Characterisation of the CspA paralogues of *Salmonella* Typhimurium

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Declaration

I declare that the work presented in this thesis is my own, except where stated otherwise.

Signed

J. L. Reyner

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Abstract

In cold temperatures, the survival of *Salmonella enterica serovar* Typhimurium (*S.* Typhimurium) requires the action of cold shock protein A (CspA) paralogues. These are thought to melt misfolded ribonucleic acids, facilitating their translation at low temperatures. However, through phenotypic analysis of our SL1344 *csp* null mutant (lacking all CspA paralogues), it has been shown that CspA paralogues function during other environmental stresses, outwith temperature reduction, and play an essential role in colony formation of an SL1344 *rpoS* mutant at 37°C.

The general stress σ subunit, RpoS, plays an important role in adapting cells to a number of stresses including oxidative stress, temperature changes, low pH and stationary phase. Under such conditions, RpoS acts as an 'emergency co-ordinator', subsequently inducing the transcription of necessary stress response genes. In *Escherichia coli*, RpoS is regulated post-transcriptionally by at least three small RNAs (sRNAs): OxyS, DsrA and RprA; that require interactions with the Sm-like RNA chaperone, Hfq. In *S.* Typhimurium, the stability of the RpoS protein itself is regulated by ClpXP, an ATP-dependent protease responsible for RpoS degradation, and a specific recognition factor that targets RpoS to this protease, MviA.

The present study has shown that the CspA paralogues of *S.* Typhimurium are involved in the expression of RpoS and aims to elucidate the role of these proteins in RpoS production. Comparative phenotypic tests were carried out in strains carrying mutations in *rpoS*, *hfq* and the *csp* genes to gain insight into the interactions of Hfq and CspA paralogues, with respect to RpoS expression. Both significant phenotypic overlaps, such as peroxide sensitivity, and phenotypes unique to certain mutant strains, such as cold acclimation in the *csp* null strain, were observed. CspA paralogues and Hfq are functionally distinct, not only in their involvement in RpoS expression, but also in RpoS-independent processes, such as cold acclimation, motility and to some extent, growth at 37°C.

The roles of Hfq and the CspA paralogues, in RpoS expression, were also assessed at the molecular level. A combination of qRT-PCR analysis, transcriptional fusions and immunoblotting (with anti-σ antibodies) has shown that DsrA and RprA are not essential for RpoS expression in *S.* Typhimurium, during stationary phase or exponential cold shock, and do not require Hfq under these conditions. Contrary to reports in *E. coli*, DsrA is not induced upon cold shock in SL1344. Northern blots have shown that neither Hfq nor the CspA paralogues are involved in regulating *rpoS* transcription during either stationary phase at 37°C or cold shock in exponential phase. Immunoblotting and translational fusions have identified different pathways for the regulation of RpoS during stationary phase at 37°C and cold shock in exponential phase. Hfq is involved during the former condition only, whilst CspA paralogues are involved in both. Protein stability experiments have shown that the CspA paralogues do not play a major role in stabilising RpoS protein against degradation. Together, these results have pointed to a role for both the CspA paralogues and Hfq in facilitating the efficient translation of *rpoS* mRNA.

An SL1344 *csp* null *rpoS* mutant is unable to form colonies on LB agar at 37°C, a phenomenon found when introducing combinations of mutations to SL1344 for phenotypic assessment. A conditional *rpoS* mutant revealed that the SL1344 *csp* null *rpoS* strain is viable but non-culturable. From the *csp* gene family, only *cspA* and *cspB* were able to restore colony forming ability to the *rpoS* mutant. Further complementation experiments pointed to faulty cell division, due to abnormal RNase E activity, as the cause.

Abbreviations used

5'UTR	5' untranslated region
Amp	ampicillin
ATR	acid tolerance response
cAMP	cyclic AMP
CAP	cAMP receptor protein
CbpA	curved DNA-binding protein A
cfu	colony forming units
CIRCE	controlling inverted repeat of chaperone expression
CSD	cold shock domain
CsdA	cold shock DEAD-box protein A
CspA	cold shock protein A
Cm	chloramphenicol
CTAB	hexadecyltrimethylammonium bromide
DAPI	4', 6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EP	exponential phase
EPS	exopolysaccharide
IPTG	isopropyl β-D-thiogalactopyranoside
Fis	factor for inversion stimulation
GASP	growth advantage in stationary phase
GFP	green fluorescent protein
HSP	heat shock protein
HU	heat-unstable nucleoid protein
IHF	integration host factor
IVs	intervening sequences
Kan	kanamycin
LB	Luria-Bertani
LPS	lipopolysaccharide
MOI	multiplicity of infection

MOPS	4-morpholinopropanesulfonic acid
OD	optical density
ONPG	o-nitrophenyl-β-D-galactosidase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming units
PNPase	polynucleotide phosphorylase
ppGpp	guanosine tetraphosphate
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RBS	ribosome-binding site
RMF	ribosome modulation factor
RNA	ribonucleic acid
RNase E	ribonuclease E
SDS	sodium dodecyl sulphate
S. Enteritidis	*Salmonella enterica* serovar Enteritidis
SP	stationary phase
SPI2	*Salmonella* pathogenicity island 2
sRNA	small non-coding RNAs
SSR	starvation-stress response
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
Tet	tetracycline
VBNC	viable but non-culturable
Xgal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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Chapter 1

Introduction

1.1 *Salmonella enterica*

Salmonellae are gram-negative, facultative anaerobic, motile, non-lactose fermenting rods belonging to the family of *Enterobacteriaceae* (Mastroeni *et al.*, 2000). Members of this genus have evolved through the acquisition of several genetic elements that enhance bacterial virulence and enable *Salmonella* to act as facultative intracellular pathogens that can survive phagocytosis (Ygberg *et al.*, 2006, Hensel, 2004). There are more than 2000 serotypes of *Salmonella enterica* that infect a wide range of organisms including reptiles, birds and mammals by the faecal-oral route. These micro-organisms can be frequently found in sewage, sea and river water and are common contaminants of a variety of foods (Mastroeni *et al*, 2000). Some serovars infect only a narrow host range (*S. typhi* infects humans and *S. gallinarum* infects chickens) whilst others, such as *S.* Typhimurium, have a much broader host range. Even with this broad host range, the course and severity of disease caused by *S.* Typhimurium varies substantially with the host. *S.* Typhimurium causes enteric fever in mice but mainly self-limiting gastroenteritis in cattle and humans, although systemic disease can occur; furthermore, it remains as a commensal bacterium of chickens (Ahmer *et al.*, 1999). These differences in disease and host range can often be correlated with segments of DNA that have been gained by horizontal gene transfer (Groisman and Ochman, 1997), although clear differences in host susceptibility to infection also exists.

1.1.1 The economical impact of Salmonellosis

Acute and chronic forms of clinical salmonellosis cause great economic and welfare problems for the UK. Around 35,000 cases of *Salmonella*-related food poisoning are reported every year in the UK, and 119 deaths from the infection were reported in the year 2000 (WHO; DEFRA). The burden on the British economy from loss of productivity, hampered agri-production, barriers to international trade and medical treatment is estimated at £1.7 billion a year (DEFRA, 2008). Therefore, there are clear public health benefits and economic gains to be made from controlling the spread of this pathogen in the food chain. Consequently, farmers and food manufacturers need more effective means to process their products economically and safely, such as improved on-farm biosecurity and proper storage and use of animal excreta as fertiliser.

1.1.2 Pathogenesis of *Salmonella* Typhimurium

Contaminated cattle and chicken carcasses are recognized as the most important source of human infection by *S.* Typhimurium in the UK. Prior to the year 2000, this serovar emerged as the leading cause of food-borne salmonellosis in the world (Parker *et al*, 2001). The typical infective dose for humans is thought to be 10^6-10^8 colony-forming units (Humphrey, 2004). After ingestion, *S.* Typhimurium resists the low pH in the stomach, allowing passage to the intestine where the bacteria invade the mucosa and replicate in the sub-mucosa of the distal ileum. This is possible as *S.* Typhimurium out-competes resident microbial flora. In humans, this form of infection causes low grade fever, nausea and diarrhoea, from which most people can recover without antibiotic treatment. However in elderly individuals, infants or immuno-compromised people, the infection can progress from the enteric form to the systemic form of infection due to expression of the *spv* genes (mainly SPI2) clustered in the *Salmonella* chromosome (Gulig *et al.,* 1993). This form of the infection results in the bacteria reaching the phagocytes of the spleen, liver and bone marrow, and can be fatal if the individual is not promptly treated with antibiotics (Mastroeni *et al.*, 2000). See Figure 1.1.2 for a summary of the infection process.

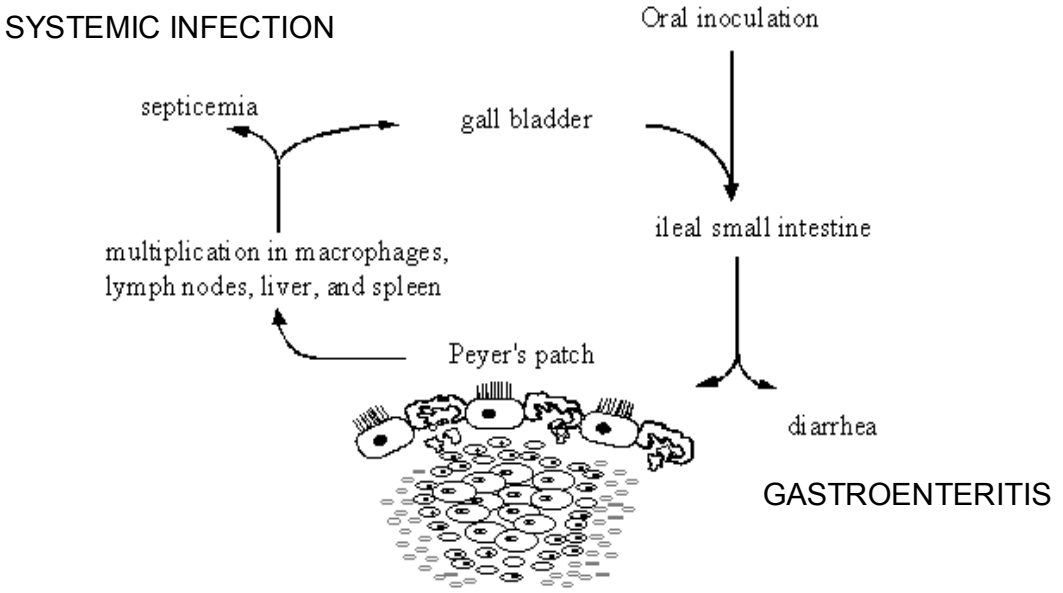


Figure 1.1.2: **The infection process of *S.* Typhimurium.** Infection usually results in gastroenteritis; however the infection can become systemic in immuno-compromised individuals. (Figure adapted from www.foodrisk.com)

1.1.3 *Salmonella* and food safety

Salmonella is the second-most common cause of food poisoning after *Campylobacter*. It is recognised that humans contract this infection by consuming contaminated products of animal origin, such as cattle, pork, poultry and eggs (FSA; see below); however, this should only pose a problem if these products are not properly handled, cooked or refrigerated. Also important is the risk from non-cooked foods, i.e. products containing raw egg (such as mayonnaise and salad dressings) and unpasteurised milk or fruits and vegetables that have been tainted by animal manure. Environmental sources of the organism include sea and river water, leading to contamination of sea food, and soil. Domestic *S.* Typhimurium outbreak investigations have revealed that the major risk factors for infection include undercooking or improper storage of raw meat, and cross-contamination from direct contact between raw and cooked foods (Humphrey, 2004). However, *Salmonella* infections can also be contracted from household pets, such as reptiles (turtles, lizards and snakes), as they can carry the bacteria on their skin (FDA) but also from cats and dogs.

An important threat to food safety is the continuing pandemic of egg-associated *Salmonella* infection. *Salmonella enterica* serovar *Enteritidis* (*S.* Enteritidis) is highly invasive in hens and persists in reproductive tissue for long periods of time, allowing contamination of egg contents, principally the albumen, under commercial conditions (Humphrey *et al*., 1996). Hence, eggs are already contaminated when they are laid, in contrast with other serovars, such as the gut commensal *S.* Typhimurium, which are present on the shell as a result of faecal contamination and enter the egg as a consequence of shell damage and/or when contents are removed. Preliminary studies indicate that tissue persistence, in chickens, is related to the expression of RpoS, an alternative sigma subunit of RNA polymerase responsible for co-ordinating a general stress response (see Section 1.4), and the structure of lipopolyssacharide (Guard-Petter, 2001). The problem of egg contamination is largely controlled in the UK by the vaccination of laying flocks before they are sexually mature, with either a live-attenuated or a dead *S.* Enteritidis vaccine; this scheme is termed the Lion Code programme (Advisory Committee on the Microbiological Safety of Food, 2001). However, outbreaks of *Salmonella* can occur through international trade of foods and food animals, as demonstrated by recent outbreaks in the UK caused by eggs (Health Protection Agency) and salad vegetables (Horby *et al*., 2003) imported from Europe.

The ability of *S.* Typhimurium to survive in the food chain is primarily due to its ability to adapt to and survive environmental changes (Humphrey, 2004). *S.* Typhimurium must survive

outside the host for long periods of time, during which cells are exposed to changes in temperature, pH and in levels of available oxygen and water (Rees *et al.,* 1995). Bacterial cells may also be exposed intermittently to sunlight and darkness (Humphrey, 2004). After ingestion, the bacterium must resist the antimicrobial defenses (nutrient starvation, presence of reactive-oxygen and -nitrogen species and extremes of pH) mounted by the host against infection (Seymour *et.al.,* 1996; Wilmes-Riesenberg *et.al.,* 1996; Kazmierczak, Wiedmann and Boor, 2005). Following processing, meat products are refrigerated until their consumption. Therefore, the ability of *S.* Typhimurium to survive at low temperatures is of concern. It has been reported that this pathogen can survive at 5°C for at least 8 months (D'Aoust *et al.*, 1985), due to the action of protective cold shock proteins. A major group of these proteins, and the ones studied in this project, are the CspA family. These proteins are thought to overcome the translational blockage that occurs at low temperature but may also function in other cellular processes (See Section 1.2). During its life cycle, *S.* Typhimurium must survive exposure to rapid and dramatic changes in environmental conditions. Understanding the underlying cell biology during adaptation to stress conditions will improve intervention strategies for food producers and consumers alike.

1.2 *Salmonella* stress responses

In higher organisms, many different strategies are used to minimise the effect of environmental changes at the cellular level. For single cell organisms, only direct cellular responses to external changes are possible. When the external changes are outwith the physiological range favoured by the organism, specific stress responses are activated (Horn *et al*., 2007). In addition, *Salmonella* cells must adapt to many external changes during their normal growth cycle.

1.2.1 The *Salmonella* growth cycle and the nucleoid

A bacterial cell can be viewed as being in a dynamic state that adapts readily to shifts in environmental parameters, by means of a variety of accommodations, to maintain balanced growth. These include appropriate modification of enzyme synthesis, modulation of nutrient uptake rates and re-routing of metabolic pathways to avoid blockages due to specific nutrient

limitation (Harder and Dukhizen, 1983). However, the bacterial cell cycle is often depicted as consisting of three phases: an initial lag phase, an exponential phase of balanced growth and stationary phase. When stationary-phase cells are inoculated into fresh medium, there is an initial lag phase during which the cells may be growing in volume or mass, synthesizing enzymes, proteins and RNA and increasing in metabolic activity. After some time, growth accelerates, a period of unrestricted growth begins, and the exponential phase starts. However, exponential growth cannot continue forever as population growth is limited by one of three factors: the exhaustion of available nutrients, the accumulation of inhibitory metabolites or end products, or exhaustion of space. When this occurs, cultures enter the stationary phase, growth declines and the metabolic activity is reduced (Delpy, Beranger and Kaweh, 1956; Akerlund, Nordstrom and Bernander, 1995).

In the bacterial cell, chromosomal DNA is compacted into a nucleoid which exists in association with various structural proteins. The nucleoid proteins play some functional roles, besides their structural roles, in the global regulation of essential DNA functions: replication, recombination, and transcription. The protein composition of the *E. coli* nucleoid varies dramatically depending on the growth phase. During exponential phase the nucleoid is loosely packed and consists of several hierarchical higher order structures; the 40 and 80 nm fibres and loop structures (Kim *et al*., 2004(ii)). The most abundant of the nucleoid-associated proteins during growth is a small basic protein designated HU; this most commonly occurs as a heterodimer (Krawiec and Riley, 1990). Another major constituent of the nucleoid during growth is the H-NS protein, implicated in compact organisation, which functions directly as a repressor of transcription (Ueguchi and Mizuno, 1993; Section 1.4). Fis forms a nucleoprotein complex near promoter regions to repress transcription during exponential phase (Hirsch and Elliott, 2005; Section 1.4.2.2). Hfq also associates with the nucleoid during growth but much less is known about this interaction; Hfq is a non-specific DNA binding protein that binds to both linear and super-coiled DNA (Takada *et al*., 1997; Section 1.4.4.1).

In early stationary phase, the nucleoid-associated proteins: Fis, HU, H-NS and Hfq, are replaced by Dps, IHF (integration host factor) and CbpA (curved DNA-binding protein A; unknown function). This nucleoid composition is also observed during lag phase growth. Dps is the most abundant nucleoid protein during stationary phase, sequestering iron and protecting DNA from damage (Halsey *et al*., 2004). These changes in the composition of nucleoid-associated proteins during stationary phase are accompanied by compaction of the genome DNA and silencing of the genome functions (Azam *et al*., 1999). *E. coli dps* mutants are particularly sensitive to DNA damage from UV irradiation and hydrogen peroxide stress (Almiron *et al*., 1992).

1.2.1.1 Stationary phase

Many of the ways in which *E. coli* and *S.* Typhimurium respond to environmental stresses have been extensively studied. Bacteria usually live in environments in which the abundance of nutrients is low and available nutrients are rapidly removed by competing bacterial populations. *S.* Typhimurium responds to total nutrient exhaustion by entering a state referred to as stationary phase.

The genetic and physiological changes that occur in *S.* Typhimurium, when starved of one or more essential nutrients, are referred to as the starvation-stress response (SSR). The SSR genes, whose products are required for starvation survival, are termed core SSR genes; four core SSR loci have been identified in *S.* Typhimurium: *rpoS, stiA, stiB and stiC* (Fang *et al*., 1992; Spector and Cubitt, 1992). The latter study is focused on predominately carbon starvation which induces an SSR that not only protects cells against the detrimental effects of starvation but also cross-protects against the harmful effects of other environmental stresses, such as extremes of heat and osmolarity as well as lethal concentrations of hydrogen peroxide (Matin, 1991; Seymour *et al*., 1996). Furthermore, this carbon-induced SSR is dependent upon the *rpoS*-encoded alternative sigma factor, σ^S (Lange and Hengge-Aronis, 1991; McCann, Fraley and Matin, 1991). During carbon starvation, there is an increase in transcription of the cAMP receptor protein regulon to allow use of alternative carbon sources. These systems also stimulate expression of genes involved in synthesis of flagella, motility and chemotaxis, aiding bacteria to find new environments that are more appropriate for growth (Neidhardt *et al.,* 1987).

Phosphate is an essential nutrient for cell functions and life, found in lipids, nucleic acids, proteins and sugars and is involved in biochemical reactions that rely on the transfer of phosphoryl groups. The Pho regulon is the main global regulatory circuit involved in bacterial phosphate management. Guanosine tetraphosphate (ppGpp) is a key phosphate-containing molecule that accumulates during phosphate starvation, initiating changes in global metabolism through the induction of RpoS accumulation (Lamarche *et al.,* 2008; See Section 1.4). ppGpp is also induced during glucose limitation (Brown and Elliott, 1996).

Cells in the stationary growth phase are considerably more resistant than cells in the exponential phase of growth, to a range of environmental conditions such as low and high temperature, low pH, UV light and hydrogen peroxide. Stationary phase cells remain metabolically active but become more compact as the surface/volume ratio of the cell is

decreased; the cytoplasm also becomes condensed and their periplasmic volume increases (Reeve, Amy and Matin, 1984). The composition of the cell wall is altered, becoming less fluid and permeable as unsaturated fatty acids are replaced by cyclopropyl derivatives; this favours adhesion and aggregation (Cronan, 1968; Rees *et al*., 1995). Cells can also synthesise storage substances, like glycogen and polyphosphates, and protective substances such as trehalose under appropriate conditions (Zambrano and Kolter, 1996; Saint-Ruf *et al.,* 2007; Hengge-Aronis, 1993). These differences have been attributed to regulation by an alternative sigma factor, encoded by *rpoS*, which directs RNA polymerase to transcribe a large number of stress response genes, such as *osmY, dps* and *katG* (Taylor-Robinson *et al.*, 2003; See Section 1.4). After prolonged storage, stationary phase cells may pass into a 'viable but non-culturable' state of dormancy, which can be compared to sporulation in Gram-positive bacteria (Ishihama, 1999).

At the onset of stationary phase, the overall rate of protein synthesis can drop to 20% of the maximal rate seen in exponential phase. This rate then further decreases slowly and steadily in the next 10-20h of stationary phase; after 11 days the rate is reported to be 0.05% that of a growing culture (Kolter *et al.,* 1993). The functional form of ribosomes in growing *E. coli* is the 70S monomer, comprising 30S and 50S subunits. 70S ribosomes can be converted into 100S particles in stationary phase by a small and basic protein, RMF (ribosome modulation factor); 100S particles (70S dimers) constitute around 40% of the ribosomes present during this growth phase (Wada *et al*., 1990). This dimeric form of ribosomes represents an inactive stored form of ribosomes that can be converted back into 70S monomers within minutes of addition of fresh medium (Wada *et al.,* 1990). Although this research was performed in *E. coli*, the *rmf* gene is present in *S.* Typhimurium and has been shown to dimerise 70S ribosomes (Chuang *et al*., 2008).

It has been shown for *E. coli* that, at the onset of stationary phase, the initiation of chromosome replication is inhibited by an uncharacterized extracellular factor (Withers *et al.*, 1998). However, this factor does not block ongoing replication as this would lead to stalling of replication forks, a potentially deleterious event. As mentioned earlier, the altered composition of the nucleoid-associated proteins during stationary phase leads to compaction of genomic DNA and silencing of the genome functions.

1.2.2 The heat shock response

A sudden temperature up-shift poses a serious threat to the integrity of almost all cellular macromolecules but especially proteins. The response of *E. coli* to a rapid temperature upshift from 30°C to 42°C is characterized by an induction period where the rate of heat shock protein (HSP) synthesis increases rapidly, followed by an adaptation period where the rate of HSP synthesis decreases to reach a new steady state level (Herendeen *et al,* 1979). As a result, the cellular levels of major HSPs increase about twofold upon shift to 42°C. The *E. coli* heat-shock regulon consists of over 20 genes, the products of which are constituents of the cellular machinery of protein folding, repair and degradation (Gething and Sambrook, 1992). Chaperones, such as DnaK, DnaJ and GrpE, and proteases, such as ClpP, ClpB and ClpX, either rescue misfolded proteins or promote their degradation (Bukau, 1993; (Derré, Rapoport and Msadek, 2002). The cellular content of *E. coli* HSPs, and that of other enteric bacteria, is regulated primarily at the level of transcription, involving positive control by the *rpoH*-encoded alternative sigma factor, RpoH (Yura *et al.,* 2000; explained later in section 1.3) or negative control by repressor proteins (Narberhaus, 1999; Muffler *et al*., 1997(i)). *rpoH* mRNA folds into a translationally incompetent secondary structure, which opens up on heat shock, resulting in a directly temperature-triggered translational induction of *rpoH* (Hengge-Aronis, 2002). There is strong evidence that mRNA folding is also an important parameter in the translational control of some eukaryotic heat-shock genes (Morimoto, 1998). In *E. coli*, RpoS is also induced by heat shock, due to interference with its turnover; however, it does not seem to contribute to heat adaptation but may induce cross-protection against other stresses (Muffler *et al*., 1997(i)). Induction is probably due to the titration of Clp protease, essential for RpoS degradation, as heat shock results in an increased cellular amount of irreversibly denatured proteins, substrates for Clp-mediated proteolysis.

In contrast to *E. coli*, the heat shock response in the Gram-positive soil bacterium *Bacillus subtilis* involves at least three different classes of heat-inducible genes, distinguished by their regulatory mechanisms (Hecker, Schumann and Völker, 1996). Class I genes encode classical chaperones, such as DnaK, GroES and GroEL. Their expression involves a σ^A-dependent promoter and the highly conserved CIRCE (controlling inverted repeat of chaperone expression) operator sequence (Zuber and Schumann, 1994). Class II genes are the largest class; their expression is induced by heat shock but also by general stress conditions such as exposure to salt or ethanol, or starvation for glucose, phosphate or oxygen (Hecker and Völker, 1990). Expression of these genes requires the σ^B sigma factor whose synthesis and activity is increased under stress conditions. Class III genes were defined as those devoid of

the CIRCE operator sequence and whose induction by heat shock and general stress conditions is σ^B-independent. These genes encode the ClpC and ClpX ATPases (Msadek, Kunst and Rapoport, 1994; Gerth *et al*., 1996), the proteolytic subunit ClpP (Gerth *et al*., 1998), as well as FtsH and LonA (Riethdorf *et al*., 1994; Deuerling *et al*., 1997).

1.2.3 The cold shock response

Temperature is a common environmental factor and virtually all organisms elicit a coordinated cellular response to a decrease in temperature. A drop in temperature has a profound impact on cell growth by influencing ribosomal synthesis, cytoplasmic membrane composition and fluidity, as well as the conformation and functionality of nucleic acids and proteins (Phadtare, 2004). As a protective measure, most bacteria are able to mount a cold-shock response. At least for *E. coli*, the cold shock response is not induced by a temperature decrease to a certain temperature but by any downshift of 6°C or more (Ermolenko and Makhatadze, 2002). The greater the magnitude of the temperature shift and the lower the new absolute temperature is, the stronger the response. The cold shock response is characterised by the arrest of cell growth, during which a number of genes (from the cold-shock regulon) are transiently expressed, whilst severe inhibition of general protein synthesis occurs simultaneously. This leads to adaptation which facilitates growth at the new (low) temperature (if within the growth range) and at an appropriate and reduced growth rate (Phadtare, Inouye and Severinov, 2002). A schematic view of the pattern of protein expression after cold shock is shown in Figure 1.2.3.

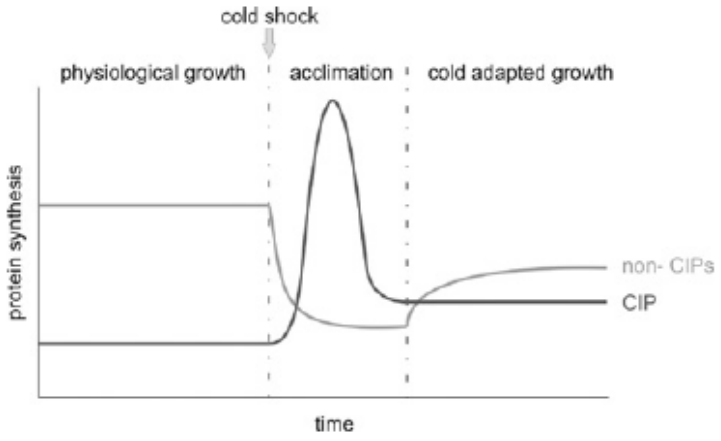


Figure 1.2.3: **Schematic view of protein expression in *E. coli* after cold shock** (taken from Horn *et al*., 2007). The acclimation phase after cold shock is characterised by a transient inhibition of protein synthesis, an increase in expression of cold-induced proteins (CIPs) and a decay of the corresponding mRNA. Following this, cold-induced protein expression declines and the bulk protein synthesis is restored with a new cold-adapted pattern.

DNA microarrays in *E. coli* have identified 297 genes (approximately 7% of the genome) whose expression is transiently up-regulated at 23°C compared to 37°C (White-Ziegler *et al*., 2008). These genes belong to the cold-shock regulon and encode proteins involved in a variety of essential functions such as transcription (*nusA*), translation (*infB* and *rbfA*), mRNA degradation (*pnp*) and recombination (*recA*) (Brandi *et al*., 1996; Wilson and Nierhaus, 2004) as discussed in more detail in Section 1.5. Following sudden temperature reduction, transcription and translation are blocked by the stabilisation of aberrant secondary structures of RNA and DNA. Some mRNAs are not simply a substrate for ribosomes but contain control elements that modulate their own expression in a condition-dependent manner. For example, RNA thermometers, common in proteobacteria, are complex mRNA structures that change their conformation in response to temperature (Narberhaus, Waldminghaus and Chowdhury, 2006). Some examples of these are the bacteriophage λ cIII gene, which controls whether bacteriophage λ enters the lytic or lysogenic cycle (Altuvia *et al*, 1989), *E. coli rpoH*, the heat shock sigma factor (Narberhaus, Waldminghaus and Chowdhury, 2006), *E. coli cspA* mRNA (Yamanaka, Mitta and Inouye, 1999) and *E. coli* DsrA (Sledjeski, Gupta and Gottesman, 1996). Most RNA thermometers are located at the 5'UTR region of mRNA and mask ribosome-binding sites by base-pairing at low temperature. Melting of the RNA secondary structure, as the temperature increases, allows ribosome access and therefore translation initiation (Narberhaus, Waldminghaus and Chowdhury, 2006).

Some bacterial cells, such as those of *Bacillus* and cyanobacteria, change the degree of saturation of membrane phospholipids, to maintain the cell membrane in a biologically functional fluid phase, in response to a lowered temperature (Thieringer, Jones and Inouye, 1998). The enzymes involved in this kind of cell response are desaturases, responsible for the synthesis of unsaturated fatty acids (Golovlev, 2003). In Gram-negative organisms, there is an additional issue. Lipopolysaccharide (LPS) is a unique constituent of the bacterial outer membrane that forms a protective barrier around the Gram-negative cell, and its fluidity was reportedly induced at low temperatures (Gao *et al*., 2006).

1.2.4 Oxidative stress

During exponential growth, *E. coli* and *S.* Typhimurium respond to oxidative stress by invoking two distinct stress responses, the peroxide response and the superoxide response, depending on whether the stress is mediated by peroxides or the superoxide anion (Farr and Kogoma, 1991). Reactive oxygen species are deleterious to cells because they can damage

proteins, DNA and membranes. Christman *et al.* (1985) have shown that in *S.* Typhimurium, 30 proteins including catalase, Dps and superoxide dismutase, are selectively induced during exponential growth by the transcriptional activator, OxyR, in response to H_2O_2 exposure. During peroxide stress in exponential phase, genomic DNA is protected by Dps, which organises the chromosome into one, tightly packed, highly ordered, crystalline structure that is resistant to oxidative and thermal stresses, low pH, UV damage and metal toxicity (Kim *et al.,* 2004). In *E. coli*, the small RNA OxyS (see section 1.4) is also dramatically induced by oxidative stress (and OxyR) during exponential growth, and in turn leads to the expression of as many as 40 proteins to protect cells against chemically induced DNA damage (Altuvia *et al*., 1997). Under these conditions, OxyS functions by Hfq-mediated RNA-RNA pairing, to *gadB* and *uphT* mRNAs (involved in the electron transport system) for example, to relieve the translational block caused by elements of secondary structure in the 5'UTR of mRNAs. In the same way, OxyS can repress translation of *rpoS* mRNA, which produces the primary alternative sigma factor (see Sections 1.3 and 1.4), by binding to and stabilising the stem-loop occluding the ribosome binding site (Wassarman, Zhang and Storz, 1999; discussed in more detail in section 1.4). During entry into stationary phase, RpoS induces expression of catalase HPII, Dps and *xthA*-encoded exonuclease III, which prevent DNA damage during oxidative stress (Tanaka, Handel, Loewen and Takahashi, 1997), so there is no need for OxyR and OxyS under these conditions as the cell is naturally more H_2O_2-resistant. Similarly, the repression of *rpoS* during exponential phase, by OxyS, inhibits functional redundancy between the OxyR/ OxyS and RpoS regulons (Wassarman, Zhang and Storz, 1999).

1.3 RNA polymerase and gene regulation

1.3.1 Structure and function of RNA polymerase

DNA-dependent RNA polymerase plays a central role in transcription (Lehninger *et al.,* 1993; Buyukuslu, 2003). In *E. coli* and *S.* Typhimurium, the RNA polymerase holoenzyme consists of 6 subunits – five forming the core enzyme (2α, β, β' and ω) and the sixth, the σ subunit (sigma factor). Despite the complexity of the core enzyme, it is incapable of promoter-specific recognition in the absence of the sigma subunit (Mooney, Darst, and Landick, 2005). The sigma factor is only associated with the RNA polymerase core enzyme transiently and the cell encodes several different interchangeable sigma factors. The function of the sigma

factor is to recognise and bind to promoter sequences in the primary DNA sequence, promoting the correct alignment of the RNA polymerase on the transcriptional unit and, in turn, initiating transcription and gene expression. Once a stable elongation complex is formed, the sigma factor dissociates from the core enzyme whilst transcription proceeds. Different sigma factors interact with the same region of the core polymerase complex (Helmann and Chamberlin, 1988).

1.3.2 Modulation of polymerase specificity by sigma subunits

Seven different species of σ subunit are known to exist in *E. coli* and *S.* Typhimurium (see Table 1.3.2) and each participates in the transcription of a specific set of genes. The alternative sigma factors contribute to DNA strand separation and provide promoter recognition specificity to RNA polymerase by outcompeting the housekeeping sigma factor (σ^{70}) under specific stress conditions (Kazmierczak *et al.,* 2005; Fang, 2005; Hengge-Aronis, 1999). Most of the genes expressed in exponentially growing cells are transcribed by the holoenzyme containing σ^{70} (the *rpoD* gene product), whilst the holoenzyme $E\sigma^{S}$ mediates transcription of most of the stationary phase specific genes (Hengge-Aronis, 1993; Ishihama, 2000). *In vivo*, these two sigma factors clearly control expression of different genes yet they are structurally and functionally very similar: they recognise overlapping DNA sequences (Hengge-Aronis, 1999). *In vitro*, RpoS binds less efficiently to core RNA polymerase than RpoD (Kusano *et al*., 1996). In association with RNA polymerase, RpoS directs transcription of as much as 10% of the *S.* Typhimurium genome, including genes necessary for stress resistance and virulence (Hirsch and Elliott, 2005).

As mentioned above, each holoenzyme recognises and transcribes a different set of genes but transcription of these genes requires, in addition, accessory proteins or nucleotide factors. In *E. coli*, functional specificity of the RNA polymerase holoenzyme is modulated through interaction with one of around 150 different transcription factors (Ishihama, 2000). These factors are classified based on their target subunits on RNA polymerase. Many stationary phase genes are transcribed by two different systems depending on growth conditions: one is RpoS dependent but transcription factor independent, and the other is RpoD and transcription factor dependent. For this reason such genes carry multiple promoters to be recognised by each form of the transcription apparatus (Ishihama, 2000). In sharp contrast to the RpoS-dependent system, the promoters under the control of the 'minor' sigma subunits, i.e. promoters for late exponential phase flagellar genes and genes involved in periplasmic stress

responses, cannot be recognised by the $E\sigma^{70}$ holoenzyme, and their holoenzymes are unable to transcribe RpoD-dependent genes.

Table 1.3.2: ***E. coli* and *S.* Typhimurium sigma factor nomenclature** (adapted from Neidhardt *et al*., 1987). The housekeeping sigma factor is termed σ^{70} or RpoD. The stress sigma factors are also known as alternative sigma subunits.

Name/ synonms	Gene	Functions
σ^{70} / σ^{D}	*rpoD*	EP σ factor
σ^{38} / σ^{S}	*rpoS* *katF*	SP, oxidative and osmotic stresses, virulence
σ^{32} / σ^{H}	*rpoH* *htpR*	transcription, HSP, cytoplasmic stress
σ^{28} / σ^{F}	*fliA* *flaD*	late EP flagellar genes chemotaxis
σ^{24} / σ^{E}	*rpoE*	periplasmic stress
σ^{54} / σ^{N}	*rpoN* *ntrA*	nitrogen metabolism phage shock response
σ^{19} / FecI	*fecI*	iron citrate transport

Mostly, alternative sigma factors work independently of each other to control discrete subsets of genes in response to different environmental stimuli. However, a sigma cascade has been discovered in *Salmonella* suggesting that RpoE and RpoH enhance the translation of RpoS by increasing expression of the RNA-binding protein, Hfq. In this instance, the activation of one sigma factor by another allows the integration of diverse environmental signals to result in expression of a more extensive direct stress response (Fang, 2005; Hengge-Aronis, 1999).

1.4 General stress σ subunit of RNA polymerase (RpoS or σ^{S})

The *rpoS* gene of *E. coli* encodes an alternative primary sigma factor for RNA polymerase with a molecular mass of 37.9kDa. RpoS can be found in most proteobacteria, namely enteric bacteria and *Vibrio* species, but is also found in actinobacteria and cyanobacteria. Homologues of RpoS have been reported in T4-like viruses and some fungal species, such as *Aspergillus niger* (NCBI Entrez Protein Database: taxonomic group search). Amongst enteric bacteria, including *S.* Typhimurium and *E. coli*, RpoS is highly conserved (see Table 1.4).

The *rpoS* gene was first reported in 1984 to map to 58.9 min on the *E. coli* genetic linkage map and to be required for the synthesis of the catalase hydroperoxidase (HPII) and the induction of catalase-peroxidase HPI (Loewen and Hengge-Aronis, 1994; Ivanova, Miller, Glinsky and Eisenstark, 2006). The expression of RpoS, which is also called σ^S or σ^{38}, increases in response to a number of stress signals: osmotic shock, nutrient depletion (i.e. stationary phase) and low temperature or any conditions that reduce growth rate (Cunning and Elliott, 1999; Jones *et al*., 2006). This increase of protein concentration leads to RpoS outcompeting vegetative sigma factors in binding to core RNA polymerase, increasing the transcription of at least 60 genes mediating stress responses and stationary phase resistance (Hirsch and Elliott, 2005). This general stress response renders cells broadly resistant in a way that damage is avoided rather than needing to be repaired. Early studies in *S.* Typhimurium by Fang *et al.* (1992) showed that a truncated mutation of *rpoS* causes pleiotropic phenotypes including reduced resistance to nutrient starvation, oxidative stress, acid stress and DNA damage, conditions which are relevant to the intraphagosomal environment of host macrophages. Similarly, RpoS is directly relevant to food microbiology as bacteria defective in RpoS production are highly sensitive to food processing procedures (Rees *et al*., 1995). Hengge-Aronis *et al*. (1993) examined the effect of two commonly used bacteriostatic food preservatives (nitrite and sulphite) on RpoS expression in *E. coli* and found that these treatments rapidly induced RpoS, with expression eventually becoming co-linear with exponential growth.

Table 1.4: RpoS amino acid identities among Gram negative bacterial species (taken from Ferreira *et al.*, 1999).

	1	2	3	4	5	6	7	8	9	10	11
1. *Escherichia coli* O157:H7	100	98·6	98·3	90·4	91·8	93·4	91·5	92·3	63·7	63·5	62·1
2. *Salmonella dublin*		100	99·7	90·9	92·0	93·9	91·7	91·7	63·6	63·6	62·0
3. *Salm. typhimurium*			100	90·6	91·7	93·7	91·5	91·5	63·4	63·6	62·0
4. *Salm. typhi*				100	75·6	77·4	75·6	88·4	51·2	51·5	50·2
5. *Yersinia enterocolitica*					100	94·3	91·3	84·4	66·9	66·4	65·2
6. *Serratia entomophila*						100	92·8	85·5	66·7	66·9	65·4
7. *Erwinia carotovora*							100	83·6	66·4	66·4	65·4
8. *Shigella flexneri*								100	57·3	57·3	55·6
9. *Pseudomonas aeruginosa*									100	90·0	88·1
10. *Ps. fluorescens*										100	92·0
11. *Ps. putida*											100

RpoS expression is a complex process and studies of *E. coli* (Hirsch and Elliott, 2005; Hengge-Aronis, 2002) have shown that it has multiple levels of regulation, including: transcriptional regulation, post-transcriptional control of its synthesis and proteolysis

inhibition (Cunning and Elliott, 1999) that occur during different growth phases. For a summary of RpoS regulation, see Figure 1.4. Bacterial survival of most stresses that stimulate transcription or translation of *rpoS*, is not dependent on a rapid emergency response. However, stresses that occur very suddenly tend to reduce or prevent RpoS proteolysis and thus allow very rapid adjustments in cellular RpoS levels.

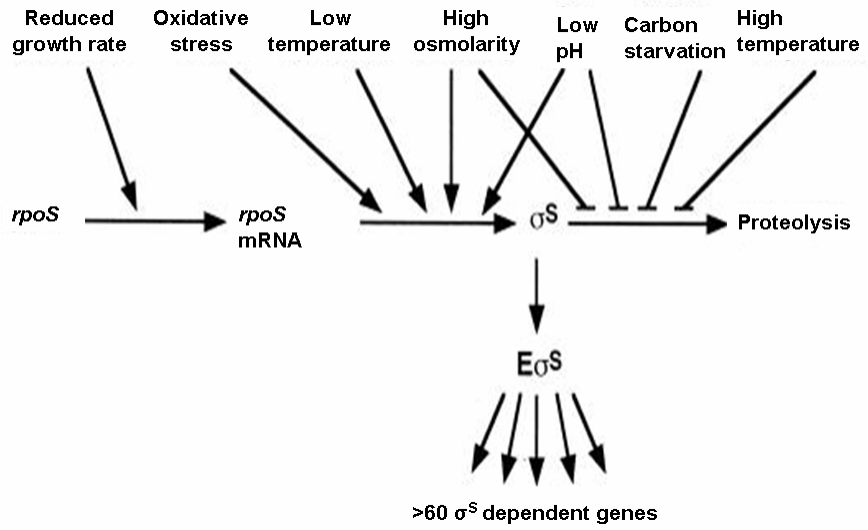


Figure 1.4: Multiple levels of RpoS regulation are differentially affected by various stress conditions (adapted from Hengge-Aronis, 2002). An increase in RpoS levels can be obtained by either stimulating synthesis at the level of transcription or translation or by inhibiting proteolysis. The most stringent and rapid response is achieved by a combination of these processes.

1.4.1 RpoS regulated genes

Genes under RpoS control are involved in providing stationary phase cells with resistance to a number of stress factors. Much work has been done into the identification of RpoS-regulated genes in *Salmonella* as it can lead to characterisation of novel factors involved in the persistence of the pathogen in the environment or within hosts (Ibanez-Ruiz *et al*., 2000). Hengge-Aronis *et al*. have shown that *rpoS* mutant strains exhibit a loss of stationary phase thermotolerance, which has been attributed to loss of trehalose synthesis (1993). *otsA* and *otsB* encode enzymes that synthesise trehalose, involved in thermo- and osmo-protection, and are induced upon osmotic up-shift and entry into stationary phase; both types of regulation are dependent on *rpoS*. Stationary phase cells also exhibit strong *rpoS*-dependent resistance mechanisms to H_2O_2 which include: induction of catalases HPII and HPI, expression of *xthA*-encoded exonuclease III, to repair damaged DNA, and expression of *dps* which encodes a

novel histone-like protein that forms highly structured complexes with DNA to prevent against attack from free radicals (Loewen, Switala and Triggs-Raine, 1985; Hengge-Aronis, 1993). Dps continues to be synthesised even in late (3 day old) stationary phase cells and also controls the synthesis of as many as 23 strongly starvation-induced proteins (Almiron *et al*., 1992). The SbcCD nuclease complex plays an important role in elimination and repair of DNA secondary structure and the maintenance of genome stability and transcription of the complex is dependent on starvation and the RpoS protein (Darmon *et al.,* 2007). *rpoS* mutants are also unable to synthesise glycogen. The glycogen synthetic genes, *glgC* and *glgA*, are induced in stationary phase under the control of cAMP but are not dependent on *rpoS*. However, another gene involved in glycogen synthesis, *glgS*, is strongly induced on entry into stationary phase and has both RpoS- and cAMP-dependent promoters (Hengge-Aronis and Fischer, 1992). A subset of RpoS-controlled genes also exhibit hyper-osmotic induction during exponential phase (Lange and Hengge-Aronis, 1994).

Some RpoS-dependent genes, such as *bolA*, influence cell morphology. Overexpression of *bolA*, which encodes the penicillin-binding protein PBP6 that plays a role in cell-wall synthesis at the septum, causes cells to become more compact and osmotically stable, typical of stationary phase cells (Van der Linden *et al*., 1992). RpoS is also responsible for the increased *ftsQAZ* expression required for septum formation during stationary phase, as one of the promoters, $ftsQ1_p$, displays reduced growth-rate dependent activity and is reliant on the increase of RpoS (see Section 1.8).

Fis is a global regulatory protein that prevents transcription initiation by trapping RNA polymerase containing σ^{70} at the promoter region, preventing elongation of new mRNA. The evolutionary distribution of this protein matches that of RpoS, as it is mainly found in proteobacteria, with homologues in eukaryotes and viruses. However, unlike RpoS, Fis is also found in archaea. Fis shows peak expression (>40,000 dimers per cell) in the early logarithmic phase of growth, followed by a sharp decrease through mid-exponential phase until it is almost undetectable at the onset of stationary phase. In *E. coli*, RpoS has been implicated in the repression of Fis expression, and the induction of Fis in the absence of aeration is facilitated by a reduction in RpoS levels under these conditions (Croinin and Dorman, 2007; Grainger *et al*., 2008). However, the reverse has also been argued that it is in fact Fis that represses transcription of *rpoS* (Hirsch and Elliott, 2005).

In addition, RpoS contributes to virulence in *S.* Typhimurium (Swords *et al.*, 1997) as it mediates the expression of *Salmonella* virulence plasmid *spv* genes during starvation; these

genes are essential for virulence in mice, invasive disease in humans and opportunistic infection in patients with aids (Norel *et al.,* 1992; Fang *et al*., 1992). In fact, an *rpoS* mutant of *S.* Typhimurium is highly attenuated for virulence in susceptible, BALB/c mice and tissue load and distribution is affected (Kazmierczak, Wiedmann and Boor, 2005; Swords, Cannon and Benjamin Jr., 1997). The role that RpoS plays in human typhoid is not known as the *spv* regulon is not found in *Salmonella typhi*; however the attenuated vaccine strain *S. typhi* Ty21a has been shown to have a mutant *rpoS* allele, likely to contribute to its attenuation (Robbe-Saule, Coynault and Norel, 1995).

1.4.2 Transcriptional regulation of *rpoS*

1.4.2.1 *rpoS* promoters

Transcription of the *rpoS* gene occurs throughout growth. However, protein is produced at lower levels in *S.* Typhimurium due to the presence of a TTG initiation codon compared to the ATG start site of *E. coli* (Jones *et al.,* 2006). *rpoS* is the second gene in a bicistronic operon together with the *nlpD* gene which encodes a lipoprotein essential in cell wall formation (see Figure 1.4.2.1). The two closely spaced *nlpD* promoters produce bicistronic *nlpD-rpoS* mRNAs to a basal level throughout cell growth. Monocistronic *rpoS* mRNA originates from a separate promoter, $rpoS_p$, within the *nlpD* gene and contains an unusually long 5'UTR region of around 600 nucleotides (Hengge-Aronis, 2002). This 5'UTR is important as deletions within it reduce RpoS expression. Primer extension, Northern blotting and *lac* fusion experiments suggest that, in both *E. coli* and *S.* Typhimurium, the monocistronic mRNA is the major transcript ($rpoS_p$ is responsible for more than 75% of *rpoS* mRNA production) and that its transcription is activated under appropriate conditions of stress where growth retardation occurs (Neidhardt *et al.,* 1987; Hirsch and Elliott, 2005; Loewen and Hengge-Aronis, 1994). The increase in transcription on growth retardation is apparent in complex medium only (Moreno *et al*., 2000). The *lac* fusion experiments also indicated that RpoS is not involved in auto-regulation during stationary phase.

The bulk of RpoS regulation occurs post-transcriptionally as dramatic changes in RpoS protein levels have been reported which are coupled with little or no increase in *rpoS* mRNA levels. The inverse is also true, as the level of RpoS protein is very low in exponentially growing cells, even though relatively high levels of *rpoS* mRNA are present (Hengge-Aronis, 2002).

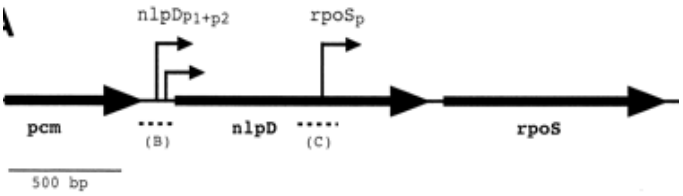


Figure 1.4.2.1: The *rpoS* gene of *E. coli* and its several promoters. The stress response promoter of *rpoS* transcription is designated $rpoS_p$ (adapted from Neidhardt *et al*., 1987).

1.4.2.2 Regulators of *rpoS* gene expression

Transcription of the *rpoS* gene is controlled by the cAMP receptor protein and through the signalling of ppGpp and polyphosphate. Transcriptional *rpoS::lacZ* fusions have shown that the levels of *rpoS* transcription are already high during exponential phase in a *crp* mutant of *E. coli* indicating that cAMP-CRP is a negative regulator of *rpoS* transcription. However, not much is known about the mode of action of cAMP-CRP but it seems to depend on growth phase, as recent studies have indicated that during entry into stationary phase, cAMP-CRP positively controls *rpoS* transcription (Hengge-Aronis, 2002). Inorganic polyphosphate often accumulates in stationary phase or under stress conditions. Transcriptional *rpoS::lacZ* fusions have indicated that polyphosphate somehow stimulates *rpoS* transcription, thereby contributing to stationary phase induction of RpoS (Hengge-Aronis, 2002). The small nucleotide ppGpp, synthesised from GTP by RelA and SpoT proteins, acts as a global regulator of gene expression in bacteria (Magnusson, Farewell and Nystrom, 2005). ppGpp levels in *E. coli* strongly increase in response to amino acid limitation or starvation for carbon, nitrogen and phosphorous sources. ppGpp has been shown to stimulate *rpoS* transcription, as shown by *rpoS::lacZ* transcriptional fusions, but does not seem to target the promoters involved. It has been suggested that the alarmone, ppGpp, may affect the transcript stability of *rpoS* rather than transcriptional elongation (Hengge-Aronis, 2002; Dodd and Aldsworth, 2002). A *relA spoT* double mutant that is unable to synthesise ppGpp has a strongly reduced cellular RpoS content and exhibits a pleiotropic phenotype similar to *rpoS* strains (Gentry *et al*., 1993). At present, it is unclear whether this ppGpp effect is direct or indirect.

Fis, a DNA binding protein, can act as a repressor of *rpoS* transcription during exponential growth in *S.* Typhimurium (Croinin and Dorman, 2007). Hirsch and Elliott report that a *S.* Typhimurium *fis* mutant showed a ninefold increase in expression from the major *rpoS* promoter ($rpoS_P$) during exponential growth, whilst expression during stationary phase was unaffected (2005). Using gel shift analysis, Hirsch and Elliott characterised Fis binding at the P_{rpoS} region and concluded that Fis forms a nucleoprotein complex near the promoter region to repress transcription during exponential phase (2005).

1.4.3 RpoS proteolysis

Even cells that grow in the relative absence of stress demonstrate a basal rate of RpoS synthesis; however, the cellular RpoS level remains low due to rapid degradation. The half-life of RpoS protein during exponential phase has been determined for *E. coli*, by pulse-chase experiments, to be between 1 min and several minutes depending on the carbon source but increases more than seven-fold, to around 10.5 min, at the onset of starvation (Hengge-Aronis, 2002; Lange and Hengge-Aronis, 1994). This stabilisation is reminiscent of RpoH, which has a short half-life at low temperatures but is stabilised in response to heat shock. RpoS degradation depends on ClpXP, a complex ATP-dependent barrel-shaped exoprotease consisting of proteolytic (ClpP) and chaperone (ClpX) subunits that form a proteasome-like assembly. This machinery accounts for up to 80% of protein degradation in the cell (Thomsen *et al*., 2002). Perhaps surprisingly, RpoS degradation by ClpXP would appear complete as no stable degradation products have been found. In addition, a two-component response regulator, RssB (termed MviA in *S.* Typhimurium) is essential for RpoS turnover. Hirsch and Elliott (2005) have shown that there is around a five-fold increase in the expression of *rpoS::lacZ* fusions in log phase cells of both *S.* Typhimurium *clpP* and *mviA* mutants compared to the wildtype. Similar results were also observed by Schweder *et al*. (1996) in *E. coli clpP* mutants. Single amino acid substitutions in RpoS have shown that the turnover element is localised around a crucial lysine residue, K173, as this is critical for binding of RssB (Becker *et al.,* 1999; Hengge-Aronis, 2002; Schweder *et al*., 1996). In *E. coli*, RssB must be phosphorylated by acetyl phosphate before it can bind to this turnover element in RpoS and present RpoS to the ClpXP machinery, where it is unfolded and completely degraded. This turnover element is not present in RpoD (Hengge-Aronis, 1999). MviA is thought to sequester RpoS during exponential phase, inhibiting its activity, even if its proteolysis is blocked (Moreno *et al*., 2000).

After RpoS is delivered to the protease, RssB is recycled and it has been estimated that one molecule can initiate the degradation of six molecules of RpoS per minute (Pruteanu and Hengge-Aronis, 2002). It is unknown whether RssB is dephosphorylated during its catalytic cycle. The activation of MviA, the equivalent of RssB in *S.* Typhimurium, does not rely on acetyl phosphate but is activated by an as yet unknown stimulus through phosphorylation on D58, which substantially increases its ability to bind to RpoS (Hirsch and Elliott, 2005; Moreno *et al*., 2000). RpoS stimulates transcription of *mviA* during SP, constituting an auto-regulatory loop in which the concentration of MviA is a limiting factor for the rate of RpoS degradation *in vivo* (Pruteanu and Hengge-Aronis, 2002). It is not yet known why RpoS becomes more stable in stationary phase as there is no decrease in cellular concentration of ClpP or ClpX. However, there may be a decrease in proteasome activity or chaperones could be protecting RpoS against degradation (Schweder *et al*., 1996).

PhoP (a DNA-binding transcriptional regulator that is induced by growth in low Mg^{2+} or Mn^{2+} (Lejona *et al*, 2003)) has been implicated in RpoS proteolysis in *S. enterica*. PhoP induces the *iraP* gene, encoding a factor that interacts with MviA, therefore preventing it from targeting RpoS to the ClpXP protease for degradation (Tu *et al*., 2006). Mutation of the *phoP* gene renders *S.* Typhimurium as sensitive to hydrogen peroxide as an *rpoS* mutant, but only if grown in low Mg^{2+} medium. However, this is not the case in *E. coli*, as low Mg^{2+} leads to only a modest stabilisation of RpoS and *iraP* is not regulated by the PhoP/PhoQ system.

1.4.4 Post-transcriptional regulation of *rpoS* mRNA

Initial evidence for the post-transcriptional regulation of *rpoS* was provided by clearly different patterns of expression from transcriptional and translational *rpoS::lacZ* fusions in *E. coli*. The *rpoS* mRNA is translated at low levels due to the energetically stable hairpin structure that occludes the Shine-Dalgarno sequence (see Figure 1.4.4.1). The rate of *E. coli* RpoS synthesis has been determined throughout the growth cycle, by pulse-labelling and study of translational *rpoS::lacZ* fusions, showing a five-fold increase during late exponential phase with maximal synthesis occurring at the onset of starvation (Neidhardt *et al*., 1987; Lange and Hengge-Aronis, 1994). There is no corresponding increase in the activity from *rpos::lacZ* transcriptional fusions, suggesting that the primary determinant of RpoS expression is by post-transcriptional control, such as the stabilisation of precise *rpoS* mRNA secondary structures which thereby facilitate translation. Muffler *et al.* (1997(i)) have shown that, in *E. coli*, the stability of *rpoS* mRNA increases during entry into stationary phase or

under conditions of stress, such as osmotic and acid stresses. This is modulated by a cascade of interacting factors including Hfq, HU, H-NS and, by the sRNAs (small non-coding RNAs): DsrA, RprA and OxyS.

1.4.4.1 sRNAs

Bacterial small, untranslated RNAs function as central regulators in response to diverse environmental growth conditions, allowing integration of multiple environmental signals to co-ordinate regulatory outputs. They can regulate both the synthesis of proteins (by affecting mRNA translation and stability), such as DsrA regulation of RpoS, and the activity of specific proteins, such as 6S regulation of RNA polymerase (Masse *et al*., 2003). Over 70 sRNAs have been discovered so far in *E. coli*, in regions between ORFs (Vogel and Wagner, 2007), most of which (around 4 in every 5 sRNAs) are conserved in *S.* Typhimurium (Zhang *et al*., 2004). Several bacterial sRNAs regulate expression of target genes at the post-transcriptional level, including DsrA, OxyS and RprA, each of which have been shown to influence RpoS synthesis in *E. coli*. These sRNAs initially bind to the Hfq protein and then use RNA base pairing interactions to regulate the expression of their target mRNAs (Wassarman, 2002).

In *E. coli*, OxyS RNA has been shown to repress *rpoS* translation via stimulation of mRNA degradation, whereas DsrA and RprA act as positive regulators (Masse *et al*., 2003). DsrA and RprA RNA have complimentary sequences that allow them to pair with the upstream leader region of *rpoS* mRNA, which normally occludes the ribosome-binding site of the transcript, thus relieving translational inhibition (Figure 1.4.4.1). Although DsrA and RprA were initially discovered and characterised in *E. coli*, their gene sequences are around 90% identical in *S. enterica* (Jones *et al.*, 2006).

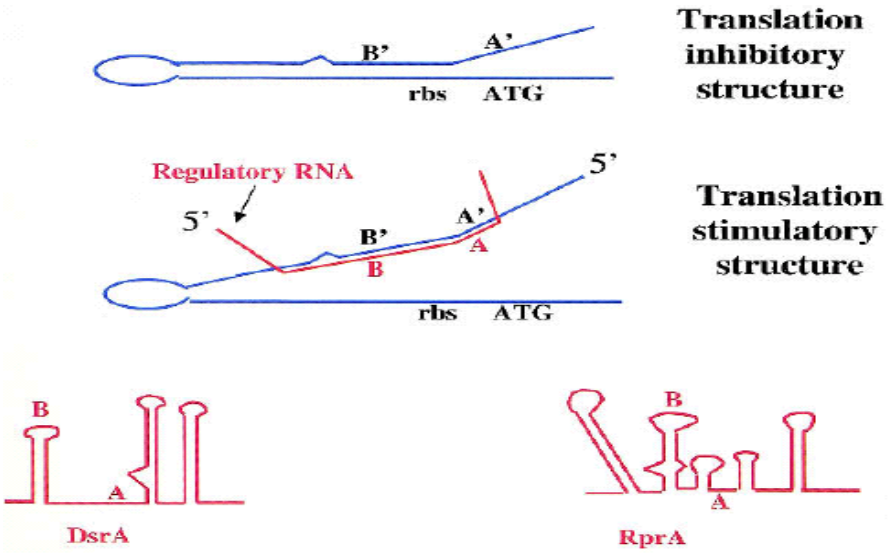


Figure 1.4.4.1: Positive regulation of RpoS expression by DsrA and RprA in *E. coli* (adapted from Gottesman, 2002). DsrA and RprA are proposed to stimulate *rpoS* translation by opening the inhibitory secondary structure in the *rpoS* mRNA leader that normally occludes the ribosome binding site (RBS) as represented by the translation inhibitory and stimulatory structures in the diagram. The regulatory RNAs, DsrA and RprA, are shown and the stem-loop regions responsible for binding to *rpoS* mRNA secondary structure are indicated (**A** and **B**). The *rpoS* mRNA regions that bind to the stem-loops of the sRNAs are highlighted **A'** and **B'**.

1.4.4.1.1 dsrA

In *E. coli*, normal RpoS expression at low temperatures depends on the non-translated RNA DsrA. Studies in *E. coli* have shown that the *dsrA* promoter (90% identical in nucleic acid sequence to that of *S.* Typhimurium) is only active at low temperatures (below 25°C), allowing expression of DsrA (an 87 nucleotide RNA) that, in turn, leads to the translation of RpoS during incubation at the reduced temperature. Repoila and Gottesman have shown, by Northern blotting of exponential phase cultures (OD_{600} of 0.3), that the amount of *E. coli* DsrA decreases 25-fold at 37°C, and 30-fold at 42°C, compared to the amount at 25°C (Repoila and Gottesman, 2001). Levels of RpoS protein are substantially lowered in an *E. coli dsrA* mutant. In addition to regulating *rpoS* mRNA translation, DsrA inhibits translation of the transcription modulator H-NS by binding to a complementary region in *hns* mRNA and blocking ribosome binding (Altuvia, 2004; Brescia *et al*., 2003). Sledjeski *et al*., have shown that *E. coli* DsrA acts post-transcriptionally to regulate RpoS, as mutations in *dsrA* decrease the expression of an *rpoS::lacZ* translational fusion, but not a transcriptional fusion (1996). Resch *et al* (2008) have shown that DsrA also stabilises the *E. coli rpoS* transcript against

degradation, by binding to and changing the conformation of the 5'UTR, therefore abolishing the RNase III cleavage site.

DsrA is predicted to form a structure with three stem-loops (as seen in Figure 1.4.4.1). The first of these stem-loops (B in Figure 1.4.4.1) is necessary for RpoS translation, and functions by an 'anti-antisense' mechanism that disrupts the natural intramolecular base-pairing which occurs around the SD region of *rpoS* mRNA, converting the sequence into a translationally active structure. The second of the stem-loops (A in Figure 1.4.4.1) is also essential for antisilencing, as it provides a target for the transcriptional silencer H-NS, and is less critical for RpoS translation (Majdalani *et al*., 1998; Hengge-Aronis, 2002).

As the sequences of DsrA and the 5'-UTR of RpoS are conserved between *E. coli* and *S.* Typhimurium, their regulatory functions also seem likely to be conserved (discussed in more detail in Chapter 4.2). However, a recent study by Jones *et al*. (2006) suggests that DsrA is not required for optimum RpoS expression in *S.* Typhimurium. This study shows that loss of *dsrA* has little effect on RpoS expression during exponential phase at 37°C. DsrA was also overexpressed in *S.* Typhimurium LT2 carrying an artificial pBAD promoter, but showed little effect on RpoS expression during both stationary phase and exponential phase at 18°C. However, this could be due to additional higher-order structures which interact (or fail to interact) to give elevated expression in this instance. Also, the impact of arabinose (the inducer) degradation via metabolism on pBAD expression of *dsrA* was not addressed in the study.

1.4.4.1.2 rprA

Both RprA (a 106 nucleotide regulatory RNA) and DsrA sRNAs have been shown to regulate RpoS translation in *E. coli* during osmotic stress (Wassarman, 2002). An *rprA* mutant does not show any obvious physiological defects, such as reduction of growth in rich media or reduced RpoS protein levels (Majdalani *et al*., 2001), suggesting that RprA is not essential or that there may be a level of genetic redundancy in the system. However, RprA was found to suppress the effect of a *dsrA* deletion during cell surface stress, namely osmotic shock, allowing the activation of RpoS translation in *E. coli* (Repoila, Majdalani and Gottesman, 2003). The Argaman lab has shown that the level of *E. coli* RprA increases with increasing cell density and is most abundant in stationary phase (Argaman *et al.*, 2001). This was later shown to be due to the stabilisation of RprA RNA during stationary phase, rather than increased synthesis of RprA (Majdalani *et al*., 2002).

In both *E. coli* and *S.* Typhimurium, RprA does not appear to have an extensive region of complementarity to the RpoS leader, leaving its mechanism of action unclear (Majdalani *et al*., 2001). This will be discussed in more detail later in Chapter 4.2.

1.4.4.1.3 oxyS

OxyS is a 109-nucleotide regulatory RNA that folds into a similar secondary structure to that of DsrA. The transcription of *E. coli* OxyS is rapidly induced (< 1min) after exposure to 60μM hydrogen peroxide during exponential growth. During oxidative stress, OxyS regulates the expression of around 40 genes in *E. coli* to help protect against oxidative damage. No induction of OxyS was observed under other stress conditions such as heat shock, cold shock or changes in osmolarity (Altuvia *et al*., 1997). Altuvia *et al.* used immunoblots in *E. coli*, probed with antiserum against RpoS, to show that RpoS translation was repressed by OxyS during exponential phase only (1997). During exponential growth, the transcriptional regulator, OxyR, induces the expression of several proteins in response to oxidative stress. Zhang *et al.* (1998) showed that OxyR also induces the expression of OxyS, which represses RpoS translation. This repression may be to avoid functional redundancy, as RpoS and OxyR activate some of the same antioxidant genes. The negative regulation of RpoS is thought to be caused by the rather long A-rich single-stranded region between stem-loops 2 and 3 of OxyS (Hengge-Aronis, 2002). However, Zhang *et al*. found that OxyS does not repress RpoS expression in *hfq* mutants (1998) and Hfq has been shown to co-immunoprecipitate with OxyS. This suggests that either OxyS represses *rpoS* translation by altering Hfq activity or that Hfq is required to facilitate their interaction.

1.4.4.2 The Hfq protein

As mentioned above, one of the functions of sRNAs is to regulate bacterial genes involved in adaptation to environmental stresses. In most cases, this RNA regulation requires Hfq, a bacterial Sm-like protein that stabilises sRNAs and enhances RNA-RNA interactions (Lease and Woodson, 2004). Hfq is widely distributed and, like RpoS, is found in all proteobacteria, actinobacteria and cyanobacteria. The eukaryotic homologues of this protein, the LSm proteins, can be found in viruses and eukaryotes, including mammals, fungi and green plants and function in cytoplasmic mRNA turnover and snRNA splicing (NCBI Entrez Protein Database: taxonomic groups). Hfq was first identified for its role in replication of the RNA phage Qβ (Franze de Fernandez *et al.*, 1968) but has more recently been identified as one of the most abundant *E. coli* proteins, with an estimated 50,000 to 60,000 copies per cell

throughout the growth cycle (Ishihama, 2000). 80 to 90% of Hfq is found in the cytoplasmic fraction in association with ribosomes and the remaining intracellular Hfq is associated with the nucleoid (Brennan and Link, 2007). Minimal, *in vitro* binding site studies have shown that *E. coli* Hfq preferentially binds AU-rich sequences adjacent to double-stranded regions (Mikulecky *et* al., 2004) and is considered an RNA chaperone that 'melts' secondary structure. Tsui and co-workers report that the disruption of the *hfq* gene, in *E. coli* K-12, causes pleiotropic phenotypes such as decreased growth rate, higher osmosensitivity, increased cell length and sensitivity to oxidants and UV light (1994). These phenotypes resemble some of those of an *rpoS* mutant (Fang *et al*., 1992; Munro *et al*., 2005). Studies in *S*. Typhimurium, using *rpoS::lacZ* protein fusions, have shown that an *hfq* mutant has a four- to sevenfold reduction in expression of *rpoS* during stationary phase. This can be attributed primarily to a defect in translation as pulse-labeling studies have shown that the rate of RpoS synthesis is significantly decreased in an *hfq* mutant (Brown and Elliott, 1996). However, Hfq also has physiological functions that are independent of RpoS; in particular it stimulates the degradation of mRNA encoded by *miaA, mutS*, *ompA* and also its own RNA, by targeting them to RNase E (see section 1.7), and is thought to recruit ribosomes to mRNA (Nogueira and Springer, 2000; Soper and Woodson, 2008). Also, Hfq can protect sRNAs and mRNA, such as DsrA and RyhB, from degradation by RNase E, as Hfq-binding sites and the RNase E cleavage site are structurally similar and overlap (Moll *et al*., 2003). Hfq has also been shown to protect mRNAs, such as that encoded by *rpsO*, from decay by binding to the poly(A) tail, protecting the message from the enzymes PNPase and RNase II which are involved in exonucleolytic mRNA degradation (Hajnsdorf and Regnier, 2000; Vassilieva and Garber, 2002; Brennan and Link, 2007). Sittka *et al*. (2007) have shown that an *S.* Typhimurium *hfq* mutant is highly attenuated in mice after both oral and intraperitoneal infection. It also shows a severe defect in invasion of epithelial cells and a growth defect in both epithelial cells and macrophages *in vitro*. These phenotypes were largely RpoS-independent and were associated with loss of cell motility, reduced adhesion and abrogated effector protein secretion.

The mechanism(s) of how Hfq mediates sRNA interactions, and regulates RpoS expression, is/ are currently unknown. Electron microscopy and crystallographic studies have shown that *E. coli* Hfq forms a homo-hexameric ring of identical 11.2 kDa subunits (Lease and Woodson, 2004). The central hole in this ring is sufficient to accommodate single-stranded but not double-stranded RNA (Pannone and Wolin, 2000). Lease and Woodson (2004) have also provided evidence that *E. coli* Hfq can bind DsrA and *rpoS* RNAs independently, and can also form ternary complexes between OxyS and Spot42 RNAs and their target mRNAs. In addition, Hfq has been shown to co-immunoprecipitate with *rpoS* mRNA. Thus, Hfq may stabilise a semi-stable *rpoS* mRNA secondary structure, which can easily open up when some

additional stimulating factor (e.g. sRNAs) is induced or activated. However, Hfq does not necessarily have to affect *rpoS* mRNA secondary structure directly, but may act as a 'platform' that recruits additional factors involved in RpoS translational control (Hengge-Aronis, 2002). In fact, Arluison *et al*. (2007) have used nuclease footprinting to show that Hfq recognizes several sites on *rpoS* mRNA without changing the secondary structure in the region that inhibits translation (discussed in more detail in Chapter 4.2). Lease and Woodson also showed that Hfq has only a modest effect on the rate of base-pairing between *E. coli* DsrA and *rpoS* mRNA (2004). This raises the question of whether other factors also contribute to this interaction *in vivo*. This idea is supported by the findings of Brescia *et al*., who report that not all RNA helices bind Hfq with equal affinity (2003). Therefore, it seems likely that higher-order structures, perhaps involving CspA (Cold shock protein A) paralogues, may be involved in these interactions.

