UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Biological Sciences

CHARACTERISING THE REGULATORY MECHANISM OF THE GLOBAL REGULATOR BIPA

By

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ABSTRACT

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Previous studies uncovered a novel GTPase termed BipA that regulates a wide range of cellular processes in bacteria such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. These processes include flagella-mediated cell motility, resistance to a host defence protein, acid toae, capsule synthesis, growth at low temperatures and the characteristic ability of enteropathogenic forms of *E. coli* to rearrange the actin cytoskeleton of infected host cells. BipA resembles a number of known ribosome-binding GTPases and occupies a site on the ribosome that coincides with that of EF-G. Further, its GTPase activity is sensitive to high GDP/GTP ratios, but is stimulated by the presence of 70S ribosomes. These observations raise the possibility that BipA functions as a translator factor and that it interacts with a specific mRNA.

The primary aim of this study was to investigate further the relationship of BipA and and a possible regulatory cascade involving the protein. Studies with reporter gene fusions revealed that *bipA* expression was growth rate-regulated. Maximum transcription occurred in the early-exponential phase of growth, with transcription activity proportional to the richness of the growth medium. These findings prompted suggestions that BipA might share control of critical regulatory targets with the global regulator Fis, which has regulatory and expression profiles similar to BipA. BipA was discovered to be involved in Fis-mediated changes in DNA topology in response to a number of cellular stresses, as well as in the expression of critical componenets for glucose uptake and utilisation.

The results of this study may begin to unravel the mechanism of action of a novel global regulatory protein, BipA. The evidence suggests that BipA provides a regulatory link between nutritional availability and the ability of the bacterial cell to commit itself to a number of energy and resource intensive tasks. The study also introduces the possibility that BipA and Fis may work together in a shared and wide spread regulatory control mechanism.

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Thank you all, it was very much appreciated.

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ABBREVIATIONS

aa	amino acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATR	Acid tolerance response
BFP	Bundle-Forming Pili
BipA	BPI inducible protein A
BPI	Bactericidal/Permeability-Increasing
B. subtilis	*Bacillus subtilis*
cAMP	Adenosine 3'5'-cyclic monophosphate
cat	Chloramphenicol acetyltransferase
Csr	Carbon Storage Regulator
CRP	cAMP Receptor Protein
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EAF	EPEC Adherence Factor
EF-G	Elongation Factor G
EF-Tu	Elongation Factor Tu
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EMSA	Electrophoretic-Mobility Shift Assays
EPEC	Enteropathogenic *Escherichia coli*
ERIC	Enterobacterial Repetitive Intergenic Consensus sequence
esc	*E. coli* secretion
esp	*E. coli*-secreted protein
E. coli	*Escherichia coli*
Fis	Factor for Inversion Stimulation
FSB	Final Sample Buffer
GAP	GTPase Activating protein
GDI	Guanosine nucleotide Dissociation Inhibitor

GDP	Guanosine diphosphate
GEF	Guanine Exchange Factor
GNBP	Guanosine Nucleotide Binding protein
GNRP	Guanosine Nucleotide Release protein
GTP	Guanosine triphosphate
Hfq (HF-1)	Host Factor for Q RNA bacteriophage, a.k.a. Host Factor-1
H-NS	Histone-like nucleoid structuring protein
HRP	Horseradish Peroxidase
IF	Initiation Factor
IHF	Integration Host Factor
IM	Intermediate superhelical plasmid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kan	Kanamycin resistance gene
kb	kilobase
kDa	kiloDalton
LEE	Locus of Enterocyte Effacement
Ler	LEE-encoded regulator
LIN	Linear plasmid
MLD	mRNA-like Domain
mRNA	messenger Ribonucleic acid
NAD	Nicotinamide Adenine Dinucleotide
OD	Optical Density
ONPG	2-Nitrophenol β-D-galactopyranoside
ORF	Open Reading Frame
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
Per	Plasmid-encoded Regulator
ppGpp	Guanosine 5'-diphosphate 3'-diphosphate
QS	Quorum-sensing
RBS	Ribosome-binding Site
RNA	Ribonucleic acid
r-protein	Ribosomal protein

rRNA	Ribosomal Ribonucleic acid
saRNA	small antisense Ribonucleic acid
sRNA	small Ribonucleic acid
SC	Supercoiled plasmid
S-D	Shine-Dalgarno
SDS	Sodium Dodecyl Sulphate
sep	secretion of *E. coli* proteins
S. typhimurium	*Salmonella enterica* serovar Typhimurium
TLD	tRNA-like Domain
TIR	Translation Initiation Region
Tir	Translocated Intimin Receptor
tmRNA	transfer messenger Ribonucleic acid
TRAP	*trp* RNA-binding Attenuation Protein
tRNA	transfer Ribonucleic acid
TTSS	Type III Secretion System
UPEC	Uropathogenic *Escherichia coli*
5' UTR	5' Untranslated Region

CHAPTER 1

Introduction

CHAPTER 1:Introduction

1.1 General Introduction and Overview

Bacteria tend to encounter many environments and frequently face fresh challenges to their survival. In the case of *Escherichia coli* (*E. coli*), it has a preference for existing in the animal gut. However, before the microbe can reach its environmental niche, it must first endure transit from one host to another and then passage through the inhospitable gastro-intestinal tract. As a consequence, the microbe can encounter a variety of cellular stresses, such as sudden and dramatic changes in temperature, acidity and osmolarity or even exposure toxic chemicals. In addition to the threat of these harmful conditions or substances, the microbe faces constant fluctuations in levels of available nutrients and long periods of non-growth. When it eventually does find itself in optimal growth conditions within the gut, a microbe like *E. coli* would have to be able to react quickly to any sudden increase in available nutrition. This would enable it to take maximum advantage of such fortuitous opportunities before other competing bacteria can do likewise.

The response made by *E. coli*, and other similarly versatile bacteria, to changes in their immediate environment (beneficial or detrimental) is generally called adaptation. In order to adapt, a cell has to recognise the situation it is in and quickly produce the appropriate proteins and other components needed for an effective response. Being unicellular, and having only limited resources, it is essential for a microbe that adaptation is an efficient and effective process, and only occurs when it is absolutely required. To ensure this precision, the microbe has evolved a vast and complex regulatory network that is able to perceive changes in the surrounding environment and co-ordinate the expression of necessary genes known as effectors, whilst at the same time suppressing those genes that are no longer relevant to the cell and the new circumstances it finds itself in.

Accordingly, there are a range of mechanisms within the cell's global regulatory network that decide whether the effector genes are switched on or off- or their activity

increased or decreased- to match the new conditions the microbe is experiencing. As Figure 1.1 shows, throughout the process of gene expression and protein production there are a number of key stages where regulation can occur.

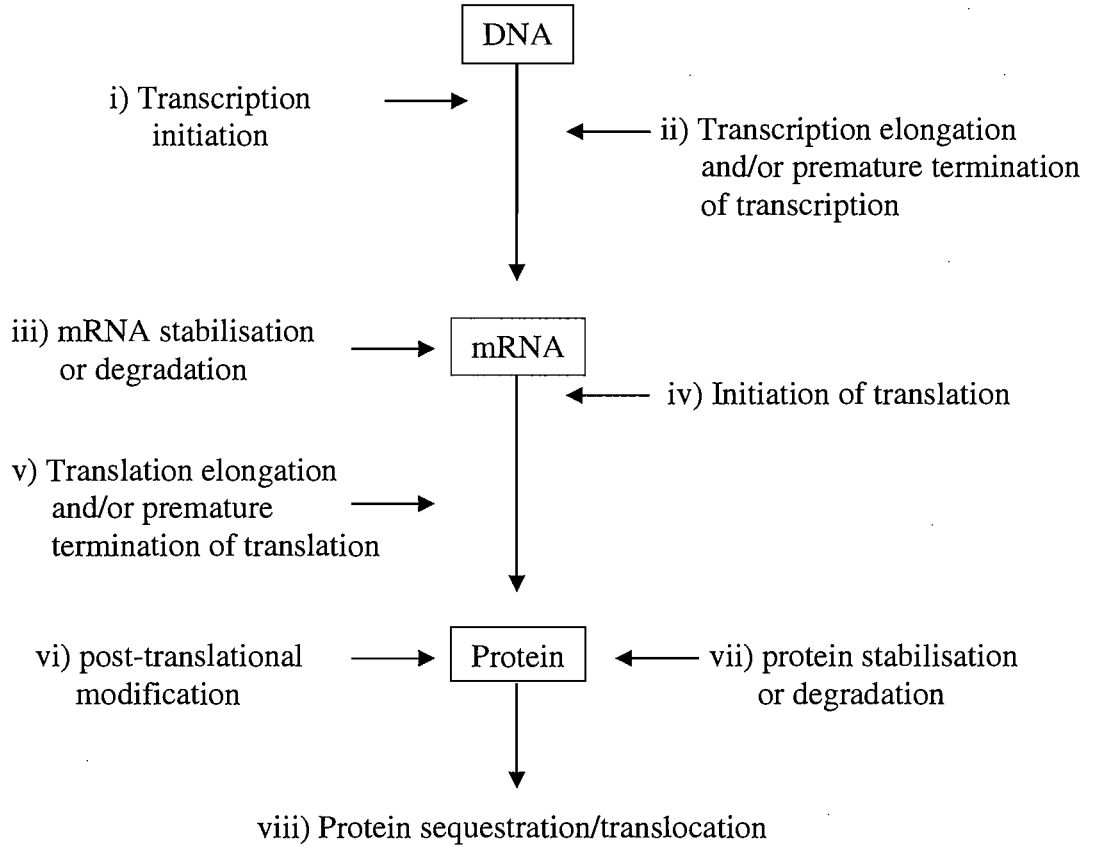


Figure 1.1 A diagram indicating the key points of regulation during gene expression and protein synthesis.

Until recently, it was the accepted opinion that the most important point of regulation was the process of the RNA polymerase interacting with the gene promoter during transcription initiation. An obvious reason for selecting transcription initiation for the key point of gene regulation would be the immediate prevention of inappropriate gene

activity, thereby totally avoiding the unnecessary waste of cellular resources (particularly the hydrolysis of valuable ATP). Furthermore, promoter-RNA polymerase interactions are affected by a multitude of others factors, these factors themselves influenced by changes in the cell's physiology, brought about by alterations in the surrounding environment and so can be incorporated into the global regulatory mechanism. For instance when a microbe experiences an increase in nutrient availability, changes in DNA topology, the production of alternative sigma factors (which direct RNA polymerase to genes specific to the cell's immediate needs), and the activation of a host of transcription factors occur. As a result of different combinations of transcription factors working together, co-regulation of functionally linked genes can occur, allowing the cell great flexibility to tune its response to a given stimuli. Interestingly, half of the known and predicted transcription factors in *E. coli* are capable of binding small metabolites, and are associated with the regulation of genes needed for the consumption or utilisation of the metabolite. A further 10% of transcription factors bear a CheY-like domain for use in a two-component signal transduction system (Madan Babu and Teichmann, 2003).

The emphasis put on regulation of transcription control as the primary point of regulation is also reflected by the shortlist of established global regulators that can be drawn up from the literature. Indeed, this list contains a select group of seven proteins, all of which act as transcription factors or modulate transcription: cAMP receptor protein (CRP), Integration Host Factor (IHF), Histone-like nucleoid structuring protein (H-NS), Factor for Inversion Stimulation (Fis), Leucine responsive regulatory protein (Lrp) and the two, two-component systems NarL and ArcA (Martinez-Antonio and Collado-Vides, 2003). Interestingly, approximately 49% of the genes in *E. coli* are regulated by more than one transcription factor, whilst the remaining 51% of genes can be directly regulated by just one out of list of seven global regulators (Martinez-Antonio and Collado-Vides, 2003).

Most of the previous research into gene regulation has therefore been on transcription initiation. Post-transcriptional regulation was largely considered to be associated with fine-tuning expression or where translation regulation did affect expression it was not

linked to a global phenomenon but restricted to a very limited number of genes (for example, the *trp* RNA-binding attenuation protein (TRAP) in *B. subtilis* (Babitske, 2004). However, over the last decade there have been a growing number of regulatory mechanisms identified and characterised that have an impact on gene expression at the posttranscriptional/translational level. Importantly, it has been observed that several of the transcription global regulators are themselves regulated at the translation level, e.g. DsrA control of H-NS expression (Lease *et al.,* 1998; Lease and Belfort, 2000). In addition, there has been the realisation that gene regulation is not limited to protein-based mechanisms, but can also be mediated through manipulation of messenger RNAs or by non-coding regulatory RNAs and their RNA-binding protein accomplices (riboregulation).

This thesis concerns the molecular dissection of a new type of regulatory mechanism and in particular studies to identify the direct target of a regulatory GTPase protein termed BipA (BPI inducible protein A). BipA was originally discovered during previous proteomic studies when it was seen to be strongly up-regulated when *Salmonella enterica* serovar Typhimurium cells (hereafter called *S. typhimurium*) were exposed to the bactericidal/ permeability-inducing protein (BPI), a cationic host defence component produced by human granulocytes (Elsbach and Weiss, 1993; Elsbach, 1998). Subsequent experiments with a BipA homologue in an enteropathogenic strain of *Escherichia coli* (EPEC) indicated that BipA has a crucial role in the regulation of a range of cellular processes (Grant *et al.,* 2003). Amongst the list of processes regulated by BipA include, flagella-mediated cell motility, regulation of group 2 capsule gene clusters, cold shock, resistance to BPI, tolerance to acid and the characteristic ability of EPEC to induce actin accumulation in host cells beneath adherent bacteria (Farris *et al.,* 1998; Barker *et al.,* 2000; Rowe *et al.,* 2000; Grant *et al.,* 2001; Pfennig and Flower, 2001; Grant *et al.,* 2003).

Recent data from the O'Connor laboratory has indicated that the protein associates with, and has its GTPase activity stimulated by ribosomes (R. Owens, PhD thesis). BipA therefore bears all the hallmarks of being a novel form of global regulator, one that is predicted to act on target RNA molecules. To date, there are a limited number of

global regulatory proteins that are known to mediate control through interacting with specific RNA molecules. Thus the regulatory mechanism of a new type of RNA-interacting regulator is of great interest and significance.

The results of this study strongly suggest that BipA operates by a novel mechanism on one specific mRNA target. As this is the case, it is perhaps relevant to consider what is currently known about bacterial regulatory processes that involve mRNA-protein and mRNA-RNA interactions.

1.2 Regulation at the level of post-transcription

1.2.1 Transcription attenuation by TRAP

In *Bacillus subtilis* constitutive initiation of the *trpEDCFBA* operon appears to be negatively regulated by transcription attenuation. Regulation is brought about by through the activity of the *trp* RNA-binding attenuation protein (TRAP) (Gollnick, 1994; Babitzke, 1996). The TRAP molecule is a ring-shaped structure comprising of eleven identical subunits (Antson *et al.,* 1995). The ability of TRAP to bind RNA is activated by the co-operative binding of 11 tryptophan residues to each one of the TRAP subunits. The mechanism by which this mass binding of tryptophan actually activates TRAP still remains unknown although it is believed that TRAP remains as an eleven-strong subunit complex even in the absence of tryptophan (Antson *et al.,* 1995; Li *et al.,* 2002).

Within the leader region of the *trpEDCFBA* mRNA transcript there are several inverted repeat sequences that can either form an intrinsic terminator or an altogether different antiterminator structure (Shimotsu *et al.,* 1986). When cellular levels of tryptophan are low the TRAP does not bind to the leader region of the nascent mRNA, therefore the antiterminator secondary structure is left intact and transcription continues on to completion. However, if the cell is growing in the presence of high levels of tryptophan TRAP binds to a series of eleven trinucleotide repeats that consist of 7 GAGs and 4

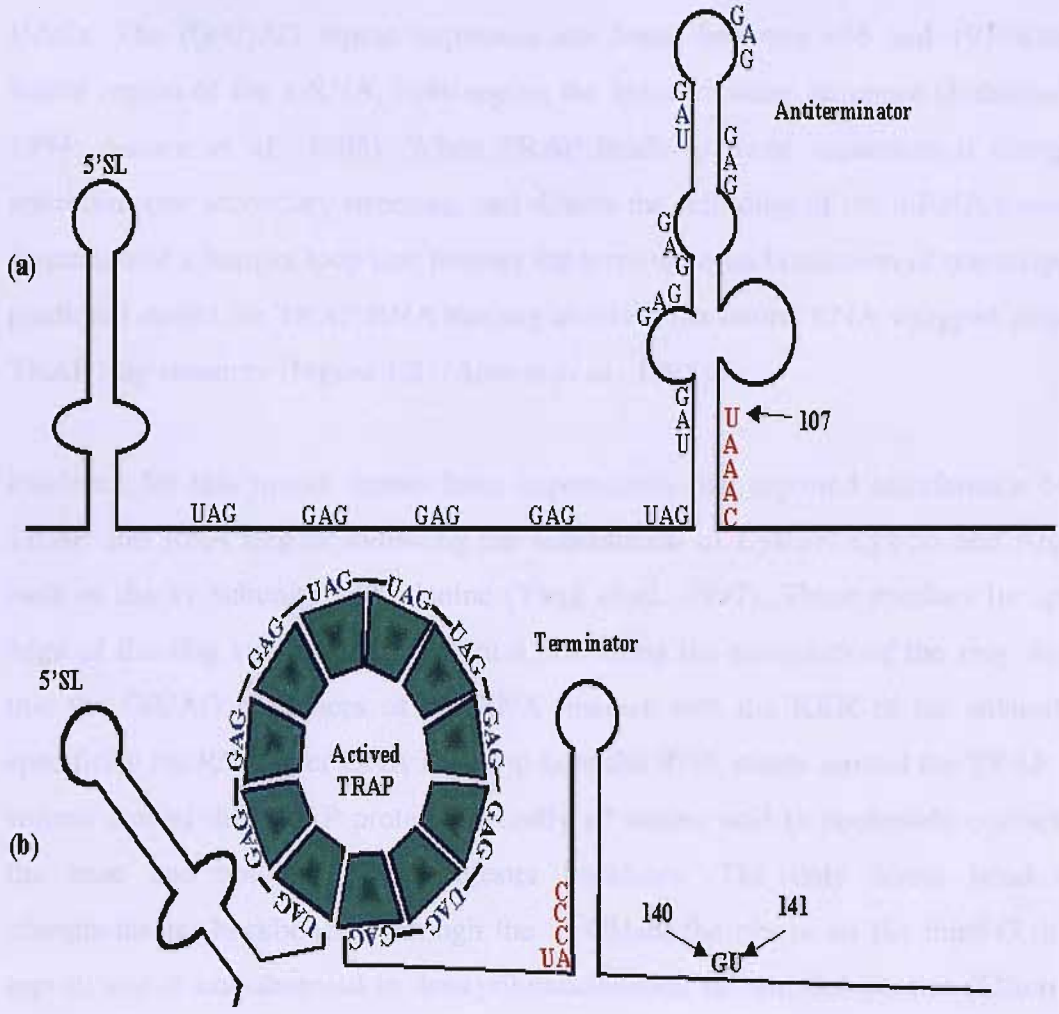


Figure 1.2 A model of *trp* operon transcription attenuation by TRAP.

