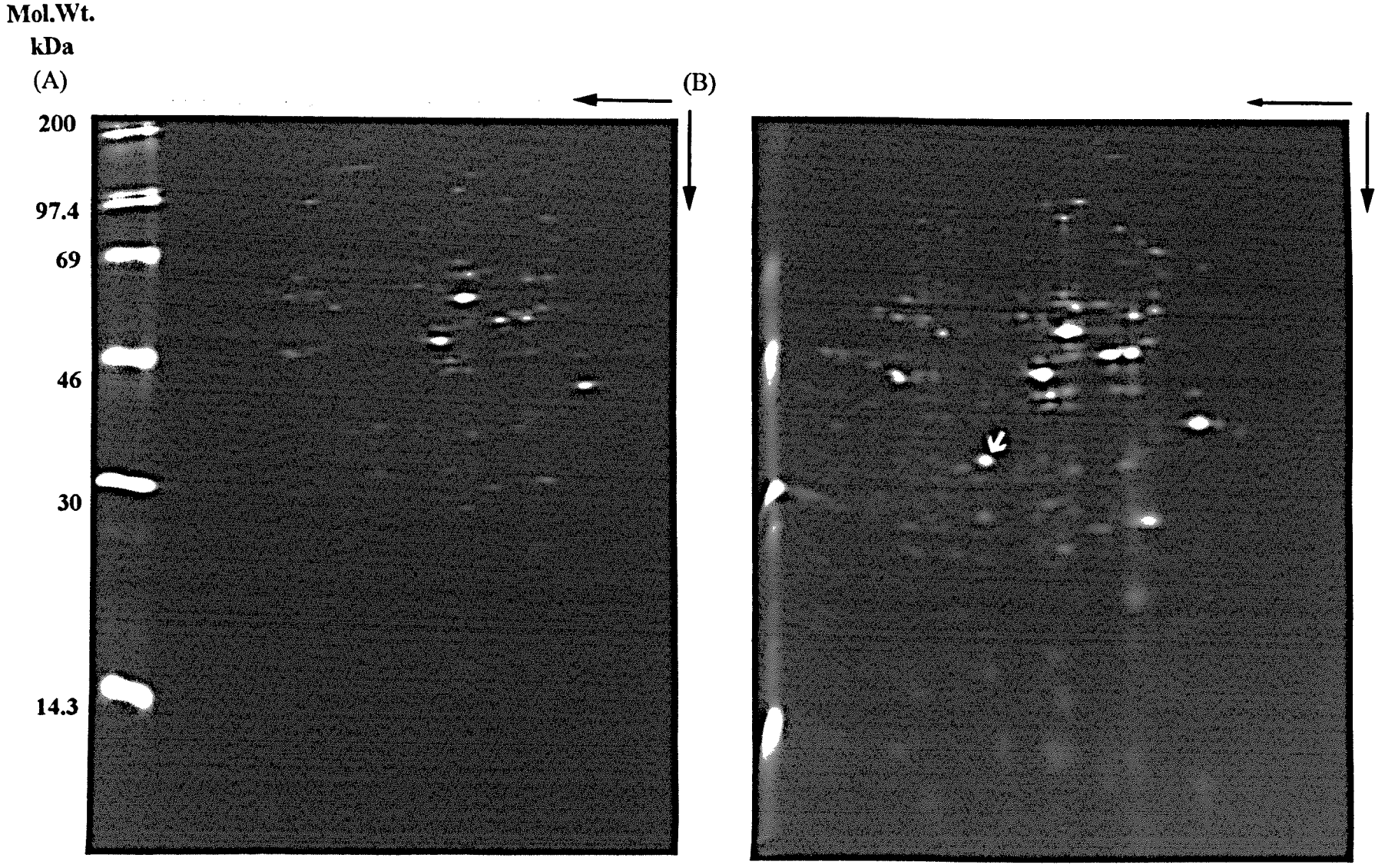


Figure 4.10. Soluble, cytoplasmic membrane and outer-membrane proteins of an 18 month-starved *E. coli* were prepared and separated on 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels and the proteins visualised by silver-staining. 20µl of sample were loaded per track. Track 1 - molecular weight markers; track 2 - outer-membrane proteins; track 3 - cytoplasmic proteins; track 4 - cytoplasmic-membrane proteins.


Mol.Wt.
kDa
(A)
200
97.4
69
46
30
14.3
(B)

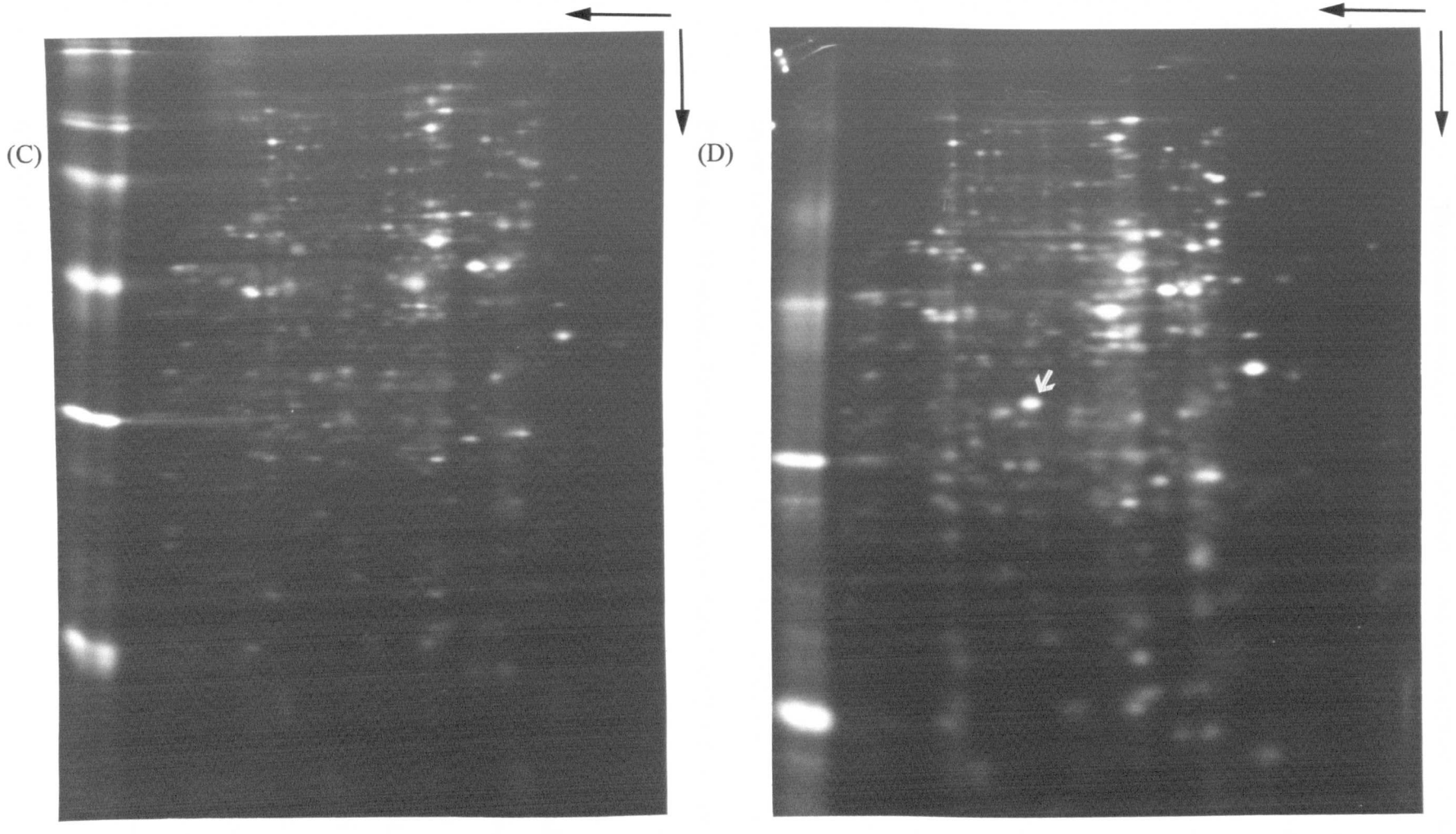


Figure 4.11. Long-term-starved cells were radio-labelled with ^{35}S-methionine (280μCi ml^{-1}) for 3hrs. Samples were removed at various times and proteins were separated by two-dimensional SDS-PAGE using 12% (w/v) polyacrylamide gels in the second dimension. 10μl of protein sample were loaded onto the first dimension. Gels were dried and autoradiography carried out. Labelling times - (A) 30 minutes; (B) 1 hr; (C) 2 hrs; (D) 3 hrs.

4.2.2. RNA synthesis

The speed at which ^{35}S-methionine was incorporated into proteins in 18-month-starved cells suggests that protein synthesis is taking place during starvation, before the addition of the ^{35}S-methionine, or that preformed messenger RNA exists in starved cells. It has been shown that protein synthesis increases rapidly in starved marine *Vibrio* species S14 when nutrients are supplied and is independent of new RNA synthesis (Albertson *et al.*, 1990b). To investigate the synthesis of RNA, an 18-month-starved culture was supplied with 0.3μM unlabelled methionine and radio-labelled at various intervals with ^{3}H-uridine (1μCi ml^{-1})and analysed using a scintillation counter, as described in Section 2.9.3.

It was found that in the first 5 minutes, ^{3}H-uridine incorporation showed no significant increase when compared to the negative control. At 10 minutes, the level of incorporation increased sharply, as shown in Figure 4.12. The peak in ^{3}H-uridine incorporation coincided with the appearance of visible bands in Figure 4.3. However, the rate of ^{35}S-methionine incorporation, demonstrated by SDS-PAGE, suggested that protein synthesis was very slow compared with actively growing cells. After 15 minutes uridine incorporation dropped rapidly to its previous level. Since the previous experiments showed that protein synthesis was increasing and the pattern of synthesis was altering in the presence of methionine, it must be concluded that this low level of uridine incorporation represented the turnover rate of messenger RNA. It is probable that the RNA and some proteins synthesised between 10 and 20 minutes are utilised to synthesise ribosomes. It has been shown that starving cells degrade their ribosomes, presumably to provide themselves with macromolecules which they can utilise in starvation survival (Dawes, 1976) and it has been suggested that viability is eventually lost due to ribosome depletion (Davis *et al.*, 1986). Before vegetative growth can resume, adequate ribosomes must be available to allow the synthesis of proteins necessary for growth and division. The low number of ribosomes

within starving cells was also suggested by the phase-contrast microscopy images (Section 5.2.2.1).

In addition, the alteration in the level of RNA synthesis when methionine was supplied to long-term-starved cells provides additional evidence that methionine alters the metabolism of these cells, in particular protein synthesis. In view of this it is suggested that analysis of protein synthesis patterns in long-term-starved cells cannot be investigated using ^{35}S-methionine.

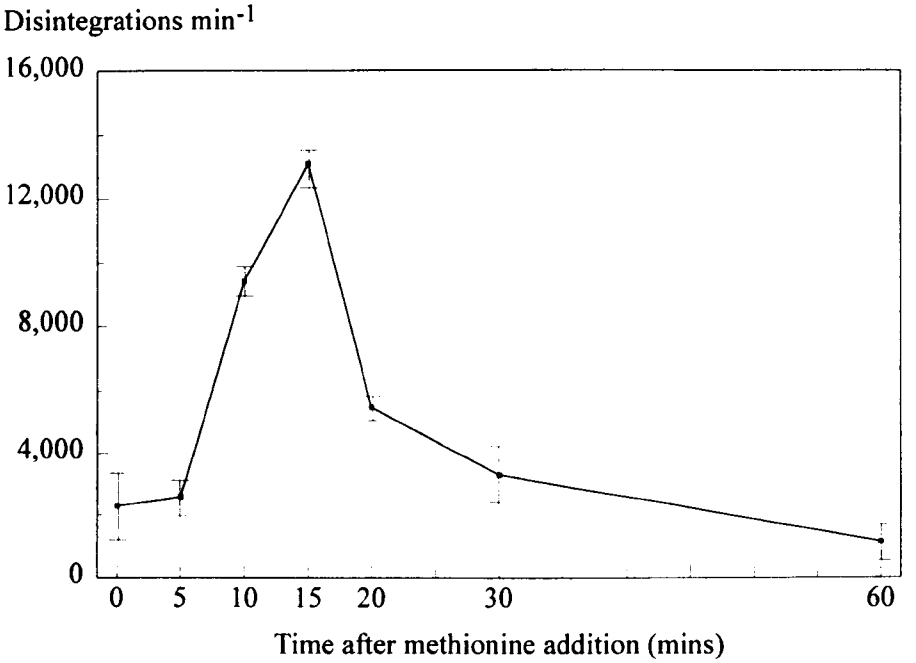


Figure 4.12. The rate of ^{3}H-uridine incorporation. Three cultures of 24 month-starved cells were supplemented with 0.3μM unlabelled methionine and ^{3}H-uridine was added to a final concentration of 1μCi ml^{-1}. At various time intervals triplicate samples were removed and the TCA insoluble material collected on GF/F glass fibre filter discs. The amount of isotope incorporation was determined by liquid scintillation counting. The data represent the mean of nine samples and error bars represent ± one standard deviation.

In order to investigate the synthesis of RNA in starving cells, cultures incubated for 61 days and 24 month-starved cells were labelled with ^{3}H-uridine, as described in Section 2.9.3, without the addition of methionine. Total and viable counts were carried out as described in Sections 2.3.1. and 2.3.2. The data are shown in Table 4.3.

Again, the results showed the same pattern as when ^{35}S-methionine and ^{3}H-leucine had been used to label cells during long-term starvation. The incorporation rate determined by viable counts showed an increase as the incubation continued, while those determined by total particle counts were not higher than the control (boiled) cells except at 2 days incubation. Since it was suggested by SDS-PAGE that protein synthesis rates were more accurate using total particle counts, the same is assumed in this experiment. This assumption is open to question and further experiments would be necessary to determine it's validity.

The incorporation of ^{3}H-uridine suggested that very little or no RNA synthesis was carried out during prolonged incubation. This agrees with the finding that very little protein synthesis was carried out. It provides additional evidence that the peak in RNA synthesis seen when cells were supplemented with methionine was possibly ribosomal RNA and mRNA coding for ribosomal proteins.

Table 4.3. ^{3}H-uridine incorporation during prolonged incubation and long-term starvation

Incubation time	dpm cell^{-1} as determined by total particle count	dpm cell^{-1} as determined by viable count
2 days	2.06×10^{-5} (5.5)	1.99×10^{-5} (5.3)
8 days	1.13×10^{-5} (8.1)	1.26×10^{-5} (4.4)
13 days	9.34×10^{-6} (5.6)	1.13×10^{-5} (12.4)
29 days	8.04×10^{-6} (10.2)	2.76×10^{-5} (6.6)
47 days	2.5×10^{-5} (6.1)	2.88×10^{-3} (9.7)
61 days	6.26×10^{-6} (4.5)	2.92×10^{-3} (3.4)
24 months	9.52×10^{-6} (11.7)	1.92×10^{-2} (6.8)
boiled cells	1.03×10^{-5} (8.9)	

^{3}H-uridine incorporation during prolonged incubation. Triplicate samples from three cultures were radio-labelled with ^{3}H-uridine (1μCi ml^{-1}) for 30 seconds. TCA insoluble material was collected on Whatman GF/F glass fibre filter discs and the amount of isotope incorporated was determined by liquid scintillation counting. The data represent the mean of nine samples and the standard deviations, given in brackets, are expressed as a percentage of the mean.

4.2.3. DNA synthesis

The rate of DNA synthesis was determined by labelling cells at various stages of starvation with ^{3}H-thymidine, 1μCi ml^{-1}, as described in Section 2.9.2 and the samples treated as for RNA synthesis estimation, (Section 4.2.1). Total and viable counts were carried out as described in Sections 2.3.1 and 2.3.2. The results are shown in Table 4.4.

The rate of ^{3}H-thymidine incorporation seen during prolonged incubation and long-term starvation indicated that little or no DNA synthesis occurred. This result was expected since no cell division has been found to occur under these conditions. Again some of the samples had lower isotope-incorporation rates than the boiled, control cells when incorporation was calculated using total particle counts. It is possible that a very low level of DNA synthesis takes place during starvation in order to repair damaged portions of the chromosome, but, other than this, any DNA synthesis in starving cells would represent an additional burden on the cells which they would not be able to cope with metabolically and which would be unnecessary, since they are unable to grow and divide in this state.

It is probable that the procedure used in this experiment is not sensitive enough to detect DNA repair since the isotope incorporation period was only 2 minutes. By increasing this time it may be possible to detect thymidine incorporation into non-replicating DNA, but the possibility of alterations occurring due to the presence of the label itself must be borne in mind.

Table 4.4. ^{3}H-thymidine incorporation during prolonged incubation and long-term starvation

Incubation time	dpm cell^{-1} as determined by total particle count	dpm cell^{-1} as determined by viable count
2 days	1.56×10^{-5} (6.6)	1.76×10^{-6} (11.4)
7 days	2.44×10^{-5} (3.2)	2.95×10^{-5} (12.2)
23 days	1.63×10^{-5} (4.1)	5.66×10^{-5} (4.4)
41 days	1.26×10^{-5} (8.1)	1.67×10^{-3} (5.1)
55 days	9.78×10^{-6} (6.4)	4.40×10^{-3} (8.9)
24 months	7.38×10^{-6} (10.2)	2.28×10^{-3} (1.2)
boiled cells	1.00×10^{-5} (10.0)	

^{3}H-thymidine incorporation during prolonged incubation. Triplicate samples from three cultures were radio-labelled with ^{3}H-thymidine, (1μCi ml^{-1}) for 2 minutes. TCA-insoluble material was collected on Whatman GF/F glass fibre filter discs and the amount of isotope incorporated was determined by liquid scintillation counting. The data represent the mean of nine readings and the standard deviations, given in brackets, are expressed as a percentage of the mean.

4.2.4. Changes in penicillin-binding proteins

To determine if cell division occurred in stationary-phase cells as they entered starvation conditions, the pattern of penicillin-binding proteins within cells at various stages of starvation was investigated. It has been reported that PBP6 shows an increase during the first 10-12 hours of stationary phase and that during the first 2 hours a doubling in cell numbers is seen (Buchanan & Sowell, 1982), suggesting that a cell division is taking place. It is thought that this increases the amount of substrate available for PBP3. PBP3 is involved in synthesising new peptidoglycan at corners, i.e. where septa form (Donachie & Begg, 1990). It has also been reported that, in the stationary phase, *E. coli* possesses two copies of the chromosome $cell^{-1}$ which is then reduced to one copy $cell^{-1}$ when the culture is incubated overnight (Boye *et al.*, 1983). This suggests that either one copy is degraded, or a cell division occurs. Stationary-phase cells have been shown to stabilise their peptidoglycan by increasing the level of Dap-Dap cross-links (Glauner & Holtje, 1990) and the same is seen in amino acid-starved cells (Goodell & Tomasz, 1980). It has been suggested that PBP6 may play a role in this process (Van Der Linden *et al.*, 1992). In addition these authors speculate that this is analogous to the role of the sporulation-specific PBP5a from *Bacillus subtilis* . In an attempt to determine whether a cell division was occurring, PBPs in stationary phase cells were estimated and total particle counts were carried out.

Samples for PBP analysis were prepared as described in Section 2.7. The PBPs were isolated in an active form by sonicating the cells, harvesting the membrane component by centrifugation and removing the outer membrane fraction by solubilisation of the cytoplasmic membrane followed by centrifugation. ^{14}C-penicillin was added to the cytoplasmic membranes. The proteins were separated on 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels as described in Section 2.5.2. and fluorography used to detect the

^{14}C-penicillin-PBP complexes as described in Section 2.5.8. It was found that the method was not sensitive enough to detect changes in PBP levels in cells during prolonged incubation or long-term starvation (Figure 4.13). Although bands could be seen on autoradiography, they were so faint that quantification could not be carried out with any degree of confidence. At 2 and 4 hours after stationary-phase was reached bands were seen which corresponded in size to PBPs 2, 5 and 6. At 6 hours after stationary-phase was reached, the intensity of all three bands was reduced and the band corresponding to PBP6 was not visible. PBP6 levels have been reported to reach maximum at 6-8 hours after stationary-phase transition (Buchanan & Sowell, 1982), but this was not evident in the present experiment. At 16 hours post stationary-phase the three bands previously seen were again visible and a fourth band was seen, corresponding in size to PBP3. PBP3 is a septation-specific peptidoglycan transpeptidase responsible for the formation of the septum (Park, 1987) and the appearance of the band suggests that septation may be taking or be about to take place.

In an attempt to increase the sensitivity of detection and allow quantification, increased sample loadings were employed, as was an increase in the amount of ^{14}C-penicillin used, but with no improvement. This was not unexpected since the amount used initially represented saturation of the PBPs with antibiotic. It was reported that the increase in PBP6 seen as cells are in the stationary-phase is less marked when cells are grown in minimal medium than when a rich medium is employed (Buchanan & Sowell, 1982). In addition it is possible that, for some reason, the method of preparation resulted in the inactivation of the PBPs, leaving them unable to bind the radio-label. This possibility is suggested by the fact that the visualisation of PBPs in the present experiment seems to be independent of the number of molecules present, for example PBP2 can be seen in all samples, yet it is the least abundant of the PBPs while PBP4 which is present at levels over 5 times greater than PBP2 was not

seen in any of the samples. The number of molecules of each PBP estimated to be in an *E. coli* cell during active growth is shown in Table 4.5.

It has been shown that the rapid release of penicillin by PBPs 5 and 6 which occurs in the presence of 2-mercaptoethanol can be prevented by the denaturation of the proteins with 1% Sarkosyl, demonstrating the enzymatic nature of the release (Spratt, 1977). It is probable that DTT used in the present experiment has the same effect as its function is the same as that of 2-mercaptoethanol, i.e. keeping the disulphide bonds of the proteins reduced. It is possible that some penicillin was released during Sarkosyl denaturation of the proteins, since the process can take around 20 minutes. In addition the use of DTT may have some bearing on this.

It had been hoped that this experiment would provide verification of transmission electron microscopy and total particle count evidence that cell division occurred during prolonged incubation. On an empirical basis, the appearance of PBP3 in the 16 hour post stationary-phase sample is suggestive of the ability of the cell to carry out septation, but the poor resolution of the PBPs meant that densitometric quantitation could not be carried out. One of the major difficulties encountered when densitometry was carried out was the high background on the autoradiographs. This was due to unbound penicillin and was particularly evident toward the bottom of the autoradiograph and, to a lesser extent, at the top. Densitometer plots are shown in Figure 4.14.