1.4.4.3 H-NS

H-NS is widely distributed in gram-negative, eukaryotes and viruses and was originally found to be one of the abundant proteins, with a molecular mass of around 15kDa, associated with *E. coli* DNA (Varshavsky *et al.,* 1977). H-NS has a strong affinity for intrinsically curved DNA, usually specified by AT-rich motifs, commonly found at bacterial promoters. It contains an amino-terminal oligomerisation domain, a carboxy-terminal nucleic-acid-binding domain and a flexible linker that connects these two functional modules (Yamada *et al.,* 1991; Dorman, 2007). H-NS exists primarily as a dimer at low concentrations but can multimerise into higher order complexes that can form bridges between DNA helices (Fang and Rimsky, 2008). In this way, H-NS influences the degree of negative super-coiling of DNA and can compact DNA, implicating H-NS in chromosome organisation (Owen-Hughes *et al.,* 1992). H-NS functions as a transcriptional repressor by physically blocking access of RNA polymerase to the promoter region (Rimsky *et al.,* 2001). Recently, H-NS has been implicated in the process of xenogeneic silencing, where it represses the transcription of foreign genes, such as pathogenicity islands, acquired by horizontal transfer (Stoebel, Free and Dorman, 2008).

In *E. coli*, studies have shown that the *hns* gene is auto-regulated at the transcriptional level, maintaining a constant H-NS to DNA ratio throughout normal growth. However, during the first 3-4 h of cold shock, the concentration of H-NS increases three- to four-fold due to stimulation of *hns* gene transcription by the cold shock protein, CspA (La Teana *et al.,* 1991; Dorman, 2007, See section 1.5). The RNA chaperone Hfq contributes to the post-

transcriptional control of *hns* expression via the negatively-acting antisense RNA, DsrA (Dorman, 2007; mentioned in section 1.4.4.1).

The phenotypes of *E. coli hns* mutants are highly pleiotropic: slow-growing, mucoidal colony formation, increased osmosensitivity and a prominent lag, before outgrowth, upon shift to low temperature (Atlung and Ingmer, 1997). H-NS has been shown to reduce the transcription of around 300 genes in *E. coli* and the level of more than 30 proteins has been shown to increase in *hns* mutants during exponential phase (Barth *et al.,* 1995; Yamada *et al.,* 1991; Brescia *et al.,* 2004; White-Ziegler and Davis, 2009). However, 2-D gel electrophoresis has shown that at least 22 of these proteins are in fact RpoS-dependent (Barth *et al.,* 1995). Many *hns* mutant strains also carry spontaneously arising secondary mutations in the *rpoS* gene, as constitutive expression of RpoS-regulated genes throughout all growth phases can be detrimental to the cell (Desai and Mahadevan, 2006).

Immunoblotting has shown that the cellular content of RpoS in *E. coli hns* mutants is increased more than 10-fold during the exponential growth phase (Barth *et al.,* 1995; Hengge-Aronis, 2002). In the same study, it was observed that the expression from *rpoS::lacZ* transcriptional fusions was not significantly altered (less than a 2-fold increase) in the *hns* mutant background. However, the expression from translational fusions was stimulated more than sevenfold under the same conditions. Nuclease foot-printing has shown that low concentrations of H-NS enhanced the degradation of the 5'UTR region of *rpoS* mRNA *in vitro* (Brescia *et al.,* 2004). Together, these results suggest that H-NS interferes with RpoS expression during exponential phase by a mechanism that acts at the post-transcriptional level of *rpoS* regulation. Interestingly, a dramatic (more than tenfold) increase in the stability of newly synthesised RpoS has also been shown in *hns* mutants of *E. coli* during exponential phase; these strains also exhibited an increased thermotolerance during logarithmic growth correlating with RpoS function (Yamashino, Ueguchi and Mizuno, 1995). This stabilisation of RpoS is independent of the stabilisation of other ClpXP substrates which may suggest the effect is specific to the RssB-dependent degradation pathway (Zhou and Gottesman, 2006).

The mechanism by which H-NS regulates RpoS expression is not yet fully known. Many explanations have been offered: H-NS may negatively regulate the transcription of a gene encoding a putative factor that is either responsible for stabilising RpoS or is involved in the efficient translation of *rpoS* mRNA. Alternatively, H-NS may bind directly to *rpoS* mRNA, resulting in inhibition of translation, as H-NS is known to bind RNA as well as DNA (Brescia, Kaw and Sledjeski, 2004). It is of interest to note that *E. coli* also contains H-NS

paralogues, StpA for example (Zhang and Belfort, 1992). Yet, these do not appear to offer significant genetic redundancy in an *hns* negative background.

1.5 Cold shock proteins

After exponentially growing *E. coli* cells are transferred to 15°C, there is a growth lag period that is characterised by a high-induction of cold shock proteins that are essential for cellular adaptation at low temperature. This occurs despite a dramatic reduction in total protein synthesis. Upon cold shock, protein synthesis is limited by the inhibition of transcription and translation due to stabilisation of aberrant RNA secondary structures (Neupert, Karcher and Bock, 2008; Waldminghaus *et al*., 2008; as described in section 1.2). Also, some bacterial organisms limit protein synthesis by storing inactive ribosomes so that they can be rapidly reactivated when conditions improve (Wilson and Nierhaus, 2004). *E. coli* protein Y inhibits translation initiation during cold shock by filling the tRNA and mRNA binding channel of the small ribosome subunit (30S), thereby stabilising intact ribosomes, but is quickly released once normal growth conditions are restored (Vila-Sanjuro *et.al.,* 2004; Wilson and Nierhaus, 2004). Protein Y is also associated with the ribosome during stationary phase where it blocks translation (Phadtare, 2004). In addition, RbfA is bound to the ribosomal 30S subunit and enhances the capability of ribosomes for translation at low temperatures, thus initiating the whole system of cell response to cold shock (Golovlev, 2003). Both protein Y and RbfA are widely distributed in bacteria; however, no homologues have been reported in eukaryotes (NCBI Entrez Protein Database: taxonomic groups). It should be noted that cold shock also suppresses protein synthesis at the level of transcription initiation in *E. coli*. However, once the synthesis of a polypeptide chain has started, its elongation will continue irrespective of the cold shock conditions (Golovlev, 2003).

In *E. coli*, expression of cold shock proteins reaches a maximum during the cold shock adaptation or acclimation phase (see Figure 1.2.3). Following this, synthesis of cold shock proteins declines and a new steady-state level of protein expression is established (Ermolenko and Makhatadze, 2002). Genes in the cold shock regulon, such as *hns*, *gyrA* and *pnp*, encode proteins involved in a variety of essential functions such as transcription, translation and mRNA degradation respectively (La Teana *et* al., 1991; Brandi *et al*., 1996; summary of the *E. coli* cold shock regulon in Appendix Table 1). Protein CspA was found to act as a cold shock transcriptional enhancer of this cold shock regulon *in vitro* and, consistent with this,

appears to be the first cold shock protein synthesised (Brandi *et al*., 1996; see sections 1.5.1 and 1.6). H-NS is one of the prominent proteins expressed during cold shock (La Teana *et al.,* 1991) and its transcription is induced shortly after a shift from 37°C to 10°C. The induction is dependent on the major cold-shock protein CspA, and *in vitro* studies have provided evidence that it binds to sequences located close to +10 in the *hns* promoter region (Brandi *et al.,* 1994). This leads to the concentration of H-NS increasing three- to four-fold during the first 3-4 h after a shift to low temperature and promotes chromosome condensation (La Teana *et al*., 1991; as mentioned in Section 1.4.4.3).

The *E. coli* polynucleotide phosphorylase (PNPase), an exoribonuclease involved in RNA degradation, is essential for growth of acclimated cells at low temperatures. Although the induction of cold shock proteins was normal in a *pnp* mutant of *E. coli*, their production was no longer auto-regulated and were maintained at high levels throughout cold shock. This resulted in cell growth arrest and a reduction of colony-forming ability below 25°C (Yamanaka and Inouye, 2001). PNPase therefore degrades mRNAs that code for cold shock proteins to allow the resumption of bacterial replication after acclimation to a low temperature environment (Ygberg *et al.,* 2006). PNPase is believed to regulate its own expression by binding its own mRNA at or near the SD sequence and interfering with mRNA interaction with 30S ribosomes. It has been suggested that changes in the efficiency by which PNPase competes with the 30S subunit for *pnp* mRNA binding may play a role in the cold-shock induction of this gene (Zangrossi *et al*., 2000).

The cold shock DEAD-box protein, CsdA, was first identified as a cold shock induced RNA helicase in *E. coli* and was found to be involved in ribosomal biogenesis of 50S subunits (Charollais, Dreyfus and Iost, 2004). CsdA was found to co-purify and interact physically and functionally with RNase E (Prud'homme-Genereux *et al*., 2004). As CsdA acts to unwind double-stranded RNA molecules at low temperatures, it facilitates both the metabolism of RNA by the degradosome, the multi-enzyme complex responsible for major RNA degradation, and the efficient translation of mRNA previously hindered by extensive secondary structure (Jones *et al*., 1996; Prud'homme-Genereux *et al*., 2004). The cold shock degradosome, and the involvement of CsdA, is described in more detail in section 1.7. In *E. coli*, knockout mutants of *csdA* demonstrate wild-type cell growth at 37°C but exhibit retarded growth at 15°C (Jones and Inouye, 1996).

1.5.1 The CspA family

Brandi *et al.* (1996) have reported that the first protein induced upon a temperature downshift was CspA of *E. coli* (a 7.4kDa cytoplasmic protein); its production alone constitutes more than 10% of the total cellular protein synthesis at 18°C (Bae *et al.*, 1999; Goldstein, Pollitt and Inouye, 1990). *In vitro* studies have shown CspA to be an RNA chaperone, which binds to and destabilises RNA secondary structures, crucial for the efficient mRNA translation at low temperatures (Jiang, Hou and Inouye, 1997). Disruption of the nucleic acid melting capacity of CspE, by mutation, abolishes its cold acclimation functions (Neidhardt *et al.*, 1987). The ability of the CspA family to destabilise RNA secondary structure has been implicated in transcription antitermination at ρ-independent terminators, and has pointed to a role as transcriptional enhancers of the cold shock regulon (Ermolenko and Makhatadze, 2002; Bae *et al.*, 2000). Eight more proteins homologous to CspA, named in alphabetical order from CspB to CspI, have been identified in *E. coli* and all preferentially bind pyrimidine-rich regions of ssDNA and ssRNA but not dsDNA. Yamanaka *et al.* suggest that the CspA family originated from a number of gene duplications and, after evolutionary adaptation, resulted in specific genes which respond to different environments (1998). None of the CspA homologues of *E. coli* appear to be singularly responsible for cold shock adaptation as deletions in any one of the *csp* genes does not result in cold sensitivity. In a triple deletion strain (Δ*cspA* Δ*cspB* Δ*cspG*) the CspE protein, normally constitutively expressed at 37°C, accumulated at low temperature. This suggests that the regulation of members of the CspA family may be coordinated and that members may functionally substitute for each other during cold acclimation (Phadtare, Inouye and Severinov, 2002). It appears that proteins of the CspA family not only play a major role during cold shock adaptation but are also important under normal growth conditions, as almost all are expressed at some point of growth under non-cold shock conditions (Horn *et al.*, 2007). CspC and CspE of *E. coli* were also shown to be involved in the regulation of the expression of *rpoS* during normal growth at 37°C (Phadtare and Inouye, 2001).

More than 900 homologues of CspA have been identified in bacteria. Three CspA-like proteins have been identified in *Bacillus subtilis*: CspB, CspC and CspD. CspB and CspC are induced upon cold shock and upon entry into stationary phase. CspB plays the most important role during cold stress and has a similar function to CspA of *E. coli*. The existence of at least one of these proteins is essential for cell viability (Horn *et al.*, 2007). CspA is also highly homologous to a domain called the CSD (cold-shock domain) of eukaryotic Y-box proteins, which are involved in regulation of gene expression at the level of transcription or translation (Bae *et al.*, 2000; Horn *et al.*, 2007). Contrary to prokaryotes, all eukaryotic CSD proteins

characterised thus far contain additional C-terminal auxiliary domains such as Arg-Gly repeats and zinc fingers (Graumann and Marahiel, 1998; Nakaminami, Karlson and Imai, 2006). To date, only four proteins are reported to contain a CSD within the plant kingdom: Arabidopsis AtGRP2 and AtGRP2b, tobacco NtGRP and wheat WCSP1. However, using comparative GenBank searches, highly conserved CSDs were identified within 19 genera that represent lower plants, monocots, dicots and woody plants (Karlson and Imai, 2003). It was discovered that plants also contain complete ORFs that encode putative CSD proteins that are nearly identical to prokaryotic CSPs in size and sequence. Interestingly, cyanobacteria and archaea lack CSD protein homologues; however, they do contain a family of cold-inducible RNA-binding domain proteins that may perform the same function (Phadtare, 2004).

1.6 The CspA paralogues of *Salmonella* Typhimurium

There are only 6 chromosomal members of the CspA family found in *S.* Typhimurium: CspA through CspE, and CspH. There is also a plasmid copy of CspA on a naturally isolated camphor-resistance plasmid (NCBI Database). Although these proteins are conserved (over 60% amino acid identity in most cases; amino acid comparison in Table 1.6), their cellular abundance and control of their expression differs. One of the unique features of several members of the CspA family is the unusually long 5' untranslated region (5'UTR). In *S.* Typhimurium, CspA has a 5'UTR of 145 nucleotides long, whilst that of CspB is 162 nucleotides long. However, it is reported that CspH has a 5'UTR of only 23 nucleotides long (Kim *et al*., 2001). Phadtare *et al*. have shown, by deletion analysis, that in *E. coli* the 5'UTR is responsible for the extreme instability of *cspA* mRNA at 37°C and the stability of this transcript at low temperatures (1999).

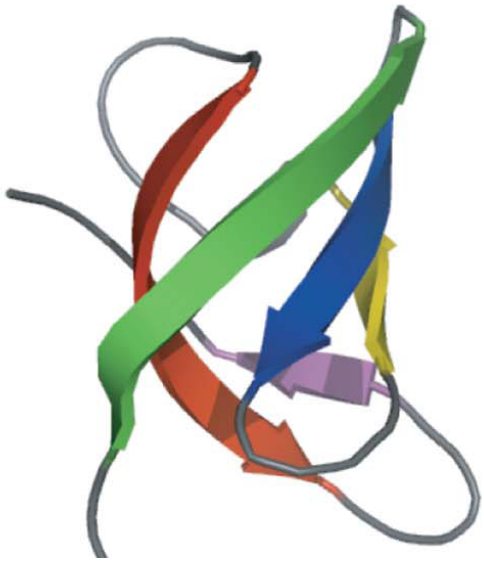
Table 1.6: Conserved amino acid identities of the *S.* Typhimurium CspA family. Values represent % positive amino acid identity as determined by NCBI Blast analysis.

CspB	CspC	CspD	CspE	CspH	
85	80	64	85	61	**CspA**
	77	66	79	61	**CspB**
		67	89	59	**CspC**
			68	48	**CspD**
				62	**CspE**

1.6.1 The 3D structure of CspA paralogues

The family of bacterial CspA proteins is characterised by a conserved sequence of 65-75 amino acid residues which form a 3-D structure consisting of five anti-parallel β-sheets, with a classical Greek key topology, named the cold shock domain fold (Newkirk *et al.*, 1994; Lopez and Makhatadze, 2000). A domain fold similar to a cold shock domain fold was found in ribosomal protein S1 and several different cold-inducible proteins: NusA, PNPase and IF1 (Ermolenko and Makhatadze, 2002). Figure 1.6.1 shows the 3-D structure of CspE from *S.* Typhimurium. There are two RNA-binding motifs, RNP1 and (a degenerate) RNP2, located on beta-strands 2 and 3, which have seven aromatic residues on the surface (Figure 1.6.1(b): shown in yellow) to allow hydrophilic interactions (Figure 1.6.1(b): dashed red lines) between the protein and nucleic acids (Phadtare *et al*., 1999) or between protein monomers (Morgan *et al*., 2009). These residues are conserved among the CspA family members, except for CspD and CspH, and are required for protein function and stability (Phadtare *et al*., 2002). Hillier *et al*. have shown, by substitution mutation, that removal of any of these aromatic residues destabilises CspA, with Phe18 most critical for maintaining stability (1998). In the presence of nucleic acid ligands, the CspA family of *Bacillus subtilis* become less sensitive to proteolytic degradation *in vitro*, suggesting that the high stability of CspA paralogues *in vivo* is mediated by nucleic acid binding (Schindler *et al*., 1999).

(a)



(b)

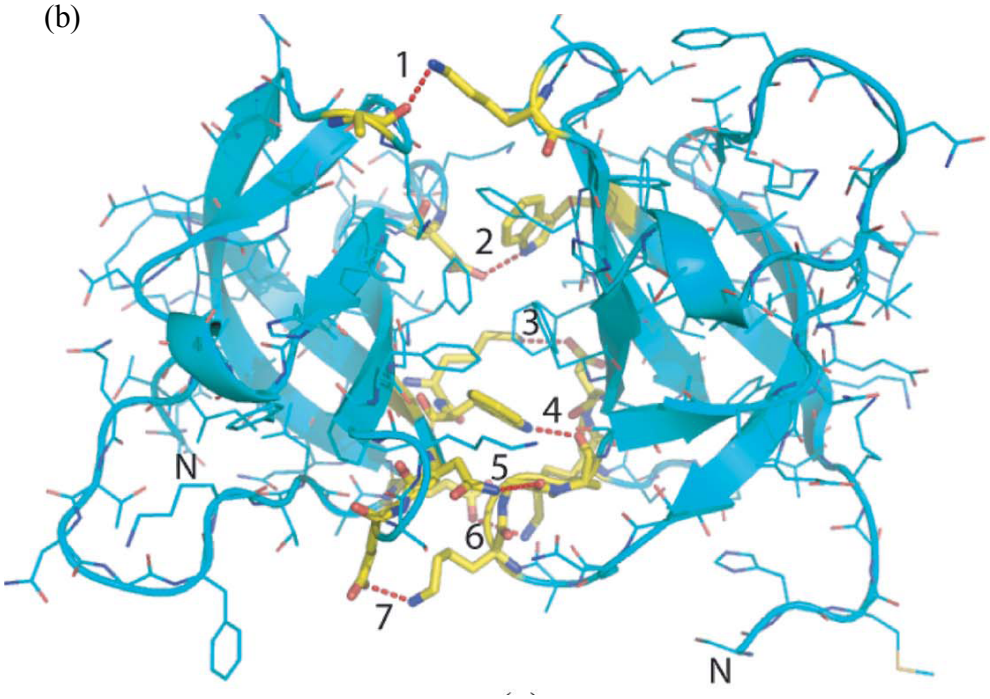


Figure 1.6.1: The X-ray crystal structure of CspE from *S.* Typhimurium (Figure taken from the PDB database (3i2z), Morgan *et al*., 2009).

(a) A three-dimensional ribbon diagram of CspE forming a classic five-stranded β-barrel structure. The five β-strands have been coloured to highlight their positions: β-1 (green), β-2 (blue), β-3 (yellow), β-4 (red) and β-5 (magenta).

(b) The amino acids (shown in yellow) which form potential hydrogen bonds (dashed red lines) between two CspE monomers or bind to nucleic acids, as calculated using the PISA program (Krissinel & Henrick, 2005). The hydrophilic residues which form hydrogen bonds are (1) Ala58, (2) Asp28, (3) Lys9, (4) Trp10, (5)Asn12, (6) Glu46 and (7) Glu13.

1.6.2 Functions of CspA paralogues

1.6.2.1 CspA and CspB

The CspA and CspB proteins are cold shock induced in *S.* Typhimurium. As in *E. coli*, the *cspA* gene is actively transcribed even at 37°C. However the *cspA* mRNA is highly unstable at this temperature (Bae *et al.*, 1999). Studies in *E. coli* have shown that the *cspA* mRNA has a half-life of less than 12 seconds at 37°C, but becomes more stable as the temperature is lowered (Phadtare *et al.*, 1999). Brandi *et al*. report that the half life of *E. coli cspA* mRNA increases to more than 20 minutes at 15°C and a stem-loop structure at the 5' terminus of the *cspA* mRNA is critical for its stabilization following cold shock (1996). These findings agree with previous studies in our lab in *S.* Typhimurium (Craig *et al*, 1998). This mRNA stability is transient at 15°C and the mRNA destabilizes once the cells are adapted to low temperature (Goldenberg, Azar and Oppenheim, 1996). Downregulation of *E. coli cspA* expression after cold acclimation appears to be mediated by a highly conserved unique 11 bp sequence termed the 'cold box', located in the 5'-UTR region of the *cspA* mRNA (Bae *et al*., 1999). Jiang *et al*. report that CspA can negatively regulate its own gene expression through this cold box region which has the potential to form a stem-loop structure and thus may be a natural transcriptional pause signal (1996); deletion of this 5'UTR region results in constitutive expression of CspA at 37°C. The 5'UTR is conserved in *S.* Typhimurium *cspA* mRNA.

Previous studies in *E. coli* have emphasised that expression of CspA is activated by a temperature downshift, usually of 6°C or more, and occurs within 30 min of this shift to low temperature (Jones and Inouye, 1996; Goldenberg, Azar and Oppenheim, 1996). However, *cspB::*Mu*dlux* experiments have shown that a large temperature downshift is not required for the induction of *cspB* in *S.* Typhimurium, as substantial gene expression occurs at or below a temperature threshold of 22°C, irrespective of the initial starting temperature. Primer extension analysis confirmed that light expression correlates with *cspB* mRNA levels, as there was a clear *cspB* band evident after a downshift to 22°C or lower but is absent at higher temperatures. Northern blotting over a time-course proved that *cspB* mRNA is highly stable at 10°C, but rapidly becomes unstable when the temperature is raised towards 37°C (Craig *et al*., 1998), even in the presence of rifampicin. Brandi *et al*. (1996) have shown a similar result as *E. coli cspB* mRNA is absent at higher temperatures but accumulates below a threshold of approximately 20°C.

1.6.2.2 CspC and CspE

Whilst traditionally named as cold shock proteins because of the high level expression of CspA at low temperatures, it is of note that not all CspA paralogues are highly induced at low temperatures. LacZ fusions have shown that in *E. coli*, two members of the CspA family, CspC and CspE, are constitutively expressed at 37°C (Bae *et al.,* 1999). Phadtare and Inouye have also shown that CspC and CspE proteins of *E. coli* are not significantly induced by high osmolarity, high or low temperature stress or extremes of pH (2001). The amount and stability of *rpoS* mRNA, the general stress sigma subunit of RNA polymerase, is greatly enhanced by the overexpression of CspC and CspE (Phadtare and Inouye, 2001). Western blot analysis, using monoclonal anti-RpoS antibodies, has shown that overexpression of *E. coli* CspE resulted in a 4-fold upregulation of RpoS (Phadtare, Inouye and Severinov, 2002). This group has also demonstrated that CspE affects *rpoS* transcription by preventing the formation of nascent RNA secondary structures (Phadtare *et al*., 2002). Proteomic studies from our lab have also shown that CspC and CspE proteins are constitutive and abundant in *S.* Typhimurium (FSA project report B01008, 2001).

Bae *et al*. have shown that the *E. coli* CspE functions as a negative regulator for *cspA* expression during the lag period of growth at 37°C, as inactivation of *cspE* results in an overproduction of CspA (1999). This is paralleled by an increase in *cspA* mRNA levels and is probably due to CspE interacting with the transcription elongation complex at the *cspA* cold box region. Binding assays have shown that CspE may bind to the poly(A) tails at the 3'end of mRNAs, thus interfering with their degradation by PNPase and RNase II at 37°C (Gualerzi, Giuliodori and Pon, 2003; Horn *et al*., 2007).

Bae *et al*. (2000) have demonstrated that CspC and CspE of *E. coli* are transcription antiterminators. Overexpression of cloned CspC and CspE at 37°C was sufficient to induce transcription of the *metY-rpsO* operon, located downstream of multiple transcription terminators. Many different methods have been used to test whether there was a direct interaction between CspE and RNA polymerase core enzyme or holoenzyme but none was detected (Hanna and Liu, 1998; Bae *et al*., 2000). This suggests that the effect of CspE on transcription termination may be caused by preventing the formation of secondary structures on the nascent mRNAs. CspC and CspE of *E. coli* have also been implicated in nucleoid condensation, as their overproduction leads to resistance against camphor treatment, by preventing the unfolding of the nucleoid normally caused by camphor (Ermolenko and Makhatadze, 2002).

1.6.2.3 CspD and CspH

Less is known about CspD and CspH, the most divergent of the paralogues in *E. coli*, although CspD is thought to be expressed in response to glucose starvation. CspD is also induced during stationary phase at 37°C independently of RpoS. ppGpp was shown to be a positive regulator of *cspD* expression in *E. coli* (Yamanaka and Inouye, 1997; Phadtare *et al.,* 2006). Giangrossi *et al* (2001) report that the *E. coli* CspD is associated with the nucleoid in the late exponential phase of growth. CspD of *E. coli* exists as a dimer *in vitro* and binds non-cooperatively ssDNA and RNA but not dsDNA (Yamanaka *et al*., 2001). Sittka *et al*. (2007) have predicted the subcellular localization of CspD, by 2D analysis, to be cytoplasmic.

Kim *et al.* reported that the *cspH* gene of *S.* Typhimurium is induced in response to a temperature downshift during early exponential phase and within 1 h of cold shock; *cspH* mRNA remained stable with a half-life of approximately 10 min (2001). This group later found that the promoter of *cspH* is active during exponential phase at 37°C, and that *cspH* mRNA is more stable at this temperature than the other *csp* mRNAs due to its unusually short 5'UTR of only 23 nucleotides and therefore low susceptibility to cellular RNases (2004). This group has also shown that the mRNA level of *cspH*, in *S.* Typhimurium, increases when stationary cells are subcultured into a rich medium, i.e upon nutrient upshift (2001). DNA gyrase is a molecular machine that uses the energy of ATP hydrolysis to introduce essential negative supercoils into DNA (Gore *et al*., 2006). The increase in the ATP/ADP ratio through nutritional upshift activates DNA gyrase, as more targets are provided for the enzyme. *cspH* expression was shown to be blocked by DNA gyrase inhibitors, such as novobiocin and coumermycin, suggesting that the *cspH* promoter is sensitive to alterations in DNA superhelical density through DNA gyrase (Kim *et al*., 2004).

1.6.3 Regulation of *cspA* and *cspB* mRNA by the endonuclease RNase E

As described previously, the gene expression of *cspA* is tightly regulated at several steps: transcription, mRNA stability and translational efficiency. Upon cold shock, the stability of *cspA* mRNA increases 150-fold, causing a corresponding increase in translation (Nogueira and Springer, 2000). The RNA degradosome is a multi-enzyme complex responsible for general RNA decay in the bacterial cell (Hankins *et al*., 2007; described in more detail in Section 1.7). Cold stress-induced change of the RNA degradosome, i.e. the replacement of the RhlB helicase with CsdA, results in a drastic stabilisation of cold shock transcripts (Gualerzi,

Giuliodori and Pon, 2003). Temperature-dependent differences in the stability of *cspA* and *cspB* transcripts represent a major control mechanism for regulating CspA and CspB production in *S.* Typhimurium.

RNase E, an essential endoribonuclease, plays a major role in the degradation of *cspA* mRNA at 37°C. Potential recognition elements have been identified in the 5'UTR of *cspA* mRNA and deletion of the 5'UTR of *cspA* leads to constitutive expression of CspA at 37°C (Fang, Xia and Inouye, 1999). Further evidence is provided by Fang *et al*. (1997) as a three-base substitution mutation at the putative RNase E site resulted in dramatic stabilisation of the mRNA, allowing a high production of CspA even at 37°C. Also, *cspA* mRNA expressed from a plasmid is stabilised in a strain carrying a temperature-sensitive *rne* mutation (Hankins *et al*., 2007).

It has been suggested that a pathway of mRNA decay may be transiently inactivated during the initial phases of cold shock, somehow sparing *cspA* mRNA. The finding that the RNA degradosome, isolated from cold shocked cells, is modified due to inclusion of the DEAD box helicase, CsdA, suggests that this may be the case (Hankins *et al*., 2007). PNPase only is held responsible for the degradation of the *cspA* transcript during cold acclimation (Gualerzi, Giuliodori and Pon, 2003). However, it is possible that alternative secondary structures may also form in the 5'UTR of *cspA* mRNA at low temperature.

1.7 The RNA degradosome

In eubacteria, mRNA degradation is an important mechanism for regulating gene expression and results from the sequential action of endo- and exo-ribonucleases (Diwa *et al*., 2002). At least three of the endoribonucleases identified in *E. coli*: RNase E, RNase III and RNase G, have been shown to initiate RNA decay. A critical step in the decay of most mRNAs in *E. coli* is cleavage at one or more internal sites by RNase E; this is generally believed to be the rate-limiting step in mRNA degradation (Carpousis, 2007). The broad cleavage site specificity of RNase E is characterised by a preference for single-stranded regions of RNA that are AU rich (Diwa *et al*., 2002). The cleavage products are then further degraded by a combination of endonucleolytic and 3'-exonucleolytic digestion (Kemmer and Neubauer,

2006). Translating ribosomes usually protect mRNA from endonucleolytic attack and mutations in the RBS have been shown to accelerate mRNA degradation (Komarova *et al*., 2005).

In *E. coli*, the exoribonuclease PNPase, the RhlB RNA helicase and the glycolytic enzyme enolase, assemble on the C-terminal region of the endoribonuclease RNase E to form a multi-enzyme complex termed the RNA degradosome. The RNA degradosome is organised as helical filamentous structures that coil around the length of the cell (Taghbalout and Rothfield, 2007) and is believed to act as a general RNA decay machine in which its components cooperate during mRNA decay. However, degradosome proteins can remain unattached to RNase E, as, in *E. coli*, only 5-10% of cellular enolase and 10-20% of PNPase are estimated to be present in the complex (Bernstein *et al*., 2004). RNase E and PNPase are also important enzymes for the maturation of stable RNA, as well as the degradation of mRNA. In view of its important function in RNA processing and decay, it is not surprising that RNase E is an essential *E. coli* protein, the underproduction or overproduction of which can impair cell growth (Apirion, 1978; Goldblum and Apirion, 1981). *E. coli* knockout mutants of RNase E cannot be cultured as the strain is unable to form colonies on solid medium but does grow in liquid as filaments. A minimum RNase E concentration of 10 to 20% of the wildtype level has been shown to be sufficient to allow normal growth of *E. coli* on solid medium (Kemmer and Neubauer, 2006).

RNase E is a large, 1060 amino acid multi-domain protein with two halves: the N-terminal half containing the catalytic domain, structurally related to DNase I, and the C-terminal 'scaffold' domain that has no known catalytic activity (Carpousis, 2007). The N-terminal portion of RNase E is sufficient to support cell growth in the absence of the full length protein (Kido *et al*., 1996). Other important features include a S1 RNA binding domain, a site forming a pocket that interacts with the terminus of 5'-monophosphorylated RNA, and a channel that permits access to the catalytic site of single-stranded RNA but not double-stranded RNA. The C-terminal region of RNase E contains two RNA binding sites and three protein interaction sites involved in the assembly of the degradosome. To maintain cellular levels of RNase E within a narrow range, *E. coli* cells have evolved a feedback mechanism for controlling its synthesis, by modulating the longevity of RNase E (*rne*) mRNA in response to changes in cellular RNase E activity (Diwa *et al*., 2002). Also, a mutation in *rne* increases *rne* mRNA stability at 37°C (Prud'homme *et al*., 2004). In *E. coli*, there is a significant sequence similarity between RNase G and the catalytic core of RNase E and both endonucleases cleave similar single-stranded regions of mRNA specifically. However, RNase G lacks the extended non-catalytic region found in RNase E and is therefore unable to

associate with the RNA degradosome (Carpousis, 2007). For this reason, RNase G is unable to substitute for RNase E. RNase III is specific for double-stranded RNA and plays multiple roles in the processing of rRNA and mRNA (Viegas *et al.*, 2007).

Studies have found that Hfq copurifies with RNase E to form specialised ribonucleoprotein complexes distinct from the degradosome and the C-terminal scaffold domain of RNase E has been identified as the Hfq binding site (Morita, Maki and Aiba, 2005; Brennan and Link, 2007). The presence of RNase E-Hfq-sRNA complexes explains why sRNAs and their mRNA targets are rapidly degraded by RNase E in a concerted manner. However, Moll *et al* (2003) have shown that the binding site of Hfq and the RNase E cleavage sites on mRNA and sRNAs tend to be coincident as they both comprise AU-rich regions next to elements of secondary structure. In this way Hfq can protect these RNAs from RNase E degradation.

PNPase acts as a 3'-exoribonuclease in *E. coli*. It degrades RNA from the 3'-end, releasing nucleotides in a progressive 3' to 5' degradation. PNPase is a phosphorylase i.e. it uses phosphate to cleave phosphodiester bonds. A functional interaction between PNPase and RhlB has been shown in *E. coli* but is dependent on the binding of these enzymes to the scaffold region of RNase E (Carpousis, 2007). PNPase specifically acts on single-stranded RNA substrates, pausing at stem-looped regions, but these are unwound by the ATP-dependent RNA helicase activity of RhlB (Vanzo *et al*., 1998).

Enolase is a glycolytic enzyme that is abundant in *E. coli* and catalyses the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate (Carpousis, 2007). However, its role in mRNA decay is yet to be elucidated. Plausible functions include the regulation of degradosome activity in response to growth conditions (Vanzo *et al*., 1998).

Several studies suggest that there are alternative forms of the RNA degradosome depending on growth conditions. For example, a direct interaction of CsdA with RNase E, PNPase and other components of the RNA degradosome has been detected under cold-shock conditions, forming what has been termed the 'cold shock degradosome'. Furthermore, CsdA is able to replace the resident RNA helicase of the degradosome, RhlB. In addition, under cold shock conditions, CsdA displays RNA-dependent ATPase activity (Prud'homme-Genereux *et al*., 2004) and is required for efficient decay of a model mRNA at low temperatures (Morita *et al*., 2005).

1.8 Septation, cell division and mini cell formation

This section is necessary to understand the reasoning behind the cell morphology and non-culturability of some strains described in future chapters. Cell division in bacterial cells occurs through the concerted action of a number of division proteins localised at the division site. FtsZ appears to play a central role in regulating the timing and frequency of septum formation and is the target of all known endogenous cell division inhibitors (Cam, Cuzange and Bouche, 1995; Cam *et al*., 1996). During cell division, the FtsZ protein, which is a GTPase, polymerises and forms a Z-ring scaffold for the assembly of proteins at the division site, causing invagination of the cell envelope layers to form a division septum (Harry, Monahan and Thompson, 2006; Tamura *et* al., 2006).

ftsZ is part of a cluster of genes involved in cell division and peptidoglycan synthesis. It is preceded by two other essential cell division genes, *ftsQ* and *ftsA*. The expression of *ftsZ* is growth rate and growth phase dependent. The transcription pattern of the gene is complex in *E. coli*, with at least 6 promoters identified in the 3 kb interval upstream of *ftsZ* (pictured in Figure 1.8(a)) and these have only been shown to account for around 60% of its expression (Aldea *et al*., 1990). One of these promoters, $ftsQ1_p$, has growth rate dependent activity and depends on RpoS (see Section 1.4). No transcription terminator has been found in the entire region. Two RNase E cleavage sites have been identified: one in the *ftsA* coding region and one in the *ftsA-ftsZ* intergenic region, resulting in mRNA decay. A ratio of FtsZ to FtsA between 50 and 100 to 1 is necessary for correct cell division to occur in *E. coli* and RNase E dysfunction decreases the ratio of these transcripts (Cam *et al*., 1996). Cleavage by RNase E is required for the efficient expression of the *ftsZ* gene. The decreased level of FtsZ, and resulting imbalance in the FtsZ to FtsA ratio, is responsible for the non-culturability of RNase E-deficient cells (Tamura et *al*., 2006).

In *E. coli*, the localisation of FtsZ depends on the MinCDE system (see Figure 1.8(b)). The *min* locus is composed of three genes, of which two (*minCD*) act together to inhibit cell division and a third (*minE*), which gives the inhibitor specificity (Bi and Lutkenhaus, 1990). Analysis of (GFP) green fluorescent protein fusions have shown that regulation by MinE is achieved by inducing MinCD to oscillate from pole to pole without allowing occupation of the mid-cell. MinCD inhibits FtsZ polymerising at the cell poles, therefore the FtsZ-ring localises at the cell mid-point. The FtsZ ring provides a scaffold for recruiting other cell division proteins, such as FtsA, FtsQ and ZipA, required for constriction of the FtsZ-A ring.

This leads to invagination and ingrowth of the peptidoglycan layer and, in turn, cytokinesis (Pichoff and Lutkenhaus, 2001). In a normal cell, the rate limiting factor in cell division is DNA replication and chromosomal partitioning, as septation cannot occur through an un-segregated nucleoid (Sun, Yu and Margolin, 2002).

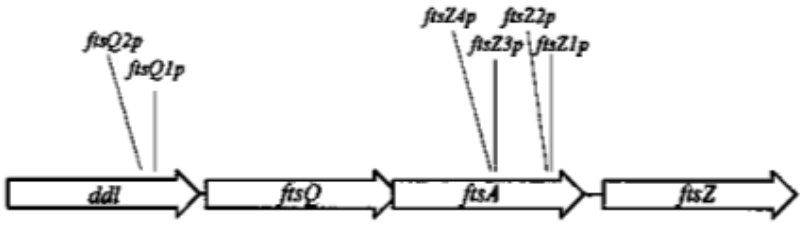


Figure 1.8(a): Genetic organisation of the *E. coli ftsQAZ* region showing the location of the six identified promoters (taken from Cam *et al*., 1996).

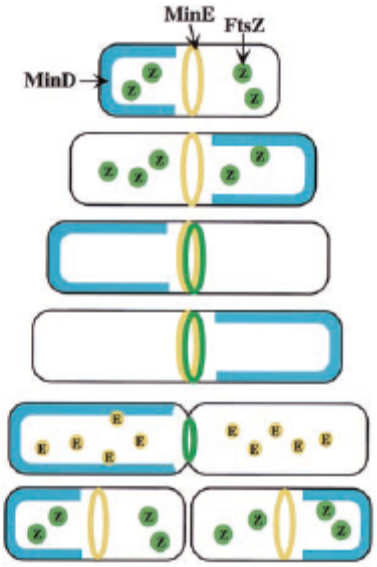


Figure 1.8(b): Model for division site selection in *E. coli* (diagram adapted from Jacobs and Shapiro, 1999). In *E. coli*, MinE localises to a ring-like structure at or near the middle of the cell early in the division cycle. MinD alternates from pole to pole and accumulates at the membrane periphery on either side of the MinE ring to prevent the formation of the FtsZ ring at the cell poles. The FtsZ ring therefore forms at the mid-cell and provides a scaffold to assemble the other cell division proteins required for constriction of FtsZ-A ring. This leads to invagination and ingrowth of the peptidoglycan layer and cytokinesis. The presence of MinE prevents MinD inhibitory activity at the cell centre and dissociates before completion of constriction.

In *E. coli*, localization studies have shown that RNaseE and the other known degradosome components (RhlB, polynucleotide phosphorylase and enolase) are organized as tubulin-like helical filamentous structures that coil around the length of the cell, resembling the localisation of MinCD structures. RNase E has been shown to interact with Min D using yeast two-hybrid systems but the tubulin-like distribution of RNase E, along the membrane, was maintained in the absence of Min proteins (Taghbalout and Rothfield, 2007).

Mini cells are small, round, anucleate cells, i.e. they do not contain chromosomal DNA, and are produced by polar divisions. There are two possible causes for mini cell production in *E. coli*: a loss of Min CD function or an increase in FtsZ levels, both of which lead to polymerisation of FtsZ at the cell poles. Mini cell formation has been reported for *E. coli hfq* mutants and is due to a fourfold increase in FtsZ levels; this is independent of RpoS (Takada, Wachi and Nagai, 1999). Also, an overproduction of Hfq inhibits cell division in *E. coli*, suggesting that Hfq is a negative regulator of FtsZ production (Takada, Nagai and Wachi, 2005). This group used Western and Northern blot analysis to show that Hfq plays a role in FtsZ production at the translational level, by preventing RNase E-mediated processing of *ftsZ* mRNA. A deletion in the *min* locus also produces mini cells and the divisions appear to occur at the expense of medial divisions, as the nucleated daughter cells have a longer average cell length than wildtype cells (Pichoff and Lutkenhaus, 2001).

1.9 Aims and Objectives

Despite the fact that the CspA proteins have been extensively studied over the last two decades, their exact functions and their *in vivo* targets, both at normal temperatures and under cold shock conditions, are not yet fully elucidated. The major aim of this study was to characterise the CspA paralogues of *S.* Typhimurium and elucidate their individual roles in stress responses. Recent studies in *E. coli* have shown that CspA paralogues are involved in the expression of RpoS, however at present the mechanism is unknown. This problem was further explored during the course of this study. From research performed in *E. coli*, before and during the course of this study, it is known that small RNAs (in particular DsrA, OxyS and RprA) regulate the production of RpoS, to allow adaptation to various environmental conditions, and that this involves interaction with Hfq. However, Hfq does not bind all RNA helices with equal affinity, suggesting the involvement of other mechanisms or higher-order structures in this interaction. We know that CspA paralogues function as nucleic acid binding proteins that regulate RpoS, but at what level? Do CspA paralogues assist in the interaction between Hfq and *rpoS* mRNA, or *rpoS* mRNAs and sRNAs, or a combination of these, or by some other indirect mechanism? Mutants that lack the CspA paralogues, Hfq, or RpoS; or a combination of these proteins, were used to give insights into the interactions involved in RpoS production. Figure 1.9 shows a proposed model of the interactions of CspA with Hfq, RpoS and the sRNAs.

During the course of constructing these mutants, it was discovered that the CspA paralogues (CspA and CspB in particular) are necessary for the successful introduction of an *rpoS* mutation into *S.* Typhimurium. The cause of the apparent non-culturability of the SL1344 *csp* null *rpoS* strain, and the mechanism involved, was investigated using a conditional *rpoS* mutation.

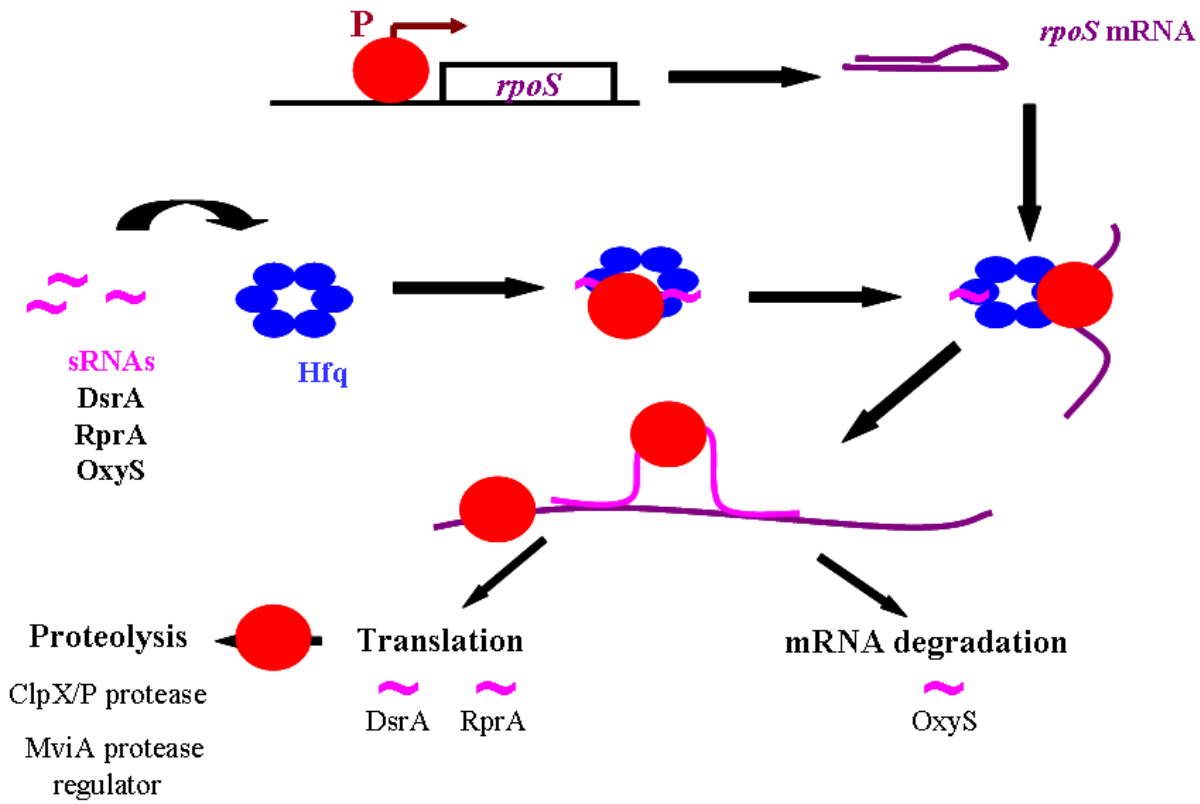


Figure 1.9: Proposed model for the CspA regulation of RpoS production. Possible binding sites of CspA paralogues are denoted by red circles.

Key: = CspA paralogues = Hfq = sRNAs

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Oligonucleotides

Primers for sequencing and PCR reactions were obtained from either Sigma-Genosys Biotechnologies Ltd. or Eurogentec S.A., diluted to 100µM and stored at -20°C. Oligonucleotides are listed in Table 2.1.1.

2.1.2 Bacterial strains and plasmids

Glycerol stocks of bacterial strains were stored at -80°C in cryovials (NUNC). Cells were recovered by streaking a sample on LB agar (1% Difco Bacto tryptone, 0.5% Difco Bacto yeast extract, 1.5% Difco agar, 1% NaCl, dH_2O, pH 7.5), containing the appropriate antibiotic, and incubating overnight at 37°C. These plates were wrapped in Parafilm and transferred to 4°C for short-term storage. The genotypes and sources of bacterial strains used in this study are shown in Table 2.1.2(a).

All plasmids were stored, in nuclease-free water, at -20°C. Plasmids were defrosted on ice before use. The plasmids used in this study are listed in Table 2.1.2(b).

2.1.3 Growth media and conditions

All bacterial strains, shown in Table 2.1.2(a), were grown routinely by inoculating 5ml Luria-Bertani (LB) medium (1% Difco Bacto tryptone, 0.5% Difco Bacto yeast extract, 1% NaCl, dH_2O, pH 7.5), containing the appropriate antibiotic, with a single bacterial colony. The cultures were then incubated overnight (16h) at 37°C in an orbital shaker (200rpm), unless otherwise stated. Table 2.1.3 details the final concentrations of antibiotics used.

Table 2.1.1: Oligonucleotides used in this study. Restriction enzyme recognition sites are written in **bold**. Oligonucleotide sequences that are identical to the SL1344 sequence are written in *italics* and their location in relation to either the SL1344 genome (Wellcome Trust Sanger Institute), or the appropriate plasmid, is reported.

Purpose	Primer	Sequence	RE site	Location on genome/ plasmid
Overexpressing ompA RNaseE sites	ompA + RNaseE site (for)	CG**CCATGG***GGTGCTCA GCATAAGCCGTA*	NcoI	comp (1162115 - 1162134)
	ompA - RNaseE site (for)	CG**CCATGG***GATTGCAG TGGCACTGGCTG*	NcoI	comp (1161988 - 1162007)
	ompA (rev)	CGG**AAGCTT***CCTGCGG CTGAGTTACCACG*	HindIII	1160942 - 1160961
Overexpressing dsrA	dsrA (for)	CG**GAATTC***ACATCAGAT TTCCTGGTGTAAC*	EcoRI	comp (2026300 - 2026269)
	dsrA (rev)	CGG**AAGCTT***GCTGAAG CATTCGAGCTGG*	HindIII	2026151 - 2026170
Transcriptional Fusions	*rpoS (*for)	GG**CCCGGG***GCCGACG CAGCAGAGCAAG*	SmaI	comp (3067127 - 3067135)
	rpoS + sRNA site (rev)	GGG**GGATTC***CCGCCGA TTTATCGCTGC*	BamHI	3066541 - 3066559
	rpoS - sRNA site (rev)	GGG**GGATTC***CTGGTTC CGGTGCTACCCAT*	BamHI	3066625 - 3066645
Sequencing	pBAD24	CCTGACGCTTTTTATCG CAA	n/a	pBAD24: 1247 - 1258
	rpoS (1)	*GCGCGTCGCGCACTGC GTGG*	n/a	comp (3066228 - 3066249)
	rpoS(2)	*CTGGCCAGCACTTCAC GCTG*	n/a	3065670 - 3065690
	rpoS (3)	*GCTGGCGACCACTACA CG*	n/a	3066312 - 3066330
	rpoS fusion (for)	GTATCACGAGGCCCTTT CG	n/a	pRS 415: 9869 - 9887
	rpoS fusion (rev)	GGGATGTGCTGCAAGG C	n/a	pRS 415: comp (76 - 92)
Northern Blotting	*rpoS* (for)	*GCAGGCAGTAAGGGAC AGGC*	n/a	comp (3066817 - 3066837)
	rpoS (rev)	*GCTTCGATATTCAGCCC CTGC*	n/a	3065457 - 3065478

Table 2.1.2(a): *E. coli* and *S.* Typhimurium strains, and their derivatives, used in this study. The genotypes and the source of each strain are reported.

Strain	Genotype	Source
E.coli DH5α	*supE*44 D*lacU*169 (Φ80 *lacZ*DM15) *endA*1 *hsdR*17 *recA*1 *gyrA*96 *thi*-1 *relA*1	Lab stock
SL1344	*his*	Lab stock
SL1344 *rpoS*	*rpoS*	Fang *et. al.*, 1992
MPG 551	SL1344 Δ *csp* B C D E::Kan H	Lab stock
MPG 552	SL1344 Δ *csp* A C D E::Kan H	Lab stock
MPG 553	SL1344 Δ *csp* A B D E::Kan H	Lab stock
MPG 554	SL1344 Δ *csp* A B C E::Kan H	Lab stock
MPG 556	SL1344 Δ *csp* A B C D E::Kan	Lab stock
MPG 557	SL1344 Δ *csp* A B C D H	Lab stock
MPG 558	SL1344 Δ *csp* A B C D E::Kan H	Lab stock
TE 3147	*put PA1303::*Kan-*hemA-lac*[pr] *hfq-1::*Mu*d::*Cam	Cunning and Elliott, 1999
MPG 601	SL1344 *hfq-1*	This study
MPG 603	MPG 557 *hfq-1*	This study
MPG 605	MPG 558 *hfq-1*	This study
MPG 607	SL1344 *hfq-1 rpoS*	This study
MPG 609	MPG 557 *hfq-1 rpoS*	This study
MPG 611	MPG 551*rpoS*	This study
MPG 612	MPG 552 *rpoS*	This study
MPG 613	MPG 551*hfq-1*	This study
MPG 614	MPG 552 *hfq-1*	This study
MPG 615	MPG 553 *hfq-1*	This study
MPG 616	MPG 554 *hfq-1*	This study
MPG 617	MPG 556 *hfq-1*	This study
MPG 618	MPG 558 *hfq-1 rpoS*	This study
MPG 619	MPG 551 *hfq-1 rpoS*	This study
MPG 620	MPG 552 *hfq-1 rpoS*	This study
MPG 621	MPG 553 *hfq-1 rpoS*	This study
MPG 622	MPG 554 *hfq-1 rpoS*	This study
MPG 623	MPG 556 *hfq-1 rpoS*	This study
MPG 624	MPG 557 *cspB*::Mu*dlux*	This study
MPG 625	SL1344 Δ *csp* A C D H *rpoS*	This study
MPG 626	MPG 557 *cspB*::Mu*dlux rpoS*	This study
MPG 627	MPG 558 pZAQ *rpoS*	This study
MPG 628	MPG 558 pUBAD::*rpoS rpoS*	This study
MG 1655 *hfq*	*hfq*	This study
MG 1655 *rpoS*	*rpoS*	This study
SL1344	SL1344 *hns*::Kan	Gift from Charles Dorman
MPG 630	SL1344 *hns*::Kan	This study
MPG 631	MPG 558 *hns*::Kan	This study
MPG 632	SL1344 *hfq-1 hns*::Kan	This study
MPG 645	MPG 557 *hns*::Kan	This study
JF 2892	UK1 *mviA*::Kan	Moreno *et. al.,* 2000
MPG 633	SL1344 *mviA*::Kan	This study
MPG 634	MPG 558 *mviA*::Kan	This study
MPG 635	SL1344 *hfq-1 mviA*::Kan	This study
MPG 646	MPG 557 *mviA*::Kan	This study
JF 3487	UK1 *clpP1*::Tn10	Moreno *et. al.,* 2000
JF 3644	UK1 *clpP2*::Tn10	Moreno *et. al.,* 2000
MPG 636	SL1344 *clpP1*::Tn10	This study

MPG 637	MPG 558 *clpP1*::Tn10	This study
MPG 638	SL1344 *hfq-1 clpP1*::Tn10	This study
MPG 647	MPG 557 *clpP1*::Tn10	This study
JF 1899	LT2 *phoP*::Tn10	Bearson *et. al. ,*1998
MPG 642	SL1344 *phoP*::Tn10	This study
MPG 643	MPG 558 *pho*::Tn10	This study
MPG 644	SL1344 *hfq-1 phoP*::Tn10	This study
MPG 648	MPG 557 *phoP*::Tn10	This study
MPG 649	SL1344 Δ *csp* C D E H *hns*::Kan	This study
MPG 650	SL1344 Δ *csp* A B D H *hns*::Kan	This study
MC4100	Mu*dlux* P1Tn*9clr* 100::Cam	Lab stock
CH463	*galE503*, *bio-561*	Lab stock
MPG 651	MPG 558 *cspB*::Mu*dlux*	This study
MPG 652	MPG 558 *cspB*::Mu*dlux*	This study
CC1024	SL1344 *rpoS::lacZ* (fused to 177th codon)	Chen *et al*., 1996
MPG 558 *rpoS::lacZ*	SL1344 *csp* null *rpoS::lacZ*	This study
MPG 601 *rpoS::lacZ*	SL1344 *hfq rpoS::lacZ*	This study

Table 2.1.2(b): **Plasmids used in this study**. The source of each plasmid, along with distinguishing features and purpose of use, is reported.

Plasmid	Features	Purpose	Source
pBAD24	pBAD promoter Amp R	Expression vector for *dsrA*	Lab stock
pRS415	Transcriptional lacZ fusion Amp R	*rpoS::lacZ* fusion	Simons *et al.,* 1987
pUBAD::*rpoS*	pBAD promoter derivative of pBAD18 Tet R (insertion in Amp cassette)	Complementation Growth studies Overexpression of ompA	Volkert *et al*., 1994
pZAQ	overexpression of FtsZAQ derivative of pBR322 Kan R Tet R	complementation growth studies	Bi *et al*., 1990

Table 2.1.3: Concentrations of antibiotics used to supplement growth media. Stock solutions of antibiotics (Melford) were made 1000 times more concentrated than final concentration required.

Antibiotic	Final Concentration (μg/ml)
Carbenicillin	50
Chloramphenicol	34
Kanamycin	50
Tetracycline	10

For cold shock experiments, stationary phase cells were diluted 100-fold into fresh LB medium and grown to exponential phase (OD_{600} = 0.3) at 37°C in an orbital shaker. These strains were then transferred to a shaking refrigerated (15°C) incubator for 1.5h prior to harvesting.

Minimal medium used for growth studies was prepared according to the following recipe: 1 X M9 salts, 0.2% glucose, 20mM $MgSO_4$, 1mM $CaCl_2$, dH_2O and 0.02% Histidine. Solutions were all autoclaved separately and then filter sterilised on addition to dH_2O.

Blue/white selection was used during production of *rpoS::lacZ* fusions, to select for positive colonies. A 0.1M stock solution of isopropyl β-D-thiogalactopyranoside (IPTG) (Melford) was filter sterilised and added to LB agar at a final concentration of 0.1mM. A 20mg/ml stock solution of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (Xgal) (Melford) was made by diluting powder in dimethyl sulfoxide (DMSO) and then added to LB agar at a final concentration of 40µg/ml. If antibiotic was required for selection, this was added to the LB agar at this time.

Motility agar plates were used to determine the swimming ability of strains. The recipe used was adapted from Harunur-Rashid *et.al.* (2000): 1% Difco Bacto tryptone, 0.5% NaCl, 0.3% (w/v) Difco agar, dH_2O. The solution was autoclaved and left to cool; 20ml of agar was poured into each Petri-dish. Following a method described by Sittka *et al.* (2007), motility agar plates were inoculated with 1µl stationary phase culture and incubated at 37°C for 4h.

2.1.3.1 Growth curves

Cultures were grown overnight to stationary phase (for approximately 16h; as described in Section 2.1.3) in media corresponding to that used for the subsequent growth curve. Cultures were then diluted 100-fold in fresh media and incubated at the appropriate temperature. OD_{600} readings were then taken at various time points and plotted against time (min) to produce growth curves. The approximate length of the lag phase, defined as the time prior to balanced growth, was calculated by extrapolating the lines of both the plateau before growth and the exponential phase of growth. The point at which these lines cross is the estimated lag phase in minutes. The doubling time in peak exponential growth is calculated from how long the culture takes, in minutes, to double its OD_{600} during logarithmic growth (i.e. the length of time to double from an OD_{600} of 0.2 to that of 0.4).

2.1.4 Solutions

Most solutions were made up according to recipes described in LAB FAQs 2nd Edition (Roche Applied Science, Germany) unless stated otherwise.

2.1.4.1 Solutions for DNA techniques

10 X TBE buffer: 0.9M Tris, 0.9M boric acid, 20mM Na_2 EDTA (pH 8.0), dH_2O
DNA sample buffer: 50mM Tris-HCl (pH 7.6), 60% (v/v) glycerol, 0.001% (w/v) bromophenol blue (Melford), dH_2O
SOC buffer: 0.5% yeast extract, 2% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 0.4% glucose, dH_2O
Phage buffer: 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl
Top agar: 1% Difco Bacto tryptone, 0.5% NaCl, 0.6% Difco agar, dH_2O
Permeabilisation solution: 200mM Na_2HPO_4, 20mM KCl, 2mM $MgSO_4$, 0.8mg/ml CTAB (hexadecyltrimethylammonium bromide), 0.4mg/ml sodium deoxycholate, 5.4µl/ml β-mercaptoethanol
Substrate solution: 60mM Na_2HPO_4, 40nM NaH_2PO_4, 1mg/ml ONPG (o-nitrophenyl-β-D-galactosidase, 2.7µl/ml β-mercaptoethanol, 20µl/ml CTAB, 10µl/ml sodium deoxycholate

2.1.4.2 Solutions for Protein techniques

2 X SDS sample buffer: 10% glycerol, 10% SDS, 125mM Tris-HCl (pH 6.8), β-mercaptoethanol, bromophenol blue
10 X Protein running buffer: 0.25M Tris, 2M glycine, dH_2O
Transfer buffer: 25mM Tris-HCl (pH 8.0), 200mM glycine, 20% methanol, dH_2O
Antibody buffer: 1% marvel milk powder, 1 X PBS, 0.05% Tween 20

2.1.4.3 Solutions for RNA techniques

Phenol-extraction mix: 0.1% SDS, 1% phenol, 19% ethanol, dH_2O
10 X MOPS solution: 0.2M MOPS, 0.05M Na-acetate-3, 0.01M EDTA (pH 7.0, NaOH), dH_2O
Sample loading buffer: 1 X MOPS, 0.5% Formamide, 18.5% Formaldehyde, 4% Ficoll 400, dH_2O, bromophenol blue
20 X SSC: 3M NaCl, 0.3M NaCitrate, dH_2O

100 X Denhardt's solution: 2% Ficoll 400, 2% polyvinylpyrrolidone (MW 360000), 2% BSA Fraction V, dH_2O

Prehybridisation solution: 4 X SSC, 50% Formamide, 5 X Denhardt's, 1% SDS, 1% 10mg/ml DNA (Salmon sperm DNA or yeast tRNA)

Hybridisation solution: 4 X SSC, 5 X Denhardt's, 50% Formamide, 1 % SDS, 5% dextransulfate

Stripping solution: 5mM Tris-HCl (pH 8.0), 2mM EDTA, 0.4% SDS, dH_2O

2.2 Methods

2.2.1 DNA manipulation

2.2.1.1 Cloning

2.2.1.1.1 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 50µl volumes consisting of 1µg template DNA, 5µl 10 X PCR buffer (Roche), 0.2mM each dNTP (Roche), 1mM 5'-primer, 1mM 3'-primer, 0.5µl *Taq* polymerase/ *Pfu* polymerase (Roche), nuclease-free water. Reactions were carried out in a PCR machine (Techne) as per manufacturer's instructions. Correct size of PCR products was confirmed by agarose gel electrophoresis.

Linear DNA fragments were purified using a QIAquick™ PCR Purification Kit (Qiagen) before use in downstream techniques.

2.2.1.1.2 Agarose gel electrophoresis

Routinely, DNA samples were resuspended in loading buffer and resolved using a 1% (w/v) agarose gel in 0.5 X TBE buffer. Ethidium bromide (Sigma) was included to a final concentration of 0.5µg/ml to allow visualisation of DNA. Agarose gels (8cm X 12cm X 1cm) were run in an electrophoresis chamber (Pharmacia) at 100V for 1-2 hours or until the dye-front approached the bottom of the gel. DNA was visualised using a UV light box (300-360nm) and photographed using a digital camera (UVP Laboratory products).

Where appropriate, DNA was recovered from agarose gel by visualising and excising the band under UV light. The DNA was then purified from the gel piece using a QIAquick™ Gel Extraction Kit (Qiagen).

2.2.1.1.3 Restriction enzyme digest of DNA

Digestion was carried out in 10µl volumes consisting: 1µl appropriate buffer, 20 units of restriction enzyme (Roche or NEB), 1µg DNA and nuclease-free water. Reactions were

incubated for an hour at the appropriate temperature. In situations where two restriction enzymes were incompatible in the same buffer, the product of the first digestion was purified using the PCR Purification Kit (Qiagen) before a second digestion was set up as described. Restriction enzymes were heat inactivated at 65°C for 20min where appropriate.

2.2.1.1.4 Dephosphorylation of plasmid DNA

Dephosphorylation of the 5'-end of digested vector DNA, using SAP (Shrimp Alkaline Phosphatase), prevents unwanted re-ligation. Reactions were carried out in 20 µl volumes containing 0.2 - 10µg DNA, 1 X Alkaline Phosphatase buffer (Roche), 1 unit of SAP (Roche) and nuclease-free water, and then incubated for an hour at 37°C. The SAP enzyme was subsequently inactivated at 65°C for 20min.

2.2.1.1.5 Ligation of vector and insert

Ligations were performed in 10µl volumes containing vector and insert DNA at appropriate ratios (routinely, 1:3 with sticky-ended DNA and 1:5 with blunt-ended DNA), 1 X T4 DNA ligase buffer and 1 unit of T4 DNA ligase (Roche). Reactions were incubated overnight (16h) at either 20°C (for blunt-ended DNA) or 15°C (for sticky-ended DNA).

2.2.1.1.6 Sequencing of clones

DNA sequencing reactions were carried out in 10µl volumes using a BIGDYE v3.1 sequencing kit (Applied Biosystems). Sequencing primers (see Table 2.1.1) were complimentary to flanking regions of plasmid inserts, ~80bp either side of the gene of interest. Plasmid DNA (500ng) was added to 2µl of BIGDYE, 2µl BIGDYE buffer, 1mM sequencing primer and nuclease-free water. Reactions were carried out in a PCR machine (Techne) following manufacturer's instructions. Samples were analysed by the sequencing service within the ICMB, University of Edinburgh, and the sequence information received was evaluated using two computer programs: Vector NTI v10 (Invitrogen) and Bio Edit (Ibis Biosciences).

2.2.1.1.7 Arabinose induction of plasmids

The induction of pBAD24 (and pUBAD) constructs, and the subsequent expression of *dsrA*, *ompA* and *rpoS*, was performed on both stationary phase cultures (grown for 16h at 37°C) and

exponential cultures (OD at 600nm of 0.4). Once grown to the desired growth phase, medium was supplemented with 0.2% arabinose and the cells were incubated for 2h at the desired temperature (cannot supplement medium whilst cells are still growing as *S.* Typhimurium is able to metabolise arabinose). For overexpression of RpoS throughout growth, from pUBAD::*rpoS*, 2% of arabinose was added to the growth medium to ensure arabinose supply was not exhausted.

2.2.1.1.8 β-galactosidase (Miller) assay

This assay uses β-galactosidase levels as a read-out for transcriptional and translational efficiency of *rpoS::lacZ* under different conditions. ONPG is cleaved by β-galactosidase to yield galactose and a yellow product, o-nitrophenol. The production of yellow colour per unit time is proportional to enzyme concentration. The method used was adapted from Miller (1972) and Zhang and Bremer (1995). Cultures were grown to the appropriate growth phase and their OD_{600} noted. 20µl aliquots of the cultures were taken and added to 80µl permeabilisation solution. 600µl substrate solution was added to each mixture and incubated at 30°C until sufficient yellow colour had developed. 700µl 1M sodium carbonate (stop solution) was added and samples were harvested by centrifugation (14,000rpm) for 5-10 min and the OD_{420} of the supernatant recorded. Miller units were then calculated as follows:

$$1 \text{ Miller unit} = 1000 \times \frac{OD_{420}}{(t \times v \times OD_{600})}$$

OD_{420nm} is absorbance of the yellow o-nitrophenol
t is reaction time in minutes
v is volume of culture assayed in ml
OD_{600} reflects cell density

2.2.1.2 Transformation

2.2.1.2.1 Preparation of electrocompetent cells

A stationary phase culture was diluted 100-fold into 1l of fresh LB and grown to an OD_{600} of 0.6 to 0.8 at 37°C in an orbital shaker. Cells were buried in ice for 30min and harvested by centrifugation (4,000rpm at 4°C in a Beckman JA-14 rotor). Cells were then very gently resuspended in sterile, ice-cold 10% glycerol. Cells were then washed 3 further times as detailed and finally resuspended in an equal volume of ice-cold 10% glycerol. 50µl aliquots of cells were snap-frozen using dry ice/ethanol for long term storage at -80°C.

2.2.1.2.2 Electroporating

Expected efficiency is 10^9-10^{10} transformants per µg of mini-prepped plasmid DNA. Cuvettes were chilled and cells were thawed on ice for around 20min. 1µg of DNA was added to the cells and both transferred to an ice-cold cuvette. Cells were then electroporated in a Gene Pulser (Bio-Rad) using the following settings: 2.5V, 200Ω and 25µFD. 0.8ml of pre-warmed SOC buffer was added to the cuvette and cells were transferred to an eppendorf and incubated at 37°C in an orbital shaker for 45min to allow cells to recover. Cells were then harvested by centrifugation (14,000rpm), resuspended in 200µl SOC buffer and spread on LB agar plates containing the appropriate antibiotic for selection.

N.B: sometimes the cells will produce a spark when electroporated. Transformants will still be recovered but efficiency will be low. The spark is caused by one of the following: improperly prepared competent cells, DNA contains salts, more than 1µg of DNA was added to the cells, cuvette contains salts or cuvette is not cold enough.

2.2.1.2.3 Preparation of chemically competent cells

A stationary phase culture was diluted 100-fold into 100ml of fresh LB and grown at 37°C to an OD_{600} of between 0.2 and 0.4 in an orbital shaker. Cells were washed 3 times in sterile, ice-cold 0.1M $CaCl_2$ and were stored overnight at 4°C to increase efficiency.

2.2.1.2.4 Transformation of chemically competent cells

2µg DNA was incubated with 100µl of chemically competent cells on ice for 15min. Cells were then incubated at 37°C for 2 min, before being chilled on ice for 15min. Cells were

recovered by adding 100µl of LB and incubating for 30min at 37°C in an orbital shaker. Cells were harvested by centrifugation (14,000rpm), resuspended in 200µl LB and spread on agar plates containing the appropriate antibiotic for selection.

2.2.1.3 P22 Transduction

2.2.1.3.1 Preparation of P22 lysates

A stationary phase culture of the donor strain was diluted 100-fold into 10ml LB and incubated for 45min at 37°C in an orbital shaker. Dilutions of P22 lysate were added (to achieve a multiplicity of infection (MOI) of 1.0) and incubated at 37 °C for 10-16h, at which point cultures should contain visible cell debris. 200µl chloroform was added to cultures, prior to vortexing and refrigeration for 2h at 4°C. Cell debris was pelleted in a bench top centrifuge (MSE Minstrel 1000) for 20min at 3,000g and the supernatant transferred into a sterile tube. As before, 200µl chloroform was added and solutions refrigerated for 1h. The solutions were then centrifuged as before and the cleared lysate transferred to sterile tubes. Finally, 200µl chloroform was added and the lysate vortexed. This could then be stored at 4°C for several months.

2.2.1.3.2 Determination of plaque forming units of lysate

100µl of an overnight culture of SL1344 was mixed with 3-5ml of top agar, poured onto a plain LB plate and allowed to solidify for 10min. Serial dilutions of the phage lysate were made in phage buffer and 10µl volumes spotted onto the surface of the top agar. Plates were incubated overnight at 37°C and plaques were counted to determine the titre.

2.2.1.3.3 Introduction of mutation to recipient strain

100µl volumes of serially diluted phage lysate were added to 100µl aliquots of a stationary phase culture of the recipient *Salmonella* strain. 100µl volumes of P22 lysate or recipient strain only were added to 100µl LB as controls. These mixtures were then incubated at 30°C for exactly 30min and plated on antibiotic-selective LB agar plates. Plates were incubated overnight at 37°C. Transductants were then passaged 4-5 times on fresh LB agar plates (containing appropriate antibiotic) to ensure removal of residual P22 before making permanent freezer stocks.

S. Typhimurium *galE* mutants are susceptible to both P22 and P1 phages. For P22 transduction, media for growth is supplemented with 0.4% galactose and 0.4% glucose to ensure the presence of P22 phage receptors on the host cells. These sugars are eliminated from the growth medium when P1 phage receptors are required.