RNA polymerase pauses at U107. In tryptophan limiting conditions the TRAP is not activated and does not bind to the trp mRNA. RNA polymerase resumes activity and continues transcription. If tryptophan is in excess TRAP is activated and whilst the RNA polymerase is paused at U107, binds to the G/UAG repeats as the mRNA is produced. This binding prompts the RNA polymerase to continue, but also prevents the anti-terminator structure forming. Instead a terminator is produced that causes transcription termination at either G140 or U141.

(Figure adapted from Babitzke, 2004).

UAGs. The (G/U)AG repeat sequences are found between +36 and +91 within the leader region of the mRNA, overlapping the antiterminator sequence (Babitzke *et al.*, 1994; Antson *et al.*, 1995). When TRAP binds to these sequences it disrupts the antiterminator secondary structure, and directs the refolding of the mRNA towards the formation of a hairpin loop that favours the terminator and cessation of transcription. A predicted model for TRAP:RNA binding involves the bound RNA wrapped around the TRAP ring structure (Figure 1.2) (Antson *et al.*, 1995).

Evidence for this model comes from experiments that reported interference between TRAP and RNA targets following the substitution of Lys-37, Lys-56 and Arg-58 of each of the 11 subunits with alanine (Yang *et al.*, 1997). These residues lie upon the edge of the ring structures, and form a line along the perimeter of the ring, implying that the G/UAG sequences of the RNA interact with the KKR of the subunits. The specificity for RNA over DNA is due to how the RNA winds around the TRAP. When wound around the TRAP protein, virtually all amino acid to nucleotide contact is via the base and not the phosphodiester backbone. The only direct bond to the phosphodiester backbone is through the 2' OH of the ribose on the third G of every repeat, and is not observed in deoxyribonucleioside substitution studies (Elliott *et al.*, 1999).

1.2.2 Translation-mediated transcription antitermination

Many of the aminoacyl tRNA synthetase genes and amino acid biosynthetic operons in gram-positive bacteria are regulated by a mechanism that uses tRNA antitermination. Low levels or starvation for a particular amino acid induces expression of the corresponding biosynthetic genes and also the aminoacyl-tRNA synthetase genes. Upregulation of the aminoacyl-tRNA synthetase genes is presumably to produce more tRNAs that correspond to the amino acid in short supply, this could allow increased tRNA charging efficiency (Grandoni *et al.*, 1992; Henkin, 1994; Condon *et al.*, 1996).

At the 5' end of the mRNAs from the aminoacyl tRNA synthetase genes and amino acid biosynthetic operons there are large UTR of around 300 nucleotides in length

(Grundy and Henkin, 1993). Within these UTRs there are several conserved features that include three stem loop structures immediately upstream of an intrinsic terminator. The terminator is the default secondary structure and is maintained when the inducer (such as high levels of the corresponding amino acid) is present. Also within the leader sequence is a conserved 14-nucleotide sequence termed the T-box and a specific triplet sequence that corresponds to the codon of the particular amino acid. The triplet sequence, which is always located on a bulge on stem loop I, is also known as the specifier sequence and is very important for the regulatory mechanism (Grundy and Henkin, 1993).

Studies with the *tyrS* gene (for tyrosyl-tRNA synthetase) have shown that altering the sequence to match the codon of another amino acid changes the regulatory response to starvation of tyrosine to that of the new amino acid. Further experiments reported that translation of the codon was not a prerequisite for gene induction, and confirmed that an uncharged tRNA molecule was the actual inducer (Grundy and Henkin, 1993). The important sequence of the leader region, the T-box, is involved in forming the antiterminator structure in the absence of the amino acid. This antiterminator is stabilised by an interaction between a UGGN sequence in the T-box with a complementary sequence, NCCA, located at the 3' end of the tRNA molecule (Grundy and Henkin, 1994). A model for tRNA antitermination regulation has recently been proposed. When the cell experiences starvation conditions for a certain amino acid, the uncharged tRNAs linked to the amino acid interact with the leader region of nascent mRNA of the required biosynthetic/tRNA synthetase gene. This binding induces the formation of an antiterminator structure that allows complete transcription to occur. Aminoacetylation of the tRNA interferes with the CCA end of the tRNA, leaving the molecule incapable of interacting with the T-box in the mRNA leader regions. This would encourage reversion back to the default mRNA secondary structure and the formation of the terminator (Condon *et al.,* 1996; Gollnick and Babitzke, 2002; Henkin and Yanofsky, 2002). Interestingly genomic studies have revealed that there are up to 300 transcription regulation mechanisms, which are based on the T-box set-up (Grundy and Henkin, 2003; Grundy and Henkin, 2004).

1.2.3 Transcription attenuation by antisense RNA

Antisense RNAs are often small non-coding RNAs that are able to base-pair with target genes or mRNAs to either augment or interfere with genetic activity. An example is the antisense RNA III that regulates the copy number of the streptococcal plasmid pIP501 by attenuation (Brantl *et al.,* 1993; Brantl and Wagner, 1994; Brantl and Wagner, 1996). RNA III inhibits expression of the *repR* gene, which encodes for the vital RepR initiator protein. RNA III binds to leader sequence of the nascent *repR* mRNA (RNA II) and through a 5′ YUNR motif (Y=pyrimidine, R=purine), a motif which is often found in RNA recognition loops in antisense RNA-regulated gene systems. The YUNR sequence motif specifies two intraloop hydrogen bonds forming U-turn structures in many anticodon-loops and all T-loops of tRNAs, the hammerhead ribozyme and in other conserved RNA loops. This structure creates a hairpin loop that forms the intrinsic terminator structure (Franch *et al.,* 1999; Heidrich and Brantl, 2003) (Figure 1.3). The interaction between the two RNAs begins with what is termed a 'kissing complex', leading to the formation of a complete intermolecular helix (Brantl and Wagner, 1994; Brantl and Wagner, 1996). Although both RNAs have a great deal of complementarity it has been shown that transcription inhibition is at a higher priority than complete and stable bonding. A reason for this could be that base-pairing RNAs often stop further transcription whilst they have reached a stage of stable binding intermediates (Brantl and Wagner, 1994; Malmgren *et al.,* 1996).

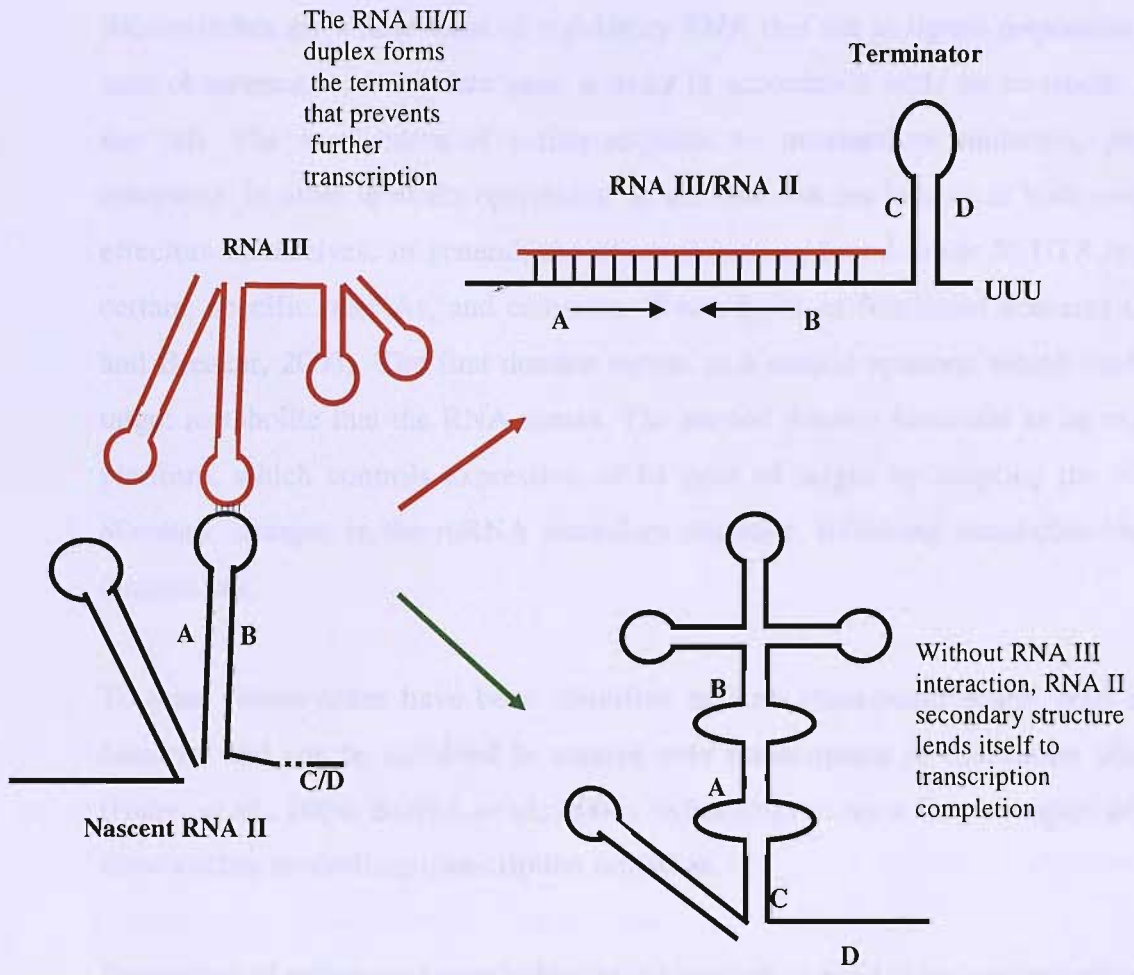


Figure 1.3 A model of the plasmid-encoded *repR* transcription attenuation. Adapted from Gollnick and Babitzke, 2002.

1.2.4 Transcription termination/antitermination by riboswitches

Riboswitches are a new class of regulatory RNA that act as ligand-responsive genetic control elements and regulate gene activity in accordance with the metabolic state of the cell. The mechanism of action requires no intermediate molecule, protein or otherwise, in order to direct operations, as the riboswitches behave as both sensors and effectors themselves. In general, the riboswitches are found in the 5'-UTR regions of certain, specific mRNAs, and comprise of two types of functional domains (Winkler and Breaker, 2003). The first domain serves as a natural aptamer, which binds to the target metabolite that the RNA senses. The second domain functions as an expression platform, which controls expression of its gene of origin by adapting the effects of allosteric changes in the mRNA secondary structure, following metabolite binding to domain one.

To date, riboswitches have been identified in both gram-positive and gram-negative bacteria, and can be involved in control over transcription or translation attenuation (Nahvi *et al.,* 2004; Barrick *et al.,* 2004). What follows are a few examples of mRNA riboswitches controlling transcription initiation.

Regulation of amino-acid metabolism by riboswitches e.g. Lysine-sensing riboswitches

The *lysC* gene of *Bacillus subtilis* (*B. subtilis*) encodes for the first specific enzyme of lysine biosynthesis process, and is under the control of a lysine-sensing riboswitch mechanism (Mandal *et al.,* 2003). The *lysC* gene is negatively controlled by the availability of lysine, which is sensed by a putative antiterminator/anti-antiterminator domain in the mRNA, called the Lys-box. Full-length copies of the *lysC* mRNA are formed when the cells are starved of the amino acid. When lysine is in plentiful supply, a truncated, 270 nucleotides long version of the mRNA is produced instead of the complete original (Kochhar and Paulus, 1996). Transcript mapping analysis revealed that the shorter version corresponded to the upstream region of the *lysC* leader sequence, running from the transcription initiation site to a hypothetical intrinsic terminator site. Further analyses have shown that the presence of lysine does not affect

the numbers of *lysC*-specific mRNAs, rather the amino acid forces the replacement of full length with truncated versions of the mRNA. As no protein or tRNA factor has been attributed to this lysine-induced change in the full length to truncated mRNA ratio, it has been postulated that *lysC* is regulated by a feedback transcription attenuation mechanism, mediated by the antiterminator and anti-antiterminator Lys-box (Figure 1.4a). Mutations within the Lys-box of the *B. subtilis lysC* gene resulted in expression, irrespective of lysine levels, whilst constitutive expression was witnessed following mutations of the Lys-box in the *E. coli* homologue (Patte *et al.,* 1998). Experiments trying to deduce the exact mechanism are currently ongoing (Nudler and Mironov, 2004).

Regulation of purine metabolism by riboswitches

It has recently been discovered that in *B. subtilis* the five operons involved with purine biosynthesis, inter-conversion and transport are all regulated by riboswitches (Mandal *et al.,* 2003; Nudler and Mironov, 2004). The *pur* and *xpt-pbuX* operons, which are involved with the synthesis of inosine monophopshate and the phosphoribosylation and uptake of xanthine respectively, are both regulated by transcription initiation and attenuation (Sonenshein *et al.,* 2002). Contained within each of the leader regions of the operons there are the terminator-antiterminator-anti-antiterminator formations that are characteristic of riboswitch regulation. There is also a conserved region known as the G-box, which can be found in the 5'-leader regions of related, purine-associated operons such as *pbuG* (hypoxanthine transporter), *nupG* (purine nucleoside transporter) and *pbuE* (purine efflux pump) (Johansen *et al.,* 2003; Mandal *et al.,* 2003). Regulation of both of these operons is governed by the presence of hypoxanthine and guanine (Christiansen *et al.,* 1997). Indeed, recent experimental analyses have determined that the G-box is a direct sensor for the two purines, and is able to form tight, specific interactions with either molecule (Mandal *et al.,* 2003). When an interaction between the G-box and either hypoxanthine or guanine occurs, a change in the secondary structure of the mRNA leader sequence is induced. This leads to the disruption of the default antiterminator formation, and refolding to form an anti-antiterminator, which immediately brings a halt to transcription (Johansen *et al.,* 2003). The one exception to

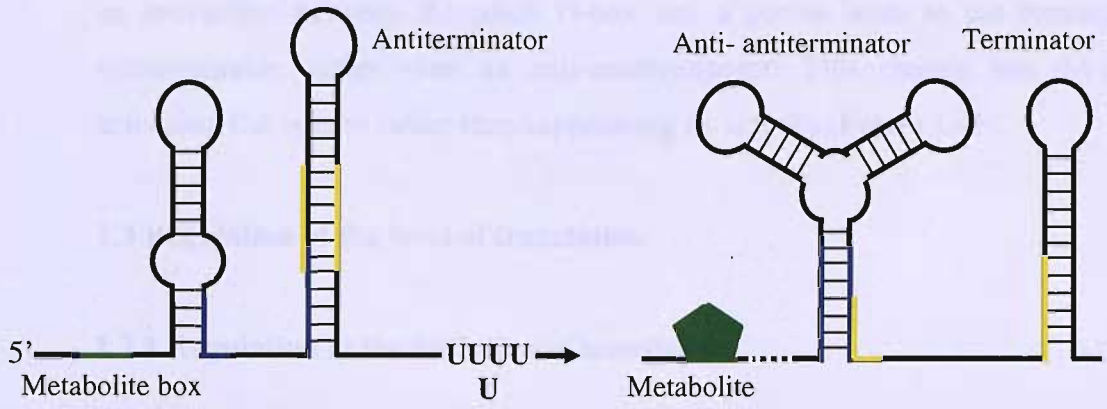


Figure 1.4 (a) Transcription termination by riboswitches. The antiterminator formation in the UTR of the nascent mRNA is the default setting, thus transcription continues normally in the absence of the specific metabolite. However when levels of the specific metabolite are elevated, metabolite molecule bind to the specific box (e.g. rfn-box and FMN). This binding interaction changes the secondary structure by disrupting the complementary sequences within the antiterminator (yellow). The interacting metabolite also stabilises the formation of the terminator by encouraging base-pairing between the second string of complementary bases (blue), thereby halting transcription.

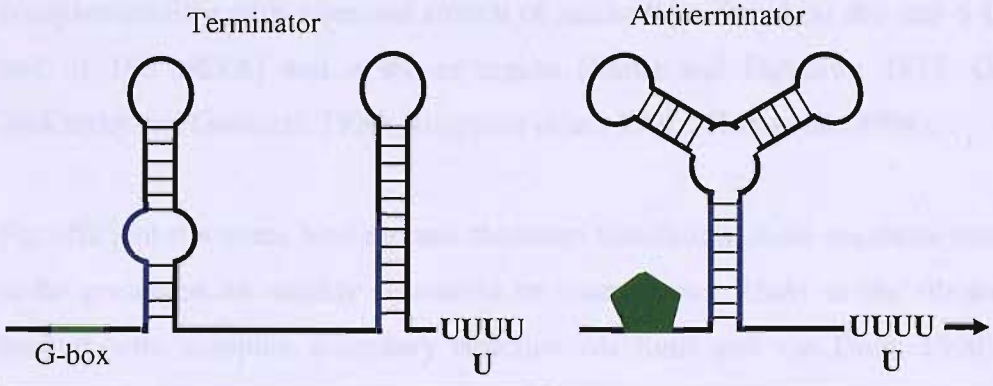


Figure 1.4 (b) Transcription antitermination by riboswitches. The terminator formation in the UTR of the nascent mRNA is the default setting, thus transcription does not proceed unless the specific metabolite is present (e.g. adenine and the *pbuE* operon). However when levels of the metabolite are elevated, the molecule binds to the specific box (e.g. G-box). This binding interaction changes the secondary structure by disrupting the complementary sequences within the terminator and stabilises the formation of the antiterminator by encouraging base-pairing between the second string of complementary bases (blue).

Figures adapted from Nudler and Mironov, 2004

this G-box rule is *pbuE*, which is able to sense adenine rather than guanine. In addition, an interaction between the *pbuE* G-box and a purine leads to the formation of an antiterminator, rather than an anti-antiterminator. This change has the effect of activating the operon rather than suppressing its activity (Figure 1.4b).

1.3 Regulation at the level of translation

1.3.1 Regulation at the initiation of translation

In both eukaryotes and prokaryotes, translational control frequently occurs at the initiation stage, thereby providing the cell with a direct and rapid way to alter the activity of an mRNA in response to a perceived change in the environment. In prokaryotes, translation is initiated from a well-defined translation initiation region (TIR). A typical TIR would consist of a ribosome-binding site (RBS), an initiator codon, the Shine-Dalgarno region (S-D) (a sequence of nucleotides that show complementarity with a second stretch of nucleotides found on the anti-S-D on the 3' end of 16S rRNA) and a spacer region (Shine and Dalgarno 1975; Gold, 1988; McCarthy and Gualerzi, 1990; Ringquist *et al.*, 1992; Chen *et al.*, 1994).

For efficient ribosome binding, and therefore translation, these sequence elements have to be presented on weakly structured or unstructured RNA, as the ribosome cannot interact with complex secondary structure (de Smit and van Duin, 1990). However there are, of course, exceptions to any rule, and back-up systems that are capable of rescuing the situation when things go wrong. Here are a few examples of these exceptions to the translation initiation rule:

Non-canonical mechanism of translation control – the S1 ribosomal protein

One of the most interesting of exceptions to the translation initiation rule is the *rpsA* mRNA of *Escherichia coli*, which encodes the ribosomal protein (r-protein) S1. The mRNA is of note, as it does not contain an authentic Shine-Dalgarno sequence in its 5' UTR. Other mRNAs encoding r-proteins do contain a recognisable S-D, with, at the

very least, four contiguous nucleotides that are complementary to the anti-S-D region of the 16S rRNA. The *rpsA* transcript contains only three such nucleotides in a GAAG sequence, and is extremely inefficient at driving translation (Dunn *et al.,* 1978, Schwartz *et al.,* 1981). Despite this difference, translation of the *rpsA* mRNA occurs at a high frequency, often at a higher rate than other r-protein encoding mRNAs that bear larger S-D sequences (Boni *et al.,* 2000).