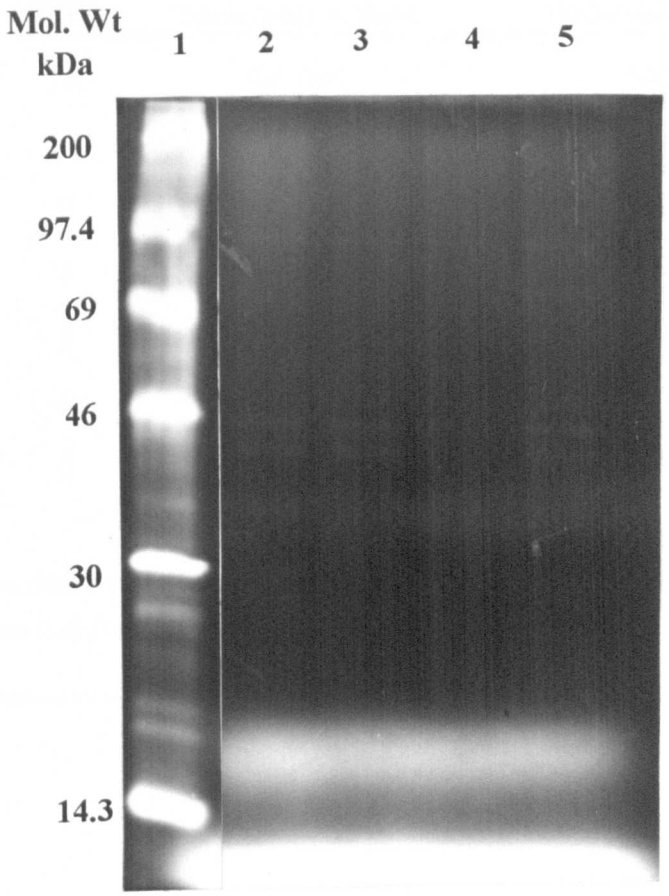


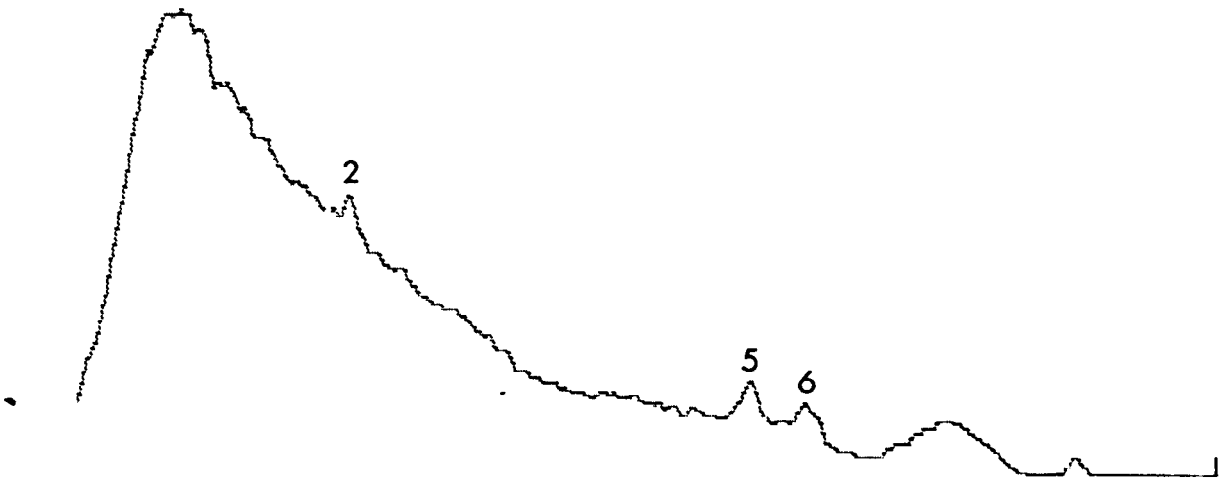
Figure 4.13. Cytoplasmic membranes were isolated and PBPs were complexed with ^{14}C-penicillin then separated using SDS-PAGE using 12% (w/v) resolving gels with 4.5% (w/v) stacking gels. 20µl of sample were loaded per track. PBPs were visualised by fluorography. Track 1 - molecular weight markers; tracks 2, 3, 4 and 5 - 2, 4, 6 and 16 hours post stationary-phase transition respectively.

Table 4.5. Penicillin-binding proteins of *E. coli*

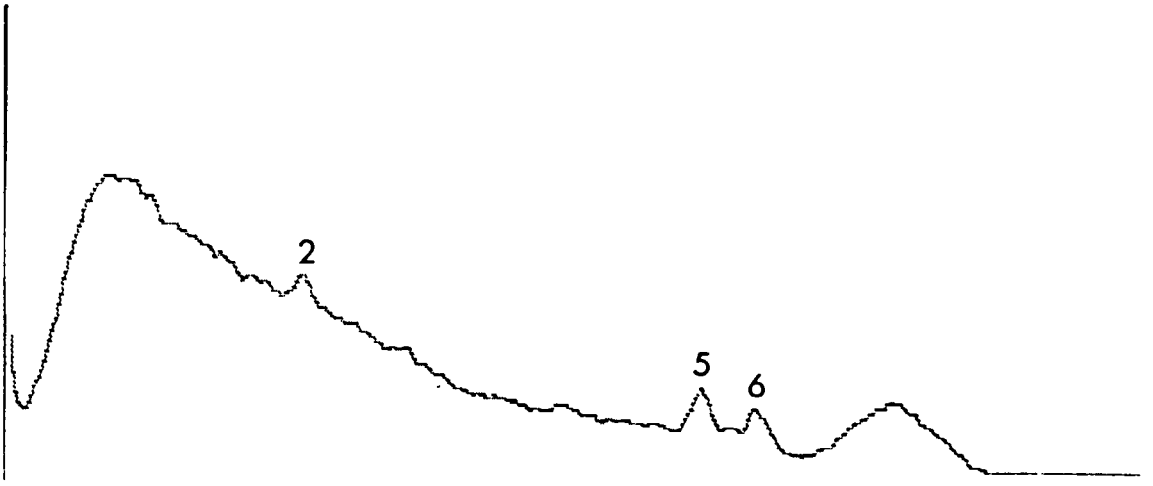
PBP	Molecular weight	Molecules cell^{-1}
1A	92, 000	100
1B	90,000	120
2	66, 000	20
3	60, 000	50
4	49, 000	110
5	42, 000	1, 800
6	40, 000	600

Only those penicillin-binding proteins which have been studied are included in the table. Table modified from Park (1987).

2 hours post stationary-phase transition



4 hours post stationary-phase transition



6 hours post stationary-phase transition

18 hours post stationary-phase transition

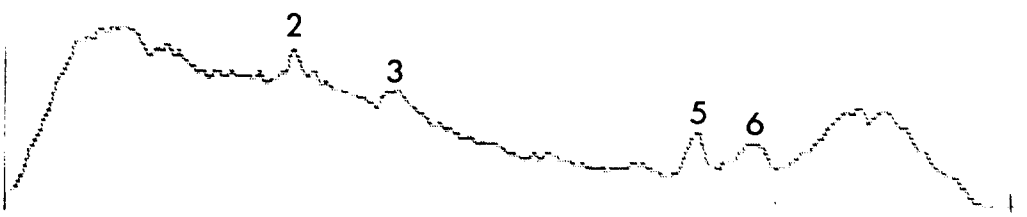


Figure 4.14. Densitometer plots obtained from the autoradiograph shown in Figure 4.13. Cytoplasmic-membrane proteins were isolated and 14C-penicillin complexed to PBPs. Proteins were separated on a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel and the PBPs visualised by fluorography. The position of the PBPs are labelled with arrows.

4.2.5. ATP content of starving cells

Since cells were starved for carbon and energy, the ATP content of the cells was measured, as described in Section 2.15, to determine how much energy was available to starved cells in the form of ATP, the energy currency of the cell.

It was found that replicate samples gave non-uniform results and the amount of ATP $cell^{-1}$ could not be calculated with any degree of confidence.

The measurement of ATP content of cells is a difficult analysis to carry out. One of the major problems encountered is degradation of ATP during sample preparation. To overcome this, duplicate samples are employed, one of which contains an internal spike of a known amount of ATP added from a stock solution before any sample preparation is carried out. The amount of luminescence due to cells in the sample can be subtracted from the luminescence value obtained for the spiked sample and the remainder represents luminescence due to the spike. By comparing this value with the expected value for the amount of ATP in the spike, the percentage degradation of ATP during sample preparation can be calculated. The luminescence values obtained for the unspiked samples can then be corrected. However, this procedure takes no account of the different stabilities of pure ATP standard solutions, which are very unstable, and of ATP released from cells.

To overcome these problems, a commercially available kit was employed. The methodology of the kit involved treating standard solutions for the calibration curve in the same way as the samples and thus internal spiking is not necessary, but may be advisable. To determine the stability of the ATP stock solution supplied with the kit, standard solutions were prepared using dilution buffer and a calibration curve obtained. This was then repeated using dilution buffer and adding an equal volume of lysis buffer, since this was the treatment to be used with the cells. The ATP was then measured as before.

It was found that the amount of ATP degradation was very small, suggesting that the sample preparation method would not significantly alter the

ATP in the samples. This was expected since the ATP standard supplied with the analysis kit is stabilised. However, the ATP from the samples is not stabilised in this way and thus the validity of this particular kit system is questionable.

It was found that replicate samples from the same population of cells showed quite wide variation with the standard deviation being up to 40% of the mean values. The variation increased when the experiment was repeated with different populations of cells sampled at the same stages of incubation. Figure 4.14 shows the ATP content of starving cells from two separate cultures. The wide variation between populations is evident, but a general pattern seems to be present. The amount of ATP within cells appeared to increase after 48 hours incubation then decrease between 7 and 8 days incubation. After 13 days incubation, ATP levels again rose.

When 18 month-starved cells were assayed no ATP was detected when compared the controls. This was unexpected since colonies arose when the culture was sub-cultured onto starvation plates. This result, coupled with the wide variations seen in Figure 4.14 led to the investigation of the lysis efficiency of the cell lysis buffer supplied with the kit.

The cell lysis buffer supposedly exhibits good lysis efficiency with a number of cell types with very little influence on luciferase activity. However, bacterial cells subjected to prolonged incubation and long-term-starvation tend to be more resistant to a number of cell surface-modifying techniques (See Chapter 5). In addition it has been shown that a starved marine *Vibrio* is more resistant to sonication (Nystrom & Kjelleberg, 1989) and the same was found for starved *E. coli* in the present work (data not shown). When these cells were exposed to lysis buffer from the kit according to the manufacturer's instructions and then plated out on starvation plates as described in Section 2.3.1 it was found that the viable counts had not dropped appreciably, suggesting that inefficient lysis was interfering with the procedure. A second, more efficient method of lysis,

recommended by the manufacturer involved incubation of cells at 100°C for 2 minutes in 100mM Tris, 4mM EDTA, pH 7.75. Stock solutions of stabilised and unstabilised ATP were subjected to this treatment (Figure 4.15). When compared to calibration curves of untreated solutions the percentage degradation could be calculated. It was found that this method resulted in excessive loss of unstabilised ATP. Although the ATP stock supplied with the kit exhibited very little degradation, unstabilised stock solutions gave degradation values of 95.1-97.4%.

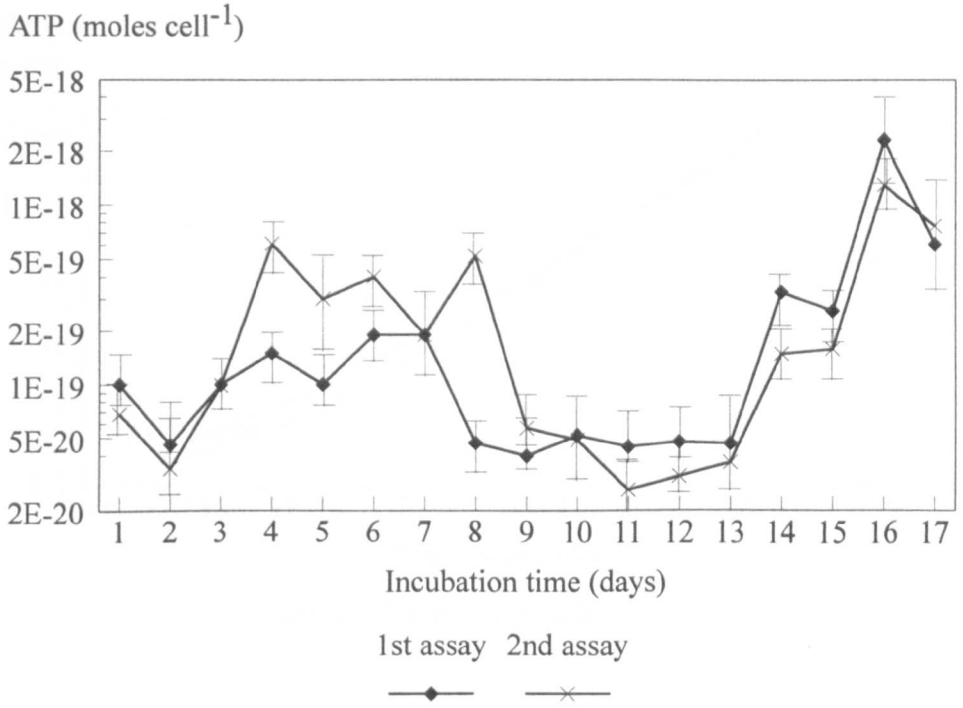


Figure 4.14. ATP content of cells during prolonged incubation. The graph shows two representative cultures. Samples from cultures were assayed in triplicate. The data represent the mean of three readings and the error bars represent ± one standard deviation.

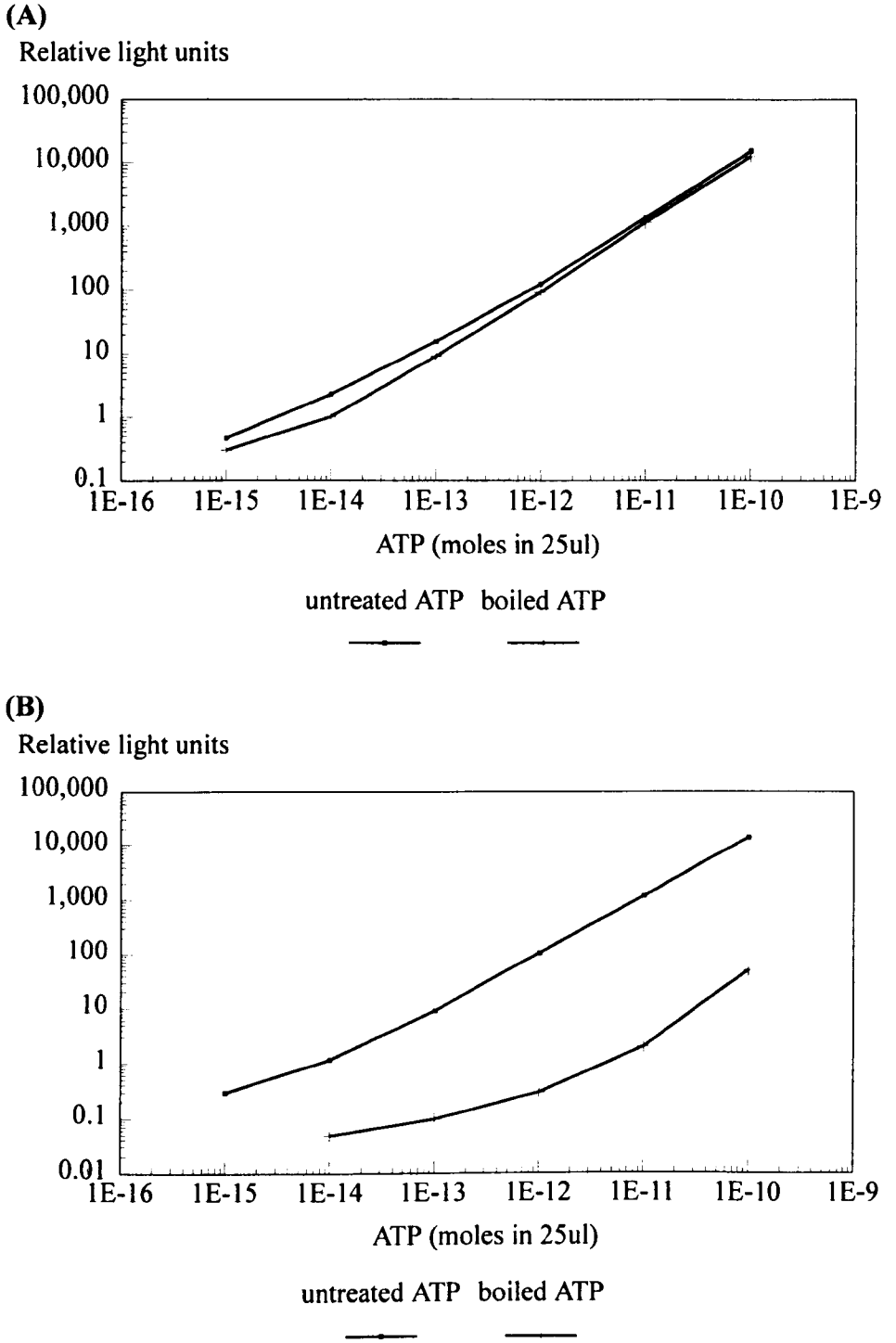


Figure 4.15. The stability of two types of ATP standard solutions were compared. Graph (A) illustrates the calibration curves obtained when stabilised ATP solution supplied with the commercial kit was assayed before and after boiling. Graph (B) illustrates the calibration curves obtained when pure ATP was used to make up the standard solutions and the solutions were treated as for Graph (A)

The comparable stability of ATP released from cells is not known. When lysis buffer alone is used, the ATP released from the lysed cells is reported to be stable, according to the manufacturers. However, the inefficiency of lysis found in the present experiments dictates that this method cannot be used. The more efficient method of lysis, while not affecting the stability of the stabilised ATP supplied with the kit, does significantly affect the stability of ATP stock solutions made with pure ATP.

The heterogeneous nature of populations of carbon-starved *E. coli* cells may have affect the results obtained. It is possible that those cells which lysed belonged to one distinct subpopulation which may have been in a different metabolic state from any other subpopulation(s). Until a method has been found which allows the separation of cell types and more efficient lysis of cells, assays such as this will be prone to misleading results.

4.3. Conclusions

Long-term-starved cells do not exhibit the same macromolecular synthesis patterns as actively growing or stationary phase cells. After 48 hours very little synthesis of macromolecules takes place in these cells. However, the cells do retain the ability to begin synthesis of these molecules very quickly after the addition of a carbon and energy source.

The rate of protein synthesis in long-term-starved cells is much slower than in actively growing and short-term stationary phase cells and is estimated to be 0.05% of that of actively growing cells (Kolter *et al.*, 1993). The pattern of protein synthesis is also different, suggesting that cells exist in a different metabolic state, possibly in a state of semi-dormancy. The alteration of protein synthesis profiles seen when cells are radio-labelled with methionine, but not when radio-labelled with leucine, suggests that supplementation of long-term-starved cells with methionine represents a nutrient upshift and this is supported by the finding that cell numbers increase in the presence of methionine, but not leucine. The suggested mechanism which may explain this is unproven, the evidence being circumstantial. The starvation regime to which cells are subjected may be important in the changes which occur in protein synthesis profiles, as discussed in Section 4.2.1.

DNA and RNA synthesis was seen to be much reduced during prolonged incubation and long-term-starvation. DNA synthesis was not expected to occur, since replication of the chromosome would represent an unnecessary burden on carbon-starved cells. In swarmer cells of *R. vannielii* no ribosomal RNA synthesis occurs (Dow *et al.*, 1983) and it has been shown that the condensed nucleoids of *C. crescentus* swarmer cells do not sustain ribosomal RNA synthesis (Swoboda *et al.*, 1982a).

It is possible that long-lived messenger RNA species are produced in these conditions as in a marine *Vibrio* species (Albertson *et al.*, 1990a). This

allow protein synthesis to occur with lower levels of mRNA synthesis than normal.

The question of whether cell division occurs at the onset of starvation has not been resolved, however circumstantial evidence presented in Chapter 3 and discussed in Section 4.2.4 suggests that their is a strong possibility that cell division is occurring as cells enter starvation conditions. The number of chromosomes present in stationary-phase cells has been shown to decrease from 2 to 1 during overnight incubation (Boye *et al.*, 1983) which is consistent with cell division or DNA degradation occurring in stationary-phase.

Of considerable interest is the ATP content of long-term-starved cells. The energy charge of a cell is very delicately controlled by the respective concentrations of the three adenylate species, ATP, ADP and AMP as follows;

$$\text{Energy charge} = \frac{[ATP]+[ADP]/2}{[ATP] + [ADP] + [AMP]}$$

Any decrease in the energy charge of the cell inhibits the utilisation of ATP and stimulates it's regeneration (Neidhardt *et al.*, 1990). Consequently, the energy charge of the cell is maintained. It has been shown in *R. vannielii* that ATP levels within swarmer cells are very high, compared with that found in vegetative cells and the marine *Vibrio* species ANT300 demonstrates an initial decrease followed by an increase in ATP levels during starvation (Amy *et al.*, 1983). This has not been observed in *E. coli*, but long-term-starved cells have not been adequately analysed. Further investigation of assay methodologies, especially the methods of lysis of these cells, together with methods for obtaining homogenous samples of subpopulations from carbon-starved heterogeneous cultures may prove interesting.

Overall, macromolecular synthesis rates in carbon-starved *E. coli* appear to be very low, although the cells retain the ability to respond rapidly to changes

in nutrient status and resume vegetative growth. This is analogous to survival cell types of differentiating organisms.

Chapter 5. The physiology of cells during long-term starvation.

5.1. Introduction

The survival cells produced by differentiating bacteria are more resistant to various forms of stress than the vegetative cells. *Bacillus* endospores, which are produced in response to nutrient deprivation, are also extremely resistant to heat and chemical damage. The swarmer cells of *Rh. vannielii* are more able to survive nutrient deprivation than the parent cell type and have been shown to be more resistant to rifampicin (Scott *et al.*, 1987).

The physiology of endospores and swarmer cells is very different, but one thing they seem have in common is reduced permeability. The insolubility of the protein in the spore coat prevents entry of polar and non-polar solvents and the resistance to rifampicin in swarmer cells of *R. vannielii* was shown to be independent of the antibiotic target molecule, RNA polymerase and it was suggested that decreased permeability was responsible (Scott *et al.*, 1987).

Starvation for carbon has been shown to induce resistance to numerous stresses including heat-shock (Grossman *et al.*, 1985; Jenkins *et al.*, 1988; Volker *et al.*, 1992; Wild *et al.*, 1993), peroxide-shock (Morgan *et al.*, 1986; Jenkins *et al.*, 1988) and salt-shock (Volker *et al.*, 1992). The mechanism by which this is achieved is not known, but the diversity of substances and conditions to which the cells become resistant suggests that an overall exclusion system may be operating to prevent entry of potentially damaging substances.

The mechanism of heat-resistance in endospores seems to be dependent on the dehydration of the DNA environment, with water being replaced with dipicolinic acid and the highly condensed state of the DNA. This condensation is brought about by the binding of α and β small acid-soluble proteins which have been shown to carry out the same function when overexpressed in *E. coli*.

The physiological differences between starving and non-starving cells is investigated in this chapter, together with the differences in their response to other forms of stress.

5.2. Results and discussion

5.2.1. Cell sedimentation characteristics

During centrifugation of starving *E. coli* K12 cultures, it was observed that the longer cells were starved for carbon the greater the optical density of the resulting supernatant. To investigate this phenomenon, a long-term starved culture (18 months) was centrifuged at 1, 500*g* for 30 minutes. The pellet was collected and the supernatant was re-centrifuged at 4, 000*g* for 30 minutes and a second pellet was obtained. The pellets were prepared for one-dimensional SDS-PAGE as described in Section 2.5.1.1 and the samples were electrophoresed on 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels, (Section 2.5.2).

Figure 5.1 shows the resulting protein profiles after silver staining, as described in Section 2.5.4. Five proteins found in the cells from the second pellet, were not present, or present in lower amounts in the cells from the first pellet.

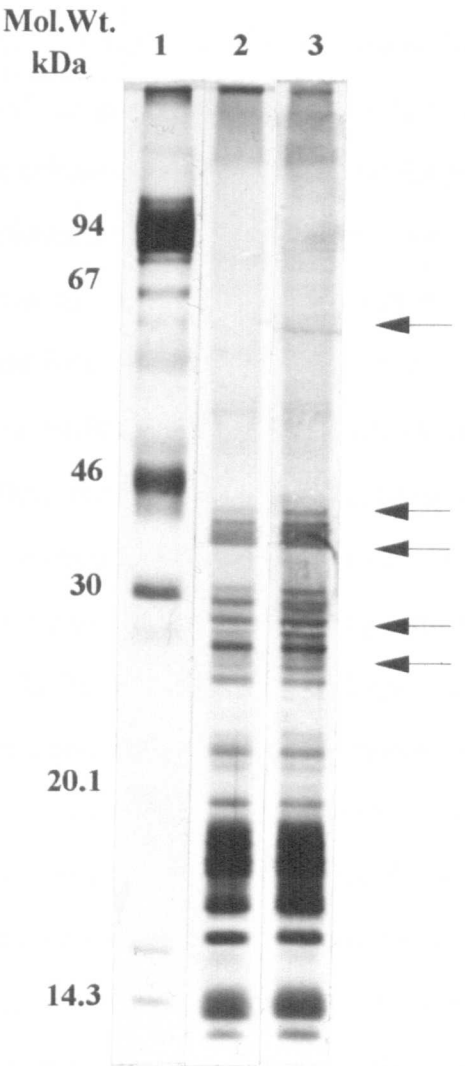


Figure 5.1. Total proteins from the first and second pellets obtained by centrifugation were separated on 12% (w/v) resolving gels with 4.5% (w/v) stacking gels. The first pellet was obtained by centrifugation at 1 500*g* and the second by centrifugation of the resulting supernatant at 4, 000*g*. 20µg of protein were loaded per track. Track 1 - molecular weight markers, track 2 - first pellet, track 3 - second pellet. The proteins marked with arrows were found only in samples prepared from the second pellet or showed alterations in the amount present.