2.2.1.4 P1 Transduction

2.2.1.4.1 Preparation of P1 lysates

A stationary phase culture of the donor strain was diluted 100-fold into 10ml fresh LB medium and grown to an OD_{600} of 0.8 at 37°C in an orbital shaker. 1ml of cells were harvested by centrifugation (14,000rpm) and resuspended in 1ml of fresh LB. 100µl volumes of this sample was added to 100µl 50mM $CaCl_2$, 100µl 100mM $MgSO_4$ and P1 phage (1µl pure phage or 5µl of P1 lysate) and incubated in a water bath at 37°C for 25min. These solutions were then added to 3-5ml volumes of top agar, mixed vigorously and spread on LB agar plates containing 0.5mM $CaCl_2$. The plates were then incubated overnight face-up at 37°C. Successful lysis should give small plaques in the bacterial lawn. To harvest the phage, 2.5ml phage buffer was added to each plate and incubated at room temperature for 15min. The top agar was then removed using sterile slides and collected in sterile falcon tubes. 2.5ml chloroform was added to each tube, vortexed well and incubated at room temperature for 30min. Samples were then mixed again and centrifuged in a bench-top centrifuge (MSE Minstrel 1000) at 3,000g for 15min. The supernatant was transferred to a sterile tube and 10% chloroform added. Centrifugation was repeated and again 10% chloroform was added. P1 lysates could then be stored at 4°C for several months.

2.2.1.4.2 Preparation of a P1 Tn9 clr 100 lysate

Method adapted from Silhavy *et al.* (1984). A stationary phase culture of the donor strain was diluted 200-fold into 10ml fresh LB medium and grown to an OD_{600} of 0.2 at 30°C with aeration. The culture was then incubated at 45°C for 30 min in a shaking water bath before incubation for around 2h (or until cells lyse) at 42°C with aeration. 0.2ml chloroform was added to the lysate and vortexed before centrifugation at 3000g for 20min to pellet cell debris. The lysate was then transferred to a sterile falcon tube and 0.1ml of chloroform added.

2.2.1.4.3 Introduction of mutation to recipient strain

A stationary phase culture of the recipient strain was diluted 100-fold into 10ml fresh LB and grown to an OD_{600} of 0.4 at 37°C in an orbital shaker. 1ml of the culture was pelleted by centrifugation (14,000rpm) and resuspended in 1ml LB. 100µl of cells were transferred into 3 tubes containing 100µl 50mM $CaCl_2$, 100µl $MgSO_4$. 1µl of neat P1 lysate, and two ten-fold dilutions, were added to each of the tubes and mixed gently before incubation for 20min in a water bath at 37°C. 200µl of 1M Na Citrate and 500µl LB was added to each reaction and tubes were incubated for a further 30min at 37°C. Cells were then pelleted as before, resuspended in 100µl LB and plated on antibiotic-selective LB agar. Plates were incubated overnight at 37°C and transductants passaged 3 times to remove residual phage.

2.2.2 Protein techniques

2.2.2.1 1D SDS-PAGE

The apparatus (Mini Protean II) was sterilised with ethanol, rinsed in dH_2O and assembled according to manufacturer's instructions (Bio-Rad). Single concentration gels (70 X 85 X 0.5mm) were used consisting of 12.5% separating gel and 5% stacking gel, as described by Laemmli (1970). OD_{600} was used to calculate the volume of each sample that should be loaded to ensure equal protein concentrations, and therefore a fair comparison. Samples were resuspended in equal volumes of 2 X SDS loading buffer and boiled for 10min prior to loading. To separate the proteins, a constant current of 200mA was applied to the gel for 1h, or until the dye-front had run off the bottom of the gel. 10µl of See Blue Plus 2 pre-stained protein marker (Invitrogen) was loaded on each gel to allow an estimate of band sizes.

2.2.2.2 Western Blotting

After electrophoresis, proteins were transferred to nitrocellulose membrane using a transblotter (Bio-Rad Trans-Blot Semi-Dry Transfer cell) according to the manufacturer's protocol. The transblotter was run at 15V for 1h at 500mA (8°C). The blots were then soaked overnight in blocking solution (5% marvel milk powder, 0.25% (v/v) Tween 20, 1 X PBS), to block non-specific binding sites on the membrane. Blots were washed 4 times (5min) in 1 X PBS + 0.05% Tween 20 at room temperature on a Lucham's rotating shaker.

The blots were incubated with monoclonal antibody to the RpoS subunit of *E. coli* RNA polymerase (Neoclone) at a dilution of 1:1000, for 2 hours at room temperature. Following this, the blots were washed (as before) and incubated with HRP-anti mouse IgG antibody (Sigma), at a dilution of 1:1000, at room temperature for 1h. Blots were then washed 4 times as before and developed with ECL Plus (GE Healthcare) unless otherwise stated. Blots were imaged with a SRX-101A (Konica) camera, using a darkbox, and images were examined with Labworks software (UVP).

2.2.3 RNA techniques

2.2.3.1 Creating a ribonuclease-free environment

Sterile techniques were observed when handling reagents and gloves were worn at all times to prevent accidental contamination of samples with ribonucleases. To ensure non-disposable glassware was RNase-free, it was baked overnight at 200°C and rinsed with 0.1M NaCl, 1mM EDTA. Gel apparatus was soaked in 1M NaOH overnight and rinsed in RNase-free water. All solutions, excluding Tris buffers, were treated with 0.1% DEPC (diethyl pyrocarbonate) to inactivate ribonucleases.

NB. DEPC reacts rapidly with amines and cannot be used to treat Tris buffers

2.2.3.2 RNA extraction

A single colony was used to inoculate fresh LB and cultures were grown to the appropriate growth phase in an orbital shaker. 10^9 bacterial cells were then harvested by centrifugation (14,000rpm), resuspended in 1ml phenol-extraction mixture to stabilise bacterial RNA and incubated on ice for 30min (method adapted from Eriksson *et. al,* 2003). RNA was extracted using Promega SV Total RNA Isolation System (Promega) and the yield of total RNA was determined spectrophotometrically (1 absorbance unit (OD_{260}) = 40µg ssRNA/ml) or by Nanodrop.

2.2.3.3 Denaturing gel electrophoresis

This is used to assess RNA quality and ensure equal quantities between samples. The agarose gel solution (1% agarose, 1 X MOPS, H_2O_{DEPC}) was heated until completely dissolved and

cooled to 65°C before the addition of 0.6M Formaldehyde, 10µl Ethidium Bromide (100µg/ml). The gel solution was poured immediately and allowed to harden for 30min. Samples for loading, containing 10µg total RNA (brought to equal volumes with H_2O_{DEPC}, add same volume loading buffer), were denatured by heating to 65°C for 10min and then snap cooling on ice. The gel was run in an electrophoresis chamber (Pharmacia) at 100V for around 2h (until loading dye is near bottom of gel) in denaturing running buffer (1 X MOPS, 0.2M Formaldehyde, dH_2O). RNA was visualised using a UV light box (300-360nm) and gel photographed using a digital camera (UVP Laboratory products).

2.2.3.4 Northern blotting

2.2.3.4.1 Northern transfer of RNA

The gel was washed 3 times (5min) in H_2O_{DEPC} to remove formaldehyde and then capillary transferred overnight (16h) in 10 X SSC transfer buffer. Capillary transfer set up: 2 X wet Whatman paper, gel face up, N^+ nylon membrane, 2 X wet Whatman paper, 2 X dry Whatman paper, 5cm thick paper towels, glass plate, weight. The membrane was removed and UV-crosslinked at 245nm in a Stratalinker (Stratagene).

2.2.3.4.2 Synthesis of radioactive probes

Internal fragments of the desired gene were amplified by PCR (random prime labelling) using High Prime DNA Labelling Kit (Roche Applied Science). 25µg template DNA was denatured by heating to 95°C for 10min and chilling quickly on ice. This was then mixed with 4µl High Prime Mix (random primer mixture, Klenow polymerase), 3µl dNTP mix (dATP, dGTP, dTTP), 5µl 50µCi[α^{32}P]dCTP (3000Ci/mM) and incubated at 37°C for 30min. The reaction was stopped by adding 2µl 0.2M EDTA prior to incubation at 65°C for 10min.

2.2.3.4.3 Hybridisation

The membrane was pre-hybridised for 3h at 42°C in a hybridisation oven (Techne), to block non-specific background binding of probe. The radioactive probe was denatured by heating at 95°C for 3min and immediate cooling on ice. Pre-hybridisation solution was replaced by hybridisation solution and the denatured probe added. The membrane was then incubated overnight at 42°C. The membrane was washed in reducing concentrations of SSC buffer in 15min stages until background was low. The membrane was then exposed, under Saran Wrap

(Sigma), to a Storage Phosphor screen (Amersham Biosciences) and the image developed in a Storm Phosphorimager (Amersham Biosciences). Images were then analysed using the ImageQuant TL v 2005 program (Amersham Biosciences).

2.2.3.4.4 Stripping membrane

To dissociate the radioactive probe from the RNA, the membrane was washed in stripping solution at 80°C for 1-3h, or until no more radioactivity could be detected. This was checked by exposing the membrane to a Storage Phoshor screen for 72h and analysing the image. After air-drying, the membrane can be stored at room temperature.

2.2.3.5 qRT-PCR (Quantitative reverse transcription polymerase chain reaction)

This method is used to assess the quantity of a specific RNA in a sample by first converting RNA to DNA by reverse transcription and then amplifying the gene of interest with specific DNA primers. SYBR Green is incorporated into each of the synthesised gene fragments, therefore provides a read-out relative to the original amount of RNA in the sample.

2.2.3.5.1 First strand cDNA synthesis

A ribonuclease-free environment must first be created as described above. RNA is then extracted from cultures at the appropriate growth phase, quantified spectrophotometrically and assessed by denaturing gel electrophoresis. Superscript III (Invitrogen) was used to synthesise cDNA from 1µg total RNA, following the manufacturer's instructions. The reaction mixture was made as follows: 2µl Invitrogen Superscript III enzyme, 10µl 2 X RT mix and 6.5µl dH_2O_{DEPC}. This was then incubated under the following conditions in a PCR machine (Techne): 10min at 25°C, then 30min at 50°C, followed by 5min at 85°C. The reaction was then chilled on ice and 1µl *E. coli* RNaseH was added, before incubation for a further 20min at 37°C.

2.2.3.5.2 qPCR reaction

Each reaction was carried out in triplicate using SYBR GreenER qPCR Super mix (Roche) as per manufacturer's instructions. A no-RT (no first strand synthesis) and a no template control were run alongside all experiments to ensure results were not affected by contamination by genomic DNA. To normalise data, cDNA was quantified using absorbance at 260nm and a

reaction containing 16S primers performed for each RNA sample. The qPCR reactions were carried out in a BioRad iCycler using the following conditions: 1 cycle of 2min at 50°C (UDG incubation), then 8min at 95°C; followed by 40 cycles of 15s at 95°C, then 60s at 60°C. This was followed by melting curve analysis of 1min at 95°C, 1min at 55°C and 80 cycles of 10s at primer annealing temperature +0.5°C.

The induction ratio was calculated as follows:

$$\text{Induction ratio } \frac{T_2}{T_0} = \frac{1 \times 2^{-ct(T2)}}{1 \times 2^{-ct(T0)}}$$

NB. Ct value is the time at which fluorescence of sample crosses the background threshold and becomes exponential

2.2.4 Microscopy

2.2.4.1 Phase contrast

This technique is used to examine the morphology of individual cells. Cells were grown to the appropriate growth phase in LB medium and 10^8 bacterial cells harvested by centrifugation (14,000rpm). The cells were then washed in 1 X PBS prior to resuspension in 0.5ml 1 X PBS. 10µl volumes of the culture were spotted in duplicate onto a sterile slide, allowed to air-dry and methanol-fixed. Cells were rehydrated with 10µl dH_2O and covered with a cover-slip before imaging. Cells were viewed under a 100 X objective, with oil immersion, using light microscopy (Zeiss Axioskop) and imaged using a digital camera (Zeiss Axiocam) linked to the microscope. Cell size measurements were performed using Openlab software (courtesy of Dr. Garry Blakely).

2.2.4.2 DAPI staining

This technique is used to visualise DNA localisation within cells. Cells were grown to the appropriate growth phase in LB medium and 10^8 bacterial cells were aliquoted into sterile eppendorf tubes. Chloramphenicol was added (final concentration of 100µl/ml) and samples were incubated in a water bath at 37°C for 15min. Cells were then harvested, washed in PBS and fixed as above for Phase Contrast microscopy. To rehydrate cells, 15µl of DAPI (1µg/ml)

was added to the spot directly and covered with a cover-slip. Cells were viewed under a 100 X objective, with oil immersion, using a fluorescent filter with an excitation maximum of 345nm and an emission maximum of 458nm (Zeiss Axioskop). As with Phase contrast microscopy, images were obtained using the digital camera connected to the microscope. Images were analysed using the AxioVision software provided with the microscope.

Chapter 3

Phenotypic characterisation of *csp*, *hfq* and *rpoS* mutants of *Salmonella* Typhimurium

3.1 Background

As detailed in Section 1.9, the primary goal of this study was to characterise the CspA paralogues of *S.* Typhimurium and elucidate their function in RpoS-mediated stress responses. Recent studies in our lab (I.W. Hutchinson, PhD submitted to U of E, 2005) have produced a suite of SL1344 *csp* monogene mutants, with all but one of the CspA paralogues deleted from the chromosome; and a SL1344 *csp* null mutant (MPG 558), lacking all 6 chromosomal CspA paralogues. Each of the cold shock gene deletions were constructed *in vitro* by crossover PCR then cloned into the suicide vector (pTOF24), using *PstI* and *SalI* restriction sites, to replace the kanamycin resistance (Km^R) gene on the vector. The Km^R replacement cassette was cloned into the *NotI* site of the PCR fragment in the pTOF24 vector. Clones were selected for on LB-Km agar plates at 30°C. Integrants were tested for on the same medium at 42°C, colony purified and cultured overnight at 30°C in LB medium to allow resolution. The resolved cells were grown on LB-sucrose-Km plates at 37°C to allow plasmid counter selection as cells with a functional levan sucrase gene (*sacB*) are killed in the presence of sucrose. Km selects for the retention of the replacement cassette. Deletions were confirmed by PCR. This suite of *csp* mutants was used to show that the CspA paralogues of *S.* Typhimurium differ in their ability to facilitate growth at low temperatures and convey stationary phase resistance to oxidative stress. Previous studies in *E. coli* have shown that CspA paralogues are involved in RpoS expression and that RpoS is induced on growth retardation. Therefore, the question arises whether the differences observed in the SL1344 *csp* mutants are due to variations in the level of RpoS expressed in each *csp* mutant.

Another component in RpoS regulation is the RNA binding chaperone, Hfq. It has been shown that many of the phenotypes observed in an *hfq* knockout strain of *E. coli* have been attributed to defects in RpoS expression (Muffler *et al.,* 1997(ii)). However, Sittka *et al*. (2007) have shown that there are many RpoS-independent phenotypes seen in an *hfq* mutant of *S.* Typhimurium. Can these phenotypes be related to those observed in the SL1344 *csp* null mutant?

The aim is to show, by comparative analysis, whether *hfq, csp* null and *rpoS* mutants have (partly) overlapping or distinct phenotypes. This will provide an insight into whether Hfq and the CspA paralogues are interacting or working separately to regulate RpoS expression and may lead to the discovery of more roles for the CspA paralogues.

3.2 Mutation of *rpoS* and *hfq* in *S.* Typhimurium

The original *rpoS* mutation, which was transduced into the SL1344 strain, was constructed by Fang *et al.* (1992) in a virulent *S.* Typhimurium strain, ATCC 14028s. An internal PCR-derived *Salmonella rpoS* gene fragment (600 bp) was cloned into a suicide vector, conjugally transferred into ATCC 14028s and selected for resistance to penicillin. Southern hybridisation, with the *rpoS* fragment, was used to confirm the truncation of the *rpoS* gene. Once transduced into SL1344, the extent of the truncation was found using flanking oligonucleotides (see Table 2.1.1) for PCR, followed by DNA sequence analysis as part of this study. This confirmed that the resulting RpoS protein would be non-functional as the domain that binds to RNA polymerase (Swiss-Prot P0A2E5: RPOS_SALTY annotated sequence) is disrupted by insertion of the β-lactamase (*bla*) gene (shown in Figure 3.2).

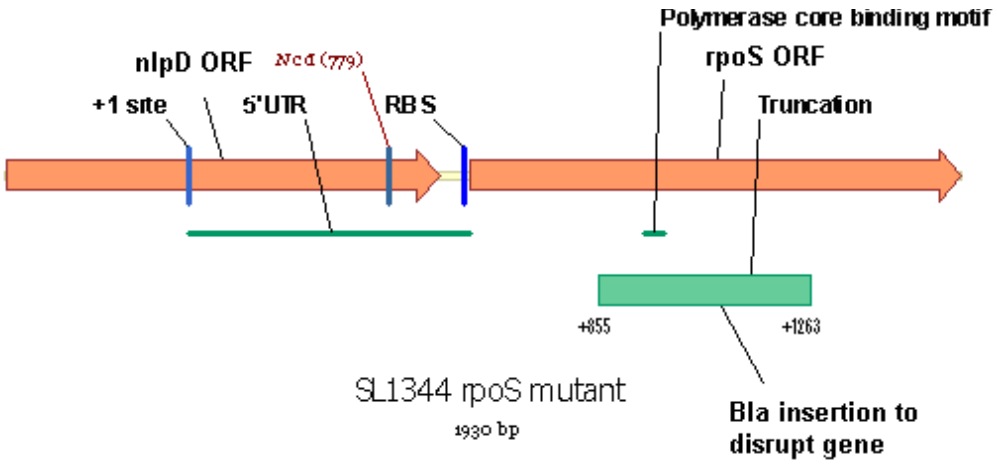


Figure 3.2: **Schematic diagram of the truncation in the SL1344 *rpoS* strain** (constructed with Vector NTI version 10, Invitrogen)**.** The insertion disrupts the polymerase core binding motif of the SL1344 *rpoS* mutant strain, therefore the RpoS protein expressed from this strain is non-functional as it can no longer direct transcription machinery. Bases disrupted by Bla insertion are stated in relation to +1 site of *rpoS* transcription (from base +855 to +1263; amino acids 85 to 222 of RpoS are displaced by the insertion).

The original *hfq-1*::Mu*d*-Cam mutation, which was transduced into the SL1344 strain, was constructed by Brown and Elliott (1996) in a virulent strain of *S.* Typhimurium, ATCC 14028s. Their *hfq* mutant, TE5314, was defective in bubble production after challenge with H_2O_2.

3.3 Defining the growth characteristics and stress resistance of a *csp* null mutant of *S.* Typhimurium and the relative importance of RpoS and Hfq

3.3.1 Lag phase, exponential growth rate and stationary phase at 37°C

Past studies have shown that the CspA paralogues of *S.* Typhimurium differ in their ability to convey resistance to oxidative stress and to allow growth at low temperatures, but are there differences in their growth profiles at 37°C? Bacterial growth is directly linked to the cell's capacity for protein synthesis. During stationary phase, the level of protein synthesis drops to around 20% of that seen when the cell is actively growing (Kaczanowska and Ryden-Aulin, 2007). As mentioned in Section 1.2, when cells enter stationary phase 70S ribosome monomers are converted and stored as inactive 100S dimers and the nucleoid becomes condensed by Dps, which is known to be induced by RpoS, as cell division is no longer possible. Following nutritional upshift, the lag phase occurs in which cells must convert from this inactive form to one capable of rapid growth. Biosynthesis rates are high, as RNA and proteins necessary for rapid growth are synthesised, but no cell division occurs (Garrido *et al*., 1993). Exponential growth occurs once the cell has adapted and is therefore capable of cell division. The time limiting factor during exponential growth is the replication of DNA. Once nutrients are depleted, the growth rate plateaus and the cell enters stationary phase again.

Are CspA paralogues involved in adapting to new nutrient environments and preparing the cell for growth? RpoS plays a central role during retardation in growth rates, therefore is RpoS required for entry into stationary phase? To address these questions, growth curves were performed in both nutrient-rich (LB) and minimal M9 media containing histidine (0.02%).

In each of the experiments, cultures were grown to stationary phase as detailed in Section 2.1.3 and then diluted 100-fold into fresh medium. No differences in the OD_{600} and cfu/ml of each stationary phase culture (after 16h) were observed when grown in an equivalent medium, so any initial differences observed in lag phase must reflect biological differences within the strains. The experiments were performed in triplicate, with separate inocula, to ensure accuracy and reproducibility in each instance.

3.3.1.1 MPG 558 has an increased lag phase but an equal growth rate compared to SL1344

Figures 3.3.1.1 (a) and (b) allow for comparison of the lag phase and growth rate of MPG 558 (SL1344 *csp* null mutant) with that of its isogenic parent at 37°C in both rich and minimal media. Strains were grown to stationary phase (for approximately 16h) in media corresponding to that used for subsequent growth curves. The approximate length of lag phase, defined as the time prior to balanced growth, and a calculation of doubling time in peak exponential growth are given for each strain in Table 3.3.1.1. These values were calculated as described in Section 2.1.3.1. Absorbance readings are provided in electronic form (supplementary data CD) and data points used for calculations are indicated.

Table 3.3.1.1: Comparison of the length of lag phase and doubling time of MPG 558 and its isogenic parent, SL1344, at 37°C. In these experiments, lag phase is defined as the time prior to balanced growth and doubling time is calculated to the nearest whole minute. Absorbance measurements are provided on the supplementary data CD.

	Rich medium		Minimal medium	
Strain	**Lag phase (min)**	**Doubling time (min)**	**Lag phase (min)**	**Doubling time (min)**
SL1344	20	29	80	58
MPG 558	60	36	120	94

The graphs (Figures 3.3.1.1(a) and (b)) show that the MPG 558 strain has an extended lag period in both rich and minimal media when compared to its isogenic parent, suggesting that CspA paralogues may be involved in optimising RNA and/ or protein biosynthesis after nutrient upshift. Ribosomes must be reconstituted during the lag phase before protein synthesis can proceed (Wada *et al.,* 1990; Chapter 1.2). The rate-limiting factor in ribosome reconstitution is the melting of stable inactive RNA by RNA chaperones and helicases as ribosomal RNA is structurally promiscuous and needs only 50% complementarity to form double helices (Kaczanowska and Ryden-Aulin, 2007). The RNA chaperone activity of CspA paralogues may help the RNA out of 'kinetic traps' in the folding pathway by destabilising and resolving RNA structure.

The doubling time calculated for the MPG 558 strain is similar to that of SL1344 when grown in rich medium. In minimal medium, the doubling time for the MPG 558 strain is 1.5-fold longer than that of its parental strain (94 min compared to 58 min), suggesting that the CspA paralogues of *S.* Typhimurium are required for optimal growth in minimal medium. DNA replication and chromosomal partitioning are the time-limiting factors during division, as septation cannot occur through an un-segregated nucleoid (Sun, Yu and Margolin, 2002). Therefore, does this suggest a role for CspA paralogues in DNA replication in minimal medium?

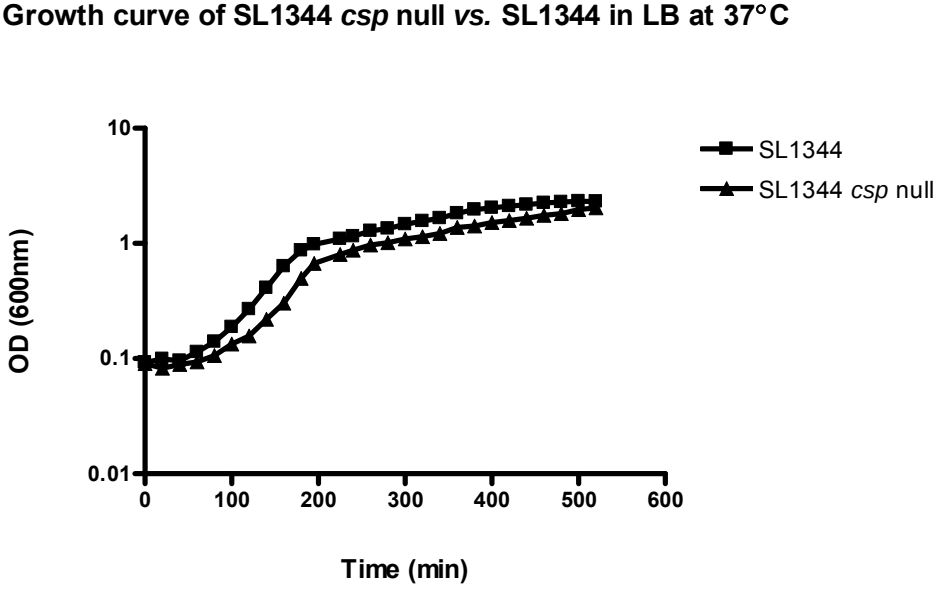


Figure 3.3.1.1 (a): Growth curve comparing the SL1344 *csp* null mutant with its isogenic parent, SL1344, at 37°C in rich medium. Strains were grown to stationary phase (16h) in LB at 37°C, with aeration. Cultures were then diluted 100-fold into fresh LB medium and incubated, with aeration, at 37°C. The OD_{600} was then recorded every 20 minutes until stationary phase was reached, i.e. no further increase in OD_{600} was observed. Growth curves were plotted from the average of three separate inocula. Samples varied less than 3%.

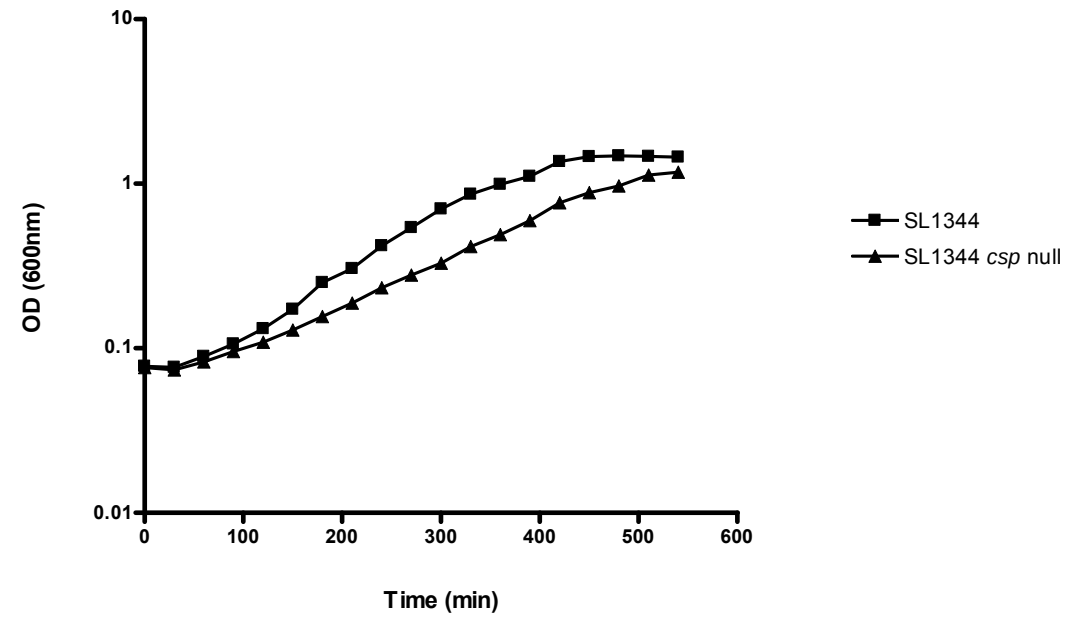


Figure 3.3.1.1 (b): Growth curve comparing the SL1344 *csp* null mutant with its isogenic parent, SL1344, at 37°C in minimal medium. Cultures were grown to stationary phase (24h) in minimal medium at 37°C, with aeration, prior to dilution into fresh minimal medium. Cultures were incubated at 37°C, with aeration, and the OD_{600} recorded every 30min. Curves were plotted from the average of three separate inocula. Samples varied less than 4%.

The MPG 558 strain is able to reach a similar final cell density to its isogenic parent at stationary phase in both media. The growth profiles of MPG 558 at 37°C suggests that CspA paralogues of *S.* Typhimurium are necessary for optimal adaptation to fresh medium, but are not essential for viability, at 37°C. This is contrary to reports by Horn *et al* (2007) which state that the presence of at least one of the CspA-like proteins is essential for cell viability at 37°C in *Bacillus subtilis.* However, all three Csps are dispensable for growth at 37°C in *Listeria monocytogenes* (Schmid *et al*., 2009).

Proteomic studies from our lab (FSA project report B01008, 2001) have shown that CspC and CspE are constitutively expressed in *S.* Typhimurium at 37°C, and studies in *E. coli* have suggested that these proteins are involved in transcription antitermination and nucleoid condensation (Chapter 1.6). Kim *et al.* (2001) have reported that levels of *S.* Typhimurium *cspH* mRNA dramatically increase when stationary phase cells are subcultured into fresh media. Are these proteins involved in converting inactive stationary phase cells into actively growing cells on nutrient upshift? Are these proteins required for optimal growth in minimal medium? This will be investigated in the following sections.

3.3.1.1.1 Which CspA paralogues are required for optimal growth at 37°C?

Differences in the lag period (in both rich and minimal media) and in the growth rate (in minimal media only) have been observed for the MPG 558 strain when compared to its isogenic parent. Can any of the CspA paralogues complement the growth limitations of the MPG 558 strain and return it to wildtype levels? To answer this question, the growth profiles of the *csp* monogene mutants were examined.

Figures 3.3.1.1.1 (a) and (b) illustrate the growth profiles of the *csp* monogene strains in comparison to their isogenic parent, SL1344. In each case, the approximate length of the lag phase and doubling time are given in Table 3.3.1.1.1. As before, the lag phase is defined as the time prior to balanced growth and the doubling time is calculated during peak exponential growth. Absorbance readings are provided in electronic form, on the supplementary data CD, and the data points used for calculations are highlighted.

In general, a similar pattern was observed for each mutant in both rich and minimal media. The doubling times of the *csp* monogene mutants are not significantly different to that of their parent strain in rich medium; however, doubling rates do vary in minimal medium depending on the CspA paralogue present. In both rich and minimal media, there is a range of lag periods, between that of the parent and MPG 558 strains, observed for the SL1344 *csp* monogene strains.

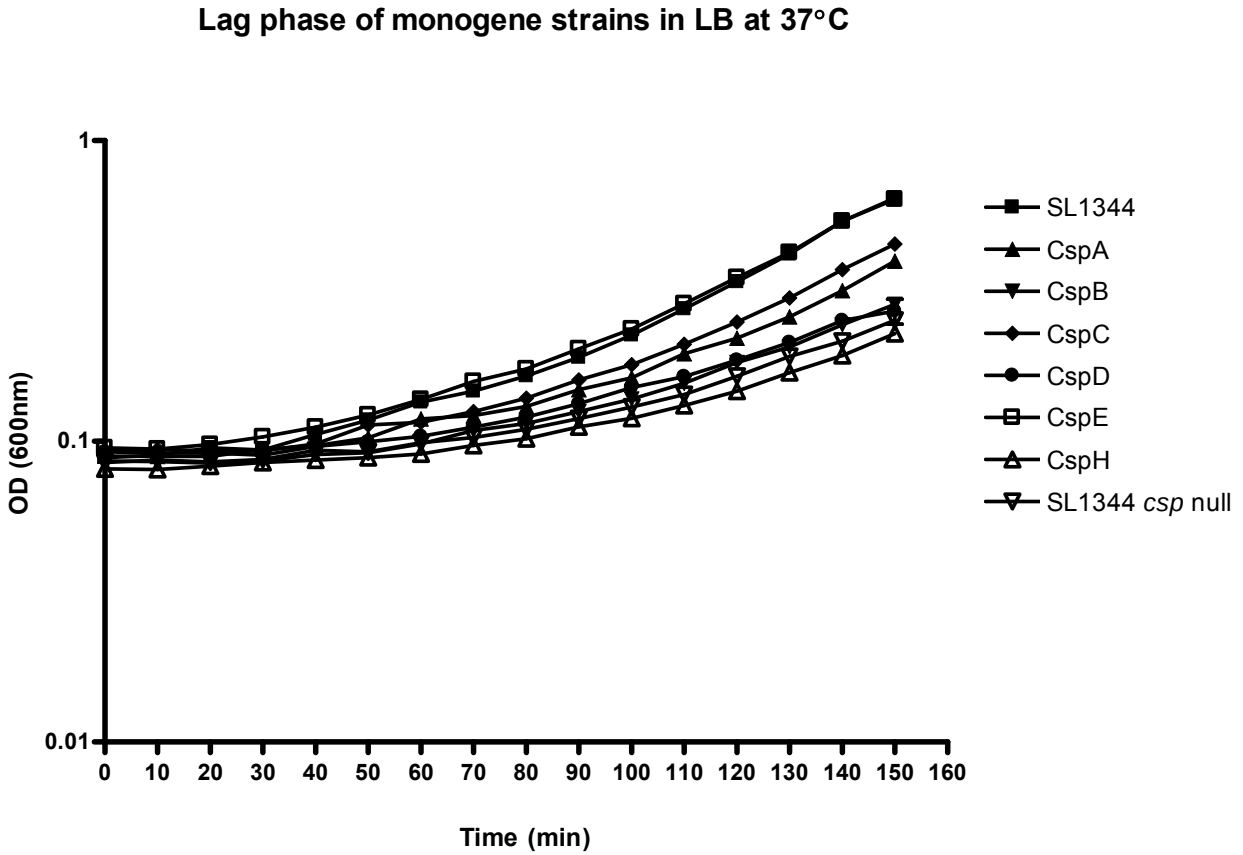


Figure 3.3.1.1.1(a): Graph comparing the lag phase and growth rate of the *csp* monogene mutants with that of the *csp* null mutant and SL1344 at 37°C in rich medium. Stationary phase cultures, grown for 16h in LB at 37°C, were diluted 100-fold into fresh LB and the OD_{600} monitored every 10min. The curves were plotted from the average of three separate inocula and the sample variance was less than 5%.

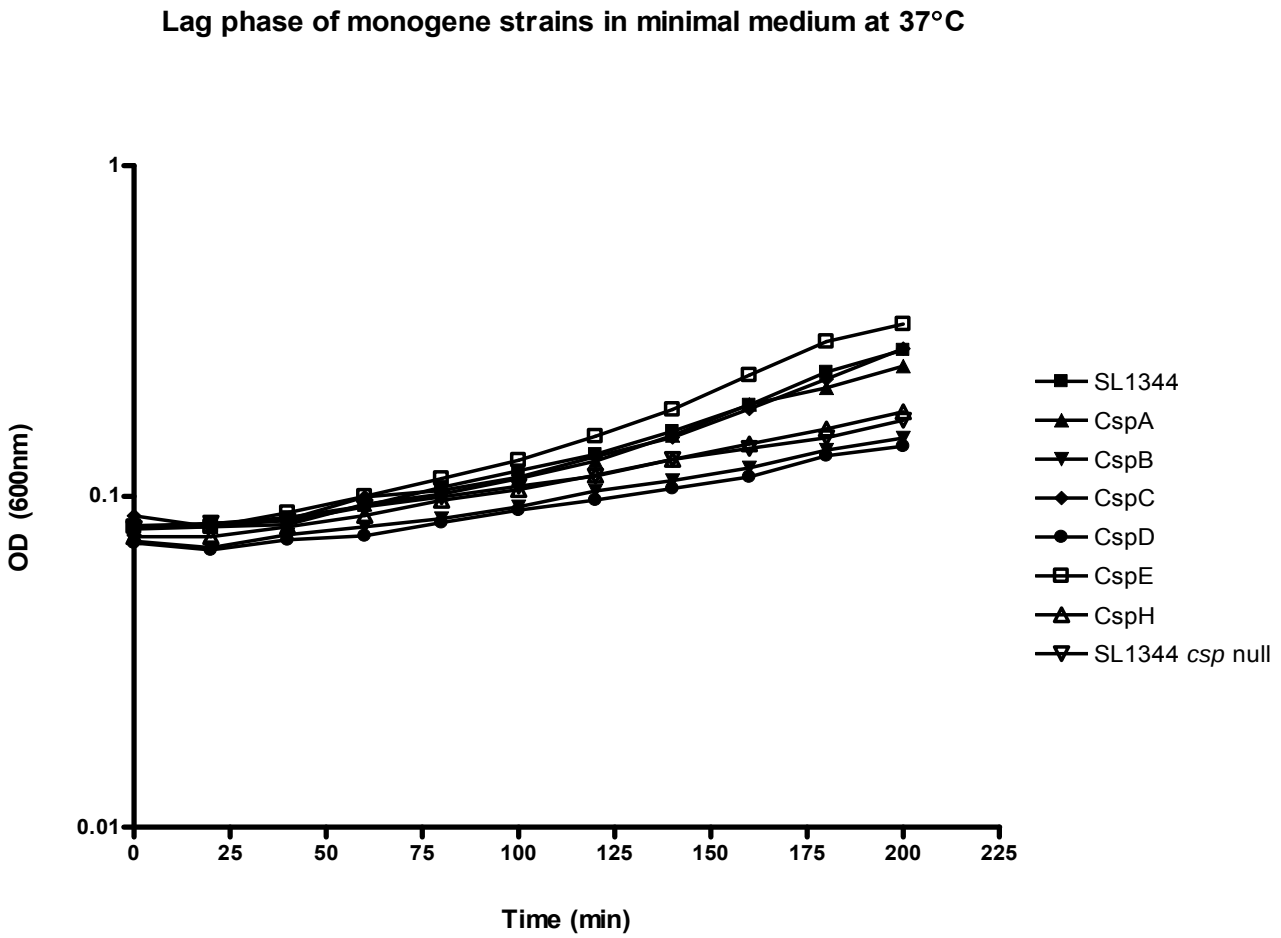


Figure 3.3.1.1.1(b): Graph comparing the lag phase and growth rate of the *csp* monogene mutants with that of the *csp* null mutant and SL1344 at 37°C in minimal medium. Stationary phase cultures, grown for 24h in minimal medium at 37°C, were diluted 100-fold into fresh minimal medium and their growth monitored. The curve was plotted from the average of three separate inocula and the sample variance was less than 3%.

Table 3.3.1.1.1: The length of lag phase and the estimated doubling time of the *csp* monogene mutants compared to those of MPG 558 and their isogenic parent strain at 37°C. Lag phase is estimated from nearest time point and doubling time is calculated to the nearest whole minute. Data points used for calculations are highlighted on the supplementary data CD accompanying this study.

	Rich medium		Minimal medium	
Strain	Lag phase (min)	Doubling time (min)	Lag phase (min)	Doubling time (min)
SL1344	20	29	80	58
MPG 551 (A)	60	33	90	64
MPG 552 (B)	60	34	120	89
MPG 553 (C)	20	32	100	61
MPG 554 (D)	50	35	120	93
MPG 557 (E)	<10	30	60	59
MPG 556 (H)	60	37	120	89
MPG 558 (null)	60	36	120	94

At 37°C, the doubling times of MPG 553 (CspC) and MPG 557 (CspE) are approximately equal to their isogenic parent in both rich and minimal media. This suggests that either CspC or CspE is enough to convert the growth of MPG 558 to wildtype in minimal medium, therefore may play a role in DNA replication. This concurs with what is known about these proteins in *E. coli*, as they have been shown to be constitutively expressed at 37°C and would therefore be present throughout growth. Proteomic studies in our lab have also shown that these proteins are constitutive and abundant in *S.* Typhimurium at 37°C (Sienkiewicz *et al.*, FSA project report B01008, 2001). Bae *et al.* (1999) have shown that deletion of *E. coli cspE* does not result in a difference in growth rates in either rich or minimal medium, confirming that the other paralogues can functionally substitute for CspE, with regard to its role in exponential growth.

However, the lag phases of the mutants differ from that observed for SL1344. MPG 557 (CspE) exhibited a much shorter lag phase than SL1344 in both media (10 min compared to 20 min in LB and 60 min compared to 80 min in minimal medium). Bae *et al.* (1999) have shown that when stationary phase cultures are diluted into fresh medium, the lag period of an *E. coli cspE* mutant was 2.5 times longer than in the wildtype. This group has also shown that during the lag period, the expression from a *cspE::lacZ* fusion in the wildtype was transiently increased, suggesting that CspE plays an important role in re-growth of the nutritionally deprived cells. The time limiting factor during lag phase is perceived to be the accumulation of active 70S ribosomes. This suggests a possible role for CspE of *S.* Typhimurium in the re-activation of 100S ribosome dimers in the absence of the other paralogues. Phadtare and Inouye (2001) found that overexpression of CspE had no effect on growth rate in *E. coli*; however, these experiments were performed in otherwise wildtype strains, i.e. with all

paralogues present. MPG 553 (CspC) exhibited a longer lag period than its isogenic parent in minimal media only (100 min compared to 80 min).

The growth profile of the MPG 558 strain is mimicked by MPG 552 (CspB), MPG 554 (CspD) and MPG 556 (CspH) in both rich and minimal media, suggesting that CspB, CspD and CspH of *S.* Typhimurium are not involved in adaptation to fresh medium, or cell growth in minimal medium, at 37°C. These findings agree with what is known about CspB in *S.* Typhimurium, as it is only induced on a temperature downshift and is not present at 37°C (Craig *et al*., 1998). This result also agrees with what is known about CspD in *E. coli* as, although it is present at 37°C, it is not induced until stationary phase and is therefore unlikely to affect the lag phase or exponential phase of growth (Phadtare *et al*., 2006). Conversely, the growth profile of MPG 556 conflicts with a previous study by Kim *et al*. (2001) that showed a dramatic increase in *S.* Typhimurium *cspH* mRNA upon nutrient upshift at 37°C, i.e. when stationary phase cells are subcultured into rich medium as is seen here. However, the importance of CspH during nutrient upshift has not yet been elucidated.

Figures 3.3.1.1.1(a) and (b) show that the growth profile of MPG 551 (CspA) is different from those of the other monogene mutants, as it partly mimics MPG 558 in rich medium but its isogenic parent strain, SL1344, in minimal medium. In rich medium, MPG 551 has a long lag period (60 min) equal to that of MPG 558, suggesting that CspA is not involved in adaptation to nutrient upshift in rich medium at 37°C. This is as expected due to the negative regulation of CspA production, by RNase E-mediated mRNA degradation, at this temperature (Bae *et al*., 1999; Phadtare *et al*., 1999). However, this strain shows optimal growth in minimal medium, as the lag period and doubling time of MPG 551 is all but equal to that of the parent strain. This suggests that CspA can partially substitute for CspC or CspE during growth in minimal medium at 37°C. This may seem unexpected as *E. coli* CspA is not normally present in the cell at 37°C as its mRNA is highly unstable at this temperature. However, Bae *et al*. have shown that the *E. coli* CspE functions as a negative regulator for *cspA* expression at 37°C, and that inactivation of *cspE* results in an overproduction of CspA in lag phase (1999), therefore CspA could functionally substitute for CspE in its absence.

3.3.1.1.2 Neither RpoS nor Hfq is required for growth at 37°C

Fang *et al.* (1992) reported that their original *S.* Typhimurium *rpoS* mutant has increased susceptibility to oxidative and acid stresses and DNA damage. Several of these phenotypes have previously been found for the MPG 558 strain (I.W. Hutchinson, PhD submitted to U of E, 2005). Also, Phadtare and Inouye have shown that overexpression of CspC and CspE in *E. coli* results in an upregulation of RpoS (2001). Can the differences in the growth profiles be attributed to differences in RpoS levels or is this an RpoS-independent phenotype? Also, does an SL1344 *hfq* mutant exhibit the same growth defects, or is this a distinct phenotype for the MPG 558 strain? To answer these questions, the growth profiles of SL1344 *rpoS* and *hfq* mutants were compared to those of their isogenic parent, SL1344, in both rich and minimal media at 37°C.

Table 3.3.1.1.2: Comparison of the length of lag phase and doubling time of SL1344 *hfq* and *rpoS* mutants with those of MPG 558 and their isogenic parent, SL1344, at 37°C. Lag phase is defined as the time prior to balanced growth and is estimated from the nearest time point. The doubling time, during peak exponential growth, is calculated to the nearest whole minute. The data points used for the calculations are highlighted in the supplementary data provided in electronic form.

	Rich medium		Minimal medium	
Strain	**Lag phase (min)**	**Doubling time (min)**	**Lag phase (min)**	**Doubling time (min)**
SL1344	20	29	80	58
SL1344 *hfq*	40	30	100	62
SL1344 *rpoS*	20	29	80	58
MPG 558	60	36	120	94

The lag phase and doubling times of each strain, in both rich and minimal media, are summarised in Table 3.3.1.1.2. Figures 3.3.1.1.2(b) and (d) show that there is no difference in the early and mid stages of the SL1344 *rpoS* mutant growth profile in either rich or minimal medium, compared to its isogenic parent strain, indicating that RpoS is not essential for growth at 37°C. This is reasonable as RpoS is not present in the cell during rapid growth at 37°C and is only induced on entry into stationary phase. The *rpoS* mutant enters stationary phase at the same point as SL1344, suggesting that RpoS is not the cell's trigger to cease rapid growth. Hengge-Aronis (1999) notes that *E. coli rpoS* mutants do not exhibit any growth disadvantage at 37°C. This is confirmed for *S.* Typhimurium by McMeechan *et al.* (2007). Also Smith, Masters and Donachie note that the inactivation of the *rpoS* gene in *E. coli* had no detectable effect on growth rates during log phase (1993). The growth profile also shows that the growth defects apparent in the MPG 558 strain at 37°C are not dependent on RpoS.

The growth curves (Figures 3.3.1.1.2(a) and (c)) show that the *hfq* mutant has the same doubling time as the SL1344 parent strain in both rich and minimal media, indicating that Hfq is not involved in cell mass accumulation and DNA replication at 37°C in *S.* Typhimurium. The SL1344 *hfq* mutant strain reached stationary phase at a similar time and optical density as that of its isogenic parent. This result is contrary to results seen previously, as Tsui *et al*. (1994) report that *E. coli hfq* mutants exhibit a reduced growth rate and growth yield compared to the parent strain. Previously, *S.* Typhimurium *hfq* mutants have been shown to have a much slower growth rate in minimal medium when compared to the wildtype (80 min doubling time compared with 49 min for the wildtype). However, this may be due to strain differences as ACC14028s was the parent strain used in this instance (Brown and Elliott, 1996), and the doubling times reported in their study are much faster than those observed for the parent strain, SL1344, in minimal medium.

Table 3.3.1.1.2 shows that the SL1344 *hfq* strain has a lag phase 20 min longer than its isogenic parent in both rich and minimal media, although this is shorter than the MPG 558 strain. This suggests that Hfq is involved in exit from stationary phase on nutrient upshift. Hfq is an RNA chaperone that enhances RNA-RNA interactions and is thought to recruit ribosomes to mRNA (Nogueira and Springer, 2000). In *E. coli*, 80 to 90% of Hfq has been shown to be in the cell's cytoplasmic fraction in association with ribosomes and the remaining intracellular Hfq is associated with the nucleoid (Brennan and Link, 2007). This would explain the requirement for Hfq during the lag phase which involves the biosynthesis of RNA and proteins that are necessary for rapid growth. Our results concur with those of Sittka *et al*. (2007) who also observed a longer lag phase for an *S.* Typhimurium *hfq* mutant at 37°C, after inoculation into fresh medium. Unlike findings reported by Sittka *et al.* (2007), this study found that cells reached stationary phase at a similar optical density to their parent. In their study, parallel determination of viable counts showed that cell viability was uncompromised; therefore differences may have been due to individual cell mass, i.e. shorter cells may have formed earlier.

Approximately 40% of the proteins that have altered synthesis in an *E. coli hfq* mutant are not affected by a mutation in *rpoS*, indicating that RpoS is not the only target for regulation by Hfq (Tsui, Leung and Winkler, 1994). This is corroborated by the longer lag phase phenotype seen in SL1344 *hfq* that is not observed in the SL1344 *rpoS* strain.

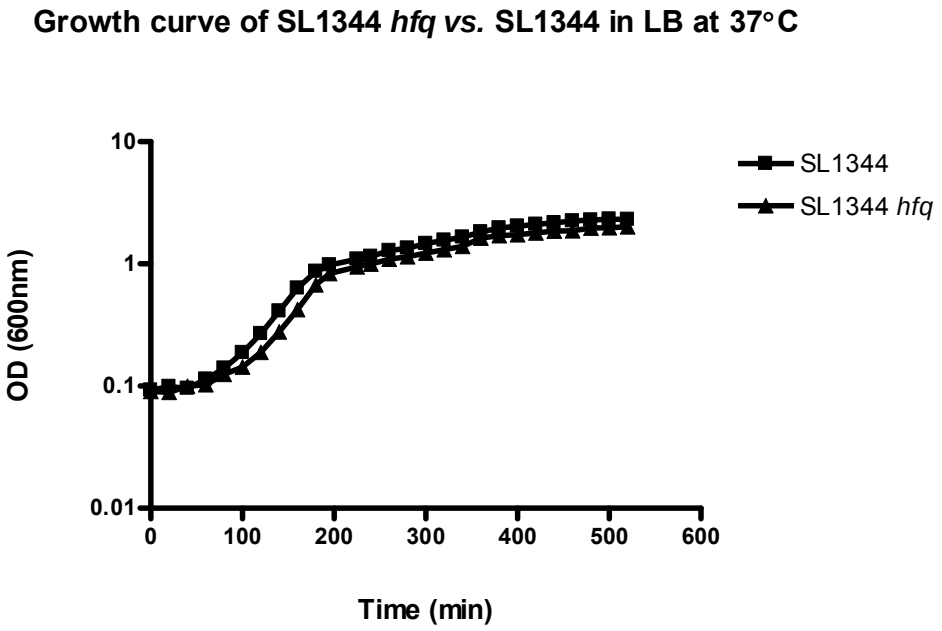


Figure 3.3.1.1.2(a): Growth curve comparing the SL1344 *hfq* mutant with its isogenic parent, SL1344, at 37°C in rich medium. Cultures were grown to stationary phase (16h) in LB medium at 37°C, with aeration, prior to 100-fold dilution into fresh LB medium. Growth was monitored at 37°C. The curves were plotted from the average of three separate inocula. Samples varied less than 5%.

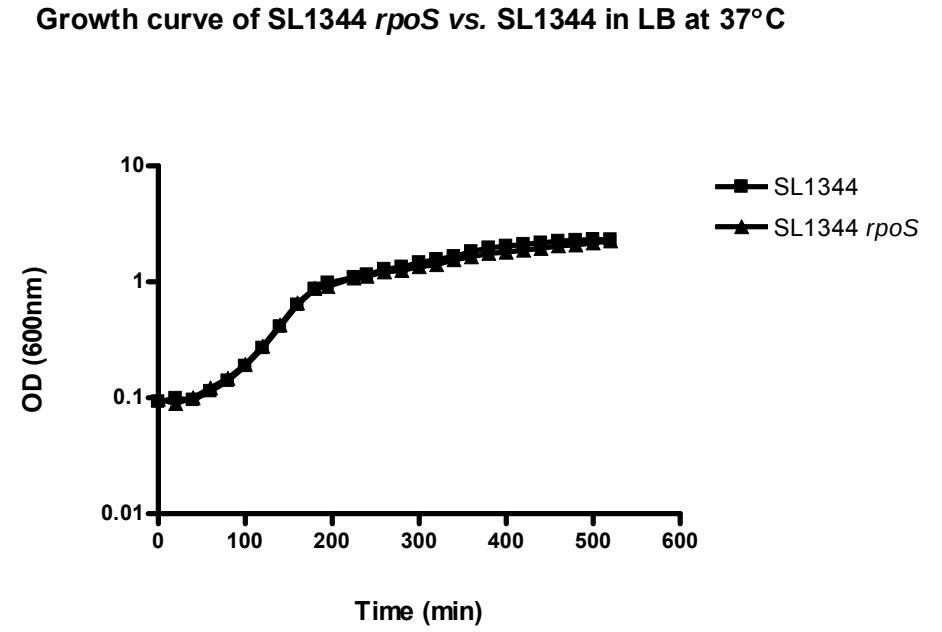


Figure 3.3.1.1.2(b): Growth curve comparing the SL1344 *rpoS* mutant with its isogenic parent, SL1344, at 37°C in rich medium. Cultures were grown to stationary phase (16h) in LB at 37°C, with aeration, prior to dilution into fresh LB medium. Growth was monitored at 37°C. Curves were plotted from the average of three separate inocula. Samples varied less than 3%.

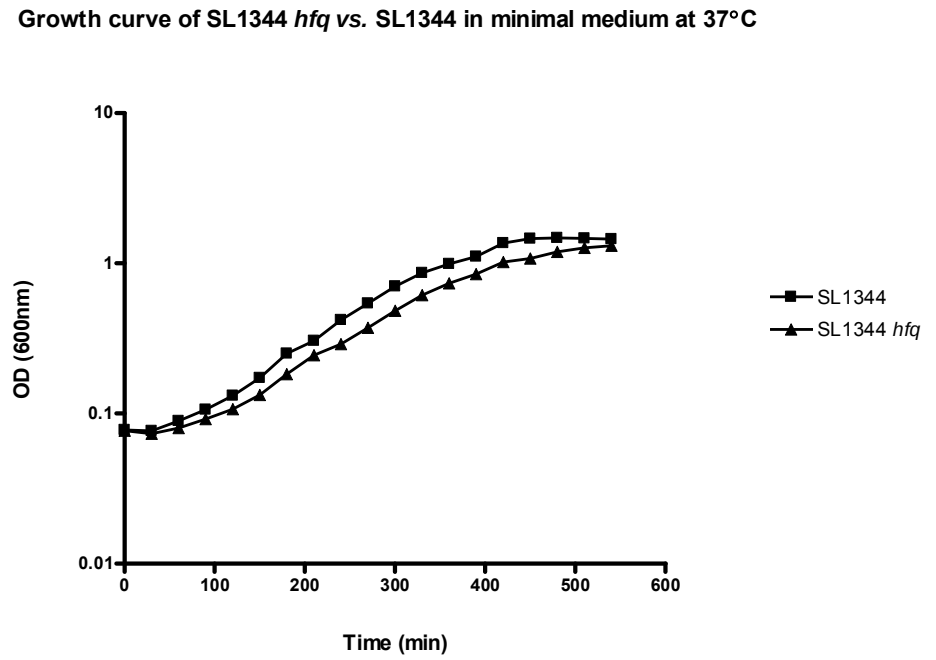


Figure 3.3.1.1.2(c): Growth curve comparing the SL1344 *hfq* mutant with its isogenic parent, SL1344, at 37°C in minimal medium. Cultures were grown to stationary phase (24h) in minimal medium at 37°C, with aeration. Samples were diluted 100-fold into fresh minimal medium and OD_{600} was monitored at 37°C. Curves were plotted from the average readings from three separate inocula. Samples varied less than 2%.

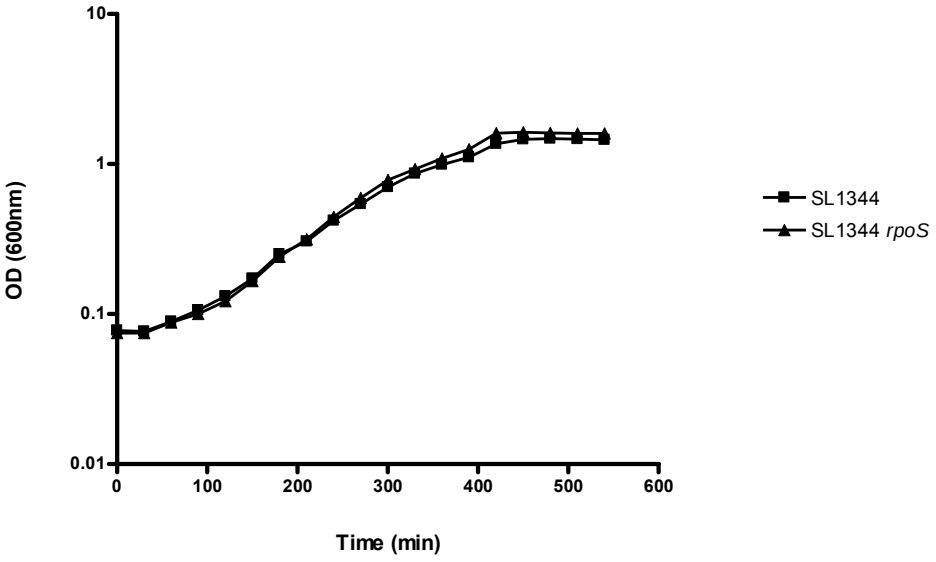


Figure 3.3.1.1.2(d): Growth curve comparing the SL1344 *rpoS* mutant with its isogenic parent, SL1344, at 37°C in minimal medium. Cultures were grown to stationary phase (24h) in minimal medium at 37°C, prior to dilution into fresh minimal medium. Growth, at 37°C, was monitored over time. Curves were plotted from the average OD_{600} readings from three separate inocula. Samples varied less than 4%.

3.3.1.2 CspA paralogues, RpoS and Hfq are required for long-term survival during stationary phase

Muffler *et al*. (1997(ii)) report that Hfq is required for the expression of RpoS-dependent genes in *E. coli* and because of this an *E. coli hfq* mutant is starvation-, and multiple stress-sensitive. In the study, the group reports that *E. coli hfq* and *rpoS* null mutants die off rapidly under conditions of prolonged starvation. After 6 days in minimal medium at 37°C, the viability of the *E. coli hfq* showed a tenfold reduction and *rpoS* mutants exhibited a hundredfold reduction when compared to the viability of the parent strain. Is this the case for *S.* Typhimurium, and is this phenotype shared by the SL1344 *csp* null mutant? To test this, the experiment was paralleled using the SL1344 mutant strains. In this experiment, the maximum viability was determined as the cfu/ml after 24h of growth (i.e. in early stationary phase); this did not vary significantly between the strains (between 2.0 and 3.0 x 10^9 cfu/ml).

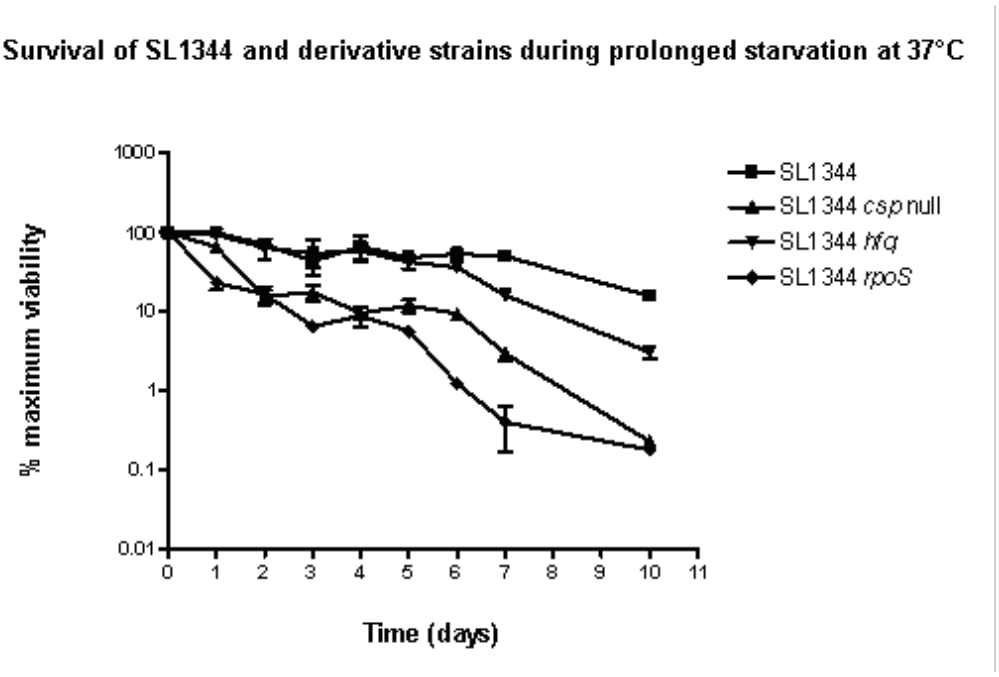


Figure 3.3.1.2: **Survival of SL1344 *rpoS*, *hfq* and *csp* null mutants compared to their isogenic parent, SL1344, in minimal medium at 37°C.** Each strain was grown to stationary phase (24h, T_0) in minimal medium (plus histidine at 0.02%) at 37°C, with aeration, and further incubated under the same conditions for 10 days. The cfu/ml was determined by plating dilutions on LB plates and was then converted into % maximum viability, using cfu/ml after 24h (T_0) as 100% viability. Each experiment was performed in triplicate, with separate inocula, and the average viability plotted on the graph. Error bars indicate the standard error within results at each time point.

This survival graph parallels that of the *E. coli hfq* and *rpoS* mutants reported by Muffler *et al*. (1997(ii)), suggesting that these proteins play the same role in *S.* Typhimurium as in *E. coli* during prolonged starvation at 37°C. The SL1344 strain was able to survive prolonged starvation for 10 days without a significant drop in cell viability, i.e. its viability after 10 days

was less than one log lower than when it originally entered stationary phase. The SL1344 *rpoS* mutant was much less resistant to prolonged starvation as the viability declined steadily throughout the study and after 10 days had decreased to 0.1% of that recorded as the strain entered stationary phase. These results are confirmed by Lange and Hengge-Aronis (1994) who report that *E. coli rpoS* mutants die off rapidly under starvation conditions and do not develop the multiple stress resistance so characteristic of stationary phase cells. Wilmes-Riesenberg *et al.* (1997) report that there was an eightfold reduction in the percentage of *Salmonella* UK-1 *rpoS*::Ap mutants recovered after 5 days of incubation in Lennox broth at 37°C. During stationary phase, cells synthesise storage substances, like glycogen and polyphosphates, and protective substances such as trehalose; the production of which have been attributed to RpoS (Zambrano and Kolter, 1996; Saint-Ruf *et al.,* 2007; Hengge-Aronis, 1993). In *E. coli*, RpoS is also responsible for the transcription of the cAMP regulon that allows the use of alternative carbon sources for energy. Studies in *E. coli* have found that another RpoS-dependent protein, Dps, is still synthesised in late (3 day old) stationary phase cells and controls the synthesis of as many as 23 strongly starvation-induced proteins (Almiron *et al*., 1992).

The SL1344 *hfq* strain does not die off as rapidly as the *rpoS* mutant, as the viability matches that of its isogenic parent until around 6 days of starvation. Following this, there is a steady decline in the viability from around 80% to less than 10% after 10 days of starvation. Although the end result of the experiment agrees with the findings of Muffler *et al* (1997(ii)), who report that their *E. coli hfq* mutant declines to around 10% of maximum viability after 8 days, the decline of the SL1344 *hfq* mutant appears to occur in two stages, rather than as a steady decline as reported with the *E. coli* mutant. As this mutant is tenfold more viable than the SL1344 *rpoS* mutant after 10 days of incubation, the results suggest that resistance to prolonged starvation is mainly RpoS-dependent and that the production or function of RpoS is not solely reliant on Hfq. This is confirmed by Muffler *et al*. (1997(ii)) who state that the effects on stationary phase resistances are somewhat less dramatic in an *E. coli hfq* mutant than those observed for *rpoS* mutants. Does this suggest a role for CspA paralogues?

The decline in viability of the MPG 558 strain resembles that of the *rpoS* mutant but occurs 24h later. As seen with the SL1344 *rpoS* mutant, the viability after 10 days was around 0.1% of that recorded on entry into stationary phase. This suggests an RpoS-dependent phenotype of the SL1344 *csp* null mutant that is distinct from the SL1344 *hfq* mutant. It remains possible that decline was influenced by the histidine auxotrophy of the cells. However, this might be considered a minor point as all the strains exhibited the same auxotrophy.

3.3.2 Cold adaptation and growth at low temperatures

On temperature decrease, all biochemical reactions are affected, influencing many cellular processes. There is a decrease in membrane fluidity, which hampers protein secretion and solute transport. However, Shaw and Ingraham (1967) have shown that *E. coli* can grow at 10°C with the same fatty acid composition as at 37°C; therefore altered fatty acid composition is not a prerequisite for growth at low temperatures. Transcription is hampered at low temperatures by an increase in the super-helical density of DNA. Most importantly, abnormal RNA secondary structure is formed thus creating a major translational blockage. These effects must be rectified before growth can resume. It is well documented that CspA paralogues of *E. coli* and *S.* Typhimurium are required for adaptation to low temperatures and for RpoS expression at 37°C, and that a retardation of growth (as seen upon cold shock) induces RpoS expression. As mentioned above (Section 3.1), previous studies in our lab have shown that the CspA paralogues differ in their ability to adapt to and allow growth at low temperatures. Is it that differences in RpoS expression are responsible for the differing ability of the SL1344 *csp* monogene mutants to grow at low temperatures?

3.3.2.1 CspA paralogues regulate RpoS expression at low temperatures

Previous studies have shown that MPG 558, the SL1344 *csp* null mutant, is unable to grow after cold shock at 10°C. However, CspA and CspE can complement this phenotype and return the growth of the MPG 558 strain to wildtype levels. Is this related to the ability of these proteins to regulate RpoS expression? In order to address this, RpoS expression in each monogene mutant strain was examined, using monoclonal antibodies raised against RpoS from *E. coli* (Neoclone), after the strains had undergone cold shock during exponential phase (as described in Section 2.1.3).

The levels of RpoS detected in each strain following cold shock are compared in Figure 3.3.2.1. The immunoblot shows distinct differences in the RpoS expression of the *csp* monogene and null mutants when compared to their isogenic parent. There is clear RpoS expression in SL1344, whilst no RpoS band can be detected in the *csp* null mutant. There are strong bands detected in the CspE and CspA monogene mutants and a faint band is detected in the CspC monogene strain. Little or no RpoS bands were observed in the other *csp* monogene strains. This indicates that CspA and CspE, and to some extent CspC, play a role in RpoS expression after cold shock in exponential phase and that these proteins may functionally substitute for each other in this instance. There is unspecific binding of the antibodies, which has also been observed by others, as immunoblots by Cunning and Elliott

(1999) detected a secondary band at around 25kD. However, an explanation was not given for the cause. Also Neoclone, the manufacturer of the antibodies, report that the antibodies react with an unspecific protein of around 50kD. These unspecific bands were also observed by Brown and Elliott (1996) although the antibodies used in this case were a personal gift (Nguyen *et al*., 1993). However, the inclusion of the SL1344 *rpoS* strain, with a truncated RpoS protein of 32kD (as described in Figure 3.2), ensures that the band denoted is indeed RpoS.

Previous studies in our lab (Craig *et al*, 1998) have reported that CspA of *S.* Typhimurium is cold induced and that CspE is constitutively expressed at 37°C. It may be that CspE is also constitutively expressed at low temperature. These results indicate that CspA and CspE may underpin RpoS expression at low temperatures. Upon cold shock, RNA secondary structure is stabilised creating a major translational blockage due to ribosome stalling. The 5'UTR of *rpoS* mRNA also forms a stable secondary structure that may occlude its own ribosome-binding site. CspA paralogues are RNA chaperones that are involved in binding to and melting aberrant RNA secondary structure, thus allowing translation at low temperatures. Consequently, it is tempting to speculate that CspA and CspE facilitate the translation of RpoS at low temperatures by melting the intrinsic secondary structure in the *rpoS* mRNA 5'UTR.

The immunoblot (Figure 3.3.2.1), when compared to previous results in our lab, shows that there is a correlation between the ability of SL1344 *csp* monogene strains to grow at low temperature and their RpoS expression. Does this mean that RpoS expression is required for adaptation and growth at low temperatures?

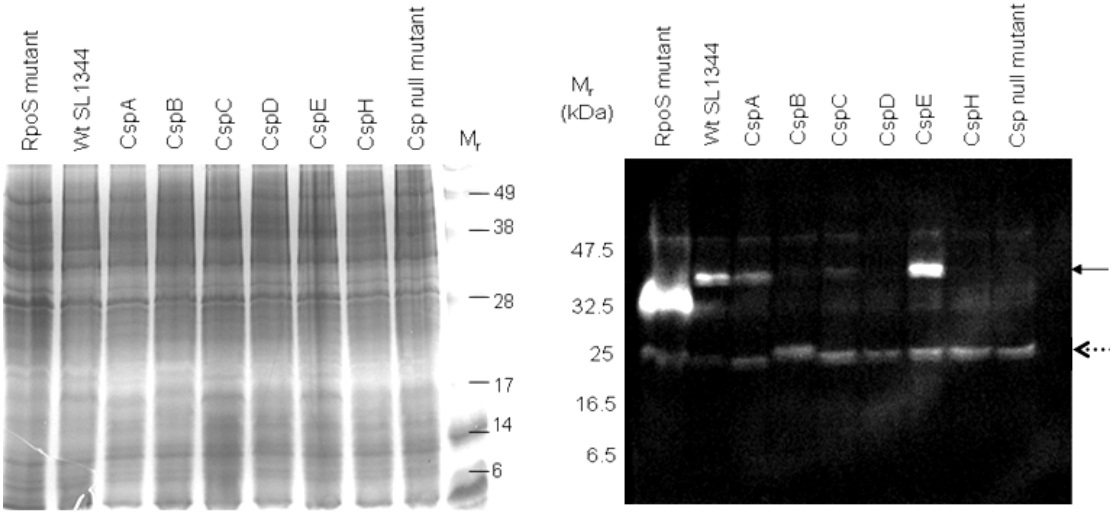


Figure 3.3.2.1: Immunoblot comparing levels of RpoS detected in the SL1344 *csp* null strain, *csp* monogene mutants and their isogenic parent strain after cold shock at 10°C. Stationary phase cultures, grown for 16h in LB at 37°C, with aeration, were diluted 100-fold into fresh LB medium and grown to an OD_{600} of 0.2 at 37°C. Cultures were then incubated at 10°C in an air-cooled orbital incubator, with aeration, for 2h. Cells were harvested and subjected to SDS-PAGE and electroblotting, as described in Chapter 2.2.2. Blots were probed with monoclonal RpoS antibodies (Neoclone). The band corresponding to RpoS is denoted (⟶). The positions of the molecular weight markers (M_r) are shown. Coomassie blue stained gel is shown to demonstrate equal loading of samples. The experiment was repeated in triplicate and the blot shown is representative of all results. A cross reacting band recognised by the antiserum is also shown (⇠). The inclusion of the SL1344 *rpoS* mutant, producing a truncated RpoS protein, shows the band indicated as RpoS is correct (the site of Bla insertion in SL1344 *rpoS* gene, resulting in a truncated protein, is shown in Figure 3.2).