S1 binds to the ribosome through its N-terminal domain, while it uses its central and C-terminal domains to interact with mRNAs readying them for translation (Subramanian, 1983; Subramanian, 1984; Bycroft, 1997). The protein belongs to the OB family of proteins, which are highly specific for single-stranded nucleic acids. Although the protein does not have any strict sequence specificity, it does have a high affinity for sequences of polypyrimidines RNAs (Subramanian, 1983; Draper and Reynaldo, 1999). Similar to other r-proteins, S1 regulates its own synthesis by acting as an autogenous repressor (Subramanian, 1983; Subramanian, 1984; Roberts and Rabinovitz, 1989; Skouv *et al.,* 1990; Boni *et al.,* 1991; Sorensen *et al.,* 1998; Boni *et al.,* 2000). A mechanism for the autoregulation of *rpsA* was recently proposed by Boni *et al.* (2004). The components of the *rpsA* TIR are spaced apart from one another and present a non-contiguous ribosome-binding region. According to the model in the Boni paper, in place of a S-D sequence, translation requires the TIR to fold to form a long, stable hairpin structure. This folding would bring together the components of the TIR, thereby completing the ribosome-binding region and optimising translation. Furthermore, it is suggested that this hairpin may present a tertiary structure that promotes S1 binding during its role as a component of the 30S subunit or by free S1 proteins. The binding of free S1 proteins to the mRNA disrupts these hairpins, preventing ribosome binding. However, free S1 protein cannot compete with 30S subunits for interaction with *rpsA* (Boni *et al.,* 2000). Thus regulation of *rpsA* translation by S1 can only occur when the mRNA is in excess. The Boni group therefore concluded that equilibrium between active and inactive (S1-bound) *rpsA* mRNAs, together with the level of active ribosomes, dictated whether S1 would act as an autorepressor or promote translation.

Translation of leaderless mRNAs by 70S ribosomes

It is the accepted opinion that translation initiation in prokaryotes proceeds with the binding of 30S ribosomes to a mRNA, through specific interactive RNA sequences (the Shine and Dalgarno (S-D) sequence on mRNA and the anti-SD sequence located at the 3'end of 16S rRNA (Shine and Dalgarno 1975; Gold, 1988; McCarthy and Gualerzi, 1990; Ringquist *et al.,* 1992; Chen *et al.,* 1994; Kozak, 1999). Three initiation factors (IF1–IF3) control the 30S-mRNA–initiator–tRNA ternary complex and enable correct codon selection. Thus the ribosome is attached to the mRNA by both the SD–anti-SD interaction and the codon–anti-codon interaction (Gualerzi and Pon, 1990). There is experimental evidence that suggest an additional control mechanism for a second *t*ranslation *i*nitiation *p*athway (*tip*). This involves a 30S-initiator–tRNA complex, which is recruited to canonical mRNAs (Wu *et al.,* 1996). The IF2 appears to act as a carrier for initiator–tRNA to the ribosome, aiding the formation of the 30S-initiator–tRNA complex (Mayer *et al.,* 2003).

However, there are a number of studies have shown that IF3 prevents 30S initiation complex formation with leaderless mRNAs *in vitro*, and that leaderless mRNA translational efficiency is negatively correlated with the concentration of IF3 *in vivo* (Balakin *et al.,* 1992; Tedin *et al.,* 1999; Grill et al., 2001; O'Donnell and Janssen, 2002). Interestingly, it has been shown that 70S ribosomes, in contrast to 30S ribosomal subunits, show a high preference for the 5' terminal AUG of leaderless phage λ*c*I repressor mRNA (Balakin *et al.,* 1992). Additionally, Moll *et al.* (2004) showed that cells that possessed IF3-deficient 30S subunits, were able to initiate translation of leaderless mRNAs from intact 70S ribosomes under normal and extreme physiological conditions. This mechanism may be involved in certain *Streptomyces* species that produce several leaderless mRNAs that encode proteins conferring resistance to multiple antibiotics. Although each of the antibiotics is structurally different, all have the ribosome as a common target. The ability to promote translation of leaderless mRNAs during physiological ideal conditions, but in the absence of working 30S subunits would provide the bacteria with a possible alternative strategy (Janssen, 1993).

Secondary translational initiation regions (STIRs)

In bacteria mRNAs can exist either in mono- or polycistronic forms, and although each individual cistron of a polycistronic message has its own TIR, in some cases, cistrons can be translationally coupled wherein a single TIR controls multiple cistrons (Inokuchi *et. al.*, 2000). There are occasions where a cistron can have more than one functional TIR (Laursen *et. al.*, 2002). Indeed, it was recently shown that monocistronic *rpoS* mRNA has two translation initiation regions (Subbarayan and Sarkar 2004b). The *rpoS* gene is highly polymorphic, with a common mutation being a C-T transition at position 97 (Atlung *et. al.*, 2002; Subbarayan and Sarkar 2004a). This mutation results in an amber codon (CAG-TAG; *rpoSAm*). Consequently, σ^S is prematurely terminated, and suppressor-free *rpoSAm* mutants would have no σ^S activity. However, it was observed that several *rpoSAm* strains exhibited only reduced σ^S activity and not a total shutdown. This was found to be due to the presence of an STIR in the *rpoS* gene (Subbarayan and Sarkar 2004a; Subbarayan and Sarkar 2004b; Subbarayan and Sarkar 2004c). Normally, the translating ribosomes would proceed from a primary TIR overriding the STIR. Translation from the STIR can be activated by two pathways; first, when ribosome disassembly occurs ahead of the STIR (as in *rpoSAm* mutants) or, second, when there is an absence of complete translation initiation from the primary TIR (Subbarayan and Sarkar 2004c). A similar mechanism known as internal ribosome entry sites (IRES) can be found in eukaryotes, where the expression of a protein is regulated by translation initiation from an internal start sites, rather than the first AUG codon the ribosome detects within the coding region (Cornelis *et. al.*, 2000).

1.3.2 Translation repression/attenuation

There are a number of examples of translation repression. Each mechanism, involves a common theme where a cluster of macromolecular molecules combine to interact and interfere with the formation of the translation initiation complexes. It is often the case that the level of gene repression/activation is governed by the rate and duration of the reversible interactions between the blocking complexes and the mRNA.

Translation repression by autorepressor, the ribosome proteins

Autogenous regulation is common among proteins that are incorporated into macromolecular assemblies, an example of which is the regulation of ribosomal protein expression. Ribosomes constitute about half the mass of a bacterial cell, and their formation is based upon a coordinated control of synthesis of the constituent ribosomal proteins (r-proteins) and RNA. The genes for the r-proteins are organised into several polycistronic operons, the expression of which is controlled at the translational level (Nomura *et. al.,* 1984; Lindahl and Zengel, 1986; Keener and Nomura, 1996). This regulation is a form of autorepression, as it is carried out by several of the r-proteins encoded by the very same polycistronic mRNA they are produced from (Keener and Nomura, 1996). In what is termed the Nomura model, the protein binding site on the mRNA, the translational operator, is structurally similar to the target the r-protein binds to on the ribosome. The molecular mimicry between the two target sites would be used to adjust the translation of ribosome proteins to the level of transcription of the rRNA. Thus, in the presence of excess unbound rRNA, the repressor r-protein would be displaced from its mRNA operator site, and translation of the mRNAs would proceed. Examples of this mechanism have been isolated in a number of cases in *E. coli*, and include the L1, S8 and S15 proteins (Gregory *et al.,* 1988; Said *et al.,* 1988; Phillippe *et al.,* 1990). In all three cases, a common recognition pattern, G(U/G)C, can be found in the one of the two participating binding sites (Scott and Williamson, 2001; Serganov *et al.,* 2002; Serganov *et al.,* 2003). Confirmation that molecular mimicry is a key feature of this form of regulation can be found with S15, as this familiar binding motif is also involved with the protein binding to the 16S rRNA (Agalarov *et al.,* 2000; Nikulin *et al.,* 2000). A more recent mechanism has been reported, which proposes that the L20 protein can form a complex of two proteins and either bind to, and therefore regulate translation of, its own mRNA, or interact with a similarly structured binding site on the 23S rRNA (Raibaud *et al.,* 2003).

Translation repression by competition: threonyl-tRNA synthetase expression

A third example of translation repression is the control of the expression of threonine-tRNA synthetase (ThrRS) in *E. coli*. Threonine-tRNA synthetase represses its own translation by binding to the leader region of its own transcripts. This has the consequence of blocking the ribosome from binding to the mRNA. In order for this system to work it has been deduced that the presence of four structural domains on the mRNA transcripts are of great importance (Moine *et al.,* 1988). Indeed, the repressor ThrRS recognises the domains as two pairs, each pair structurally mimicking the anticodon arm of tRNAThr. Each pair therefore acts as a competitive inhibitor of aminoacylation by the ThrRS, suggesting that the mRNA and tRNAThr compete with one another for the available ThrRS. Both the domains in the mRNA contain an anti-codon type loop that is crucial to their ability to regulate effectively. Experiments where the CGU loop was replaced with CAU, the anti-codon loop from tRNAMet, resulted in methionyl-tRNA synthetase molecule behaving as the repressor (Romby *et al.,* 1992). Regulation of translation occurs only when there is an excess of threonine-tRNA synthetase molecules available to bind to the *thrS* mRNA transcripts. When a protein:mRNA complex is formed, the 30S ribosomal subunit is unable to locate and bind to the ribosome binding site on the mRNA.

It was calculated that threonine-tRNA synthetase and 30S have overlapping binding sites on the *thrS* mRNA, and were therefore in direct competition with one another (Comer *et al.,* 1996). Evidence for a competition-based regulatory system was proven experimentally. Increases in 30S concentration lead to a rapid increase in translation initiation complexes, and more threonine-tRNA synthetase molecules. Furthermore, an increase in tRNAThr caused derepression of *thrS* mRNA translation, alleviating the competition between *thrS* mRNA and tRNAThr for binding to the threonine-tRNA synthetase repressor (Comer *et al.,* 1996). These experimental findings would serve to explain how the expression of ThrRS is growth rate related. An increase in growth rate leads to an increase in *thrRS* expression and an increased synthesis of threonine-tRNA synthetase. This increase in synthesis is expected to be the result of a number of factors. Firstly, during rapid growth, ribosomes are accumulated and tRNAThr levels are

elevated. Under these conditions a greater proportion of the threonine-tRNA synthetase molecules can take part in the aminoacylation of tRNA, resulting in more free *thrS* mRNA molecules and an increased rate of translation initiation (Comer *et al.,* 1996).

Translation repression by the *trp* RNA-binding attenuation protein (TRAP)

The ability of the protein known as TRAP (*trp* RNA-binding attenuation protein) to regulate the transcription of the *trp* operon has already been discussed, however it should be mentioned that the protein is also capable of regulating the translation of *trpE*. When TRAP binds to a *trp* operon transcript, the RNA adopts an alternative hairpin structure, within which the *trpE* S-D sequence is hidden (Meirino *et al.,* 1995; Du and Babitzke, 1998). In contrast, when TRAP is not bound, the *trp* leader transcript adopts a large secondary structure in which the *trpE* S-D sequence is single-stranded (Meirino *et al.,* 1995; Du and Babitzke, 1998; Schaak *et al.,* 2003). A NusA-stimulated RNA polymerase pause site was identified at U144 (Yakhnin and Babitzke, 2002). U144 is 3–4 nucleotides downstream from the *trp* leader termination points (G140 and U141), and it is suggested that pausing at U144 is crucial for the *trpE* translation control mechanism. Pausing at U144 occurs in *trp* operon readthrough transcripts, encourages the antiterminator structure to be formed. However, the antiterminator must unfold before the large secondary structure can form, as all of the nucleotides that comprise the antiterminator are present within the 5′ half of the large secondary structure. If the antiterminator does not unfold in time, the large secondary structure never forms. Thus, pausing at U144 may provide additional time for the RNA to refold such that the *trpE* S-D blocking hairpin only forms when TRAP is bound to the transcript (Babitzke, 2004). TRAP translation control doesn't end with *trpE*. It has also recently been determined that TRAP also blocks the ribosome from binding to the *trpP* transcript by itself binding to a specific region containing nine triplet repeats sequences, found within the UTR of the target transcript (Yakhnin *et al.,* 2004).

Translation attenuation regulation of chloramphenicol acetyltransferase (CAT) genes

The chloramphenicol (Cm)-inducible *cat* and *cmlA* genes are regulated by a process termed translation attenuation (Lovett, 1990). Bacterial resistance to chloramphenicol can result from either of two genetically dominant resistance mechanisms. The first mechanism is through alterations in permeability to the antibiotic, whilst the second, and most common method, is antibiotic inactivation (Burns *et al.,* 1981; Bissonette *et al.*, 1991). Antibiotic inactivation is due to the presence of a plasmid- or chromosome-borne *cat* gene, which encodes for the chloramphenicol acetyltransferase (CAT). CAT acetylates chloramphenicol, which renders the antibiotic unable to interfere with translation (Bissonette *et al.*, 1991). In gram-positive bacteria, the vast majority of *cat* genes identified are all expressed following induction, with chloramphenicol acting as the inducer. Two of these genes, *catA86* and *catA112*, have been demonstrated to respond to a type of translation attenuation regulation. The *cat* genes are continuously transcribed but protein is not constantly produced because the mRNA transcripts are not translated. This is due to the ribosome-binding site for the *cat* coding sequence being hidden away within the secondary structure of the mRNA (Lovett, 1990). Furthermore, a short open reading frame (ORF), called the leader ORF, is immediately upstream from and partially included in the region of secondary structure.

The first five codons of the gene *catA86* specify a peptide that has the sequence MVKTD, which is believed to have RNA-binding properties. This particular pentapeptide targets and is an *in vitro* inhibitor of peptidyltransferase, which is the catalytic activity of the ribosome that goes on to form peptide bonds. By binding to the peptidyltransferase, the peptide alters the conformation of the IV and V domains of the 23S rRNA, which has the effect of pausing translation (Rogers and Lovett 1994). The 23S rRNA is the target for chloramphenicol, and it is suggested that chloramphenicol can bind to the inhibitory peptide sequence. The binding of the antibiotic to the pentapeptide is thought to cause ribosomal stalling in the leader ORF that then destabilises the RNA secondary structure. This destabilisation exposes the RBS for the *cat* coding sequence, resulting in its translation (Gu *et al.*, 1993; Moffat *et al.*, 1994).

1.3.3 Regulation of translation by small RNA molecules

The existence of small RNAs (sRNAs) has been known for more than three decades, but it is only relatively recently that a large number of chromosome-encoded sRNAs have been discovered and their importance in gene regulation towards environmental adaptation appreciated (Wassarman *et al.,* 2001; Gottesman, 2002; Wassarman, 2002).

There are many regulatory mechanisms associated with sRNAs, however, the riboregulators can be categorised into one of two groups, depending upon their mechanism of action (Gottesman, 2002; Storz *et al.,* 2004). The first group includes RNA regulator base pairing with another nucleotide, usually another RNA molecule. These small antisense RNAs (saRNA) can change the secondary structure of the RNA molecules that they bind to, and/or block RNA-binding proteins (such as those in the ribosome) from interacting with the target RNA. Depending upon the system, this interaction between saRNA and target RNA could lead to enhanced stability or degradation rate (Gottesman, 2002). The second group features sRNAs that target and have an effect on a specific nucleotide-binding regulatory protein action. In this case the sRNA acts as a molecular decoy, where the sRNA, or some part of the sequence, mimics the binding site of the actual target molecule (Gottesman, 2002). There is a third type of sRNA molecule that does not fall into either of the two regulatory groups, which is called tmRNA. This intriguing molecule will be discussed along with this review of the two main groups of sRNAs.

Group 1: Regulation by small RNAs acting as molecular decoys

The post-transcriptional global regulatory system Csr (carbon storage regulator) was originally identified in *E. coli* cells. The system comprises of a RNA-binding protein (CsrA) and two sRNAs (CsrB and CsrC). The CsrA molecule is a 61-amino acid protein and consists primarily of a single KH motif. This KH motif is a single stranded RNA-binding domain that is a characteristic of a particular subset of eukaryotic and prokaryotic RNA-binding proteins. This group of molecules includes NusA,

polynucleotide phosphorylase and ribosomal protein S3 (Romeo, 1998). In *E. coli*, CsrA was found to be an activator of glycolysis, acetate metabolism, flagellar synthesis and motility, and acted as a repressor of gluconeogenesis, glycogen synthesis and biofilm formation (Romeo *et al.,* 1993; Liu *et al.,* 1995; Sabnis *et al.,* 1995; Liu and Romeo, 1997; Wei *et al.,* 2001; Baker *et al.,* 2002). CsrA homologues can be found in a wide range of bacteria, but principally within the eubacterial domain. Examples of CsrA homologues can be found in highly adapted pathogens such as *Helicobacter pylori* (Barnard *et al.,* 2004), *Erwinia* spp. (Murata *et al.,* 1994; Cui *et al.,* 1995), Pseudomonas spp. (Blumer *et al.,* 1999; Heeb *et al.,* 2002) and *S. typhimurium* (Altier *et al.,* 2000; Lawhon *et al.,* 2003).

Certain unknown characteristics of the target mRNA is thought to somehow determine whether CsrA behaves as a repressor or activator. In its repressor role, the protein was predicted to facilitate the degradation of target mRNAs, by binding close to the ribosome-binding site (Liu and Romeo, 1997). Indeed this is the case of CsrA regulation of the *glgCAP* mRNA, where CsrA first binds to the Shine-Dalgarno sequence and then to a second site in the mRNA leader sequence, preventing translation (Baker *et al.,* 2002). A similar observation was made by Dubey *et al.* (2003), who used the leader sequence of the *glgCAP* mRNA to search for other mRNAs that would possibly interact with CsrA. They found that translation of mRNA transcripts from carbon starvation gene *cstA*, was prevented by the activities of CsrA. Analysis showed that CsrA was binding to up to four sites along the leader sequence, of the *cstA* transcript. As some of these binding sites overlapped with the Shine-Dalgarno sequence they concluded that CsrA physically prevented ribosomes from attaching to the *cstA* transcripts. Little is known about how CsrA is able to act as an activator, however CsrA does appear to bind to target mRNAs such as the flagellar synthesis gene *flhDC*, in much the same way as it would with *glgCAP*. This binding appears to stabilise the *flhDC* transcript rather than promote its degradation, perhaps by preventing endonucleolytic attack, or by preventing the activities of a negative regulator (Wei *et al.,* 2001).