Cells were subjected to prolonged incubation over a period of 6 months and at various time intervals samples were removed and Percoll density gradient centrifugation was carried out, as described in Section 2.4.2.

It was found that cultures incubated for 1-6 weeks produced a single, tight band, while cultures incubated beyond 6 weeks produced a more diffuse band. Cells were collected from the bands and analysed in parallel with short-term-stationary phase cells and long-term-starved cells. It was found that cells from cultures incubated for 1-6 weeks were uniform in size and resembled cells from a 48 hour culture. Cells from the diffuse band appeared to contain two cell types. One type resembled those from the tight band and were found predominantly at the top of the band. The second type resembled long-term-starved cells and were found predominantly at the bottom of the band. Figure 5.2 shows the cell-size profiles obtained, as described in Section 2.3.2. However, it was found that a homogeneous suspension of each cell type could not be obtained.

The cells from the top and the bottom of the diffuse band were prepared for SDS-PAGE, as described in Section 2.5.1.1 and the proteins were separated on 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels as described in Section 2.5.2. After silver-staining, it was found that there were no obvious differences in the protein profiles of the samples, (Figure 5.3).

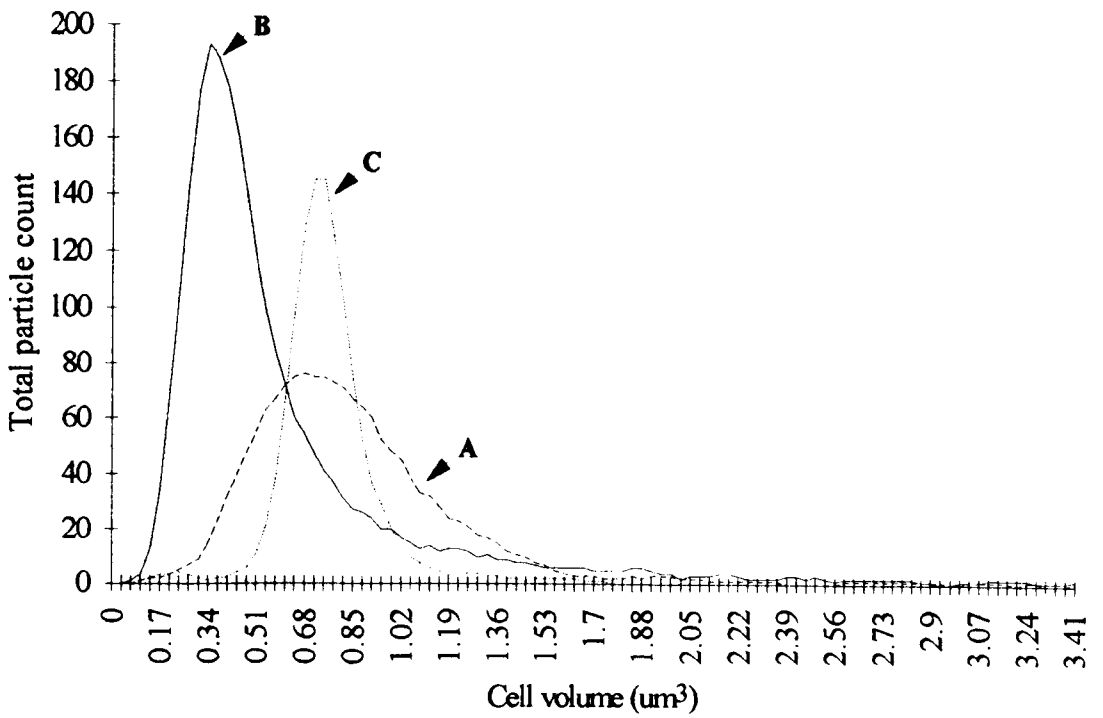


Figure 5.2. Cell size profiles of cells from the tight band (A) the top (B), and bottom (C), of the diffuse band obtained by Percoll density gradient centrifugation. The tight band was obtained when cells incubated for less than 6 weeks were centrifuged and the diffuse band when cells incubated for more than six weeks were centrifuged.

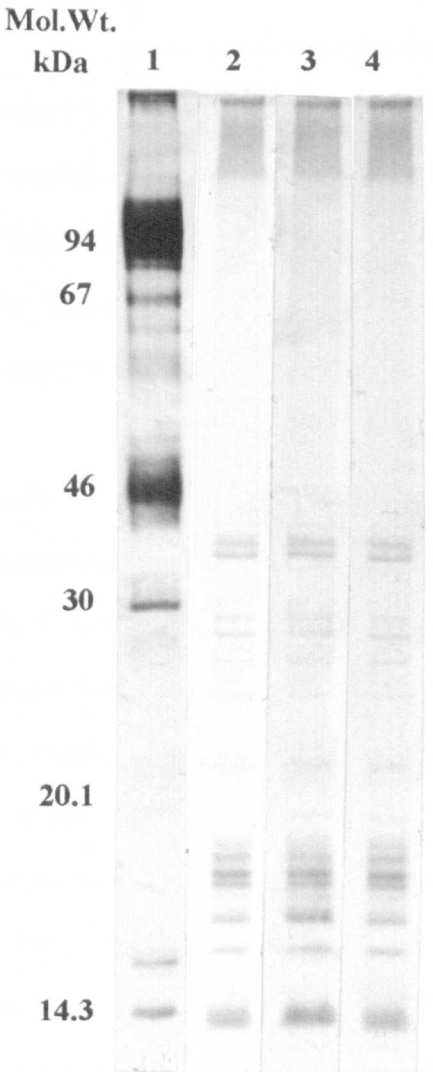


Figure 5.3. Cells obtained from Percoll density gradient centrifugation were separated by SDS-PAGE using 12% (w/v) resolving gels with 4.5% stacking gels. Track 1 - molecular weight markers; track 2 - single, tight band obtained from cells incubated for 1-6 weeks; track 3 - cells from the top of the diffuse band obtained from cells incubated for 10 weeks; track 4 - cells from the bottom of the diffuse band. Proteins were visualised by silver-staining. 20μg of protein were loaded per track.

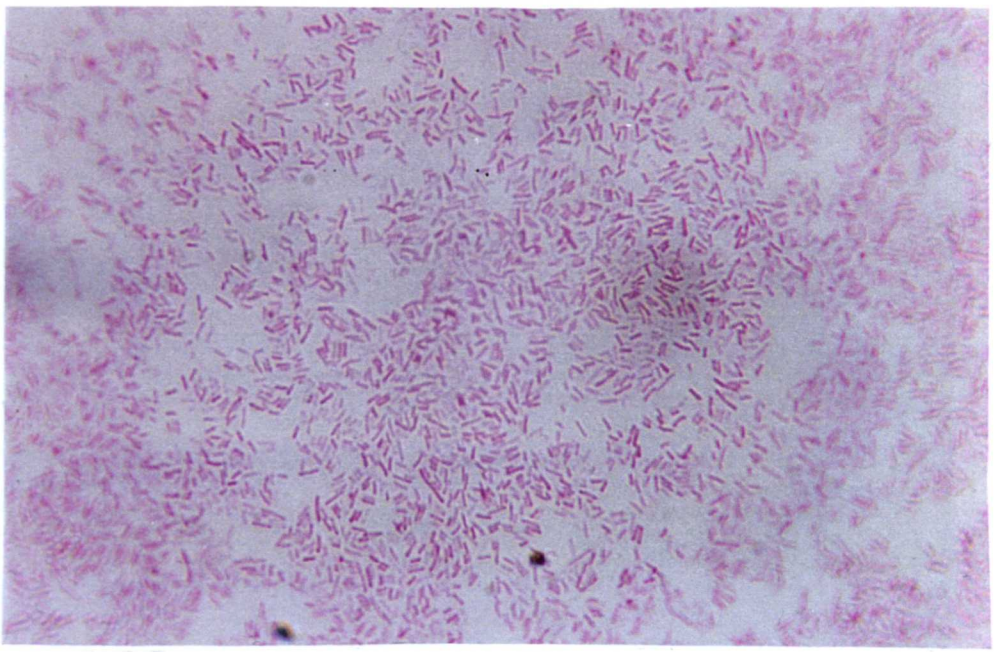
5.2.2. Staining characteristics

It was found that long-term-starved cells were very difficult to stain with a wide range of standard dyes. This was also true of small cells collected from the top of the diffuse band from Percoll density gradient centrifugation. Therefore, in the following sections, the results obtained for long-term-starved cells also refer to these small cells. The staining procedures were modified (detailed in individual sections) in an attempt to improve their efficiency.

5.2.2.1. Gram stain

Long-term-starved cells showed the same Gram reaction as non-starving cells. It was noticed however, that the long-term-starved cells showed greater accumulation of basic fuchsin counterstain at the poles of the cell, or at specific points along their length, (Figure 5.4). This corresponded to the phase contrast images seen of these cells. When cells are observed by phase-contrast microscopy, the cytoplasm usually shows a uniform darkness and the nucleoid cannot be seen. This is due to the presence of large numbers of ribosomes within the cell which have a refractive index close to that of DNA. If cells are suspended in a mounting fluid with the same refractive index as the cytoplasm or treated with polyvinylpirollidone (Donachie & Robinson, 1987), the nucleoid can usually be seen as a lighter area. In long-term-starved cells it was found that regions of the cell had a much reduced refractive index and consequently appeared much lighter. Dark areas could be seen along the length of the cells. Figure 5.5 shows phase contrast images of 24 hour cells and long-term-starved cells. Basic fuchsin has an affinity for the acidic DNA and RNA and it was thought that a change in the conformation of the DNA or the amount of RNA present as ribosomes may be the cause of the bipolar staining seen in the long-term-starved cells.

(A)



(B)

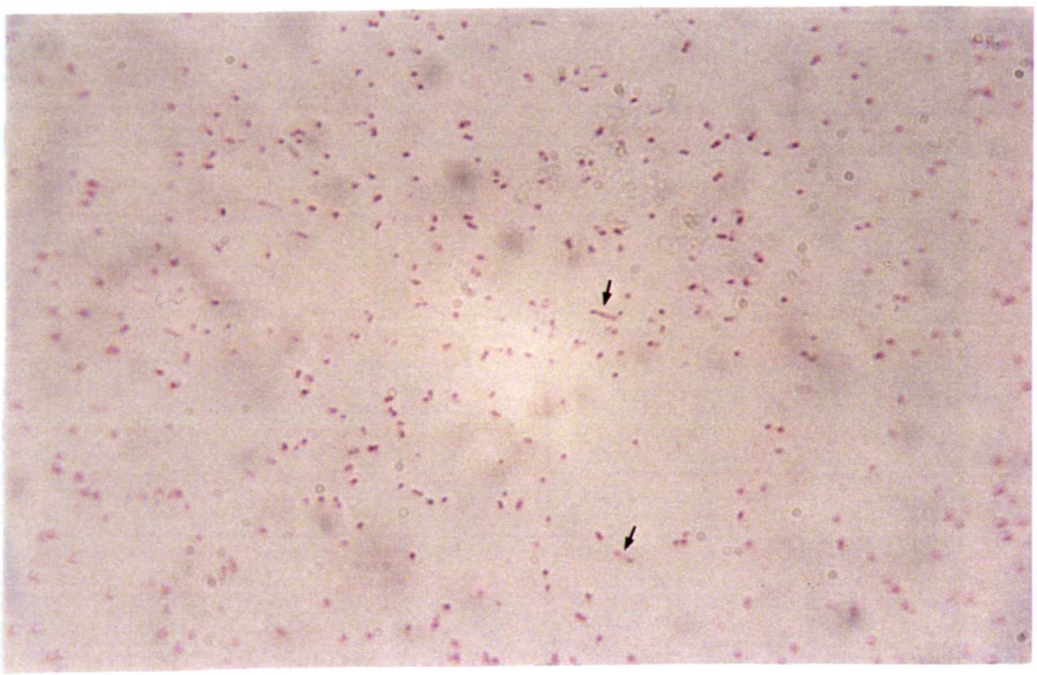
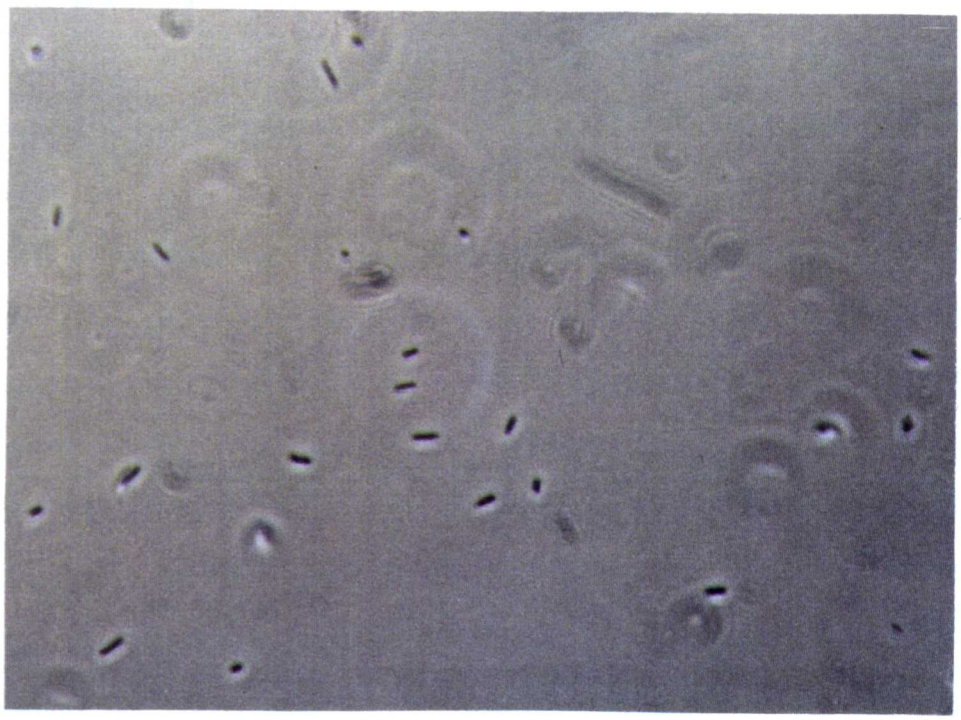


Figure 5.4. 48 hour-incubated, (A) and long-term-starved cells, (B), stained by Gram's method. Arrows indicate cells showing extreme bipolar staining. Magnification x 1000

(A)



(B)

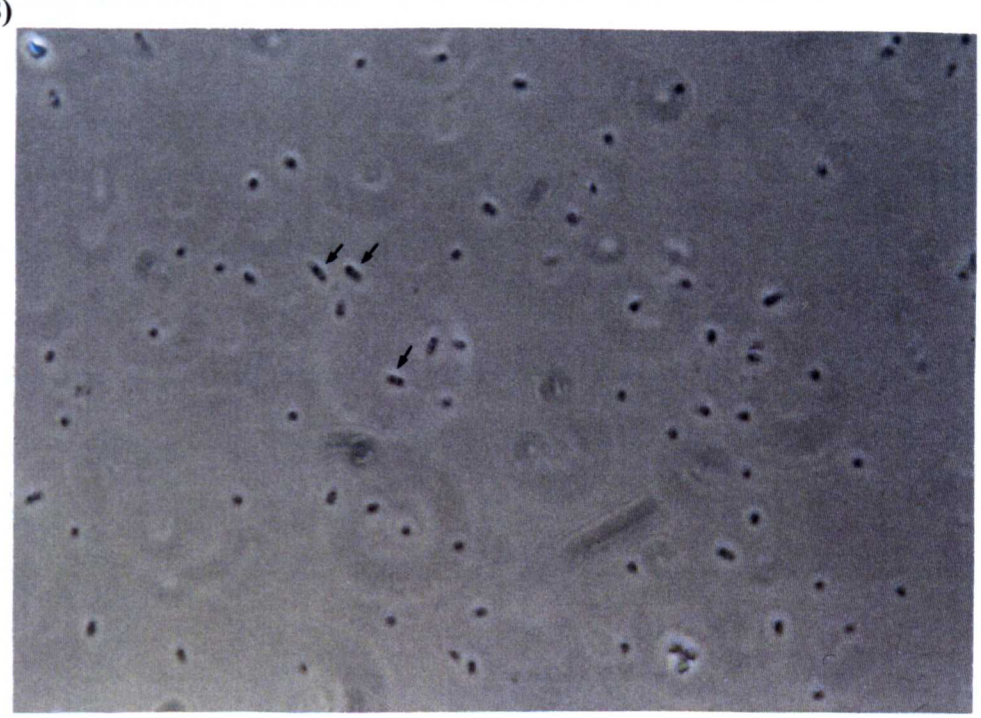


Figure 5.5. 48 hour-incubated, (A), and long-term-starved cells, (B) seen using phase contrast microscopy. Arrows indicate cells showing marked increased refractivity at the poles. Magnification x 1000

5.2.2.2. DAPI stain

DAPI has been used to determine the total number of bacteria in populations from environmental samples as staining is not dependent on viability and has taken over from acridine orange in recent years as the most popular method for determining total bacterial counts, especially in aquatic and soil samples (Kepner & Pratt, 1994). DAPI is a non-intercalating DNA stain which binds preferentially to A-T rich regions within the DNA. When bound to DNA and excited by light with a wavelength of 365nm it emits at 390nm, producing a bright blueish-white fluorescence. When unbound or bound to non-DNA molecules it fluoresces over a range of yellow colours which is easily distinguished from DNA-associated fluorescence. This stain was employed to determine the conformation of the nucleoid and to determine if the bipolar staining seen using Gram staining was due to changes in nucleoid conformation (Hiraga *et al.*, 1989; Eliasson *et al.*, 1992).

Using phase contrast and UV illumination together, as described in Section 2.10.6, it was possible to determine the shape and size of the nucleoid in relation to the whole cell. It could be seen that the areas of the long-term-starved cells which appeared light on phase-contrast microscopy corresponded to the position of the nucleoid within the cell (data not shown). The areas which did not stain with DAPI corresponded to the areas which appeared dark on phase-contrast microscopy and basic-fuchsin-stained regions when Gram stain was employed. The nucleoid of 24 hour-incubated cells tended to occupy more of the cell volume than that of the long-term-starved cells (Figures 5.6 and 5.7) suggesting that the nucleoid of the long-term-starved cells was much more condensed than that of stationary-phase cells.

It was found that, as starvation continued, fewer and fewer cells exhibited the ability to either take up DAPI or allow it's intercalation with the nucleoid. In long-term-starved cultures it was found that very few cells showed any evidence of staining. Figure 5.6 shows phase contrast and UV images of the same field of

48 hour-incubated cells and Figure 5.7 shows long-term-starved cells. This suggests that the fixation and permeabilisation stage of the staining process was not as efficient in long-term-starved cells. The fixation in -20°C methanol for 10 minutes was extended to 30 minutes and it was found that the proportion of cells stained by DAPI increased. This suggests that the membrane of long-term-starved cells was more resistant to alcohol permeabilisation. The failure of DAPI to stain cells which are visible with acridine orange has been reported previously, especially in samples containing fine sediments (Kepner & Pratt, 1993; Suzuki *et al.*, 1993).

When total particle counts and viable counts were carried out on long-term-starved cultures it was found that 10% of the total cell population were capable of forming colonies and were obviously viable. However, the proportion of cells which showed DAPI staining was much less than both the total count and viable count, suggesting that cells were failing to stain even when viable. This suggested that DAPI staining could not be used as a reliable way of assessing total counts or culture viability.

In view of this it is possible that the conformation of the nucleoids seen represents only a small proportion of the viable cells and other conformations may be present.

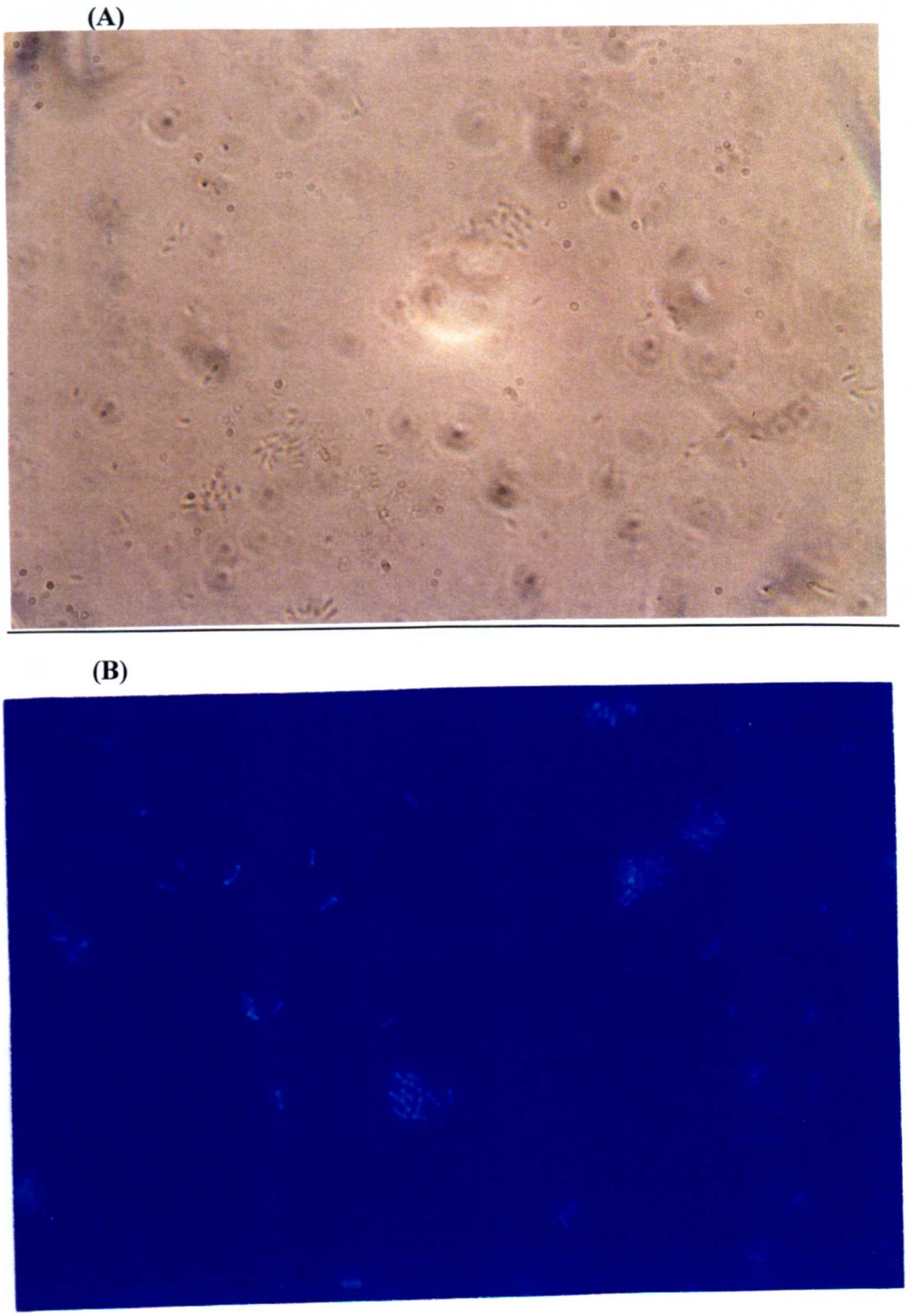
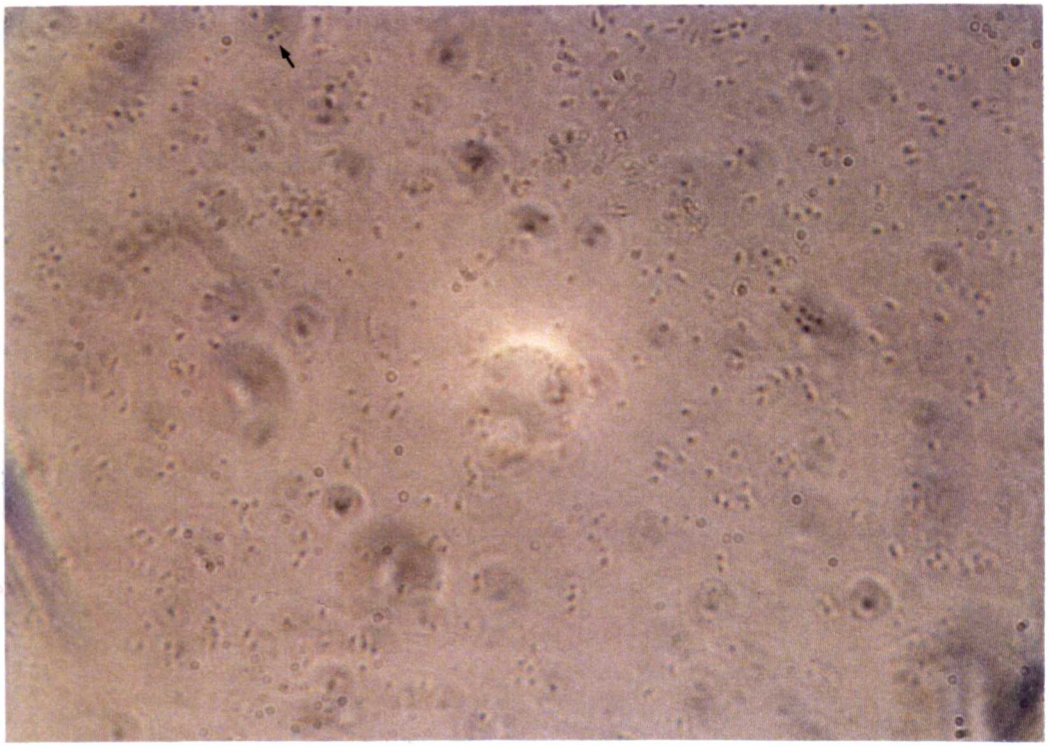