3.3.2.1.1 Neither RpoS nor Hfq are required for growth at low temperatures

Figures 3.3.1.1.2(b) and (d) have shown that RpoS is not required for optimal growth in rich or minimal medium at 37°C. However, Figure 3.3.2.1 has shown a correlation between RpoS expression and growth at low temperatures. Studies in *S.* Typhimurium have shown that an *hfq* mutant has a four- to seven-fold reduction in expression of *rpoS* (Brown and Elliott, 1996). So, the questions remained, is RpoS required for growth at low temperatures and does the SL1344 *hfq* mutant share the same growth phenotype as the MPG 558 strain at low temperature? To address these questions, growth of the SL1344 *rpoS* and *hfq* strains was monitored at 10°C and compared to that of the *csp* null and isogenic parent strains. The raw data, used to construct the growth profiles, can be found in electronic form, on the supplementary data CD, accompanying the text.

Figure 3.3.2.1.1(a) compares the colony-forming ability of the SL1344 *rpoS* and *hfq* strains with that of MPG 558 and their isogenic parent on solid agar following incubation at 15°C for 72h. These results show that, like their isogenic parent, both the *rpoS* and *hfq* mutant are able

to grow and form single colonies at low temperatures. These results also reaffirm that the *csp* null mutant is unable to grow at low temperatures. This shows that RpoS and Hfq are not required for colony-forming ability at low temperatures. However, these results only tell us that these strains have reached stationary phase by 72h, but not how fast they are able to adapt to and overcome the biochemical alterations that occur at low temperatures.

Figure 3.3.2.1.1(b) compares the growth profiles of the SL1344 *rpoS* and *hfq* mutants with their parental strain, SL1344, after cold shock at 10°C (as described in Section 2.1.3 and in the figure legend). This shows that there is no major difference in the growth rates (estimated at around 120 min; see supplementary CD for data) of the *rpoS* and *hfq* mutants when compared to SL1344. Therefore, RpoS and Hfq are not required for DNA replication and cell mass accumulation at low temperatures. Sledjeski *et al*. (1996) have shown that RpoS is not essential for growth of *E. coli* at low temperatures in rich or minimal medium, as there is no difference in growth rate between the wildtype and *rpoS* or *dsrA* mutants. This finding was confirmed by White-Zieger *et al.* (2008) who found that, despite changes in gene expression for many RpoS-dependent genes, experiments assessing growth rate at 23°C and viability at 4°C did not demonstrate significant impairment in *rpoS::*Tn*10* or *dsrA::cat* mutant strains compared to the wildtype. To confirm that a lack of RpoS is not the reason for the low temperature growth phenotype of the SL1344 *csp* null strain, complementation experiments were performed, over-expressing *rpoS* from the arabinose-inducible plasmid pUBAD::*rpoS*. The overexpression of RpoS did not result in growth of MPG 558 on LB agar at 15°C or in LB medium at 10°C (data not shown).

Contrary to these results, Muffler *et al*. (1997(ii)) report that that an *E. coli hfq* null mutant exhibits a reduced growth rate and yield when grown in rich medium at 20°C. This is confirmed by Sittka *et al.* (2007) who report that their SL1344 *hfq* mutant grows much slower at room temperature when compared to the wildtype. As observed in this study, their SL1344 *hfq* strain reaches stationary phase at a lower OD than the parent strain. Parallel determination of viable counts demonstrated that cell viability was uncompromised.

These results indicate that, although RpoS expression correlates with CspA paralogue activity, RpoS is not essential for growth at low temperatures. CspA paralogues must therefore mediate low temperature adaptation and growth through other means. Upon cold shock, CspA paralogues are required to overcome the translational blockage that occurs due to the aberrant stabilisation of RNA secondary structures, and allow cells to adapt for growth at low temperatures. RpoS expression may be a consequence of the effect that CspA and CspE have on the overall protein synthesis at low temperatures.

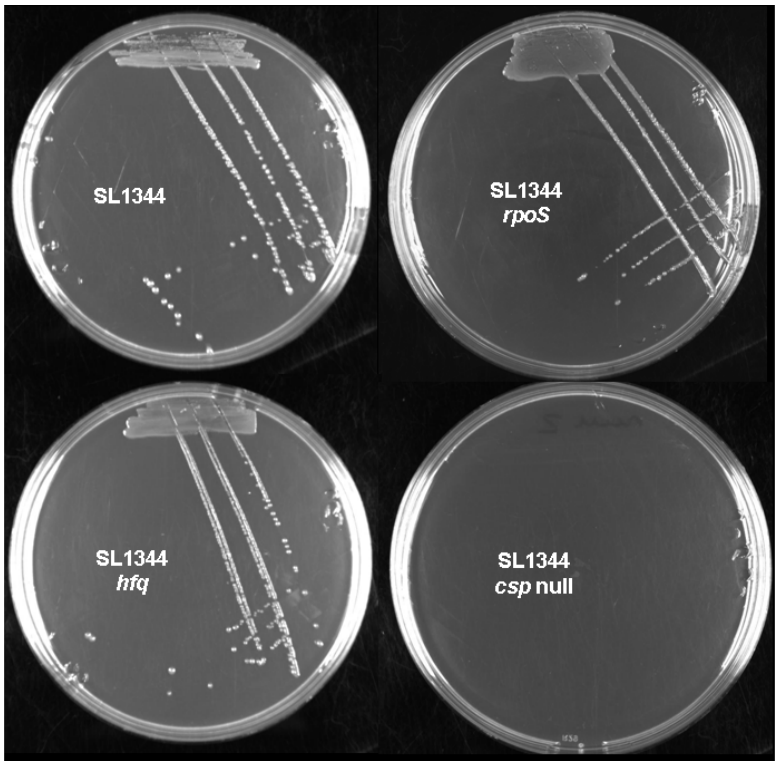
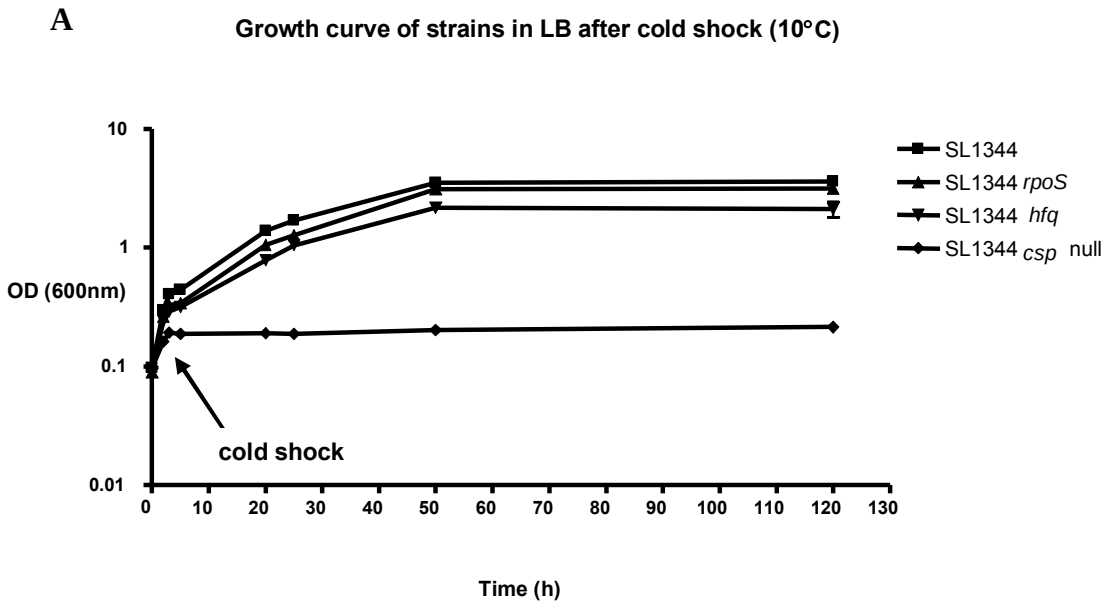


Figure 3.3.2.1.1(a): Colony forming ability of strains on solid agar plates following incubation for 72h at 15°C. Cultures were grown to stationary phase (16h) in LB at 37°C. 10µl of each culture was added to the plate and used to streak for single colonies. Plates were then incubated for 72h in a static incubator at 15°C. The experiment was repeated in triplicate, from separate inocula, and the images shown are representative of all results.



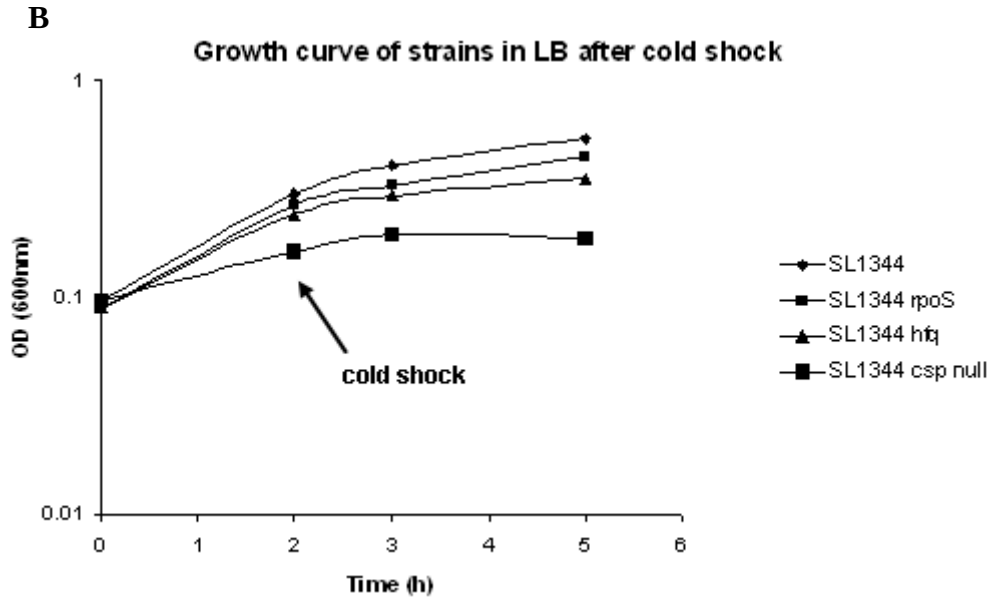


Figure 3.3.2.1.1(b): Growth profiles of the SL1344 *hfq* and *rpoS* mutants in comparison to the *csp* null strain and their isogenic parent strain in rich medium at 10°C. A For each strain, a 1 in 100 dilution of stationary phase culture (grown for 16h in LB at 37°C) was added to fresh LB medium (T_0) and grown for 2h at 37°C. The cultures were then incubated at 10°C, with aeration, and the OD_{600} taken at various time points. Each experiment was repeated in triplicate, with separate inocula, and the points shown are an average of the OD_{600} measurements. The maximum variation in samples was 3%. **B** Enlargement of first 5h of experiment with point of cold shock indicated.

3.3.2.2 The *csp* null mutant is able to grow if the temperature is gradually reduced to 10°C

Horn *et al*. (2007) reported that the majority of cellular processes observed after fast, large temperature shifts also occur during the acclimation to naturally occurring small, slow temperature downshifts, as long as the temperature is within the physiological range. Figure 3.3.2.1.1(b) has shown that the CspA paralogues, but not RpoS or Hfq, are required for growth at low temperatures, likely due to the melting of aberrant mRNA secondary structure and thus facilitating translation. However, if the temperature was gradually reduced would there be the same initial ribosome-jamming effect of cold shock? If the cell was gradually adapting to decreases in temperature, would the role of CspA paralogues be redundant? These questions were addressed by examining the colony-forming ability (cfu/ml) and OD_{600} of MPG 558 after 72h at 10°C, following various rates of decline to this temperature. Stationary phase cultures, grown in LB at 37°C with aeration, were diluted 1000-fold into fresh medium and grown for 2h at 37°C. These cultures were then placed in a refrigerated PCR machine and subjected to a gradual temperature decline to 10°C at one of three rates: 1°C/min, 2°C/min or 3°C/min. Subsequent growth, following 72h incubation at 10°C, was monitored by serial dilution and plating at 37°C. Each experiment was repeated in triplicate, with separate inocula, to ensure accuracy and reproducibility. Following each experiment, the MPG 558 culture was re-streaked on LB agar plates and incubated at 15°C for 72h. None of the samples grew on agar plates at 15°C, ensuring the results recorded were real and not due to contamination or outgrowth of mutants with advantageous secondary mutations.

Figure 3.3.2.2(a) shows the OD_{600} of the MPG 558 strain after gradual decline in temperature compared to the readings after cold shock or during continued incubation at 37°C. As there is a substantial increase in OD (from 0.1 to 1.0) following the three decline rates tested, this experiment shows that the MPG 558 strain can grow if the temperature is gradually reduced to 10°C. Also, the results suggest that the slower the rate of decline, the more capable the *csp* null mutant is to grow at low temperature. This aligns with an observation by Jones and Inouye (1994) that the magnitude of Csp induction is dependent on the range of temperature shift; the larger the temperature shift, the more pronounced the response. In the experiment, the final OD never equalled that observed for the *csp* null strain in stationary phase when left at 37°C, even at the slowest rate of temperature decline; however, a previous experiment (Figure 3.3.2.1.1(b)) has recorded an OD_{600} of 1.8 for the SL1344 parental strain following cold shock compared to that of 2.8 during stationary phase at 37°C.

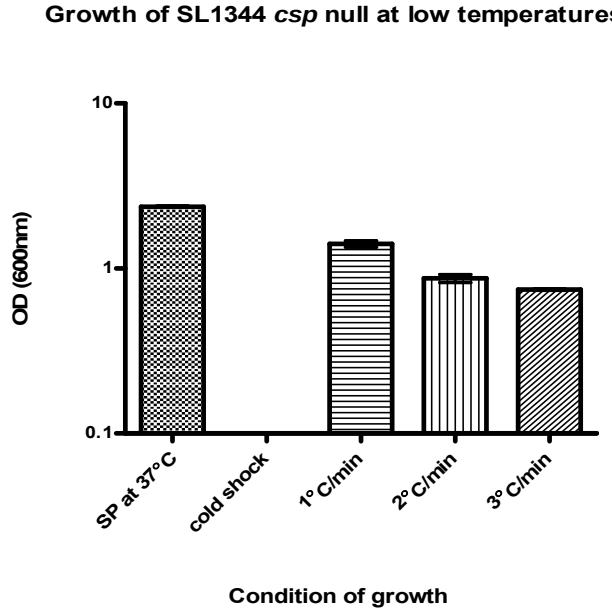


Figure 3.3.2.2(a): Graph comparing the OD_{600} of the SL1344 *csp* null mutant after incubation at 10°C for 72h, following various rates of temperature decline from 37°C. Stationary phase cultures, grown in LB at 37°C for 16h, were diluted 1000-fold into fresh LB and transferred to a PCR machine. Cultures were then chilled to 10°C, at different rates of temperature decline, and incubated for 72h. The results shown are the average of triplicate experiments from separate inocula. SP refers to stationary phase cultures incubated for 72h at 37°C. Cold shock refers to a sample incubated at 10°C in a refrigerated incubator.

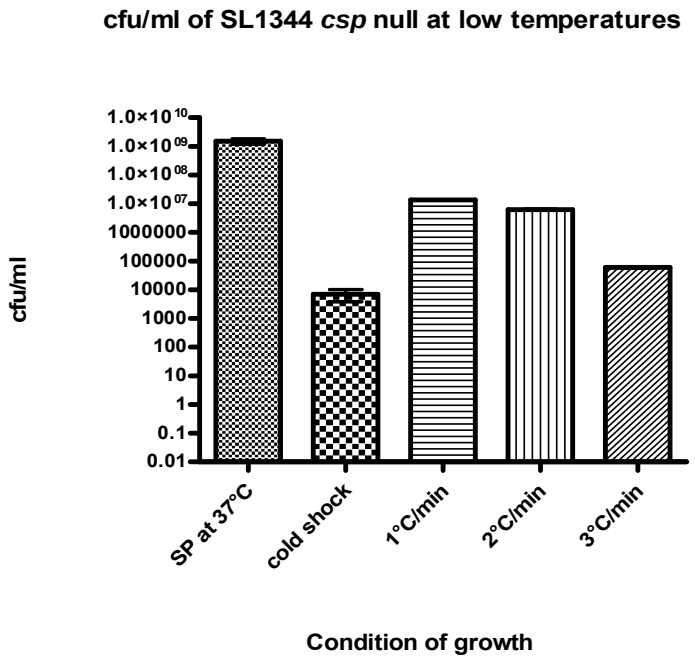


Figure 3.3.2.2(b): Graph comparing the colony forming ability of the SL1344 *csp* null after incubation at 10°C for 72h, following various rates of temperature decline from 37°C. Stationary phase cultures, grown in LB at 37°C for 16h, were diluted 1000-fold into fresh LB and transferred to a PCR machine (or a refrigerated incubator, at 10°C, for cold shock). The temperature was then reduced to 10°C at the rate stated. Following this, cultures were incubated at 10°C for 72h. The results shown are the average of triplicate experiments from separate inocula. Cold shock cfu/ml represents the number of viable cells in a sample subjected to 10°C, without gradual decline in temperature, for 2h during exponential growth.

An increase in optical density of a sample is a good indication of an increase in cell density; however, OD does not necessarily relate to growth as filamentation also results in an increase in OD_{600}. For this reason, the colony-forming ability of the strain after 72h incubation at 10°C, following the three decline rates, was also examined. Figure 3.3.2.2(b) shows the cfu/ml of the MPG 558 strain at various growth conditions. This experiment confirms that the *csp* null mutant is indeed able to grow at 10°C, and multiply to reach 10^7 cells/ml, if the temperature is gradually reduced. However, there is a hundredfold reduction in the cfu/ml of the *csp* null strain after a temperature decline of 1°C /min when compared to that of a culture when continuously incubated at 37°C. There is little difference in the colony forming ability of MPG 558 between a temperature reduction rate of 1°C/min and 2°C/min. However, there is a further hundredfold reduction in cfu/ml after a decline in temperature of 3°C/min. This confirms that the strain is more capable of growth following a slower decline in temperature. The SL1344 *csp* null strain clearly has the ability to grow at low temperatures but not to overcome the biochemical changes that occur upon cold shock (i.e. rapid temperature reduction).

According to the earlier growth rate experiments at 37°C (in Section 3.3.1), the MPG 558 strain has a peak doubling time of 36 min in rich medium and the longest decline from 37°C to 10°C is 27 min, at a rate of 1°C/min. Therefore, the growth observed in this experiment is not due to a doubling at 37°C before the low temperature is reached. Unfortunately, the equipment could not provide accurate rates of temperature decline faster than 3°C/min. It would be interesting to examine what the minimum rate of temperature decline is which results in the cold shock growth phenotype. Cold shock, as termed in this study (Figures 3.3.2.2(a) and (b)), results in a culture's decline from 37°C to 10°C in less than 7 min, as crudely measured by thermometer in the refrigerated orbital incubator.

Together these results suggest that CspA paralogues are not essential for the acclimation phase after cold shock but are required to overcome the initial translational blockage that occurs due to the stabilisation of mRNA secondary structure.

3.3.3 Differences in cell size and morphology of the *csp* null, *rpoS* and *hfq* mutants

Cell growth relies on the accumulation of cell mass, which triggers DNA replication, termination of which triggers septum formation and cytokinesis. Figures 3.3.1.1(a) and (b) have determined that the SL1344 *csp* null strain (MPG 558) takes longer to adapt to new growing conditions at 37°C, i.e. to start accumulating mass, than its parental strain. The doubling time of the MPG 558 strain is much slower than its parent strain in minimal media, although doubling times are similar in rich media (Table 3.3.1.1). Also, this strain is not able to grow following cold shock but is able to grow if the temperature is gradually reduced. At which point in cell growth is MPG 558 experiencing difficulty? To address this question, the cell morphology of the MPG 558 strain was compared to that of its isogenic strain. In a wild-type cell, RpoS plays a role in the increased expression of FtsZ prior to stationary phase, leading to smaller, more compact cells than observed during exponential phase. For these reasons, the morphologies of SL1344 *rpoS* and *hfq* cells were also examined. In *S.* Typhimurium there is a close relationship between length, cell volume and growth rate (Aldea, Herrero and Trueba, 1982; Shannon and Rowbury, 2007). Therefore, as the peak exponential growth rates of these strains is comparable in LB at 37°C, the mean cell size should be similar during exponential phase. In *E. coli*, different strains may change their diameter or length according to growth rate (Donachie, 1968; Addinall and Holland, 2002).

3.3.3.1 A SL1344 *csp* null mutant forms minicells at 37°C

Growth curves have shown that the MPG 558 strain takes longer to adapt to new growing conditions, and start accumulating mass, than its parental strain at 37°C. Also, a reduced exponential growth rate is observed for MPG 558 in minimal media at 37°C. The cell morphology of the MPG 558 strain was compared to its isogenic strain, during exponential phase at 37°C, to provide insights into the problems experienced by the strain during cell growth. Phase contrast microscopy was used to image cells as described in Chapter 2.2.4 and revealed that the MPG 558 strain formed minicells. Figure 3.1.1.1(a) shows an image of an MPG 558 cell forming a minicell. For phase contrast images see Appendix (Figures 3a and 3b).

As mentioned in Section 1.8, the ability to place the cell division site at a specific position within the cell is a fundamental property of most living organisms. In prokaryotic rod-shaped organisms, the division septum forms at the mid-point of the cell. To accomplish this, the mid-cell must be selected in preference to other potential division sites at the cell poles. If the residual sites are not inactivated, septa are formed near the cell poles giving rise to minicells

that lack chromosomal DNA (de Boer *et al*., 1991). To date only two possible ways to form minicells have been discovered. Which one is likely to be responsible for minicell formation in the MPG 558 strain? The site-specific inhibition of septum formation at the poles is governed by the products of two genes in the *minB* operon: *minC* and *minD*. In the absence of MinC or MinD, the polar sites are not inactivated and minicell formation occurs at the expense of divisions at the mid-cell (de Boer, Crossley and Rothfield, 1989). Bi and Lutkenhaus (1990) demonstrate that there is a broad cell length distribution, seen with a *min* deletion strain in *E. coli*, ranging from normal-length cells to long filaments. The broader cell distribution in *min* mutants is thought not only to be due to increased frequency of polar septa, but also to disturbed nucleoid segregation (Akerlund, Gullbrand and Nordstrom, 2002).

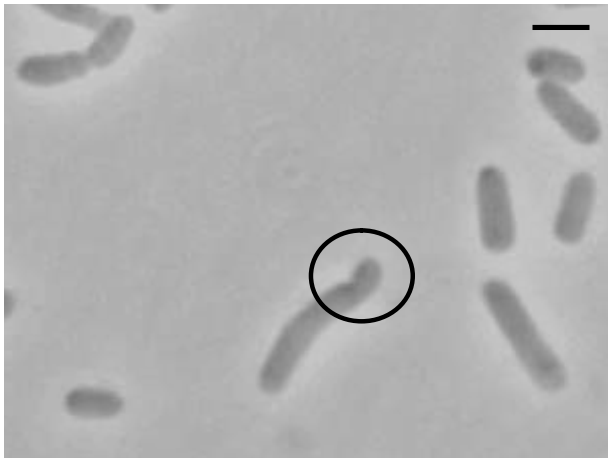


Figure 3.3.3.1(a): Phase contrast image of MPG 558 cells during exponential growth in LB at 37°C. Cells were grown to stationary phase (16h) in LB at 37°C and diluted 100-fold in fresh LB medium. Cells were grown to an OD_{600} of 0.2, then harvested, washed and resuspended in PBS. 10µl cell samples were fixed onto microscope slides using ice-cold methanol. Black circle denotes minicell prior to septation. Scale bar represents 1µm. Image is representative of triplicate experiments from separate inocula.

As described in Chapter 1.8, FtsZ (a GTPase) regulates the timing and frequency of septum formation by providing a scaffold for the assembly of division proteins. Ward and Lutkenhaus (1985) have shown that a two- to seven-fold increase in the level of FtsZ protein results in induction of the minicell phenotype. However, this differs from that seen in a *min* mutant, where minicell divisions occur at the expense of normal divisions, as minicell divisions, following FtsZ overexpression, occur in tandem with normal divisions and result in a much narrower size distribution.

The cell size distribution of the MPG 558 strain was compared to that of its parent strain to determine which of these pathways was responsible for the formation of minicells in this strain. Minicells are too small to be measured accurately for cell length distribution

experiments, so the consensus is to not include them but note their appearance. From manually comparing phase contrast images with DAPI-stained images of MPG 558 cells, the occurrence of anucleate minicells is around 5% (14 of 300 cells) during exponential phase and 12% (24 of 200 cells) during stationary phase. Figures 3.3.3.1(b) and (c) compare the size distribution of MPG 558 cells with those of its parent strain, during different growth phases. The cell length measurements are provided in electronic form on the CD accompanying the text. Phase contrast images of each strain are shown in Appendix, Figures 3a and 3b.

Figure 3.3.3.1(b) compares the cell size distribution of both strains during exponential phase. The average size of a cell of the SL1344 strain is 2.3µm during exponential growth. The twofold cell distribution seen in SL1344 is consistent with that seen in *E. coli* wildtype cells as reported by Akerlund, Gullbrand and Nordstrom (2002) to represent a 'normal' growth phenotype. The MPG 558 strain has elongated cells during exponential phase, with a mean length of 3.4µm, compared to its parent but still displays a twofold size distribution. The mean cell lengths are statistically different as determined by a two-tail student T-test (p value of less than 0.01). As mentioned earlier, there is a close relationship between cell length and growth rate in *S.* Typhimurium, i.e. cells with equal doubling rates should be similar in length; this is clearly not the case here (Aldea, Herrero and Trueba, 1982).

Figure 3.3.3.1(c) compares the cell size distribution of the MPG 558 strain and its isogenic strain during stationary phase at 37°C. As noted for exponential phase, a twofold cell size distribution is observed for both strains. The average length of an SL1344 cell during stationary phase is 0.98µm. Similar to observations made during exponential phase, the MPG 558 strain is slightly elongated (by around 7%), although more than half of the cells fall within the same bin range as that of its parent (from less than or equal to 1µm to 1.5µm). The average cell size in the MPG 558 strain is 1.05µm, similar to the parent strain, during stationary phase. These sizes are not significantly different as determined by a two-tailed student T-test on 200 cell length measurements of each strain.

The mean cell sizes show that the elongation of MPG 558 is around 1.5 cell lengths (of SL1344) during exponential phase. Ward and Lutkenhaus (1985) have calculated that the average cell size in a *min* mutant is increased by 70%. Also, a *min* minicell phenotype would result in a much wider cell size distribution which is not seen with MPG 558. Therefore, the cause of minicell formation in the MPG 558 strain could be due to an increase in FtsZ levels. Takada, Wachi and Nagai (1999) have shown that RNase E activity is increased in an *E. coli hfq* mutant, which leads to more efficient cleavage of the *ftsQAZ* transcript and therefore more efficient translation, and increased levels, of FtsZ (described in detail in Chapter 1.8). This increase in FtsZ results in minicell formation but has little effect on cell length.

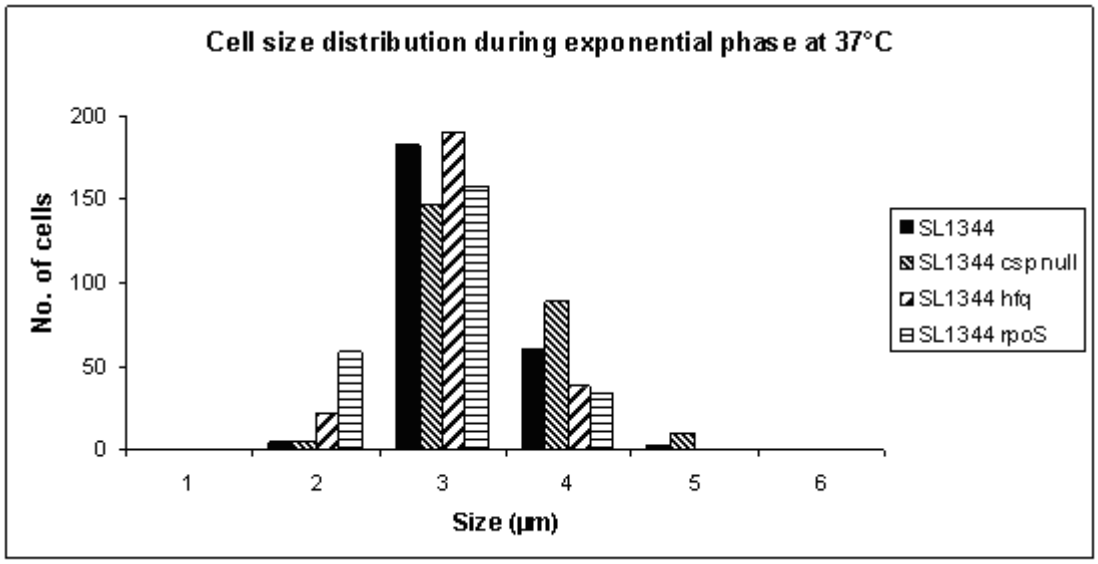


Figure 3.3.3.1(b): Cell size distribution comparing the *csp* null, *rpoS* and *hfq* mutants with their parental strain, SL1344, during exponential phase at 37°C. Stationary phase cultures, grown for 16h in LB at 37°C, were diluted 100-fold into fresh LB and grown to an OD_{600} of approximately 0.2. Cells were harvested, washed and resuspended in PBS and 10µl volumes fixed to microscope slides using ice-cold methanol. Experiments were performed in triplicate and phase contrast images were evenly sampled to measure 250 cells. Raw measurement data for this graph is provided in electronic form.

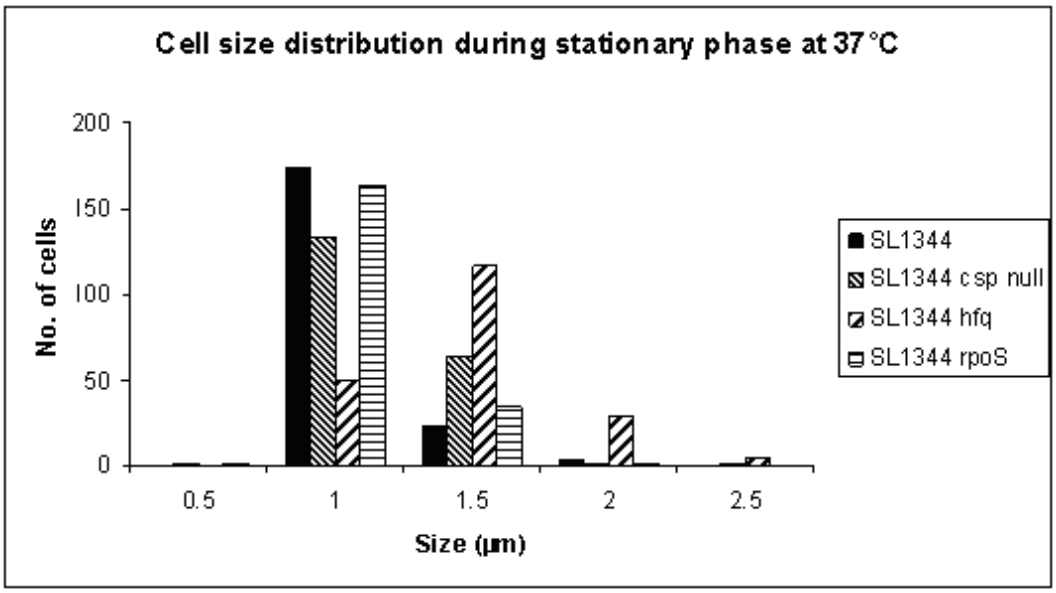


Figure 3.3.3.1(c): Cell size distribution of the mutant strains compared to that of SL1344 during stationary phase at 37°C. Cultures were grown to stationary phase (16h) in LB at 37°C, with aeration. Cells were harvested, washed and resuspended in PBS prior to methanol-fixation to microscope slides. 200 cell lengths were measured using phase contrast microscopy as described in Chapter 2.2.4. Images were randomly chosen from triplicate experiments. Raw measurement data is provided in electronic form accompanying the text.

Feng *et al*. (2001) report that inhibition of endonucleolytic digestion by RNase E was increased by addition of *E. coli* CspE due to the melting of mRNA secondary structure elements, normally recognised by RNase E. Therefore the absence of CspE in the MPG 558 strain may lead to an increase in the cleavage of the *ftsQAZ* transcript by RNase E, resulting in an increase in functional FtsZ levels. There is a persistence of elongated rod-shaped cells observed for *E. coli rpoS* mutants during stationary phase, as RpoS induction of *bolA* is required to form shorter cells (Van der Linden *et al*., 1992). Previous studies in *E. coli* have shown that the CspA paralogues are involved in RpoS expression (Phadtare and Inouye, 2001). Does the cell size distribution of the MPG 558 strain mirror that of the SL1344 *rpoS* or the *hfq* mutant?

3.3.3.1.1 An hfq mutant also forms minicells but independently of RpoS

As one of the aims of this study is to find distinct phenotypes for the SL1344 mutant strains, the question of whether the cell size distribution of MPG 558 was comparable to that of the *rpoS* and *hfq* strains was addressed. Again, phase contrast microscopy was used to compare cell morphologies and measure cell size distribution. Sample phase contrast images of each strain are located in the Appendix (Section 3) and cell length measurement data is provided on the supplementary data CD, accompanying the text. Figures 3.3.3.1(b) and 3.3.3.1(c) show the cell size distribution of each strain during exponential and stationary phase at 37°C. The cell size distribution of the SL1344 and MPG 558 strains has been discussed previously. Table 3.3.3.1.1 summarises the mean cell size of each strain, calculated from 250 cell measurements during exponential phase, and 200 cell measurements during stationary phase, at 37°C.

Table 3.3.3.1.1: The mean cell sizes of SL1344 strains in LB, during both exponential growth and stationary phase at 37°C. Cultures were grown to stationary phase (16h) in LB, with aeration, at 37°C. Also, stationary phase cultures were diluted 100-fold into fresh LB medium and grown to an OD_{600} of 0.2 at 37°C. Cells from both growth phases were harvested, washed and resuspended in PBS and 10µl volumes were methanol-fixed onto microscope slides. Each experiment was performed in triplicate, with separate inocula, and images from each experiment were chosen randomly to measure 200 cell lengths for stationary phase and 250 cell lengths for exponential phase.

		mean cell size (µm)	
Strain	Doubling time	Exponential phase	Stationary phase
SL1344	29	2.3 ± 0.45	0.98 ± 0.12
SL1344 *hfq*	30	2.4 ± 0.24	1.32 ± 0.33
SL1344 *rpoS*	29	2.4 ± 0.19	1.07 ± 0.23
MPG 558	36	3.4 ± 0.58	1.05 ± 0.41

Phase contrast images show that the SL1344 *hfq* strain forms minicells at a frequency of around 4% (12 cells in a sample of 300), whilst no minicells were detected in the SL1344 *rpoS* strain. Takada, Wachi and Nagai (1999) observed that their *E. coli hfq* mutant strain formed minicells at a high frequency (6%). This effect was also more pronounced during stationary phase and in poor medium, and was due to an increase in FtsZ levels. This group also examined the effect of *rpoS* mutations on cell morphology and discovered that the minicell phenotype of *E. coli hfq* mutants is RpoS-independent. Figure 3.3.3.1(b) shows that the cell size distribution of the SL1344 *hfq* strain mirrors that of SL1344 during exponential phase, with an average cell length of 2.4µm (compared to 2.3µm for the parent strain). This observation is consistent with that of Ward and Lutkenhaus (1985) who found that increased FtsZ levels result in minicell formation without a dramatic change in cell size distribution, as mid-cell divisions proceed as normal. As expected, the cell distribution of the SL1344 *rpoS* strain mirrors that of its parental strain during exponential phase, as RpoS is not expressed until entry into stationary phase; no minicells were observed. The mean cell size for the SL1344 *rpoS* strain during exponential phase is 2.4µm. Lange and Hengge-Aronis (1991) observed that both wildtype and *E. coli rpoS* strains exhibited similar rod-shaped morphology during exponential growth.

Figure 3.3.3.1(c) shows the distribution of cell size during stationary phase at 37°C. The SL1344 *hfq* strain exhibits a two-fold distribution of sizes, as similarly demonstrated by its parental strain. However, the SL1344 *hfq* strains are elongated compared to the SL1344 strain, with a mean cell size of 1.3µm compared to 0.98µm. This is contrary to what was found during exponential phase, showing that the differences in cell size caused by increased FtsZ levels are more pronounced in stationary phase. This is in agreement with the report by Takada, Wachi and Nagai (1999), who state that the minicell phenotype of *E. coli hfq* mutants was much more apparent in stationary phase cultures. The cell size distribution of the SL1344 *hfq* mutant was narrower than that of the MPG 558 strain during stationary phase, but more widespread during exponential phase, suggesting that the role of Hfq and CspA paralogues in FtsZ regulation may differ. Hfq-binding sites and the RNase E cleavage site are structurally similar and overlap, therefore it is thought that Hfq can protect mRNA from RNase E degradation (Moll *et al*., 2003). CspE binds to and destabilises areas of mRNA rich in secondary structure, possibly preventing recognition by RNase E (Feng *et al*., 2001). On the other hand, one of the major 'sinks' of RNase E activity at 37°C is the 5'UTRs of *cspA* and *cspB* mRNA (Fang, Xia and Inouye, 1999) which is absent in the SL1344 *csp* null strain. The absence of this sink may well impact on septation through altered *ftsQAZ* mRNA processing.

Figure 3.3.3.1(c) shows that, during stationary phase, the cell size of the SL1344 *rpoS* strain exhibits a two-fold distribution, as also seen with its parental strain. In the former, the mean

cell size is 1.07µm compared to 0.98µm of its parent strain. These sizes are not significantly different according to a two-tailed student T-test (p value of 0.115). In contrast to this observation, Loewen and Hengge-Aronis (1994) noted that there is a persistence of elongated cells in *E. coli rpoS* mutants during stationary phase as RpoS is required for induction of *bolA*. There is an increase in average cell length in the SL1344 *hfq* strain compared to its parent strain (1.32µm and 0.98µm respectively) during stationary phase. Previous experiments in *S.* Typhimurium have shown that Hfq is required for the optimal expression of RpoS during stationary phase (Brown and Elliott, 1996). However, this is unlikely to be the cause of the increase in length observed in the SL1344 *hfq* strain as Table 3.3.3.1.1 shows that, despite reports in *E. coli* (Loewen and Hengge-Aronis, 1994), a mutation in *rpoS* has little effect on the mean cell length of SL1344 in stationary phase. Therefore, the increase in cell length of the SL1344 *hfq* strain during stationary phase, and MPG 558 during exponential phase, appears to be RpoS-independent. The minicell phenotype observed in the MPG 558 and SL1344 *hfq* strains also appears to be completely independent of RpoS.

Ward and Lutkenhaus (1985) report that an increase in FtsZ levels between two-fold and ten-fold result in the minicell phenotype, and this matches well with observations for the SL1344 *csp* null and *hfq* mutant strains. However, this group also report that an increase in FtsZ levels of more than twelve-fold will result in a complete inhibition in cell division, as revealed by filamentation. The question may therefore be posed as to what will happen if the *csp* null and *hfq* mutations are combined.

3.3.3.1.2 *An SL1344 csp null hfq mutant has aberrant cell morphology but does not form minicells*

Cell size distribution graphs (Figures 3.3.3.1(b) and (c)) indicate that the minicell phenotype observed in the SL1344 *csp* null strain (MPG 558) may be due to an increase in FtsZ levels, possibly induced by increased cleavage of the *ftsQAZ* transcript by RNase E. However, a minicell phenotype can also be due to a mutation in the *min* operon. Bi and Lutkenhaus (1990) showed that overexpression of FtsZ could increase the total division potential of *minB E. coli* cells so that extra divisions take place at nonpolar sites, resulting in an almost complete return to a wild-type cell length distribution. Increased FtsZ has also been shown to return cell division towards normal in otherwise filamentous *E. coli minC* cells, resulting in normal cell morphology (de Boer, Crossley and Rothfield, 1992). The minicell phenotype of *E. coli hfq* strains is known to be due to an increase in FtsZ levels (Takada, Wachi and Nagai, 1999), therefore can this mutation rescue the *csp* null cell morphology phenotype and return it to normal or does it result in an additive effect on cell division. The *hfq* mutation was transduced into the *csp* null strain as described in Chapter 2.2.1 and mutants, containing multiple deletions, selected by resistance to both kanamycin and chloramphenicol. As described previously, phase contrast images were taken of the strain during both exponential and stationary phase and used for cell size measurements. Figures 3.3.3.1.2(a) and (b) compare the cell size distribution of this strain with the distribution of the MPG 558 and SL1344 *hfq* strains. For phase contrast images of the SL1344 *csp* null *hfq* strain during exponential and stationary phase, see Appendix (Section 3).

From examining the phase contrast images, no minicells were detected in the *csp* null *hfq* mutant strain. Figure 3.3.3.1.2(a) and (b) shows that the cell size distribution of the multiple mutant strain was much broader than that of the SL1344 *hfq* strain but similar to that of MPG 558 under both growth conditions tested, showing that an *hfq* mutation, and the theoretical increase in FtsZ levels, does not return the SL1344 *csp* null mutant to a normal growth phenotype. The mean cell size of the SL1344 *csp* null *hfq* strain is equivalent to that of the MPG 558 strain during both exponential phase (3.3µm compared to 3.4µm) and stationary phase (1.1µm compared to 1.05µm). This would suggest that the minicell phenotype of the MPG 558 is not due to a defective *minCD* system. Ward and Lutkenhaus (1985) report that a 12-fold increase in FtsZ levels results in the inhibition of both minicell and normal divisions and leads to filamentous growth. On examination of phase contrast images of the *csp* null *hfq* mutant strain (Appendix, Figure 3(e)) no minicells were found suggesting that there may be a large increase in FtsZ levels; however, elongation was only observed for a small proportion of the cells (7%) during exponential phase.

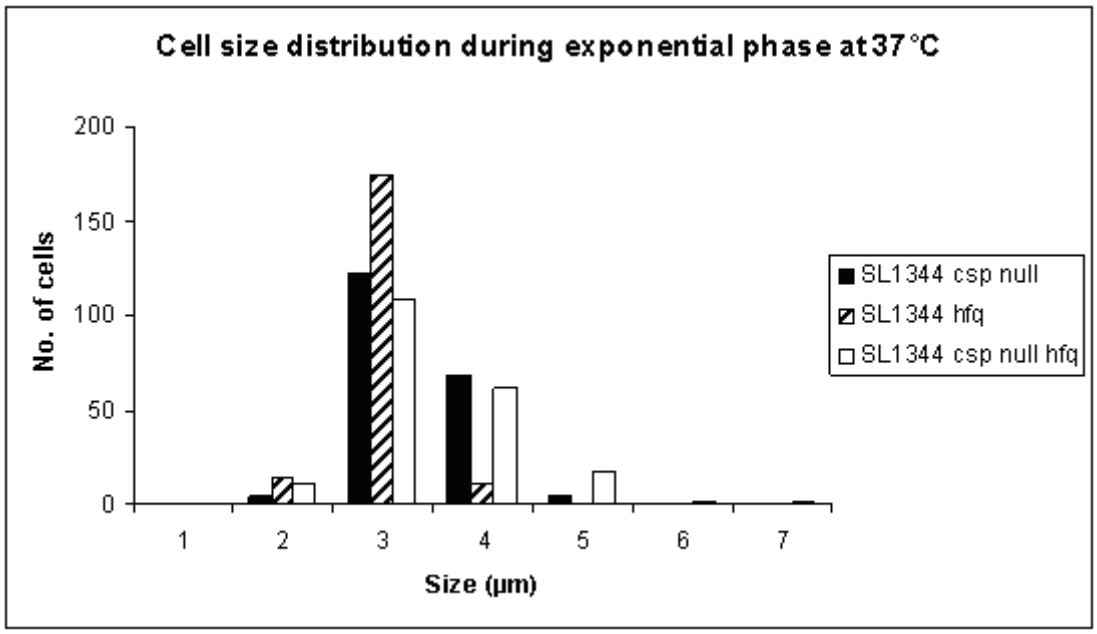


Figure 3.3.3.1.2(a): Cell size distribution of SL1344 *csp* null *hfq* strain compared to the SL1344 *csp* null and SL1344 *hfq* mutants during exponential phase at 37°C. Stationary phase cultures, grown for 16h in LB at 37°C, were diluted 100-fold into fresh LB and grown to an OD_{600} of 0.2. Cells were harvested, washed and resuspended in PBS prior to imaging. 200 cells, randomly chosen from triplicate experiments, were measured as described in Chapter 2.2.4. Cell length measurements are provided in electronic form.

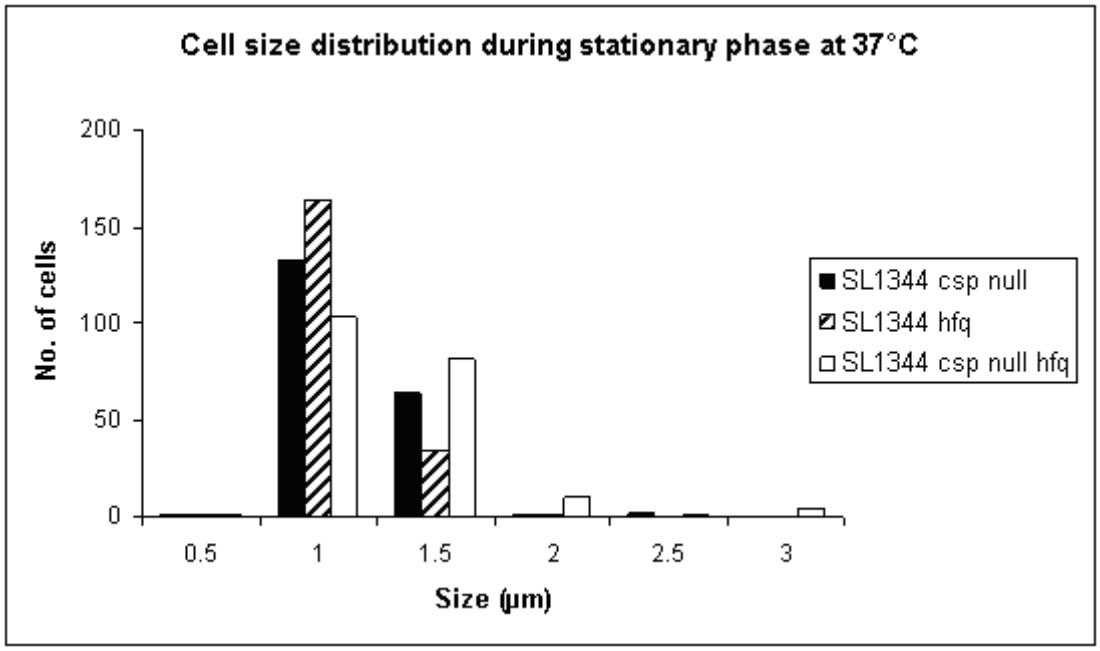


Figure 3.3.3.1.2(b): Cell size distribution of the SL1344 *csp* null *hfq* strain compared to that of the SL1344 *csp* null and SL1344 *hfq* mutants during stationary phase at 37°C. Cells grown to stationary phase, for 16h in LB at 37°C, were harvested and resuspended in PBS prior to imaging. As before, 200 cells taken from triplicate experiments were used for cell length measurements. Measurements are provided in electronic form on the CD accompanying the text.

3.3.3.2 An SL1344 *csp* null mutant elongates at low temperature

Figures 3.3.3.1.1(a) and (b) have shown that the cell morphology of the SL1344 *csp* null mutant (MPG 558) is aberrant at 37°C, possibly due to an increase in FtsZ levels. As this strain is unable to resume cell division at low temperature after cold shock, does looking at its cell morphology at low temperature provide clues to where the division cycle is faulty? To test this, the cell size distribution of the MPG 558 strain was compared to that of its parental strain following cold shock. Phase contrast images were used to measure cell sizes and can be seen in the Appendix (Figure 3f). Cell length measurements are provided in electronic form on the supplementary CD.

Earlier growth curves following cold shock (Figure 3.3.2.1.1(b)) have shown that SL1344 has already adapted to low temperatures and resumed growth 5h following cold shock. However, MPG 558 is unable to grow at this time point. Figure 3.3.3.2.1 compares the cell size distribution of the *csp* null strain and its parent 5h following cold shock at 10°C. This graph shows that SL1344 has a two-fold distribution which demonstrates 'normal' growth, with a mean cell length of 2.24µm. This size corresponds to that of its mean cell length during exponential growth at 37°C (2.3µm), as shown in Table 3.3.3.1.1. However, the size distribution of the SL1344 *csp* null strain is dramatically more widespread, with a mean cell size of 4.5µm. The mean cell size of MPG 558 cells during exponential phase at 37°C is 3.4µm. The MPG 558 strain elongates upon cold shock to around 1.5 times the mean cell length that of its parent strain under the same conditions. MPG 558 cells also elongate to 1.3 times their own mean cell length prior to cold shock and this elongation is accompanied by a 1.5-fold increase in cell mass within the same period (from OD_{600} of 0.23 to 0.34 as shown in Figure 3.3.2.1.1(b)). This suggests that the accumulation of cell mass is not grossly affected upon cold shock, but that the final invagination and cytokinesis phase is faulty. FtsZ causes the invagination and ingrowth of the peptidoglycan cell wall. How can this be so when MPG 558 forms minicells at 37°C, a condition which is likely due to increased FtsZ levels? What is the reason for the inability of the SL1344 *csp* null strain to divide and multiply after cold shock?

3.3.3.2.1 *Elongation at low temperature is overcome by overexpression of FtsQ, FtsA and FtsZ*

A lack of functional FtsZ leads to the formation of nonseptate filaments in *E. coli* (de Boer, Crossley and Rothfield, 1992). On average, the SL1344 *csp* null strain elongates by 50% following cold shock. Ward and Lutkenhaus report that pZAQ causes a sevenfold increase in FtsZ and leads to minicell formation in *E. coli* and *Salmonella* species (1985). Figure 3.3.3.2.1 shows that the over-expression of FtsQ, FtsA and FtsZ in the MPG 558 strain prior to cold shock, allows cell division and septation to occur in the time frame examined after cold shock, and returns cell morphology towards a 'normal' cell size distribution, i.e. that of its parent. However, tracking the OD and cfu/ml of the SL1344 *csp* null pZAQ strain (results not shown) shows that although the strain is able to divide (2-fold increase in OD accompanied by a doubling in cfu/ml at 5h after cold shock) it cannot multiply (no further increase in cfu/ml), i.e. initiate a new division cycle. Therefore, invagination of the peptidoglycan wall and cytokinesis appears to be the first step of the division cycle inhibited in the MPG 558 strain following cold shock. The pZAQ plasmid has no effect on the cell size distribution of the MPG 558 strain during exponential phase at 37°C prior to cold shock (mean cell length of 3.5µm compared to 3.4µm; Appendix, Figure 3g).

It has already been shown that the MPG 558 strain can grow if the temperature is gradually reduced to 10°C (Figures 3.2.2.2(a) and (b)), therefore the CspA paralogues are required for overcoming the initial ribosome 'jamming effect' that occurs following cold shock and not for long-term adaptation to low temperatures. RNA secondary structure is stabilised in response to temperature reduction (Narberhaus, Waldminghaus and Chowdhury, 2006). Most secondary structure is located at the 5'UTR region of mRNA and can mask ribosome-binding sites (Chowdhury *et al*., 2003). Melting of the RNA secondary structure by RNA chaperones, such as the CspA paralogues, allows ribosome access and therefore translation initiation (Narberhaus, Waldminghaus and Chowdhury, 2006).

However, a further problem may be that ribosomes are not fully mature in the *csp* null mutant. Evidence from the longer lag phase observed with this strain at 37°C compared to the parent (Table 3.3.3.1), suggests that ribosomes may take longer to form and mature even at optimal temperature. Ribosomes must be reconstituted, from inactive 70S dimers, during the lag phase before protein synthesis can proceed. The rate-limiting factor in ribosome reconstitution is the melting of stable inactive RNA by RNA chaperones and helicases as ribosomal RNA is structurally promiscuous and needs only 50% complementarity to form double helices (Kaczanowska and Ryden-Aulin, 2007). The secondary structure of ribosomal

RNA will be further stabilised at low temperature. At low temperatures, RbfA is bound to the ribosomal 30S subunit and enhances the capability of ribosomes for translation, thus facilitating the whole system of cell response to cold shock (Golovlev, 2003). *rbfA* is part of the *metY-rpsO* operon and, in *E. coli*, its cold shock induction is dependent on transcription antitermination mediated by CspA, CspC and CspE (Bae *et al*., 2000).

Together these results suggest that not only are the CspA paralogues required to destabilise the secondary structure formed in mRNA at low temperatures to allow ribosome access but may also be required for ribosome assembly at both low and optimal temperature.

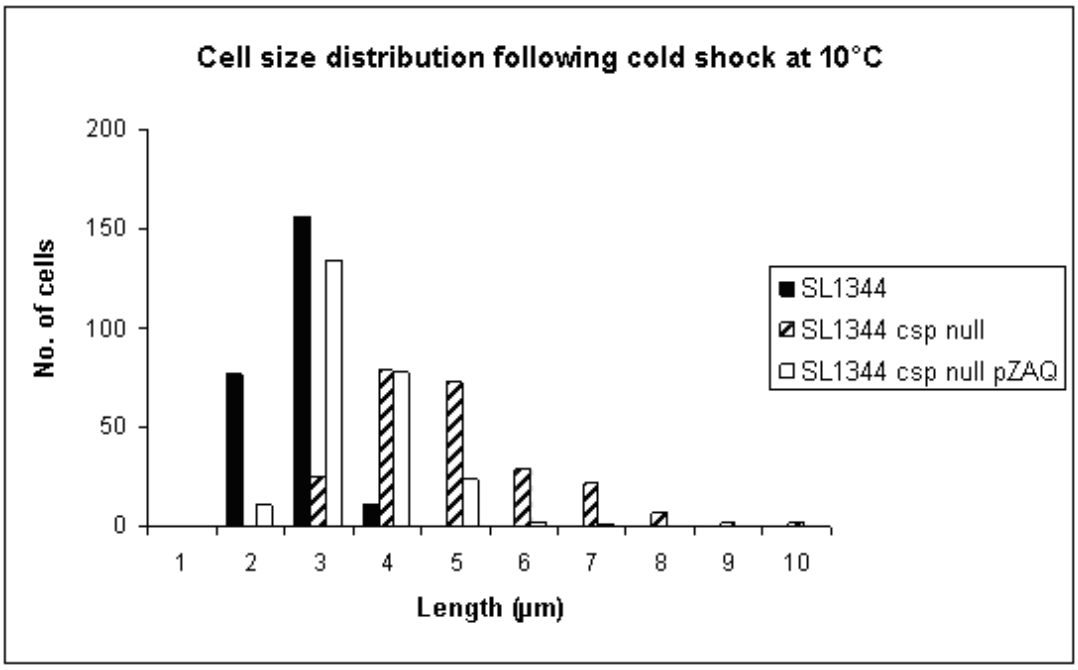


Figure 3.3.3.2.1: Cell size distribution of SL1344 *csp* null strain (with/ without overexpression of FtsZ from pZAQ plasmid) compared to its parent strain 5h following cold shock at 10°C. Stationary phase cultures, grown in LB for 16h at 37°C, were diluted 100-fold into fresh LB medium and grown to an OD_{600} of 0.2 at 37°C. Samples were then incubated at 10°C for 5h in a shaking refrigerated incubator. Cells were harvested, washed and resuspended in PBS and 10µl volumes fixed to microscope slides using ice-cold methanol. 250 cells, randomly chosen from triplicate experiments, were imaged and measured as described in Chapter 2.2.4. Cell length measurements are provided in electronic form, on the CD supplementary to the text.

3.3.3.3 An SL1344 *csp* null mutant has impaired motility but this is independent of RpoS

The motility and chemotaxis of bacteria allow cells to find nutrients, associate with surfaces or move away from stressful environments. Motility and the expression, synthesis, assembly and function of flagella require the expression of more than 50 genes, constituting a large and co-ordinately regulated flagellar regulon. Transcription of most of these genes is under the control of the RNA polymerase holoenzyme RpoF (Makinoshima *et al*., 2003). In stationary phase, there is a decrease in the number of flagella per cell and this correlates with the decrease in the level of RpoF, which occurs in parallel with the increase in RpoS (Jishage *et al*., 1996). The expression of FliC, a phase 2 flagellin protein, was strongly reduced in an *S.* Typhimurium *hfq* mutant and resulted in impaired motility (Sittka *et al*., 2007). FliC is the most prominent protein secreted into the culture medium by *S.* Typhimurium (Komoriya *et al*., 1999). Can the phenotype of an *hfq* mutant be exploited to further dissect the interaction between Hfq and CspA paralogues in RpoS expression? Is motility dependent on RpoS expression and does the SL1344 *csp* null mutant have altered motility? To test this, motility assays were carried out paralleling the experiment performed by Sittka *et al*. (2007), as described in Chapter 2.1.3. The motility of each of the mutant strains was compared to that of their isogenic parent strain at 37°C.

Figure 3.3.3.3 first compares the motility of the SL1344 *hfq* mutant with that of its parent at 37°C. The experiment shows that SL1344 cells are motile and formed concentric rings of 36 ± 3mm around the point of inoculation. In contrast, the SL1344 *hfq* null mutant displayed impaired motility as judged by the smaller motility ring of 21 ± 2mm formed from the point of inoculation. This is consistent with what has previously been reported for *Salmonella* and *E. coli hfq* mutants (Sittka *et al*., 2007; Kuleus *et al*., 2008). This figure also shows that the motility of SL1344 is unaffected by a mutation in *rpoS*, as the SL1344 *rpoS* strain forms a similar-sized concentric ring around the point of inoculation (32 ± 4mm) to SL1344. The motility assay demonstrates that the SL1344 *csp* null strain also has impaired motility as there is greatly reduced outward migration from the point of inoculation (18 ± 1mm). As mentioned above, the reason for reduced motility in the SL1344 *hfq* strain is that Hfq is required for the expression of FliC, an important flagellin protein, essential for motility (Sittka *et al*., 2007). Microarray studies of CspC and CspE deletion mutants in *E. coli* have shown that the Csp proteins are involved in RpoS-independent expression of the flagellar proteins MotB, FlhA and FlhB (Phadtare *et al*., 2006). In *E. coli*, MotB links the flagellar machinery to the cell wall, thus enabling flagellar motor rotation. Temperature sensitive *E. coli motB* mutants are non-motile under the non-permissive temperature as, although the flagella can assemble, flagellar filament structures cannot rotate (Berg, 2003). FlhA and FlhB are essential flagellar

biosynthesis proteins in *Salmonella*, necessary for the export of flagellar subunits across the cytoplasmic membrane for assembly (Minamino and MacNab, 2000). This suggests that although both the CspA paralogues and Hfq are involved in motility, they may play different RpoS-independent roles in the pathway.

Wet mounts were examined by phase contrast microscopy to confirm these motility results. SL1344 and SL1344 *rpoS* strains were motile and exhibited a 'run and tumble' motion. However, the SL1344 *csp* null and *hfq* strains showed impaired motility and exhibited mainly 'twitching motility'. The SL1344 *csp* null has previously been shown to have reduced swarming and swimming ability at 37°C (I.W. Hutchinson, PhD submitted to U of E, 2005).

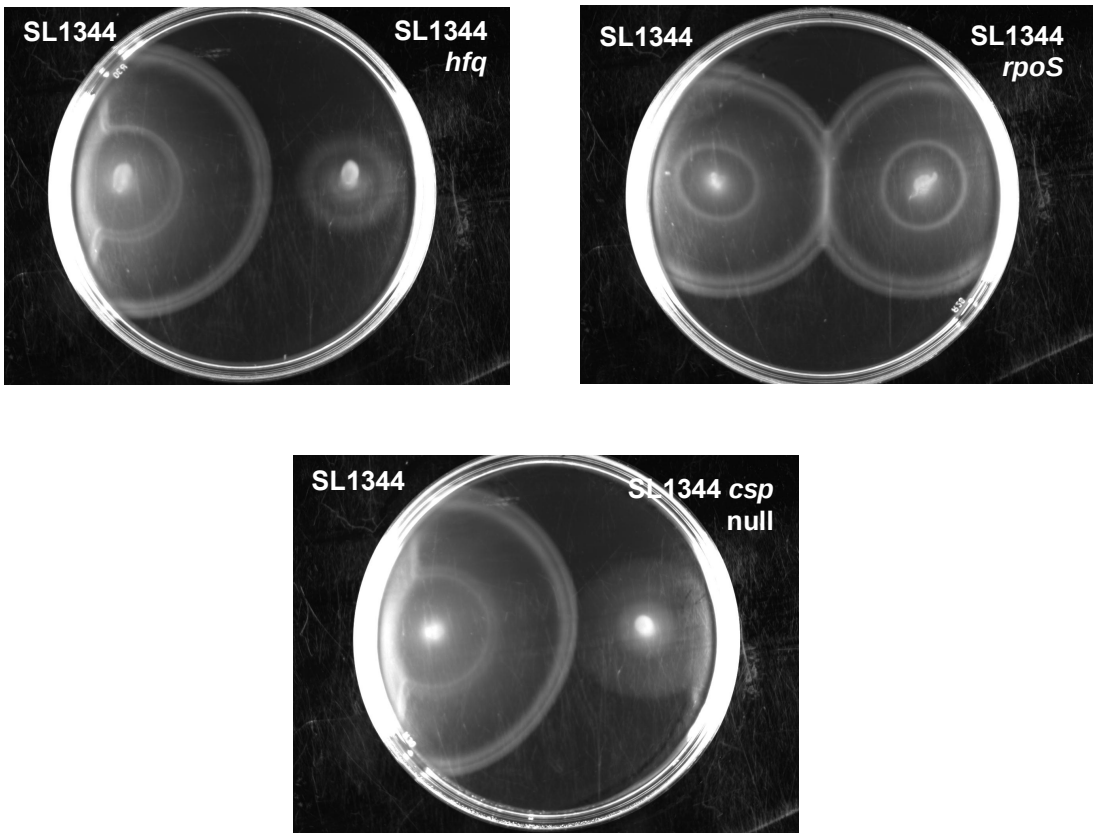


Figure 3.3.3.3: Motility assays comparing the motility of SL1344 *csp* null, *hfq* and *rpoS* mutants with the motility of their parental strain. 1µl volumes of stationary phase cultures, grown for 16h in LB at 37°C, were used to inoculate motility plates. The cfu/ml of each strain was measured and found to be equal prior to inoculation (around 3 X 10^9 cfu/ml). Plates were incubated at 37°C for 4h. The diameter of the motility ring, from the point of inoculation, was measured for each strain. These images are representative of experiments performed in triplicate with separate inocula. An equal volume (20ml) of agar was added to each petri-dish. Experiment as described in Chapter 2.1.3.

3.4 CspC or CspE is required for both RpoS expression and peroxide resistance during stationary phase

Resistance to oxidative stress is important in the ability of *Salmonella* to withstand oxygen-dependent killing mechanisms in phagocytic cells. Previous studies have shown that *Salmonella rpoS* mutants are highly susceptible to hydrogen peroxide treatment during stationary phase due to reduced catalase, and in particular *katE*, activity (Fang *et al.*, 1992). Are CspA paralogues or Hfq involved in RpoS-mediated resistance to oxidative stress?

3.4.1 Stationary phase resistance to oxidative damage correlates with RpoS levels

Previous studies in our lab (I.W. Hutchinson, PhD submitted to U of E, 2005) have shown that CspA paralogues differ in their ability to convey resistance to oxidative stress (Appendix, Figure 3(h)). This data shows that 80% of stationary phase SL1344 cells survived exposure to 10mM H_2O_2 for 1h whilst less than 1% of SL1344 *csp* null (MPG 558) cells survived this treatment. Monogene strains CspC and CspE were equally as resistant to the peroxide stress as their parent strain. The other monogene strains exhibited intermediate phenotypes. Does this resistance correlate with RpoS expression during stationary phase? Figure 3.4.1 shows the amount of RpoS detected from each strain during stationary phase, when grown in LB, at 37°C before peroxide treatment. The blot showed RpoS expression in the SL1344 strain whilst little or no RpoS was detected in MPG 558. Also, it is clear that the CspC and CspE monogene strains were able to express RpoS to a detectable level and it was these strains that survived the peroxide treatment to a level similar to that of the parental strain. There is little or no RpoS detected from the peroxide sensitive strains. These results show that CspA paralogue-mediated RpoS expression correlates with resistance to peroxide stress during stationary phase in *S.* Typhimurium. As mentioned previously, Bae *et al*. (1999) have shown that CspC and CspE are constitutively expressed at 37°C in *E. coli* and proteomic studies in our lab have shown that the same is true for *S.* Typhimurium (Sienkiewicz *et al*., FSA project report B01008, 2001). Phadtare and Inouye (2001) have shown that CspC and CspE are involved in the regulation of RpoS in *E. coli*.

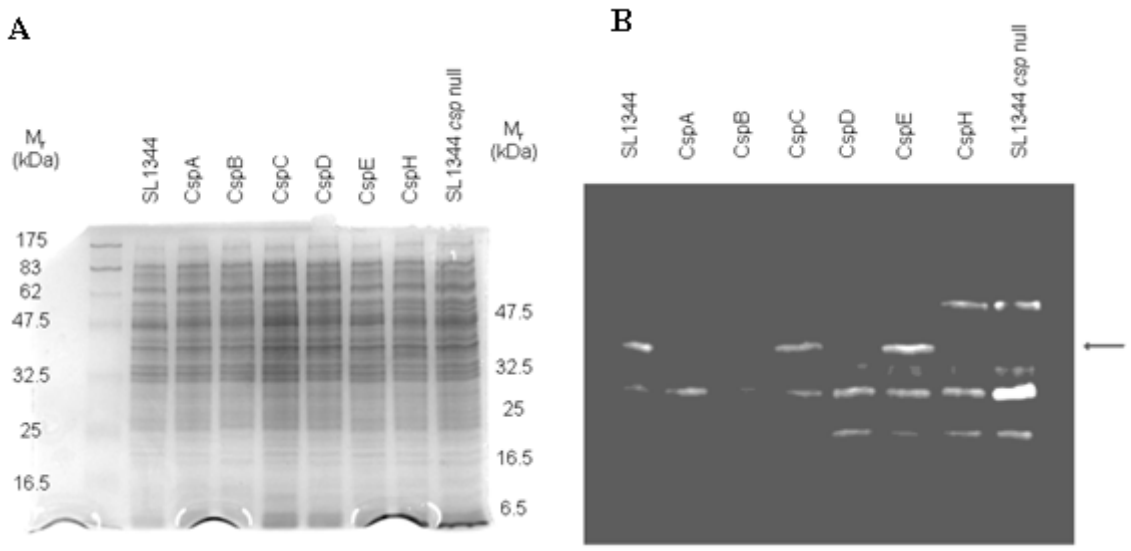


Figure 3.4.1: **Western blot comparing levels of RpoS detected in the SL1344 *csp* null strain, *csp* monogene mutants and their isogenic parent strain on entry into stationary phase at 37°C.** Immunoblotting was carried out as previously described in Section 2.2.2. Cells were grown aerobically to stationary phase (16h) in LB at 37°C and harvested by centrifugation. **B** The correct band corresponding to RpoS (38kDa) is denoted (⟶). The positions of the molecular weight markers (M_r) are shown. **A** Coomassie blue stained gel is shown to demonstrate equal loading of samples. The blot shown is representative of triplicate experiments performed.

3.4.2 CspA paralogues only partially regulate RpoS-mediated peroxide resistance

CspA paralogues are involved in RpoS expression and this correlates with resistance to peroxide stress during stationary phase. Does this mean that the SL1344 *csp* null strain is as sensitive to hydrogen peroxide as the SL1344 *rpoS* strain? To test this, peroxide sensitivity tests were carried out paralleling previous lab experiments. 20mM H_2O_2 was added to 1ml volumes of stationary phase cultures (grown aerobically at 37°C in LB), vortexed and incubated statically for 1h at 37°C. Serial dilutions were then plated on agar and the cfu/ml was calculated and compared to the cfu/ml before treatment.

Figure 3.4.2(a) confirms results from previous experiments showing that around 80% of the SL1344 cells resisted exposure to peroxide, whilst the resistance of the SL1344 *csp* null is reduced by 2-$\log_{10}$. Interestingly, the resistance of the SL1344 *rpoS* strain exhibited a further tenfold reduction. This suggests that the CspA paralogues of *S.* Typhimurium only partially regulates RpoS expression and peroxide resistance during stationary phase, and therefore other factors must be involved.

To address whether the stationary phase peroxide sensitivity of the SL1344 *csp* null strain was entirely RpoS-dependent, complementation studies were performed, using a plasmid to over-express RpoS. The plasmid, pUBAD::*rpoS*, containing the *rpoS* gene under the control of an arabinose-inducible promoter, was introduced to the SL1344 *csp* null strain. As before, cultures were grown aerobically to stationary phase (16h) in LB at 37°C. To ensure over-expression of RpoS, 2% arabinose was added to the growth medium prior to inoculation, as *S.* Typhimurium is able to metabolise arabinose. Following this, 20mM H_2O_2 was added to each culture and samples were incubated statically for 1h at 37°C. The cfu/ml after treatment was then measured and compared to the cfu/ml prior to peroxide exposure, allowing a % survival to be calculated. Figure 3.4.2(b) shows that the sensitivity of the SL1344 *csp* null strain to peroxide is completely abolished by over-expression of RpoS, highlighting the importance of the CspA paralogues in mediating RpoS-dependent peroxide resistance in stationary phase.