The two other components of the Csr mechanism are both untranslated RNA molecules called CsrB (360-nucleotides) and a slightly shorter version called CsrC (245 nucleotides long). Interestingly, CsrA cannot autoregulate expression from its own gene, however this could be compensated by the indirect regulation of the *csrB* and *csrC* genes by CsrA through the response regulator UvrR (Gudapaty *et al.*, 2001; Suzuki *et al.*, 2002). These two molecules regulate the activity of CsrA by imitating the leader sequences of mRNAs. Both CsrB and CsrC contain a series of imperfect repeat sequences that closely resemble Shine-Dalgarno sites (18 for CsrB and 9 for CsrC) (Liu *et al.*, 1997; Weilbacher *et al.*, 2003). It is through these repeat sequences that these RNAs bind and sequester CsrA molecules (Figure 1.5). The half-lives of both of the RNAs are relatively short, being around two minutes. With such short half-lives the levels and activity of CsrA could constantly change and respond rapidly to conditions that affect turnover of CsrB and CsrC, such as changes in cellular energy demands and growth conditions. Also, CsrA activates stationary phase associated processes, occurring when cellular resources are potentially at their lowest. Thus a regulatory system based upon RNA molecules, which are comparatively cheaper to synthesise than a host of regulatory proteins, would be an energy efficient mechanism of control over the level of active CsrA (Liu *et al.*, 1997; Weilbacher *et al.*, 2003).

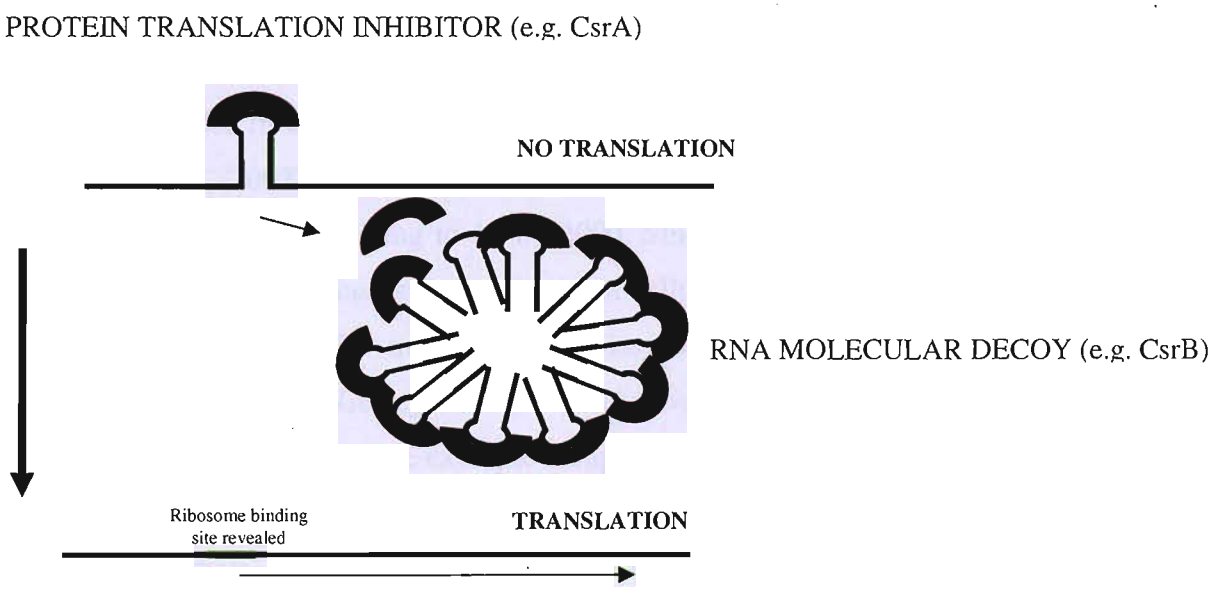


Figure 1.5 A diagram illustrating the principle behind a RNA molecular decoy mechanism. In this diagram the decoy relieves translation inhibition by binding to multiple copies of the inhibitor (in reality CsrB would be able to bind 18 copies of CsrA, while CsrC could potentially bind 9). Figure adapted from Gottesman, 2002.

Group 2: Translation regulation by small antisense RNAs (saRNAs)

The early antisense mRNA molecules to be discovered regulated only one target, and were encoded in *cis* from the opposite DNA strand of the target nucleotide. These *cis*-encoded antisense RNAs were commonly found in plasmid replication (e.g. RNAI controlling ColE1 plasmid copy numbers) and antitoxin strategies (e.g. *sok* RNA preventing the toxic *hok* translation). These antisense RNAs were often small (between 50 to 400 nucleotides is size), diffusible, untranslated, and highly structured (one to four stem-loops) molecules (Wassarman *et al.*, 1999). They bind to target regions on sense DNA or RNA molecules, altering downstream replication, transcription or translation. Detailed analyses have revealed that *cis*-encoded antisense RNAs generally have 5-8 nucleotide GC-rich loops. Stem structures in mRNA are important for metabolic stability and are often interrupted by bulges, which prevent enzymatic degradation by dsRNase and also facilitate melting upon antisense/sense RNA interaction. Furthermore, recognition loops of the antisense RNA or the sense RNA often contain a 5'-YUNR motif, which is proposed to form a U-turn structure that provides a scaffold for the rapid interaction with the complementary RNA (Franch *et al.*, 1999).

There has been a recent increase in the numbers of *trans*-encoded saRNAs discovered, which are encoded for by chromosomal genes. These saRNA genes are usually positioned far from the target genes, and the saRNAs have multiple regulatory targets (Gottesman, 2002). The first *trans*-encoded saRNAs were discovered three decades ago, with the number rising to 13 by 2000. Since then the number has swelled to 50, suggesting that, in *E. coli* at least, the list of cellular processes that involve some degree of regulation by RNA molecules may be larger than first appreciated. With the discovery of new RNA regulators, several new global post-transcriptional regulators have duly emerged, a prime example being the Hfq protein. Originally discovered in *E. coli*, the Hfq protein was identified as a necessary host factor for the initiation of plus-strand synthesis by the replicase of the Q RNA bacteriophage (Franze de Fernandez *et al.*, 1968). It is known that the conserved RNA-binding protein belongs to the eukaryotic and archaeal Sm/Sm-like (Lsm) family of proteins. In eukaryotic cells,

members of this family form ring-shaped heteroheptamers that interact with uridine-rich sequences within target mRNAs, and participate in mRNA splicing and degradation reactions. Interestingly, Hfq recognition sites are remarkably similar to RNase E binding sites, in that they seek AU-rich, unstructured sequences within single stranded RNA. This could mean that Hfq directly competes with RNase E for the free saRNA molecules (Moller *et al.,* 2002; Schumacher *et al.,* 2002; Zhang *et al.,* 2002; Brescia *et al.,* 2003).

small antisense RNA mediated translation activation

The importance of Hfq was first realised after it was reported that the complete disruption of the *hfq* gene led to defects that were similarly observed following the deletion of the *rpoS* gene. These phenotypes included decreased growth rate, increased cell length and an increase in sensitivity to ultraviolet radiation (Tsui *et al.,* 1994). It was later discovered that in *E. coli* and *S. typhimurium,* Hfq was an essential component in the efficient translation of the *rpoS* mRNA (Brown and Elliott, 1996; Muffler *et al.,* 1996). Interestingly, when the RNA-binding regulatory molecule Hfq was deleted, translation of *rpoS* mRNA was severely affected (Brown and Elliott, 1996; Muffler et al., 1997; Moller *et al.,* 2002; Zhang *et al.,* 2002). The region of *rpoS* mRNA that was crucial to Hfq activity was pinpointed to a hairpin loop located just upstream of the AUG site. It was determined that this secondary structure prevented ribosome binding to the transcript, thereby preventing translation (Brown and Elliott, 1997). Three saRNA molecules, DsrA, RprA and OxyS, were all discovered to have a role in regulating *rpoS* expression (Sledjeski *et al.,* 1996; Zhang *et al.,* 1998; Majdalani *et al.,* 2001) and require Hfq for activity (Zhang *et al.,* 1998; Majdalani *et al.,* 2001; Sledjeski *et al.,* 2001). Both DsrA and RprA have complementary sequences with *rpoS* mRNA and activate its translation (Majdalani *et al.,* 1998; Majdalani *et al.,* 2002) whilst OxyS modulates *rpoS* expression through Hfq but through an unknown mechanism (Zhang *et al.,* 1998). It is hypothesised that by having multiple regulators and forms of regulation, the *rpoS* gene is able to respond to a number of different environmental stimuli (Hengge-Aronis, 2002; Repoila *et al.,* 2003). DsrA levels increase at lower temperatures and activate *rpoS* following a temperature downshift. DsrA basepairs with

the *rpos* mRNA, thereby preventing the formation of the hairpin structure in the 5' UTR. This promotes the translation of the mRNA by revealing the ribosome binding site (Lease *et al.,* 1998) (Figure 1.6).

small antisense RNA mediated translation inhibition

An example of antisense inhibition of translation can be found with the osmoregulation of the *ompF* and *ompC* genes. The two genes each encode outer membrane proteins that act as pores that allow the passage of small hydrophilic molecules (Nikaido, 1992). When bacteria experience increased osmolarity, the expression of *ompC* increases and the expression of *ompF* decreases. This regulation is mainly through the *envZ/ompR* two-component system, which detects fluctuations in osmolarity (Pratt *et al.*, 1996). There is, however, a second regulatory system in the form of the product of the *micF* gene. *micF* is a stress response gene found in *E. coli* that post-transcriptionally controls expression of *ompF* gene. *micF* encodes a non-translated 93 nucleotide antisense RNA, which is produced when the cell experiences a number of external events or stresses such as, changes in nutritional availability, increases in osmolarity of the growth environment and temperature fluctuations (Anderson *et al.,* 1989; Coyer *et al.,* 1990). MicF RNA is highly complementary to a continuous portion of 5' untranslated region of the *ompF* transcripts, so when produced, the sRNA seeks out and binds to its target *ompF* mRNA very rapidly. The region of complementarity includes the ribosomal binding site and the translational start codon of the *ompF* mRNA. Approximately one third of the MicF RNA binds to the *ompF* transcript, and shields the Shine-Dalgarno sequence from any ribosomes, thereby inhibiting translation and inducing degradation of the *ompF* mRNA (Mizuno *et al.*, 1984; Delihas and Forst, 2001) (Figure 1.7).

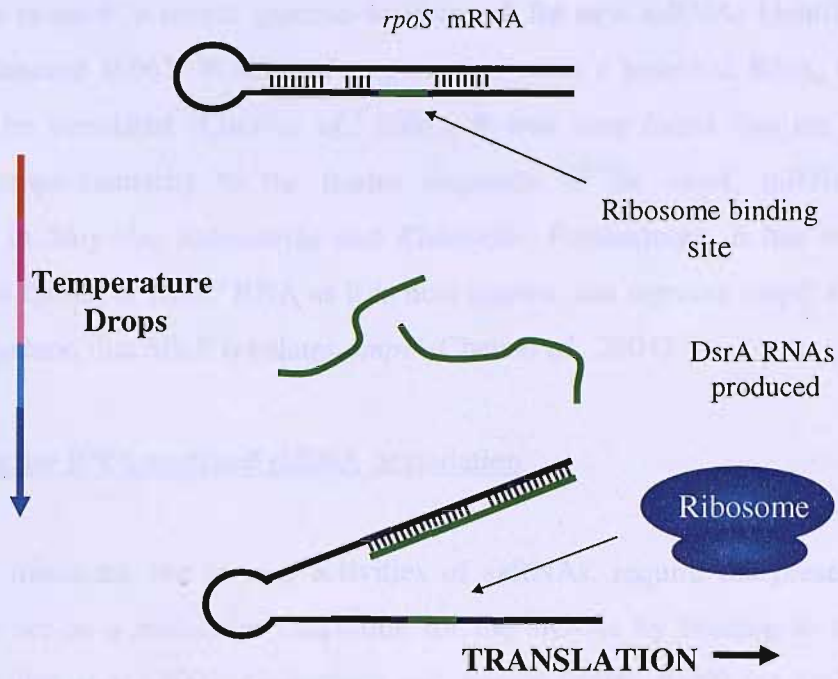


Figure 1.6 A diagram illustrating the DsrA RNA-mediated regulation of *rpoS* translation, following a drop in temperature. Figure adapted from Storz *et al., 2004*.

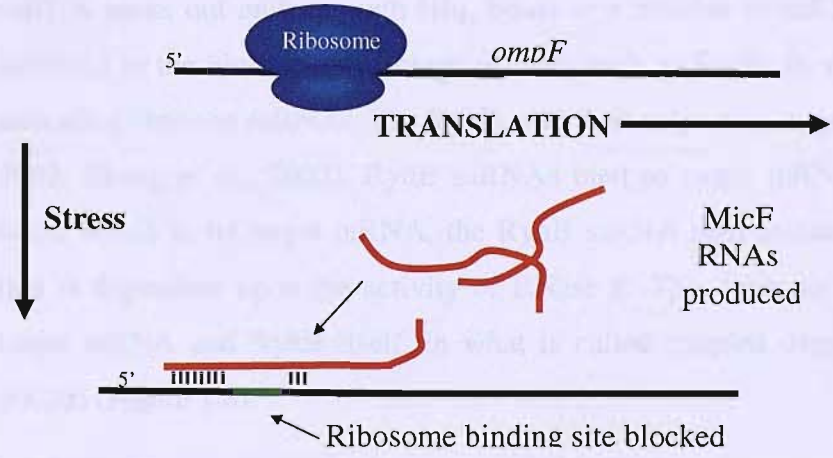
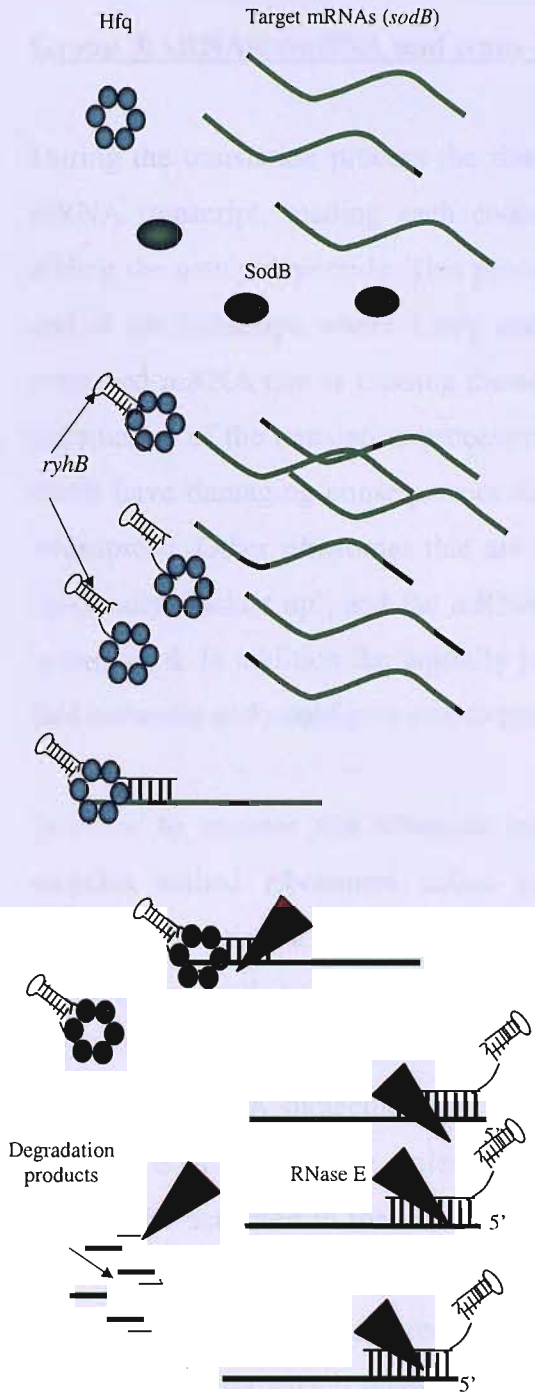


Figure 1.7 Following a perceived stress, MicF is produced which then binds to the 5' UTR of *ompF* mRNA. This blocks the ribosome binding site, preventing translation, triggering the eventual degradation of the mRNA. Figure adapted from *Storz et al.,* 2004

In addition to *micF*, a recent genome-wide search for new saRNAs identified a region of DNA denoted IS063. Within this region there was a potential RNA, but one that could not be translated (Chen *et al.,* 2002). It was later found that the small RNA showed complementarity to the leader sequence of the *ompC* mRNA, and was conserved in *Shigella*, *Salmonella* and *Klebsiella*. Furthermore, it has been recently proven that IS063, or MicC RNA as it is now known, can regulate *ompC* expression in the same fashion that MicF regulates *ompF* (Chen *et al.,* 2004).

small antisense RNA mediated mRNA degradation

In several instances, the *in vivo* activities of saRNAs, require the presence of Hfq, which can act as a molecular chaperone for the sRNAs by binding to their mRNA targets (Moller *et al.,* 2002; Geissmann and Touati, 2004). RyhB (or SraI) is a small antisense RNA (saRNA) molecule, the gene for which is regulated by the activities of the Fur (ferric uptake regulation) repressor. When cellular iron levels are low, transcription of the *rhyB* gene is derepressed (Masse and Gottesman, 2002; Masse *et al.*, 2003a). The synthesised RyhB saRNA binds the RNA-binding regulatory protein Hfq, which protects the saRNA from degradation. In this Hfq:RyhB complex, the saRNA seeks out and, through Hfq, binds to a number of mRNA molecules that are involved in the binding and storage of iron, such as SodB. In vitro, Hfq promotes the annealing between saRNAs, like RyhB, and their respective target mRNAs (Moller *et .,* 2002; Zhang *et al.,* 2002). RyhB saRNAs bind to target mRNAs in a 1:1 ratio, and when bound to its target mRNA, the RyhB saRNA then initiates a degradation event that is dependent upon the activity of RNase E. This leads to the degradation of the target mRNA and RyhB itself, in what is called coupled degradation (Masse *et al.*, 2003b) (Figure 1.8).



1. Normal Growth Conditions, little or no sRNA produced and what is produced is rapidly degraded, maximum translation from target gene mRNA.

2. Rapid Increase in sRNA synthesis following stress (E.g. *ryhB* and Iron depletion) sRNAs bound by Hfq to prevent early degradation.

3. Hfq bound sRNA is introduced to the target mRNA. Base-pairing between the RNAs occurs, triggering RNase E-mediated coupled degradation sRNA production continues so long as stimulus is present, i.e. iron limitation.

4. So long as the stress stimulus is present and the target mRNA accumulates, the sRNA is produced and degradation continues. As soon as the stress is over, the sRNA is used up through coupled degradation and translation of the target mRNA is permitted.

Figure 1.8 An illustration depicting the main events that occur during coupled degradation with the RyhB RNA & *sodB* mRNA. Figure adapted from Masse *et al.*, 2003.

Group 3: sRNAs: tmRNA and trans-translation

During the translation process the ribosome continues in a stepwise manner along the mRNA transcript, reading each codon, receiving the correct aminoacyl-tRNAs and adding the new polypeptide. This procedure continues until the ribosome reaches the 3' end of the transcript, where a stop codon terminates translation. However a defective, truncated mRNA that is missing the stop codon causes the ribosome to stall as proper termination of the translation process is prevented (Vioque and de la Cruz, 2003). This could have damaging consequences for the cell, especially if the defective mRNA is widespread. Other ribosomes that are translating the same defective mRNA would be essentially 'locked up', and the mRNA would continue to trap other ribosomes until it is degraded. In addition the partially produced polypeptide chain would not be able to fold correctly and could give rise to potentially toxic aggregates (Haebel *et al.,* 2004).