Figure 5.6. Phase contrast, (A), and UV illumination, (B), of the same field of DAPI-stained 48 hour-incubated cells. Magnification x 1000

(A)



(B)

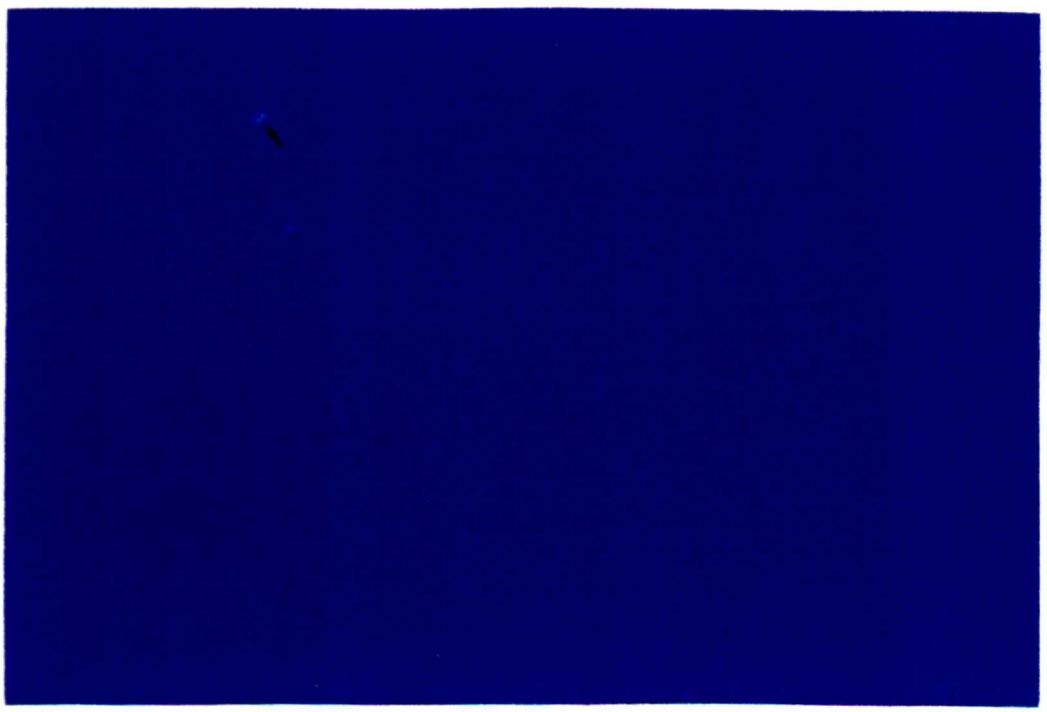
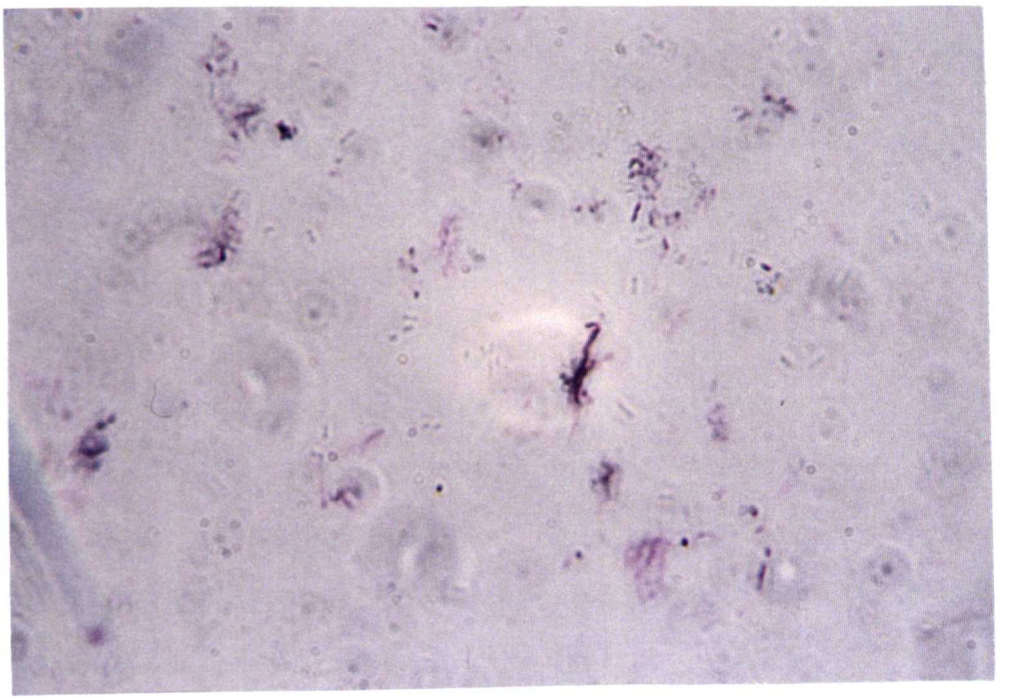


Figure 5.7. Phase contrast, (A), and UV illumination, (B), of the same field of DAPI-stained long-term-starved cells. The arrows mark the same cell under the different illuminations. Magnification x 1000

5.2.2.3. Geimsa stain

In order to overcome the problems found with DAPI staining, Geimsa stain was employed as described in Section 2.10.2. This stain has been used successfully to observe the nucleoid conformation of swarmer cells of *R. vannielii* and *C. crescentus*. Again, difficulties were encountered in staining the long-term-starved cells. Geimsa stain appeared unable to penetrate the cells. The incubation time in 1M HCl was increased, but no increase in staining efficiency was seen. The 48 hour-incubated cells did stain with Geimsa and the nucleoid could be observed, indicating that the staining method was valid in *E. coli*, but permeability was again the problem (Figure 5.8).

(A)



(B)

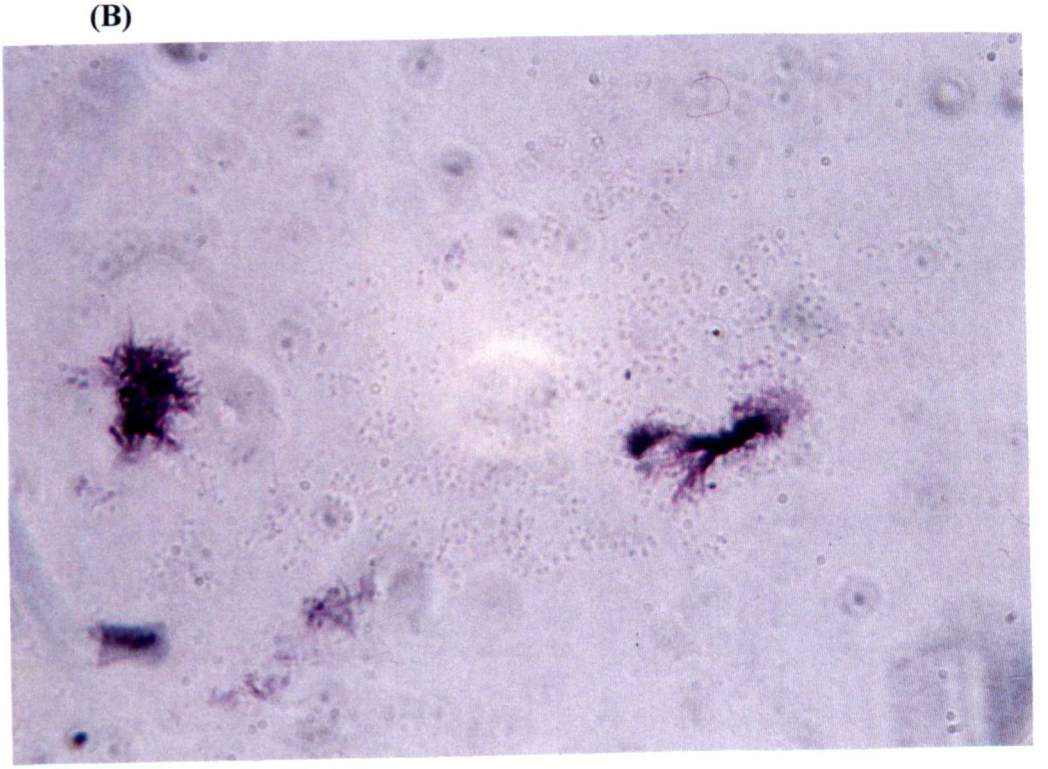


Figure 5.8. Geimsa stain of 48 hour-incubated, (A), and long-term-starved cells,(B). Magnification x 1000

5.2.2.4. β polyhydroxybutyrate stain

Cells were stained by this method because it is useful for staining extremely hydrophobic intracellular molecules and it was thought that this stain might prove more able to penetrate the cells and to allow visualisation of any storage granules. Black-stained storage granules are visible on a saffranin-stained background. A 48 hr culture showed no evidence of hydrophobic inclusion bodies and the pink colouration of the cytoplasm was obvious. When an 18 month-starved culture was stained, inclusion bodies were seen (Figure 5.9). However, no pink colouration of the cytoplasm was seen. This suggested that the water-soluble saffranin had not penetrated the cells, but that the hydrophobic sudan black in ethylene glycol had.

The xylene fixation step in the staining procedure, as described in Section 2.10.3 was extended from 5 minutes to 1 hour in 15 minute increments. Saffranin staining of the cytoplasm was visible only after a 1 hour fixation step (Figure 5.10). This suggests that the envelope of the long-term-starved cells is able to exclude polar molecules which actively growing and stationary phase cells allow to cross into the cytoplasm. The non-polar nature of the xylene fixative causes damage to the outer membrane of Gram negative cells and it is this which allows the stains to enter the cell. The success of the extended fixation step suggests that the hydrophobicity of the cell envelope in long-term-starved cells is much greater than that of the control cells and is much more resistant to chemical damage.

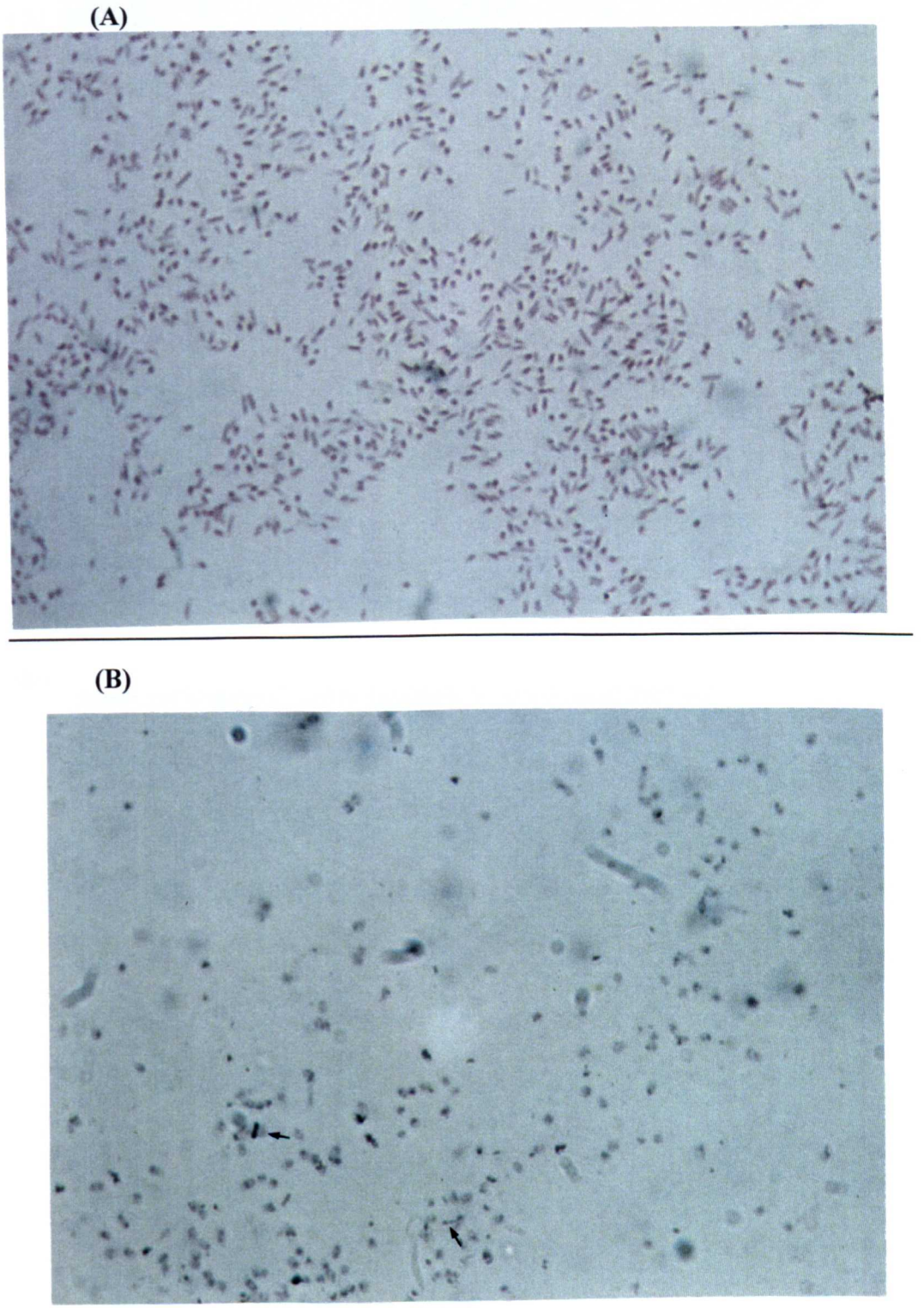
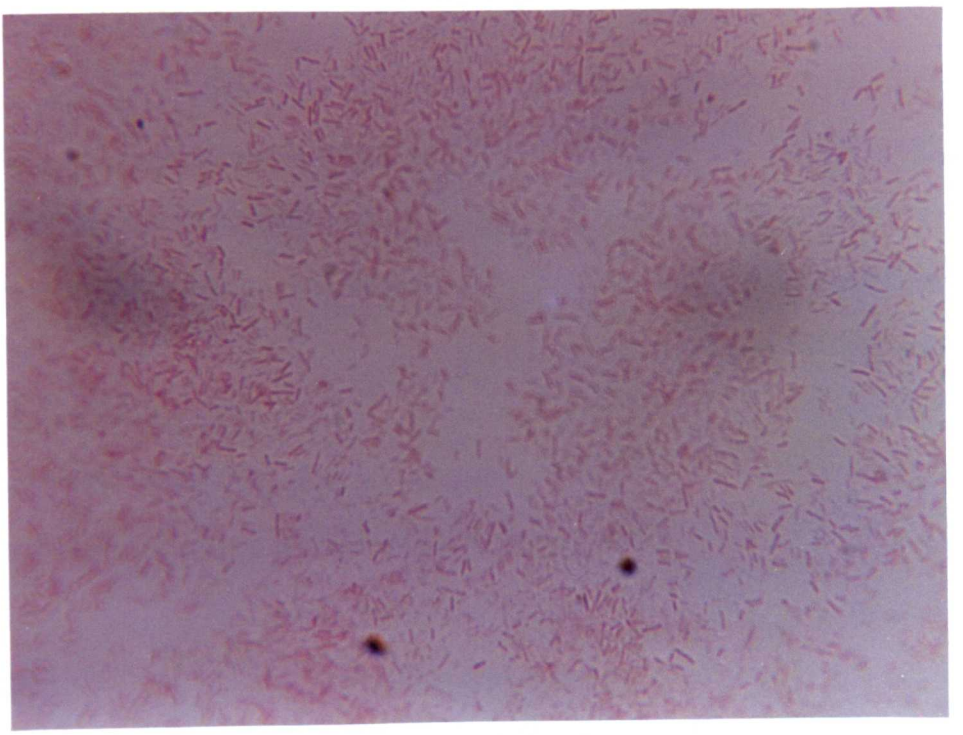


Figure 5.9. β-poly hydroxybutyrate staining of 48 hour-incubated, (A), and long-term-starved cells, (B), employing a 5 minute xylene-fixation stage. Magnification x 1000.

(A)



(B)

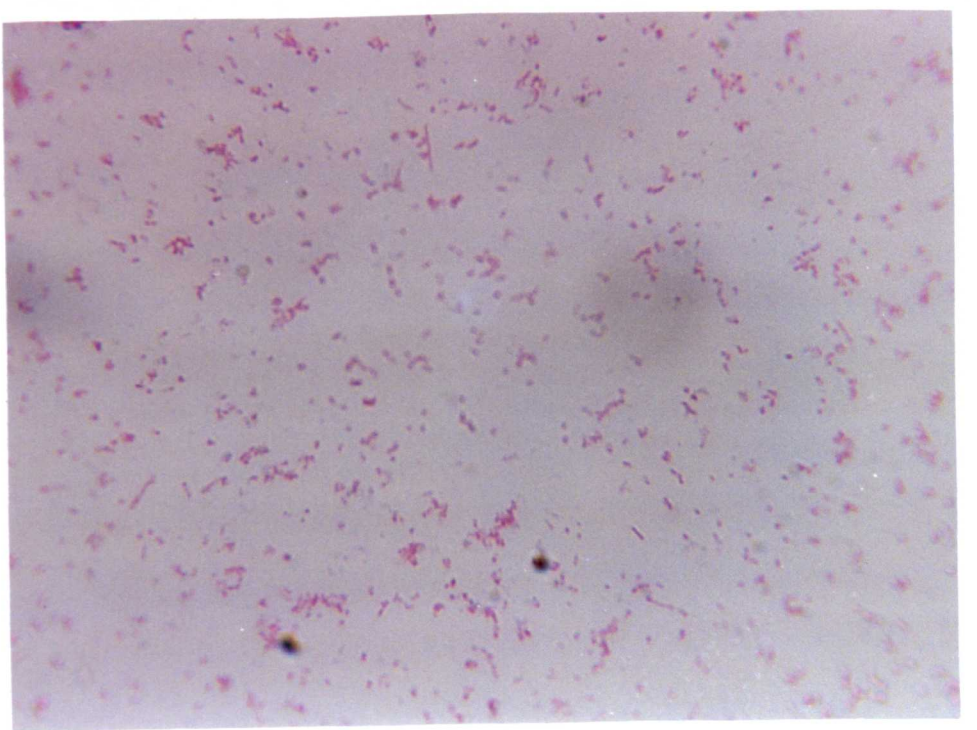


Figure 5.10. β-polyhydroxybutyrate staining of 48 hour-incubated, (A), and long-term-starved cells, (B), employing a 1 hour xylene fixation stage. Magnification x 1000.

5.2.3. Colonial morphology of starving cells

During incubation of starvation plate viable counts (Section 2.3.1), colonies showed papillae formation which became more accentuated and frequent as the starvation time increased. This was independent of the temperature of incubation. During the first 5 days of starvation, no papillae arose on plates incubated at 30°C for 48 hours then incubated at room temperature for a further 10 days. When cells were starved for longer than five days, papillae formed during incubation at room temperature after 48 hours incubation at 30°C. Using a straight wire, papillae were sub-cultured onto starvation plates, nutrient agar and MacConkey agar plates. It was found that the colonies which arose from the papillae were extremely mucoid and those subcultured from the portion of the colony with an entire edge were rough and showed an irregular edge on MacConkey agar plates, as did the original inoculum (Figure 5.11). The two colony types retained their respective morphologies on further subculture to all three media.

(A)

(B)

Figure 5.11. Colonies arising from papillae, (A), and normal portion of colony, (B).

When subcultured to liquid M9 medium with 0.05% (w/v) glucose, cells from rough colonies remained rough when recovered on solid media. Cells from mucoid colonies, however, showed reversion to the rough form. Initially, at stationary phase, all colonies arising were of the mucoid type. After 7 days incubation the proportion of rough colonies arising was 93.7% (standard deviation 3.6%). This suggested that reversion to a rough colony type was occurring and that the rough form was better able to survive starvation than the mucoid form. In contradiction to this, it was found that when rough type cells were subjected to long-term starvation, initially with 0.05% (w/v) glucose, the population arising on recovery showed both colonial morphologies (Figure 5.12).

To ensure that the mucoid colonies were, in fact, an *E. coli* K12, a range of biochemical tests were carried out on the two forms using the API 20E system. The profiles obtained are shown in Table 5.1. Both colony types gave identical profiles which identified them as *E. coli*. Although this did not rule out the possibility that the two colony types were different strains of *E. coli*, it suggested that they had arisen from the culture inoculum and neither was a contaminant.

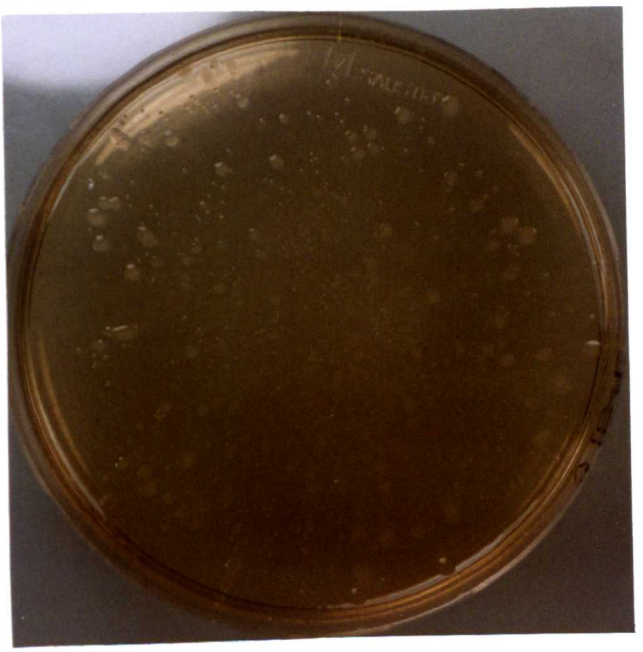


Figure 5.12. Long-term-starved cells from liquid culture showing both colonial morphologies.