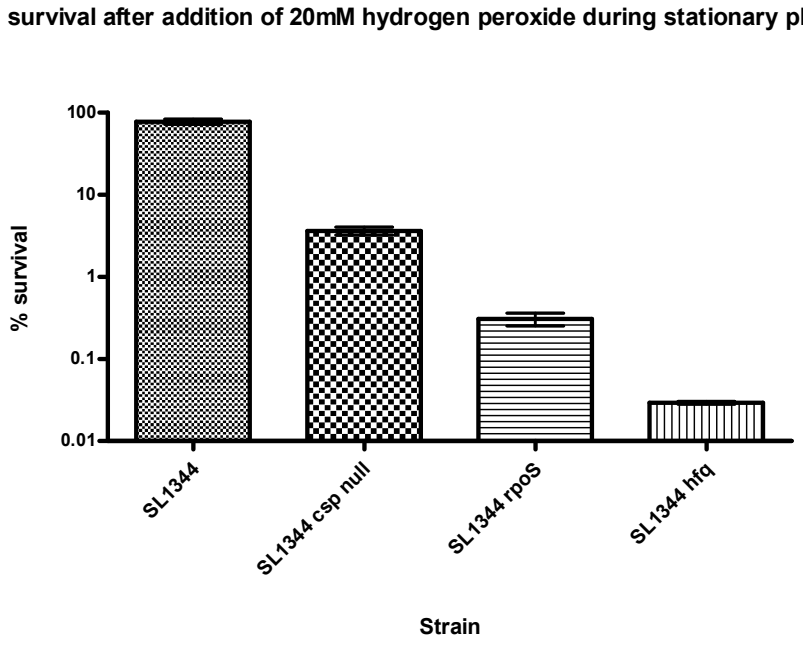


Figure 3.4.2(a): Graph comparing the % survival of SL1344 *csp* null, *hfq* and *rpoS* strains with their isogenic parent after 1h exposure to 20mM H_2O_2 during stationary phase at 37°C. Cells were grown aerobically to stationary phase (16h) in LB at 37°C then 20mM H_2O_2 added to each culture prior to static incubation for 1h at 37°C. Cells were then serially diluted in PBS and 100µl volumes plated on LB agar. After overnight incubation at 37°C, the cfu/ml was calculated. The cfu/ml of stationary phase cultures prior to challenge with H_2O_2 was calculated for each strain and used to represent 100% survival. Experiments were performed in triplicate, from separate inocula, and the average result used for plotting the graph. Error bars are shown.

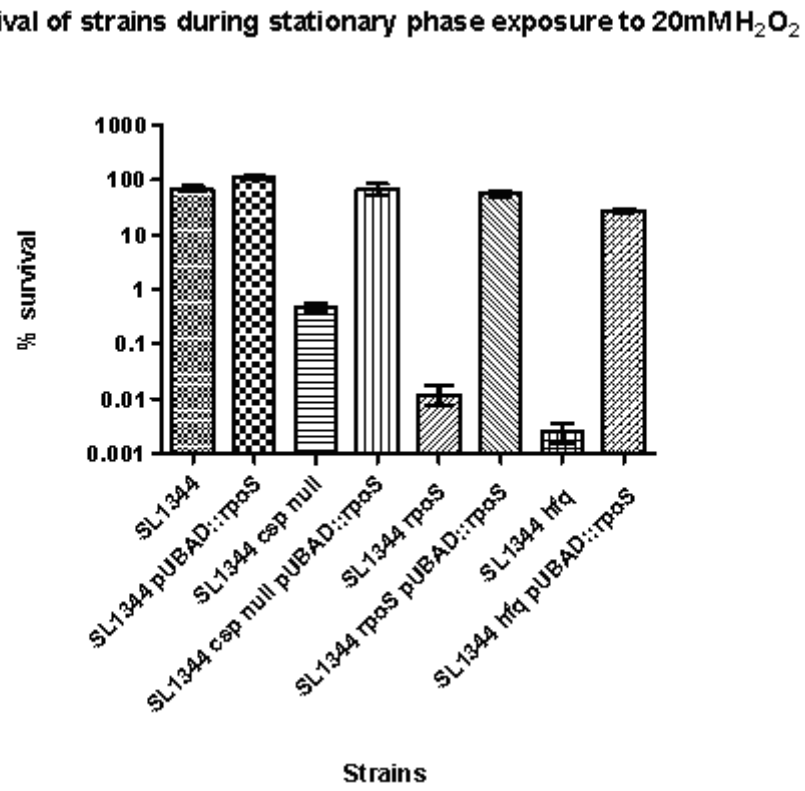


Figure 3.4.2(b): Graph showing the complementation of peroxide sensitivity of each strain by overexpression of RpoS from the pUBAD::*rpoS* plasmid. Cells were grown aerobically to stationary phase (16h) at 37°C in LB containing 2% arabinose, to induce RpoS expression. 20mM H_2O_2 was added to each culture and samples were incubated statically for 1h at 37°C. Cells were then serially diluted in PBS and 100µl volumes plated on LB agar. After overnight incubation at 37°C, the cfu/ml was calculated. The cfu/ml of stationary phase cultures prior to challenge with H_2O_2 was calculated for each strain and used to represent 100% survival. Experiments were performed in triplicate, starting from separate inocula, and the average survival was plotted on the graph. Error bars are shown and indicate reproducibility.

3.4.2.1 Hfq-dependent RpoS expression correlates with stationary phase peroxide resistance

Although RpoS expression correlates with the pattern of resistance of Csp monogene strains to peroxide, CspA paralogues have been shown to only partially regulate RpoS-mediated peroxide resistance. The SL1344 *csp* null strain showed an intermediate phenotype compared to that of the *rpoS* mutant strain and their parent strain. The question therefore arose as to whether Hfq is involved in RpoS expression during stationary phase and whether Hfq is responsible for RpoS-mediated peroxide resistance?

To test the involvement of Hfq in RpoS expression during stationary phase, Western blots were carried out as described previously (Chapter 2.2.2). Figure 3.4.2.1 shows an immunoblot

comparing the amount of RpoS detected from the SL1344 *hfq* strain to that of the *csp* null and their isogenic parental strain. This blot correlates with previous findings, as a clear RpoS band was detected from the parent strain, whilst a faint band was detected from the *csp* null strain. The blot also showed that little RpoS was detected in the SL1344 *hfq* mutant strain; therefore Hfq is required for optimal RpoS expression during stationary phase in *S.* Typhimurium. This agrees with previous studies by Brown and Elliott (1996) who, using *rpoS::lacZ* protein fusions in *S*. Typhimurium strain ATCC 14028s, showed a four- to seven-fold reduction in expression of *rpoS* in an *hfq* mutant. This can be attributed primarily to a defect in translation, as their pulse-labeling studies have shown that the rate of RpoS synthesis is significantly decreased in an *hfq* mutant. However, does a mutation in *hfq* render the cell equally as susceptible to peroxide stress as the SL1344 *rpoS* mutant?

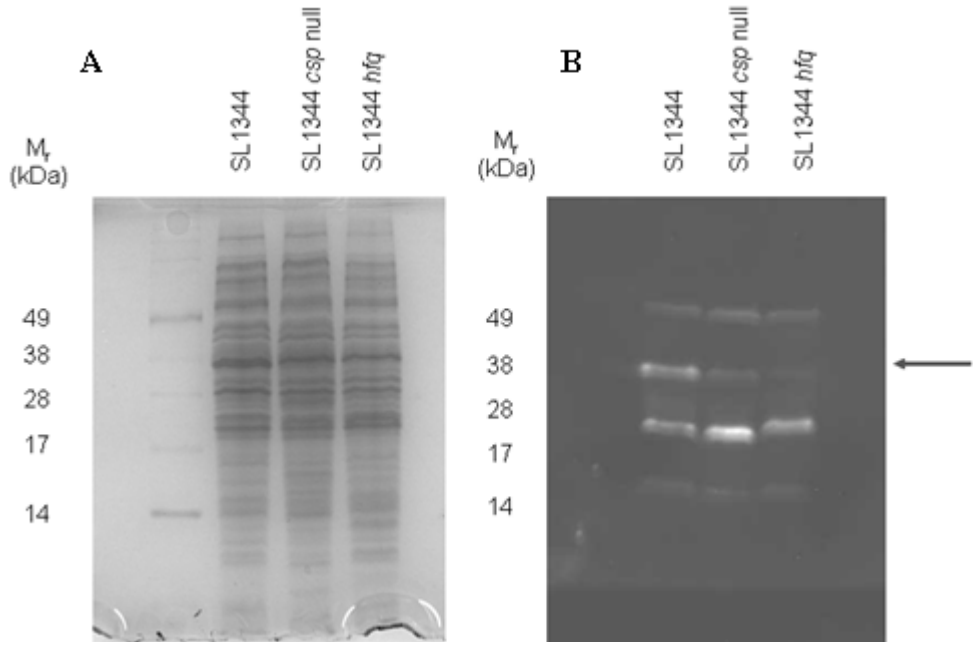


Figure 3.4.2.1: Western blot comparing levels of RpoS detected in the SL1344 *csp* null strain, the SL1344 *hfq* strain and their isogenic parent strain on entry into stationary phase at 37°C. Cells were grown, aerobically in LB, to stationary phase (16h) at 37°C and harvested by centrifugation. Immunoblotting was performed as described previously in Chapter 2.2.2. **B** The band corresponding to RpoS is denoted (⟶). The positions of the molecular weight markers (M_r) are shown. **A** Coomassie blue stained gel is shown to demonstrate equal loading of samples. The experiment was repeated in triplicate and the blot shown is representative of all results.

Figure 3.4.2(a) shows the susceptibility of the SL1344 *hfq* strain to exposure to 20mM H_2O_2 for 1h during stationary phase, compared to that of the SL1344 *rpoS* mutant. Less than 1% of SL1344 *rpoS* cells were found to be resistant to the peroxide, whilst approximately 5% of cells survived for the SL1344 *csp* null strain. However, the SL1344 *hfq* was found to be more susceptible to peroxide stress than the SL1344 *csp* null strain, therefore Hfq and the CspA paralogues do not play equivalent roles in peroxide resistance. This correlates with a previous report of Brown and Elliott (1996) who found that the level of *katE::lacZ* fusion activity detected in an *hfq* mutant background, in stationary phase, was about 25% of that observed in the parent strain. Muffler *et al* (1997(ii)) have shown that *E. coli hfq* mutants have impaired resistance to H_2O_2 during stationary phase but this was less dramatic than observed for *rpoS* mutants. This is contrary to what was found here for *S.* Typhimurium as the *hfq* mutant is a tenfold more susceptible to peroxide stress than the *rpoS* mutant strain. An *E. coli hfq* mutant enhances expression of outer membrane proteins, FepA and FhuE, which participate in transporting iron ions into the cell. As a result, iron is accumulated, hydroxyl radicals may be produced, and cell sensitivity to hydrogen peroxide increases (Vassilieva and Garber, 2002). This shows that susceptibility of an *E. coli hfq* mutant to oxidative damage is not solely RpoS-dependent. This also appears to be the case for the SL1344 *hfq* strain, as over-expression of RpoS does not completely restore resistance to peroxide stress, i.e. only 75% survival is achieved (Figure 3.4.2(b)).

3.5 Discussion

In light of previous observations (Muffler *et al*., 1997(ii); Sittka *et al*., 2007) indicating that many of the pleiotropic phenotypes observed for *Salmonella* and *E. coli hfq* mutants could be attributed to RpoS expression, comparative phenotypic tests were carried out in strains carrying both of these mutations. As the CspA paralogues of *E. coli* have been shown to control RpoS expression, a SL1344 *csp* null strain was included in the phenotypic tests to identify whether observed phenotypes were RpoS-dependent or independent. The aim was to give further insight into the interactions of Hfq and the CspA paralogues with respect to RpoS expression. Accordingly, in the assays both significant phenotypic overlaps and phenotypes unique to certain mutant strains were observed. These are summarised in Figure 3.5.

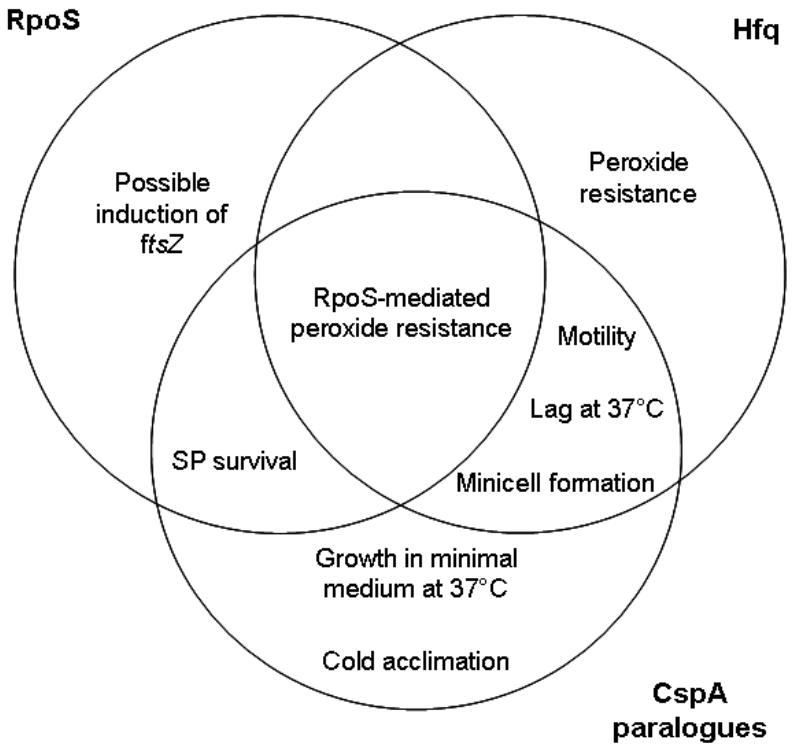


Figure 3.5: Summary of overlapping and distinct phenotypes, attributable to each of the proteins, which have been discovered in this study.

A common role has been identified for RpoS, Hfq and the CspA paralogues in peroxide resistance during stationary phase. Immunoblots have shown that both Hfq and the CspA paralogues are required for optimal RpoS expression during stationary phase. However, there seems to be a further RpoS-independent role for Hfq in peroxide resistance. Studies in *E. coli* have shown that this is due to the overexpression of outer membrane proteins (FepA and FhuE) that transport iron ions into the cell, leading to iron accumulation within the cell (Vassilieva and Garber, 2002). This can cause hydroxyl ion production that would render the cell more susceptible to peroxide stress. Gene expression analysis or immunoblotting could be used to confirm whether these genes or proteins were also up-regulated in the SL1344 *hfq* strain or whether an alternative explanation may exist.

Growth curves have shown that the increased lag phase observed in the SL1344 *csp* null mutant at 37°C, and its inability to grow after cold shock to 10°C in exponential phase, are not dependent on RpoS. The growth curves of the SL1344 *rpoS* strain resembled those of its parent strain under both of the above conditions. Yet, Western blots of the SL1344 *csp* monogene strains, both at 37°C and after exponential cold shock, have shown a correlation between RpoS expression and those strains exhibiting wildtype-like growth phenotypes (CspE and CspC during stationary phase; CspA and CspE during cold shock). These growth phenotypes were also distinct from the SL1344 *hfq* strain. This shows a role for CspA paralogues, in adapting the cell for growth, independent of both RpoS and Hfq. Measurements of cell length have determined that MPG 558 cells elongate to around 1.5 times the length of SL1344 cells upon cold shock in exponential phase. Therefore, the CspA paralogues are not required for the accumulation of cell mass under these conditions. However, under the same conditions, the MPG 558 strain is unable to undergo septation and cytokinesis and begin a new division cycle. The CspA paralogues appear not to be required for the acclimation period that follows cold shock, as the SL1344 *csp* null mutant can grow at 10°C if the temperature is gradually reduced (by 1°C or 2°C/min). This aligns with an observation by Jones and Inouye (1994) that the magnitude of *E. coli* Csp induction is dependent on the range of temperature shift; the larger the temperature shift, the more pronounced the response. These results support the view that the major role of the CspA paralogues during cold shock is to overcome the initial translational blockage, caused by aberrant stabilisation of mRNA secondary structure, and that these proteins are not required during growth at the new low temperature.

Two RpoS-independent phenotypes were shared by the SL1344 *csp* null and the SL1344 *hfq* strains. The minicell phenotype of these strains, observed when cells were grown in LB with aeration at 37°C, is independent of RpoS and may be caused by an increase in FtsZ levels. Examination of FtsZ levels in the MPG 558 strain compared to that of its parental strain

would confirm whether this is in fact the case. The minicell phenotype has been observed previously in *E. coli hfq* mutants and is known to reflect increased RNase E activity, leading to additional cleavage of the *ftsQAZ* transcript and the production of more functional FtsZ protein (Takada, Wachi and Nagai (1999)). For the MPG 558 strain, it can be hypothesised that increased RNase E activity could result from the absence of *cspA* and *cspB* mRNA, which are known RNase E 'sinks' at 37°C (Fang, Xia and Inouye, 1999). This question could be addressed by examining the cell morphology of the MPG 558 strain following the overexpression of supplementary RNase E targets, such as 6S RNA, *ompA* mRNA or the *papAB* primary transcript.

Motility assays have highlighted RpoS-independent roles for Hfq and the CspA paralogues, suggesting another common role for these RNA chaperones outwith RpoS expression. Hfq has been shown to regulate FliC expression, a flagellin protein essential for motility, in *S.* Typhimurium (Sittka *et al*., 2007). As yet, no link has been reported between CspA paralogues and FliC regulation. However, microarray studies in *E. coli* have shown that CspC and CspE regulate the expression of MotB, FlhA and FlhB, proteins essential for the assembly and export of flagella (Phadtare *et al*., 2006). Immunoblots could be used to confirm whether these proteins were down-regulated in the MPG 558 strain. In addition, motility assays using the SL1344 *csp* monogene strains would determine which of the CspA paralogues were responsible for cell motility in SL1344. These results suggest that, although both components are required for optimal cell motility, Hfq and the CspA paralogues may have distinct roles in this process.

This study has presented phenotypic data suggesting that the CspA paralogues of *S.* Typhimurium are functionally required for both efficient cold and oxidative stress adaptation responses. This has significant implications in view of practical food microbial control techniques. The combined or sequential exposure of *S.* Typhimurium cells to both refrigeration and food preservatives may induce cross-protection responses, causing antimicrobial treatments to be less effective.

This study has identified clear, distinct phenotypes that can be exploited for each of the SL1344 *hfq*, *csp* null and *rpoS* mutants. This study has also shown that CspA paralogues and Hfq are functionally distinct, not only in their involvement in RpoS expression, but also in RpoS-independent processes, such as cold acclimation, motility and to some extent, growth at 37°C. However, the particular roles of these proteins in RpoS expression is no better understood. This problem can be assessed at the molecular level using Northern analysis and use of *rpoS::lacZ* transcriptional and translational fusions. RpoS protein stability can also be examined.

Chapter 4

Dissecting the role of CspA paralogues and Hfq in RpoS expression

4.1 Background

As described previously, the main aim of this study is to characterise the CspA paralogues of *S.* Typhimurium and elucidate their function in RpoS-mediated stress responses. Phadtare and Inouye (2001) have shown that the overexpression of CspC and CspE, in *E. coli*, greatly increases the amount and stability of *rpoS* mRNA and consequently, the level of RpoS itself. Previous results presented in this study (Figures 3.3.2.1 and 3.4.2.1) have shown that the MPG 558 (SL1344 *csp* null) strain is deficient in RpoS expression during stationary phase at 37°C and after cold shock in exponential phase. Figure 3.4.2.1 has also shown that an SL1344 *hfq* mutant is deficient in RpoS expression during stationary phase at 37°C.

Studies using *rpoS::lacZ* protein fusions in *S.* Typhimurium have shown that an *hfq* mutant has a four- to sevenfold reduction in expression of *rpoS*, which is attributed primarily to a defect in translation as pulse-labeling studies have shown that the rate of RpoS synthesis is significantly decreased (Brown and Elliott, 1996). In *E. coli*, it has been shown that the sRNAs, DsrA, RprA and OxyS bind to Hfq and use RNA base pairing interactions to regulate the expression of their target mRNAs, in this case RpoS (Wassarman, 2002; Masse *et al*., 2003). Lease and Woodson (2004) have shown that Hfq has only a modest effect on the rate of base-pairing between *E. coli* DsrA and *rpoS* mRNA. This raises the question of whether other factors contribute to this interaction *in vivo*. This idea is supported by Brescia *et al*. (2003) who report that not all RNA helices bind Hfq with equal affinity. Therefore, it seems likely that higher-order structures may be involved in these interactions. Phadtare, Inouye and Severinov (2002) have demonstrated that CspE is a transcription anti-terminator and Giangrossi *et al*. (2001) have shown that *E. coli* CspD is associated with the nucleoid. As CspA paralogues have the ability to bind DNA at least *in vitro*, they could be acting at the transcriptional level to influence *rpoS* mRNA production. Equally, not much is known about CspA paralogue protein-protein interactions therefore the question arises, could these proteins be involved in stabilizing RpoS against degradation by the ClpX/P protease?

The previous chapter was concerned with the comparative phenotypic analysis of the SL1344 *csp* null, *hfq* and *rpoS* mutants to provide further insight into the interactions of Hfq and the CspA paralogues with respect to RpoS expression. These tests demonstrated both significant phenotypic overlaps and phenotypes unique to individual mutant strains. Phenotypic analysis did prove useful in showing that the CspA paralogues and Hfq are functionally distinct with regards to cold acclimation, motility and to some extent, growth at 37°C, but their individual

roles in relation to RpoS expression are no further elucidated. Therefore, this must be assessed at the molecular level.

4.2 2 distinct pathways for the post-transcriptional control of RpoS expression

As discussed previously, results in Chapter 3 have shown that MPG 558 is deficient in RpoS expression during stationary phase at 37°C and after cold shock in exponential phase. However, this does not give any clues to the precise role of CspA paralogues in RpoS regulation. To investigate this, the production of *rpoS* mRNA, and the role of DsrA and RprA in its translation, will be examined in MPG 558. As results in Chapter 3 have shown that the SL1344 *hfq* mutant is also deficient in RpoS expression in stationary phase, the effect of Hfq on RNA levels will also examined. Also, the question arises of whether RpoS regulation is the same during both stationary phase and cold shock, or are there distinct pathways for expression under different conditions?

4.2.1 CspA paralogues and Hfq are required for RpoS expression during stationary phase at 37°C but do not function at the transcriptional level

RpoS is expressed on entry into stationary phase, in *E. coli* and *S.* Typhimurium, and is responsible for long term adaptation to nutrient starvation (Taylor-Robinson *et al*., 2003; Hengge-Aronis, 1993). Previously, Figure 3.4.2.1 has shown that both MPG 558 and the SL1344 *hfq* strain are deficient in RpoS expression during stationary phase at 37°C. CspA paralogues have the ability to bind DNA (Phadtare, Inouye and Severinov, 2002), therefore do these proteins facilitate *rpoS* transcription, or do they act post-transcriptionally? To assess this, Northern analysis was utilised, as described in Chapter 2.3.3, to compare the levels of *rpoS* mRNA in MPG 558 in relation to the parent strain. The level of *rpoS* mRNA was also analysed in the SL1344 *hfq* strain to ensure that the role of Hfq is, in fact, post-transcriptional in *S.* Typhimurium.

Figure 4.2.1 illustrates that *rpoS* mRNA is present and that the levels are approximately equal in MPG 558 and the SL1344 *hfq* strain, when compared to their parent strain SL1344, during stationary phase at 37°C. This indicates that the CspA paralogues and Hfq are not involved in the transcriptional regulation of *rpoS* under these conditions; therefore their role must be post-transcriptional. This agrees with findings by Cunning, Brown and Elliott (1998) who report that *hfq* mutants of both *S.* Typhimurium and *E. coli* have substantially reduced expression of RpoS and that this effect is post-transcriptional. In the same study, it was also shown that Hfq regulation of RpoS expression occurs independently of changes in activity of native *rpoS* promoters.

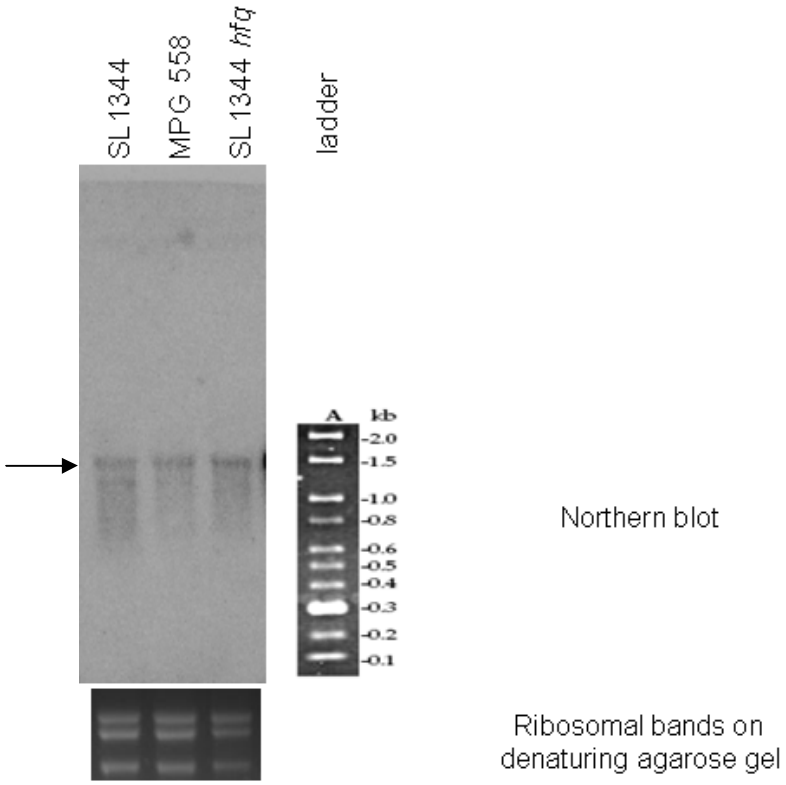


Figure 4.2.1: Northern analysis comparing *rpoS* mRNA levels in MPG 558 and the SL1344 *hfq* strain with their parent strain in stationary phase at 37°C. Cultures were grown to stationary phase in LB, with aeration, at 37°C and total RNA was extracted. RNA concentration was analysed on the Nanodrop and equal levels were confirmed by denaturing agarose gel electrophoresis (as shown). RNA was transferred to nylon membrane and probed with a ssDNA *rpoS* probe (encompassing the whole *rpoS* gene including 5'UTR sequence; bases +1 to +1382) labelled with [α^{32}P]dCTP. Arrow indicates full-length *rpoS* transcript. Results shown are indicative of triplicate experiments, with separate inocula.

4.2.2 RpoS is induced during cold shock in exponential phase and requires the action of CspA paralogues but not Hfq

Results in Chapter 3.3.2 have shown that, although RpoS is not essential for growth at low temperature, RpoS is produced upon cold shock and that this production relies on the action of the CspA paralogues. It was shown in the previous section that CspA paralogues are involved post-transcriptionally in the control of RpoS production during stationary phase (Figure 4.2.1). Do these proteins function in the same way during cold shock? Also, Hfq has been shown to act post-transcriptionally in the control of RpoS expression at low temperatures in *E. coli*, due to its involvement in pairing DsrA to *rpoS* mRNA (Masse *et al*., 2003). Is this the case in *S.* Typhimurium? To exclude a transcriptional role for the CspA paralogues and Hfq in *rpoS* expression during cold shock, the levels of *rpoS* mRNA were examined in the mutant strains and compared with those from their parent strain.

Figure 4.2.2(a) illustrates that the levels of *rpoS* mRNA, after cold shock, are similar in the MPG 558 and SL1344 *hfq* strains when compared to their parent strain. This eliminates the possibility of a major transcriptional role for CspA paralogues and Hfq during cold shock, demonstrating that these proteins must function at the post-transcriptional level to regulate RpoS expression. This would be logical due to the RNA chaperone activities of these proteins, crucial for efficient mRNA translation (Jiang *et al*., 1997, Lease and Woodson, 2004). However, it also remains possible that they may function indirectly.

Interestingly, in a study by Paesold and Krause (1999), more than one band was detected in each RNA sample by the *rpoS* probe, contrary to what has previously been seen with *E. coli rpoS* mRNA (Resch *et al*., 2008). Northern blots from *Salmonella dublin* have also shown multiple *rpoS* transcripts: the most intense signal was provided by the 1.6 kb message, corresponding to the whole *rpoS* gene including the 5'UTR; and a lower band around 1 kb corresponding to the coding sequence of the *rpoS* gene. Further studies have shown that the shorter fragment was unable to produce functional RpoS (Paesold and Krause, 1999). This smaller fragment has not been described for *E. coli* and may represent a stable degradation product from an endonucleolytic cleavage which does not arise in *E. coli*. In the present study, multiple transcripts were detected by the *rpoS* probe in each strain in Figure 4.2.2(a). The most intense band occurred around 1.5kb, as indicated with a solid arrow, and relates to the full length, functional transcript. The shorter fragments, indicated with unfilled arrows, are likely to reflect stable intermediates in the degradation process. This may suggest that neither CspA paralogues nor Hfq is involved in a major way in *rpoS* mRNA stability or degradation under the conditions examined.

Although it has been determined (Figures 4.2.1 and 4.2.2(a)) that the CspA paralogues do not appear to function directly in the regulation of *rpoS* transcription, the exact function of these proteins has yet to be elucidated. These proteins may be involved in sRNA-*rpoS* mRNA interactions or interact with *rpoS* mRNA directly. Due to the multiple fragments observed by Northern blot analysis, relating to putative stable *rpoS* mRNA degradation products, it seems unlikely that these proteins play a key role in stabilising *rpoS* mRNA against nuclease activity, since a similar pattern is observed for the SL1344 parent strain. Also, although unlikely due to their characterisation as RNA chaperones, it is possible that the CspA paralogues may function directly or indirectly to stabilise RpoS against degradation by the ClpX/P protease.

As reported previously in *E. coli*, Hfq has been shown to act post-transcriptionally during RpoS expression at low temperature (Masse *et al*., 2003). Is this the same for SL1344? Although results (Figure 3.4.2.1) have shown that Hfq is involved in RpoS expression during stationary phase in SL1344, it has not yet been tested whether this protein plays a role during cold shock. To test this, immunoblotting was carried out, with anti-RpoS antibodies, on the same samples as used for Northern analysis. Figure 4.2.2(b) illustrates the levels of RpoS detected in the SL1344 *hfq* mutant when compared to the SL1344 *csp* null strain, MPG 558, and their isogenic parent strain, SL1344. This blot reaffirms that RpoS is expressed upon cold shock in exponential phase and that MPG 558 is deficient in RpoS expression. Surprisingly, RpoS is detected in the SL1344 *hfq* strain, to a similar level as that observed in the parental strain. This suggests that Hfq is not required for RpoS expression in *S.* Typhimurium at low temperatures, contrary to what has been found in *E. coli* (Masse *et al*., 2003).

The role of Hfq at low temperatures in *E. coli* is to aid in the interaction of DsrA with *rpoS* mRNA to promote translation (Wassarman, 2002; Majdalani *et al*., 1998; Masse *et al*., 2003). Does this mean that DsrA is not required for translation of RpoS during cold shock in *S.* Typhimurium, and if not, does this suggest a possible role for the CspA paralogues in this instance? In such a scenario, the role of CspA paralogues in *E. coli* and *S.* Typhimurium would then appear to differ and may reflect an evolutionary divergence.

The results from Figures 3.4.2.1 and 4.2.2(b) suggest that there are 2 different pathways for the post-transcriptional regulation of RpoS expression in *S.* Typhimurium; one during cold shock involving the CspA paralogues alone and the other during stationary phase involving both the CspA paralogues and Hfq. The reason for the lack of involvement of Hfq during cold shock, and the possible functional replacement by the CspA paralogues, requires to be investigated.

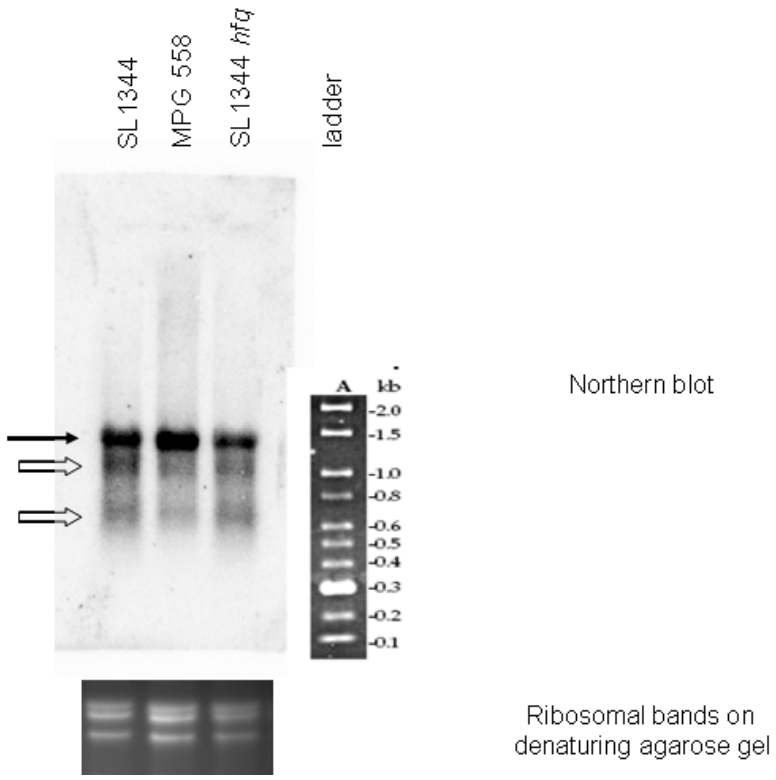


Figure 4.2.2(a): Northern analysis comparing *rpoS* mRNA levels in MPG 558 and the SL1344 *hfq* strain with their parent strain following cold shock in exponential phase. LB medium was inoculated with a 1 in 100 dilution of stationary phase cultures and grown at 37°C, with aeration, to an OD_{600} of around 0.2. These cultures were then cold shocked at 10°C for 2h with aeration. Following this, total RNA was extracted as described in Chapter 2.2.3. RNA concentration was analysed on the Nanodrop and equal levels were confirmed by denaturing agarose gel electrophoresis (as shown). RNA was transferred to nylon membrane and probed with a ssDNA *rpoS* probe, encompassing the whole *rpoS* gene including the 5'UTR (bases +1 to +1382), labelled with [α^{32}P]dCTP. Solid arrow indicates full-length *rpoS* transcript. Unfilled arrows indicate putative stable degradation products. Results shown are indicative of triplicate experiments, with separate inocula.

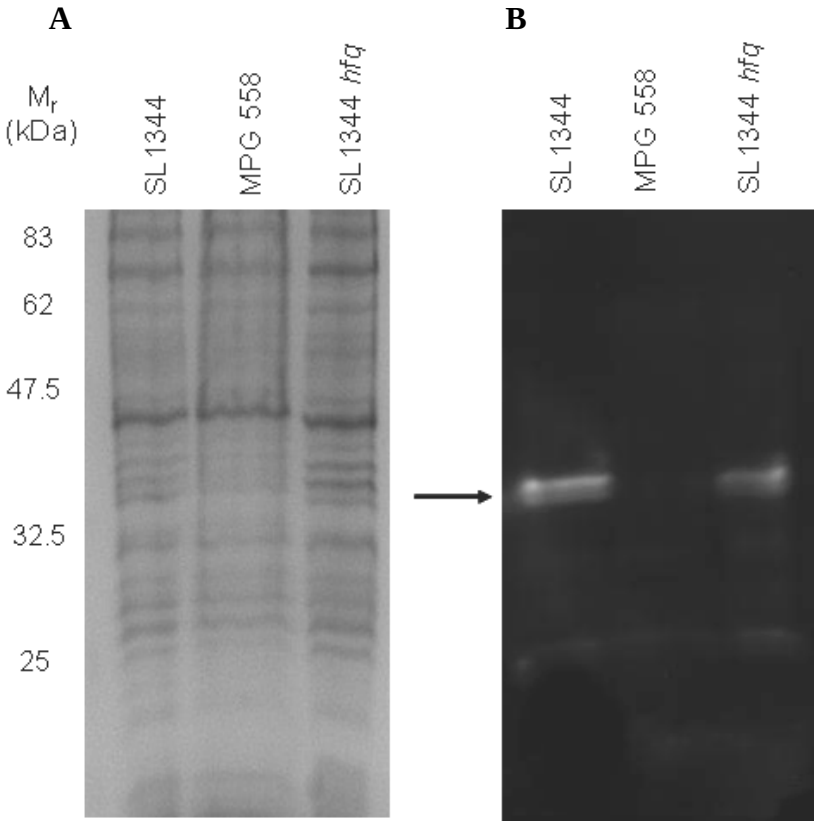


Figure 4.2.2(b): Immunoblot comparing levels of RpoS detected in the SL1344 *hfq* strain to MPG 558 and their isogenic parent strain after cold shock to 10°C in exponential phase. Cells were harvested from the same samples as used for Northern analysis (Figure 4.2.2(a)) and used for SDS-PAGE electrophoresis (panel **A**). Proteins were then transferred to nitrocellulose membrane by electroblotting and probed with anti-RpoS antibodies (panel **B**). The band corresponding to RpoS is indicated with an arrow. The positions of the molecular weight markers (M_r) are shown. The Coomassie blue stained gel is shown to demonstrate equal loading of samples (panel **A**). The experiment was repeated in triplicate and the blot shown is representative of all results.

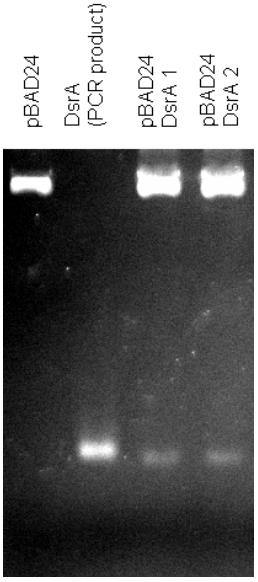
4.2.3 DsrA is not involved in RpoS expression during cold shock in *Salmonella* Typhimurium

Results (Figure 4.2.2(b)) have shown that, in *S.* Typhimurium, Hfq is not involved in RpoS expression during exposure to low temperature in exponential growth, contrary to what has been reported for *E. coli.* Masse *et al*. (2003) have shown that DsrA and RprA act as positive regulators of *rpoS* mRNA translation in *E. coli* at low temperatures and that their action requires Hfq. The gene sequences of DsrA and RprA are 94% and 80% identical, respectively, between *E. coli* K12 and SL1344 (see Figures 4.2.4.2(b) and (c) for sequence alignments), therefore do these sRNAs function in a similar way in *S.* Typhimurium? Is the role of Hfq redundant during cold shock in *S.* Typhimurium, and if so, do the CspA paralogues perform the role of facilitating sRNA to *rpoS* mRNA interactions or do they have some other indirect influence?

To investigate whether DsrA functioned in a similar way in SL1344 compared to *E. coli*, the transcribed region of *dsrA* was cloned into pBAD24 so that its expression could be modulated by the presence (or absence) of arabinose; the chromosomal locus remained intact. The plasmid was then transformed into SL1344 to assess the effect of arabinose-mediated DsrA expression on RpoS levels. This experiment paralleled that of Sledjeski, Gupta and Gottesman (1996) who induced DsrA in *E. coli*, under various growth conditions, and measured the effect on RpoS expression. In order to determine the optimal time of arabinose induction for DsrA, an induction curve was performed on cultures of SL1344 pBAD24 *dsrA* in exponential phase (OD_{600} of 0.2 at T_0) following incubation at low temperatures (10°C), as this is the condition under which *E. coli* normally expresses DsrA (Repoila and Gottesman, 2001). RpoS levels were used as a read-out (see Figure 4.2.3(b)). The induction curve illustrated that maximal RpoS expression occurred around 1h after incubation at low temperatures during exponential phase, therefore a 1h induction time was used in future experiments.

Figure 4.2.3(b) also illustrates the levels of RpoS detected in the SL1344 pBAD24 *dsrA* strain in either the presence or absence of arabinose at 10°C. However, no major difference was observed. This could suggest that DsrA is not essential for RpoS expression during cold shock in *S.* Typhimurium; but *dsrA* mRNA levels must be measured to prove that plasmid induction actually occurred. To confirm this, DsrA levels were measured using qRT-PCR. This immunoblot also confirms the induction of RpoS following cold shock in exponential phase in SL1344.

A



B

CCCNCCTGAGTTAGGACANAACCCGTTCGGGAGTCGNANTTCTGAAACCN
GGGTAGCAGGAGGAATTCACATCAGATTTCCTGGTGTAACGAATTNNCTA
NAGGTTCTTGCATAAGCAAGTTTGATCCCGACCCGCTNCGGCCGGGATTT
TTTTCGGCCATTTTTAACGGTTTACTTCTGTTACACTAAAATCATGAATT
CCAGCTCGAATGCTTCAGCAAGCTTGGCTGTTTTGGCGGATGAGAGAAGA
TTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAA
CAGAATTTGCCTGGNGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCC
GAACTCAGAAGTGAAACGCCGTAGCGCCNATGGTAGTGTGGGGTCTCCCC
CATGCGAGAGTNGGGAACTGCCAGGCATTNNAATAAAACNAAAGGCTCAG
TCGGAAAGACTGGGTCTTTTCNTTTTNTNCTGTTNNCTGTCGGTGAANGC
CCNCCTGAGTTAGGACANAACCCGTTCGGGAGTCGNANTTCTGAAACCNT
TGCTNAAGNNNTNNNNCCNGGNANGGTNGTNNCNGNNNNGACNCCCCNNN
CNNTNCCCCGGNCNNNNNCNATTCCNCNTTTNNCNATGNNNCGGCCCANN

Figure 4.2.3(a): Sequence and restriction analysis confirming insertion of *dsrA* in pBAD24. Region of *dsrA* cloned relates to bases 2026151-2026300 of the SL1344 sequence (Sanger website) and encompasses the whole gene (bases +1 to +149). *dsrA* is cloned under the control of the pBAD promoter using restriction sites *EcoRI* (1810) and *HindIII* (1862). Restriction analysis (using *EcoRI* and *HindIII* to release *dsrA* fragment; see panel **A**) was used to confirm that the vector contained insert. Sequencing (primer described below) was used to confirm that the pBAD24 constructs were correct (see panel **B** for resulting sequence). The sequence identical to that of SL1344 *dsrA* is highlighted in yellow and the *HindIII* sequence for insertion into pBAD24 is highlighted in blue. Some of the nucleotides within the highlighted sequence were not recognised due to AT-rich regions resulting in slippage; however, these were worked through by eye to confirm identity.

Sequencing primer (pBAD24): *CCTGAGCTTTTTATCGCAA;* the sequence identical to that of pBAD24 is highlighted in *italics*.

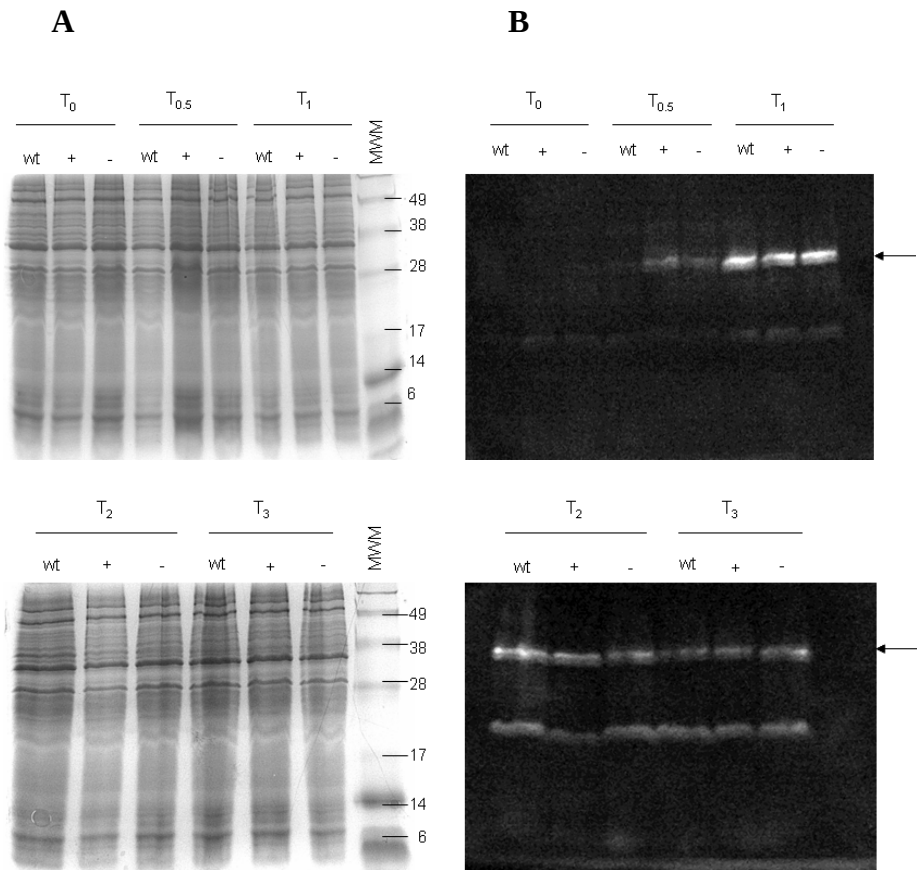


Figure 4.2.3(b): Induction curve to assess optimal induction of *dsrA*. Stationary phase cells were diluted 100-fold into fresh LB medium and grown to an OD_{600} of around 0.2 at 37°C, with aeration. Medium was then supplemented with 0.2% arabinose and incubated at 37°C for 20min to induce *dsrA* expression. Following this, cells were incubated at 10°C and samples taken at various times. The OD_{600} of each sample was taken and used to estimate total protein concentration. Equal protein concentrations of each sample, confirmed by Coomassie staining (panel **A**), were used for SDS-PAGE. Following electroblotting, samples were probed with anti-RpoS antibodies. The RpoS protein detected is highlighted by an arrow (panel **B**). Experiments were carried out in triplicate with separate inocula. The blots shown are representative of three experiments.

wt refers to SL1344 containing pBAD24 with no insert; +/- refers to SL1344 pBAD24 *dsrA* in the presence/absence of arabinose**;** T denotes the length of time, in hours, of incubation at 10°C following addition of arabinose.

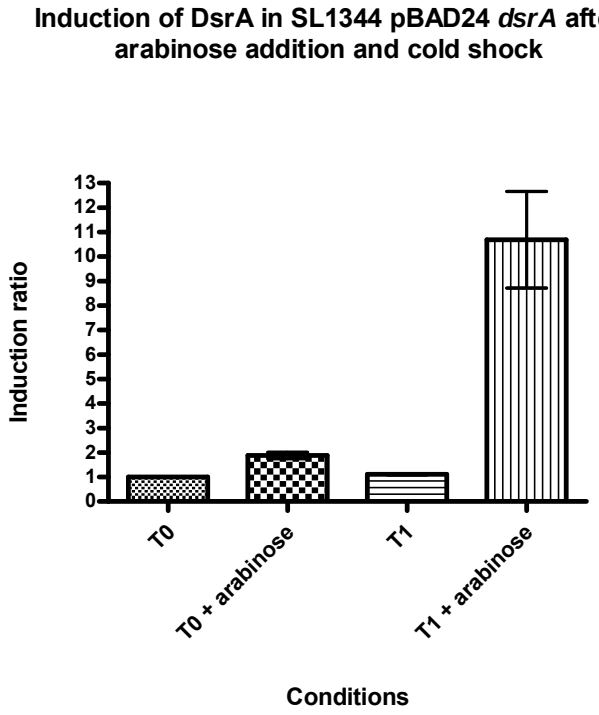
Figure 4.2.3(c) (panel A) illustrates the levels of DsrA present in SL1344 pBAD24 *dsrA* in the presence and absence of arabinose, before and after cold shock, as measured by qRT-PCR. The natural level of DsrA in *E. coli* (MG1655) and *S.* Typhimurium SL1344, before and after cold shock, was also measured under the same conditions as a control (panel B). The results show that the levels of DsrA increased three-fold after cold shock in the *E. coli* strain MG1655 (formula used for calculating ratio as described in Chapter 2.2.3.5). Surprisingly, although this study has shown an increase in RpoS production following 1h of cold shock in SL1344 (carrying pBAD24 with no insert; Figure 4.2.3(b) (panel B)), no substantial increase in synthesis of DsrA was detected under these conditions (Figure 4.2.3(c) panel B). This suggests that DsrA is not necessary for RpoS expression during exponential cold shock in *S.* Typhimurium. In contrast, an eleven-fold induction of *dsrA* mRNA was observed in SL1344 pBAD24 $dsrA_{st}$, after the addition of arabinose (Figure 4.2.3(c) panel A), despite no correlating increase in RpoS expression (Figure 4.2.3(b) panel B). This result is in agreement with that of Jones, Goodwill and Elliott (2006) who reported that DsrA is not required for optimal RpoS expression in *S.* Typhimurium, as a *dsrA* knockout mutant produces a similar amount of RpoS to its parent strain during exponential phase at 15°C. Jones and co-workers also reported that the overexpression of plasmid-borne *S.* Typhimurium *dsrA* was able to complement an *E. coli dsrA* chromosomal mutation by restoring RpoS expression to parental levels. Together these results suggest that *S.* Typhimurium DsrA, although apparently functional structurally, is not required for optimal RpoS expression during cold shock in exponential phase in SL1344.

Using Northern analysis and transcriptional fusions, Repoila and Gottesman (2001) reported that transcriptional responses to low temperature result in a four- to six-fold increase in DsrA synthesis at 25°C compared to that at 42°C, due to natural activation of the temperature sensitive *dsrA* promoter; the increase in synthesis at 25°C compared to 37°C was not reported. This group also reported an increase of 30-fold in the steady state levels of DsrA at 25°C compared to 37°C, as a result of stabilisation of functional full length transcript against degradation. In the present study the primers designed for qRT-PCR detect both full length and truncated non-functional transcript, i.e. the degradation product from the full length transcript, not including the first stem-loop responsible for RpoS regulation (see Figure 1.4.4.1). Thus, only increases in synthesis or induction of DsrA are accounted for and function is not addressed.

Earlier immunoblots have shown that Hfq is not required for RpoS expression during cold shock in *S.* Typhimurium and now it appears that DsrA also does not exert a significant influence on RpoS expression under these conditions. As a major role of Hfq during cold

shock in *E. coli* is to facilitate the interaction of DsrA with the *rpoS* mRNA leader sequence, these results could explain why the role for Hfq appears to be redundant in *S.* Typhimurium at low temperatures. Nevertheless, CspA paralogues are required for effective RpoS expression at low temperatures (Figure 3.3.2.1). Do these results point to a possible role for CspA paralogues in binding to and allowing efficient translation of *rpoS* mRNA? Equally, it remains possible that the effects of the CspA paralogues are indirect. Although its role in RpoS expression appears redundant, DsrA is still present in *S.* Typhimurium therefore it remains possible that it may play a role in RpoS expression under different conditions or interact with other RNA targets in the cell.

A



B

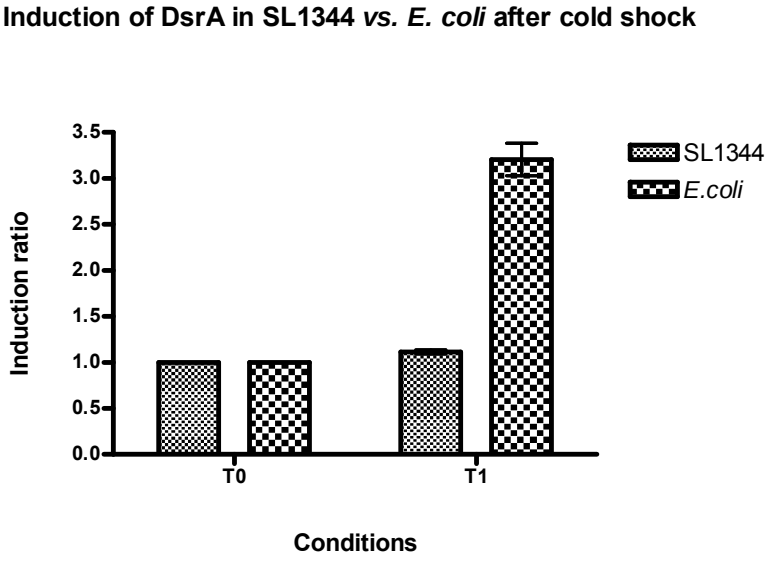


Figure 4.2.3(c): Natural and plasmid-mediated induction of DsrA in SL1344 and MG1655 upon cold shock. Panel **A** shows the arabinose-mediated induction of *dsrA* from pBAD24 $dsrA_{st}$ before and after cold shock for 1h at 10°C. Panel **B** shows the natural induction of *dsrA* in *E. coli and S.* Typhimurium following cold shock for 1h at 10°C. Stationary phase cultures were diluted 1 in 100 into fresh LB medium and grown to an OD_{600} of 0.2. RNA was extracted before (T_0) and after 1h induction (T_1) of DsrA expression at 10°C, quantified by Nanodrop and equal RNA concentrations confirmed by denaturing agarose electrophoresis (not shown). Copy DNA was prepared, by reverse transcription, and used for qRT-PCR analysis with primers (see Table 2.1.1 for oligonucleotide sequences) to amplify DsrA. Experiments were performed in triplicate and results show the average induction of DsrA. Error bars show the standard error calculated from the results. Induction ratio refers to the level of DsrA present after treatment, divided by the levels present at T_0, i.e. ratio before treatment (T_0) is 1.

4.2.4 DsrA and RprA have redundant roles in *S.* Typhimurium RpoS expression

Previously, qRT-PCR analysis (Figure 4.2.3(b) panel B) has shown that DsrA is not induced upon cold shock in *S.* Typhimurium and has no effect on RpoS expression under these conditions. In addition, immunoblots have shown that Hfq is not required for RpoS expression during cold shock in exponential phase (Figure 4.2.2(b)). However, as DsrA is still present in *S.* Typhimurium does it play a role under other growth conditions or is its role in RpoS expression completely redundant? Another sRNA, RprA, has been shown to regulate RpoS expression during osmotic stress in *E. coli* (Wassarman, 2002). However, an *E. coli rprA* mutant does not show any obvious physiological defects (Majdalani *et al*., 2001) suggesting that RprA is not essential or that there may be genetic redundancy in the system. Nevertheless, this group did show that RprA was capable of suppressing the effect of an *E. coli dsrA* deletion, allowing the activation of RpoS translation. The question then arises, could RprA replace DsrA in facilitating RpoS translation during cold shock in *S.* Typhimurium? Jones, Goodwill and Elliott (2006) have reported that DsrA and RprA are not required for optimal RpoS expression in *S.* Typhimurium; however, this was only tested at low temperatures (10°C) during exponential phase, i.e. under the conditions during which these sRNAs are known to function in *E. coli*. The Argaman lab (2001) have shown that levels of *E. coli* RprA increase with increasing cell density and are most abundant in stationary phase therefore it seems possible that this sRNA could be functional during stationary phase in *S.* Typhimurium.

To answer the question of whether DsrA functions under a different growth condition or is in fact redundant in *S.* Typhimurium with respect to RpoS regulation, and whether RprA can substitute for DsrA, transcriptional *rpoS::lacZ* fusions, both with and without the sRNA binding sites, were constructed on plasmids and their expression characteristics were examined in SL1344.

4.2.4.1 Construction of *rpoS::lacZ* transcriptional fusions

To assess the effect of DsrA and RprA on *rpoS* translation in *S.* Typhimurium, firstly the sRNA binding regions must be identified on the *rpoS* 5'UTR. Majdalani *et al*. (2001) showed that in both *E. coli* and *S.* Typhimurium, RprA does not have an extensive region of complementarity to the *rpoS* leader sequence, leaving its mechanism of action unclear. However, since then, Majdalani, Hernandez and Gottesman (2002) have used mutational analysis in both *E. coli rprA* and *rpoS* mRNA to pin point the region of interaction. Sequence

alignment was used to identify the corresponding RprA binding region on the SL1344 *rpoS* mRNA (indicated in Figures 4.2.4.1(a) and (b), for *rprA* alignment see Figure 4.2.4.2(b)).

DsrA is predicted to form a structure with three stem-loops and the first of these is necessary for RpoS translation and is complementary to the *rpoS* 5'UTR region (shown in Figure 1.4.4.1). Mutations in the *rpoS* 5'UTR and compensating mutations in DsrA confirmed that this predicted pairing is necessary for DsrA-mediated RpoS translation in *E. coli* (Majdalani *et al*., 1998). Again, sequence alignment was used to identify the corresponding DsrA binding sequence in SL1344 *rpoS* (indicated in Figures 4.2.4.1(a) and (b), for *dsrA* alignments refer to Figure 4.2.4.2(c)).

Regions of the *rpoS* 5'UTR were amplified by PCR using primers (as indicated by double-headed arrows in Figure 4.2.4.1(a); primers described in Table 2.1.1), with and without DsrA and RprA binding sites, and were cloned into the plasmid pRS415, upstream of the RBS of *lacZ*, using restriction sites *BamHI* and *SmaI*. Positive (blue) colonies were identified by blue/white screening on LB agar, containing ampicillin, and correct clones were confirmed by amplification of the insert using plasmid sequencing primers (Figure 4.2.4.1(c); primers described in Table 2.1.1) and then DNA sequencing of the vector (resulting sequences in Figure 4.2.4.1(d)). The resulting plasmids were then transformed into SL1344, by electroporation, to assess the effect of both DsrA and RprA on RpoS translation in *S.* Typhimurium under various growth conditions.

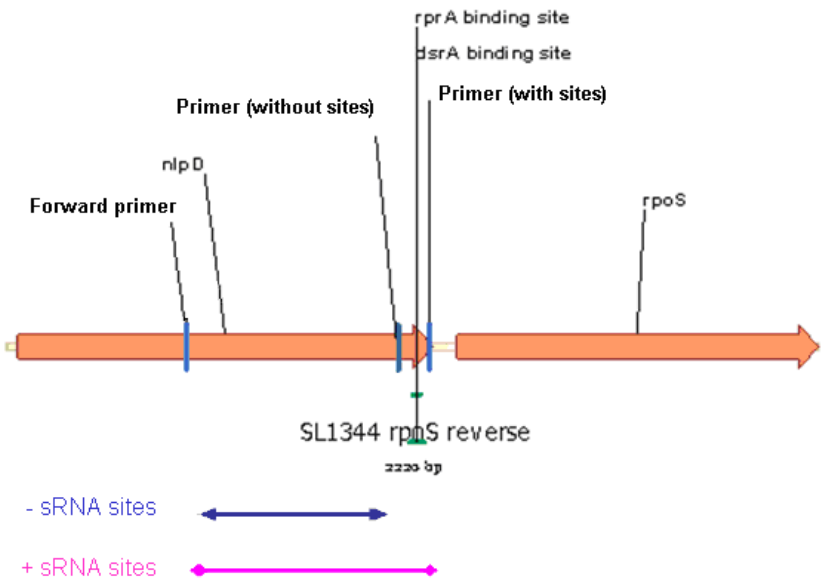


Figure 4.2.4.1(a): Diagram showing regions of *rpoS*, +/- sRNA binding sites, amplified for use in *rpoS::lacZ* transcriptional fusions (diagram constructed using Vector NTI version 10, Invitrogen). Diagram is to scale and represents 2220bp (from complement 3066784 to 3062344 of the SL1344 sequence; Sanger Institute). Site of primers are indicated: *rpoS* forward primer (3067127-3067135), primer without DsrA and RprA binding sites (3066625-3066645) and with binding sites (3066541-3066559). The areas amplified by each set of primers are indicated by double-ended arrows. Location of RprA and DsrA binding sites are highlighted (sequences indicated in Figure 4.2.4.1(b)).

Primer sequences: *rpoS* (forward) GG**CCCGGG***GCCGACGCAGCAGAGCAAG*, *rpoS* (without sites) GGG**GGATTC***CTGGTTCCGGTGCTACCCAT* and *rpoS* (with sites) GGG**GGATTC***CCGCCGATTTATCGCTGC*. Restriction sites are identified in **bold** text and sequence corresponding to SL1344 sequence is highlighted in *italics*.

rpoS (forward): 230bp upstream

121 gttaaggcgg ggcaaaaaat cgctactatg ggtagcaccg gaaccagctc tacacgcttg

primer: *rpoS* (without sites)

181 catttt**gaaa ttcgttaca**a g**gggaaatc**c gtaaacccgc tgcgttattt accgcagcga

sRNA binding (red: DsrA only, blue: both DsrA and RprA)

primer: *rpoS* (with sites)

241 taaatcggcg gaaccaggct ttgacttgct agttccgtca agggatcacg ggt**aggag**cc

start codon **RBS**

301 acctt**ttg**ag tcagaatacg ctgaaagttc atgatttaaa tgaagacgcg gaatttgatg

Figure 4.2.4.1(b): DNA sequence of SL1344 *rpoS* highlighting regions bound by DsrA and RprA (as retrieved from the Sanger Institute assembled SL1344 sequences). The sequence of *rpoS* that binds to DsrA and RprA, as described in section 4.2.4.1, is highlighted (DsrA binding only is underlined in red; both RprA and DsrA binding is double-underlined in blue). The oligonucleotide sequences, used to amplify regions of *rpoS* for transcriptional fusions (with and without sRNA binding sites) are indicated. The RBS and translational start point are also indicated. This sequence is 100% conserved with that of *E. coli* MG 1655. RpoS binding sequence was reported for *E. coli dsrA* by Majdalani *et al*. (1998). Sequence of *rpoS* responsible for binding to RprA was identified for *E. coli* by Majdalani, Hernandez and Gottesman (2002).

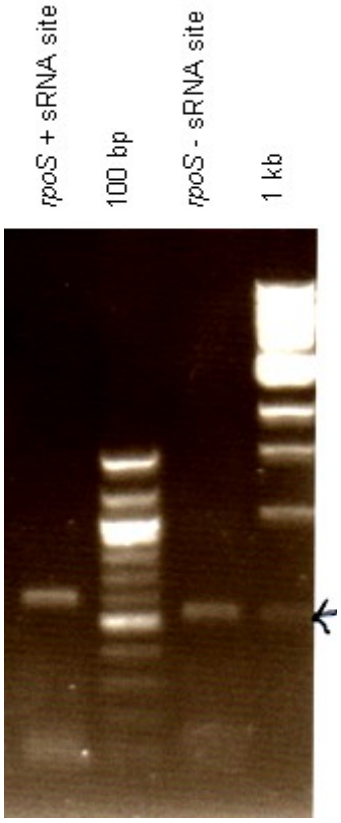


Figure 4.2.4.1(c): Amplification of *rpoS* ± sRNA binding site inserts from plasmid pRS415. The vector pRS415 has the RBS for LacZ present and was used to assess whether DsrA and RprA are functional in SL1344. Region containing sRNA sites relates to bases 3066541-3067135 of the SL1344 sequence. Region without sRNA sites relates to bases 3066625-3067135. These regions contain the *rpoS* promoter and were cloned, in frame with the RBS of LacZ, into the vector using restriction sites *SmaI* and *BamHI*. Colonies were screened using blue/white colony assays and vectors with inserts were identified by PCR amplification of the plasmid prep using plasmid-specific sequencing primers around 60bp either side of multiple cloning site (described below). These products were then used for sequencing with the same primers (sequences shown in Figure d).

Primer sequences: *rpoS* fusion (forward) *CTATCACGAGGCCCTTTCG*; *rpoS* fusion (reverse) *GGGATGTGCTGCAAGGC*. The sequences identical to pRS415 are highlighted in *italics*.

A

>012_G01 JR_seq rpoS minus

GGNGTGGTNNGAAACCGAAGGNNGNNATTACNNNAAANGAACNGGAGNNNGTAGGGGTATNCCCCCCTT
NNNGAAAANGANCNNTNTTGGNTAAATACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGAT
ATCGAATTCCTGCAGCCCGGGCGACTGAGGGCAANGTGATCGAAACCTTAGGCGCTTCTGAGGGCCGCA
ACAAGGGGATTGATATCGCAGGCAGCAAAGGACAGGCAATTATCGCGACCGCAGATGGCCGCGTTGTTT
ATGCTGGTAACGCGCTGCGCGGCTACGGTAATCTGATTATCATCTAACATAATGATGATTACCTGAGTG
CCTACGCCCATAACGACACAATGCTGGTCCGGGAACAACAAGAAGTTAAGGCGGGGCAAANAATAGCGA
CCATGGGTAGCACCGGAACCAGGGATTCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCT
TTTGTTCCCTTTAGTGATNNACCNCNANNANTNNNACAGANCNCTCNNGTCTTNNNNGATGGTGTANNN
ANTNCNNNCNNGTNTNCATNNGTCCCAGNGGNCAGNGNNGTNNNNNANNCCTN

B

>018_H02 JR_seq rpoS plus

CTCCGACATTNAANTACGCNCGGNNNANNCNTCNTNATNTNCATNNGTCCCAGNGGNCAGNGNNGTTGG
GTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGCGAC
TGAGGGCAAAGTGATCGAAACCATTGGCGCTTCTGAGGNNGGCAACAAGGGGATTGATATCGCAGGCAG
CAAAGGACAGGCAATTATCGCGACCGCAGATGGCCGCGTTGTTTATGCTGGTAACGCGCTGCGCGGCTA
CGGTAATCTGATTATCATCAAACATAATGATGATTACCTGAGTGCCTACGCCCATAACGACACAATGCT
GGTCCGGGAACAACAAGAAGTTAAGGCGGGGCAATNAATAGCGACCATGGGTAGCACCGGAACCAGTTC
AACACGCTTGCATTTNGAAATTCGTTACAAGGGGAAATCCGTAAACCCGCTGCGTTATTTGCCGCAGCG
ATAAATCGGCGGGGATTCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCT
TTAGTGANGTNNNNTNCTNNGNNCGCTGTTCTNNCNANCNTNAANNNGTGANATCCTCCTNNNCNTANT
TTGGNCTATNNNNNTNGTTTNNANNAGNNCNATTTTCNCNGNANNAGNTCNATTGCCNCNNANCANNCC
NNNCNCTGTGACGGCGGNNTTCTCCNNNANCTCCGACATTNAANTACGCNCGGNNNA

Figure 4.2.4.1(d): Sequence analysis of pRS415 *rpoS* transcriptional fusions. Sequencing (primers described below) was used to confirm that the pRS415 transcriptional constructs were correct. The sequence identical to that of SL1344 *rpoS* is highlighted in yellow and the restriction site sequences used for insertion into pRS415 are highlighted in blue. Some of the nucleotides within the highlighted sequence were not recognised due to AT-rich regions resulting in slippage; however, these were worked through by eye to confirm identity. Panel **A** shows the sequence for the *rpoS* fusion without sRNA binding sites. Panel **B** shows the sequence for the *rpoS* fusion containing the sRNA binding site.

Primer sequences: *rpoS* fusion (forward) *CTATCACGAGGCCCTTTCG*; *rpoS* fusion (reverse) *GGGATGTGCTGCAAGGC*. The sequences identical to pRS415 are highlighted in *italics*.

4.2.4.2 DsrA and RprA are not required for *rpoS* translation

Immunoblots have shown that the overexpression of DsrA has little effect on RpoS expression in *S.* Typhimurium during cold shock in exponential phase (Figure 4.2.3(b)). Jones, Goodwill and Elliott (2006) have also reported that DsrA and RprA are not required for optimal RpoS translation in exponential cultures of *S.* Typhimurium at low temperatures in rich medium. However, is the role of DsrA, and RprA, totally redundant with respect to RpoS regulation or do they regulate RpoS under other growth conditions? It is conceivable that these sRNAs may also interact with other mRNA targets.

Figure 4.2.4.2(a) shows the activity of the *rpoS::lacZ* transcriptional fusions in SL1344, with and without DsrA and RprA binding sites, under various growth conditions. On entry into stationary phase, the activity of the SL1344 *rpoS::lacZ* transcriptional fusion, with these two sRNA sites, was 130 Miller units. A similar level of activity (120 Miller units) was also observed for the transcriptional fusion with the DsrA and RprA binding sites absent from the *rpoS* leader sequence. This represents a relatively low expression of *rpoS* mRNA. However the basal expression level, measured from the SL1344 strain without the plasmid, was found to be 8 ± 3 Miller units. Paesold and Krause (1999) reported that in contrast to *E. coli,* no significant induction of *rpoS* transcription was observed when *S.* Typhimurium cells entered stationary phase. Hengge-Aronis (2002) reported that, upon entry of *E. coli* into stationary phase, there is a large increase in RpoS protein expression coupled with little or no increase in *rpoS* mRNA levels; this is primarily due to the stabilisation of RpoS against ClpX/P degradation.

In the present study (Figure 4.2.4.2(a)), the activity of the SL1344 *rpoS::lacZ* fusion, with DsrA and RprA binding sites, was found to be less than 10 Miller units during exponential phase at 37°C, a level similar to that of basal activity in SL1344, suggesting little or no induction of *rpoS* under these conditions. This is not surprising as RpoS is not usually expressed during exponential phase at optimal growth conditions. However, following cold shock during exponential phase, the activity of the *rpoS::lacZ* transcriptional fusion increased nearly 30-fold to 270 Miller units, showing a dramatic increase in expression of *rpoS* mRNA upon cold shock. This occurred independently of the putative sRNA binding sequences.

Figure 4.2.4.2(a) shows that removing the binding sites for both DsrA and RprA has no effect on the activity of the *rpoS::lacZ* transcriptional fusions under any of the growth conditions tested. This result not only confirms that DsrA and RprA are not required for optimal *rpoS*

mRNA levels in exponential phase at low temperature in *S.* Typhimurium, but also shows that they do not function in RpoS expression under other growth conditions tested. As both of these sRNAs have been shown to require Hfq to form RNA-RNA interactions with *rpoS* mRNA in *E. coli* (Muffler, Fischer and Hengge-Aronis, 1996; Sledjeski, Whitman and Zhang, 2001; Brescia *et al*., 2003; Majdalani *et al*., 2001) and these sRNAs are not required for RpoS translation at low temperatures, there may be little need for Hfq under these conditions. This may also suggest that the CspA paralogues can substitute for the sRNA-Hfq complex at low temperatures, although the possibility of an indirect action also exists.