In order to counter this situation bacteria have developed a complex system that recycles stalled ribosomes called trans-translation (Keiler *et al.,* 1996). Trans-translation requires a stable type of RNA called tmRNA (also known as 10Sa RNA or Ssr RNA), so called as the molecule acts as a transfer and a messenger RNA (Williams, 2002). The precursor of tmRNA, which is encoded for by the *ssrA* gene, can in vivo form a half tRNA molecule with an amino acid acceptor arm and a TΨC stem-loop (Komine *et al.,* 1994). The molecule can therefore act as a tRNA through these features and can be activated in the same way to a tRNA. This includes 5' end processing by RNase P and is amino-acetylated by alanyl-tRNA synthetase (Komine *et al.,* 1994). The trans-translation system also requires the presence of a small basic protein called SmpB (small protein B), which binds tightly to the tRNA-like domain (TLD) of the tmRNA. Binding of SmpB stimulates tmRNA aminoacetylation by stabilising the region of the tmRNA that accepts the amino acid (Barends *et al.,* 2001; Williams, 2002; Someya *et al.,* 2003).

Keiller *et al.* (1996) first proposed a model for trans-translation, which begins with a ternary complex consisting of a GTP, EF-Tu and the alanylated tmRNA:SmpB

complex which interacts with EF-Tu through the TLD of tmRNA. The EF-Tu then delivers the tmRNA:SmpB to the A site of the stalled ribosome in a manner similar to how a charged tRNA is brought to the ribosome. Once the tmRNA is installed into the ribosome A site the EF-Tu is released after a GTP hydrolysis event, which is possibly triggered by a SmpB-stimulated conformational change in the ribosome (Haebel *et al.*, 2004). Following EF-Tu dissociation the CCA end of the tmRNA is accepted by the peptidyl transferase centre of the ribosome. During the next cycle of elongation, the non-coded alanine is incorporated into the nascent polypeptide chain and the mRNA-like domain (MLD) of tmRNA replaces the mRNA in the ribosome (Muto *et al.*, 1998). The ribosome protein S1 possibly aids the replacement of the defective mRNA with the MLD of tmRNA (Valle *et al.*, 2003). The mRNA-like domain of tmRNA is then used as the template, which contains an ORF that encodes for several codons followed by a correct stop codon. These additional amino acids are added to the C-terminus of the nascent polypeptide in successive rounds of translation. The internal stop codon in the MLD directs translation termination and the release of the polypeptide (Keiler *et al.*, 1996).

The extra amino acids that were encoded for by the MLD and that were added to the truncated polypeptide, now act as a tag. This tag is recognised by specific proteases such as ClpAP and ClpXP, and possibly RNase R, which rapidly degrade the polypeptide (Gottesman *et al.*, 1998; Karzai and Sauer, 2001). It has become apparent that tmRNA-mediated rescue of stalled ribosomes does not just occur with truncated mRNAs. Indeed, tmRNA activity has been observed in situations where the ribosome stalls due to the effect of translation targeting antibiotics that induce misreading such as kanamycin. Alternatively, tmRNA can become involved when the ribosome comes across a rare codon or a codon that that has a low abundance of associated aminoacyl-tRNA (Roche and Sauer, 1999; Thompson *et al.*, 2002). In addition tmRNA can compete with release factor in order to terminate translation when the ribosome reaches a stop codon (Roche and Sauer, 2001). (Figure 1.9) The tmRNA and SmpB molecules are found throughout the Bacterial kingdom, but generally the presence of tmRNA in bacteria is not essential for cell viability and neither do tmRNA knock out mutants show any discernible difference in growth rates to that of wild type cells (Oh and

Apirion, 1991; Komine *et al.*, 1994). However for reasons still unknown, both *Neisseria gonorrhoeae* and *Mycoplasma* species exhibit a dependence upon tmRNA for continued survival, and in addition tmRNA is required for full virulence with *S. typhimurium* (Huang *et al.*, 2000; Julio *et al.*, 2000).

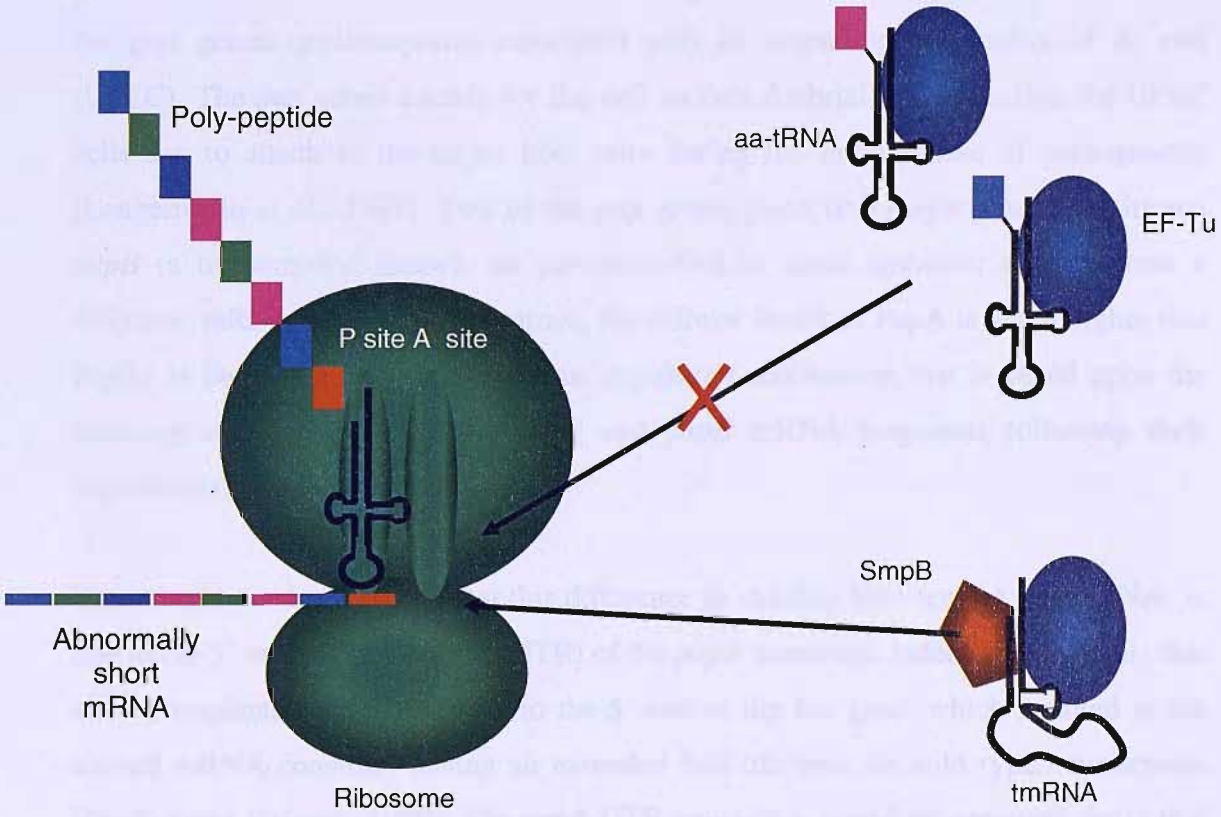


Figure 1.9 Trans-translation activity of tmRNA is triggered by the stalling of a ribosome. This stalling can be due to a number of reasons ranging from ribosome targeting antibiotics, rare codons or, as depicted here, a curtailed mRNA with no normal stop codon. Shortened mRNAs can be due to earlier damage or a fault during the transcription process. Adapted from Vioque and de la Cruz, 2002.

1.4 Regulation through the manipulation of mRNA

1.4.1 mRNA stability influencing gene expression

e.g The papAB genes

The stability of mRNA can be increased by the inclusion of stem-loop structures in the secondary structure of the mRNA, which has the effect of disrupting the activity of PNPase and RNase II. An example of this arrangement can be found with the mRNA of the *pap* genes (pyelonephritis-associated pili) in uropathogenic strains of *E. coli* (UPEC). The *pap* genes encode for the cell surface fimbrial structures that the UPEC cells use to attach to the target host cells during the initial phase of pathogenesis (Langermann *et al.,* 1997). Two of the *pap* genes, *papA* (the major pilus subunit) and *papB* (a transcription factor), are co-transcribed in equal amounts, as they form a dicistroic mRNA transcript. In contrast, the cellular levels of PapA is much higher that PapB, as there is a post-transcriptional regulatory mechanism that is based upon the differing stability levels of the *papA* and *papB* mRNA fragments following their separation (Baga *et al.,* 1988).

Recent analyses have shown that this difference in stability between the two mRNAs is due to the 5' untranslated region (UTR) of the *papA* transcript. Indeed, experiments that have transplanted the *papA* UTR to the 5' end of the *bla* gene, which resulted in the altered mRNA construct having an extended half-life over its wild type counterparts (Bricker and Belasco, 1999). The *papA* UTR contains a stem-loop structure (hp1) that when deleted, results in a three times faster rate of degradation of the mRNA molecule (Bricker and Belasco, 1999). It is believed that this stem loop, as with other forms of UTR base-pairing, deters RNase E from attacking the mRNA molecule (Mackie, 1998).

1.4.2 Regulation through induced alterations in mRNA secondary structure

e.g. Temperature sensing and the cspA gene

When *E. coli* cells expreriences a sudden temperature downshift there is a strong induction of a number of proteins involved with acclimatising to the lower temperatures, including CsdA, RbfA, NusA, PNPase and members of the Csp family (Phadtare *et al.,* 1999; Gualerzi *et al.,* 2003). By far the best characterised of the cold shock proteins is CspA, which is principal gene regulator under cold shock conditions (Jiang *et al.,* 1997). Although the primary targets of CspA are currently unknown, it is appreciated that CspA binds to target mRNAs and acts as a molecular chaperone. This binding is done without any apparent sequence specificity (Phadtare *et al.,* 2000). Following successful binding, CspA then destabilises the mRNA secondary structure, thereby allowing efficient translation to occur at low temperatures (Phadtare and Inouye, 1999).

The expression of *cspA* during cold-shock is attributed to two mechanisms at the post-transcriptional and translation level. Immediately after a downshift in temperature, the stability of the *cspA* mRNA increases by a factor of 150-fold, which leads to a dramatic increase in the rate of *cspA* mRNA translation. The cause of this increase in stability has not yet been determined. However, the *cspA* mRNA has a 159 nucleotides long 5' untranslated region, which is believed to also influence the induction of *cspA* expression. Experiments involving deletion mapping revealed that the UTR was responsible for altering the levels of *cspA* expression. First of all, a *cspA* transcript that lacked the first 80 bases (Δ1-80) corresponding to the 5′ half of the UTR had a substantially longer half-life compared to the wild-type transcript both at high and at low temperature (Brandi *et al.,* 1996; Goldenberg *et al.,* 1996). A smaller change, like the substitution of three bases around the SD sequence, was found to stabilize the *cspA* mRNA 150-fold, while complete deletion of the 5′ UTR was reported to cause the constitutive expression of *cspA* at 37 °C (Fang *et al.,* 1999).

The complete 5′ UTR of *cspA* was found to confer to *cat* mRNA an instability ($t_{1/2}$<30 seconds) similar to that of *cspA* mRNA and greater than that conferred by the truncated (Δ1-80) 5′ UTR (Brandi *et al.,* 1999). Furthermore, stability at 37 °C of *cat* mRNA preceded by the 5′ UTR of *cspA* was found to be somewhat growth cycle-dependent, decreasing during the transition from early to mid–late exponential growth. Current models for the expression of *cspA* indicate that at low temperatures, the UTR of *cspA* mRNA alters in secondary structure and allows ribosome loading and translation. Thus depending upon the temperature experienced, the *cspA* mRNA can act as a thermosensor (Yamanaka *et al.,* 1999; Nogueira and Springer, 2000).

1.5 The discovery and initial analysis of BipA

Initial investigations into the response by *S. typhimurium* challenged with the host defence protein BPI, led to the discovery of the ribosome-binding BipA GTPase. A proteomic analysis comparing the protein expression profiles of BPI-exposed and non-BPI-exposed *Salmonella*, indicated that a 72 kDa protein was upregulated 7-fold in the former cells (Qi *et al.*, 1995). Due to its strong induction in *Salmonella* on addition of BPI, the previously unnamed protein was termed BPI inducible protein A or BipA. Concurrent research by Freestone *et al.* (1995) in Enteropathogenic *E. coli* (EPEC) led to the discovery of a phosphoprotein that they termed TypA or Tyrosine phosphorylated protein A, which was later shown to co-purify with RNA polymerases (Freestone *et al.*, 1998). Comparisons of sequence revealed that BipA and TypA were in fact two names for the same protein, as they both shared amino terminal region sequence homology with the same hypothetical *E. coli* K-12 protein encoded by the predicted gene *yihK* (also termed *orf 591*) located on the K-12 chromosome at 88 minutes (Qi *et al.*, 1995). Further inspection of BipA's sequence led to the discovery that the protein belonged to a family of GTPases. It is therefore pertinent to review some of the relevant properties of these enzymes.

1.6 GTPases and the GTPase cycle

GTPases, also known as Guanosine nucleotide binding proteins (GNBPs), are known to bind and hydrolyse guanine nucleotides (Bourne *et al.*, 1991). GTPases are often poor catalysts, but they do form stable complexes with both the substrate (GTP) and the converted product (GDP). There at least 150 known GTPases that have been characterised, all of which are involved in many cellular processes, including signal transduction, protein biosynthesis, protein targeting (Takai *et al.*, 2001). Often these G-proteins are involved in cells as so-called molecular switches, where activation is achieved by the binding of GTP (the 'on' position) whereas deactivation is achieved by the hydrolysis of GTP to GDP (then 'off' position) (see Figure 1.10).

Activation of GTPases requires the dissociation of the bound GDP in exchange for GTP, which is in some situations accelerated by the activity of guanine exchange factors (GEFs) or guanosine nucleotide release proteins (GNRPs). GEF proteins are often large, multi-domain proteins that have a large interaction area with the GTPases, in order to stabilise the transient and very unstable nucleotide-free form of GTPase (Zhang *et al.*, 2000). Binding of GTP triggers conformational changes in the enzyme's molecular structure, which is often necessary to enable the enzyme to carry out its main biological function. Switching off the GTP involves the hydrolysis of the GTP to GDP. This process can also be accelerated by other factors known as GTPase activating proteins (GAPs). When a GAP associates with the G-domain of the GTPase, the GAP stabilises the intermediate state of the hydrolytic reaction by enhancing the rigidity of the G-domain in a way that correctly orientate the catalytic residues, thereby increasing the rate of enzyme activity (Scheffzek *et al.*, 1996). The cycle is completed once the GTP is hydrolysed, and the structural conformation of the GTPase reverts to the 'off' state. This conformation can be maintained by the binding of guanosine nucleotide dissociation inhibitors (GDIs) e.g. the RabGDI and RhoGDI proteins, which prevent nucleotide exchange (Shalk *et al.*, 1996). Otherwise the cycle begins again with the GEF/GNRP interaction.

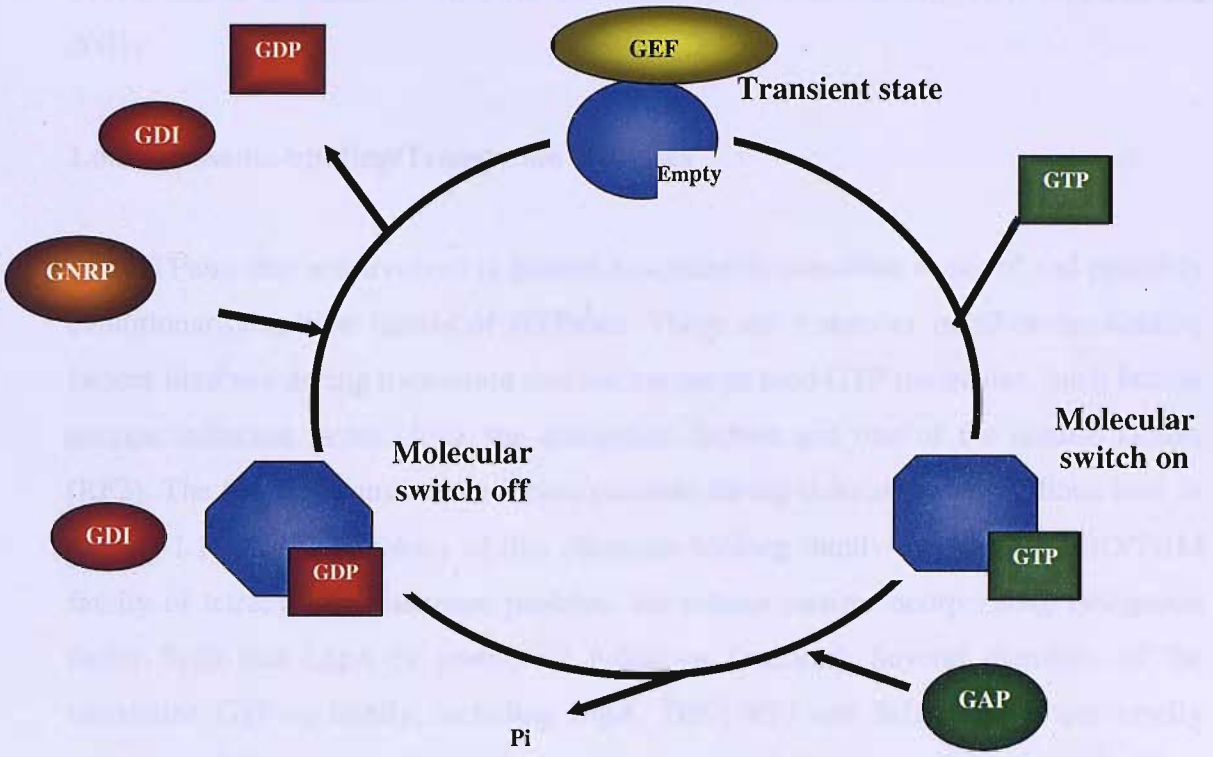


Figure 1.10 The GTPase cycle with the three conformational states of the GTPase enzyme. Adapted from Bourne *et al.* (1991).

1.6.1 Bacterial GTPases

Until quite recently there were very few bacterial GTPases known apart from those involved in translation. However, it is now known that bacterial species that have large genomes contain approximately 20-30 GTPases, whereas bacteria with relatively smaller genomes contain as few as 11 examples. The eleven universally conserved GTPases are: EF-G, EF-Tu, IF-2, FtsY, Ffh, Era, ThdF/TrmE, EngA, YchF, Obg and LepA. The phylogenetic analysis of the grouping of these eleven GTPases showed that there were four distinct sub clusters of GTPases. The first group containing EF-G, EF-Tu, IF-2 and LepA, whilst Era, ThdF/TrmE and EngA form a second, YchF and Obg a third and Ffh and FtsY form the fourth group. Emerging data suggests that all eleven GTPases perform a role that are either necessary for ribosome function or for the

transmittal of information from the ribosome to downstream targets (Caldon *et al.*, 2001).