Table 5.1. Biochemical properties of rough and smooth colony-forming cells

	Rough	Smooth
β-galactosidase	+	+
Arginine dihydrolase	-	-
Lysine decarboxylase	+	+
Ornithine decarboxylase	-	-
Citrate utilisation	-	-
H_2S production	-	-
Urease	-	-
Tryptophan desaminase	-	-
Indole production	+	+
Acetoin production	-	-
Gelatinase	-	-
Glucose utilisation	+	+
Mannitol utilisation	+	+
Inositol utilisation	-	-
Sorbitol utilisation	+	+
Rhamnose utilisation	+	+
Sucrose utilisation	-	-
Melibiose utilisation	+	+
Amygadalin utilisation	-	-
Arabinose utilisation	+	+
Oxidase	-	-

5.2.4. Protein profile analysis of mucoid and rough colony types

The rough and mucoid colonies were harvested from MacConkey agar plates and total protein and outer-membrane proteins were prepared as described in Section 2.5.1.3 and separated using SDS-PAGE on 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels, as described in Section 2.5.2. It was found that there were no differences in the profiles of the two cell types (data not shown).

5.2.5. Development of cross-protection

5.2.5.1. Biocide challenge

To investigate the development of cross-protection during carbon starvation, cells at various stages of incubation were exposed to varying concentrations of biocides. The biocides used were Dimochlor Triple X, (Dimex House), a chlorine based laboratory disinfectant, absolute ethanol and Stericol (Lever Industrial), a phenolic disinfectant used in laboratories and hospitals. The minimum inhibitory concentration of each biocide used was determined as described in Section 2.17.2 and this concentration was used subsequently in assessing rates of death in cultures at varying stages of starvation. The results obtained in the MIC are shown in Table 5.2. It was found that after 30 seconds no colonies arose on plating suggesting that those concentrations which killed cells did so very rapidly.

Table 5.2. Effect of starvation on bacteriocide resistance of *E. coli* K12

	Bacteriocide		
Incubation time	Dimochlor	Ethanol	Stericol
24 hour	1.4	1/192	1/24
48 hour	1.4	1/192	1/24
72 hour	2.9	1/24	1/12

E. coli K12 was incubated in batch culture for various times before being exposed to varying dilutions of each biocide. The figures refer to the minimum concentration which prevented growth. In the case of Dimochlor Triple X, the figure refers to chlorine in parts per million, for ethanol the dilution factor of absolute ethanol and for Stericol the dilution factor of a 1% (v/v) solution.

The MIC method relies on the ability of cells to grow in the presence of the biocide. Since this involved all cells entering exponential growth, it was expected that all cultures would give the same MIC value and that this value would be equal in sensitivity to exponential cells. This was not the case. It was found that between 2 and 3 days incubation, the resistance of the cells to all three biocides increased. The evidence suggests that the cells have a "memory" of their previous culture conditions and that the increased resistance to the biocides seen in starved cells is, in some unidentified way, carried over into exponential growth.

This phenomenon has been reported in stationary phase survival of cells, (Kolter, 1992; Siegele & Kolter, 1992) and the data suggest that the exponential growth of previously starved cells differs from that of cells which have not recently experienced starvation. It is possible that the difference in the cells is due to some process which occurs during stationary phase/starvation which is not

fully reversed during the first cycle of active growth which the cells encounter. The nature of this process is still to be explained, but it may involve alterations in the outer membrane of the cells which decrease their permeability and sensitivity to biocides. In particular, since the biocides used affect protein conformation and function, it is not unreasonable to suggest that starving cells synthesise a unique outer membrane protein(s) which confers increased resistance to stresses. When the cells begin active growth, this protein(s) is no longer produced, but is still present, either being degraded slowly or not at all. The reduction in the amount of this protein would therefore depend on the dilution effect of new membrane synthesis. If the number of cell divisions during batch culture is limited, either by competition from a second organism, as in Kolter's experiments (Kolter, 1992; Siegele & Kolter, 1992), or a low level of carbon source as in the present experiments, very few cells would arise which were fully sensitive to stress. The evidence for this mechanism is circumstantial at present and the hypothesis remains to be tested.

5.2.5.2. Heat resistance

48 hour-incubated, exponential and long-term-starved cells were subjected to heat shock by incubation at 60°C as described in Section 2.17.1. Samples were removed at two minute intervals and viable counts and total particle counts were carried out as described in Sections 2.3.1 and 2.3.2. Viable and total particle counts were carried out on each culture before the experiment was begun and survival was expressed as a percentage of the initial count.

It was expected that 48 hour-incubated cells would show a greater degree of resistance to heat shock than exponential cells, but this was not the case. After 4 minutes at 60°C the viable counts of the 48 hour-incubated cells dropped rapidly to 0 at 6 minutes. The exponential cells exhibited a slower decline throughout, but reached 0 at 12 minutes the decline being slightly faster between 6 and 10 minutes. Long-term-starved cells showed an initial increase in viable

counts after which their decline was less rapid than that of the other two cell types (Figure 5.13). 26.1% of the initial count showed the ability to form colonies at 10 minutes. After 12 minutes incubation at 60°C, no colonies arose on starvation plates as had been seen for the other cell types. Prolonged incubation showed no further colonies arising. With all three cell types, at each sampling time 0.1ml of sample was placed in half-strength nutrient broth. This was done in order to determine if those samples showing no growth contained recuscitable cells,. Only those samples which showed no colonies arising on viable count plates were relevant. It was found that no growth occurred in nutrient broth after up to 14 days incubation (data not shown).

The total particle counts of exponential and 48 hour-incubated cells remained unchanged throughout the incubation time, while the long-term-starved cells showed an increase in total count (data not shown). It was thought that survival may depend on the ability of the cells to produce specific heat-shock proteins at the onset of temperature challenge. Exponential cells have an adequate supply of carbon in the form of glucose and are thus better equipped to deal with heat shock. Although certain of the carbon-starvation-induced proteins are heat-shock proteins, the full complement of heat-shock proteins is not produced during carbon-starvation. Consequently, the 48 hour-incubated cells may have been unable to elicit the complete heat-shock response and gain full heat-shock resistance. In addition, the sudden demand for protein synthesis in already stressed cells may have proved too much for the cells to cope with and accelerated their death rate. Prolonged incubation of viable count plates (up to 7 days) demonstrated that no further colonies arose.

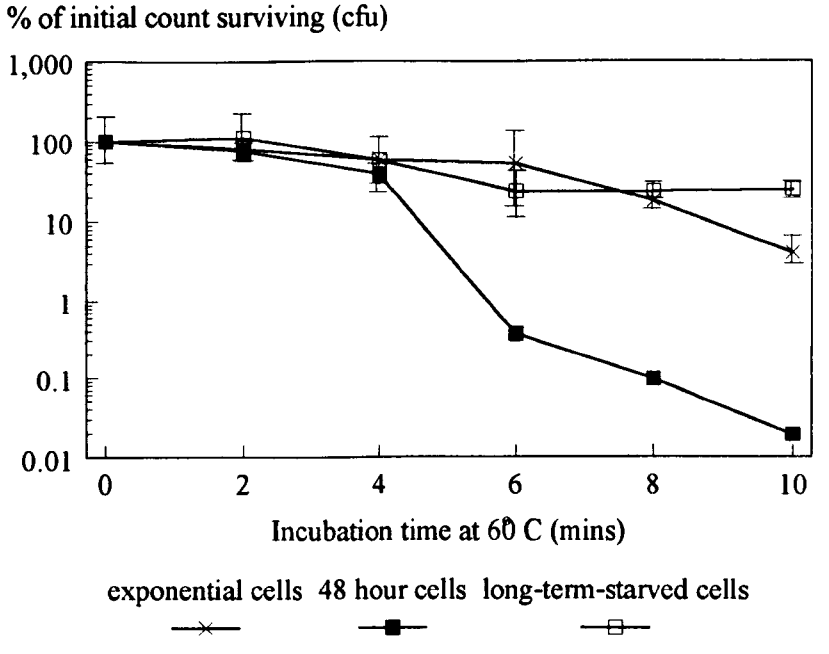


Figure 5.13. Survival of exponential, 48 hour-incubated and long-term-starved cells when exposed to 60°C. Samples were removed at two minute intervals and viable counts were carried out on starvation plates. Duplicate cultures were sampled in triplicate and the values plotted represent the mean of six samples. The error bars represent ± one standard deviation

The long-term-starved cells showed two colony types, rough and mucoid. During exposure to 60°C, it was found that the two colony types did not exhibit the same rate of death. The mucoid colony type initially showed an increase in viable cells. After 2 minutes at 60°C they then declined much more rapidly than the rough (Figure 5.14). Initially, the mucoid cells represented 41.1% of the total cells, but by 10 minutes represented only 16.8%. The increase in overall total count indicates that the increase in mucoid colony type arose by cell division rather than reversion of rough to mucoid colony types.

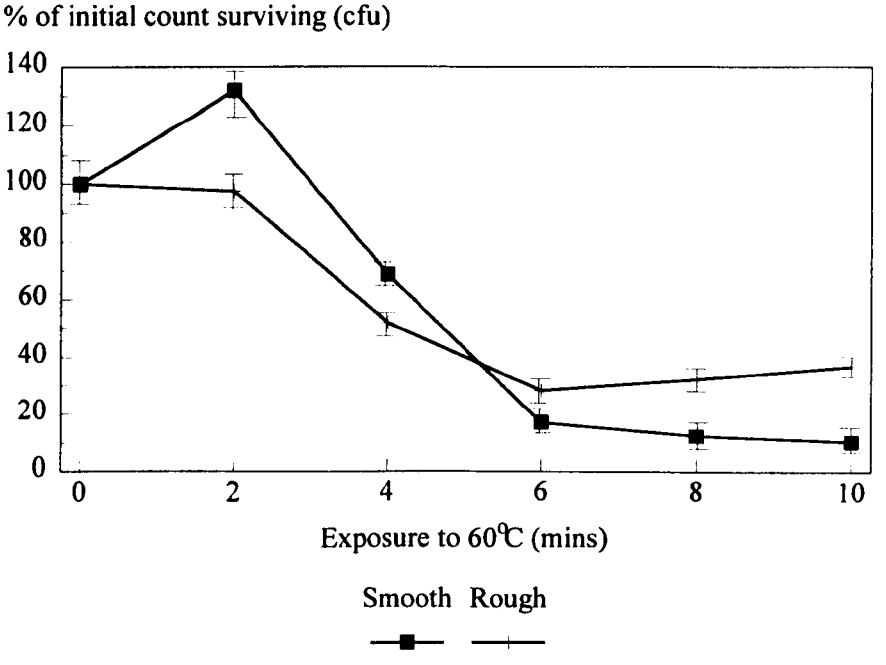


Figure 5.14. Comparison of the survival of rough and mucoid colony-forming types during incubation at 60°C. Samples were removed at two minute intervals and viable counts were carried out on starvation plates. Duplicate cultures were sampled in triplicate and the values plotted represent the mean of six samples. The error bars represent ± one standard deviation

5.2.6. Nucleoid conformation

To determine if the nucleoid of long-term-starved cells existed in a more condensed state than in actively growing cells, nucleoids were isolated from cells at daily intervals from exponential phase until 15 days incubation, 6 month and 12 month incubated cells, centrifuged on a 10-30% (w/v) sucrose gradient, the supercoiling of the DNA being maintained by the presence of 0.1mM $MgCl_2$ and the gradients collected as described in Section 2.13.

It was found that the peak absorbance at 260nm occurred at the same point in the gradient in all samples for the first 14 days, suggesting that there was no difference in the sedimentation co-efficient of nucleoids (Figure 5.15). The 15-day-incubated culture, however, showed a second peak, further down the gradient than the first (Figure 5.16). This second peak remained present throughout starvation of long-term-starved cells.

In some samples, a peak was seen to occur at the bottom of the gradient. Initially this was thought to represent a second band of very dense DNA (Figure 5.16). However, close examination of the collection procedure revealed that this represented the remainder of the first peak which had remained uncollected. This occurred when the distance between the meniscus and the band was less than the distance between the collection apertures and the meniscus detector of the probe. Ethidium bromide was added to all the fractions and spots were placed on Fresh-cling on a Transilluminator. Those fractions which contained the peak, as measured by absorbance at 260nm showed orange fluorescence, typical of ethidium bromide intercalation with DNA. Those fractions which did not contain the peak showed no fluorescence. This indicated that the peak in absorbance was due to DNA and not protein alone. The ratio of absorbance at 254:280 to determine protein could not be used as the nucleoids isolated were membrane attached and thus contained protein. The results suggest that a second, more condensed form of nucleoid was present in cells incubated for longer than 14 days.

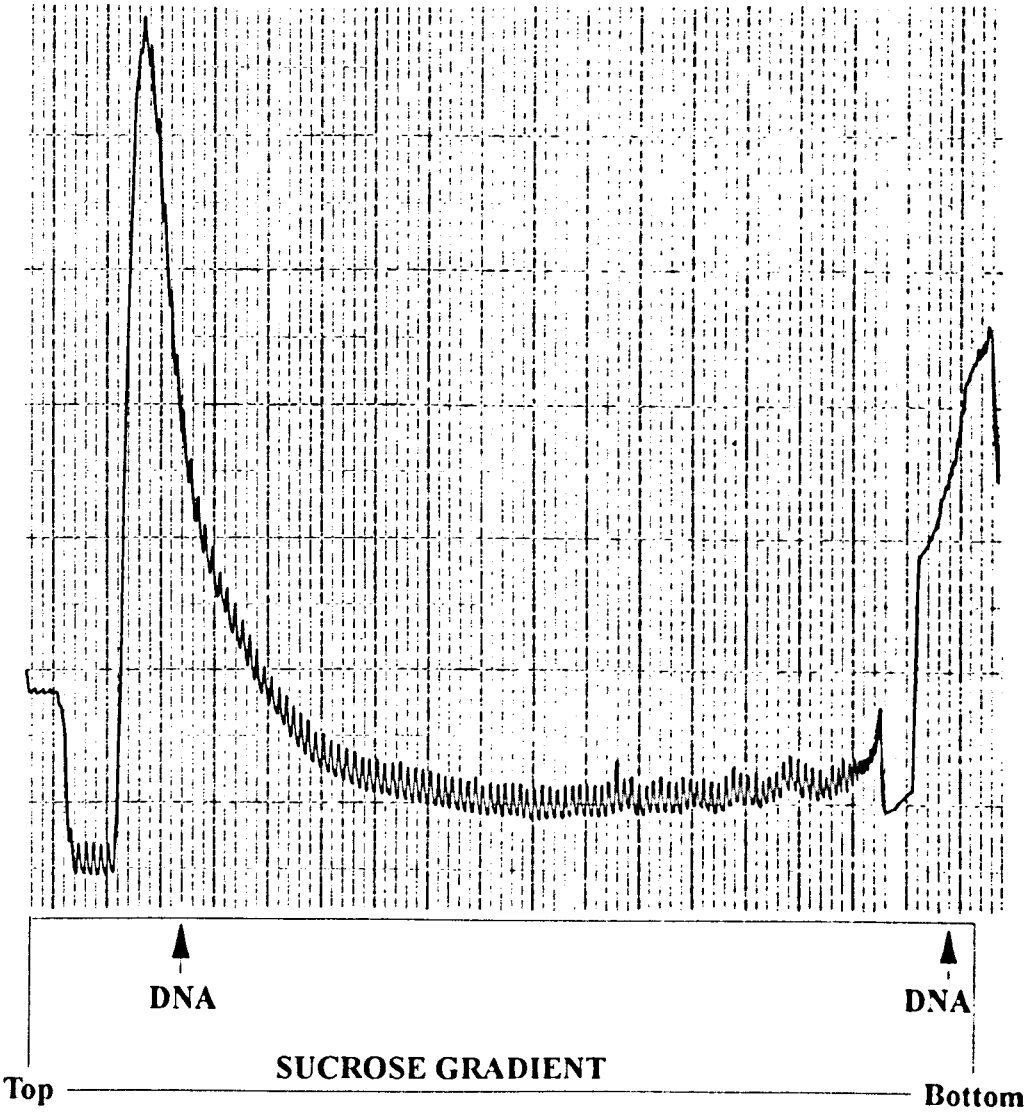


Figure 5.15. Membrane-attached nucleoids from cells incubated for 1-14 days were centrifuged on a 10-30% (w/v) sucrose gradient and the absorbance through the gradient at 260nm was measured. The position of the DNA is marked with an arrow. The second peak occurring near the bottom of the gradient was found to be an artefact of the collection procedure.

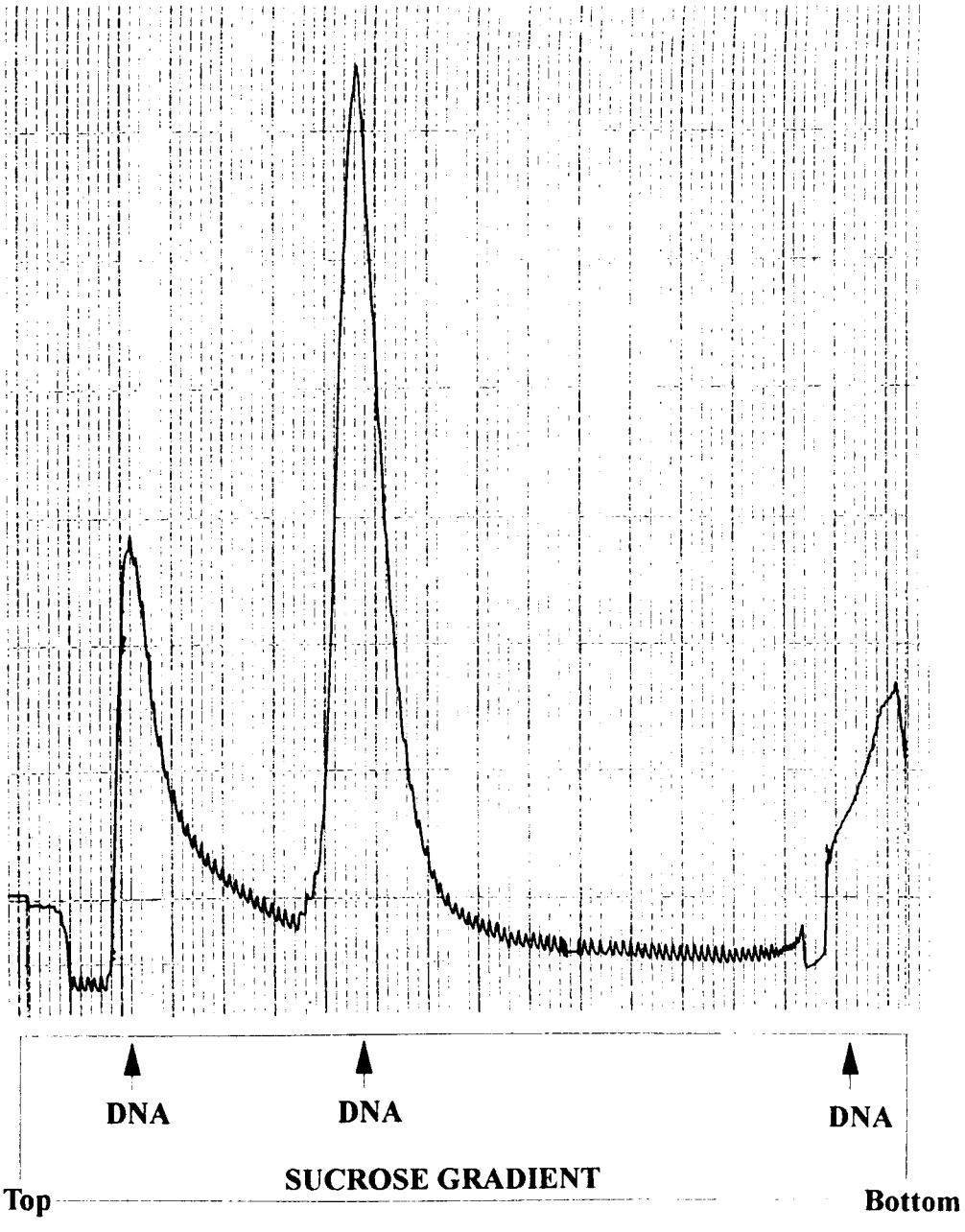


Figure 5.16. Membrane-attached nucleoids from cells incubated from 15 days up to 12 months were centrifuged on a 10-30% (w/v) sucrose gradient and the absorbance through the gradient at 260nm was measured. The positions of the DNA are marked with an arrow.

5.2.7. Electron microscopy of starving cells

Starving cells were examined by transmission electron microscopy. Whole cells were visualised by negative staining as described in Section 2.11.1. 48 hour-incubated cells showed the typical rod-shaped morphology associated with *E. coli* K12 (Figure 5.17 (a)) As starvation continued, cells showed a tendency to shorten, with bulging of the side-walls and areas of polar transparency (Figure 5.17 (b)). This is similar to the morphology seen in starving *Campylobacter jejuni* (Rollins & Colwell, 1986), *Vibrio cholerae* (Baker *et al.*, 1983) and the marine *Vibrio* species ANT-300 (Novitsky & Morita, 1976), suggestive of a polar retraction of the cytplasmic membrane and an increase in the size of the periplasm. This morphology is also seen in PBP6-overproducing mutants (Van Der Linden *et al.*, 1992) and in wild-type cells, PBP6 is present at levels 2-10 fold higher in stationary-phase than in exponentially growing cells (Buchanan & Sowell, 1982). This suggests that PBP6 may be involved in the morphological changes seen in starving cells. As discussed in Chapter 1, PBP6 has been implicated indirectly in septum formation prior to cell division. Cells were observed which suggested that some form of cell division was occurring, although the point of division was polar in position and minicell formation was indicated (Figure 5.17 (c)) In addition, by 15 days starvation, small, coccoid forms were observed (Figure 5.17 (d)). These coccoid forms appeared to possess fimbrii which the original inoculum did not possess. It was not clear whether the small, coccoid cells arose from the suggested cell division or were formed by shrinkage of cells during starvation. Cell size profiles of starving cultures showed a shift of the peak cell volume to the left, suggesting cell shrinkage. If asymmetrical cell division was occurring, two distinct peaks had been expected. However, it is possible that asymmetric division of a cell population of non-uniform size would give rise to the peak shift to the left.

To attempt to answer this question the ultra-structure of starving cells was examined by ultra-thin sectioning as described in Section 2.11.2.2. It was found

that the cells again resembled those seen in starving *Campylobacter jejuni* (Rollins & Colwell, 1986), *Vibrio cholerae* (Baker *et al.*, 1983) in that electron transparent areas were seen throughout the cells (Figure 5.18 (a)). In addition, areas varying in electron density were observed (Figure 5.18 (b)). In many cases, the areas of different density seemed to be separated from each other by a double membrane structure (Figure 15.18 (c)). The vast majority of cells appeared to be intact and had retained integrity of their membrane and wall. This suggests that, even after 18 months starvation, the cells within these particular cultures have not only retained the ability to grow and divide when nutrients are supplied, but are carrying out cell division during starvation. The electron transparency seen throughout the cytoplasm of the cells suggested that ribosomes were very few in number, which agreed with the observations made using phase-contrast microscopy.