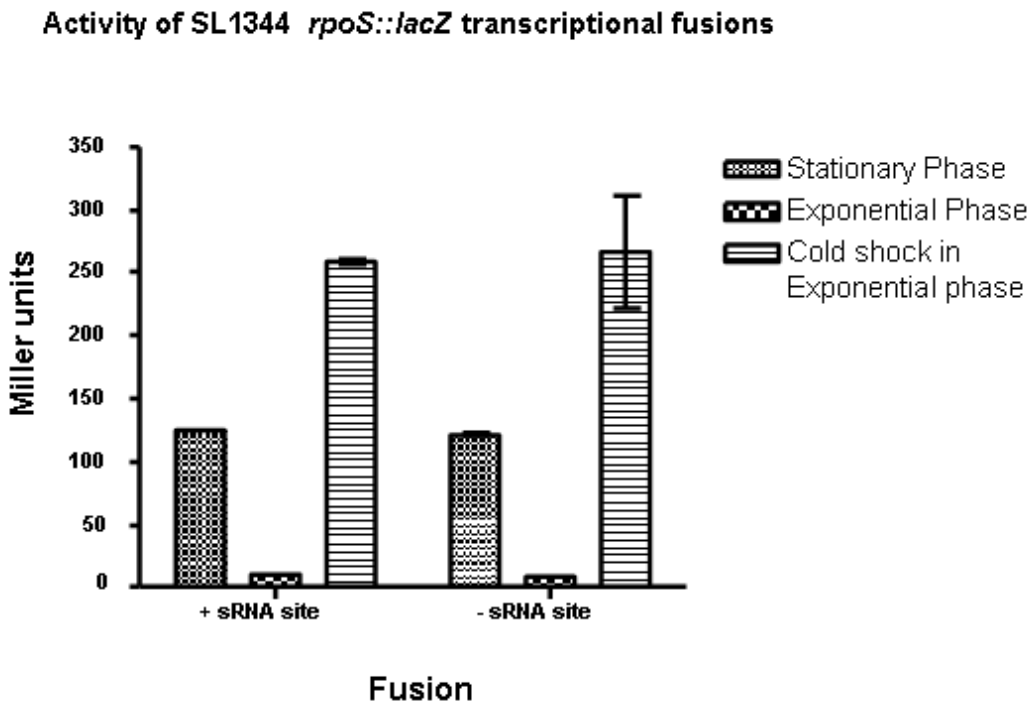


Figure 4.2.4.2(a): Activity of SL1344 *rpoS::lacZ* transcriptional fusions to assess the effect of DsrA and RprA on RpoS expression under various growth conditions. Cultures were grown to stationary phase (16h) in LB medium at 37°C with aeration. Following this, cultures were diluted 100-fold into fresh LB medium and grown at 37°C, with aeration, to an OD_{600} of 0.2. Cultures were then incubated for 1h at 10°C in a shaking, refrigerated incubator. Samples were taken from growth phases described and β-galactosidase (Miller) assays performed as described in Chapter 2.2.1. Experiments were performed in triplicate with separate inocula and the results shown are an average activity. Error bars correspond to the standard error between each set of triplicate experiments.

Why do DsrA and RprA appear to be redundant, with respect to RpoS expression, in *S.* Typhimurium? What is the difference between DsrA and RprA in *E. coli* and *S.* Typhimurium? In aligning the sequences of SL1344 and *E. coli* K12, it was found that the 5'UTR of *rpoS* is 100% conserved between the two species. The difference must therefore be in the sequence of the sRNAs: RprA is 93% identical and DsrA is 80% identical, at the DNA sequence level, between the two species; alignments are shown in Figure 4.2.4.2(b) and Figure 4.2.4.2(c) respectively. Majdalani, Hernandez and Gottesman (2002) have utilized secondary structure prediction and mutagenesis to identify the RprA sequence necessary for binding to the *rpoS* mRNA in *E. coli*. Figure 4.2.4.2(b) demonstrates that this sequence is unaltered in *S.* Typhimurium and therefore is not the cause for the redundancy of RprA in SL1344. Majdalani *et al*. (1998) have also identified the sequences within the DsrA mRNA of *E. coli* required to bind to *rpoS* mRNA. Figure 4.2.4.2(c) demonstrates that there are three DNA sequence differences between *E. coli* and SL1344 *dsrA*, in the primary stem-loop region of DsrA (underlined in Figure 4.2.4.2(c)), which may affect binding to the stem-loop structure of *rpoS* mRNA. Brescia *et al*. (2003) have demonstrated that these sequences are also within the region which binds to Hfq. Alignments have shown that there is a fourth base difference between the Hfq/RpoS binding sequence of *E. coli* and SL1344. Deletion and mutagenesis experiments have shown that an AAYAA sequence (underlined in Figure 4.2.4.2(c)) in DsrA is essential for Hfq binding in *E. coli,* and in turn is critically important for the formation of the *rpoS* RNA-DsrA binary complex (Soper and Woodson, 2008). Two of the differences in sequence in SL1344 lie within this consensus as highlighted in Figure 4.2.4.2(c).

However, the differences that reduce the AT-rich stem regions of these sRNAs seem unlikely to be the cause of their redundancy in RpoS regulation of SL1344, as Jones, Goodwill and Elliott (2006) have shown that the DsrA of *S.* Typhimurium is able to complement an *E. coli dsrA* mutant and therefore must be functional. Is there a further component present in *E. coli* that is involved in these interactions? As there is no induction of *dsrA* in *S.* Typhimurium at low temperatures (Figure 4.2.3(b) panel B), this may suggest that the unknown component is involved in the transcription of *dsrA* in *E. coli*. This seems unlikely, however, as arabinose-induced overexpression of *dsrA* from pBAD24 did not increase RpoS levels in SL1344 (Figure 4.2.3(b)). However, the possibility that these sRNAs are functional under another growth condition not yet discovered, or interact with another mRNA target, cannot be disregarded. These results lead to the conclusion that the CspA paralogues of *S.* Typhimurium are not involved in mediating the interaction of DsrA or RprA sRNAs with *rpoS* mRNA at low temperatures, since the latter appear not to affect RpoS expression (Figure 4.2.4.2(a)). Therefore, the CspA paralogues must function directly or indirectly either to stabilise RpoS

protein against degradation by ClpX/P protease or to melt the secondary structure of the 5'UTR of *rpoS* mRNA to facilitate its translation.

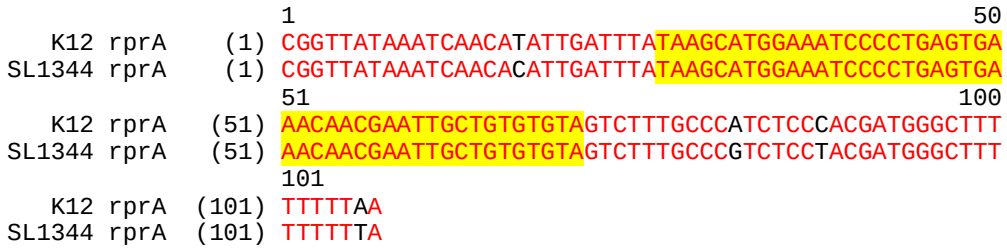


Figure 4.2.4.2(b): Sequence alignment of *rprA* from *E. coli* K12 against that of SL1344 (aligned in Vector NTI version 10, Invitrogen). Identity between these two organisms is highlighted by red text (93%). Sequences involved in RpoS-binding are highlighted in yellow, as identified for *E. coli rprA* by Majdalani, Hernandez and Gottesman (2002). No differences in the RpoS-binding sequences (yellow) were observed between the species.

Figure 4.2.4.2(c): Sequence alignment of *dsrA* from *E. coli* K12 against that of SL1344 (aligned in Vector NTI version 10, Invitrogen). Common identity between these two organisms is highlighted by red text (80 %). Sequences involved in Hfq-binding are highlighted in yellow and RpoS-binding sequences are highlighted in blue. The regions that form a stem structure are underlined. Differences in these sequences, between the species, are indicated in **bold**. Hfq binding sequence as those identified for *E. coli* DsrA by Brescia *et al.* (2003). RpoS binding sequence was reported for *E. coli dsrA* by Majdalani *et al.* (1998). The AAYAA sequence essential for Hfq binding is indicated (AAYAA).

4.3 CspA paralogues are not involved in stabilising RpoS against degradation

Northern blots have determined that neither the CspA paralogues nor Hfq are necessary for the transcription of *rpoS* during stationary phase at 37°C and cold shock in exponential phase (Figures 4.2.1 and 4.2.2(a)) and are unlikely to be responsible for the stability of *rpoS* mRNA. Transcriptional *rpoS::lacZ* fusions and qRT-PCR analysis have shown that both DsrA and RprA are dispensable with regards to RpoS expression in *S.* Typhimurium under the afore-mentioned conditions (Figure 4.2.4.2(a)), and immunoblotting has shown that Hfq is not required for RpoS expression at low temperatures (Figure 4.2.3(a)), in contrast to *E. coli*. Therefore, of these components, only the CspA paralogues alone appear involved in RpoS expression at low temperatures. Their most likely role seems either to stabilise the RpoS protein against degradation or to melt the secondary structure in the 5'UTR of *rpoS* mRNA that normally occludes its translation. Due to their role as RNA chaperones, the latter function is more likely; however the involvement of CspA paralogues in RpoS stability must be investigated.

4.3.1 Mutating components in the RpoS degradation pathway

To investigate the role of CspA paralogues in RpoS expression, a mutation that would allow RpoS protein to be detected in MPG 558 can be utilised. Is MPG 558 unable to produce RpoS, or is the process just less efficient? There are many other components involved in general transcription and translation processes and proteolysis which may effect RpoS expression; therefore analysis of mutants that might increase RpoS levels was undertaken.

Moreno *et al.* (2000) observed that *S.* Typhimurium *clpP::*Tn*10*d mutants produce increased amounts of RpoS because its proteolysis was prevented. This study also observed that an *mviA::kan* mutation rendered the strain deficient in RpoS proteolysis as MviA is required to deliver RpoS to the ClpX/P protease. These results were confirmed in a study by Hirsch and Elliott (2005) who reported increased activity of *rpoS::lacZ* fusions during log phase in these mutants. In the present study, these mutations were introduced to the SL1344, MPG 558 and SL1344 *hfq* strains by P22 transduction as described in Chapter 2.2.1. A mutation n *hns* has been shown to increase the cellular content of *E. coli* RpoS by around 10-fold during

exponential phase and that this is a post-transcriptional effect (Barth *et al.,* 1995; Hengge-Aronis, 2002). The cellular content of *E. coli rpoS* mRNA has been compared previously and shows equal levels in a wildtype strain compared to that of an *hns* mutant background (Yamashino, Ueguchi and Mizuno, 1995). In order to assess the effect of H-NS or MviA on RpoS production, an SL1344 *hns::kan* deletion mutant, received as a gift from Charles Dorman's lab, or an UK-1 *mviA::kan* deletion mutant (Moreno *et al*., 2000), was used for the introduction of mutations into MPG 558 and the SL1344 *hfq* strains by P22 transduction. In order for antibiotic resistances to be compatible, the kanamycin gene, which is flanked by FRT sites, was excised from MPG 558. This was facilitated by the introduction of the pCP20 helper plasmid containing FLP recombinase (Merlin, McAteer and Masters, 2002). The plasmid was selected for by growth on LB agar containing ampicillin at 30°C, a temperature at which the plasmid replicates. Colonies were streaked on pure LB agar and then incubated at 42°C to simultaneously cure the plasmid and express FLP recombinase, therefore excising the kanamycin marker at the FLP recognition sites, the flanking FRT sequences (method developed by Cherepanov and Wackernagel, 1995; experiment described in more detail in Chapter 5.3.2 with PCR confirmation of marker excision). The *hns::kan* or *mviA::kan* mutations were then transduced into the modified MPG 558 strain. After introducing the mutations to each strain, and removing the excess P22, notable differences in the colony morphologies were observed (summarised in Table 4.3.1(a)).

Table 4.3.1(a): Genotypes and colony morphologies of new mutants. Mutations were introduced into each strain by P22 transduction and selected for on LB agar containing the appropriate antibiotics. *hns::kan* and *mviA::kan* mutations were selected for on LB agar containing kanamycin. *clpP::*Tn*10*d mutations were selected for on LB agar containing Tetracycline.

(*) To ensure that the variations in colony morphology were due to *hns* mutations and not secondary mutations, P22 lysates were made from these strains and transduced back into the SL1344 parent strain. Mutations were selected for on LB agar containing kanamycin and resulted in the small and gloopy colony phenotype previously observed for the SL1344 *hns::kan* mutant. Phenotypes have been observed in triplicate experiments.

Strain	Genotype	Phenotype
MPG 630	SL1344 *hns::kan*	small, gloopy
MPG 631	SL1344 *csp* null *hns::kan*	normal (*)
MPG 632	SL1344 *hfq hns::kan*	normal, gloopy (*)
MPG 633	SL1344 *mviA::kan*	small, non-gloopy
MPG 634	SL1344 *csp* null *mviA::kan*	small, non-gloopy
MPG 635	SL1344 *hfq mviA::kan*	small, non-gloopy
MPG 636	SL1344 *clpP::* Tn*10*d	small, non-gloopy
MPG 637	SL1344 *csp* null *clpP::* Tn*10*d	small, non-gloopy
MPG 638	SL1344 *hfq clpP::* Tn*10*d	small, non-gloopy

H-NS represses colanic acid expression in *E. coli*, an exopolysaccharide (EPS) common to many enterobacteria, including *S.* Typhimurium (Sledjeski and Gottesman, 1995). Previously, mucoidal colony morphologies have been observed by *S.* Typhimurium *hns* mutants due to a 20-fold increase in EPS production, this is an RpoS-independent phenotype (Ledeboer and Jones, 2005). This mucoidal phenotype was also observed in the SL1344 *hns* (MPG 630) and SL1344 *hfq hns* (MPG 632) mutants. However it was absent in the MPG 558 *hns* (MPG 631) strain. Does this mean that the CspA paralogues are involved in EPS or colanic acid production? To investigate this, the *hns::kan* mutation was introduced into SL1344 strains with various combinations of CspA paralogues present; Table 4.3.1(b) shows the resulting colony morphologies. MPG 650 (CspC and CspE *hns*) has a colony morphology equivalent to that of MPG 630 (SL1344 *hns*) suggesting that CspC or CspE are required for the overproduction of EPS at 37°C. In contrast, MPG 649 (CspA and CspB *hns*) has a colony forming morphology similar to that of MPG 631 at 37°C; CspA and CspB are not thought to be produced at 37°C to any significant level. However, when MPG 649 was grown on LB agar at 15°C for 72h, the colony morphology observed was small and gloopy, equivalent to MPG 630. These results suggest that CspA or CspB are required for the overproduction of EPS at 15°C.

Table 4.3.1(b): Genotypes and colony morphologies of SL1344 *csp hns* mutants. *hns::kan* mutations were introduced into each strain by P22 transduction and selected for on LB agar containing the appropriate antibiotics. Phenotypes reported have been observed in triplicate experiments. LB agar plates were either incubated overnight (16h) at 37°C or for 72h at 15°C where appropriate.

Strain	Genotype	Phenotype
MPG 630	SL1344 *hns::kan*	small, gloopy
MPG 631	SL1344 *csp* null *hns::kan*	normal
MPG 632	SL1344 *hfq hns::kan*	normal, gloopy
MPG 645	MPG 557 (CspE) *hns::kan*	small, gloopy
MPG 649	MPG Δ *csp* CDEH (CspAB) *hns::kan*	normal at 37°C
MPG 649	MPG Δ *csp* CDEH (CspAB) *hns::kan*	small, gloopy at 15°C
MPG 650	MPG Δ *csp* ABDH (CspCE) *hns::kan*	small, gloopy

E. coli hns mutants demonstrate an increase in the translation efficiency of *rpoS* mRNA, especially during exponential phase, which results in a slow growth phenotype and the formation of small colonies on solid medium (Barth *et al.*, 1995; Yamashino, Ueguchi and Mizuno, 1995). Immunoblots have shown that MPG 558 and the SL1344 *hfq* strains are deficient in RpoS production, which may explain the normal colony size when an *hns* mutation is introduced to these strains. A normal colony size was also observed for MPG 649

at 37°C but colonies were small at 15°C. Immunoblots (Figure 3.3.2.1) have shown that CspA and CspB are involved in RpoS expression upon cold shock in exponential phase but not in stationary phase at 37°C. This would explain why the small colony morphology, resulting from overexpression of RpoS, would only occur at low temperature.

In a study by Webb *et al.* (1999), a *S.* Typhimurium *clpP* mutant displayed small-colony morphology due to a reduced growth rate at 37°C. This resembles the small colony phenotype of the *hns* mutants caused by overproduction of RpoS. Interestingly, this group reports that large-colony mutants arose with high frequency; many large colonies were also observed for the *clpP* strains in this study. Less large colonies were observed when *clpP* was introduced to the MPG 558 and SL1344 *hfq* strains; this may be due to the reduced expression of RpoS previously observed for these strains. The small colony morphology was also observed for the *mviA* mutants; this was expected as MviA and ClpXP act as a two-component system for RpoS degradation (Moreno *et al*., 2000).

From looking at colony morphologies, a possible role for the CspA paralogues in EPS production has been discovered. To substantiate this, hydrolysates of EPS preparations of the MPG 558 strain could be analysed chromatographically to determine sugar composition compared to that of its parent strain (Grant, Sutherland and Wilkinson, 1969). EPS may also have an impact on biofilm formation as a mutation in the *S.* Typhimurium colanic acid biosynthetic gene, *wcaM*, was found to disrupt biofilm formation on HEp-2 cells and chicken intestinal tissue (Ledeboer and Jones, 2005). In addition, a mutation in the cellulose biosynthetic gene, *yhjN*, was found to disrupt biofilm formation on HEp-2 cells, chicken intestinal epithelial cells and on plastic surfaces. (Ledeboer and Jones, 2005). These findings may suggest that the *S.* Typhimurium *csp* null mutant could have impaired attachment to, and growth in, eukaryotic cells due to deficiencies in EPS production.

It has also been determined that the overexpression of RpoS results in small colony morphology in SL1344, as similarly observed previously for *E. coli* (Barth *et al*., 1995; Yamashino, Ueguchi and Mizuno, 1995).

4.3.2 The SL1344 *csp* null strain is capable of accumulating RpoS if its proteolysis is prevented

Is the MPG 558 strain unable to produce RpoS protein, or is its translation just less efficient? As mutations that should increase RpoS levels have been introduced to the MPG 558 strain, are any of these new strains able to produce detectable amounts of RpoS protein? Does this provide any clues as to the role of the CspA paralogues in RpoS regulation, during both stationary phase and cold shock, and any interactions involved? These mutations were also introduced to the SL1344 *hfq* strain, to investigate the role of Hfq in RpoS regulation during stationary phase at 37°C, and to the SL1344 parent strain to act as a control for each result.

4.3.2.1 RpoS expression during stationary phase at 37°C

Immunoblots have shown that both Hfq and the CspA paralogues are required for optimal RpoS expression during stationary phase in *S.* Typhimurium when grown in LB at 37°C (Figure 3.4.2.1). However, little knowledge of how they mediate this effect is available. For this reason, the mutations which are known to increase RpoS levels were introduced to these strains and RpoS expression was examined during the stationary phase of growth. Figure 4.3.2.1(a) shows the RpoS levels detected in the new *clpP* strains, compared with the parent strains during stationary phase at 37°C. This blot reaffirms that both the CspA paralogues and Hfq are required for optimal RpoS expression in *S.* Typhimurium during stationary phase. The experiment also shows that the levels of RpoS detected in the SL1344 sample are substantially increased when a *clpP* mutation is introduced; this is expected as the *clpP* mutation renders the cell unable to degrade RpoS, allowing the protein to accumulate. This blot also shows that, when *clpP* is mutated, MPG 558 and SL1344 *hfq* strains are able to produce RpoS to the same levels as their parent strain SL1344.

Figure 4.3.2.1(b) demonstrates the RpoS levels in the *mviA* and *hns* strains compared with the parent strains during stationary phase in LB at 37°C. Unlike as observed with the *clpP* mutant, there is no increase in RpoS levels in the SL1344 *mviA* sample. This is surprising as MviA is responsible for targeting RpoS to the ClpX/P protease, therefore major degradation of the protein should not be taking place. Does the ClpX/P protease function to some extent in the absence of MviA in *S.* Typhimurium? It is of note that there is a strong band of around 32kDa detected in the MPG 634 (MPG 558 *mviA*) and MPG 635 (SL1344 *hfq mviA*) mutants that is not present in the parent strains (SL1344, MPG 558 and SL1344 *hfq*). Does this band, indicated by an unfilled arrow in Figure 4.3.2.1(b), represent a stable RpoS degradation intermediate? It is unlikely that this band corresponds to a truncated non-functional RpoS

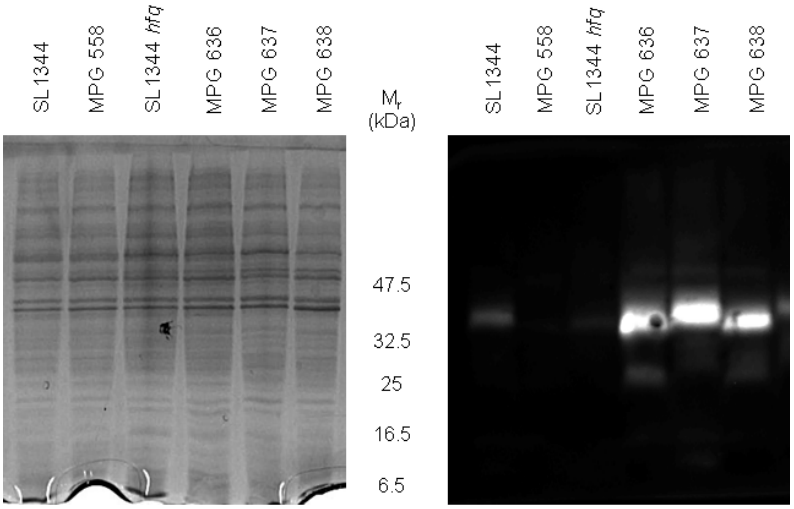


Figure 4.3.2.1(a): Immunoblot comparing RpoS levels in *clpP* mutant strains to their parent strains during stationary phase at 37°C. Cultures were grown to stationary phase (16h) in LB, with aeration, at 37°C. Cells were harvested and used for SDS-PAGE and electroblotting; blots were probed with anti-RpoS antibodies. Protein concentration was estimated spectrophotometrically and equal loading was confirmed by Coomassie staining as shown. The experiment was repeated in triplicate and the blot shown is representative of all results. An estimate of the band sizes is indicated. Strain genotypes: MPG 558 (SL1344 *csp* null), MPG 636 (SL1344 *clpP*), MPG 637 (MPG 558 *clpP*) and MPG 638 (SL1344 *hfq clpP*).

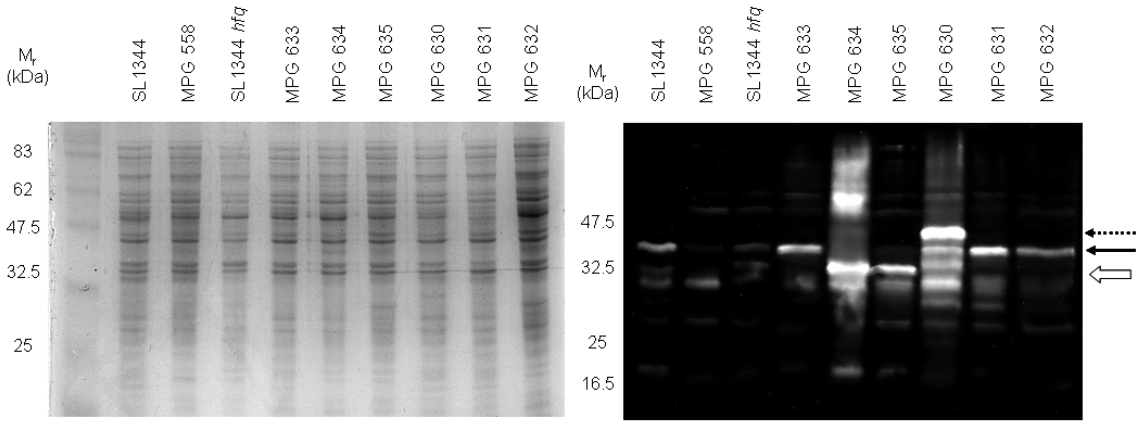


Figure 4.3.2.1(b): Immunoblot comparing RpoS levels in *mviA* and *hns* mutant strains to their parent strains during stationary phase at 37°C. Cultures were grown to stationary phase (16h) in LB at 37°. Cells were harvested and used for SDS-PAGE and electroblotting; blots were probed with anti-RpoS antibodies. Protein concentration was estimated spectrophotometrically and equal loading was confirmed by Coomassie staining as shown. The experiment was repeated in triplicate and the blot shown is representative of all results. An estimate of the band sizes is indicated as determined with a Pre-stained protein marker (New England Biolabs). The band representing RpoS is indicated with an arrow. The larger band, indicated by a dotted arrow, may be a modified version of the RpoS protein. The lower band, indicated by a hollow arrow, is a putative stable degradation product (32kDa). Strain genotypes: MPG 558 (SL1344 *csp* null), MPG 633 (SL1344 *mviA*), MPG 634 (MPG 558 *mviA*), MPG 635 (SL1344 *hfq mviA*), MPG 630 (SL1344 *hns*), MPG 631 (MPG 558 *hns*) and MPG 632 (SL1344 *hfq hns*).

protein, which is usually degraded by an MviA-ClpXP complex, as it is not observed in the MPG 558 *clpP* strain. Moreover, a study by Zhou *et al*. (2001) has reported that, in *E. coli*, the degradation of RpoS by the ClpX/P protease is complete, i.e. no stable degradation products have been observed. Single amino acid substitutions in *E. coli* RpoS have shown that the turnover element in RpoS is a single lysine residue, K173, as this is the binding site for RssB (the equivalent of MviA) (Becker *et al.,* 1999; Hengge-Aronis, 2002; Schweder *et al*., 1996). In *E. coli*, RssB must be phosphorylated by acetyl phosphate before it can bind to this turnover element in RpoS and present RpoS to the ClpXP machinery, where it is unfolded and completely degraded. MviA, in *S.* Typhimurium, does not rely on acetyl phosphate but is activated by an as yet unknown stimulus through phosphorylation on D58. This substantially increases its ability to bind to RpoS (Hirsch and Elliott, 2005; Moreno *et al*., 2000). However, it remains possible that the Clp protease could have residual activity in the absence of MviA, i.e. ClpX/P can make an initial cleavage but cannot completely degrade the protein. As the stable degradation intermediate is only present in the MPG 558 *mviA* and SL1344 *hfq mviA* strains, a possible role for the CspA paralogues and Hfq may be in modulating a second protease.

In Figure 4.3.2.1(b), the RpoS protein appears to be modified in the SL1344 *hns* strain, as represented by a larger band of around 42kDa; this is not apparent in the other *hns* mutants. This is unlikely to be an upstream translational start point or downstream read-through as there is a STOP codon immediately upstream of the 5'UTR of the *rpoS* sequence and terminator sequences at the 3' end of the transcript. Therefore, the size increase is likely due to protein modification. The post-transcriptional involvement of H-NS in RpoS expression is yet to be fully understood but its action is thought to be indirect by negatively regulating the transcription of a gene encoding a putative factor that is either responsible for stabilising RpoS or is involved in the efficient translation of *rpoS* mRNA (Yamashino, Ueguchi and Mizuno, 1995; Dorman, 2004). More recently, Zhou and Gottesman (2006) have suggested that in an *E. coli hns* mutant, RssB is present but inactive thus preventing degradation. Does H-NS negatively regulate the transcription of a protein that blocks access of RssB to RpoS or a protein that facilitates the formation of the RssB-ClpXP complex? If the latter was true, the larger band may represent an MviA-RpoS complex. MviA is thought to sequester *S.* Typhimurium RpoS during stationary phase, inhibiting its activity, even if its proteolysis is blocked (Moreno *et al*., 2000). Many *hns* mutant strains also carry spontaneously arising secondary mutations in the *rpoS* gene, as constitutive expression of RpoS-regulated genes can be detrimental to the cell (Desai and Mahadevan, 2006). However, secondary mutations are unlikely to be the cause of the larger band as only small colonies, caused by overexpression of

functional RpoS, were chosen for experiments. A slow growth phenotype was also confirmed, by OD_{600} readings, during incubation at 37°C in LB.

The introduction of an *hns* mutation in MPG 558 and SL1344 *hfq* strains resulted in the detection of RpoS; levels were similar to those observed in the SL1344 parent strain (Figure 4.3.2.1(b)). These results have shown that the MPG 558 and SL1344 *hfq* strains are able to produce RpoS protein during stationary phase at 37°C when the degradation pathway is disrupted. However, this does not clarify the individual role of the CspA paralogues or Hfq in RpoS regulation. Possible roles for these proteins lie in the efficient translation of *rpoS* mRNA, or the protection of the protein against proteolysis.

4.3.2.2 RpoS expression in exponential phase after cold shock

Immunoblots have shown that the CspA paralogues alone, and not Hfq, are involved in the regulation of RpoS following cold shock in *S.* Typhimurium (Figure 3.3.2.1). Are CspA paralogues functioning in the same way at low temperatures and during stationary phase at 37°C? The major role of the CspA family during cold shock is thought to be that they melt mRNA secondary structure, which have been aberrantly stabilised by low temperatures, therefore allowing translation or degradation. Is it that without the CspA paralogues, *S.* Typhimurium cannot produce RpoS at low temperatures? In order to investigate this, the levels of RpoS produced in the mutants, each with a disrupted RpoS degradation pathway, were compared to those observed in their isogenic parent strains.

Figure 4.3.2.2(a) indicates the levels of RpoS produced by the strains carrying a *clpP* mutation compared to their parental strains, SL1344 and MPG 558, following cold shock for 2h at 10°C in exponential phase (OD_{600} of around 0.2 and grown in LB). This blot reaffirms that Hfq is not required for RpoS expression under these conditions. Moreover, there is no increase in the level of RpoS detected in the SL1344 *clpP* mutant compared to the parent strain, contrary to results observed in stationary phase. This suggests that ClpP may be playing a reduced role in this situation. Hirsch and Elliott (2005) have shown that there is around a five-fold increase in the expression of *rpoS::lacZ* fusions in log phase cells of both *S.* Typhimurium *clpP* and *mviA* mutants compared to the wildtype; however this refers to cells grown at 37°C. Cunning and Elliott (1999) have shown that stresses that occur during exponential phase tend to increase *rpoS* transcription and translation, whilst the increase of RpoS upon entry into stationary phase is due to an inhibition or reduction in proteolysis. Figure 4.3.2.2(a) shows that the MPG 558 *clpP* strain is able to produce RpoS protein at a

similar concentration as observed in the SL1344 *clpP* sample. However, again this does not reveal the exact role of the CspA paralogues under these conditions.

Figure 4.3.2.2(b) illustrates the RpoS levels detected in the *mviA* and *hns* mutants compared to their parental strains, SL1344 and MPG 558, following cold shock in exponential phase. The levels of RpoS detected in the SL1344 *mviA* strain are similar to those observed in the parent strain. This was as observed for the SL1344 *clpP* mutant and agrees with the view that ClpXP proteolytic degradation of RpoS is not a major factor in RpoS regulation during exponential phase. As seen previously, very little RpoS protein is detected in the MPG 558 strain during cold shock. After the introduction of an *mviA* mutation to this strain, no major increase in RpoS levels is detected; which is contrary to observations made in the MPG 558 *clpP* strain under these conditions (Figure 4.3.2.2(a)) where RpoS was found to be present at similar levels to that found in the SL1344 *clpP* strain. This could suggest an MviA-independent role for the CspA paralogues in protecting RpoS from ClpXP-dependent degradation at low temperatures. To investigate whether this is the case, protein stability could be examined in MPG 558 compared to its parent strain, SL1344, and will be described in Section 4.3.3.

Figure 4.3.2.2(b) demonstrates that a mutation in *hns* dramatically increases the levels of RpoS in the SL1344 strain during cold shock. Barth *et al*. (1995) report that the translation of *rpoS* mRNA is increased in *E. coli hns* mutants, especially during exponential phase. Also, contrary to results during stationary phase, there is no apparent modification of the RpoS protein observed in the SL1344 *hns* strain during cold shock. This mutation also increases the levels of RpoS detected in the MPG 558 strain to a similar level as observed in the SL1344 strain. As the post-transcriptional role of H-NS in RpoS regulation is not yet fully understood, no real conclusions can be drawn from this data.

Intriguingly, a smaller band of around 32kDa, which may correspond to a stable degradation product, is observed in the SL1344 *hfq hns* mutant during cold shock (highlighted by a hollow arrow in Figure 4.3.2.2(b)). It is known that Hfq contributes to the post-transcriptional control of *hns* expression via the negatively acting antisense RNA, DsrA (Dorman, 2007); however none of these components are present in this strain. This band is of the same size as that observed in the MPG 558 *mviA* and SL1344 *hfq mviA* strains during stationary phase at 37°C (highlighted in Figure 4.3.2.1(b)). Zhou and Gottesman (2006) have suggested that RssB (or MviA) is present but inactive in *E. coli hns* mutants. Previous results (Figure 4.3.2.1(b)) have suggested that ClpXP may have residual or modified activity in the absence of MviA,

possibly acting to initiate cleavage but cannot fully degrade RpoS. This may suggest an indirect role for Hfq in protecting RpoS against proteolysis.

Together these results show that the MPG 558 strain is able to produce RpoS protein if proteolysis is prevented. However, it remains unclear whether the reduced expression of RpoS protein, observed in the MPG 558 strain, is due to inefficiency in *rpoS* mRNA translation or instability of the RpoS protein. The MPG 558 *clpP* strain displayed a dramatic increase in the level of RpoS protein detected during cold shock of exponential cells; however, no such increase was observed in the MPG 558 *mviA* strain under these conditions. During stationary phase, a smaller band of around 32kDa was observed in the MPG 558 *mviA* strain, which could correspond to a stable intermediate degradation product of RpoS. No stable degradation products of RpoS have been previously reported therefore is the role of MviA to ensure complete proteolysis?

This may suggest that, in the absence of the CspA paralogues, ClpXP can degrade RpoS protein unaided by MviA. Do these results suggest that the CspA paralogues stabilise the RpoS protein against proteolysis? The involvement of the CspA paralogues in RpoS protein stability is examined in the following section.

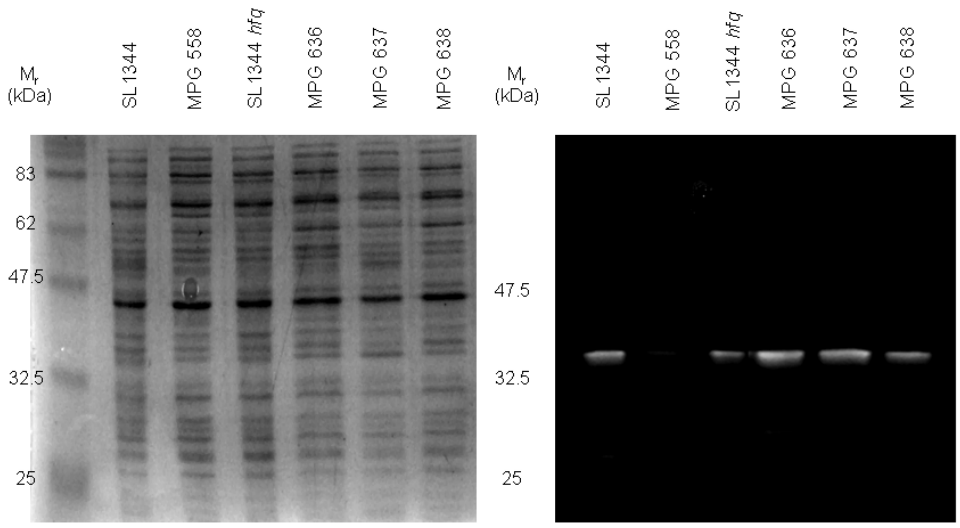


Figure 4.3.2.2(a): Immunoblot comparing RpoS levels in *clpP* mutant strains to their parent strains after cold shock in exponential phase. Stationary phase cultures were diluted 1 in 100 into fresh LB, grown to an OD_{600} of 0.2 at 37°C and then transferred to 10°C for 2h. Cells were harvested and used for SDS-PAGE and electroblotting; blots were probed with anti-RpoS antibodies. Protein concentration was estimated spectrophotometrically and equal loading was confirmed by Coomassie staining as shown. The experiment was repeated in triplicate and the blot shown is representative of all results. An estimate of the band sizes is indicated as determined by a Pre-stained protein marker (New England Biolabs). Strain genotypes: MPG 558 (SL1344 *csp* null), MPG 636 (SL1344 *clpP*), MPG 637 (MPG 558 *clpP*) and MPG 638 (SL1344 *hfq clpP*).

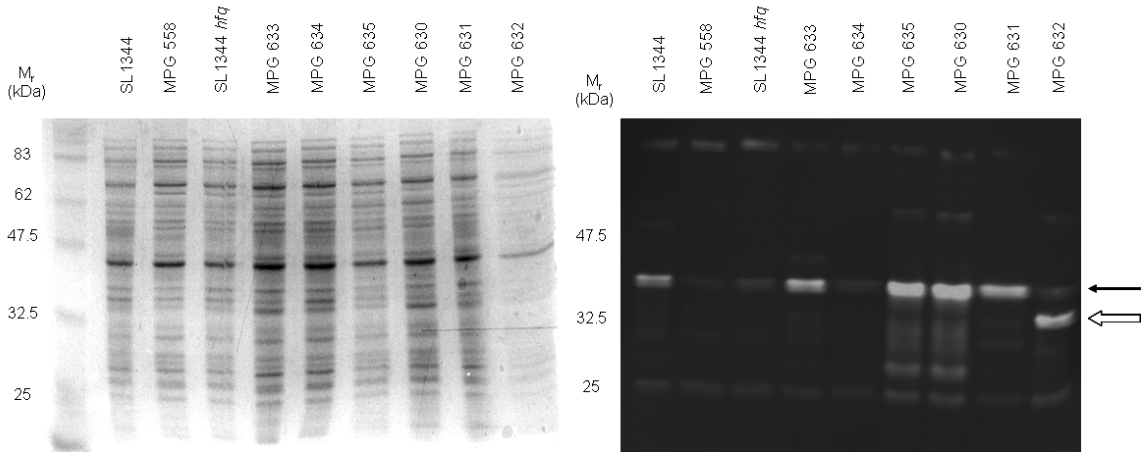


Figure 4.3.2.2(b): Immunoblot comparing RpoS levels in *mviA* and *hns* mutant strains to their parent strains after cold shock in exponential phase. Stationary phase cultures were diluted 1 in 100 into fresh LB, grown to an OD_{600} of 0.2 at 37°C and then transferred to 10°C for 2h. Cells were harvested and used for SDS-PAGE and electroblotting; blots were probed with anti-RpoS antibodies. Protein concentration was estimated spectrophotometrically and equal loading was confirmed by Coomassie staining as shown. The experiment was repeated in triplicate and the blot shown is representative of all results. The band representing RpoS is indicated with an arrow. A hollow arrow indicates a putative stable degradation product. Protein sizes are indicated as determined by a Pre-stained protein marker (New England Biolabs). The SL1344 *hfq* strains were added as a control. Strain genotypes: MPG 558 (SL1344 *csp* null), MPG 633 (SL1344 *mviA*), MPG 634 (MPG 558 *mviA*), MPG 635 (SL1344 *hfq mviA*), MPG 630 (SL1344 *hns*), MPG 631 (MPG 558 *hns*) and MPG 632 (SL1344 *hfq hns*).

4.3.3 RpoS protein is stable in the SL1344 *csp* null strain after 24 hours

The half-life of RpoS during exponential phase has been calculated in *E. coli* to be between 1 min and several minutes, dependent on the carbon source (Lange and Hengge-Aronis, 1994). However, for stationary phase the half-life of RpoS is more vague and is stated as greater than 30 min (Zhou and Gottesman, 1998). To investigate RpoS protein stability in the MPG 558 strain, the half-life of RpoS protein in this strain was compared to that observed in SL1344; stationary phase was used as the growth condition. In order to achieve detectable amounts of RpoS in the MPG 558 strain, the plasmid pUBAD::*rpoS* was introduced to the strain to allow over-expression of the protein (described in more detail in Chapter 5.2); data from section 4.3.2 clearly showed that it was possible to produce RpoS protein in mutants derived from MPG 558. The experimental procedure paralleled that of Zhou and Gottesman (2006), who used pBAD *rpoS* to allow overexpression of the protein to assess RpoS half-life in exponential phase.

Strains were grown to stationary phase at 37°C in LB, supplemented with 2% arabinose where appropriate, and then general translation was blocked by chloramphenicol. Incubation for 20 min with 20µg/ml chloramphenicol has previously been shown to block translation of GFP in SL1344 and MPG 558 during stationary phase at 37°C (S. Spragg, MPG lab results; see Appendix, Figure 4). Samples were then taken at various time points and the cells were harvested by centrifugation and used for SDS-PAGE and immunoblotting with anti-RpoS antibodies. Various concentrations of total protein from the SL1344 time zero sample were tested to ensure that an RpoS level below that of image saturation was detected in protein stability experiments; this should allow accurate visualisation of protein degradation. The levels of RpoS protein detected in the MPG 558 strain were normalised to the level of SL1344 protein levels after translational blockage, i.e. the levels of total protein were adjusted to give comparable amounts of RpoS detected in the mutant strain compared to the parent at time zero. A protein concentration equal to that used in time zero was then loaded at each time point thereafter.

Figure 4.3.3(a) demonstrates the levels of RpoS detected in SL1344 at various time points after translation was blocked by chloramphenicol. This blot reveals that the RpoS protein had not undergone proteolysis and is still stable 24h after entry into stationary phase. This is in agreement with previous reports which show a dramatic increase in RpoS levels and stability for both *E. coli* and *S.* Typhimurium on entry into stationary phase (Hengge-Aronis, 1993;

Moreno *et al.*, 2000) which is mainly achieved by reduced proteolysis (Zhou and Gottesman, 1998; Cunning and Elliott, 1999; Hirsch and Elliott, 2005).

Figure 4.3.3(b) shows the levels of RpoS detected in MPG 558, when over-expressing RpoS from the plasmid pUBAD *rpoS*, at various time points after translation was blocked by chloramphenicol. This blot illustrates that the RpoS protein is completely stable 24h after entry into stationary phase in the MPG 558 strain. This is consistent with results observed in the SL1344 parent strain, therefore the CspA paralogues appear not to be involved in protecting RpoS against degradation under these conditions. This could suggest that the CspA paralogues, if functioning directly in RpoS production, facilitate the translation of *rpoS* mRNA by melting the stem-loop structure that occludes the ribosome-binding sites. The possible role for CspA paralogues in facilitating *rpoS* translation is investigated in the following section using *rpoS::lacZ* translational fusions.

It is of note that more background bands were observed in these blots as the developing solution was changed to Immobilon Western HRP Substrate (Millipore) solution to increase detection sensitivity and decrease exposure times.

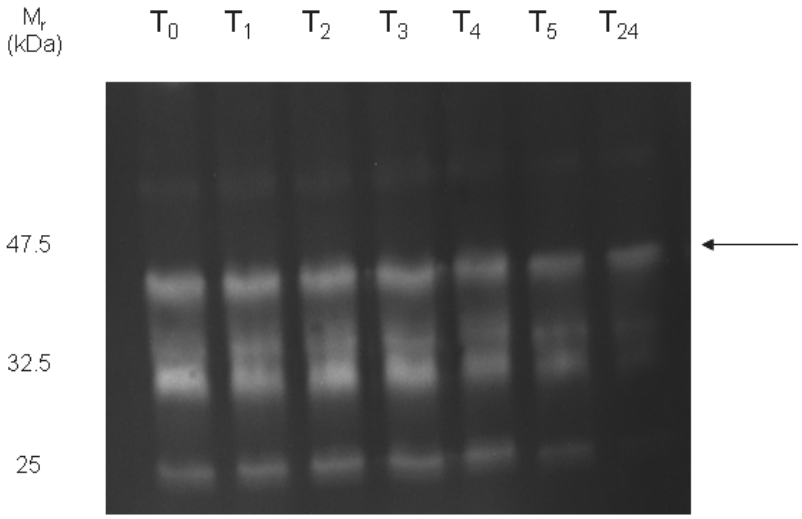


Figure 4.3.3(a): RpoS is stable for over 24h during stationary phase at 37°C in the SL1344 strain. The SL1344 strain was grown to stationary phase in LB at 37°C then incubated for 20 min after the addition of 20µg/ml chloramphenicol. Samples were then taken at various time points (T represents time in hours after translational blockage). Cells were harvested then subjected to SDS-PAGE and electroblotting; blots were probed with monoclonal anti-RpoS antibodies. The experiment was repeated in triplicate and the blot shown is representative of all results. An estimate of protein sizes is indicated as determined by a Pre-stained protein marker (New England Biolabs). The band representing RpoS is indicated with an arrow.

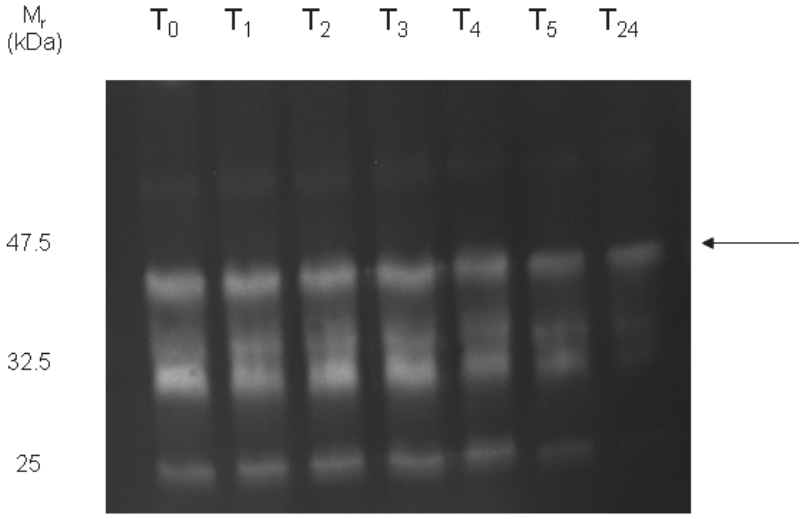


Figure 4.3.3(b): RpoS is stable for over 24h during stationary phase at 37°C in the MPG 558, over-expressing RpoS from pUBAD *rpoS*. The MPG 558 strain was grown to stationary phase in LB, containing 2% arabinose, at 37°C and then incubated for 20 min after the addition of 20µg/ml chloramphenicol. Samples were taken at various time points (T represents time in hours after translational blockage). Cells were harvested then subjected to SDS-PAGE and electroblotting; blots were probed with monoclonal anti-RpoS antibodies. The blot shown is representative of triplicate experiments. Protein sizes are indicated as determined by a Pre-stained protein marker (New England Biolabs). The band representing RpoS is indicated with an arrow.

4.4 CspA paralogues and Hfq appear to regulate RpoS at the translational level

Translational *rpoS::lacZ* fusions can actually reflect regulation of *rpoS* translation as well as proteolysis (Hengge-Aronis, 2002). A chromosomal *lacZ* translational fusion, fused in frame with the 177th codon of *rpoS* (the full length transcript) was received as a gift from Dr. Chen's lab (Chen *et al*., 1996). The *rpoS* gene contains the fully functional polymerase core binding domain, situated at amino acids 118 to 131, and the amino acid essential for ClpXP turnover of RpoS (K123), (Becker *et al.,* 1999; Hengge-Aronis, 2002; Schweder *et al*., 1996). The fusion was constructed in the chromosome in order to avoid copy number effects seen with plasmid vectors and to retain the normal DNA structure flanking *rpoS*. Their resulting *rpoS*::*lacZ* reporter strain, designated CC1024, demonstrated pronounced growth phase regulation during culture *in vitro*. This translational *rpoS::lacZ* fusion was transferred to our strains using P22 transduction. As there was no antibiotic resistance to select for, and since *S.* Typhimurium does not contain a *lac* operon, the successful transfer of the *lacZ* fusion was identified by growth on minimal agar plates using lactose as the only available carbon source. Also, X-gal was added to the agar plates to identify β-galactosidase activity as an additional control.

The recipient strains were not able to grow on the minimal lactose plates (containing 0.02% histidine) ensuring any colonies observed after transduction should contain the *rpoS::lacZ* fusions. The SL1344 *rpoS::lacZ* colonies produced a dark blue colour after growth, at 37°C, on minimal lactose agar containing histidine and X-gal, showing that *rpoS* was actively translated and present at easily detectable levels. This was as expected as immunoblots have shown that relatively high amounts of RpoS are detected in the SL1344 strain during stationary phase at 37°C (Figure 3.3.1.1(a)). The SL1344 *hfq rpoS::lacZ* colonies appeared light blue in colour on the minimal lactose agar plates containing histidine and X-gal. This suggested that there was *rpoS* translation occurring but not at the optimal level. This agrees with immunoblots, during stationary phase at 37°C, which have shown that Hfq is required for optimal RpoS expression under these conditions (Figure 3.4.2.1). The colonies produced by the SL1344 *csp* null *rpoS::lacZ* strain, although showing growth on minimal lactose agar at 37°C, were much smaller in size and appeared white in colour. This suggested that this strain was producing very low or no RpoS. However it must have expressed *lacZ* and exhibited β-galactosidase activity for growth to occur; the recipient strain was unable to grow on the minimal lactose agar plates prior to transduction. The size of the colonies suggested

this strain was struggling to grow on these plates, i.e. was only metabolising lactose at a low level. This would suggest that the blue colour normally associated with β-galactosidase activity was below the limit for human visual detection in the SL1344 *csp* null *rpoS::lacZ* strain. However, as shown later in this section, some *rpoS::lacZ* activity was detected in liquid by Miller assay.

This study has shown (Chapter 4.2) that there are 2 distinct pathways for the regulation of RpoS in SL1344. Stationary phase expression involves both the CspA paralogues and Hfq, whilst production at low temperatures relies solely on the CspA paralogues. Northern analysis has shown that neither of these components is involved at the transcriptional level. Moreover, since the pattern of transcripts is similar for SL1344 and the mutant strains, these proteins appear not to be involved in stabilising the mRNA against degradation in any major way. Immunoblots in the previous section have also shown that the CspA paralogues are not required for the stabilisation of the RpoS protein against protease activity. Therefore, these components appear likely to be working at the translational level, although an indirect role for the CspA paralogues in RpoS regulation cannot be ruled out at this point. Do the *rpoS::lacZ* translational fusions give clues to the specific roles of the CspA paralogues (and Hfq) and confirm what has previously been observed by immunoblotting?

To investigate whether the CspA paralogues, and Hfq, function at the translational level with regards to RpoS expression, the activity of *rpoS::lacZ* fusions in the *csp* and *hfq* mutant strains was compared to that of the SL1344 *rpoS::lacZ* strain during stationary phase at 37°C and following cold shock in exponential phase. Strains carrying the *rpoS::lacZ* translational fusions were grown to stationary phase (for 16h) in LB at 37°C, diluted 100-fold into fresh LB and then grown at 37°C to early exponential phase (OD_{600} of 0.2). Cultures were then transferred to a refrigerated shaking incubator at 10°C for 2h. Samples were extracted at each growth phase, the OD_{600} measured, and cells permeabilised with CTAB, a cationic detergent, for 20 min at 30°C (as described in Chapter 2.2.1.1; Miller, 1972). Substrate solution, containing ONPG, was then added to each sample and the time for each sample to turn yellow was recorded. The reaction was stopped by the addition of 1M sodium carbonate and the OD_{420} was then measured and used to calculate the Miller units for each strain under various growth phases, i.e. the activity of each *rpoS::lacZ* fusion. The Miller units of the isogenic parent strains (which are *lac* minus) were also tested to provide a basal Miller unit level to represent no activity. The basal activity ranged from 32 to 38 Miller units depending on the parent strain used.

Figure 4.4 shows the Miller units recorded for each strain under various growth conditions. The activity of the SL1344 *rpoS::lacZ* fusions during stationary phase at 37°C was 1352 ± 132 Miller units. The activity of this fusion during exponential phase at 37°C was 512 ± 35, suggesting a low expression during exponential phase, as previously observed for *S.* Typhimurium (Moreno *et al*., 2000). These results also show a 2.6-fold induction in *rpoS::lacZ* activity during stationary phase at 37°C in SL1344. This is in reasonable agreement with studies in *E. coli* which have determined that there is a 4 to 5-fold increase in RpoS synthesis on entry into stationary phase (Neidhardt *et al*., 1987; Lange and Hengge-Aronis, 1994). The activity of the *rpoS::lacZ* translational fusions following cold shock in exponential phase was 2173 ± 250 Miller units. This shows a 4.2-fold increase in *rpoS* activity upon cold shock in exponential phase. A study by Jones, Goodwill and Elliott (2006) have also reported a 3-fold increase in expression from an *rpoS::lacZ* translational fusion, in *S.* Typhimurium strain LT2, at 18°C compared to 37°C.

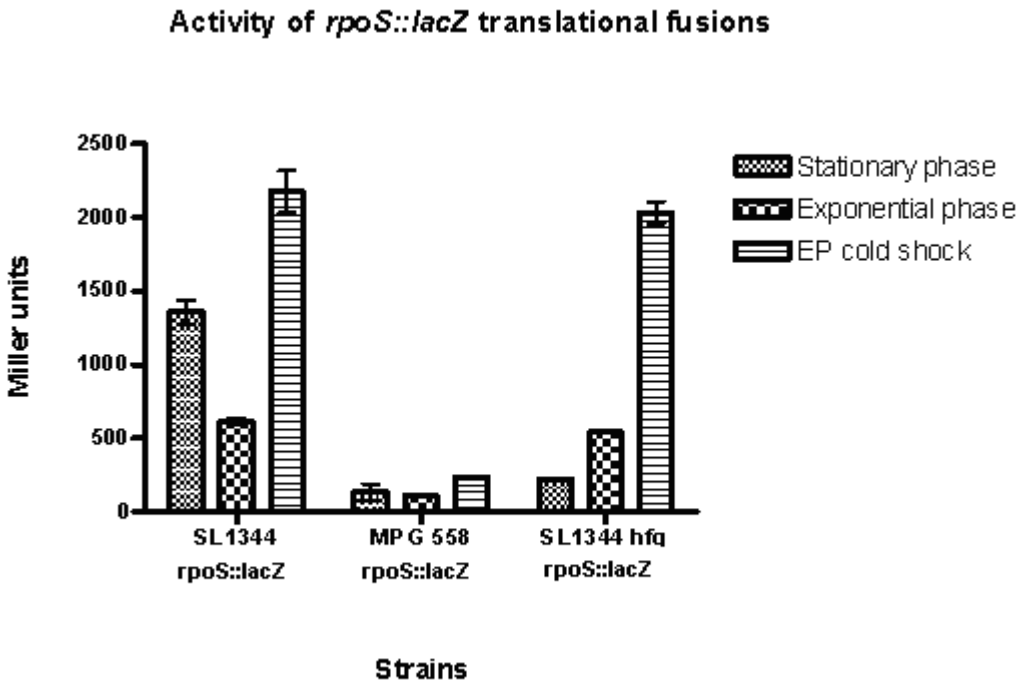


Figure 4.4: Activity of *rpoS::lacZ* translational fusions to assess the involvement of CspA paralogues and Hfq in SL1344 RpoS regulation during various growth phases. Samples were grown to stationary phase (for 16h) in LB at 37°C with aeration. Cultures were then diluted 100-fold into fresh LB and grown to an OD_{600} of 0.2 at 37°C. Cultures were then transferred to a refrigerated incubator at 10°C and cold shocked for 2h. Samples were taken at each growth phase described and used for β-galactosidase (Miller) assays as described in Chapter 2.2.1.1. Experiments were performed in triplicate from separate inocula and the average readings used to produce the graph. Error bars represent the standard deviation in each triplicate set of experiments.

Figure 4.4 shows that activity from the MPG 558 (SL1344 *csp* null strain) *rpoS::lacZ* translational fusion under various growth conditions. The activity from this fusion during stationary phase at 37°C was 192 ± 7 Miller units. This activity was 7-fold less than observed for the translational fusion in the parent strain under these conditions, suggesting that CspA paralogues are required for the synthesis of RpoS during stationary phase at 37°C. This is in agreement with earlier immunoblots (Figure 3.4.2.1) that showed little RpoS was detected from this strain under these conditions. The activity during exponential phase at 37°C was 104 ± 6 Miller units, whilst the activity following cold shock was 234 ± 8 Miller units. This shows a very slight 2-fold increase in expression from *rpoS::lacZ* translational fusions upon cold shock although the activity is 10-fold less than that observed for the SL1344 strain under the same conditions. This suggests that although there is a small induction of RpoS activity in the MPG 558 strain, the CspA paralogues are required for the efficient expression of RpoS upon cold shock. This is in agreement with impaired RpoS expression observed in the MPG 558 strain, detected by immunoblotting, following cold shock in exponential phase (Figure 3.3.2.1). This also correlates with a previous report where Western blot analysis, using monoclonal anti-RpoS antibodies, showed that overexpression of *E. coli* CspE resulted in a 4-fold upregulation of RpoS during stationary phase (Phadtare, Inouye and Severinov, 2002).

Figure 4.4 also shows the activity of the *rpoS::lacZ* translational fusions in the SL1344 *hfq* strain. During stationary phase, the activity of the *rpoS::lacZ* fusions was 218 ± 19 Miller units compared to 538 ± 15 Miller units in exponential phase. This shows a decrease of RpoS activity upon entry into stationary phase, and a 6-fold reduction compared to the parent strain, suggesting that Hfq plays a significant role in stationary phase induction of RpoS expression in SL1344. This is in agreement with previous immunoblotting experiments showing impaired RpoS expression in the SL1344 *hfq* strains during stationary phase at 37°C (Figure 3.4.2.1). Other studies using *rpoS::lacZ* protein fusions in *S.* Typhimurium have shown that an *hfq* mutant has a four- to sevenfold reduction in expression of *rpoS* during stationary phase (Brown and Elliott, 1996). This can be attributed primarily to a defect in translation as pulse-labeling studies, with S^{35}-Methonine, have shown that the rate of RpoS synthesis is significantly decreased in an *hfq* mutant (Brown and Elliott, 1996). The activity of the fusion in the SL1344 *hfq* strain during exponential phase at 37°C is in accordance with that observed for the parent strain. However, following cold shock in exponential phase, the activity of the *rpoS::lacZ* fusions increases to 2030 ± 131 Miller units, a 3.7-fold increase from the activity prior to cold shock. This is concurrent with activity levels observed from the parent strain under the same conditions. This suggests that Hfq is not required for optimal RpoS expression during cold shock in SL1344 and is in agreement with immunoblots showing similar levels of RpoS detected in the SL1344 *hfq* strain compared to the parent (Figure 4.2.2(b)). These

results are contrary to what has been reported for *E. coli* (Masse *et al*., 2003) but in agreement with results previously reported for *S.* Typhimurium (Jones, Goodwill and Elliott, 2006). Masse *et al*. (2003) have shown that DsrA and RprA act as positive regulators of *rpoS* mRNA translation in *E. coli* at low temperatures and that their action requires Hfq. However, the present study has shown that DsrA and RprA are not required for *rpoS::lacZ* transcriptional fusion expression during cold shock (Figure 4.2.4.2(a)). This is in support of findings by Jones, Goodwill and Elliott (2006) that showed that *S.* Typhimurium DsrA and RprA were not required for optimal RpoS expression at low temperature. These results also confirm what has previously been seen by immunoblotting (Figures 3.4.2.1 and 4.2.2(b)) that there are in fact 2 distinct pathways for RpoS regulation in SL1344.

It is of note that the site of integration of the *rpoS::lacZ* translational fusions was not assessed and it can not ruled out that the fusions may nave integrated elsewhere in the chromosome. However, this is unlikely as the fusions confirm what was previously seen with immunoblotting.

4.5 Discussion

The aim of this Chapter was to determine the role of the CspA paralogues and Hfq in RpoS expression in *S.* Typhimurium. Northern analysis has shown that these components are not involved in *rpoS* transcription during either stationary phase or cold shock in exponential phase as the levels of *rpoS* mRNA are similar in the MPG 558 (SL1344 *csp* null) and SL1344 *hfq* strains compared to their isogenic parent strain, SL1344, in both situations. This agrees with findings by Cunning, Brown and Elliott (1998) who reported that *hfq* mutants of both *S.* Typhimurium and *E. coli* have substantially reduced expression of RpoS but that this effect is mainly post-transcriptional.

Northern blots from *Salmonella dublin* have shown multiple *rpoS* transcripts: the most intense signal was provided by the 1.6 kb message, corresponding to the whole *rpoS* gene including the 5'UTR; and a lower band around 1 kb corresponding to the coding sequence of the *rpoS* gene. Further studies have shown that the shorter fragment was unable to produce functional RpoS (Paesold and Krause, 1999). These smaller fragments have not been described for *E. coli* and may represent a stable degradation product from an endonucleolytic cleavage which does not arise in *E. coli*. A similar transcript pattern was observed for our three strains tested;

therefore this may suggest that neither CspA paralogues nor Hfq is involved in a major way in *rpoS* mRNA stability or degradation. This finding is contrary to what has previously been found for *E. coli* as the amount and stability of *rpoS* mRNA is greatly enhanced by the overexpression of CspC and CspE (Phadtare and Inouye, 2001).

Immunoblots have shown that there are two distinct mechanistic pathways for RpoS regulation in *S.* Typhimurium as CspA paralogues are required for optimal RpoS expression during both stationary phase and cold shock, whilst Hfq is necessary for stationary phase only. This agrees with findings by Cunning, Brown and Elliott (1998) who reported that *hfq* mutants of both *S.* Typhimurium and *E. coli* have substantially reduced expression of RpoS and that this effect is post-transcriptional. However, their study only evaluated the effect of Hfq during stationary phase. The role of Hfq at low temperatures in *E. coli* is to aid in the interaction of DsrA with *rpoS* mRNA to promote translation (Wassarman, 2002; Majdalani *et al*., 1998; Masse *et al*., 2003). The present study has shown that Hfq is not required for optimal RpoS expression at low temperatures in SL1344. Immunoblots have shown that RpoS expression is also induced upon cold shock in exponential phase (Figure 4.2.3(a)) despite growth profiles proving that RpoS is not essential for growth at low temperatures (Figure 3.3.2.1.1(b)).

Immunoblotting and qRT-PCR experiments have demonstrated that DsrA is not required for optimal RpoS expression during cold shock in exponential phase in *S.* Typhimurium; contrary to results observed in *E. coli* (Sledjeski, Gupta and Gottesman, 1996). In addition, qRT-PCR results have also shown that DsrA is not induced upon cold shock in exponential phase. Transcriptional *rpoS::lacZ* fusions, comparing *rpoS* expression in the presence and absence of sRNA binding sites, suggested that neither DsrA nor RprA were required for optimal RpoS expression in SL1344 under all growth conditions tested (Figure 4.2.4.2(a)). This confirms results reported by Jones, Goodwill and Elliott (2006) who state that DsrA and RprA are not required for RpoS expression at low temperatures in *S.* Typhimurium.

Alignments comparing the sequences of these sRNAs in MG1655 with SL1344 have highlighted differences that make Hfq and *rpoS* mRNA binding regions less AT-rich. However, this seems unlikely to be the reason for the redundancy of these sRNAs in SL1344 as Jones, Goodwill and Elliott (2006) have shown that a plasmid over-expressing *S.* Typhimurium *dsrA* will complement an *E. coli dsrA* chromosomal mutation. As described previously in Section 4.2.4, there is no induction of DsrA in SL1344 upon cold shock; therefore, is there another component involved in the transcription of *dsrA* in *E. coli* that is absent in *S.* Typhimurium? These results also eliminate the requirement for CspA paralogues

in facilitating the interactions of DsrA and RprA with *rpoS* mRNA at low temperatures. This may suggest that CspA paralogues could substitute for these sRNAs and regulate *rpoS* mRNA translation by binding to and melting the stem-loop structure that usually occludes the ribosome-binding site on the transcript. It remains possible, however, that the paralogues may exert an indirect action on RpoS expression under these conditions.
The question remains why these sRNAs are present in SL1344 despite their apparent redundancy in RpoS expression. Recent studies have found that some sRNAs have multiple targets and can play previously unsuspected critical regulatory roles (Majdalani, Vanderpool and Gottesman, 2005). In *E. coli*, DsrA has been shown to both positively regulate RpoS translation and negatively regulate the transcription of *hns* (Majdalani *et al*., 1998). Therefore, it would not be surprising if further analysis revealed that DsrA and RprA regulated the translation of other target mRNAs in SL1344.

Mutations hampering RpoS degradation were introduced to the MPG 558 and SL1344 *hfq* strains to attempt to further elucidate the interactions of the component proteins. It is of note that the introduction of some of these mutations resulted in abnormal colony morphologies compared to the recipient parent strains. Small colony morphologies were observed with the SL1344 *mviA*, *hns* and *clpP* mutants. The present study has determined that the overexpression of RpoS results in small colony morphology in SL1344, due to reduced growth rate, as previously observed for *E. coli* (Barth *et al*., 1995; Yamashino, Ueguchi and Mizuno, 1995). Small colony morphology was not observed for the MPG 558 *hns* and SL1344 *hfq hns* mutants. This may be due to the reduced RpoS expression noted for the recipient strains.

Previously, mucoidal colony morphologies have been observed by *S.* Typhimurium *hns* mutants due to a 20-fold increase in EPS production and this has been shown to be an RpoS-independent phenotype (Ledeboer and Jones, 2005). This was also observed for the SL1344 *hns* and SL1344 *hfq hns* strains. However, it was not seen with the MPG 558 *hns* colonies. This suggested that the CspA paralogues may be involved in EPS production. This was verified to some extent by the introduction of *hns* mutations to SL1344 strains with various combinations of *csp* genes. MPG 650 (CspC and CspE *hns*) had a colony morphology equivalent to that of MPG 630 (SL1344 *hns*) suggesting that either CspC or CspE facilitates the overproduction of EPS at 37°C. In contrast, MPG 649 (CspA and CspB *hns*) exhibited a colony forming morphology similar to that of the MPG 558 *hns* strain at 37°C; CspA and CspB proteins are not thought to be produced at 37°C to any significant level. However, when MPG 649 was grown on LB agar at 15°C for 72h, a situation where CspA and CspB are

produced, the colony morphology observed was equivalent to MPG 630. These results suggest that CspA or CspB are required for the overproduction of EPS at 15°C.

When mutant strains, with disrupted RpoS degradation, were used for immunoblotting, higher levels of cellular RpoS were observed in some cases (Figures 4.3.2.1(a) and (b) and Figures 4.3.2.2(a) and (b)). During stationary phase at 37°C, the *clpP* mutant strains demonstrated increased RpoS levels compared to their parent strains. This was as expected as the ClpXP protease is responsible for RpoS degradation and RpoS has previously been shown to accumulate in *E. coli clpP* mutants (Zhou and Gottesman, 1998). This also showed that the MPG 558 strain was able to produce RpoS protein if its degradation was prevented.

Unlike the situation observed with the *clpP* mutant, there was no increase in RpoS levels in the SL1344 *mviA* sample. This is surprising as MviA is responsible for targeting RpoS to the ClpX/P protease, therefore major degradation of the protein should not be taking place. This suggests that ClpXP may have residual protease activity independently of MviA. This has not been reported for other species. It is of note that there is a strong, smaller band of around 32kDa detected in the MPG 634 (MPG 558 *mviA*) and MPG 635 (SL1344 *hfq mviA*) mutants that is not present in the parent strains (SL1344, MPG 558 and SL1344 *hfq*).

Figure 4.3.2.2(b) demonstrates that a mutation in *hns* dramatically increases the levels of RpoS in the SL1344 strain during cold shock. This immunoblot also showed that the cellular levels of RpoS were increased in the MPG 558 *hns* and SL1344 *hfq hns* strains under these conditions. Barth *et al*. (1995) report that the translation of *rpoS* mRNA is increased in *E. coli hns* mutants, especially during exponential phase.

Immunoblotting also revealed that there is no increase in the level of RpoS detected in the SL1344 *clpP* mutant following cold shock during exponential phase, compared to the parent strain, contrary to results observed in stationary phase at 37°C. This suggests that ClpP may be playing a reduced role in this situation. In contrast, the ClpXP protease has been shown to be the main cause of low RpoS levels in *E. coli* during exponential phase at 37°C (Hengge-Aronis, 2002; Lange and Hengge-Aronis, 1994). However, the role of the ClpXP protease has not been assessed at low temperatures in *E. coli*. Overall, these immunoblots showed that MPG 558 was able to produce RpoS if its degradation was prevented. Northern analysis and immunoblotting experiments have suggested that only two possible roles for the CspA paralogues remain: to facilitate the translation of *rpoS* mRNA or to stabilise RpoS against degradation. However, an indirect effect on RpoS regulation cannot be ruled out.

Further immunoblotting experiments have shown that RpoS is stable in MPG 558 in stationary phase cells for at least 24h, as similarly observed with the parent strain, eliminating a role for the CspA paralogues in protein stability. This is in agreement with previous reports which show a dramatic increase in RpoS levels and stability for both *E. coli* and *S.* Typhimurium on entry into stationary phase (Hengge-Aronis, 1993; Moreno *et al.*, 2000). This increase is mainly achieved by reduced proteolysis (Zhou and Gottesman, 1998; Cunning and Elliott, 1999; Hirsch and Elliott, 2005). This suggests that the CspA paralogues are most likely functioning at the RNA level to facilitate *rpoS* translation, although other indirect possibilities clearly exist.

Finally, *rpoS::lacZ* translational fusions were used to investigate RpoS expression in the MPG 558 and SL1344 *hfq* strains compared to their isogenic parent strain. These results illustrated that there was a 2.6-fold increase in RpoS expression upon entry into stationary phase at 37°C. This is in agreement with studies in *E. coli* which have determined that there is a 4 to 5-fold increase in RpoS synthesis at the onset of starvation (Neidhardt *et al.*, 1987; Lange and Hengge-Aronis, 1994). In accordance with immunoblots (Figure 4.2.3(a)), these findings have also demonstrated an induction of RpoS expression upon cold shock in SL1344. The expression of RpoS at low temperatures has previously been reported for *E. coli* (Sledjeski, Gupta and Gottesman, 1996). This induction is not dependent on DsrA in SL1344, contrary to results reported for *E. coli* (Sledjeski, Gupta and Gottesman, 1996).