1.6.2 Ribosome-binding/Translation GTPases

The GTPases that are involved in protein biosynthesis constitute a special and probably evolutionarily ancient family of GTPases. There are a number of ribosome-binding factors involved during translation that are known to bind GTP molecules. Such factors include initiation factor (IF2), the elongation factors and one of the release factors (RF3). The functions and roles of these proteins during translation are outlined later in section 1.10. Other members of this ribosome-binding family include the TetO/TetM family of tetracycline resistance proteins, the selenocysteine-incorporating elongation factor SelB and LepA (a protein of unknown function). Several members of the translation GTPase family, including BipA, TetO, RF3 and SelB, are not universally conserved. However, they contain the sequence motifs that strongly suggest that they are bacterial GTPases that may well interact with the ribosome.

1.6.3 BipA shows sequence similarity to ribosome-binding GTPases

Homologues of the BipA protein have been found in most bacteria and also in plants. Detailed examination of the sequence of BipA revealed that it is very similar to the first 130 amino acids of the proteins belonging to a superfamily of ribosome-binding GTPases, including elongation factor G (EF-G), elongation factor Tu (EF-Tu) and the TetO/TetM family of tetracycline resistance proteins (Table 1.1). Indeed, the sequence of BipA has a conserved region that resembles the ribosome interacting domain IV of EF-G. However, the most highly conserved regions between BipA and the ribosome-binding G-proteins map to the amino-terminal third of the proteins, and correspond to guanine nucleotide-binding domains.

All ribosome-binding G-proteins share a common structural design for their nucleotide-binding domains (Aevarsson, 1995; Kjeldgaard *et al.*, 1996), which when compared, show that there are four consensus sequence elements (Table 1.1). The first sequence

motif GXXXXGK(S/T) is found in many proteins that bind purine nucleoside triphosphates such as ATP synthases. This structure is known as the phosphate binding loop, the glycine rich loop or sometimes the Walker motif (Walker *et al.*, 1982; Moller and Amons, 1985). The second motif is DXXG, and is situated close to the phosphate-binding loop and is thought to be involved in the conformational change that occurs between the GTP and GDP-bound protein forms. Specificity for guanine is due to an Asp side chain in this particular motif. (Vetter and Wittinghofer, 2001). The third motif is NKXD and determines the specificity for guanine (Kjeldgaard *et al.*, 1996), and the fourth motif that contains RGITI, which is found only in all of the ribosome-binding GTPases (including the translation factors and the TetM/TetO/TetQ family).

Typically the first three of the motifs from the nucleotide-binding domain have often been used to determine putative GTPases from just their primary amino acid sequences (Farris *et al.*, 1998). In the case of BipA, as can be seen in Table 1.1 the protein contains all four motifs. This information confirmed that BipA was indeed a newly discovered member of the ribosome-binding G-protein superfamily (Bourne *et al.*, 1991; Farris *et al.*, 1998). As will become apparent, subsequent studies have established that BipA does indeed bind to ribosomes (R. Owens, Ph.D thesis, 2001)

GTPase	G1 motif	G2 motif	G3 motif	G4 motif
EF-G	IGISAHIDAGKTTTT	INIIDTPG	IAFVNKMD	EQERGITITSA
EF-Tu	VGTIGHVDHGKTTLT	QAHVDCPG	IVFLNKCD	EKARGITINTS
TetO	IGILAHIDAGKTSVT	CNIIDTPG	IVVINKVD	EKRRGITVRAS
BipA	IAIIAHVDHGKTTLV	INIVDTPG	IVVINKVD	EKERGITILAK