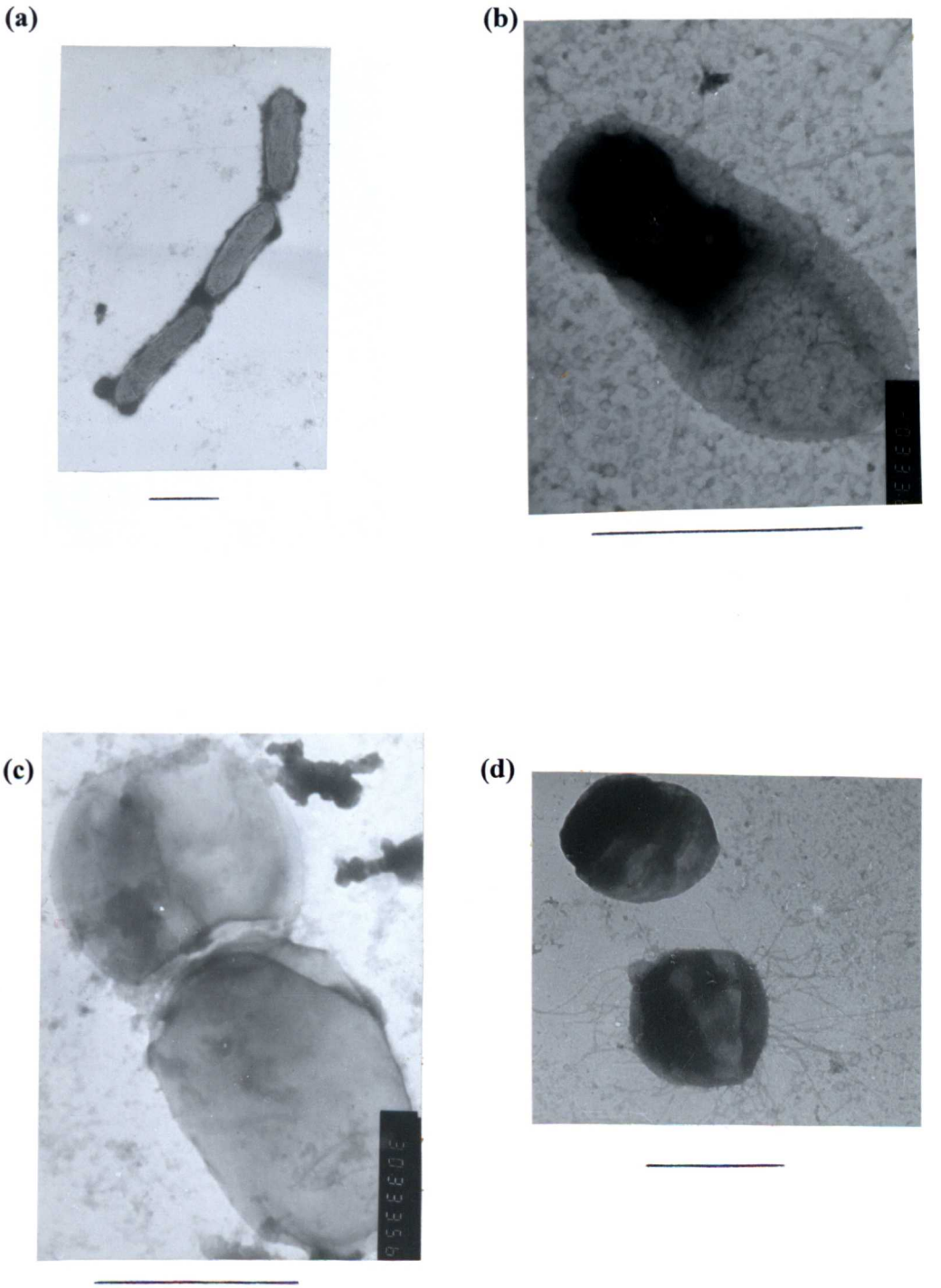


Figure 5.17. Whole cells from starving cultures were negatively stained with phosphotungstic acid and visualised by transmission electron microscopy. (a) 48 hr-incubated cells, (b) 5 day-incubated cells, (c) 9 day-incubated cells, (d) 17 day-incubated cells. The size bar beneath each photograph represents 1μm.

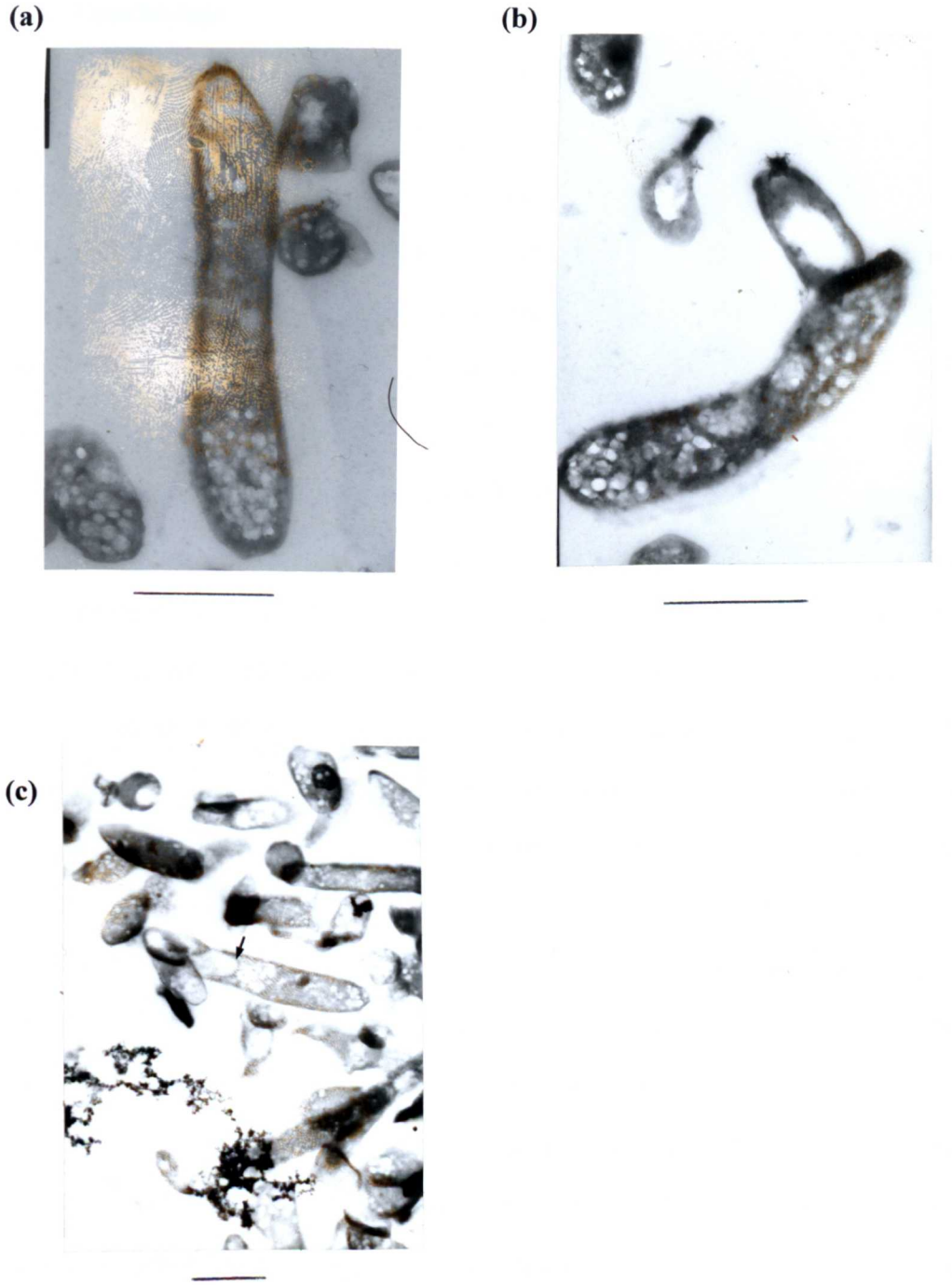


Figure 5.18. Long-term-starved cells were fixed and stained with osmium tetroxide and uranyl acetate and 60nm ultra-thin sections were cut by cryo-sectioning. The sections were observed using transmission electron microscopy. (a) vacuoles seen throughout cells, (b) photograph illustrating the electron density variations (c) double membrane structure which appears to divide the interior of the cell. The size bars beneath the photographs represents 1μm

5.3. Conclusions

During starvation for carbon, many physiological changes take place in *E. coli* which tend to enhance survival. The cell size reduction which occurs early in starvation (Chapter 3) tends to affect the sedimentation characteristics of the cells. Differences were found in the protein composition of cells with different sedimentation characteristics, suggesting that carbon-starved *E. coli* populations are heterogeneous with respect to protein profiles. This suggests that at some point during starvation for carbon cultures are also heterogeneous with respect to gene expression and protein synthesis. Investigation of protein synthesis profiles of cell types within each population was not possible since homogeneous populations were not obtained. This was not unexpected since density gradient centrifugation has proved unsuccessful at separating small cells from starved *Campylobacter jejuni* cultures (Rollins & Colwell, 1986). Although protein profiles could be examined using silver-staining which allowed the determination of differences in the presence or absence of particular proteins, possible differences in the metabolic rates of the two populations would skew any isotope-incorporation data obtained. Until a method of separating the different cell types within carbon-starved *E. coli* populations is found relative rates and patterns of protein synthesis cannot be investigated.

The staining characteristics of long-term-starved cells suggested a major change in the resistance and permeability of these cells. Various dyes proved unable to penetrate the cells after conventional and modified fixing methods. This alteration in permeability may be the cause of the enhanced resistance seen in carbon-starved cells. The phase-contrast microscopy suggested that ribosome degradation had occurred during starvation, since the refractive index of the cytoplasm of the cells had altered and light was more able to travel through the cell. This allowed visualisation of the nucleoid which, in some cases, seemed to be present in multiple copies.

The changes seen in colonial morphology during starvation suggested that exopolysaccharide may play an important dual role in starvation survival. The appearance of mucoid colonies in liquid culture suggested a major role in protection of the cell envelope. However, if the exopolysaccharide conferred enhanced survival characteristics, it would be expected that all cells within a culture would eventually become mucoid. This was not the case. Observations which have not been presented, but which may have a bearing on the role of the exopolysaccharide during this work, suggest that the availability of water may be a major switching mechanism. When agar plates were incubated for 5 days, it was seen that the agar began to dry and the large areas of exopolysaccharide diminished. This suggested that the cells were able to utilise water present in the exopolysaccharide matrix. When cells such as these were subcultured to fresh plates, it was found that they had reverted to the rough form. It is possible that in environments of high water content, such as in liquid culture, cells do not require protection against dehydration and so reversion to the rough form is seen. During incubation on agar plates, as the medium dehydrates, the availability of water decreases and the cells may not be able to afford to carry out exopolysaccharide production and so again revert to the rough format and recycle the water in the exopolysaccharide which surrounds them. The heterogeneity of colonial morphology seen when long-term-starved cells were plated out cannot be explained at present. The presence of exopolysaccharide within these liquid cultures before plating was not investigated, but it is possible that the mucoid colonies arose from cells which were already synthesising this material and continued to do so on plating. The ease with which exopolysaccharide production could be controlled by incubation conditions makes this a very interesting area for further investigation. For further information on the role of exopolysaccharide in the desiccation tolerance of prokaryotes see Potts (1994).

The differences in heat resistance of rough and mucoid colony-forming cells was unexpected, but shows that mucoid cells are less able to withstand heat-

shock than rough colony-forming cells. This may be due to the additional burden placed on the cells by exopolysaccharide production. This hypothesis suggests that exopolysaccharide production is being controlled by a mechanism which is entirely independent of the heat-shock response and, possibly, the carbon-starvation response.

The cross-protection conferred on cells by carbon-starvation seems to develop within the first 72 hours, yet the impermeability to dyes seems to take at least 6 weeks to develop. In addition, the decrease in refractive index also takes at least 6 weeks. These observations seem to be at odds with each other and cannot be explained at present.

The increased resistance to biocides seen, even though the cells entered exponential growth tends to indicate that changes which take place during carbon-starvation are not completely reversed by exponential growth. It has been shown that mutations accumulate in stationary-phase and non-growing populations. The conventional view that mutations arose from errors during DNA replication precluded the possibility of spontaneous mutation in non-growing cells. It has been suggested that stationary-phase populations had some mechanism by which they could accumulate advantageous mutations (Cairns *et al.*, 1988). Since then evidence has accumulated that this indeed occurs (Hall, 1990, 1991, 1992; Cairns & Foster, 1991; Foster, & Cairns, 1992). In addition it has been reported to occur in a eukaryotic yeast (Hall, 1992) The hypothesis proposed to explain this should be investigated. The subject was recently reviewed and considered the mechanisms by which it may occur (Foster, 1993). It has been demonstrated that the cells which tend to survive in stationary phase are mutants which manage to take over the culture and the mutation involved is in the *katF* gene which encodes the stationary-phase-specific σ^S (Zambrano *et al.*, 1993). σ^S has been shown to be a major regulator of the carbon-starvation response and has been shown to be involved in the production of a number of stress proteins (See Chapter 1). It is possible that the increased survival seen in

carbon-starved cells when subjected to biocide challenge is due to changes in the outer membrane of the cell which are controlled by σ^S and that subsequent mutation in the *katF* gene causes increased expression of the gene allowing the increased resistance to be carried over into subsequent periods of growth.

The results of the nucleoid conformation investigation suggest that, as carbon-starvation progresses, the nucleoid of the cell condenses, and indicates that very little protein synthesis is carried out during carbon-starvation. In addition the highly condensed nucleoids which gave rise to the second peak on sucrose density gradient centrifugation suggest that the nucleoids in carbon-starved *E. coli* cells are more condensed than those of stationary-phase cells. This may be for protective purposes and is analogous to the system used by *C. crescentus* and *R. vannielii* swarmer cells. The implication is that *E. coli* is able to produce a survival cell type with striking similarities to swarmer cells.

As discussed in Section 5.2.7 electron microscopy tends suggest that gross morphological changes have occurred during starvation for carbon and that these changes are seen in other species of bacteria. The polar retraction of the cytoplasm seen in these cells, in conjunction with reports in the literature on the effects of PBP6 suggest that alterations in the peptidoglycan of the cells take place and suggests that cell division may continue in long-term-starved cultures and may result in the production of a more resistant cell type.

In conclusion it can be said that the physiology of long-term-starved *E. coli* is reminiscent of the survival cell types of differentiating organisms and very different from the physiology of exponential and short-term-stationary phase cells. This suggests that *E. coli* does differentiate, but the morphology of the cells is not grossly affected and the survival cell type is not visibly distinct from the parent cell when observed by light microscopy.

Chapter 6. Surface characteristics of starved cells.

6.1. Introduction

The outer-membrane of Gram negative bacteria is in contact with the environment and as such is the organism's first line of defence against any adverse conditions it may encounter, e.g. it is known that carbon-starved cells are more resistant to other forms of stress, (Jenkins *et al.*, 1988, 1990; Volker *et al.*, 1992; Hartke *et al.*, 1994). *Bacillus* endospores are impermeable to many substances, both harmful and beneficial to the cell. This universal impermeability prevents the entry of substances which a starving cell may be unable, metabolically, to deal with. For example, resistance to some antibiotics in non-differentiating bacteria depends on the ability of the cell to pump out any antibiotic that has entered the cell before the antibiotic has time to exert its effect. Mechanisms of this type require the expenditure of energy. In carbon-starved cells, energy is limited and it may be more economical for the cell to alter its surface to prevent the entry of damaging substances. Changes in the outer-membrane composition, such as the removal of porins, may alter the permeability of the outer-membrane, while alterations in the lipopolysaccharide may prevent substances reaching the outer membrane in the first place. These changes may represent an survival form equivalent to endospores and swarmer cells of differentiating bacteria.

Changes in the hydrophobicity of the cell surface may play an important role in altering the permeability of cells. If the surface of a cell becomes more hydrophobic, harmful, water-soluble substances may be too polar to be able to penetrate. In the environment, water is a universal solute and so by increasing the hydrophobicity of the cell surface, the cell can, theoretically, increase its resistance to harmful, dissolved substances.

It has also been shown that, in an aqueous environment bacteria exhibit increased adherence to surfaces and increased cell-surface hydrophobicity (Kjelleberg & Hermansson, 1984; Hermansson *et al., 1987*). In addition, nutrient concentrations in aqueous environments tends to be higher at water-solid

interfaces allowing growth (Dawson *et al.*, 1981). In environments such as these, an increase in cell surface hydrophobicity would allow an increase in non-specific attachment to surfaces where nutrients are concentrated and the generation of biofilms. It has been demonstrated that bacteria within biofilms exhibit an increased resistance to many forms of stress, e.g. starvation, biocide challenge (Wright *et al.*, 1991; Block, 1992; Mattila-Sandholm & Wirtanen, 1992) This suggests that changes on the surface of the cell may be responsible for many of the resistance characteristics seen in starving cells

The aims of the following experiments were to examine the cell surface of long-term-starved cells with respect to protein composition, lipopolysaccharide characteristics and hydrophobicity changes. Firstly, the lipopolysaccharide of starving cells was examined to ascertain whether any alterations occurred during starvation. Secondly, the protein composition of the outer-membrane was investigated to determine whether any carbon-starvation-specific proteins were an integral part of the outer-membrane and to use any such protein for polyclonal antibody production with a view to differential staining of starving and non-starving cells. Thirdly, the hydrophobicity of the cells was assessed to determine if any changes in hydrophobicity correlated with the duration of starvation and which would enhance non-specific attachment. All of the above were compared with the characteristics of the cell surface of short-term-stationary phase and actively growing cells.

6.2. Results and discussion

6.2.1. Changes in lipopolysaccharide

The lipopolysaccharide of exponential cells, stationary phase cells and long-term-starved cells was digested as described in Section 2.12.1 and the digests separated on 15% (w/v) polyacrylamide gels containing 4M urea with 4.5% (w/v) stacking gels as described in Section 2.12.2. The lipopolysaccharide

profile was visualised by silver-staining as described in Section 2.12.3. It was found that the lipopolysaccharide of the cells was of a rough form and no typical ladder-like pattern was seen after Proteinase K digestion (Figure 6.1).

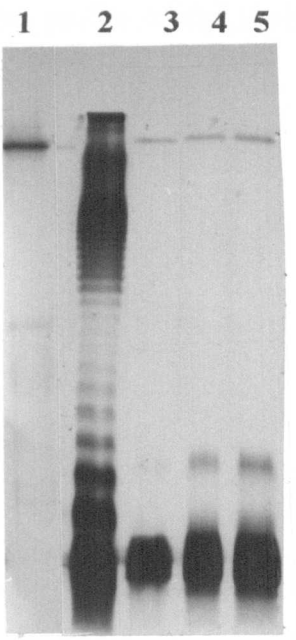


Figure 6.1.. Lipopolysaccharide samples, obtained by Proteinase K digestion of outer membrane samples, were separated on a 15% (w/v) polyacrylamide gel containing 4M urea with a 4.5% (w/v) stacking gel and visualised by lipopolysaccharide silver-staining as described in Section 2.12.3. Track 1 - Proteinase K, track 2 - *E. coli* 0111:B4 LPS, track 3 - exponential phase cells, track 4 - stationary phase cells, track 5 - long-term-starved cells. 20µl of each sample were loaded per track.

The lipopolysaccharide of mucoid cells, discussed in Chapter 5, was examined. The cells were obtained from M9 starvation plates, not liquid culture, and consequently could not be compared directly to the lipopolysaccharide profiles of exponential, stationary phase and long-term-starved cells. Rough type cells were grown on M9 starvation plates as a comparison. It was found that the rough type cells possessed the same profile as stationary and long-term-starved cells. However, the mucoid cell type possessed a profile which, while similar and lacking the typical ladder-like profile of the control, contained an extra band (arrowed in Figure 6.2).

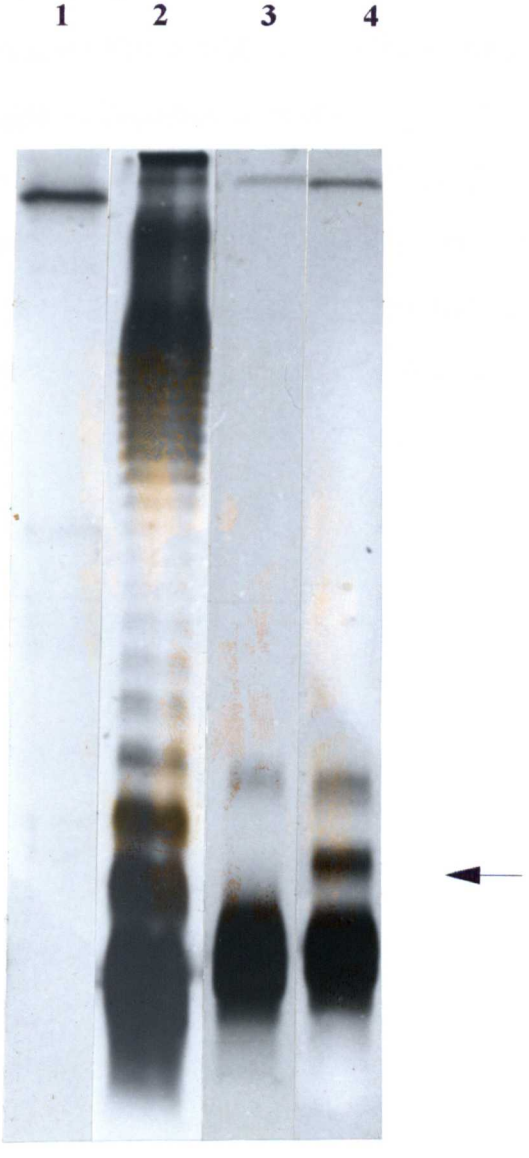


Figure 6.2.. Lipopolysaccharide samples, obtained by Proteinase K digestion of outer membrane samples, were separated on a 15% (w/v) polyacrylamide gel containing 4M urea with a 4.5% (w/v) stacking gel and visualised by lipopolysaccharide silver-staining as described in Section 2.13.3. Track 1 - Proteinase K, track 2 - *E. coli* 0111:B4 LPS, track 3 - rough type cells, track 4 - mucoid type cells.

6.2.2. Changes in outer membrane proteins

Outer membrane proteins from exponential phase, stationary phase and long-term-starved cells were isolated, as described in Section 2.5.1.3, and analysed by one-dimensional SDS-PAGE using 12% (w/v) resolving gels with 4.5% (w/v) stacking gels as described in Section 2.5.2. It was found that no major differences were evident between exponential and stationary phase outer membranes. The outer membrane of long-term-starved cells contained two proteins (arrowed, A) which did not seem to be present in the other two cell types (Figure 6.3). Exponential and stationary phase cells were radio-labelled with 50μ Ci ml^{-1} of ^{35}S-methionine for 5 minutes then immediately drop frozen in liquid nitrogen. When required, the cells were thawed, placed on ice and outer membranes were prepared as described in Section 2.5.1.3. Since ^{35}S-methionine incorporation in long-term-starved cells had been found to be very slow and longer labelling times altered the pattern of protein synthesis, (See Chapter 4), the outer membrane proteins were visualised by silver-staining in these samples (Section 2.5.4). The samples were subjected to two-dimensional-electrophoresis (Section 2.5.3). One protein was found in stationary phase and starving cells which did not appear in the exponential cell outer membrane samples, (Figures 6.4, 6.5 and 6.6). The two-dimensional analysis indicated that a major band, apparent in the one-dimensional-SDS-PAGE (arrowed, B, in Figure 6.3), contained three proteins with the same molecular weight, but different iso-electric points. The most basic of these proteins was not found in exponential phase cells. It was decided to follow the appearance of this protein as cells entered stationary phase in order to determine whether it was present uniformly throughout the outer membrane or whether it was localised, i.e. polar, suggesting that an asymmetric division was taking place.

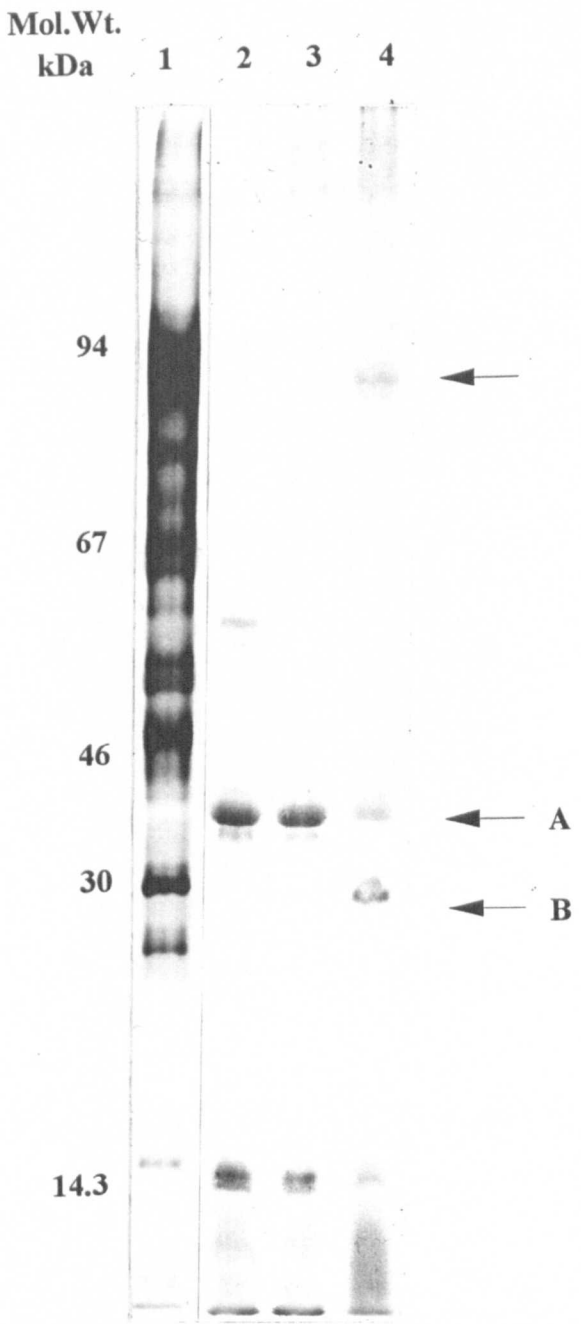


Figure 6.3. Outer membrane proteins of exponential phase, stationary phase and long-term-starved cells were separated by SDS-PAGE on a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel and visualised by silver-staining. 20μg of protein was loaded per track. Track 1 - molecular weight markers; track 2 - exponential-phase cells; track 3 - stationary-phase cells; track 4 - 18-month-starved cells.