Translational fusions also revealed that the RpoS expression from the MPG 558 strain was dramatically reduced during stationary phase at 37°C, compared to the parent strain. In addition, a small induction of RpoS expression was observed for this strain following cold shock in exponential phase, but this was dramatically reduced compared to the parent strain. These findings are in agreement with the reduced level of RpoS detected from this strain under both growth conditions shown earlier in the study. As the CspA paralogues do not play a major role in RpoS protein stability, the role of these proteins would appear to be in facilitating the translation of *rpoS* mRNA. However, the possibility of an indirect role for these proteins in RpoS regulation cannot as yet be ignored.

A sixfold reduction in expression from *rpoS::lacZ* translational fusions was observed for the SL1344 *hfq* strain compared to the parent strain during stationary phase, suggesting that Hfq may be required for the stationary phase induction of RpoS expression in SL1344. This is in agreement with previous immunoblotting experiments showing impaired RpoS expression in the SL1344 *hfq* strains during stationary phase at 37°C (Figure 3.4.2.1). Previous studies, using *rpoS::lacZ* protein fusions in *S.* Typhimurium, have shown that an *hfq* mutant has a

four- to sevenfold reduction in expression of *rpoS* during stationary phase. This can be attributed primarily to a defect in translation as pulse-labeling studies have shown that the rate of RpoS synthesis is significantly decreased in an *hfq* mutant (Brown and Elliott, 1996). However, the present study also showed that a mutation in *hfq* did not affect the activity of the *rpoS::lacZ* fusion following cold shock in exponential phase. This suggests that Hfq is not required for optimal RpoS expression during cold shock in SL1344 and is in agreement with immunoblots showing similar levels of RpoS detected in the SL1344 *hfq* strain compared to the parent strain (Figure 4.2.2(b)). This has also been shown for *S.* Typhimurium by Jones, Goodwill and Elliott (2006). However the finding is in conflict with reports for *E. coli* (Masse *et al*., 2003). Masse *et al*. (2003) have shown that DsrA and RprA act as positive regulators of *rpoS* mRNA translation in *E. coli* at low temperatures and that their action requires Hfq. However, the present study has shown that DsrA and RprA are not required for *rpoS::lacZ* transcriptional fusion expression during cold shock (Figure 4.2.4.2(a)) and is in support of findings by Jones, Goodwill and Elliott (2006). This study has also shown that DsrA is not induced upon cold shock in SL1344. These results point to a role for Hfq in *rpoS* translation during *S.* Typhimurium stationary phase only and not during cold shock of exponential cells.

A post-transcriptional role for Hfq and the CspA paralogues seems logical due to the RNA chaperone activities of these proteins, which is crucial for efficient mRNA translation (Jiang *et al*., 1997, Lease and Woodson, 2004). However, it also remains possible that they may function indirectly. Analysing *de novo* RpoS synthesis, by monitoring ^{35}S incorporation, in the MPG 558 and SL1344 *hfq* strains would confirm the importance of CspA paralogues and Hfq in *rpoS* mRNA translation. From the results in this study, the model for possible roles of CspA paralogues in RpoS regulation, proposed in Section 1.9, has now been amended (Figures 4.5(a) and (b)).

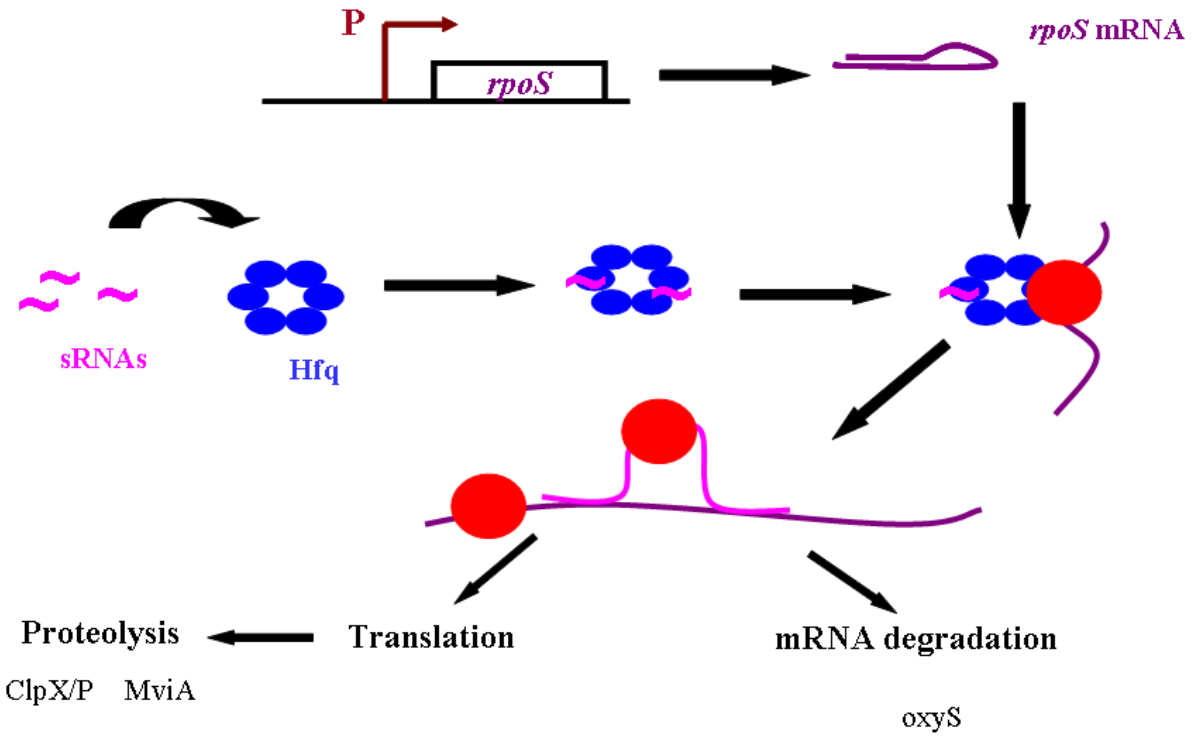


Figure 4.5(a): Amended model for the CspA regulation of RpoS production in SL1344 during stationary phase. Possible binding sites of CspA paralogues are denoted by red circles. A possible indirect role for the CspA paralogues cannot as yet be ruled out.

Key: = CspA paralogues = Hfq = sRNAs

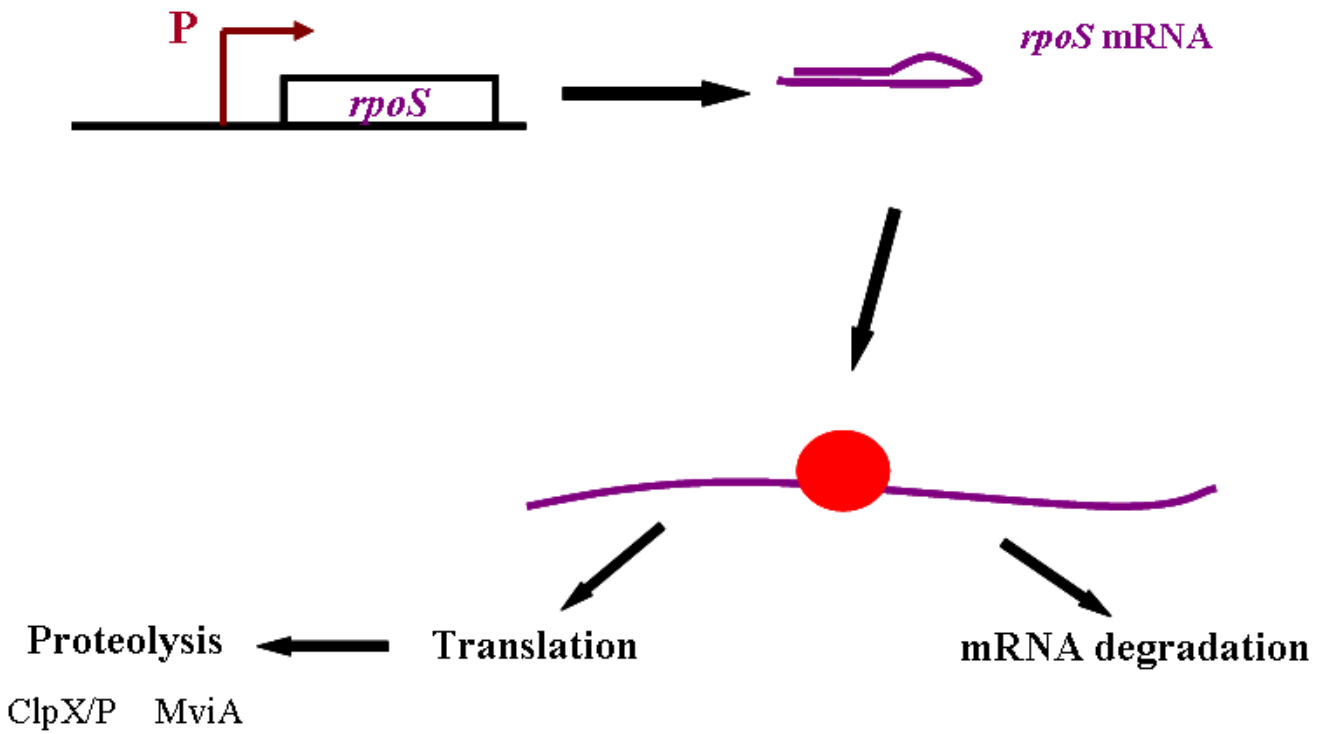


Figure 4.5(b): Amended model for the CspA regulation of RpoS production in SL1344 following cold shock in exponential phase. Possible binding sites of CspA paralogues are denoted by red circles. An indirect role for the CspA paralogues cannot at this point be ruled out.

Key: = CspA paralogues = Hfq = sRNAs

Chapter 5

The importance of *cspA* mRNA to colony formation at 37°C

5.1 Background

As with all good discoveries, the importance of *cspA* mRNA to colony forming ability, was stumbled upon by accident. As described in Chapter 3.2, *rpoS* and/ or *hfq* mutations were introduced, by P22 transduction, to the SL1344 strain in an attempt to further dissect the RpoS regulation pathway. To assess interactions between RpoS and Hfq proteins and the CspA paralogues, these mutations were also transduced into the MPG 558 (SL1344 *csp* null) strain. However the introduction of an *rpoS* mutation into MPG 558 was not possible despite numerous attempts. The SL1344 parent strain was used as a positive control to ensure the experimental procedure was effective and a range of lysate MOIs were used lest MPG 558 was more susceptible to P22 lysis. There are two possible reasons for the failure to introduce a mutation: either the *rpoS* mutation is toxic to the SL1344 *csp* null mutant, or the strain is viable but unable to form colonies. The following chapter is concerned with determining which of these possibilities is the cause and why?

5.2 An SL1344 *csp* null *rpoS* mutant is unable to form colonies at 37°C

A truncated *rpoS* mutation, introduced into the MPG 558 strain, was described previously in detail in Chapter 3.2. The original mutation was constructed by Fang *et al.* (1992) by cloning an internal PCR-derived *Salmonella rpoS* gene fragment (600 bp) into a suicide vector, which was conjugally transferred into ATCC 14028s and selected for resistance to ampicillin. In this study the *rpoS* mutation was introduced to our strains by P22 transduction. DNA sequence analysis confirmed that the resulting RpoS protein was non-functional as the polymerase binding domain was absent (highlighted in Figure 3.2) therefore the protein would be unable to direct transcription machinery.

It is unclear why a mutation in *rpoS* would be toxic to MPG 558 at 37°C, as it has previously been shown (in Chapter 3.4) that this strain is already deficient in RpoS expression and although is unable to survive certain stresses as well as its parent strain, is able to grow readily and reach stationary phase at 37°C. However, Chapter 4.3 has shown that MPG 558 is able to produce a level of RpoS when the RpoS degradation pathway is disrupted by mutations in *clpP*, *mviA* or *hns* and so MPG 558 is not fully defective in its capacity for RpoS

production. Traditionally, cell viability was determined by the ability of a strain to produce colonies. More recently, however, studies have revealed both Gram positive and Gram negative bacteria that have the ability to enter a viable but non-culturable (VBNC) state to protect themselves against environmental stresses and starvation (Brogosian and Bourneuf, 2001; Besnard, Federighi and Cappelier, 2000). At this time, cells are still viable and show metabolic activity but cannot be detected by conventional colony forming ability via plate counts (Roszak and Colwell, 1987; Bloomfield *et al*., 1998; Colwell and Grimes, 2000). VNBC cells have the ability to be resuscitated given the correct stimuli (Roszak and Colwell, 1987; Nilsson, Oliver and Kjelleberg, 1991). This could have serious medical consequences as pathogens that cannot be detected in potential sources or reservoirs of infection may still be able to initiate infection (Smith *et al*., 2002). Is it that the MPG 558 *rpoS* strain is viable but lacks colony forming ability?

An example of the VBNC phenomenon occurs with *E. coli rne* mutants, which cannot be cultured as the strain is unable to form colonies on solid medium; however is able to grow in liquid medium as filaments. RNase E plays an essential role in the maturation of mRNA and in mRNA lifetimes, and therefore has a direct impact on levels of protein synthesis. Thus, both the overproduction and underproduction of this protein can have negative effects on cell growth (Apirion, 1978; Goldblum and Apirion, 1981; Claverie-Martin *et al*, 1991). Cell morphology and size distribution experiments (in Chapter 3.3.3) have shown that MPG 558 forms minicells, suggesting that this strain may exhibit increased levels of FtsZ. An increase in FtsZ levels can be caused by increased activity of RNase E, as demonstrated by *E. coli hfq* mutants, which results in increased cleavage of the *ftsQAZ* transcript and therefore more efficient translation of FtsZ (Tamura *et al*., 2006). Is there a relationship between RpoS and RNase E or FtsZ that could explain the failure to be able to produce a MPG 558 *rpoS* mutant?

An extensive literature search has found no link between RNase E and RpoS. In *E. coli*, RNase III alone appears to be responsible for *rpoS* mRNA turnover (Resch *et al*., 2008). Mutational studies of the *S.* Typhimurium *rpoS* leader have shown an increase in RpoS expression due to inhibition or absence of RNase III cleavage. Also, the stabilisation of full length *rpoS* transcript occurs in a *S.* Typhimurium *rnc* (RNase III) mutant (Resch *et al*., 2008).

However, there is a link between RpoS and FtsZ levels, as one of the promoters that control transcription of the *ftsQAZ* region is RpoS-inducible; this is the main reason why cells are at their shortest during stationary phase when RpoS expression is highest (Aldea *et al*., 1990; See Figure 5.2). Takada, Nagai and Wachi (2005) have shown that decreased levels of FtsZ,

an essential cell division protein, results in cell filamentation and the loss of colony forming ability. This is due to a perturbation in the FtsZ/FtsA ratio essential for septation (Tamura *et al*., 2006). Vinella and D'ari (1994) reported that an *E. coli rpoB369* mutant, which contains an RNA polymerase which is less sensitive to ppGpp, exhibits a block in cell division at non-permissive temperature, similar to that of an FtsZ$^-$ phenotype. This block is relieved by either the overexpression of FtsQAZ, an increase in ppGpp concentration or by entry into stationary phase. The latter two conditions cause an induction of RpoS, which would subsequently result in an increase in transcription of the *ftsQAZ* transcript.

However, cell morphology experiments in Chapter 3.3.3 have alluded to an increase in FtsZ levels in MPG 558, despite exhibiting reduced RpoS expression. As hypothesized in Chapter 3, the formation of minicells, observed in the MPG 558 strain, may be due to an apparent increase in RNase E activity, caused by reduced target availability (*cspA* and *cspB* mRNA), and therefore increased cleavage of the *ftsQAZ* transcript. The reduction in FtsA and FtsZ levels, caused by the *rpoS* mutation, cannot be directly responsible for the inability to produce an MPG 558 *rpoS* mutant because a wildtype strain, with naturally lower FtsZ levels than MPG 558, is able to withstand an *rpoS* mutation.

CspA paralogues are RNA chaperones that melt mRNA secondary structure. RNase E has broad cleavage site specificity characterised by single-stranded regions of RNA that are AU rich adjacent to elements of secondary structure (Diwa *et al*., 2002). Feng *et al*. (2001) report that the addition of *E. coli* CspE inhibits endonucleolytic digestion by RNase E, as CspE melts mRNA secondary structure and therefore eliminates recognition sites for the enzyme. Is the absence of CspE in MPG 558 responsible for an increase in RNase E activity, and therefore the apparent increase in functional FtsZ?

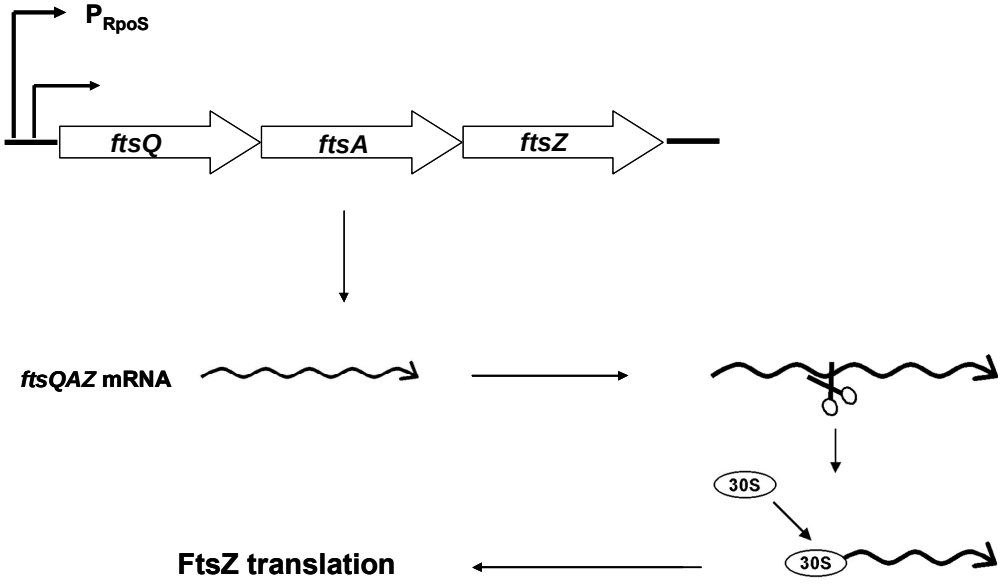


Figure 5.2: RpoS-inducible transcription of the *ftsQAZ* operon (figure adapted from Takada, Nagai and Wachi, 2005). RpoS is responsible for around 50% of the transcription of this region during stationary phase. The full length transcript must be cleaved by RNase E to allow efficient translation of FtsZ and FtsA. The scissor icon represents RNase E.

5.2.1 A mutation in *hfq* compensates for the deleterious effect of an *rpoS* mutation

As stated earlier (in Chapter 3.2), an *hfq* mutation was introduced into MPG 558 to aid in the dissection of the RpoS regulation pathway. The construction of a double *hfq rpoS* mutant in MPG 558 would be important for providing further insight into whether the component proteins were interacting directly or sequentially? Intriguingly, the current situation found that an *rpoS* mutation could be successfully introduced into MPG 558 if an *hfq* mutation was introduced first, i.e. the *hfq* mutation somehow compensates for the deleterious effect of the *rpoS* mutation. Results of the experiment to introduce an *rpoS* mutation to the strains mentioned are summarised in Table 5.2.1.

Table 5.2.1: **A summary of the success/ failure of introducing an *rpoS* mutation to different strains.** The results reported are the average of triplicate experiments. Results of special interest are highlighted in **bold** text. The transductions were carried out in parallel, with the same P22 lysates, to ensure the experimental procedure was effective. A range of lysate MOIs was tested.

Strain	Colonies?	No. of transductants		
		10^7 pfu	10^8 pfu	10^9 pfu
SL1344 *rpoS*	Yes	15 ± 4	50 ± 8	12 ± 2
SL1344 *hfq rpoS*	Yes	13 ± 2	42 ± 5	11 ± 1
MPG 558 *rpoS*	**No**	**0**	**0**	**0**
MPG 558 *hfq rpoS*	**Yes**	**7 ± 3**	**16 ± 6**	**4 ± 1**

There is a well characterised link between Hfq and RpoS in *Salmonella*. Studies using *rpoS::lacZ* protein fusions in *S.* Typhimurium have shown that an *hfq* mutant has a four- to sevenfold reduction in expression of *rpoS* during stationary phase at 37°C. This is attributed primarily to a defect in translation as pulse-labeling studies have shown that the rate of RpoS synthesis is significantly decreased in an *hfq* mutant (Brown and Elliott, 1996). In the previous chapter, *rpoS::lacZ* translational fusions have demonstrated that the SL1344 *hfq* strain has a sixfold reduction in expression of *rpoS* during stationary phase (Figure 4.4). In addition, immunoblots in Chapter 3.4 have also shown that a SL1344 *hfq* strain has reduced levels of RpoS during stationary phase at 37°C. However, this seems unlikely to be the reason why it was possible to introduce an *rpoS* mutation into the MPG 558 *hfq* strain.

Another well established link is that between Hfq, RNase E and FtsZ. Studies have found that, in *E. coli*, Hfq copurifies with RNase E to form specialised ribonucleoprotein complexes, showing that Hfq can facilitate RNA degradation by targeting mRNAs to RNase E (Morita,

Maki and Aiba, 2005; Brennan and Link, 2007). However, Folichon *et al*. (2003) reported that Hfq protects the *ftsQAZ* transcript from RNase E degradation in *E. coli*; a mutation in *hfq* leads to the formation of minicells due to increased cleavage of the *ftsQAZ* transcript, and therefore more efficient translation of FtsZ and FtsA. Takada, Nagai and Wachi (2005) have shown that the introduction of an *hfq::cat* mutation suppresses the temperature-sensitive colony formation of the *rne-1* mutant under non-permissive conditions.

Figure 5.2.1 shows the regulation of FtsZ translation by Hfq and RNase E. This suggests that in a system lacking Hfq, there would be more efficient translation of the *ftsQAZ* transcript, which may compensate for the reduction in transcription of this region caused by a mutation in *rpoS*. At this point, it is still unclear how the CspA paralogues are involved in this system. The hypothesis that CspA paralogues reduce RNase E activity by melting mRNA secondary structure and therefore eliminating RNase E recognition/cleavage sites is still possible.

Increased RNase E activity is known to be detrimental to cell growth as it plays a crucial role in mRNA maturation and mRNA lifetimes, which has a direct impact on general protein synthesis. Therefore, in a system lacking Hfq, which plays a dual role in RNase E activity as it has also been shown to target RNA to the degradosome (Morita, Maki and Aiba, 2005; Brennan and Link, 2007), the decay rate of bulk mRNAs will be reduced allowing for more efficient translation. Is it just that the introduction of an *hfq* mutation redresses the balance of RNase E activity and general RNA degradation towards 'normal' in the MPG 558 *rpoS* system? Further experiments will help to answer this question and determine the role for Hfq, RpoS and CspA paralogues in this system.

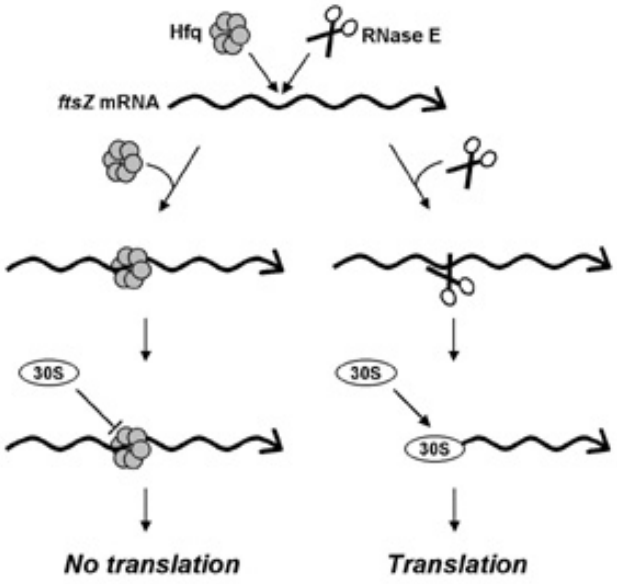


Figure 5.2.1: Regulation of FtsZ translation by Hfq and RNase E (figure taken from Takada, Nagai and Wachi, 2005). Hfq competes with RNase E to protect the *ftsQAZ* transcript from cleavage and in turn prevents efficient translation of FtsZ and FtsA.

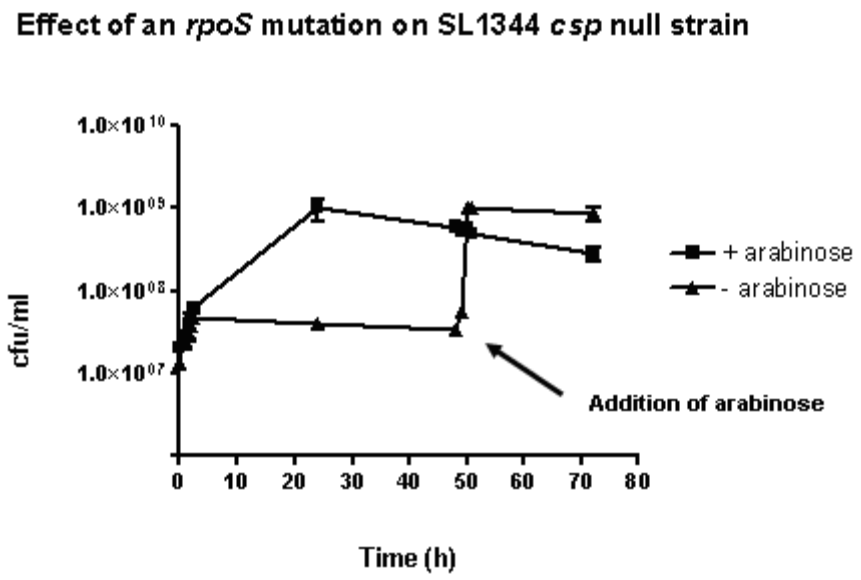
5.2.2 A conditional *rpoS* mutation in the SL1344 *csp* null mutant shows that the strain is viable but cannot form colonies

It is essential to determine whether the *rpoS* mutation is toxic to the MPG 558 strain, or simply that the strain is unable to form colonies. A plasmid that permits the inducible over-expression of RpoS in the presence of arabinose, pUBAD::*rpoS*, was used to compensate for the chromosomal *rpoS* mutation and allowed a means for the conditional production of *rpoS* in an MPG 558 *rpoS* strain. This provided a means for the successful introduction of a chromosomal *rpoS* mutation and was used to assess the effect of the *rpoS* mutation when plasmid *rpoS* was not induced. The question could then be asked whether the cells die or filament. Also, what will happen to cell morphology and cell numbers once RpoS is switched back on?

The plasmid, pUBAD::*rpoS* (Volkert *et al*., 1994), was introduced to the MPG 558 strain by electroporation as described in Chapter 2.2.1 and selected for on LB agar containing tetracycline. The MPG 558 pUBAD::*rpoS* strain was grown to stationary phase (for 16h) in LB containing 2% (w/v) arabinose, at 37°C with aeration. This is a 100-fold higher concentration of arabinose than is used standardly for promoter induction because *S.* Typhimurium can catabolise arabinose. The chromosomal *rpoS* mutation was then introduced to this strain by P22 transduction, from the SL1344 *rpoS* strain, as described in Chapter 2.2.1 and colonies were selected for on LB agar containing tetracycline and 2% arabinose. Once the strains had been passaged to remove excess P22 phage, cells were grown to stationary phase in LB containing 2% arabinose (for 16h at 37°C with aeration), harvested and washed in PBS to remove the arabinose. Cells were then resuspended in LB medium to a final concentration of 2 X 10^9 cfu/ml. Following this, 2 X 10^7 cells/ml were added to LB containing arabinose (2%) and 2 X 10^7 cells/ml added to LB without arabinose. After a period of 48h, 2% arabinose was added to the culture grown previously without arabinose. The cfu/ml was measured over time, by plating dilutions on LB agar containing arabinose, and is shown in Figure 5.2.2(a). The corresponding cell lengths, at each time point, were also measured using Phase contrast microscopy to assess what was happening to the strain (Figure 5.2.2(b)).

Figure 5.2.2(a) shows that the cfu/ml of MPG 558 *rpoS* (pUBAD::*rpoS*) grown in media containing arabinose, i.e. where the chromosomal *rpoS* mutation is compensated for, behaves similarly to MPG 558 when diluted into fresh LB and grown with aeration at 37°C. The graph shows that the plasmid containing strain reaches exponential phase within 2h, as illustrated by the dramatic increase in the cfu/ml, and reaches stationary phase by 24h, whereupon there is no further increase in cfu/ml. This follows a similar growth pattern to that of MPG 558.

A



B

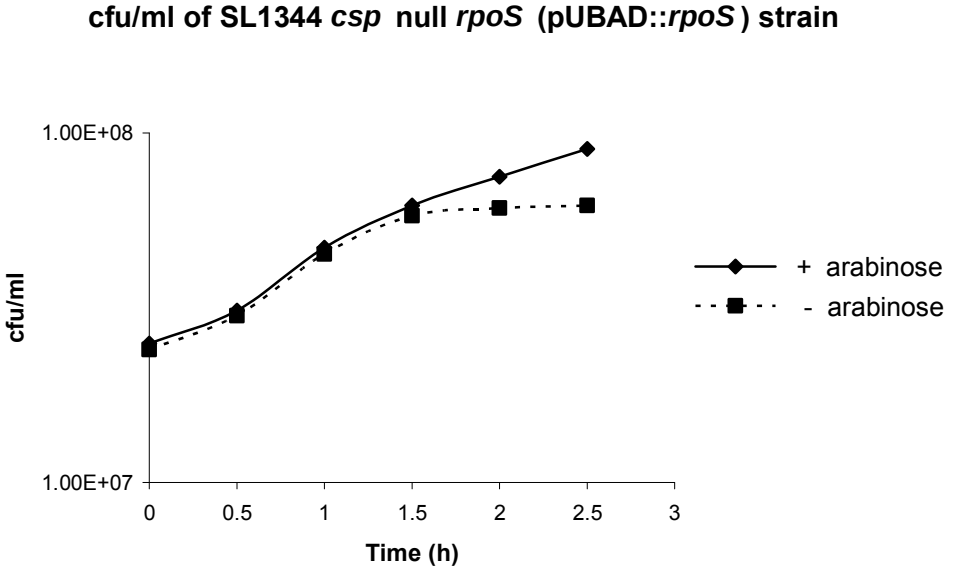


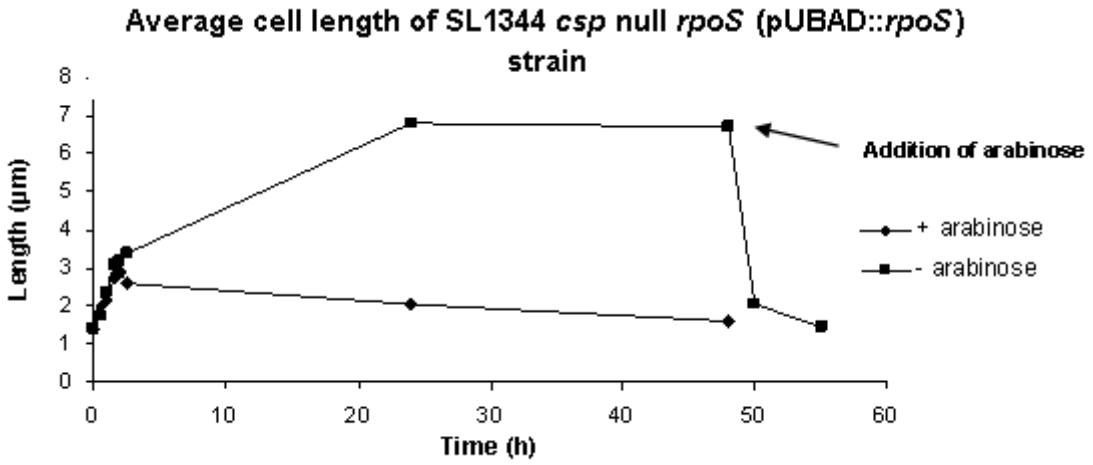
Figure 5.2.2(a): **Graphs showing how the cfu/ml of the SL1344 *csp* null *rpoS* (pUBAD::*rpoS*) conditional mutant compares in the presence or absence of arabinose (and therefore RpoS)**. Cells were grown to stationary phase in LB containing 2% arabinose (for 16h at 37°C with aeration), harvested and washed in PBS to remove the arabinose. Cells were then resuspended in LB and 2 X 10^7 cells/ml were added to LB with or without arabinose. Both cultures were incubated at 37°C, with aeration, for the length of the experiment. After a period of 48h, 2% arabinose was added to the culture grown previously without arabinose. **A** The cfu/ml was measured over time, by plating dilutions on LB agar containing 2% arabinose. The point at which arabinose is added to the strain is indicated with an arrow. The experiment was repeated in triplicate, with separate inocula, and the average result is displayed on the graph. Error bars indicate variation between experiments. **B** Enlarged version of the exponential growth phase of the culture.

Figure 5.2.2(b) shows the relative cell size for this strain at each time point. There is in an increase in the average cell size from 1.6µm to 2.9µm in the first 2h, which reconciles with exponential growth as the cells must reach double cell length before dividing. This coincides with what has previously been described for *E. coli* as the average cell size increases as an exponential function of growth rate (Donachie, 1968). In general, the cells never elongate to more than double the average cell length of the original cells on dilution to fresh LB. After the 2h time point, the average cell size begins to reduce from 2.9µm to 1.8µm (at 24h), consistent with the reduction in growth rates observed as the cells approach stationary phase. Akerlund, Nordstrom and Bernander (1995) have shown that, in rich media, *E. coli* cell size started to decrease long before the cultures left the exponential growth phase. In the present study, the cells have returned to 1.8µm, a similar cell size to that of the cells prior to dilution into fresh medium (1.6µm), by 24h. Lange and Hengge-Aronis report that size reduction in *E. coli* cells along the growth curve is a biphasic process (1991) which is also observed with this strain of *S.* Typhimurium as the cells are at their largest (2.9µm) 2h after dilution into fresh LB but only return to their original size on entry into stationary phase. On the whole, the MPG 558 *rpoS* (pUBAD*::rpoS*) strain, grown in LB containing arabinose, behaves as the MPG 558 strain at 37°C under the parameters examined.

A different story was observed for the MPG 558 *rpoS* (pUBAD::*rpoS*) strain grown in LB lacking arabinose, i.e. lacking *rpoS*. To calculate the cfu/ml for each time point, serial dilutions were plated onto LB agar plates containing 2% arabinose. Figure 5.2.2(a) shows that, in the first 1.5h following dilution into fresh medium, there is an initial increase in cfu/ml of the strain equal to that of the strain grown in medium containing arabinose. However, following this time point, there is a plateau in the cfu/ml, at around $6.0x10^7$ cells, a much lower final cell density than observed with the strain grown in arabinose. However, there is no reduction in the cfu/ml, even after 48h, suggesting that the *rpoS* mutation is not toxic to the strain but seems to force the cells into a form of 'dormancy'. Following the addition of 2% arabinose at 48h, the cfu/ml dramatically increases within 1.5h to $2.0x10^9$ i.e. that of a stationary phase culture. This increase in cfu/ml corresponds to around 5 doublings, which in an exponentially growing MPG 558 strain should take around 3h (as calculated from doubling times reported in Chapter 3.3.1). How can the cfu/ml double faster than the doubling time of the strain?

Figure 5.2.2(b) tracks the average cell length over the period of the experiment. For the first 1.5h after dilution into fresh medium, the increase in cell size mirrors that of the MPG 558 *rpoS* strain when grown in media containing arabinose. This coincides with the increase in cfu/ml seen with this strain in the first 1.5h. After this time point the cfu/ml plateaus; however

A



B

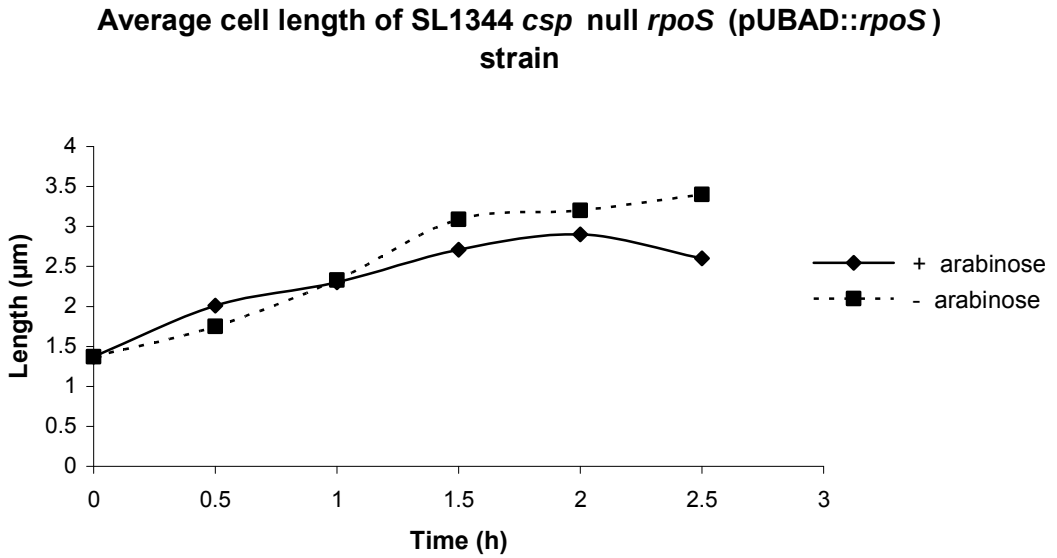


Figure 5.2.2(b): **Graphs showing the average cell length of the strain, in the presence or absence of arabinose, at each time point throughout the experiment.** Cells were grown to stationary phase in LB containing 2% arabinose (for 16h at 37°C with aeration), harvested and washed in PBS to remove the arabinose. Cells were then resuspended in LB and 2 X 10^7 cells were added to LB with or without arabinose. Both cultures were incubated at 37°C, with aeration, for the length of the experiment. After a period of 48h, 2% arabinose was added to the culture grown previously without arabinose. **A** 200 cells were measured at each time point, evenly chosen from three separate inocula, and the average results are plotted. The point at which arabinose is added to the strain is indicated with an arrow. **B** Enlarged version of the cell length during exponential phase growth of the cultures.

the average cell size continues to increase, from 2.3µm at 1.5h to 3.4µm after 2.5h, suggesting that the cells are elongating. The average cell size after 2.5h (3.4µm) corresponds to around 2.5 cell lengths (1.4µm). Cells elongate by mass doubling, therefore must undergo DNA replication; however in this case there is no septation and consequently no cell division and no increase in colony forming units. This type of phenomenon has been observed previously by Leroy *et al.* (2002) who reported that their conditional *E. coli rne131* strain was significantly filamentous under restrictive conditions. In the latter study, staining of the cells with DAPI showed DNA replication and partitioning of the nucleoid appeared normal. However, the cells failed to form septa and divide. Goldblum and Apirion (1981) also reported that there was no increase in the number of colony forming units during the 3h after the shift of the *E. coli rne* temperature-sensitive mutant to the non-permissive temperature of 43°C despite the corresponding threefold increase in the optical density of the culture. Subsequent microscopic examination revealed that there was a doubling in average cell length and the *E. coli* cells seemed to grow as filaments. 24h after dilution of MPG 558 *rpoS* (pUBAD::*rpoS*) strain into LB without arabinose, the maximum average cell size is reached of 6.8µm, or approximately 5 times the original cell size (1.4µm). No further increase in cell size occurred following this time point (24h). Phase contrast images of this strain during stationary phase (16h) at 37°C are shown in the following section (panel **B**, Figure 5.2.3). After 48h, 2% arabinose was added to the culture to induce the expression of *rpoS*. Within 2h of this point, the average cell size returned to 2µm, a similar size to that of the strain at stationary phase when grown in arabinose (1.6µm). After a further 5h, the MPG 558 *rpoS* cell size had returned to that of the original cell size at time zero.

Figure 5.2.2(a) shows an increase in cfu/ml equal to 5 doublings, after the addition of arabinose to the MPG 558 *rpoS* (pUBAD::*rpoS*) culture. Figure 5.2.2(b) shows a corresponding 5-fold increase in average cell length from that on dilution to fresh medium (1.4µm) to after 24h of growth (6.8µm). This suggests that the increase in cfu/ml on addition of arabinose occurs due to septation at potential division sites along the filament rather than due to a normal DNA replication and division cycle. This phenomenon has been reported previously after a period of division inhibition in *E. coli*, as wild-type filaments re-divided rapidly, ultimately forming normal-sized cells (Jaffe, Boye and D'Ari, 1990).

This experiment confirms that the *rpoS* mutation is not toxic to the MPG 558 (SL1344 *csp* null) strain but renders this strain viable but non culturable. This strain, like other reported VBNC strains, has the ability to be resuscitated given the correct stimuli (Roszak and Colwell, 1987; Nilsson, Oliver and Kjelleberg, 1991), i.e. in this case, if *rpoS* is re-introduced. In the absence of *rpoS*, these cells are able to elongate by mass doubling and must

undergo normal nucleoid partitioning as they are able to divide immediately on expression of *rpoS*. The limitation in ability to divide therefore must lie in septation and invagination of the peptidoglycan. The driving force in this process is FtsZ, which polymerises to form the scaffold for the recruitment of the other cell division proteins required for constriction of the FtsZ-A ring and cytokinesis to form two daughter cells (Bi and Lutkenhaus, 1990; Garriso *et al*., 1993). As mentioned previously, the promoter responsible for the majority of transcription of the *ftsQAZ* region is RpoS-inducible. It has also been shown that Hfq prevents the efficient translation of this transcript. Therefore the question could be asked, does over-expressing FtsZ and FtsA compensate for the lack of colony forming ability in an *rpoS* mutant? This question is addressed in the following section.

5.2.3 Overexpression of FtsQ, A and Z can compensate for the effect that an *rpoS* mutation has on the colony forming ability of MPG 558

Cam *et al*. report that in *E. coli* there is a doublet site for RNase E cleavage in the *ftsA-ftsZ* intergenic region (1996). Cleavage at these sites would alter the stability of the resulting mRNA and therefore affect the ratio of the resulting protein products. An FtsZ/FtsA ratio of approximately 50:1 is essential for septation to occur; an excess of either protein results in a division block that is relieved by induction of the other protein (Joseleau-Petit, Vinella and D'ari, 1999). FtsZ rings can form at division sites in the absence of FtsA but cannot contract. Expression of the *ftsQ* gene is not required to produce divisions (Donachie, 1968).

Experiments have shown that although an MPG 558 *rpoS* mutant is able to grow in liquid broth, it is unable to form colonies on solid medium. Furthermore, results (in Section 5.2.2) have suggested that the lack of colony forming ability in this strain may be due to reduced levels of FtsZ (and FtsA). Does the overexpression of FtsQ, FtsA and FtsZ rectify this problem, or is the story more complex? As mentioned in Chapter 3.3, the plasmid pZAQ (Bi *et al*., 1990) results in the constitutive overexpression of the essential cell division proteins: FtsQ, FtsA and FtsZ (Ward and Lutkenhaus, 1985) and results in minicell formation in SL1344. pZAQ was introduced into MPG 558 by transformation, as described in Chapter 2.2. The presence of the pZAQ plasmid was found to permit the subsequent introduction of an *rpoS* mutation into the MPG 558 by P22 transduction, from the SL1344 *rpoS* strain. Phase contrast microscopy was used to examine the cell morphology of this strain in liquid broth to assess the effect of the *rpoS* mutation.

MPG 558 is naturally able to form minicells, as observed in Chapter 3.3.3; however no minicells were observed after the subsequent introduction of an *rpoS* mutation to the MPG 558 (pUBAD::*rpoS*) strain. The MPG 558 (pUBAD::*rpoS*) strain had unaltered morphology compared to its parent strain when grown in LB at 37°C. Instead, the introduction of an *rpoS* mutation to MPG 558 (pUBAD::*rpoS*) caused extensive filamentation due to faulty cell division (as shown by the increase in average cell length described in the previous section). Figure 5.2.3 shows phase contrast images of the aberrant cell morphology of the MPG 558 (pUBAD::*rpoS*) *rpoS* strain and how this is rectified by the addition of the pZAQ plasmid. The introduction of the pZAQ plasmid dramatically reduces the frequency of filaments caused by the *rpoS* mutation, as the strain is now capable of cell division.

This experiment shows that pZAQ compensates for the lack of colony forming ability caused by an *rpoS* mutation in MPG 558. There are two possible explanations for this. Firstly, the plasmid-induced increase in the FtsQ, A and Z proteins could compensate for the deficit caused by decreased RpoS-dependent transcription of the *ftsQAZ* operon. Or secondly, the plasmid-induced overexpression of the *ftsQAZ* transcript provides extra targets for RNase E to compensate for the apparent increased activity of the enzyme in MPG 558, due to decreased target availability (*cspA* and *cspB* mRNA), demonstrated by minicell formation (See Chapter 3.3.3). At this point it is unclear which of these two processes is responsible for the restoration of colony forming ability. However, investigating whether any or all of the CspA paralogues can compensate for the lack of colony forming ability may give further insight into the process.

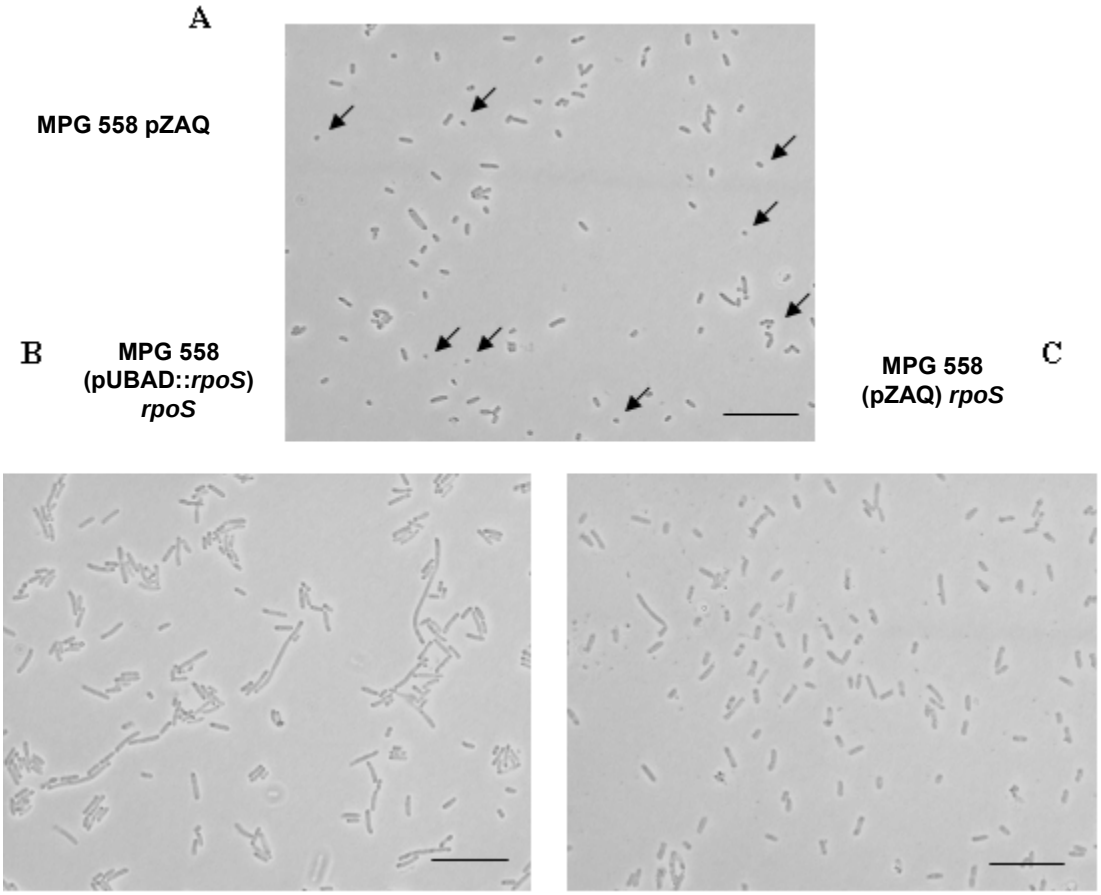


Figure 5.2.3: Phase contrast images of the three strains during stationary phase (24h) grown in LB at 37°C. **A** Cell morphology of the MPG 558 strain containing the pZAQ plasmid. Some minicells are apparent and are highlighted by arrows **B** Extensive filamentation can be seen in the MPG 558 (pUBAD::*rpoS*) strain carrying the chromosomal *rpoS* mutation when grown in LB without arabinose. **C** The pZAQ plasmid returns the cell morphology to 'normal' and restores colony forming ability, i.e. allows cell division. Images are representative of at least three experiments. Scale bars represent 10µm.

5.3 *cspA* and *cspB* mRNA mediates colony forming ability of an *rpoS* mutant

So far a mutation in *hfq* and the introduction of pZAQ, a plasmid that constitutively over-expresses FtsQ, FtsA and FtsZ, have restored the colony forming ability of the MPG 558 *rpoS* mutant. RpoS induces the transcription of the *ftsQAZ* operon and Hfq protects this transcript from RNase E cleavage. Cleavage of this transcript is required for efficient translation of the proteins and a critical ratio of FtsZ: FtsA (of 50:1) is required for cell division to proceed. Previous results (Figure 3.3.3.1(a) and Chapter 3.3.3) have pointed to an increase in FtsZ levels in the MPG 558 strain, resulting in minicell formation. This is usually a consequence of increased RNase E activity (Folichon *et al*., 2003; Takada, Nagai and Wachi, 2005). Feng *et al*. (2001) report that the addition of *E. coli* CspE inhibits endonucleolytic digestion of poly(A)-tails by RNase E *in vitro*. Together, these findings suggest that the CspA paralogues may protect mRNA against RNase E cleavage by melting the mRNA secondary structure that serves as a recognition site for the enzyme. Is this in fact the case? Is one, or all, of the CspA paralogues required for colony formation in a MPG 558 *rpoS* strain? This question is addressed in the following section.

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5.3.1 Only the CspA and CspB monogene strains are able to form colonies when carrying a mutation in *rpoS*

To test which of the CspA paralogues are responsible for the loss in colony forming ability of an MPG 558 *rpoS* mutant, the *csp* monogene strains (MPG 551 to MPG 557), with all but one of the CspA paralogues deleted, were used as parent strains. P22 transduction was used, as before, to introduce the *rpoS* mutation (from SL1344 *rpoS*) into these strains and the colony forming ability was assessed on solid LB agar at 37°C. Table 5.3.1 summarises the outcome of this experiment.

Surprisingly, it was found that *rpoS* mutations in MPG 551 (CspA) and MPG 552 (CspB) monogene strains were able to form colonies when plated on LB agar; however, *rpoS* mutations in the other monogene strains (MPG 553 (CspC), MPG 554 (CspD), MPG 557 (CspE) and MPG 556 (CspH)) were not. This shows that the previous hypothesis, that the CspA paralogues melt mRNA secondary structure and thereby protect against RNase E cleavage, is not the appropriate explanation in this instance because an MPG 557 (CspE) *rpoS*

mutant is unable to form colonies. As mentioned in Chapter 1.6, these proteins are highly related, so why aren't they interchangeable in this instance?

Figure 5.3.1: Alignment of the *cspA* 5'UTR sequences of *E. coli* K12 and SL1344. The DNA sequences are 92% identical between the two species. The start codon of *cspA* is highlighted in **bold**. The RNase E site on K12 (as determined by Fang *et al*., 1997) is highlighted in yellow. This sequence is largely conserved in SL1344. The regions that form stems are underlined. The base deletion in the *E. coli* K12 strain within the RNase E site is in a loop region therefore has no effect on secondary structure.

Table 5.3.1: Summary of the colony forming ability of *csp* monogene *rpoS* mutants on LB agar at 37°C. Results quoted were the average of triplicate experiments, the variation in each experiment set is quoted. Results of special interest are highlighted in **bold** text.

Parent strain	Genotype	Can an rpoS mutant form colonies	No. of transductants		
			10^7 pfu	10^8 pfu	10^9 pfu
SL1344	**SL1344**	**Yes**	**15 ± 2**	**50 ± 9**	**12 ± 3**
MPG 551 (A)	**SL1344 Δ *csp B C D E H***	**Yes**	**5 ± 2**	**13 ± 4**	**1 ± 2**
MPG 552 (B)	**SL1344 Δ *csp A C D E H***	**Yes**	**4 ± 1**	**7 ± 3**	**3**
MPG 553 (C)	SL1344 Δ *csp A B D E H*	No	0	0	0
MPG 554 (D)	SL1344 Δ *csp A B C E H*	No	0	0	0
MPG 557 (E)	SL1344 Δ *csp A B C D H*	No	0	0	0
MPG 556 (H)	SL1344 Δ *csp A B C D E*	No	0	0	0
MPG 558 (null)	SL1344 *csp* null	No	0	0	0

Although SL1344 CspA and CspE proteins are around 80% identical at the amino acid level, greater differences exist between the two operons at the RNA level. Both CspA and CspB have a 5'UTR, containing a putative RNase E cleavage site, which is not present in the other CspA paralogues of SL1344. Fang, Xia and Inouye (1999) have shown that *E. coli cspA* is always highly transcribed at 37°C but the mRNA is unstable at this temperature. Fang *et al*. (1997) have shown that this instability depends on the presence of an AT-rich RNase E site at the ribosome binding site, as an A to G point mutation in this area allows constitutive expression of the CspA protein at 37°C. Sequence alignment shows that this sequence is also present in the 5'UTR of SL1344 *cspA* (Figure 5.3.1). There are two single base differences between the species within this region; however, these do not disrupt the extensive AT region necessary for RNase E recognition. Craig *et al.* (1998) have shown that SL1344 *cspB* mRNA is also highly unstable at 37°C but stabilises as the temperature is decreased. Does this mean the differences observed in the colony forming ability of the monogene *rpoS* mutants could be mediated by the mRNA and the presence of the RNase E cleavage site in the 5'UTR?

5.3.2 *cspA* mRNA and not protein mediates the colony formation of an MPG 558 *rpoS* mutant

To test the hypothesis that the 5'UTR is important for mediating colony forming ability in an MPG 558 *rpoS* mutant, new strains were constructed and the impact of their genetic composition on the formation of *rpoS* mutants, was tested as before. Results have already shown that the MPG 552 (CspB) monogene strain can form colonies after the introduction of an *rpoS* mutation but that the MPG 557 (CspE) monogene strain could not. As discussed previously, a major difference between *S.* Typhimurium CspB and CspE is at the mRNA level, therefore does this mean that a functional protein is not required?

In previous studies in the lab, an SL1344 *cspB*::Mu*dlux* fusion strain was constructed which produces a *cspB* transcript containing the RNase E site, i.e. the 5'UTR is intact, but results in a non-functional CspB protein, with only 24 amino acids of CspB (Craig *et al*. 1998). This *cspB::*Mu*dlux* was transduced into the MPG 557 ($CspE^+$ monogene) strain. The resulting strain was found to permit subsequent successful introduction of the *rpoS* mutation by P22 transduction from the SL1344 *rpoS* strain. An isogenic strain MPG 625 (SL1344 Δ *csp* ACDH; $cspB^+$ and $cspE^+$) was used as an isogenic comparison strain for this experiment and an *rpoS* mutation was also successfully introduced to this strain. The fact that, following P22 transduction of *rpoS*, plating ability is not exhibited by a $cspE^+$ strain but occurs in the same genetic background in the presence of either $cspB^+$, or the *cspB::*Mu*dlux*, suggests that it is

the *cspB* mRNA and not the protein that modulates the colony forming ability of a *csp* null *rpoS* mutant. However, to clearly validate the result, the experiment required to be confirmed in a strain background free from functional Csp paralogues, i.e. in MPG 558. Unfortunately, both MPG 558 and the *cspB*::Mu*dlux* fusion carry kanamycin resistance therefore are incompatible in their current state.

To remove the kanamycin resistance gene (flanked by FRT sites) from MPG 558, the pCP20 helper plasmid (Merlin, McAteer and Masters, 2002), containing a FLP recombinase and ampicillin resistance, was transformed into this strain by electroporation. The plasmid was selected for on LB agar containing ampicillin at 30°C, a temperature at which the plasmid replicates but does not express the recombinase. Colonies were streaked on LB agar (without Amp) and incubated at 42°C to simultaneously cure the plasmid and excise the kanamycin marker at the FLP recognition sites (method developed by Cherepanov and Wackernagel, 1995). The MPG 558 strain successfully lost the kanamycin resistance gene, as confirmed by PCR (Figure 5.3.2 panel A), but remained ampicillin resistant in spite of 24h continuous exponential growth in LB at 37°C in the absence of Amp selection. The plasmid pCP20 could also be recovered from the strain (Figure 5.3.2 panel B) and therefore had not integrated into the chromosome. However, the strain was still of no use as ampicillin resistance is used to select for successful transduction of the *rpoS::bla* mutation. Therefore, it was decided to alter the antibiotic resistance of the *cspB*::Mu*dlux*.

Strain CH463, a *Salmonella galE* mutant, was used as a recipient strain as it has both P1 and P22 sensitivity (Hiles, Gallagher, Jamieson and Higgins, 1987). For P22 transduction, media for growth is supplemented with 0.4% galactose and 0.4% glucose to ensure the presence of P22 phage receptors on the host cells. These sugars are eliminated from the growth medium when P1 phage receptors are required. Strain CH463 was grown to stationary phase in LB, supplemented with galactose and glucose, and the *cspB::*Mu*dlux* fusion was introduced by P22 transduction (as described in Chapter 2.2). Following this, a P1 Tn9 *clr* 100 lysate was made (using a method adapted from Silhavy *et al*, 1984; Chapter 2.2) from the donor strain, a modified *E. coli* MC4100 which contains a tetracycline resistant mini Mu*dlux* on the chromosome. The *galE cspB*::Mu*dlux* strain was then grown to stationary phase in LB, without sugar supplement, and used as a recipient strain for P1 transduction from the modified MC4100 strain. Recombination of the Mu*dlux* was screened for by selecting for resistance to tetracycline and subsequently screening of colonies for sensitivity to kanamycin. Colonies carrying the *cspB*::Mu*dlux* fusion were also observed to be luminescent when placed in the cold room. Transfer of the *cspB*::Mu*dlux* (Tet^R) fusion to MPG 558 was achieved by P22 transduction from the *galE cspB*::Mu*dlux* (Tet^R) strain. This provided a clean background

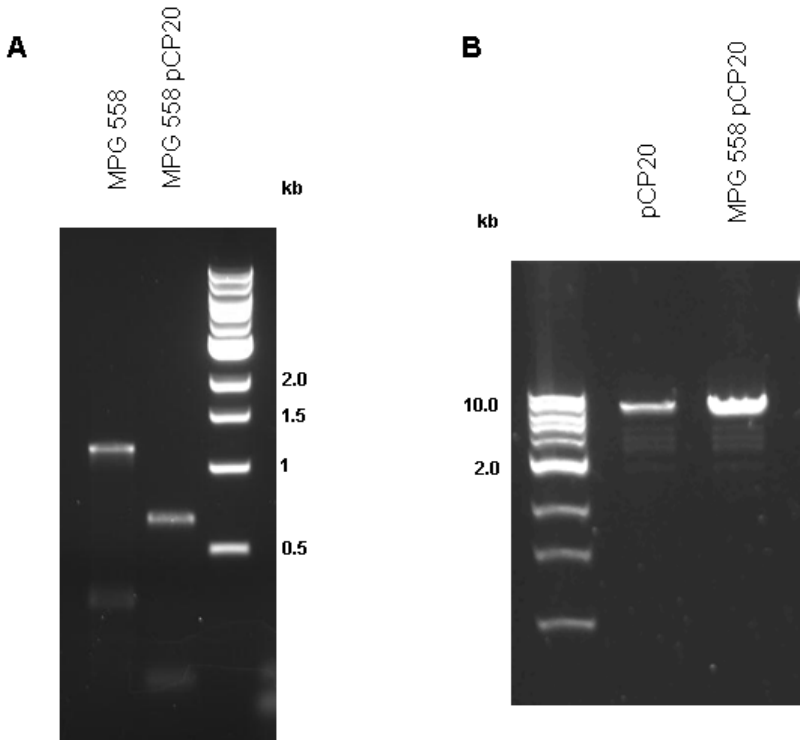

Figure 5.3.2: Successful removal of the Kanamycin resistance cassette from the MPG 558 strain. Primers (cspE forward and reverse; see Table 2.1.1) were designed for the regions flanking the *cspE* gene of SL1344, which the kanamycin cassette had replaced. PCR amplification was carried out using DNA from both the MPG 558 strain and this strain carrying the pCP20 plasmid. **A** Agarose gel electrophoresis was used to compare the sizes of PCR amplified regions of both strains and confirms the removal of the 600bp kanamycin cassette. Expected sizes of PCR products were 1.2kb (from MPG 558) and 0.6kb (after removal of cassette). **B** Agarose gel electrophoresis confirmed that the (uncut) pCP20 plasmid can be recovered from the MPG 558 strain (using a Promega Wizard SV Miniprep DNA purification kit). The original plasmid DNA was run alongside as a control to confirm plasmid size.

Table 5.3.2: A summary of the colony forming ability of *rpoS* mutants on LB agar at 37°C. Results quoted are the average of triplicate experiments. The variation within each set of experiments is indicated. Results of special interest are highlighted in **bold** text.

Parent strain	Notes	Can an rpoS mutant form colonies?	No. of transductants		
			10^7 pfu	10^8 pfu	10^9 pfu
SL1344 *cspB*	functional CspB protein *cspB* mRNA has RNase E site	Yes	4 ± 1	7 ± 2	3 ± 0
SL1344 *cspE*	**functional CspE protein no RNase E site**	**No**	**0**	**0**	**0**
SL1344 *cspB* cspE	functional CspB and CspE proteins RNase E site available	Yes	6 ± 2	11 ± 3	2 ± 0
SL1344 *cspE* *cspB*::Mu*dlux*	functional CspE protein no functional CspB protein *cspB* mRNA has RNase E site	Yes	7 ± 4	12 ± 5	4 ± 0
SL1344 *csp* null *cspB*::Mu*dlux*	**no functional Csp proteins *cspB* mRNA has RNase E site**	**Yes**	**5 ± 0**	**8 ± 2**	**3 ± 1**

to test the hypothesis that it is the *cspB* mRNA and not the protein that mediates the colony forming ability of the *rpoS* mutant. As expected, subsequently it was possible to transduce the *rpoS* mutation (from SL1344 *rpoS*) successfully into the MPG 558 *cspB::*Mu*dlux* (Tet^R) strain using P22 transduction. The effect of an *rpoS* mutation on colony forming ability was re-examined. Table 5.3.2 summarises the outcome of this experiment and the points of note for each parental strain. These results have shown that it is the presence of the *cspB* mRNA, and therefore presumably the presence of an RNase E cleavage site in the 5'UTR, that is important for the colony forming ability of the *rpoS* mutant. Fang, Xia and Inouye (1999) have shown that *cspA* mRNA is actively transcribed at 37°C but the mRNA is highly unstable due to RNase E cleavage. Craig *et al.* (1998) have shown that *cspB* mRNA is also highly unstable at this temperature but highly abundant at low temperature (10°). The question then arises of whether these two mRNAs function to provide a major cellular 'sink' for RNase E at 37°C and therefore is RNase E degradation of other transcripts escalated in their absence? As discussed earlier, the *ftsQAZ* transcript is also a target for RNase E and around 50% of its transcription relies on RpoS (Ballesteros *et al.*, 1998). If the reason for the lack of colony forming ability in the MPG 558 *rpoS* mutant is due to excessive RNase E activity, it might be predicted that the overexpression of another RNase E target (to sequester RNase E activity) would restore a normal colony forming phenotype? This issue is addressed in the next section.

5.3.3 Does the overexpression of any RNase E target compensate for the effect of an *rpoS* mutation in the SL1344 *csp* null strain?

A new hypothesis is now emerging that the lack of colony forming ability observed with the MPG 558 *rpoS* strain may be due to increased RNase E activity caused by a deficiency of targets (*cspA* and *cspB* mRNA) for the enzyme. Cam *et al*. (1996) report that RNase E dysfunction, either by deletion of the gene or by protein overexpression, has an inhibitory affect on cell division in *E. coli* due to formation of an altered ratio of FtsZ to FtsA. This leads to filamentous growth and a loss of colony forming ability. Would the overexpression of another RNase E target compensate for the deficit of *cspA* and *cspB* RNase E cleavage sites in the MPG 558 *rpoS* strain? This has already been tested to an extent by the successful introduction of an *rpoS* mutation to the MPG 558 strain whilst over-expressing the *ftsQAZ* transcript from the plasmid pZAQ (Figure 5.2.3). Two RNase E cleavage sites have been identified in the *E. coli ftsQAZ* transcript: one in the *ftsA* coding region and one in the *ftsA-ftsZ* intergenic region (Cam *et al*., 1996). However, the restoration of colony forming ability in this strain could also be explained by reinstatement of the *ftsQAZ* transcript levels that were

reduced by the loss of RpoS-inducible transcription, as opposed to providing additional RNase E sites.

As a further attempt to confirm whether the overexpression of RNase E cleavage sites would restore the colony forming ability of the MPG 558 *rpoS* mutant, another gene was utilised that is subject to RNase E regulation but is unrelated to the RpoS pathway or cell division. An arabinose-inducible promoter was also used as an additional level of control.

OmpA is a member of the OmpA-OmpF porin family and is believed to function as a selective facilitated diffusion channel that allows a variety of small solutes to cross the outer membrane of the cell (Sugawara and Nikaido, 1992). In *E. coli*, *ompA* transcription is negatively controlled by RpoE, the sigma factor which controls the extra-cytoplasmic regulon, and decreases in response to envelope stress (Rhodius *et al*., 2005). Binding of *ompA* mRNA by the sRNA, *micA*, results in degradation by RNase E (Udekwu *et al*., 2005) in an Hfq-dependent manner (Vytvytska *et al*., 2000). Moll *et al.* (2003) used nuclease foot-printing to identify 2 RNase E sites on *ompA* mRNA in *E. coli*. Sequence alignment was used to identify the corresponding RNase E sites in the SL1344 *ompA* sequence and primers were designed to amplify regions of *ompA* either with or without RNase E cleavage sites (Figure 5.3.3(a)). The regions of *ompA* sequence amplified for use in this experiment are shown in Figure 5.3.3(b)). Each amplified gene sequence was then cloned (on *NcoI* and *HindIII* sites) into pUBAD, an arabinose inducible plasmid, and the correct construct confirmed by restriction enzyme digestion and DNA sequencing. The vector was then transformed into electrocompetent MPG 558 cells. To assess the effect of expressing the *ompA* fragment on colony forming ability, each strain was grown to stationary phase, in LB with or without 2% arabinose (100-fold higher than normal plasmid induction levels, as *S.* Typhimurium can metabolise the sugar as an energy source). The *rpoS* mutation was then introduced, from SL1344 *rpoS*, by P22 transduction. The colony forming ability of each resulting strain was then assessed on LB agar either with or without arabinose. Table 5.3.3 summarises the outcome of this experiment.

This experiment has shown that the presence of additional RNase E cleavage sites, from *ompA* mRNA, compensates for the inability of the MPG 558 *rpoS* strain to form colonies. The *ompA* sequence without RNase E cleavage sites and the un-induced constructs were used as controls. As the *ompA* mRNA is unrelated to FtsZ and cell division, these results suggest that the lack of colony forming ability observed in the MPG 558 *rpoS* mutant is solely down to increased RNase E activity caused by a reduction in potential targets for the enzyme.

Figure 5.3.3(a): Alignment of *ompA* sequences of *E. coli* K12 and SL1344. There is 98% DNA sequence identity between the two species. The start codon is highlighted by **bold** text. The RNase E binding sites (identified by Moll *et al*., 2003) are highlighted in yellow and are conserved between the two species. The sequence between these RNase E sites forms extensive secondary structure, i.e a stem-loop. Underlined regions indicate primer sequences used for PCR and cloning (ompA forward (+ and – RNase E sites) described in more detail in Table 2.1.1). The reverse primer is downstream of this region (Figure 5.3.3(b)). Amplified regions of *ompA* were cloned into pUBAD using restriction sites (NcoI and HindIII). Correct clones were identified by restriction digest and DNA sequencing.

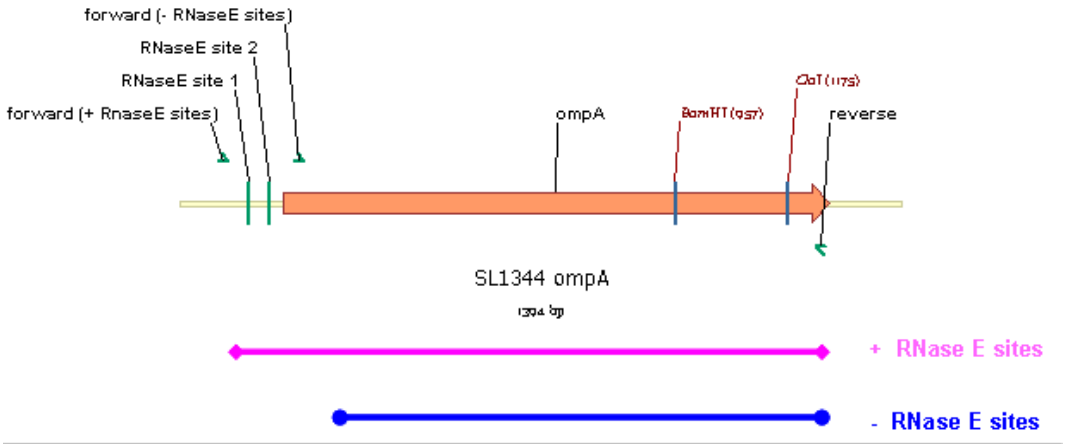

Figure 5.3.3(b): **Diagram showing sequences of *ompA*, +/- RNase E sites, amplified and used for inserts into pUBAD plasmid** (diagram constructed using Vector NTI version 10, Invitrogen). The pUBAD plasmid was used for the controllable expression of extra RNase E targets on the *ompA* transcript. Region of *ompA*, containing RNase E sites, corresponds to bases 1160942 to 1162134 of the SL1344 sequence. Region of *ompA*, without sRNA sites, relates to bases 1160942 to 1162007 of the SL1344 sequence. These regions were cloned into the pUBAD plasmid using restriction sites NcoI and HindIII. Restriction analysis (Figure 5.3.3(c)) and sequencing (Figure 5.3.3(d)) were used to identify correct clones.