Table 1.1 The consensus sequence motifs of four ribosome-associated G-proteins

```
A. thaliana      (1)MPVEVKKKQLDRRDNVRNIAIVAHVDHGKTTLVDSMLRQAKVFRDN--QV
EPEC             (1)-----------MIEKLRNIAIIAHVDHGKTTLVDKLLQQSGTFDSR--AE
S.typhimurium    (1)-----------MIENLRNIAIIAHVDHGKTTLVDKLLQQSGTFDAR--AE
EF-G             (1)-------ARTTPIARYRNIGISAHIDAGKTTTTERILFYTGVNHKIGEVH
TetQ             (1)-------------MNIINLGILAHIDAGKTSVTENLLFASGATEKCGRVD

A. Thaliana     (49)MQERIMDSNDLERERGITILSKNTSITYK-------NTKVNIIDTPGHSD
EPEC            (38)TQERVMDSNDLEKERGITILAKNTAIKWN-------DYRINIVDTPGHAD
S.typhimurium   (38)TQERVMDSNDLEKERGITILAKNTAIKWN-------DYRINIVDTPGHAD
EF-G            (44)DGAATMDWMEQEQERGITITSAATTAFWSGMAKQYEPHRINIIDTPGHVD
TetQ            (38)NGDTITDSMDIEKRRGITVRASTTSIIWN-------GVKCNIIDTPGHMD

A. thaliana     (92)FGGEVERVLNMVDGVLLVVDSVEGPMPQTRFVLKKALEFGHAVVVVVNKI
EPEC            (81)FGGEVERVMSMVDSVLLVVDAFDGPMPQTRFVTKKAFAYGLKPIVVINKV
S.typhimurium   (81)FGGEVERVMSMVDSVLLVVDAFDGPMPQTRFVTKKAFAHGLKPIVVINKV
EF-G            (94)FTIEVERSMRVLDGAVMVYCAVGGVQPQSETVWRQANKYKVPRIAFVNKM
TetQ            (81)FIAEVERTFKMLDGAVLILSAKEGIQAQTKLLFNTLQKLQIPTIIFINKI

A. thaliana    (142)DRPSARPEFVVNSTFELFIEL-----------------------------
EPEC           (131)DRPGARPDWVVDQVFDLFVNL-----------------------------
S.typhimurium  (131)DRPGARPDWVVDQVFDLFVNL-----------------------------
EF-G           (144)DRMGANFLKVVNQIKTRLGANPVPLQLAIGAEEHFTGVVDLVKMKAINWN
TetQ           (131)DRDGVNLERLYLDIKTNLSQD-----------------VL----------

A. thaliana    (163)----NATDEQCDFQAIYASGIKGKAGLSPDDLAEDLGPLFEAIIRCVPGP
EPEC           (152)----DATDEQLDFPIVYASALNGIAGLDHEDMAEDMTPLYQAIVDHVPAP
S.typhimurium  (152)----DATDEQLDFPIIYASALNGIAGLDHEDMAEDMTPLYQAIVDHVPAP
EF-G           (194)DADQGVTFEYEDIPADMVELANEWHQNLIESAAEASEELMEKYLGGEELT
TetQ           (154)----FMQT-VVDGLVYPICSQTYIKEEYKEFVCNHDDNILERYLADSEIS

A. thaliana    (209)NIEKDGALQMLAT-----------------------------------
EPEC           (198)DVDLDGPFQMQIS-----------------------------------
S.typhimurium  (198)DVDLDGPLQMQIS-----------------------------------
EF-G           (244)EAEIKGALRQRVLNNEIILVTCGSAFKNKGVQAMLDAVIDYLPSPVDVPA
TetQ           (199)PADYWNTIIDLVAKAKVYPVLHGSAMFNIGINELLDAISSFILPP-----

A. thaliana    (222)----------------------------NIEYDEHKGRIAIGRLHAGVLR
EPEC           (211)----------------------------QLDYNSYVGVIGIGRIKRGKVK
S.typhimurium  (211)----------------------------QLDYNNYVGVIGIGRIKRGKVK
EF-G           (294)INGILDDGKDTPAERHASDDEPFSALAFKIATDPFVGNLTFFRVYSGVVN
TetQ           (244)------------E----SVSNRLSAYLYKIEHDPKGHKRSFLKIIDGSLR

A. thaliana    (244)KGMDVRVCTSEDSCRFARVSELFVYEKFYRVPTDSVEAG--DICAVCGID
EPEC           (233)PNQQVTIIDSEGKTRNAKVGKVLGHLGLERIETDLAEAG--DIVAITGLG
S.typhimurium  (233)PNQQVTIIDSEGKTRNAKVGKVLTHLGLERIDSNIAEAG--DIIAITGLG
EF-G           (344)SGDTVLNSVKAARERFGRIVQMHANK---REEIKEVRAG--DIAAAIGLK
TetQ           (278)LRDIVRINDSEKFIKIKNLKTIYQGR-----EINVDEVGANDIAIVEDME

A. thaliana    (292)NIQIGETIADKVHGKPLPTIKVEEPTVKMSFSVNTSPFSGREGKYVTSRN
EPEC           (281)ELNISDTVCDTQNVEALPALSVDEPTVSMFFCVNTSPFCGKEGKFVTSRQ
S.typhimurium  (281)ELNISDTICDPQNVEALPALSVDEPTVSMFFCVNTSPFCGKEGKFVTSRQ
EF-G           (389)DVTTGDTLCDPDAPIILERMEFPEPVISIAVEPKTKADQEKMG-------
TetQ           (323)DFRIGDYLG--TKPCLIQGLSHQHPALKSSVRPDRSEERSKVIS------
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A. thaliana     (342)LRDRLNRELERNLAMKVEDGETADTFIVSGRGTLHITILIENMRREG-YE
EPEC            (331)ILDRLNKELVHNVALRVEETEDADAFRVSGRGELHLSVLIENMRREG-FE
S.typhimurium   (331)ILDRLNKELVHNVALRVEETEDADAFRVSGRGELHLSVLIENMRREG-FE
EF-G            (432)--LALGRLAKEDPSFRVWTDEESNQTIIAGMGELHLDIIVDRMKREFNVE
TetQ            (365)---ALNTLWIEDPSLSFSINSYSDELEISLYGLTQKEIIQTLLEERFSVK

A. thaliana     (391)FMVGPPKVINKRVNDKLLEPYEIATVEVPEAHMGPVVELLGKRRGQMFDM
EPEC            (380)LAVSRPKVIFREIDGRKQEPYENVTLDVEEQHQGSVMQALGERKGDLKNM
S.typhimurium   (380)LAVSRPEVIFREIDGRKQEPYENVTLDVEEQHQGSVMQALGERKGDLKNM
EF-G            (480)ANVGKPQVAYR-----ETIRQKVTDVEGKHAKQSGGRGQYGHVVIDMYPL
TetQ            (412)VHFDEIKTIYK-----ERPVKKVNKIIQIEVPPNP----YWATIG--LTL

A. thaliana     (441)QGVGS-EGTTFLRYKIPTRGLLGLRNAILTASRGTAILNTVFDSYGPWAG
EPEC            (430)NPDGK--GRVRLDYVIPSRGLIGFRSEFMTMTSGTGLLYSTFSHYDDVRP
S.typhimurium   (430)NPDGK--GRVRLDYVIPSRGLIGFRSEFMTMTSGTGLLYSTFSHYDDIRP
EF-G            (525)EPGSNPKGYEFINDIKGG--VIPGEYIPAVDKGIQEQLKAGPLAGYPVV-
TetQ            (451)EPLPLGTGLQIESDISYG--YLNHSFQNAVFEGIRMSCQSG-LHGWEVT-

A. thaliana     (490)-DISTRDLGSLVAFEDGTSTSYALASAQE--RGQMFVGSGVDVYKGQIVG
EPEC            (478)GEVGQRQNGVLISNGQGKAVAFALFGLQD--RGKLFLGHGAEVYEGQIIG
S.typhimurium   (478)GEVGQRQNGVLISNGQGKAVAFALFGLQD--RGKLFLGHGAEVYEGQIIG
EF-G            (572)-DMGIRLHFGSYHDVDSSELAFKLAASIAF--KEGFKKA-KPVLLEPIMK
TetQ            (497)-DLKVTFTQAEYYSPVSTPADFRQLTPYVFR---LALQQSGVDILEPMLY

A. thaliana     (537)IHQRPGDLGLNICKKKAATNIRSNKDVTVILDTPLTY--S-LDDCIEYIE
EPEC            (526)IHSRSNDLTVNCLTGKKLTNMRASGTDEAVVLVPPIR--MTLEQALEFID
S.typhimurium   (526)IHSRSNDLTVNCLTGKKLTNMRASGTDEAVILVPPIK--MSLEQALEFID
EF-G            (618)VEVETPEENTGDVIGDLSRRRGMLKGQESEVTGVKIHAEVPLSEMFGYAT
TetQ            (443)FELQIPQAASSKAITDLQKMMSEIEDISCNNEWCHIKGKVPLNTSKDYAS

A. thaliana     (584)EDELVEVTPSSIRMCKNQKMAKKGRQ----------------------
EPEC            (574)DDELVEVTPTSIRIRKRHLTENDRRRANRAPKDD---------------
S.typhimurium   (574)DDELVEVTPTSIRIRKRHLTENDRRRANRGQKEE---------------
EF-G            (668)QLRSLTKGRASYTMEFLKYDEAPSNVAQAVIEARGK-------------
TetQ            (593)EVSSYTKGLGVFMVKPCGYQITKGDYSDNIRMNEKDKLLFMFQKSMSSK
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Figure 1.11 A Sequence alignment of elongation factor G (EF-G) (from *Anacystis nidulans*, P18667) and the tetracycline resistance gene TetQ (from *Bacteroides fragilis*, Q08425), compared to sequences for BipA from EPEC, *S. typhimurium* and *Arabidopsis thaliana.*

Red = Residues that are conserved
Blue = Residues that are identical or conserved in at least two of the sequences
Green = Residues that are weakly similar

1.6.4 BipA interacts with the 70S ribosome

Previous studies by R. Owens with hexahistidine tagged derivatives of BipA revealed that BipA bound to and was co-eluted with 70S ribosomes. Further experiments showed that the GTPase activity of BipA was negligible in the absence of ribosomes, but was stimulated greatly by the presence of 'naked' 70S ribosomes. This GTPase activity of BipA was almost doubled by the presence of tRNA and mRNA molecules bound to the 70S ribosome. It was proposed that this increased GTPase activity was in response to the subtle changes in ribosomal architecture caused by RNA molecule interaction. Interestingly, the GTPase activity of 70S ribosome-bound BipA was inhibited by both thiostrepton and α-sarcin; both of these molecules bind to the ribosome at the GTPase associated region. Furthermore, BipA was shown to not bind to 70S ribosomes when in the presence of EF-G, suggesting that BipA and EF-G had a binding site on the ribosome that overlapped. Sequence analysis, circular dichromism and molecular modelling results suggested that the structure of BipA was broadly similar to that of domain IV of EF-G (R. Owens, PhD thesis) (Figure 1.11).

This is of interest, for despite the apparent low level of sequence homology, the crystal structures of EF-G-GDP and EF-Tu-tRNA-GTP are virtually superimposable, and hint at a possible occurrence of molecular mimicry (Nissen *et al.,* 1995; al-Karadaghi *et al.,* 1996; Rodnina *et al.,* 1997). The structural similarities of both of these components suggest that EF-G might bind to the same site on the ribosome that also interacts with the anticodon stem loop of the EF-Tu-tRNA complex. Interestingly, the molecular mimicry does not end here, for IF2 is also thought to bind to the 70S ribosome in the same manner as EF-G and EF-Tu, as well as the TetM and TetO family of tetracycline resistance proteins (Burdett, V. 1991; Dantley *et al.,* 1998; La Teana *et al.,* 2001). It has been proposed therefore that all members of this subfamily, and that now must include BipA, are mimics of the same structure, despite all having substantial differences in their biological functions (Brock *et al.,* 1998).

R. Owens also determined that BipA has an approximately 3-fold higher affinity for GTP over GDP, and that GDP greatly inhibits GTP hydrolysis by BipA, even when in

the presence of ribosomes. EF-G is known to have an approximate 10-fold higher affinity for GTP than GDP, allowing the GTPase activity of EF-G to remain unaffected by increasing GDP concentrations. This difference between BipA and the other ribosome-binding proteins in the affinity for GTP and GDP could offer an insight into how BipA functions in the cell. A possible function of BipA might be to respond to conditions where GTP concentration was high.

1.7 The BipA protein controls a number of cellular processes

Following the discovery of BipA, subsequent studies have sought to find out the role of the protein in the bacterial cell. As a result, a number of important cellular processes have been identified as to being regulated by BipA. What follows is a brief description of these BipA-regulated processes, which is also summarised by Figure 1.12.

1.7.1 Resistance to Bactericidal/permeability-increasing protein (BPI)

As mentioned earlier, part of the human immune response to bacterial infection involves the deployment of antibacterial peptides and proteins such as BPI. In order to survive in the human host, commensal and pathogenic bacteria such as *E. coli* and *S. typhimurium* develop strategies to resist these antimicrobial agents. Barker and colleagues (2000) showed formate and certain other carboxylic acids provided protection to stationary-phase *E. coli* and *Salmonella* cells, from a potent derivative of BPI and, that this protection involves BipA. EPEC *bipA*$^-$ cells were also shown to be hypersensitive to BPI, suggesting some specificity in the protective effect of BipA (Farris *et al.*, 1998).

1.7.2 Flagella mediated cell motility

During the original initial characterisation of BipA, the profiles of proteins released into the medium during growth of *bipA*$^-$ cells and parental EPEC cells were compared. In contrast to parental cells, large amounts of a 60 kDa protein were found in the culture

medium of cells lacking BipA. Additionally, levels of secretion of this 60 kDa were reduced to normal upon introduction of the *bipA* gene, *in trans* into the mutant. Amino-terminal sequencing identified this 60 kDa protein as flagellin. It was subsequently established that *bipA*$^-$ cells were markedly more motile than the parent cells and it was concluded that BipA negatively regulates the expression of flagellin and possibly other components associated with the production of flagella. (Grant *et al.,* 2003)

1.7.3 Regulation of group 2 capsules and growth at low temperatures in *E. coli*

The expression of *E. coli* group 2 capsules (K antigens) is temperature dependent, only occurring at temperatures above 20°C. Group 2 capsule gene clusters have a common organisation of three regions. A central, capsule-specific region, region 2, encoding the enzymes necessary for polysaccharide biosynthesis, which is flanked on either side by two additional regions, regions 1 and 3. These flanking regions contain the genes that encode for proteins that are required for the successful transport of capsule components out of the cell. Rowe *et al.* (2000) reported that the global regulator H-NS and also BipA are essential for maximal capsule production at 37°C, and yet both H-NS and BipA also appear to play roles in repressing production at 20°C. This suggests that BipA and H-NS play key roles in the regulation of group 2 capsule gene expression. Recent studies by A. Grant *et al.* (2001) and Pfennig and Flower (2001) have subsequently shown that BipA is required for *E. coli* growth during cold shock. BipA$^-$ mutants were capable of growth on solid media at 37°C, yet, although not fatal, the absence of BipA had a detrimental effect on growth at 27°C. BipA$^-$ mutants with plasmid borne *bipA* were again able to tolerate the drop in temperature to 27°C.

1.7.4 BipA and the regulation of EPEC pathogenesis

Previous studies by Grant *et al.* (2003) led to the discovery that BipA regulated virulence determinants by genes on the LEE by modulating the expression of the LEE-encoded regulator, Ler. Further experiments proved that the BipA protein did not regulate *ler* directly. Rather, it was proposed that BipA controlled *ler* activity by

governing the translation of the mRNA for an upstream factor 'X', which was predicted to be required for the transcription of the *ler* gene. However, the mechanism BipA uses to control the transcription of *ler* was not determined.

1.7.5 BipA and Acid Tolerance in *E. coli*

Like other gut-dwelling bacteria, *Escherichia coli* has been shown to modulate acid tolerance levels in response to changes in extracellular pH, increasing tolerance when the external pH grows more acidic. This has enabled the bacteria, particularly the pathogenic varieties, to survive in a number of acidic environments, particularly certain foodstuffs and passage through the human gut (Merrell and Camilli, 2002). Preliminary studies by Fowler (PhD thesis) suggested that *Salmonella* and EPEC cells that were unable to produce BipA, were able to survive exposure to pH3 (inorganic) acid levels for far longer periods of time during log phase, than wild-type cells. This would be of obvious interest because any gene that could singularly dictate whether a bacterial cell could tolerate high levels of acidity would have implications and consequences for the medical and food industry.

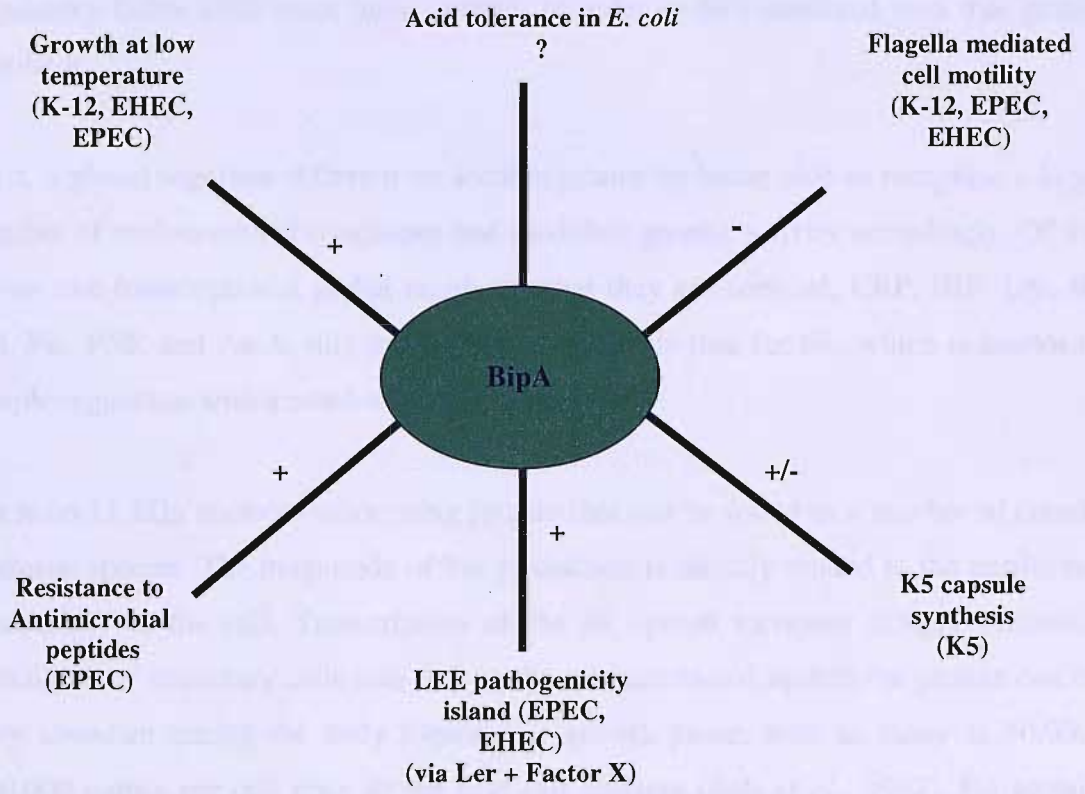


Figure 1.12 A diagram illustrating the cellular processes known to be regulated by BipA. Figure adapted from Grant *et al.*, 2003

1.8 Is BipA a global regulator?

As with other unicellular organisms, *E. coli* encounters continuous environmental challenges and fluctuations in numerous growth-related e.g. temperature, osmolarity, pH and nutrient availability, or in the case with EPEC the need to perceive the correct parameters for virulence. Bacteria can recognise and relay these stimuli through specific signal receptors and transduction pathways, which recruit the correct local and global regulatory molecules, needed to co-ordinate the activity of the necessary genes for a successful response. But what is it that constitutes a global regulator, and can BipA be classified as one? Following their observations of the activities of many gene regulators in bacteria, Martinez-Antonio and Collado-Vides (2003) state that a

regulatory factor must meet three criteria, in order to be considered as a true global regulator.

First, a global regulator differs from local regulator by being able to recognise a large number of environmental conditions and modulate genetic activity accordingly. Of the seven true transcriptional global regulators that they put forward, CRP, IHF, Lrp, H-NS, Fis, FNR and ArcA, this statement is particularly true for Fis, which is known to couple regulation with a number of conditions.

Fis is an 11 kDa nucleoid-associating protein that can be found in a number of enteric bacterial species. The magnitude of Fis production is directly related to the nutritional availability to the cell. Transcription of the *fis* operon increases sharply following subculture of stationary cells into rich media. On nutritional upshift the protein can be very abundant during the early logarithmic growth phase, with as many as 50,000-100,000 copies per cell prior to the first cell division (Ball *et al.,* 1992). Fis protein production throughout cell growth mirrors that of the *fis* transcription activity. Fis can respond to many different conditions through the input and influence of many different regulators of *fis* gene expression. The *fis* promoter is reported to be sensitive to changes in the superhelical density of DNA and is thought to respond to growth phase-dependent fluctuations in DNA topology (Schneider *et al.,* 2000). IHF stimulates *fis* expression by up to three-fold from a binding site 166 nucleotides upstream of the start codon (Pratt *et al.,* 1997). Interestingly the *fis* gene is rare in that transcription initiates with a CTP. Mutations that cause a switch from CTP use to that of ATP or GTP appear to have a detrimental effect on the growth rate dependent nature of *fis* expression (Walker and Osuna, 2002). In addition, in the absence of Fis, CRP promotes Fis production by displacing RNA polymerase from a divergent promoter from the *fis* gene that would normally reduce Fis production (Nasser *et al.,* 2001).

Fis abundance decreases by one hundred-fold as the cells approach stationary phase, with negative autoregulation by Fis blocking RNA polymerase interacting with the *fis* promoter accounting for some six-fold decrease in transcription (Ninnemann *et al.,* 1992). CRP alone is required to maintain *fis* transcriptional activity, yet CRP when in a

combination with available Fis protein, will act synergistically to repress *fis* activity (Nasser et al., 2001). Although still a contentious issue, it has been demonstrated that the *fis* gene is also strongly repressed by a regulatory mechanism that is termed stringent response (Ninnemann *et al.*, 1992; Walker *et al.*, 1998). The stringent response mechanism is activated under conditions of stress such as cell starvation and is designed to divert the cells energies away from metabolism and growth. This ability of Fis levels to vary enormously in response to changing environmental and nutritional conditions is deemed crucial to its role as a global regulator. From previous work on BipA, it is possible that to deduce that the *bipA* gene is also involved in a number of varied conditions and stresses. However, data concerning regulation of the *bipA* gene itself is limited.

The regulatory networks that direct many cellular processes are not based on a hierarchical organisation or divided into domains of influence headed by a particular global regulator. Instead a system of global co-regulation exists where global regulators work together or against one another to direct gene expression. Again using Fis as an example, out of the seven accepted global regulators, Fis comes second only to CRP regulating 296 genes in *E. coli* (220 of these candidate genes are regulated indirectly) (Madan Babu and Teichmann, 2003). Fis regulatory activity, whether it is negative or positive, is often associated with fellow global regulators such as IHF, CRP and H-NS. Most of the genes involved in the catabolism of sugars and nucleic acids regulated by Fis are also dependent on the presence of a cAMP-CRP complex (Gonzalez-Gil *et al.*, 1996). As the mechanism of action for BipA has yet to be determined in any of the processes it regulates, it is not possible to gauge the importance of the protein by the level of interaction it has with other global regulators. However, it is evident that BipA must be capable of interaction and match this criterion, as it regulates many different cellular processes. It is possible that as BipA shares the characteristics of a ribosome-binding protein, BipA could have a novel approach to interacting with the regulatory network.

Interestingly, Martinez-Antonio and Collado-Vides also state that unlike local regulators, the seven main global regulators are transcribed uncoupled from the genes

they regulate. This may seem to be stating the obvious, as they regulate so many genes, yet the distinction is made as many local regulators are encoded by genes that can be found next to the target, and are often the result of horizontal transfer, e.g. Ler and the regulation of the LEE in enteropathogenic *E. coli.*

Using Fis one last time as a prime example, the protein is associated with nutritional upshift, and yet is involved in the regulation of a multitude of processes, many having no link other than the contribution Fis has. For instance, Fis participates in the regulation of the stimulation of stable RNA synthesis (Nilsson *et al.*, 1990; Hirvonen *et al.*, 2001), modulation of DNA topology (Weinstein-Fischer *et al.*, 2000), initiation of *oriC*-directed DNA replication (Gille *et al.*, 1991; Filutowicz, 1992), carbon and nucleic acid catabolism (Gonzalez-Gil *et al.*, 1996) and more recently the protein has been associated with the regulation of the virulences genes in EPEC (Goldberg *et al.*, 2001), *S. typhimurium* (Wilson *et al.*, 2001) and for biofilm production in enteroaggregative *E. coli* (EAEC) (Sheikh *et al.*, 2001). BipA certainly fits this third criterion, as it has an equally diverse range of genes that are dependent upon its presence, as described in section 1.7. Collectively, the findings of previous studies and reports certainly indicate that BipA is a novel form of global regulator. As shall be described now, the goal of this study was to explore this fascinating protein and its regulatory mechanism further.

1.9 Current Hypotheses and Aims of this Study

BipA's activities might be explained by one of the ever-growing number of proposed translational/post transcriptional regulatory mechanisms. Alternatively, BipA might regulate through a totally novel mechanism. In order to determine BipA's true mechanism of action it is necessary to first identify a primary target gene for the protein, which can be subjected to further in-depth research. As BipA is most likely to act through the ribosome, the best candidate for a primary target for BipA must be mRNA, or more specifically a certain subset of mRNA species that the protein recognises as different from the rest of the total cellular mRNA population.

To help identify these mRNAs it might be possible to first identify the protein that is encoded by it, which is referred to as factor 'X'. There are two directions that this study will proceed, in order to try to determine the identity of factor 'X'. The first way is to characterise the regulation and expression patterns of the *bipA* gene. By doing this it is hoped that by knowing how *bipA* behaves during culture growth or other environmental stimuli where the BipA protein is prominent, it may be possible to narrow down the search parameters for factor 'X's.

In essence then there are two main questions that this study will attempt to find answers:

I. How is the *bipA* gene regulated? By finding an answer to this question it may indicate whether BipA behaves like a global regulator in that it responds to a number of environmental stimuli.

II. What other proteins/processes does BipA control through its regulatory cascade? If BipA is a novel type of global regulator, how far does its influence reach?

CHAPTER 2

Materials and Methods

CHAPTER 2: Materials and Methods

Materials and reagents

The following specialist materials were used:

Disposable fluorimetric range cuvettes (Hughes and Hughes International)
Nitrocellulose transfer membrane (Schleicher and Schuell)
NuPAGE™ Bis-Tris Gels (NOVEX)
Blue sensitive X-ray film (GRI)
Sterile disposable filters (0.22μm and 0.45μm; Sera-lab)
Qiaprep spin kits (Qiagen)

The following specialist reagents were used:

Bicinchoninic Acid (BCA) Protein Assay kit (Pierce)
DNA 1 kb ladder size marker (BRL)
ECL Western blotting Analysis system (Pierce)
Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma)
2-Nitrophenyl β-D-galactopyranoside (ONPG) (Sigma)
DL-glyceraldehyde-3-phosphate (Sigma)
Radiochemicals (Amersham-Pharmacia Biotech Inc.)
Restriction endonucleases (New England Biolabs (NEB), Promega, Stratagene)
Rnase Zap (Invitrogen)
T4 kinase (Promega)

Unless stated all other reagents were from Fisher, Promega, New England Biolabs or Sigma.

2.1 General Techniques

2.1.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids that were used in this study are listed in Tables 2.1 and 2.2, respectively. All strains were stored frozen at -70°C, typically in 66% Luria-Bertani (LB) medium, 33% glycerol (v/v). Unless otherwise stated, an overnight culture typically consisted of a single representative colony being used to inoculate a 10 ml culture, which was then grown at 37°C with agitation. Bacterial cultures were usually prepared in LB medium (+/- antibiotics), and grown at 37°C with agitation. For nutrient upshift studies 2 x YT medium was used (Schneider *et al.*, 1997). Where expression of virulence proteins was needed Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1, sodium pyruvate, glucose (0.45% v/v) and pyroxidine (Gibco BRL Life Technologies) was used. Other methods required alternative and specific growth conditions, details of which will be given when and where necessary.