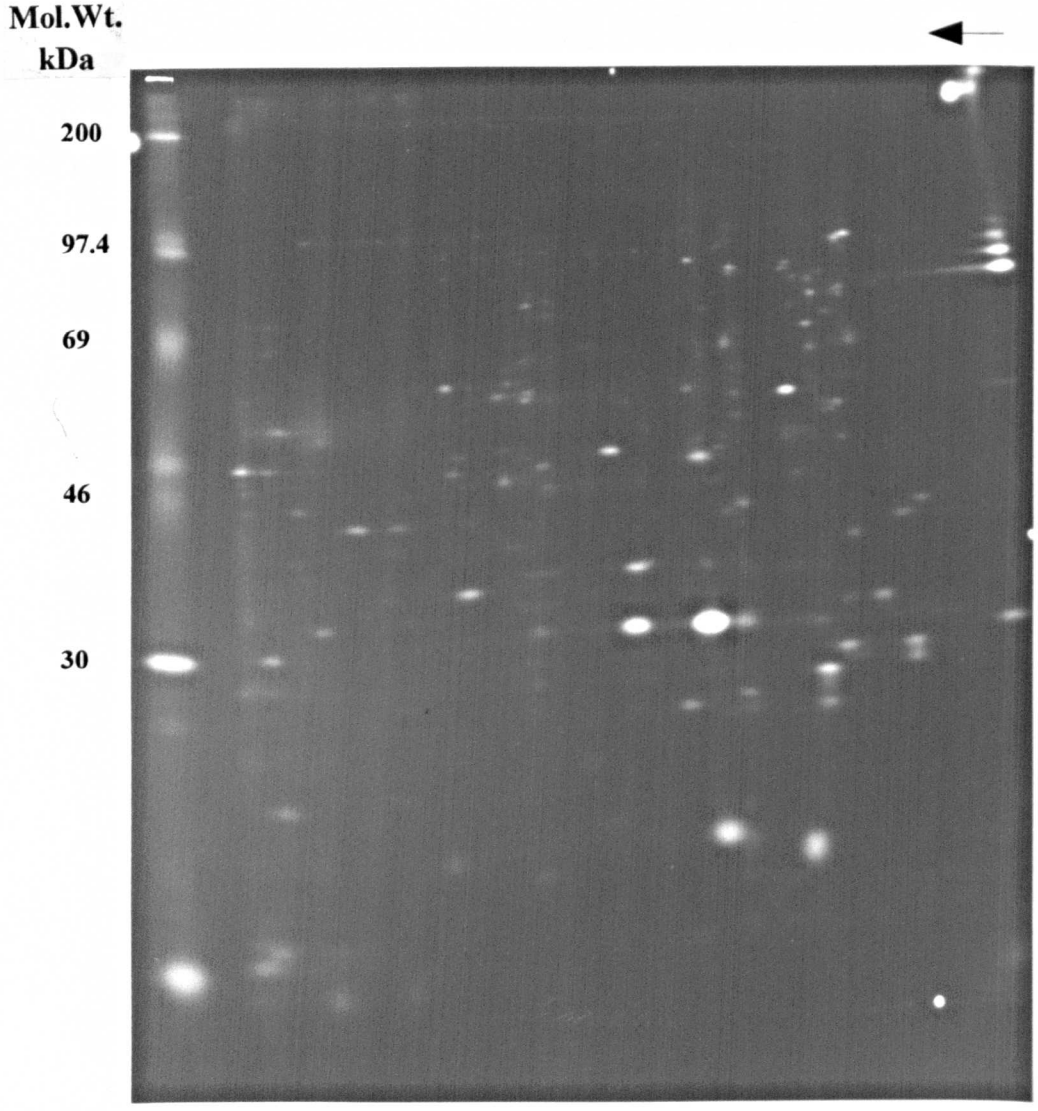


Figure 6.4. Exponential phase cells were radio-labelled with ^{35}S-methionine (50μCi ml^{-1}) and the outer membranes isolated. The outer membrane proteins were separated by two-dimensional electrophoresis with a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel in the second dimension and the proteins visualised by autoradiography. 20μl of sample was loaded onto the first dimension rod gel.

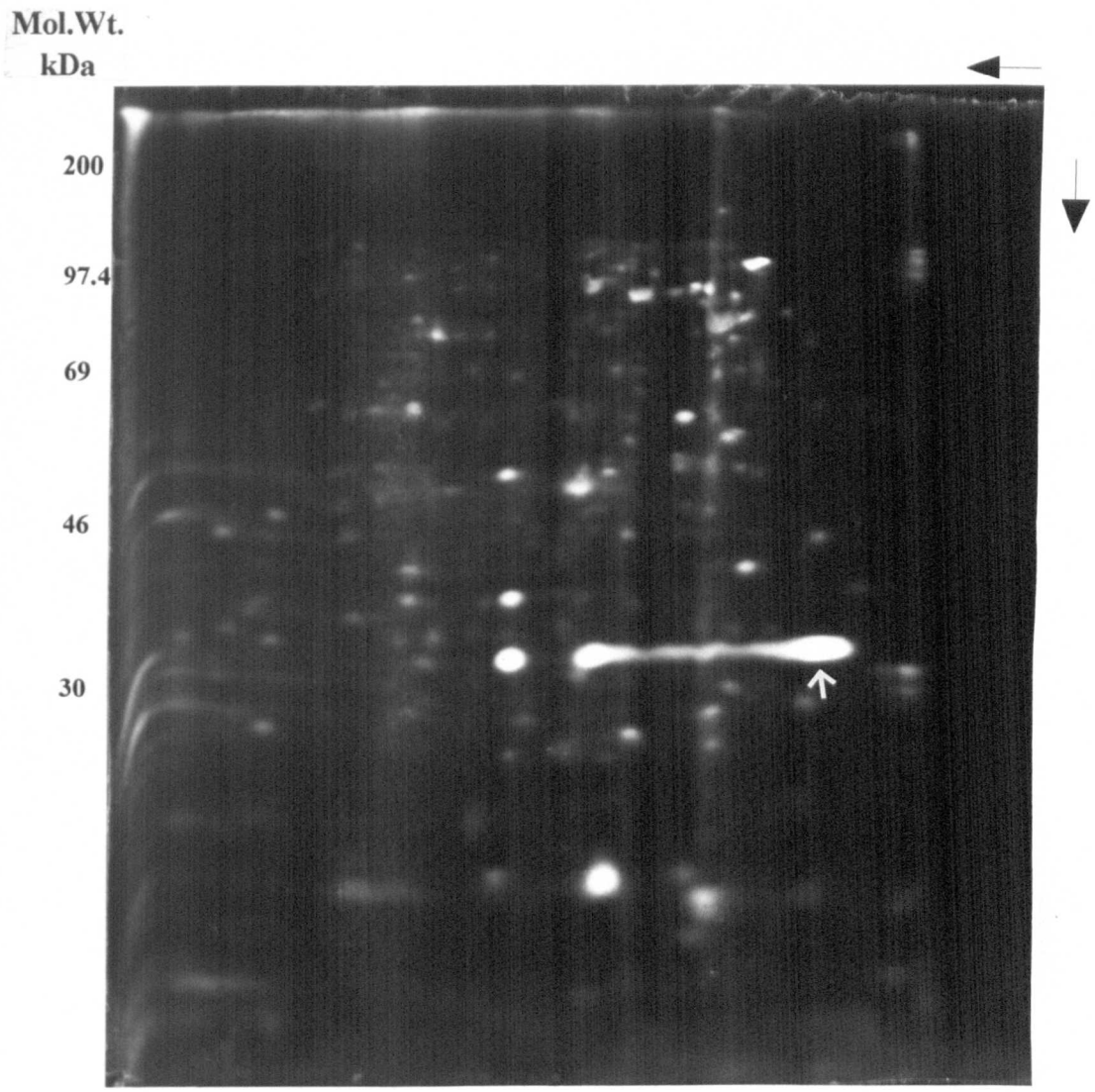


Figure 6.5. Stationary phase cells were radio-labelled with ^{35}S-methionine (50μCi ml^{-1}) and the outer membranes isolated. The outer membrane proteins were separated by two-dimensional-electrophoresis with a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel in the second dimension and the proteins visualised by autoradiography. 20μl of sample was loaded onto the first dimension rod gel.

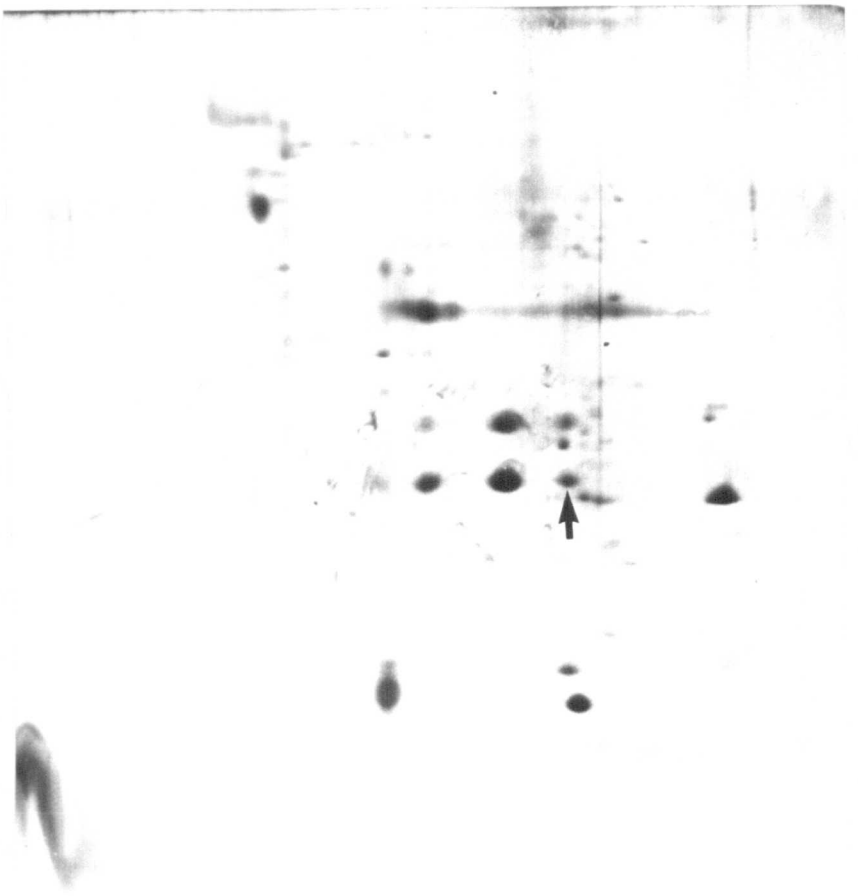


Figure 6.6. Outer membranes of long-term-starved cells were isolated and the proteins separated by two-dimensional electrophoresis using a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel in the second dimension. The proteins were visualised by silver-staining. 20μg of protein were loaded onto the first dimension rod gel.

It was decided to use an immunological approach to follow the outer-membrane protein in question (arrowed in Figures 6.5 and 6.6) by raising polyclonal antibodies and using them to immuno-label cells. Using this method it would be possible to visualise the location of the protein using immuno-gold labelling and electron microscopy.

Since proteins separated by two-dimensional-electrophoresis are difficult to stain effectively using Coomassie blue, the antigen was detected using silver-staining which is much more sensitive. However, this technique is more harsh than Coomassie blue staining. To determine whether silver-staining resulted in degradation of proteins, rendering them unsuitable for use in polyclonal antibody production, silver-stained outer-membrane proteins were destained as described in Section 2.5.5 and re-electrophoresed as described in Section 2.5.6, and the resulting gel stained with silver as described in Section 2.5.4. It can be seen from Figure 6.7 that, although some degradation had occurred, most of the proteins formed a diagonal line across the gel, indicating that their size had not altered. Although this does not give any indication of changes in antigenicity or alteration of epitopes on the proteins, it was judged that silver-stained proteins were adequate for polyclonal antibody production.

Figure 6.7. 20μg of outer membrane proteins of stationary phase cells were separated by SDS-PAGE on a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel and visualised by silver-staining. The track was cut out and destained, then proteins were re-electrophoresed on the same gel system and visualised by silver-staining. The diagonal line obtained indicated that very little degradation had occurred during the primary silver-staining process.

Using two-dimensional-electrophoresis, the outer-membrane protein identified in Figure 6.5 was isolated from a number of gels and used to immunise a half-lop rabbit, as described in Section 2.16.1. Pre-immune serum was analysed by Western blotting of outer-membrane proteins which had been separated by one-dimensional SDS-PAGE on a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel as described in Section 2.16.2. Five proteins showed the ability to bind antibody at a 1/100 dilution of the pre-immune serum, although four bound antibody only weakly (Figure 6.8). Although this was probably due to non-specific binding at a low dilution, of concern was the fact that the strongest band evident corresponded to the predicted location of the band containing the protein under investigation. To determine if antibody to this particular protein was present in the pre-immune serum, or whether the binding was due to the other two proteins known to be present in the band, outer-membrane samples were prepared for two-dimensional polyacrylamide gel electrophoresis as described in Section 2.5.1.3 and electrophoresed as described in Section 2.5.3 using a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel in the second dimension. Western blotting was carried out as described in Section 2.16.2 and antigen detected as in Section 2.16.3. The pre-immune serum was used at a dilution of 1/75 to compensate for the reduction in protein present in individual spots as compared with the three-protein bands in one-dimensional gels. It was found that no binding took place on the two-dimensional Western blot (data not shown). This suggested that non-specific binding was responsible for the appearance of bands in the one-dimensional blot and that this would not interfere with subsequent work on immune serum.

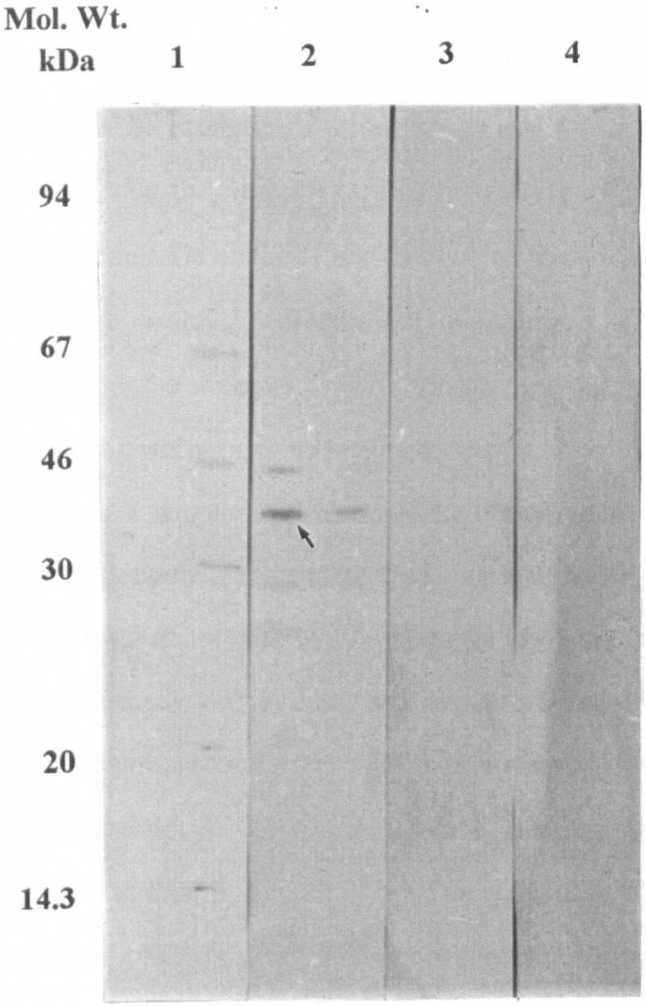


Figure 6.8. Western blot of outer-membrane proteins separated by SDS-PAGE using a 12% (w/v) resolving gel with a 4.5% (w/v) stacking gel showing the reaction obtained with pre-immune antiserum. Track 1 - molecular weight markers; track 2 - pre-immune; 1/100 dilution; track 3 - pre-immune; 1/500 dilution; track 4 - pre-immune; 1/1000 dilution.

Immune serum was collected 10 days after the first boost with the protein and a Western blot was carried out on a one-dimensional-gel of outer membrane proteins. It was found that the band containing the protein of interest was exhibiting increased antibody binding, (data not shown), suggesting that the antibody titre of the serum was increasing. After 2 boosts with antigen, the serum was checked for the presence of antibody. It was found, using outer membrane protein samples, that the antibody titre had risen (Figure 6.9). However, this did not indicate whether the antibody was binding to the correct protein, since it was known that three proteins were present in this band. In addition, two other proteins exhibited the ability to bind antibody, one of which appeared to bind antibody more efficiently than the band in question.

The protein sample was separated by two-dimensional-gel electrophoresis as described in Section 2.5.3 and blotted. It was found that all three proteins present in the band of interest bound antibody (data not shown) suggesting that they were antigenically very similar, or that the original protein preparation used for immunisation was contaminated with the other two proteins.

To determine if the proteins were similar, they were N-terminal sequenced, as described in Section 2.6. The sequences obtained were identical; alanine - proline - X - aspartic acid - asparagine - threonine. A search of the Swissprot database revealed that this sequence has 83.3% similarity with the outer -membrane protein A precursor of *E. coli*. This suggests that the different mobilities in iso-electric focusing were due to a post-translational modification of the protein, possibly acylation and that this modification does not occur until cells have entered stationary phase. It may represent activation of the protein in response to carbon-starvation. Other sequences from the database which matched were of a different size. However, it must be stated that only six residues were sequenced, one of which was unidentified, thus the identification of the proteins can only be presumptive. This is an area requiring further study.

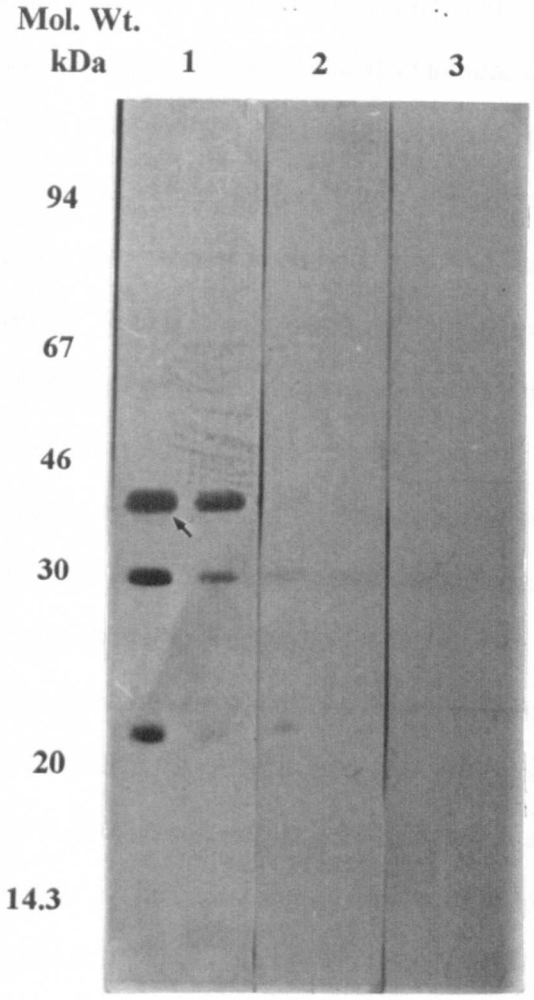


Figure 6.9. Outer membrane proteins from stationary phase cells were separated by SDS-PAGE on a 12% (w/v) resolving gel with a 4.5% (w/v) stacking gel. 20μg of protein were loaded per track. Western blotting was carried out using post-immune serum at a range of dilutions. Track 1 - 1/100; track 2 -1/500; track 3 - 1/1 000. Molecular weight marker track is not shown, but their position is marked.

6.2.3. Changes in cell-surface hydrophobicity

The hydrophobicity of cells was estimated using the bacterial adherence to hexadecane (BATH) and hydrophobic interaction chromatography, (HIC), methods as described in Sections 2.14.1 and 2.14.2 respectively. It has been shown that hydrophobic bacteria tend to adhere to surfaces more readily than less hydrophobic ones and that bacteria in starvation conditions are more likely to attach to a surface than actively growing bacteria (Kjelleberg & Hermansson, 1984). It has also been demonstrated that, in batch culture, the hydrophobicity of cells changes during the growth curve. Spencely (1994) showed that the hydrophobicity of a culture drops as cells enter the exponential phase of growth then increases as stationary phase approaches. To determine if cell-surface hydrophobicity increased in *E. coli* during starvation, exponential-phase, stationary-phase and long-term-starved cells were compared with each other and the results are shown in Table 6.1.

In general it was found that there was good correlation between the two methods. Although, in certain cases, the values obtained were different (e.g. 24 and 120 hour-incubated cultures), both methods showed the same trends in the hydrophobicity of the cells as starvation continued. The cells showed an initial, slow decrease in hydrophobicity then an increase

As a consequence of the differences in starvation-survival found when cells were grown in differing concentrations of glucose, (Chapter 3), the same experiment was repeated using cells grown in 0.5% (w/v) glucose. The results are shown in Table 6.1. Figure 6.10 shows a comparison of the hydrophobicity values of cells grown under both conditions as determined by BATH.

Table 6.1.