Primer sequences: *ompA* + sRNA sites (for) CG**CCATGG***GGTGCTCAGCATAAGCCGTA*, *ompA* – sRNA sites (for) CG**CCATGG***GATTGCAGTGGCACTGGCTG* and *ompA* (rev) CGG**AAGCTT***CCTGCGGCTGAGTTACCACG*. Restriction sites are indicated in **bold** print and the sequence identical to that of SL1344 is highlighted in *italics*.

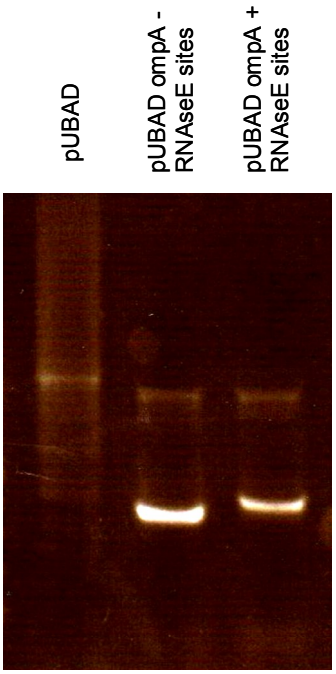

Figure 5.3.3(c): **Restriction analysis of pUBAD::*ompA* clones**. The pUBAD plasmid was digested using restriction sites *NcoI* and *HindIII* to release *ompA* fragments.

A

> JR_seq_ompA(a)5 for

NGGGCCCTGGGTNAGCNTATCNANTCNTAANACGGGATGTATNNGGCTNNGGGANNGGGCCCCCNTNTA
ANGGGGGGGGGGGGGNGGTTGTANAATGCTCAGCATAAGCCGTAGATATCGGTAGAGTAACTATTGAGC
AGATCCCCCGGTG**AAGGATTT**AACCGTGTTATCTCGTTGGAGATATTCATGGCG**TATTTT**GGATGATAA
CGAGGCGCAANNAATGAAANNGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTNNCGCTACCGTAGC
GCAGGCCGCTCCGAAAGATAACACCTGGTACGCTGGTGCTAAACTGGGCTGGTCCAGTACCATGACACC
GGCTTCATTCACAATGATGGCCCGACTCATGAAAACCAACTGGGCGCAGGTGCTATTGGTGGTTCCAGG
TTAACCCGTATGTTGGCTTTGTAATGGGCTACGACTGGTTAGGCCGTATGCCGTACAAAGGCGACAACA
TCAATGGCGCTTATAAAGCTCAGGGCGTTCAGTTGACCGCTAAACTGGGTTATCCAATCACTGACGATC
TGGACGTTTATACCCGTCTGGGTGGTATGGTATGGCGTGCAGACACCAAGTCTAACGTCCCTGGCGGCC
CGTCTACTAAAGACCACGACACCGGCGTTTCCCCGGTATTCGCGGGCGGTATCGAGTATGCTATCACTC
NNTTTTTTTTNGNANCTTTTTNAGANGTTTTTCGNAANTNTTNNTCNACTNAGNGNTATNATNTTTACN
AGGNNTNNNTTTTTACNNNNANNNNNNNATNTNNNNNTCTTNTNNNNCATNTTNTNCTTNTNNNTNTNT
NTGNCANGTNCTGNTTANGTNTGATNTNTATTTAATNATTTNATGNGNNNNNTCTANTTCGNCTACTTA
NANNTNNTATGNNGNNTANNTGATNATTTNACNNNNTANTGTANNGANTNNTGTANGT

> JR__seq_ompA(a)5 rev

TNNTTTTGTGCNTGGTATTTNAGTNANGAANTGANNNNAACCCCCCTTANNNANGGGGNGTNGGGGGNG
AGGTNGAATCANGAGGGGTTNCCCCCTTAAGCCTGCGGCTGAGTTACCACGTCTATAACGCCTTTAACT
TCGATCTCTACGCGACGATCCGGAGCCAGGCAATCGATCAGGGCAGCGCGAGGTTTCACGTTGTCACAG
GTGTTGCCGGTAACCGGGTTAGATTCGCCCATACCACGTGCGGAGATATTGTCAGACGGAATACCTTTG
GAGATCAGGTAATCAACAACAGACTGAGCACGTTTCTCGGACAGACCCTGGTTGTAAGCGTCAGAACCG
ATACGGTCAGTGAAGCCCAGAACGACAACGGAACCGTCTATCGGATCCAGGTGCTCAGCTGGCTGTACA
GCTGATCCAGAGCCTGCTGGCCTTCCGNCTTCAGGGTAGATTTGTTGAAGTTGAACAGTACGTCAGACT
TCGAAGTGAAGTGCTTGGTCTGTACTTCCGGAGCCGGAGCTGGTGCCGGAGCTACTACCGGAGCAGCTT
CTTGCTGGCCGAAACGGTAGGAANCACCTACGCTCAGCAGGCCGTTGTCCGGACGGGTGCCGATGGTGT
TGGCATCACCGATGTTGTTAGTCCACTGGTATTCCAGACGGGTTGCGATTTCANNGGTGATACGATATT
TNNGNNGGTGTAANTGAATGGTTNGGNAANGGNTNATNGNCANNNNNTNNCNCNANNAGTTAGAANATA
TCTNCNNNNNNNNNATGNTNNATNATCTNTTNTANCTANANGTAGGNGNANTGTNGNTNTNNTATATNN
NNANNNANTTNTNGTANCTT

B

>JR_seq_ompA(b)7 for

TTGGNAGGCACGANANGAATTTTATTATTNNTTTGNTTANANTNTNAGAAACTGGGGNTTAANTGTTTN
GGCGCCCCTNNNANNGGTTTAAAAAAAATTNNNNTGNGNTTANCNGTNATGCCNCCCTTNANGGGGGGT
TGNGTANGGGNGTTNTNTGTTTCAATANTGTTTCGCTACCGTAGCGCAGGCCGCTCCGAAAGATAACAC
CTGGTACGCTGGTGCTAAACTGGGCTGGTCTCAGTACCATGACACCGGCTTCATTCACAATGATGGCCN
GCTCATGAAAACCAACTGGGCGCAGGTGCTTTNGGTGGTTACCAGGTTANCCCGTATGTTGGCTTTGAA
ATGGGCTACGACTGGTTAGGCCGTATGCCGTACAAAGGCGACAACATCAATGGCGCTTATAAAGCTCAG
GGCGTTCAGTTGCCGCTANACTGGGTTATCCAATCACTGACGATCTGGACGTNNATACCCGTCTGGGTG
GTATGGTATGGCGTGCANNCACCAAGTCTAACGTCCCTGGCGGCCCGTCTACTAATGACCACGACACCG
GCGTTTCCCCGGTATTCGCGGGCGGTATCGAGTATGGTATCACCCCTGAAATCGCAACCCGTCTGGAAT
ACCAGTGGACTAACACATCGGTGATGCCAACACCATCGGCACCCGTCCGGACAACGCCTGANNGTAGCT
GNTTCCTACCNTTTTCTNTAAGTNTGTCGGNTAGNTGTATNNCNCATNNTGTNNGTGANNNNTATAGTN
ATNTNTTNANNNTNTGTNCTNCNNNNGGTCNTATNCGTNTNGNNNTTTGATANNGTANNTTNNNTNTAN
TANTNCCTGNGTANTCTNTNTTNNNATNNNTC

>JR_seq_ompA(b)3 rev

ANGCTTCNNACTTCCTCATTCACGGTCCGAGAATCCAGACCAGAGCAATTAACCCGGCGACGCGGGGTT
ANTTATCAGACGGAAACTTAAGCCTGCGGCTGAGTTACCACGTCTTTAACGCCTTTAACTTCGATCTCT
ACGCGACGATCCGGAGCCAGGCAATCGATCAGGGCAGCGCGAGGTTTCACGTTGTCACAGGTGTTGCCG
GTAACCGGGTTAGATTCGCCCATACCACGTGCGGAGATNNTGTCAGACGGAATACCTTTGGAGATCAGG
TAATCAACAACAGACTGAGCACGTTTCTCGGACAGACCCTGGTTGTAAGCGTCAGAACCGATACGATCA
GTGAAGCCCAGAACGACAACGGAACCGTCTTTCGGATCCAGGTTGCTCAGCTGGCTGTACAGCTGATCC

AGAGCCTGCTGGCCTTCCGGCTTCAGGGTAGATTTGTTGAAGTTGAACAGTAAGTCAGACTTCAGAGTG
ANGTGCTTGGTCTGTACTTCCGGAGCCGGAGCTGGTGCCGGAGCTACTACCGGAGCAGCTTCTTGCTGG
CCGAAACGGTAGGAATCACCTACGCNCAGCANGCCTGTCCCTTACTGCCTGCAATNATTATNTTCACGT
TGTTCNCCTTTACCNCGNTGCTGGGTTCAGGCAATCCAACGCTGGTATTCGNGTTCCTGTTGTTAGGCC
TGTCCCTTACTGCCTGCANTNTCN

Figure 5.3.3(d): **DNA sequence analysis of pUBAD::*ompA* clones**. Sequencing (primers described below) was used to confirm that the pUBAD::*ompA* (+/- RNaseE sites) construct were correct. The sequence identical to that of SL1344 *ompA* is highlighted in yellow. Some of the nuclotides within the sequences were not recognised due to AT-rich regions resulting in slippage; however, these were worked through by eye to confirm identity. Several mismatches are highlighted in blue but these are out-with the important RNaseE sites. Panel **A** shows the forward and reverse sequences of the *ompA* + RNaseE site construct. The RNaseE sites are intact and are highlighted by bold text. Panel **B** shows the forward and reverse sequences of the *ompA* – RNaseE site construct.

Primer sequences: *ompA* + sRNA sites (for) CG**CCATGG***GGTGCTCAGCATAAGCCGTA*, *ompA* – sRNA sites (for) CG**CCATGG***GATTGCAGTGGCACTGGCTG* and *ompA* (rev) CGG**AAGCTT***CCTGCGGCTGAGTTACCACG*. Restriction sites are indicated in **bold** print and the sequence identical to that of SL1344 is highlighted in *italics*.

Table 5.3.3: Summary of the colony forming ability of the SL1344 *csp* null mutant following P22 transduction with an *rpoS* mutation in the presence/ absence of additional RNase E cleavage sites encoded on a plasmid. MPG 558 pUBAD::*ompA* strains was grown to stationary phase at 37°C in LB, with or without 2% arabinose, and the *rpoS* mutation introduced (from SL1344 *rpoS*) by P22 transduction. Colony forming ability was assessed subsequently by growth on LB agar, with or without arabinose, as stated. Results reported are the average of triplicate experiments. The variation in each set of experiments is indicated. Results of special note are highlighted in **bold** text.

Plamid RNase E sites?	Arabinose added to LB agar?	Can an rpoS mutant form colonies?	No. of transductants		
			10^7 pfu	10^8 pfu	10^9 pfu
No	No	No	0	0	0
No	Yes	No	0	0	0
Yes	No	No	0	0	0
Yes	**Yes**	**Yes**	**9 ± 2**	**17 ± 5**	**4 ± 1**

5.4 Discussion

Experiments in this chapter have shown that an MPG 558 (SL1344 *csp* null) *rpoS* mutant is unable to form colonies on LB agar at 37°C. A conditional *rpoS* mutant has shown that the *rpoS* mutation is not toxic but that RpoS is essential for colony forming ability in a strain of *S.* Typhimurium lacking CspA paralogues. From the whole SL1344 *cspA* gene family, only the *cspA* or *cspB* genes were able to restore the colony forming ability of the *rpoS* mutant. Further experiments have shown that it is the *cspA* and *cspB* mRNA and not the protein that is necessary for colony forming ability. This suggested that faulty cell division in MPG 558 *rpoS* may result from an RNase E phenomenon and if so, that the *cspA* and *cspB* mRNAs function as a major RNase E sink and soak up most of the RNase E activity of the cell at 37°C. This view was supported by findings that additional RNase E sites, encoded by *ftsQAZ* and *ompA* mRNA, when expressed from plasmids, also restored the colony forming ability of the MPG 558 *rpoS* mutant.

Figure 5.4(a) shows the location of the 6 promoters responsible for transcription of the *ftsQAZ* region in *E. coli*. The full-length *ftsQAZ* transcript must be cleaved by RNase E to allow the efficient translation of all 3 cell division proteins. Ballesteros *et al*. (1998) reported that the growth rate dependency of the promoter, *ftsQ1p*, which normally provides around 46% of the total transcriptional potential of the *ftsQAZ* operon in *E. coli*, is abolished in a strain in which the *rpoS* gene is insertionally inactivated. The second housekeeping promoter, *ftsQ2p*, is unaffected by RpoS levels. The other known promoters in this region produce monocistronic transcripts of *ftsZ* mRNA alone.

The hypothesis proposed in this chapter is that, in a normal healthy cell, the *ftsQAZ* transcript acts as a target, and *cspA* and *cspB* mRNA act as sinks, for RNase E. In MPG 558, although there is a lack of RNase E sinks due to the deletion of the *cspA* and *cspB* loci, the *ftsQAZ* transcript is still present as a target for RNase E. This would result in increased levels of FtsZ and A proteins through RNase E processing and could explain the minicell phenotype previously observed in this strain (Chapter 3.3.3). However, the absence of *rpoS* leads to transcription of mainly *ftsZ* mRNA, rather than the polycistronic *ftsQAZ* transcript from the *rpoS*-dependent promoter (see Figure 5.4(a)), leading to a major imbalance in FtsZ and FtsA levels. A ratio of FtsZ to FtsA between 50 and 100 is necessary for correct cell division to occur in *E. coli* and RNase E dysfunction alters the ratio of these transcripts, due to altered processing of the polycistronic *ftsQAZ* transcript (Cam *et al*., 1996), resulting in the production of elongated filamentous structures with the inability to form colonies on solid media (Tamura *et al*., 2006). There may also be an impact on general protein synthesis, as

increased degradation by RNase E will shorten mRNA lifetimes and therefore generally reduce mRNA availability for translation. In *E. coli*, Hfq plays an important role in facilitating degradation by targeting mRNAs to the RNA degradosome (Morita, Maki and Aiba, 2005; Brennan and Link, 2007). If this role was disrupted in the MPG 558 *hfq rpoS* strain, it would result in increased mRNA species, providing more targets for RNase E therefore reducing RNase E processing of the *ftsZ* monocistronic transcript and, in turn, FtsZ translation. This begins to redress the balance of FtsZ: FtsA translation towards normal and subsequently allows restoration of colony forming ability. Figures 5.4 (b-e) summarise these findings in models representing what happens in a normal cell, compared to the MPG 558 *rpoS* mutant, and how this is recovered by provision of additional RNase E cleavage sites.

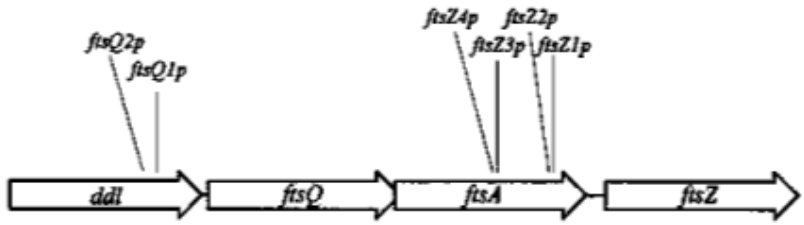

Figure 5.4(a): **Location of the 6 promoters identified for the *ftsQAZ* operon of *E. coli*** (taken from Cam *et al*., 1996). Promoter *ftsQ1p* is RpoS-inducible and is responsible for 46% of transcription of these genes. The other housekeeping promoter, *ftsQ1p*, is also responsible for transcription of the whole region. However, there are 4 promoters that induce the transcription of *ftsZ* alone.

In light of its many crucial biological functions, it is not surprising that an imbalance in *E. coli* RNase E activity can impede cell growth. To ensure a steady supply of this protein, *E. coli* RNase E auto-regulates its production by a mechanism dependent on a *cis*-acting stem-loop within the *rne* 5'-UTR. Recent mutational studies have shown that this loop is a poor target for cleavage by RNase E but does bind the catalytic domain of the enzyme and either directs its action elsewhere in the transcript or acts as a silencer against translation (Schuck, Diwa and Belasco, 2009). However, it is unknown whether the auto-regulation relies on cellular protein concentration or is controlled by enzyme activity, or both. In the MPG 558 *rpoS* strain, there is no increase in RNase E concentration, simply there are less potential targets for the enzyme and therefore the remaining targets, such as *ftsQAZ*, are degraded more quickly.

In an SL1344 cell, FtsZ: FtsA (50:1) = cell division

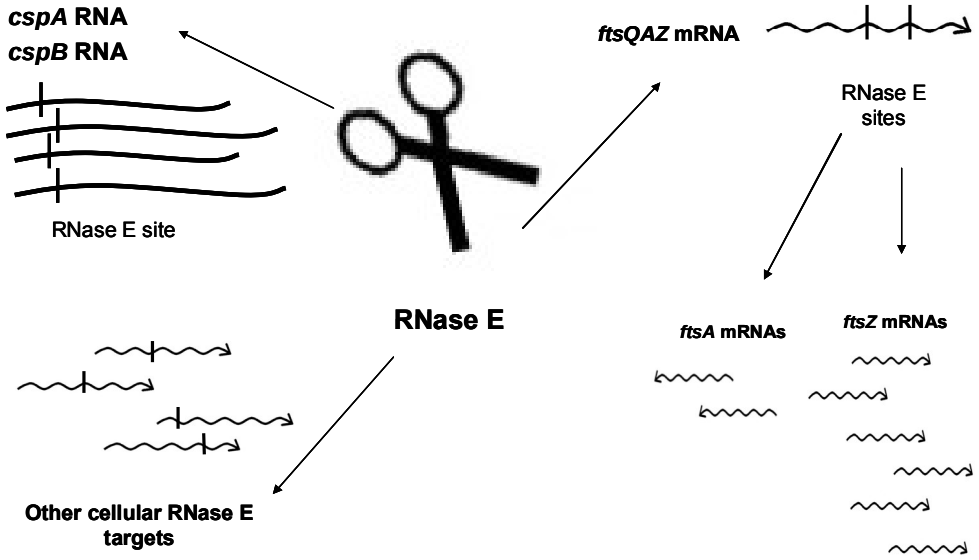

Figure 5.4(b): Possible RNase E targets in a normal SL1344 cell. RpoS induces *ftsQAZ* transcription which, along with the mRNA of *cspA* and *cspB* provides major targets RNase E. The FtsZ to FtsA ratio is around 50: 1; therefore cell division proceeds as normal.

In a MPG 558 cell, ↑FtsZ: ↑FtsA (50:1 as more mRNA is processed) = cell division

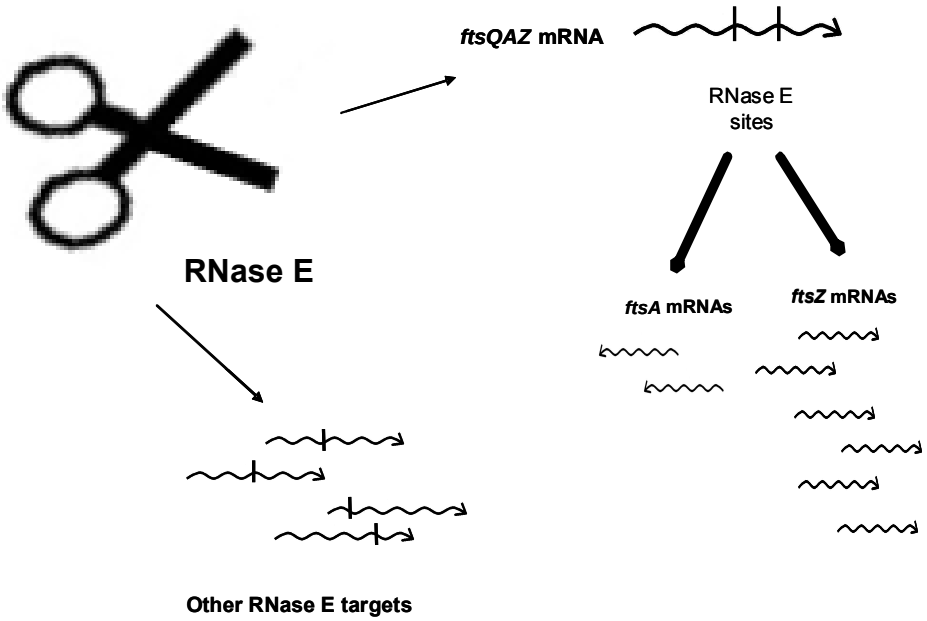

Figure 5.4(c): Possible RNase E targets in a MPG 558 cell. RpoS induces transcription of *ftsQAZ* which has 2 RNase E cleavage sites and therefore is still a major target for RNase E. However, RNase E cleavage of this transcript and other targets is increased due to the loss of *cspA* and *cspB* mRNA which are major targets for RNase E normally. Enhanced processing of *ftsQAZ* mRNA results in enhanced translation of FtsZ and FtsA, a condition known to produce minicells.

In a MPG 558 *rpoS* cell, ↑FtsZ: ↓FtsA (>50:1 due to decreased *ftsQAZ* mRNA) = no cell division

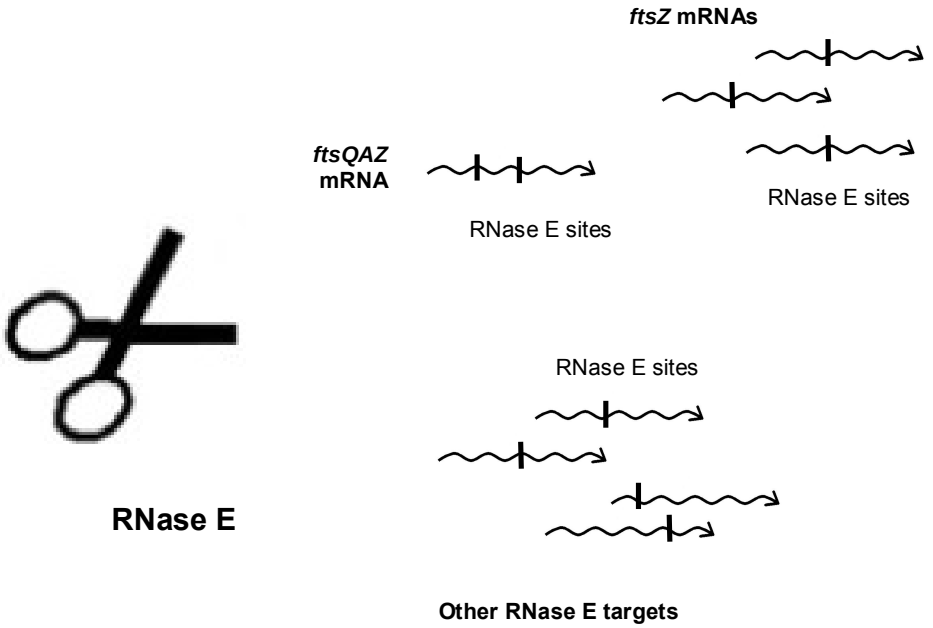

Figure 5.4(d): Possible RNase E targets in a MPG 558 *rpoS* cell. In this strain, the housekeeping promoters only are responsible for the transcription of the *ftsQAZ* region, therefore only a small amount of the full-length transcript is available for RNase E cleavage. This reduces the level of *ftsA* mRNA, and therefore insufficient FtsA is translated. Due to reduced targets for RNase E, the amount of *ftsZ* transcript processed is increased, therefore increasing translation of FtsZ. This produces a major imbalance in the FtsZ to FtsA ratio and therefore the cell is incapable of cell division.

In a MPG 558 *rpoS* cell + extra RNase E targets, FtsZ: FtsA (50:1) = cell division

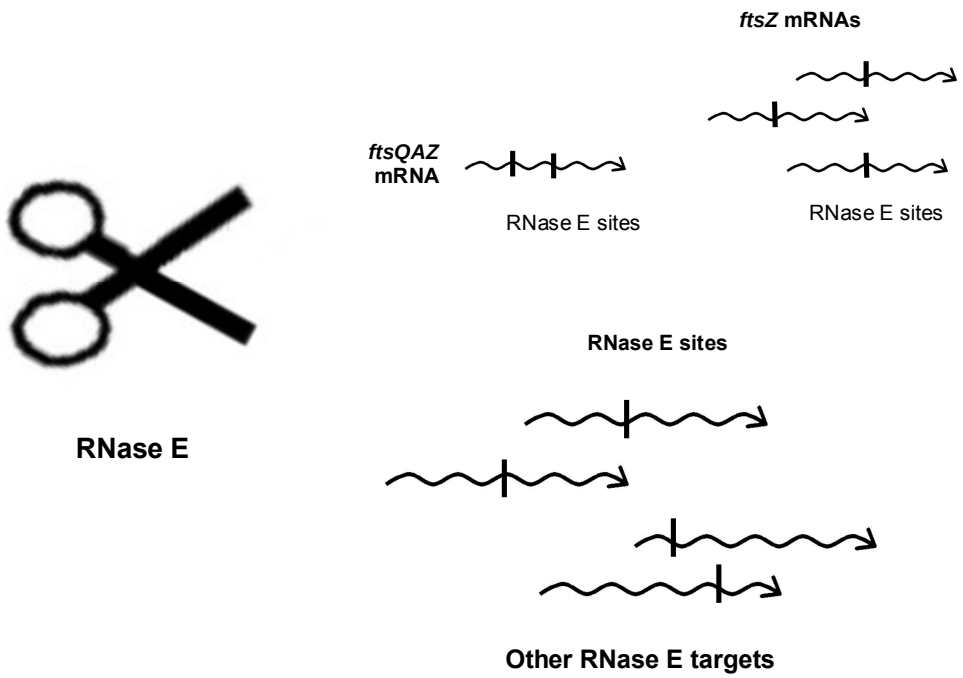

Figure 5.4(e): **Action of RNase E in a MPG 558 *rpoS* cell after the addition of extra targets for the enzyme.** The transcription of the *ftsQAZ* operon remains as it does in the previous model (d). There is still a reduction in the levels of FtsA because of reduced promoter activity. However, as there is now an overexpression of other targets for RNase E, the likelihood of *ftsZ* mRNA cleavage is reduced thereby rebalancing the FtsZ: FtsA ratio to a suitable level for cell division. This has the same effect as reducing RNase E activity.

Chapter 6

Concluding discussion

6.1 Phenotypic characterisation of CspA paralogues of *S.* Typhimurium

One of the aims of this study was to characterise the CspA paralogues of *S.* Typhimurium and elucidate their individual functions in RpoS-mediated stress responses. Studies in *E. coli* have shown that CspC and CspE up-regulate RpoS expression (Phadtare, Inouye and Severinov, 2002); this work has found the same to be true in *S.* Typhimurium during stationary phase at 37°C. In light of observations that an *S.* Typhimurium *rpoS* strain exhibits pleiotropic phenotypes including susceptibility to nutrient deprivation and oxidative stress (Fang *et al.*, 1992), comparative phenotypic analysis was carried out on the MPG 558 (SL1344 *csp* null) and SL1344 *rpoS* strains. Sittka *et al*. (2007) reported that many of these phenotypes were also observed for a *S.* Typhimurium *hfq* mutant due to impaired RpoS expression; however, RpoS-independent phenotypes were also observed by them. For the purpose of providing further insight into the interactions involved in RpoS regulation, a SL1344 *hfq* mutant was added to the group, examined in the present study, for comparative phenotypic analysis. Consequently, in the assays both significant phenotypic overlaps and phenotypes unique to certain mutant strains were observed. This suggested that although CspA paralogues and Hfq are both required for RpoS expression, each also exhibits unique functional elements and modulatory roles within the cell.

The CspA paralogues are required for growth upon cold shock in *S.* Typhimurium. Further investigation revealed that MPG 558 could grow if the temperature was very gradually reduced, suggesting that the CspA paralogues are required to overcome the initial translational blockage only and not for long-term adaptation to low temperature growth. Upon cold shock, MPG 558 filaments but is able to complete a round of septation if artificially supplied with FtsZ, FtsA and FtsQ. This suggests a key problem lies at the level of translation, maybe in ribosome 'jamming'. Potentially, complementation of MPG 558 growth at 15°C, with a plasmid library, might reveal genes which could substitute for the CspA paralogues, i.e. ribosome maturation factors etc, and suppress the inability of this strain to grow at low temperatures.

RpoS, Hfq and the CspA paralogues are required for mediating peroxide resistance in stationary phase. Resistance to oxidative stress and other RpoS-dependent responses to nutrient deprivation, acid stresses and DNA damage are also relevant for the intraphagosomal environment of host macrophages (Fang *et al.*, 1992; Van der Straaten *et al.,* 2004). In future

studies, phenotypic analysis could be extended to the survival of SL1344, MPG 558 and SL1344 *hfq* strains inside cultured macrophages, and the underlying mechanisms explored.

During the course of this study, an RpoS-independent role in *S.* Typhimurium motility was identified for the CspA paralogues. Sittka *et al*. (2007) have reported that a mutation in *hfq* impairs the motility of *S.* Typhimurium as Hfq is required for the expression of FliC, an important flagellin protein. However, no link between this protein and the CspA paralogues has been determined as yet. A recent paper by Phadtare *et al*. (2006) suggested that CspC and CspE are involved in the biosynthesis and function of flagella through the regulation of MotB, FlhA and FlhB (as described in Chapter 3.3.3). However, before this can be concluded, reduced levels of these proteins must be confirmed in MPG 558 compared to its parent strain. Studies have shown that that flagella-mediated motility accelerates, but is not absolutely required for, the invasion of epithelial cells by *S.* Typhimurium and its virulence in BALB/c mice (Lockman and Curtis III, 1990; Van Asten *et al*., 2004). The invasion of epithelial cells by the MPG 558 strain should be compared to that of its parent strain and may show that MPG 558 is deficient in adherence and infection of epithelial cells. The study may be extended to virulence studies in mice as RpoS also contributes to virulence by regulating the expression of plasmid *spv* genes, and the colonisation of Peyer's Patches and the liver, during starvation (Swords *et al*., 1997). If appropriate, monogene strains may give further insight into roles for individual CspA paralogues.

Minicell formation is a known phenotype of *E. coli hfq* mutants due to an increase in FtsZ levels (Takada, Wachi and Nagai, 1999); this was also observed for the *S.* Typhimurium *hfq* strain. This is an RpoS-independent phenotype as a persistence of elongated rod-shaped cells has been observed for *E. coli rpoS* mutants in stationary phase (Loewen and Hengge-Aronis, 1994) due to decreased *ftsZ* transcription; FtsZ mediates *bolA*, essential for regulating cell morphology (Aldea *et al*., 1988). A minicell phenotype was also observed for MPG 558 and cell size distributions have suggested that this strain has increased levels of FtsZ. This must be confirmed by immunoblotting. Cell morphology of monogene strains would determine which of the CspA paralogues are involved in septation.

Investigating other RpoS-dependent phenotypes may find further distinct phenotypes for MPG 558 or the SL1344 *hfq* strain. Exposure to low pH during exponential phase leads to *rpoS* induction and the acid shock response in *S.* Typhimurium; RpoS is not required for stationary phase acid tolerance (Bang *et al*., 2000). Hengge-Aronis *et al*. (1993) have observed that *E. coli* RpoS is involved in both osmotic stress resistance and thermotolerance. These phenotypes could be tested for each of the *S.* Typhimurium mutants to determine the

involvement of the CspA paralogues and Hfq in each of the stated stresses. Also, it could be tested whether exposure to one stress leads to cross-protection from another otherwise lethal stress, which could have significant implications in view of practical food microbial control techniques.

6.2 Molecular dissection of the role of CspA paralogues in RpoS regulation

The major aim of this study was to examine the role of the CspA paralogues in regulating RpoS expression in *S.* Typhimurium. In the study, immunoblots demonstrated that these proteins are involved in regulating RpoS during both stationary phase and cold shock in exponential phase. Contrary to results found in *E. coli*, Hfq is not required for optimal RpoS expression during cold shock. This suggested two distinct pathways for RpoS expression in *S.* Typhimurium. The presence of two distinct pathways for RpoS regulation in SL1344 was confirmed by *rpoS::lacZ* translational fusions in the MPG 558 and SL1344 *hfq* strains. qRT-PCR and immunoblotting demonstrated that DsrA was not expressed upon cold shock in SL1344. Further to this, transcriptional fusions showed that neither DsrA nor RprA are required for RpoS expression, under the growth conditions tested. This stands in contrast to what is known for *E. coli* (Masse, Majdalani and Gottesman, 2003). As these sRNAs require Hfq to facilitate binding to their target mRNA, *rpoS*, (Wassarman, 2002) their apparent redundancy in *S.* Typhimurium, in the present study, could account for Hfq being unnecessary during cold shock. This does not rule out the action of these sRNAs on alternative targets. However, over 70 sRNAs have been discovered in *E. coli* and many of these are expressed during stationary phase, and largely regulate expression of their target mRNAs via interaction with Hfq (Vogel and Wagner, 2007).

Northern analysis demonstrated that neither the CspA paralogues nor Hfq were involved in *rpoS* transcription, as similar levels of *rpoS* mRNA were detected in the mutant and parent strains, therefore these components must work at the post-transcriptional level. The detection of multiple, similar bands, which could relate to stable degradation products, in the SL1344 *hfq* and MPG 558 strains compared to their parent strain SL1344, could suggest that the CspA paralogues do not control RpoS expression by stabilising the mRNA. Comparative northern

analysis, with more precise probes to detect the 5'UTR or the coding sequence of *rpoS* mRNA only, would determine if these products were in fact stable degradation products as proposed. Also, nuclease foot-printing of the *rpoS* mRNA in the presence and absence of the CspA paralogues, and Hfq, would confirm whether these components directly interact with *rpoS* mRNA and are involved in stabilising the mRNA against degradation.

Mutations rendering cells defective in RpoS degradation were combined with the MPG 558 and SL1344 *hfq* strains and demonstrated that these strains were able to express RpoS. However, this did not show whether impaired RpoS expression resulted from less efficient translation of the *rpoS* mRNA or from protein instability. Examination of protein stability suggested that the CspA paralogues are not involved in stabilisation of RpoS against degradation by the ClpX/P protease. During the course of this study, a larger RpoS protein was detected in the SL1344 *hns* strain during stationary phase, which was not observed in the MPG 558 *hns* strain; this may well be due to modification of the protein or to transcription read-through, although sequence analysis suggests that this is unlikely. H-NS is known to function as a repressor of transcription (Rimsky *et al.,* 2001) and CspE has been implicated in transcription antitermination in *E. coli* (Phadtare *et al*., 2002). To investigate the cause of this apparent modified protein, *hns* mutations could be introduced into the *csp* monogene strains and the CspA paralogues involved in the modification determined. Also, a potential RpoS degradation intermediate was observed in the MPG 558 *mviA* mutant that wasn't observed in the SL1344 *mviA* strain. To determine which of the CspA paralogues are involved, the *mviA* mutation could be introduced to the SL1344 *csp* monogene strains.

Experiments using *rpoS::lacZ* translational fusions showed that RpoS was induced upon entry into stationary phase and upon cold shock in exponential phase in SL1344. These fusions also showed that CspA paralogues were required for the efficient translation of RpoS under both these growth conditions. However, as observed with immunoblotting (with monoclonal RpoS antibodies), Hfq was only required in stationary phase, contrary to reports in *E. coli*. Although confirming what has been seen previously, these fusions did not fully elucidate the role of the individual components in RpoS regulation. To confirm that CspA paralogues and Hfq are required for efficient translation of *rpoS* mRNA, *de novo* RpoS protein synthesis could be examined by monitoring ^{35}S-methionine incorporation. The possibility that CspA paralogues facilitate the interactions of, or stabilise, as yet unexamined sRNAs cannot be ruled out. The facilitation of sRNA interactions with *rpoS* mRNA is the likely role for Hfq during stationary phase but this will need to be confirmed.

6.3 *cspA* mRNA is essential for colony formation at 37°C in an SL1344 *rpoS* mutant

During the course of this study, it was discovered that *cspB* (or *cspA*) mRNA, containing a putative RNase E cleavage site, but not CspB protein, was essential for the colony forming ability of a SL1344 *rpoS* mutant. The addition of extra RNase E targets also restored the colony forming ability of the *rpoS* mutant. Transcription of *ftsQAZ* mRNA, usually cleaved by RNase E, is reduced in an *rpoS* mutant. Only one other promoter site induces transcription of the full *ftsQAZ* transcript, whilst 4 other promoters induce *ftsZ* transcription only. Therefore, a mutation in *rpoS* reduces the FtsA: FtsZ ratio. It is proposed that *cspA* and *cspB* mRNA provides a major sink for RNase E at 37°C and, in their absence, there are reduced targets for RNase E, therefore increased processing of remaining targets, such as *ftsZ* mRNA, in turn increasing FtsZ translation. MPG 558 produces minicells at 37°C, known to be a consequence of increased FtsZ levels. When this mutation is combined with a mutation in *rpoS*, the ratio of FtsZ: FtsA is disturbed to the extent that cell division can no longer proceed. A more than twelve-fold increase in FtsZ levels (compared to FtsA) has previously been shown to cause filamentation in *E. coli* (Ward and Lutkenhaus, 1985). Therefore to test whether this is this case, antibodies against FtsZ and FtsA could be used to compare levels in SL1344 to those in the MPG 558 and MPG 558 *rpoS* strains. To complete this study, a plasmid library would provide a complete screen of genes, or mRNAs, that restore colony forming ability to the MPG 558 *rpoS* strains.

As the addition of *rpoS* results in multiple septation events along filamentous MPG 558 *rpoS* cells, time-lapse microscopy could be used to visualise whether these events occurred simultaneously along the filament or individually at the cell mid-points. This would provide further insight into the septation process which at present remains ambiguous.

Chapter 7

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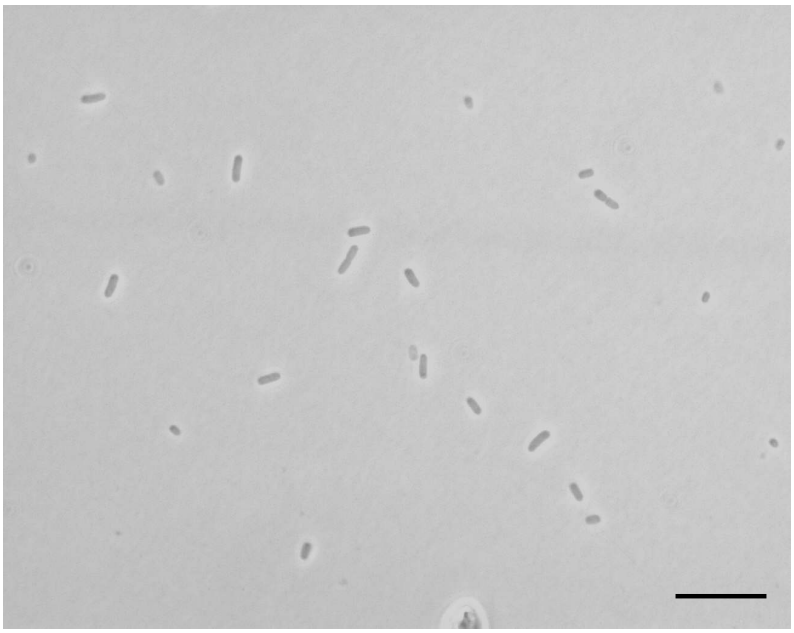
Appendix

Chapter 1

Table 1: *Escherichia coli* cold shock genes and gene products (taken from Gualerzi *et al.*, 2003).

Gene	Location (kb)	Product	Function/properties
aceE	123.0	AceE	Pyruvate dehydrogenase (decarboxylase)
aceF	125.7	AceF	Pyruvate dehydrogenase (dihydrolipoyltransacetylase)
cspA	3717.7	CspA	RNA and DNA binding (CCAAT/GGTTA); RNA chaperone; transcriptional and translational enhancer
cspB	1639.6	CspB	Unknown; RNA binding (U/T stretches)
cspE[a]	656.5	CspE	RNA binding (AU/AT rich regions); nascent RNA chaperone; transcriptional antiterminator; PNPase and RNase E inhibitor
cspG	1050.7	CspG	Unknown
cspI	1636.7	CspI	Unknown
deaD	3305.5	DeaD	RNA helicase; ribosome assembly?; RNA degradation?
dnaA	3881.4	DnaA	DNA binding and replication (initiation); transcriptional regulator
gyrA	1227.4	GyrA	DNA-binding/cleaving/rejoining subunit of gyrase
hns	1292.1	H-NS	Nucleoid protein; transcriptional repressor; DNA supercoiling
hscA	2657.9	Hsc66	DnaK homologue (Hsp70-type protein chaperone)
hscB	2657.5	HscB	DnaJ homologue
hupB[b]	460.7	HUβ	Nucleoid protein; DNA supercoiling
infA	925.7	IF1	Translation initiation, RNA binding
infB[d]	3313.7	IF2	Translation initiation; fMet-tRNA binding; protein chaperone
infC	1798.7	IF3	Translation initiation; initiation site selection; RNA binding
nusA	3315.2	NusA	Transcription elongation/termination/antitermination
otsA	1979.6	OtsA	Trehalose phosphate synthase
otsB	1980.4	OtsB	Trehalose phosphatase
pnp	3308.8	PNPase	3′-5′ Exonuclease; degradosome component
rbfA	3310.8	RbfA	Ribosome assembly/maturation
recA	2821.8	RecA	Homologous recombination; SOS response
tig	454.4	Trigger factor	Multiple stress protein; protein chaperone; ribosome binding
ves	1823.0	Ves	Unknown
yfiA	2735.2	pY	Translational (A-site) inhibitor

SL1344



SL1344 *csp* null

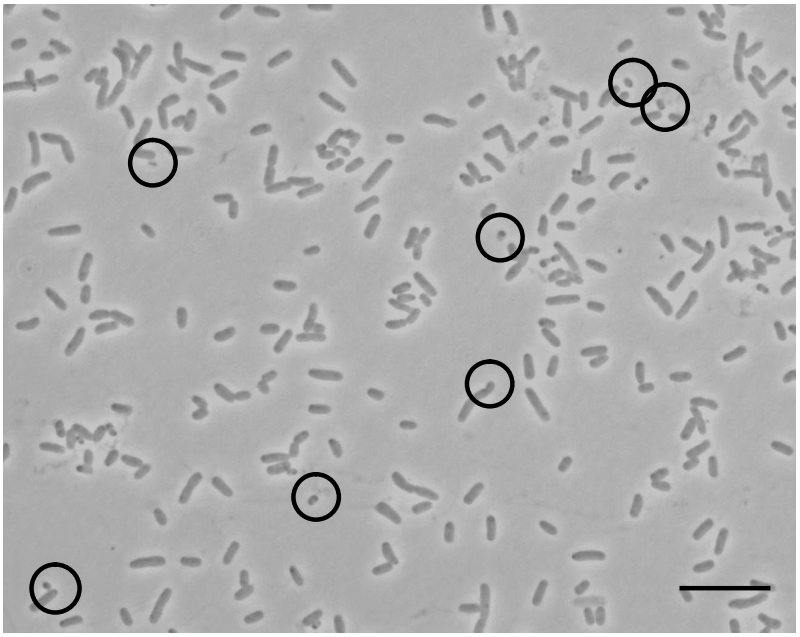
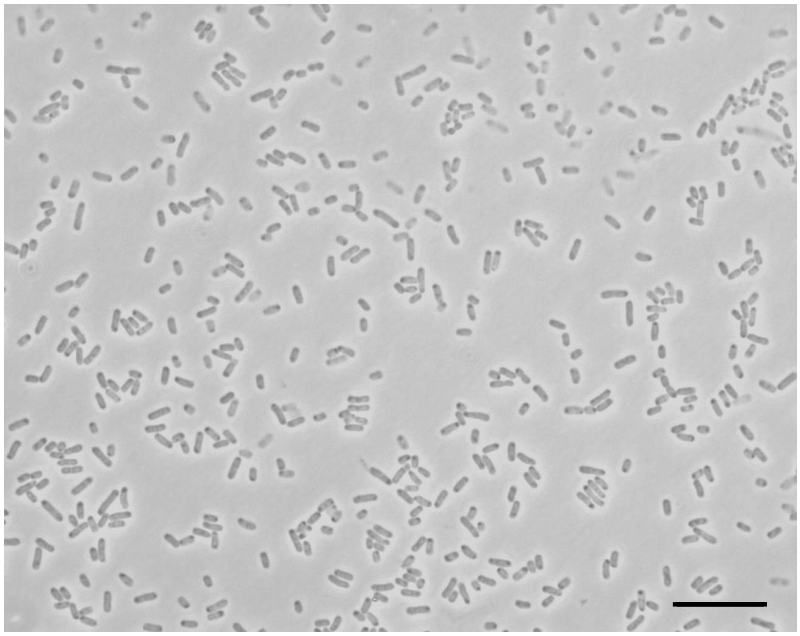


Figure 3(a): Phase contrast images of SL1344 and SL1344 *csp* null in exponential phase at 37°C. Cultures were grown to stationary phase (16h) at 37°C in LB, with aeration. Following this, cells were diluted 100-fold into fresh LB and grown to an OD_{600} of 0.2 at 37°C. Cells were harvested and washed in PBS before imaging. Images are representative of three replicate experiments. Minicells are denoted by black circles. Scale bars represent 10µm.

SL1344



SL1344 *csp* null

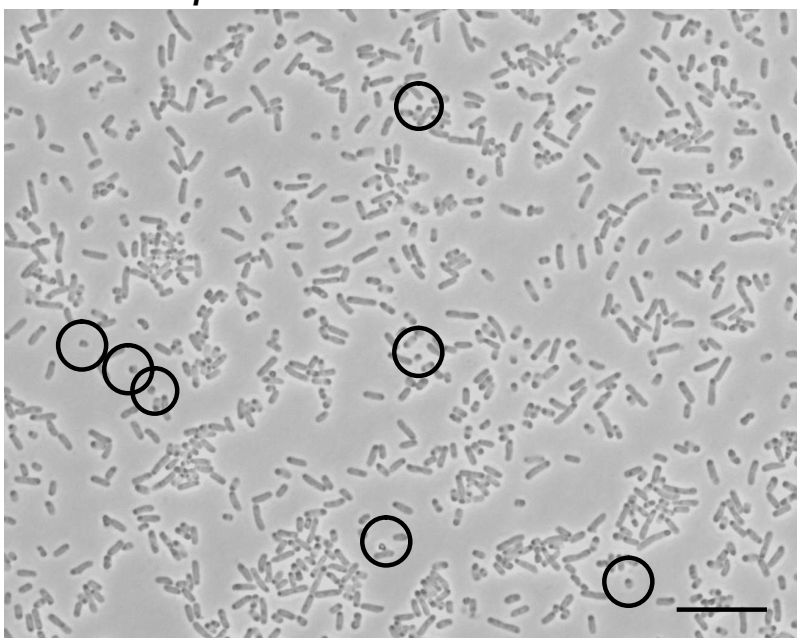
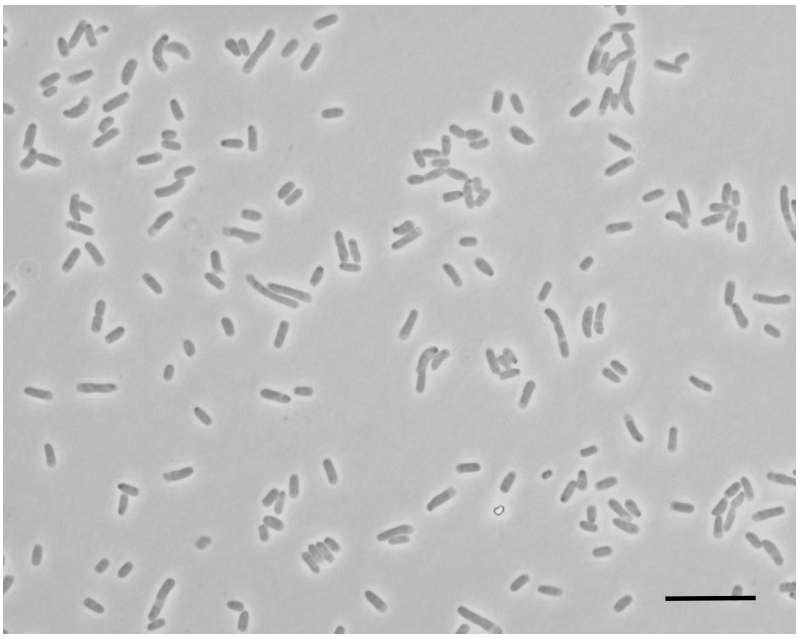


Figure 3(b): Phase contrast images of SL1344 and SL1344 *csp* null strains during stationary phase at 37°C. Cultures were grown to stationary phase (for 16h) in LB at 37°C, with aeration. Cells were harvested and washed in PBS prior to imaging. The images are representative of three replicate experiments. Minicells are denoted by black circles. Scale bars represent 10μm.

SL1344 *hfq*



SL1344 *rpoS*

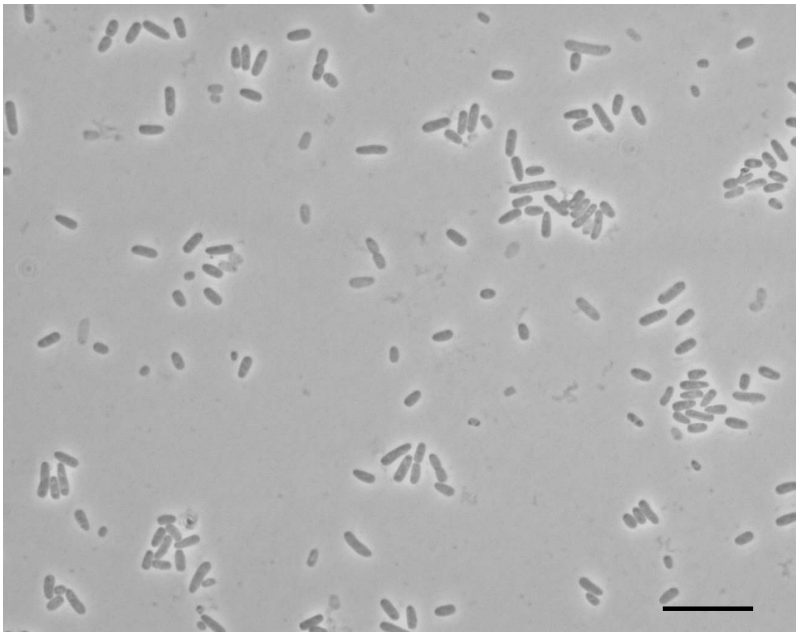
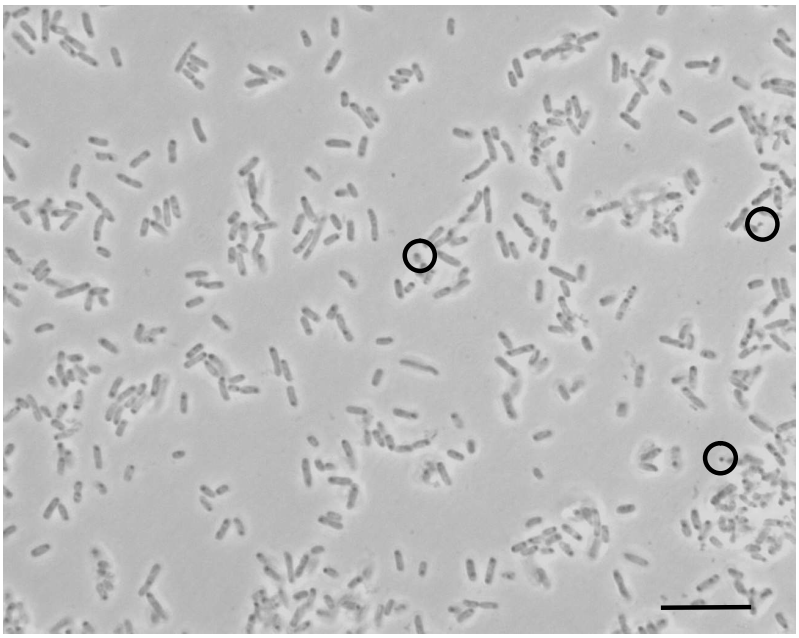


Figure 3(c): Phase contrast images of SL1344 *rpoS* and SL1344 *hfq* strains in exponential phase at 37°C. Cultures were grown to stationary phase (16h) at 37°C in LB, with aeration. Following this, cells were diluted 100-fold into fresh LB and grown to an OD_{600} of 0.2 at 37°C. Cells were harvested and washed in PBS before imaging. The images are representative of three replicate experiments. Scale bars represent 10µm.

SL1344 *hfq*



SL1344 *rpoS*

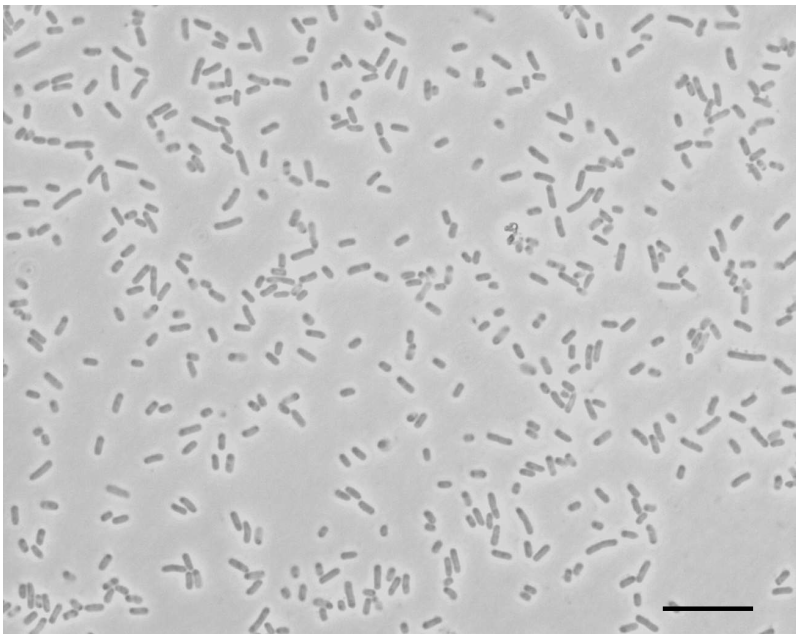
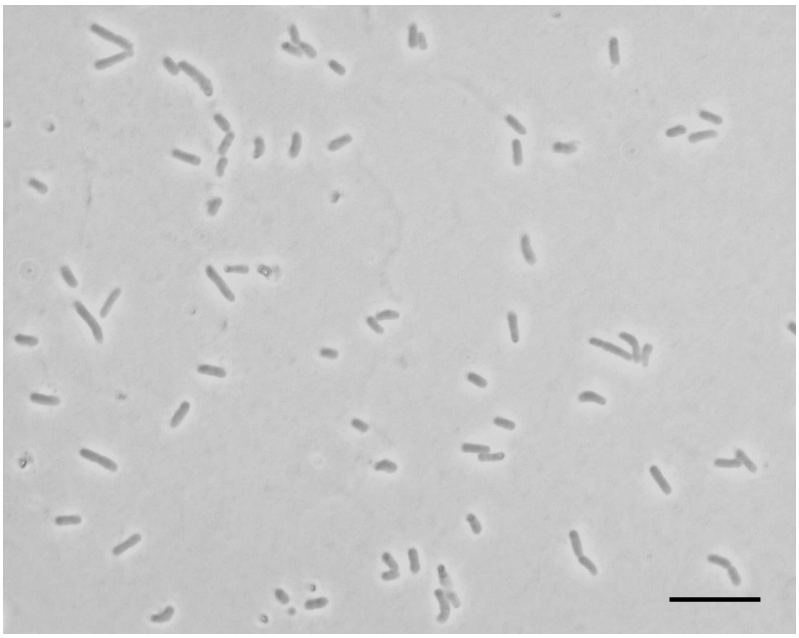


Figure 3(d): Phase contrast images of SL1344 *rpoS* and SL1344 *hfq* strains in stationary phase at 37°C. Cultures were grown to stationary phase (for 16h) in LB at 37°C, with aeration. Cells were then harvested and washed in PBS prior to imaging. Images are representative of three replicate experiments. Black circles denote minicell formation. Scale bars represent 10μm.

SL1344 *csp* null *hfq* mutant

Exponential phase



Stationary phase

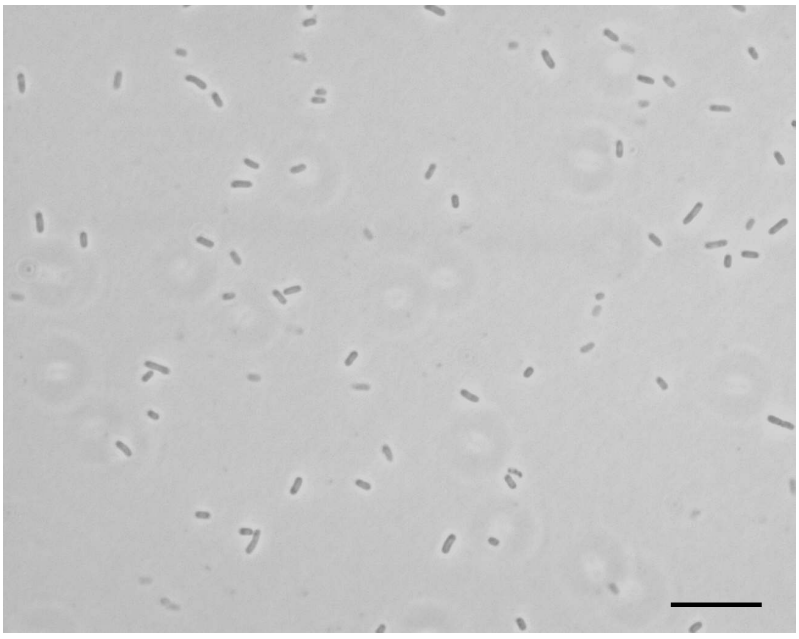


Figure 3(e): Phase contrast images of the SL1344 *csp* null *hfq* strain during both exponential and stationary phases at 37°C. Cultures were grown to stationary phase (for 16h) in LB, with aeration, at 37°C. Cells were diluted 100-fold into fresh LB and grown, at 37°C, to an OD_{600} of 0.2 Cells from both growth phases were harvested and washed in PBS prior to imaging. These images are representative of three replicate experiments. Scale bars represent 10μm.

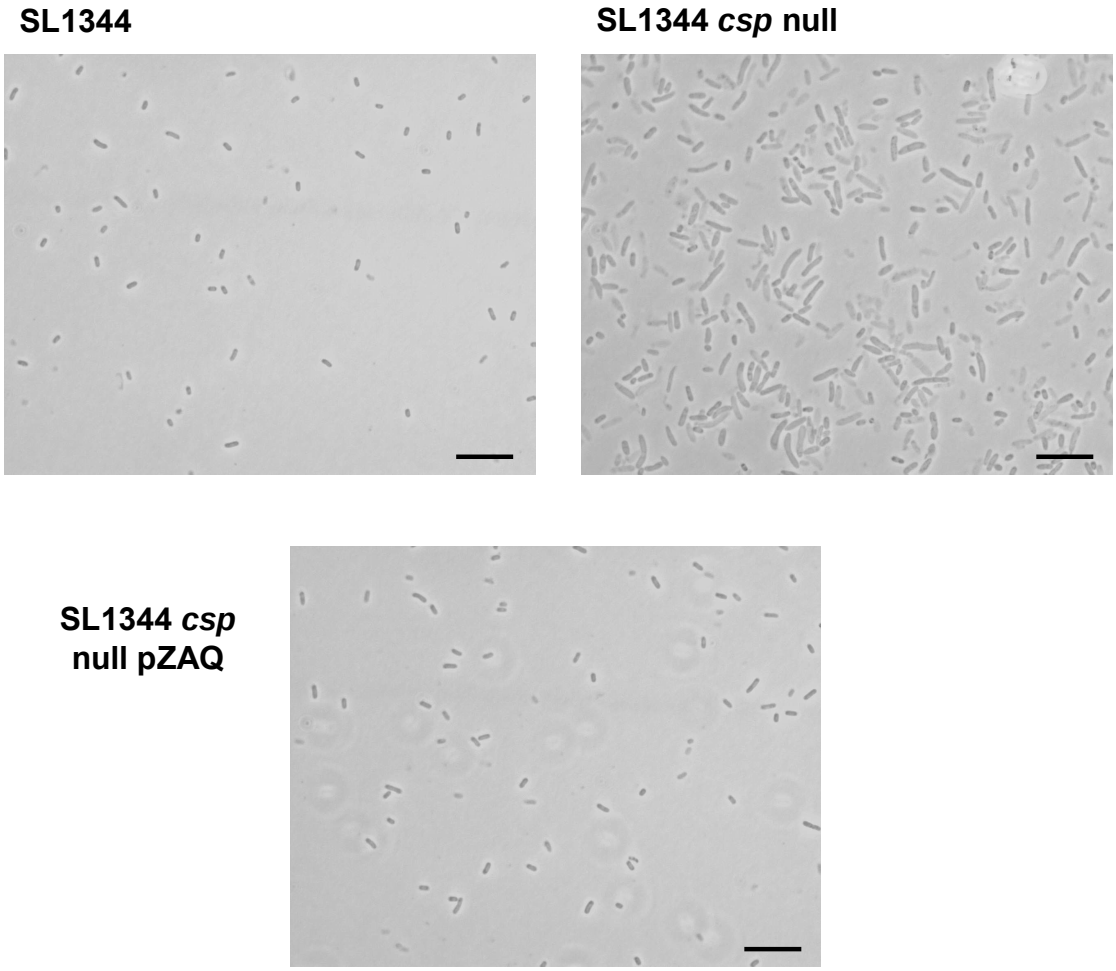


Figure 3(f): Phase contrast images comparing the SL1344 *csp* null and *csp* null pZAQ strains with their isogenic strains 3h following cold shock at 10°C. Cultures were grown to stationary phase in LB at 37°C, with aeration. Following this, cells were diluted 100-fold into fresh LB and grown to an OD_{600} of 0.2, at 37°C. Cells were then incubated, with aeration, at 10°C for 2h. Cells were harvested and washed in PBS prior to imaging. The images are representative of triplicate experiments. Scale bars represent 10µm.

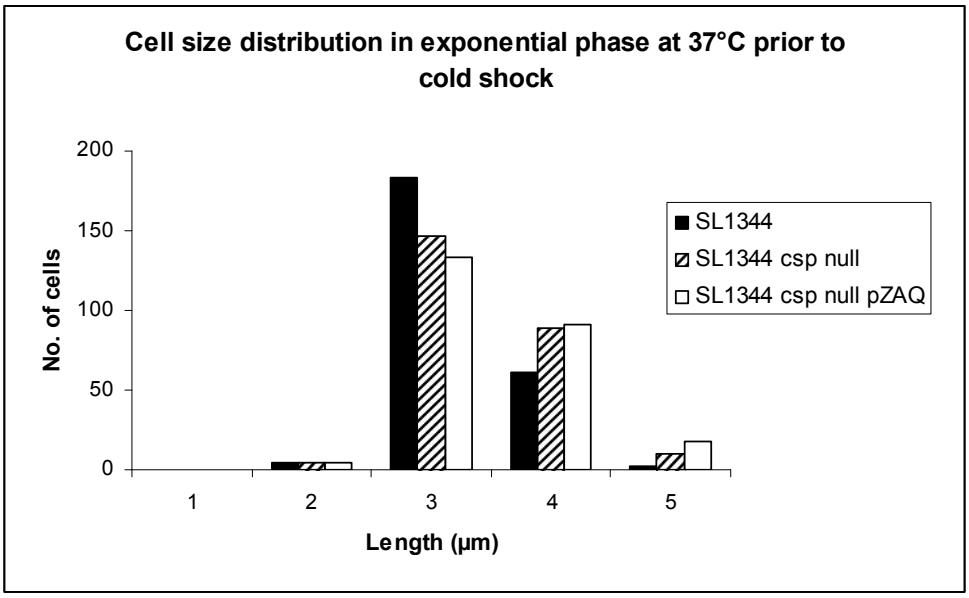


Figure 3(g): Cell size distributions of the SL1344 *csp* null strain, and this strain containing the pZAQ plasmid, compared to the isogenic parent strain during exponential phase at 37°C prior to cold shock. Stationary phase cultures, grown for 16h in LB at 37°C, were diluted 100-fold into fresh LB and grown to an OD_{600} of approximately 0.2 at 37°C, with aeration. Cells were then harvested, washed and resuspended in PBS prior to phase contrast microscopy. Experiment was performed in triplicate and 250 cells, evenly chosen from images, used for cell length measurements. This experiment was used as a control to show the effect of pZAQ plasmid on MPG 558 prior to cold shock.

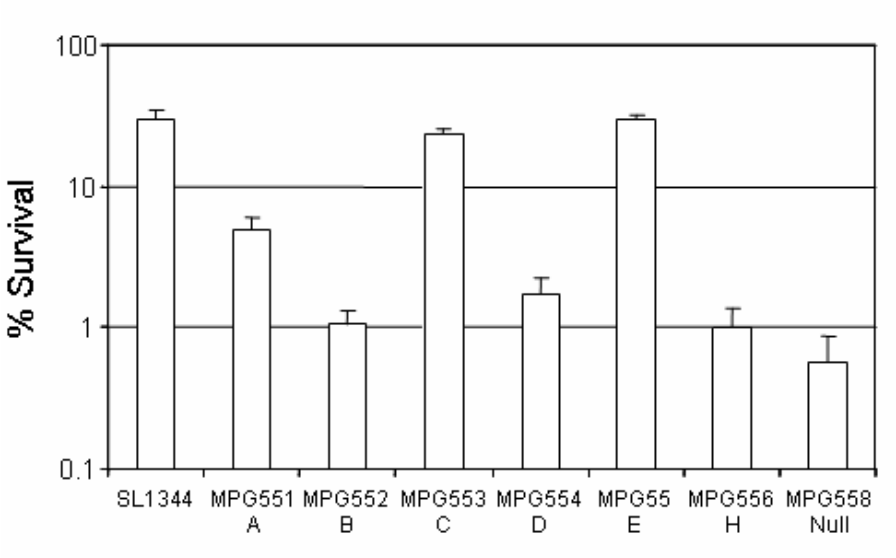


Figure 3(h): Graph showing percentage survival of the *csp* monogene strains after exposure to oxidative stress in stationary phase at 37°C. Strains were grown to stationary phase (for 16h) in LB at 37°C and exposed to 10mM H_2O_2 for 1h at 37°C. The resulting cfu/ml was compared to that before peroxide treatment. Results show the average of triplicate experiments and error bars show the standard error for each strain. Results taken from I.W. Hutchinson, PhD submitted to U of E, 2005.

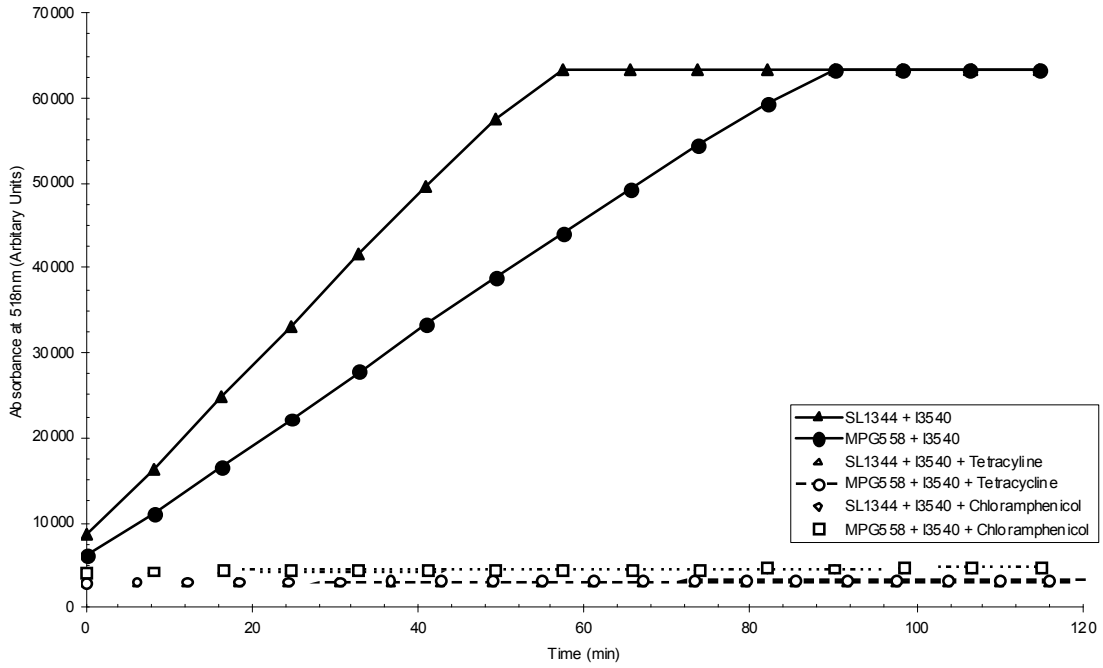


Figure 4: Determination of experimental conditions required to block translation in stationary phase cells. Stationary phase cultures of SL1344, containing the GFP-expressing plasmid I13540, were grown overnight (16h) at 37°C, in LB with aeration. The cultures were pre-treated with chloramphenicol before induction with 0.2% arabinose. Fluorescence was recorded, at 518nm, at various time points. A block in translation is implied when no further GFP induction was observed. 10 min treatment with chloramphenicol (20µg/ml) successfully inhibits translation in a stationary phase culture of SL1344.