Comparison of hydrophobicity values obtained by BATH and HIC on cells grown in M9 medium with 0.05 and 0.5% (w/v) glucose

Cell type	% adherence to n-hexadecane		% adherence to phenyl-sepharose	
	Glucose concentration (w/v)			
	0.05%	0.5%	0.05%	0.5%
Exponential cells	40.4 (2.6)	10.0 (1.1)	21 (1.4)	14.5 (3.6)
24 hour	74.6(3.7)	21.0 (3.1)	57.2 (7.1)	33.3 (4.0)
48 hour	44.5 (3.9)	51.9 (1.5)	56.7 (3.7)	51.1 (0.6)
72 hour	34.5 (1.5)	20.4 (3.3)	30.0 (8.8)	21.5 (7.2)
96 hour	58.6 (6.8)	42.8 (3.7)	49.3 (9.2)	35.3 (2.6)
120 hour	68.5 (13.9)	39.5 (10.6)	46.3 (5.8)	27.0 (6.3)
8 days	53.1 (4.2)	29.6 (4.4)	49.1 (5.9)	30.4 (3.1)
18 month-starved	56.0 (1.4)		50.2 (2.1)	

Values given are the mean value of three samples on two separate occasions and the standard deviation is given in brackets

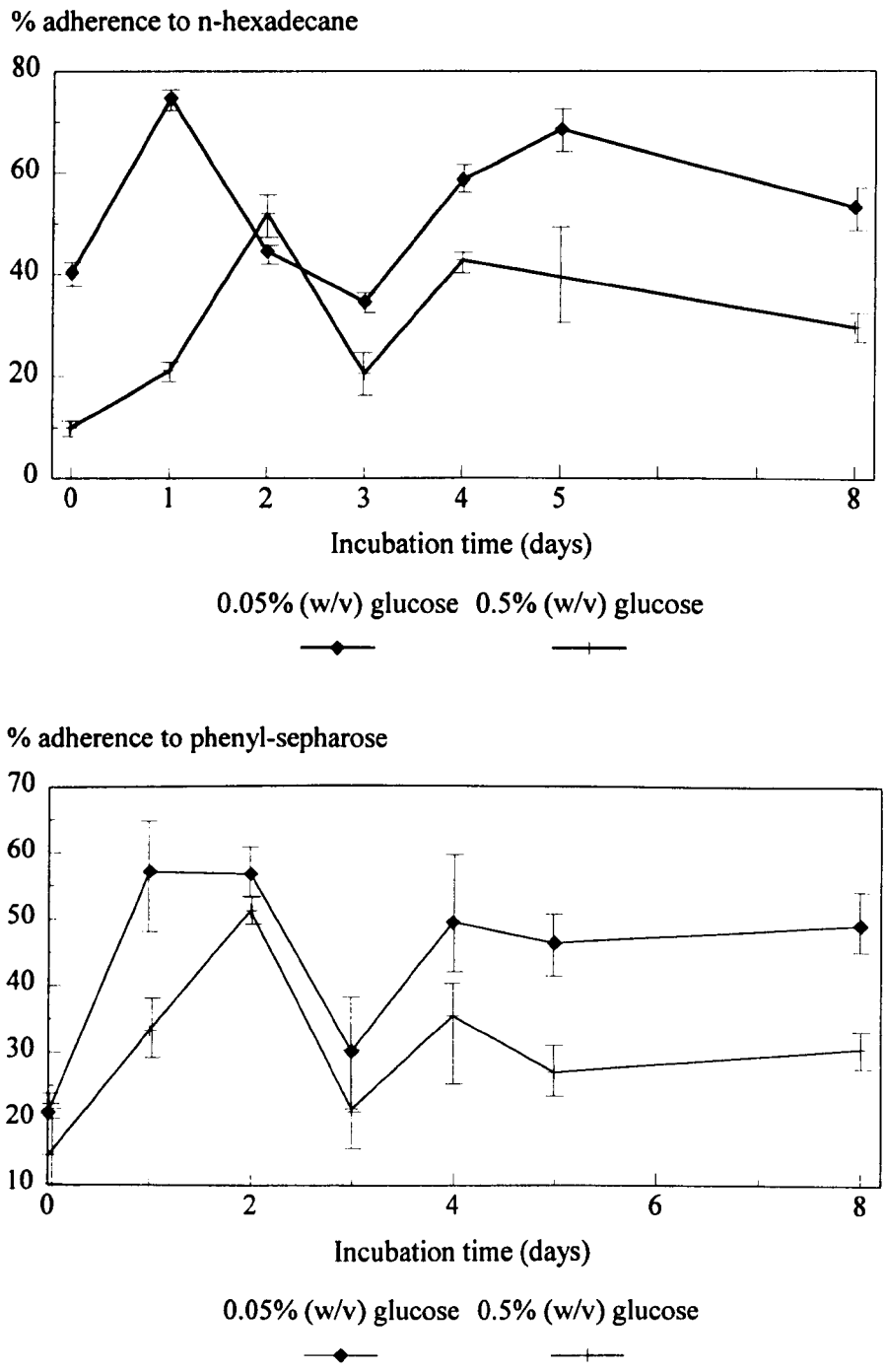


Figure 6.10. Graphical representation of data presented in Table 6.1 Cells were grown in M9 medium with glucose added to the required concentration. The error bars represent ± one standard deviation

The percentage adherence to n-hexadecane and to phenyl-sepharose of the exponential cells was very low compared with that of the stationary phase and long-term-starved cells. The higher values for the stationary phase cells as compared with the exponential phase cells was expected. It has been shown that bacterial adherence to surfaces increases the probability of finding nutrients (Dawson *et al.*, 1981) and that bacteria contained within biofilms are more resistant to many forms of environmental stress. As the cells begin to enter stationary phase nutrients are depleted and it is thought that, at this point, the bacteria begin to adhere to surfaces. Specific forms of adherence, such as pili, require the presence of a specific receptor. If the receptor is not available, the bacteria are unable to adhere. In starvation and stress survival a more non-specific form of adherence is required to allow the bacteria to adhere to whatever surfaces are available. Biofilms form on many different types of material, e.g. stainless steel, plastics, rubber, rocks and soil particles. An increased surface hydrophobicity in an aquatic environment would allow cells to attach to any surface. As starvation continued the hydrophobicity of the cells decreased, possibly representing an attempt to escape the starvation conditions either by release from any surface the cells are adhering to, or the release of progeny in order to ensure the survival of the genetic material. This theory is supported by the findings of Spencely (1994), that the peak cell volume of cells in the eluate from a phenyl-sepharose column was much smaller than the peak cell volume of the original culture and surmised that younger cells were less hydrophobic than the parent cell and therefore were eluted from the column.

The results obtained for long-term-starved cells, shown in Table6.1 indicate that the non-specific adherence ability of these cells remained high. Maximum hydrophobicity was achieved after 24 hours incubation and then decreased until 3 days incubation. This corresponded with the increase in total particle counts seen after 2-3 days incubation (Chapter 3). It is possible that this increase in total particle count and decrease in hydrophobicity represents a

starvation-induced cell division, asymmetric with respect to cell size and hydrophobicity, the daughter cells being smaller and less hydrophobic and therefore unlikely to adhere in close proximity to the carbon-starved parent cell and more likely to be dispersed into the planktonic phase. This suggests that during periods of non-growth due to a lack of carbon source, non-differentiating bacteria such as *E. coli* produce a small daughter cell which is morphologically distinct from the parent cell and it is possible that this daughter cell is a survival cell type, analogous to the endospores of *B. subtilis* and the swarmer cells of *R. vannielii* and *C. crescentus* and destined for dispersal.

To separate and obtain samples of cells which differed in surface hydrophobicity an 10ml phenyl-sepharose column was prepared as described in Section 2.4.3. A 5-day old culture of *E. coli* K12 was radio-labelled with ^{35}S-methionine (50μCi ml^{-1}) for 5 minutes at 37°C, then incorporation was stopped by the addition of 10mM unlabelled methionine. This was necessary because the method of preparation to obtain cells for the column would induce salt-shock protein synthesis and it has been shown that many of the stress proteins produced in response to a particular stress overlap with carbon-starvation proteins (Jenkins *et al.*, 1990; Volker *et al.*, 1992). The cells were harvested, washed in 1M NaCl and applied to the column as described in Section 2.4.3. 1ml fractions of the eluate were collected. Figure 6.11 shows the absorbance at 600nm of the eluate fractions. After an initial increase in absorbance, representing the initial salt washing and non-adherent cells eluting, a plateau was reached. The fractions from this plateau were pooled and the cells harvested as Eluate 1. The addition of cells to the top of the column was continued until the absorbance of the eluate increased and reached a plateau which was close to the absorbance of the initial cell preparation. At this point, the column was considered to be saturated and 1M NaCl was run down the column to remove all non-adherent cells. The hydrophobic adherence was reversed using 0.05M NaCl and the eluate collected. An increase in optical density was seen, followed by a decrease. The fractions

were again pooled and the cells harvested as Eluate 2. The column was washed with 0.05M NaCl until the absorbance reached close to zero. 0.01M NaCl was added at the top of the column and the fractions collected as Eluate 3. The cells from the three eluates were harvested as described in Section 2.4.3 and prepared for SDS-PAGE analysis as described in Section 2.5.1.1. The protein samples were loaded in duplicate onto 12% (w/v) polyacrylamide gels with 4.5% (w/v) stacking gels (Section 2.5.2). After electrophoresis, the gel was cut in half. One half was stained with silver, as described in Section 2.5.4 and the other half was dried and autoradiography carried out as described in Section 2.5.8. Cells untreated with the phenyl-sepharose column were included as a control. Figure 6.12 shows the silver-stained gel and the autoradiograph obtained.

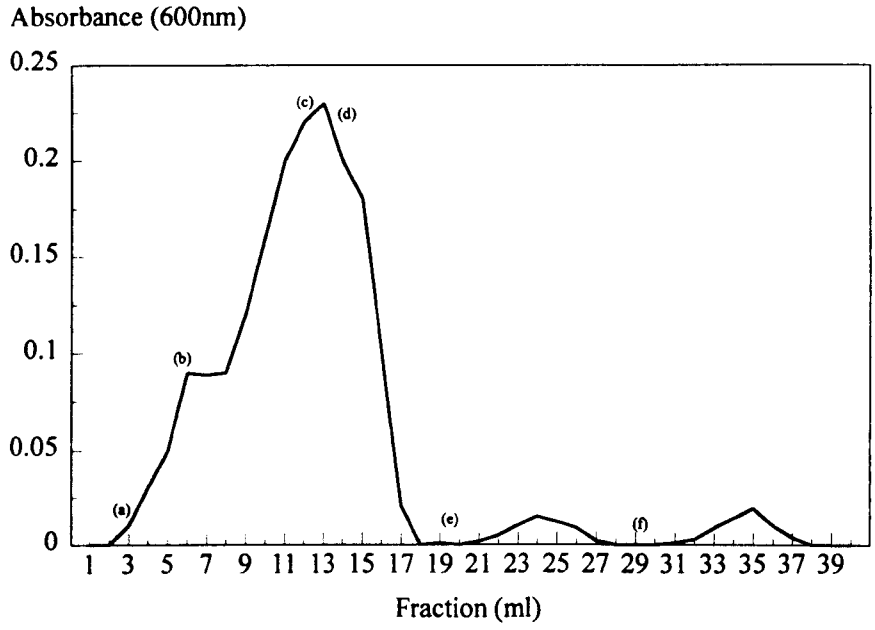


Figure 6.11. Absorbance at 600nm of the eluate from an 8cm column during washing with a discontinuous NaCl gradient, as described above. (a) Cells added to top of column; (b) plateau obtained as non-adherent cells eluted; (c) column saturation; (d) 1M NaCl wash; (e) 0.05M NaCl added; (f) 0.01M NaCl added.

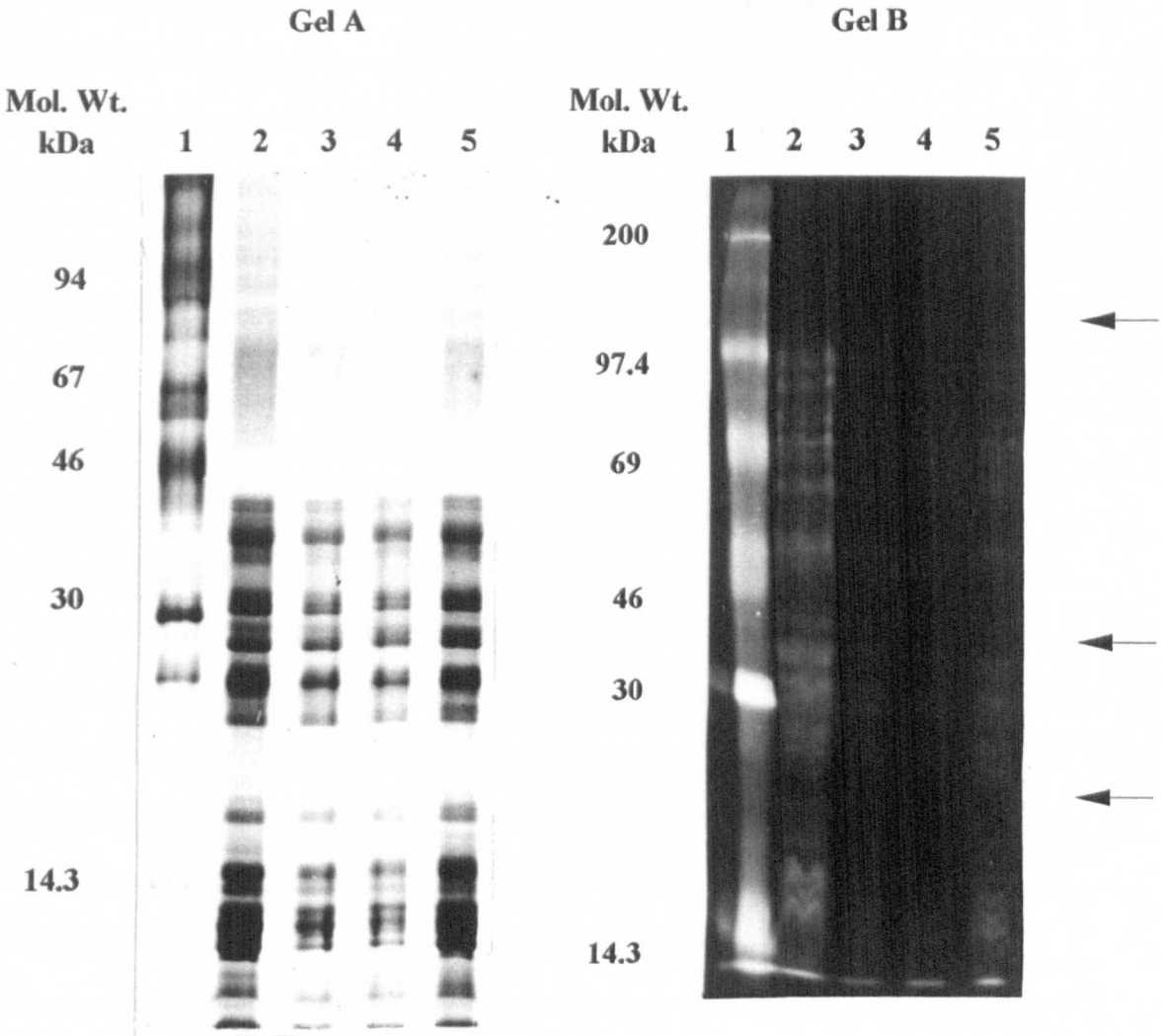


Figure 6.12. Cells were radio-labelled with 50μCi ml^{-1} ^{35}S-methionine then incorporation was stopped by the addition of 10mM unlabelled methionine before the cells were separated on a phenyl-sepharose column. Total proteins from cells eluted from the phenyl-sepharose column using differing NaCl concentrations were separated by SDS-PAGE on a 12% (w/v) polyacrylamide gel with a 4.5% (w/v) stacking gel. 20μg of protein were loaded per track. Proteins were visualised by silver-staining (Gel A) and autoradiography (Gel B). Track 1 - molecular weight markers; track 2 - control cells; pre-column; track 3 - Eluate 1; 1M NaCl; track 4 - 0.05M NaCl; track 5 - 0.01M NaCl.

No obvious differences were found between the eluates and the control cells when the proteins were visualised by silver-staining. However, the autoradiograph shows minor differences in the proteins being synthesised by the cells in the eluates. The bands marked with arrows seem to be synthesised in eluate 3 and cells untreated with the column, but not in eluates 1 or 2, i.e. the less hydrophobic cells. In addition, the cells in eluate 3 appear to have incorporated more ^{35}S-methionine than those in eluates 1 or 2, suggesting that protein synthesis is more rapid in eluate 3. It was suggested above that cells with reduced surface hydrophobicity may be produced by carbon-starved cells as a method of dispersal. The reduced isotope incorporation in cells with a lower hydrophobicity, (eluates 1 and 2) suggests that these cells are less metabolically active than those cells which have a higher cell surface hydrophobicity. It is known that *C. crescentus* and *R. vannielii* swarmer cells are mechanisms by which dispersal is achieved. The swarmer cells are metabolically inactive and no cell division occurs until a favourable environment has been encountered and differentiation into the vegetative cell type has occurred (see Chapter 1). The results presented here suggest that an analogous system may be operating in *E. coli* and that further investigation is warranted.

Although great care was taken to ensure that the same amount of protein was loaded per track (20μg) it must be considered a possibility that the difference in band intensity seen on autoradiography is due to differences in the amount of protein loaded. This possibility is due to the fact that the harvest of cells from a 10ml phenyl-sepharose column was very small. To verify the data presented in Figure 6.14 a much larger phenyl-sepharose column is required and a larger volume of culture. In addition, the hydrophobicity of cells is unlikely to change in discrete steps and a continuous NaCl concentration gradient should be used.

One of the major difficulties encountered when studying cells which are starved for carbon is the difficulty in obtaining a homogeneous population and this problem was discussed in Chapters 3 and 5. If cell-surface hydrophobicity

can be used as a method of resolving a heterogeneous population of cells, carbon-starved cells will be more amenable to study.

6.3. Conclusions

The nature of the cell surface is very important in starvation survival as it is the point of contact between the bacterial cell and it's surroundings. The results presented in this chapter demonstrate that changes to the cell surface occur when cells are starved for carbon. These changes, which may enhance carbon-starvation-survival encompass lipopolysaccharide, outer membrane proteins and cell-surface hydrophobicity. The reasons for these changes are not known, but it is possible that they are responsible, in part, for a change in cell permeability. As discussed in the introduction to this chapter, cells may alter their permeability to to exclude harmful substances which they are unable to deal with during carbon starvation. In addition the changes which take place may result in a cell surface which is more difficult to damage, e.g. membrane damage by organic solvents, preventing structural damage which the cell would be unable to repair, given the paucity of nutrients. The changes in the protein composition of the outer membrane may represent nutrient uptake systems which are not expressed when cells are not starved for nutrients and may represent scavenging systems to allow more efficient uptake of nutrients present at a very low concentration such as that seen in the marine *Vibrio* species S14 which produces a glucose-binding protein with a higher affinity for substrate than that produced when glucose is plentiful (Albertson *et al.*, 1990b). Changes in the outer-membrane proteins of stationary-phase cells have been reported previously (Alexander & St John, 1994; Kragelund & Nybroe, 1994), but the significance of these are not known. Changes in the phospholipid component of the outer membrane were not investigated, but it is known that unsaturated fatty acids in phospholipids are methylated on transition to stationary phase (Ingraham, 1987). In addition, the CFA synthase gene has been shown to be induced by σ^S (Wang & Cronan, 1994), indicating that the modification of phospholipids in the membrane is seen to be important to survival by the cell.

These changes to the surface of the bacterial cell may result in the production of a survival cell type, analogous to the swarmer cells of *C. crescentus* and *R. vannielii* and the spores of the *Bacillus* and *Clostridium* species, suggesting that *E. coli* cells may possess a non-obligate "life-cycle", although the cells produced are not visibly distinct from the normal vegetative cell.

It is not clear if the changes occurring are the result of a starving cell altering components of its surface or whether the starving cell produces a morphologically dissimilar daughter cell since the immunological studies could not be carried out, but the hydrophobicity data suggests that a division is occurring resulting in the production of a less hydrophobic daughter cell. This may occur by asymmetric division, previously only described in *E. coli* during the production of non-viable minicells. This remains an area for further study.

The results suggest that populations of carbon-starved cells are heterogeneous making the study of these cells difficult. Separation based on cell-surface hydrophobicity has been shown to be a promising tool in the investigation of different physiological cell types within a heterogeneous population.

Chapter 7. Conclusions

Non-differentiating bacteria are able to survive nutrient deprivation for surprisingly long periods and the physiological changes which take place under these conditions are of importance in many areas. The existence of the viable but non-culturable state has major implications in public heath and food safety fields. Most human bacterial pathogens are non-differentiating organisms, e.g. *Salmonella*, *Campylobacter*, *Shigella* and *Vibrio* species and enteropathogenic *E. coli* such as the 0157:H7 serotype which causes haemorrhagic colitis and haemolytic uraemic syndrome. Routine laboratory detection of these pathogens relies on standard cultivation techniques - methods which would fail to detect viable but non-culturable organisms.

The carbon-starvation response induces changes in cell physiology which render cells more resistant to various forms of stress such as bacteriocide and heat challenge which may enable them to survive routine disinfection procedures. In addition, the tendency of starving cells to adhere in clumps and to surfaces, forming biofilms, further enhances this resistance.

The results presented in this work suggest that *E. coli* survives carbon starvation extremely well and for long periods. The longest starvation period in the present work was 2 years , yet viable, culturable cells could still be recovered on starvation medium.

The survival of *E. coli* when starved for carbon has been shown to be due more to the rate of growth of the cells when starvation is encountered than the phase of the growth curve the cells are in. The increased sensitivity of cells to carbon-starvation as the lag period occurs has been demonstrated. It has been demonstrated previously that, during lag phase in fresh medium *E. coli* becomes more sensitive to various stresses and that this increased susceptibility is dependent on protein synthesis. This is also seen in the endospores of organisms such a *Bacillus* species and in the swarmer cells of *Rhodomicrobium* and *Caulobacter* species.

The increase in cell numbers seen as cultures were subjected to prolonged incubation suggests that a form of reductive division may be taking place as is seen in marine *Vibrio* species. The decrease in hydrophobicity seen as the cell numbers increase suggests that the progeny are less hydrophobic than the parent cell type and that this may be a mechanism by which the progeny will not adhere to surfaces in the immediate vicinity of the starving parent cells. Gilbert *et al.*, (1991) found that daughter cells released from a perfused filter system were less hydrophobic than the attached parent cells and Spencely (1993) demonstrated that cells which did not adhere to phenyl-sepharose were smaller than the adherent cells. The ability of *E. coli* to grow in a polar manner at very low growth rates (Donachie & Begg, 1970; Begg & Donachie, 1977) suggests that the changes seen in these conditions represent an asymmetric division, the parent cell producing a morphologically different progeny. Immunological experiments to determine if this mechanism operated could not be carried out as the antibody raised to a starvation-specific protein in the outer-membrane cross-reacted with protein present in exponential-phase cells.

Macromolecular synthesis rates in carbon-starved *E. coli* cells are different from that seen in vegetative cells or cells in early stationary-phase. The rate of bulk protein synthesis is very low, but some protein synthesis was seen to occur. The production of two proteins synthesised during carbon-starvation was seen to stop during nutrient up-shift and the proteins were swiftly degraded. This is similar to the protein produced by *R. vannielii* swarmer cells which is thought to be a repressor of swarmer cell differentiation (Porter & Dow ,1987) and may represent an analogous system preventing resumption of growth until nutrients have been available for a certain period.

The differences in protein synthesis profiles seen when leucine or methionine were provided suggest that radio-labelling of long-term-starved cells with ^{35}S-methionine may act as a nutrient upshift and therefore cannot be used to

examine the patterns of proteins synthesised during carbon-starvation. It may be more appropriate to use ^{3}H-leucine.

The rate of RNA synthesis in carbon-starved *E. coli* was seen to be very low, but resumption was achieved rapidly by the addition of methionine, indicating that the cells are able to respond rapidly to changes in nutrient availability and resume vegetative growth. The low levels of RNA synthesis is reminiscent of the swarmer cells of *R. vannielii* and *C. crescentus*. These cells do not synthesise ribosomal RNA, although messenger RNA synthesis continues at low levels (Swoboda, 1982; Scott & Dow, 1986). Very little DNA synthesis was seen to occur in long-term-starved cells, again similar to the situation seen in swarmer cells. The pattern seen in the ATP levels within starving cells suggests that they are able to accumulate ATP in the absence of an energy source - a situation seen in *R. vannielii* swarmer cells.

The changes seen in the exclusion of routine bacteriological stains suggests that long-term-carbon-starved cells have a reduced permeability which may partly explain the increased resistance to various stresses. In addition, the increased hydrophobicity of the cells reduces the ability of polar solvents to enter the cell. Reduced permeability has been demonstrated in *R. vannielii* swarmer cells as a mechanism of increased resistance to rifampicin (Scott, 1987). The condensation of the nucleoid seen to occur after 14 days incubation of cultures is yet another area of similarity with survival cell types of differentiating organisms.

When studying the physiological changes occurring in carbon-starved cells a major problem is the difficulty in obtaining a homogeneous population. With organisms such as *R. vannielii* and *C. crescentus* this is relatively easy. The present work demonstrated that separation based on surface hydrophobicity is a promising tool which may allow more detailed study of these cells.

Overall, the changes which occur in carbon-starved *E. coli* K12 bear remarkable similarities to the physiological differences seen when survival cell types of differentiating bacteria are compared with their parent cell type. The

evidence suggests that *E. coli* may be able to produce a survival cell, analogous to the endospores of *Bacillus* species and the swarmer cells of *R. vannielii* and *C. crescentus*. The visible morphology of these cells was similar to that of the parent cell type, unlike differentiating bacteria, but physiologically they appear very different.

Chapter 8. References

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