Luria-Bertani medium (per litre)(pH 7.4): 10 g tryptone, 5 g yeast extract and 5 g NaCl, which was then autoclaved at 121 °C (15 psi) for 20 minutes. Addition of 15 g of agar (1.5 %) to 1 litre of LB media yielded LB plates. A stock solution of 40 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide was used to produce a working concentration of 40 µl/ml in X-gal containing plates.

2 x YT medium (per litre) (pH 7.4): 16 g Tryptone, 10 g Yeast Extract and 5 g NaCl, which was then autoclaved at 121 °C (15psi) for 20 minutes.

Unless otherwise stated, M63 minimal medium (per liter) (pH 7.0) 13.6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.5 mg $FeSO_4$, 0.05 % casamino acids, 1 ml, 1 M $MgSO_4$, 1 µg/ml (final concentration) thiamine and 10 ml of a 40 % solution of carbon source (e.g. glucose or glycerol).

Strain name	Genotype/ Comments	Source/Reference
DH5α	F' φ80d *lacZ* Δ(*lacZYA- argF*) 169, *recA1*, *endA1*, *hsdR17* (rk^- mk^+), *sup*E44, λ^-, *thi-1*, *gyrA*, *relA*.	Hanahan *et al*, 1991
MG1655	F- λ-	(ATCC No. 47076)
MG1655 Δ*bipA*	MG1655 *bipA* mutant (Δ*bipA*)	Arigoni *et al.*, 1998
MAR001 O217:H6	EPEC clinical isolate, plasmid cured	V. Norris
MAR001 *bipA::cat*	MAR001 *bipA::cat*	M. Farris
MC4100	F- *araD139* Δ(*argF-lac*) *U169 rpsL150* (Str^r) *relA1 flbB5301 deoC1 ptsF25 rbsR*	C. Guttierez
MC4100 *fis::cat*	*fis* null mutant containing a *cat* cassette inserted into the *fis* gene.	C. Guttierez
CSH50	*F'((pro-lac), ara, thi, strA, F(proAB, lacIQZMI5, traD36))*	R. Kahmann
CSH50 *fis::cat*	CSH50 *fis* null mutant containing a *cat* cassette, encoding for chloramphenicol acetyltransferase, inserted into the *fis* gene.	R. Kahmann
MC1000	*[araD139]*, Δ(*araA-leu)7967*, Δ(*codB-lacI)3*, *galK16*, *galE15*, λ^-, *e14*, *relA1*, *rpsL150*(strR), *spoT1*, *mcrB1*	Casadaban and Cohen, 1980

Table 2.1 Bacterial strains used in this study

Plasmid name	Genotype/ Comments	Source/Reference
pACYC184	Low-copy number cloning vector Cm^r, Tc^r	Chang and Cohen
pAJG4	Plasmid bearing a full copy of the *E. coli bipA* Amp^r	A. Grant; University of Southampton.
pAJG38	Arabinose inducible plasmid bearing an inducible copy of the K-12 *bipA* gene	A. Grant
PRS415	Multi-copy fusion plasmid encoding Amp^r	Simons *et al.*, 1987
pWP1	pRS415 with all three *bipA* promoters	S. Payot; University of Southampton
pWP2	As pWP1 but with mutated CRP site at position $^-$268 (relative to *bipA* start codon)	S. Payot
pWP5	As pWP1 but with mutated NtrC site at position $^-$201 (relative to *bipA* start codon)	S. Payot
pKT38	IPTG inducible copy of the *relA* gene	K. Tedin
pKT40	IPTG inducible mutated *relA* gene (RelA protein inactive)	K. Tedin
pBip116	pRS415 bearing *bipA* promoter 3 fragment See Figure 3.1 Chapter 3	S. Payot
PDJS70.33	pRS415 bearing *bipA* promoter 2 fragment See Figure 3.1 Chapter 3	D. Studholme; University of Southampton
PDJS73.1	pRS415 bearing *bipA* promoter 1 fragment See Figure 3.1 Chapter 3	D. Studholme
pTM918	Derived from pΔ150, bears no Fis binding regions	Schneider *et al.*, 1997
pTrcfis	Plasmid bearing IPTG inducible copy of the *E. coli fis* gene Amp^r	R. Johnson

Table 2.2 Plasmids used in this study

2.1.2 Antibiotics

Stock solutions of the following antibiotics were made up using filter sterilised analytical grade water, or 100% ethanol. Where necessary they were added to autoclaved medium, once the medium had cooled to below 45°C.

Ampicillin: 100 mg/ml. Final concentration was 100 μg/ml. (water)
Chloramphenicol: 25mg/ml. Final concentration was 25 μg/ml. (ethanol)
Kanamycin: 25mg/ml. Final concentration was 25 μg/ml. (water)
Tetracycline: 12.5mg/ml. Final concentration was 12.5 μg/ml. (50:50 ethanol:water)

All stock solutions were stored at -20°C.

2.1.3 Preparation of Pre-conditioned media for Quorum-sensing studies

Quorum sensing studies involving pre-conditioned medium was prepared by growing *E. coli* MC1000 in 1 litre of LB medium. For early-exponential phase of growth cells, cultures were grown to an A_{600} = 0.3. For mid-exponential and stationary phase of growth, cultures were grown to A_{600} = 0.6 and A_{600} = 1.0, respectively. The bacteria were removed from the cultures by 2 x centrifugation steps 5000 rpm in a Beckman JA-20 for 20 minutes, and the resulting supernatant was passed through two sterile 0.2μm filters in order to remove any lingering bacterial cells. This double filtrate was termed 2 x pre-conditioned medium and was diluted 50:50 with fresh, sterile 2 x LB for use with experiments. When the medium was used a separate culture was set up, with no bacterial inoculation. This served to prove that there were no contaminating bacteria.

2.2 Techniques involving DNA

2.2.1 Plasmid DNA preparation

Cells were grown overnight in 10 ml LB medium and with correct antibiotics. The next day the cultures were used to inoculate fresh LB medium and were grown to an A_{600} of 0.4, and were then processed using Qiagen mini plasmid preparation kit. The protocol used was with the use of a microcentrifuge, as outlined by the manufacturers instruction manual. DNA was suspended in sterile analytical grade water.

2.2.2 Agarose gel electrophoresis

Samples of DNA fragments were separated by the use of horizontal agarose gels, as described by Sharp *et al.* (1973). Appropriate amounts of agarose were dissolved into TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), to which was added 0.5 µl/ml of ethidium bromide. Prior to loading, 0.2 volumes of gel loading buffer (30% v/v glycerol, 0.3% w/v bromophenol blue, all made up in TE buffer (10 nM Tris-HCl, pH 8.0, 1 mM EDTA) was added to each of the DNA samples. For DNA band size evaluation 5 µl of a 1 kb DNA ladder (Invitrogen) (Table 2.3) was used a size marker. Electrophoresis was normally carried out at 100 V for 45 minutes in TAE buffer that contained 0.5 µl/ml of ethidium bromide. DNA bands were viewed by the use of a transilluminator (UVP) with 300 nm UV light.

DNA band (1 kb Invitrogen)	Size (kbases)
1	12.216
2	11.198
3	10.180
4	9.162
5	8.144
6	7.126
7	6.108
8	5.090
9	4.072
10	3.054
11	2.036
12	1.636
13	1.018
14	0.507
15	0.396
16	0.344
17	0.298
18	0.220
19	0.201
20	0.154
21	0.134
22	0.075

Table 2.3 Invitrogen 1 kb DNA marker

2.2.3 Extraction and purification of DNA samples from agarose

DNA fragments purified from agarose gels were excised carefully by using a clean scalpel blade, whilst illuminated briefly by a UV lamp. The DNA within the excised segments of agarose was extracted by using the Qiagen Gel-Extraction kit and by following the accompanying manufacturers protocol. Following completion of the process, the DNA was ultimately eluted into sterile analytical grade water.

2.2.4 Estimation of DNA quantity

The concentrations of DNA within samples were calculated through the use of UV spectroscopy. The samples were placed into quartz cuvettes, the machine calibrated with a suitable blank, and the absorbance at 260 nm was taken. Protein contamination in the DNA samples was calculated, by comparing the ratios of absorbance at 260 nm and 280 nm.

2.2.5 Transformations

Electroporation

Cells were grown in LB medium to early mid-exponential phase (typically an absorbance of 0.4 A_{600}), and were then chilled upon ice for 10 minutes. Afterwards the cultures were then centrifuged at 6000 rpm at 4°C, for 15 minutes (Beckman JLA 10,500 rotor), in order to pellet the cells. The supernatant was carefully removed and the pellet washed by re-suspending in ice-cold sterile analytical grade water and re-spun (2 x). The pellet was then re-suspended in 25 ml of sterile ice-cold 10 % glycerol, followed by another spin at 6000 r.p.m. for 15 minutes at 4 °C in a Beckman JA-20 rotor. The supernatant was then removed and the bacteria re-suspended in the glycerol residue and aliquoted into 40 µl volumes and stored at $^{-}$70 °C or used immediately.

To transform, desalted DNA (10-100 ng) was added to the 40 µl volume of cells, and incubated on ice for 1 minute. The sample was then transferred to an ice-cold

electroporation cuvette and the bacteria shocked with a pulse set at 1.25 mV for 4.5 mseconds. The bacteria were rescued by the addition of 1 ml of sterile SOC medium (SOC, 1 litre: 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, 20 g bacto-tryptone and 5 g bactot-yeast extract). After 1 hour at 37 °C, with constant agitation, the cells were serially diluted and plated.

Calcium chloride competent cells

Cells were grown in LB medium to early mid-exponential phase (typically an absorbance of 0.4 A_{600}), and were then chilled upon ice for 10 minutes. Afterwards the cultures were then centrifuged at 6000 rpm at 4°C, for 15 minutes (Beckman JLA 10,500 rotor), in order to pellet the cells. The supernatant was carefully removed and the pellet washed by re-suspending in 10 ml of ice-cold 0.1 M $CaCl_2$. This process of washing was repeated once, where-upon the pellet was then resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$ + 50% glycerol, prior to being divided into 200 µl volumes, snap-frozen with liquid nitrogen and then stored at -70°C.

To transform, competent cells were thawed upon ice and were then slowly mixed with DNA/ligation mixture. The cells were then incubated on ice for 45 minutes and were then placed into a 42 °C waterbath for precisely 2 minutes. The cells were then put on ice for 5 minutes before addition 800 µl SOC medium and incubated at 37°C for 1 hour. After incubation the cells were serially diluted and plated on to LB plates with appropriate antibiotic and left overnight at 37°C.

2.3 Protein Techniques

2.3.1 Preparation of bacterial whole cell extracts for Western Blot studies

Cells were grown overnight, normally in 10 ml LB with appropriate antibiotics. The overnight cultures were then used to inoculate a second culture on the following day, the type of medium being dependant upon the experiment pursued. Cells were grown to an appropriate A_{600} or time following inoculation, whereupon 1.5 ml samples were

taken and centrifuged (12,000 r.p.m benchtop centrifuge). The pellet was then resuspended into 350 µl of 2x final sample buffer (FSB) (0.125 M Tris-HCl (pH6.8), 4 % SDS, 20 % glycerol, 10 % 2-Mercaptoethanol, 0.05 % bromophenol blue). Samples were boiled for 5 minutes, and the lysates cleared by centrifugation at 12,000 r.p.m. for 5 minutes at 4 °C. Samples were then stored at ⁻20 °C until needed

2.3.2 Estimation of Protein Concentration

Protein concentration was measured spectrophotometrically by the Pierce BCA assay system. Bovine serum albumin standards were used in the range 200 µg to 1200 µg, diluted from a 2mg/ml stock. All samples were always measured in triplicate. 10 µl of sample (blank, protein of interest, BSA) was pipetted into a microtitre plate well and 200 µl of BCA solution added. The plate was then incubated at 37°C for 30 minutes after which the absorbance at A_{570} was read. A standard curve was then plotted and the protein concentration of interest calculated.

2.3.3 SDS polyacrylamide gel electrophoresis of proteins

Separation of proteins was performed using 12 % SDS-PAGE gels, as previously described by Laemmlli (1970), using the Bio-Rad mini-gel electrophoresis apparatus in accordance with the manufacturer's instruction. Unless otherwise stated, NuPAGE™ 4-12 % Bis-Tris gels (Novex) were used. Protein samples were visualised by staining with Coomassie Blue (0.25 % (w/v) Coomassie Brilliant Blue R-250 dissolved in 9% (v/v) glacial acetic acid and 45% (v/v) methanol) and destained (7.5% (v/v) glacial acetic acid, 45% (v/v) methanol). The protein standard size markers used were from New England Biolabs (Table 2.5).

Protein Broad Range Molecular Marker	Molecular Mass (kDa)
Myosin	212,000
MBP-β-galactosidase	158,194
β-galactosidase	116,351
Phosphorylase b	97,184
Serum albumin	66,409
Glutamate dehydrogenase	55,561
Maltose binding protein 2	42,710
Lactose dehydrogenase	36,487
Triose phosphate isomerase	26,625
Trypsin inhibitor	20,040
Lysozyme	14,313
Aprotinin	5,617
Insulin A, B chain	2,340

Table 2.4 Protein broad range molecular weight markers (NEB).

2.3.5 Western Blotting

Protein samples for immunoblotting were resolved on 4-12% Bis-Tris NuPAGE™ gels. The proteins were then transferred to a nitrocellulose filter (Schleicher and Schuell) by using a Mini-Trans Blot Electrophoresis Transfer Cell (BioRad). Prior to blotting, the gel was equilibrated in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol) in order to minimise shrinking during transfer. The transfer was carried out for 2 hours at a constant 70 volts (250 – 350 mA). Following complete transfer the membrane was incubated in 150 ml blocking buffer (10 mM Tris pH 7.5, 133 mM NaCl, 1.75 mM Na_2EDTA pH 8, 0.5 % Triton X-100 (v/v), 0.5 % BSA (w/v)) for 2 hours (4°C). The filter was then incubated with a suitable primary antibody, (see table 2.7 for antibodies and respective dilutions into blocking buffer used) overnight, with gentle agitation (4°C). Following incubation the membrane was washed three times, 20 minutes each wash, with blocking buffer and was then incubated for 2 hours (room temperature, with gentle agitation) with the secondary antibody (Goat anti-rabbit, Autogen/Santa Cruz). This secondary antibody bearing the horseradish peroxidase (HRP) label was also diluted into blocking buffer (1:3000). The membrane was then washed three times with a Tris-saline solution (50 mM Tris-HCl (pH 7.5), 100mM NaCl), 10 minutes each wash. Finally SuperSignal chemiluminescence substrate (Pierce) was used in order for the antibody-bound HRP to enzymatically generate light.

Primary Antibody	Dilution Used	Source
Anti BipA	1:400	M. Farris

Table 2.5 Primary Antibodiy used in this study.

2.4 Techniques involving RNA

2.4.1 Total cell RNA extraction and preparation for *ptsG* analysis

Total cell RNA was isolated using a Trizol-based technique. Bacteria were grown in 50 ml LB medium (+/-) 1.0 % glucose to an OD_{600} of 0.5. Cultures were then separated into 1 ml aliquots and cells harvested by centrifugation (7,800 r.p.m. in a Biofuge bench-top centrifuge, 10 minutes at 4 °C). The supernatant was removed and the pellet resuspended in 1 ml of Trizol reagent (Gibco) by agitation with a pipette tip, followed by vortexing for 1 minute. The homogenate was then freeze-thawed by immersion into liquid nitrogen for 1 minute, followed by 3 minutes in a 50 °C waterbath. Following a second incubation period of 5 minutes at 25 °C, 0.2 ml of chloroform was added to the homogenate. The samples were shaken vigorously by hand for 15 seconds and left on ice for 10 minutes. The mixture was centrifuged at 12,800 r.p.m. in a Biofuge bench-top centrifuge at 4 °C for 15 minutes. The resulting aqueous phase was removed and incubated with 1 µl of DNAse I (Boerhinger) for 30 minutes at room temperature. 0.5 ml of isopropyl alcohol was added, and left to incubate at room temperature for 10 minutes to precipitate the RNA. The mixture was centrifuged at 12800 r.p.m. with a Biofuge bench-top centrifuge at 4 °C for 15 minutes. The supernatant was removed and the pellet washed with 1 ml of 75 % ethanol, followed by a second spin at 10,000 r.p.m in a Biofuge bench-top centrifuge, for 5 minutes at 4 °C. The RNA pellet was left to air dry for 10 minutes before being dissolved in 25 µl DEPC water. Samples were then stored at -70 °C.

2.4.2 Estimation of RNA quantity and purity

RNA concentrations were calaculated by UV spectroscopy, performing absorbance readings in quartz cuvettes at 260 nm against a suitable blank. The purity of the RNA was assessed by comparing the ratio of absorbance values at 260 nm and 280 nm. The quality of the RNA was further tested through using agarose gel electrophoresis. Gels and buffers were prepared using DEPC-treated water, and the electrophoresis apparatus was treated with RNase Zap (Invitrogen).

2.4.3 5' end labelling of primers for extension analysis

A labelling reaction mixture would contain the following: primer (100 pmoles, final concentration), 25 μCi [γ ^{32}P] dATP (Amersham), DEPC-treated water to a final volume of 28.5 μl, 3 μl of 10 x kinase buffer (pH 7.6) (70 mM Tris-Hcl, 7 mM $MgCl_2$, 5 mM DTT) and 15 units of T4 Polynucleotide kinase. The reaction was incubated at 37 °C for 30 minutes and then stopped by the addition of 470 μl of analytical grade water, followed by incubation on ice, or storage at -70 °C.

2.4.4 Primer extension reaction

The reaction mixture contained: 10 μg of RNA, 0.5 pmol of labelled primer, 1.5 μl of 5 x hybridisation buffer (250 nM HEPES (pH 7.0), 500 mM KCl in DEPC-treated water. The total volume of the reaction mixture was raised to 7.5 μl by the addition of DEPC-treated water. The following was then added to the mixture: 2.5 μl of 5 x avian myeloblastosis virus reverse transcriptase (AMV RT) buffer (Boehringer), 2 mM of each dNTP, 20 units of Rnase Inhibitor (Boehringer) and 25 units of AMV RT (Boehringer). The reaction was incubated at 42 °C for 60 minutes before being stopped by the addition of 10 μl of sequencing stop buffer (95 % formaide, 20 mM EDTA (pH 8.0), 0.05 % Bromophenol blue, 0.05 % xylene cyanol), and stored at -20 °C.

2.6 Acid Tolerance Assay

Cells were grown overnight in fresh LB (10 ml) at 37 °C. The overnights were then used to inoculate fresh LB (1:50 dilution) at either pH 7.0 or pH 5.0 (to adapt the cells to acid conditions) (+ 0.2 mM IPTG where appropriate), and were grown to mid-logarithmic phase of growth (A_{600} = 0.6). Cells were not analysed at this growth step. 50 ml samples of these cultures were then used to inoculate (1:20 dilution) fresh LB medium at either pH 3.0 or pH 7.0 (+ 0.2 mM IPTG, where appropriate). As part of the analysis, 1 ml samples were taken at the designated times, serially diluted and plated on LB agar plates. The plates were evaluated the next day and the number of survivors counted and recorded.

2.6 β-galactosidase assays

2.6.1 Nutritional up-shift studies

This assay was adapted from the one described by Miller (1972). Bacteria were grown overnight in 1 x LB medium and were then diluted in a 1:100 fashion into fresh LB. The new cultures were grown with agitation and at 37°C to an OD_{600} of 1.0 and were then re-diluted 1:100 into fresh medium (medium type depending upon the assay). This third culture was the nutrient up-shift samples that were grown with agitation and at 37°C, with 2 ml samples taken throughout the time-course, as indicated.

2.6.2 Acid exposure studies

Bacteria were grown overnight in 1 x LB medium and were then diluted in a 1:100 fashion into fresh LB. The new cultures were grown with agitation and at 37°C to an A_{600} of 0.6 and were then re-diluted 1:20 into fresh LB medium that were at either pH 7.0 or pH 5.0. These cultures were permitted to grow to stationary phase. Throughout growth, and at the time points indicated, 2ml aliquots were used for β-galactosidase analysis.

Reaction process

Each sample was processed according to Miller (1972). The samples were cooled on ice to prevent further growth. 1 ml of the sample was used to obtain the absorbance value at OD_{600}. The rest of the sample was then used in the assay. Where low levels of β-galactosidase activity was expected, 0.5 ml of the sample was added 0.5 ml of Z buffer, where high levels were anticipated 0.1 ml of sample was added to 0.9 ml Z buffer. (Z buffer (per liter): 16.1 g NA_2HPO_4, 5.5 g NaH_2PO_4, 0.75 g KCl, 0.246 g $MgSO_4$ and 2.7 ml β-mercaptoethanol. Adjusted to pH 7.0).

Two drops of chloroform and one drop of 0.1 % SDS were added to the culture-Z buffer mixture, which was then vortexed for precisely 10 seconds. The mixtures were then placed into a 28 °C waterbath for precisely 5 minutes. The reaction was started by addition of 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (at 4 mg/ml) in Z buffer, and a 5 second vortex burst before being placed back into the 28 °C waterbath. The duration of the reaction was recorded by stop-watch. The reaction was permitted up to a maximum of 20 minutes, or once the reaction mixture had taken on a yellow colour. The reaction was then stopped completely by the addition of 0.5 ml 1 M Na_2CO_3.

The OD_{420} and OD_{550} measurements of the stopped reactions were compared against a suitable blank (Z buffer plus 1 M Na_2CO_3) and were recorded. The OD_{550} measurement represents the amount of light scattering that was due to cellular debris, while the OD_{420} measurement represents the amount of light scattering due to both debris and *o*-nitrophenol. These values were entered into the formula below (devised by Miller (1972)), and the number of β-galactosidase units calculated.

β-galactosidase units = $1000 \times (OD_{420} - 1.75 \times OD_{550}) \div (t \times v \times OD_{600})$

(Miller units)

Where t = time of the reaction in minutes and v = volume of the culture used in the assay

2.7 Topoisomerase Assays

2.7.1 Nutritional up-shift assay

The assay was adapted from the paper by Schneider *et al.* (1997). Essentially, overnight cultures, grown in LB medium, were used to inoculate 500 ml of fresh, pre-warmed 2 x YT broth. These cultures were grown, with shaking at 37°C, to early stationary phase (typically at an absorbance of 1.0 OD_{600}) A 50 ml sample of the stationary phase culture was retained for processing and was kept on ice. From the remainder, 100 ml was used to inoculate a second batch of 400 ml fresh 2 x YT medium, and again was grown with shaking at 37°C. From this point (time zero) 50 ml aliquots were taken at the time-points indicated. Each sample was kept on ice until taken for processing of the cells and harvesting of the plasmids.

2.7.2 Hydrogen peroxide assay

Overnight cultures, grown in LB medium, were used to inoculate 500 ml of fresh, pre-warmed LB broth. Cultures were grown for 45 minutes before 0.01% hydrogen peroxide was added. 10 minutes after hydrogen peroxide addition, 50 ml samples were taken for plasmid isolation.

2.7.3 Acid Tolerance assay

Overnight cultures, grown in pH 7.0 1 x LB were used to inoculate 500 ml of fresh, warmed, pH 7.0 LB broth. These cultures were grown at 37 °C to mid-exponential phase of growth (OD_{600} = 0.5). At this point 100 ml of each culture was then used to inoculate a second batch of 900 ml LB broth, set at either pH 5.0 or pH 7.0. These cultures were grown at 37 °C for a further 20 minutes, whereupon the cells were harvested by centrifugation and the plasmid pTM918 extracted, as explained below.

Processing of culture samples

Culture samples were centrifuged at 12,000x *g* for 15 minutes, 4°C, in a Beckman (JA-20 rotor). The supernatant was removed and the cell pellet incorporated into the Qiagen Midi kit protocol for plasmid preparations of this scale. Plasmid DNA was resolved using a 1% agarose gel by dissolving the agarose in the TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). In place of ethidium bromide 0.3μg/ml of chloroquine was added. To the 20 μl samples from each plasmid preparation, 0.2 volumes (4 μl) of gel loading buffer was added. Electrophoresis was carried out at 100 V for 60 minutes. To visualise the DNA bands, the gel was soaked in TAE, containing ethidium bromide (at 0.6 μg/ml), for as long as required until the bands became apparent and could be photographed.

2.8 Glyceraldehyde-3-phosphate dehydrogenase assays

Bacterial strains were grown in 1 x LB medium to an OD_{600} = 0.25. Cells were harvested by centrifugation and whole cell protein extracts collected following sonication (as in 2.3.2). The concentration of protein in each sample was assessed by BCA assay, section 2.3.3. The following components were then added to a cuvette containing 2.6 ml sodium pyrophosphate/sodium arsenate buffer (0.015 M sodium pyrophosphate, 0.03 M sodium arsenate, pH 8.5), in the following order, 0.1 ml of 7.5 mM NAD (analytical grade) (Sigma), 0.1 ml of 0.1 M dithiothreitol and 0.1 ml of protein extract (each sample of equal concentration). This was incubated in a spectrophotometer at 25°C for 3 - 5 minutes to achieve temperature equilibrium and establish a blank rate. At zero time, add 0.1 ml of 0.015 M DL-glyceraldehyde-3-phosphate (Sigma) and record A_{340} for 3 - 5 minutes. Determine ΔA_{340}/minute from the initial linear portion of the curve.

CHAPTER 3

Studies on the regulation of the *bipA* gene

CHAPTER 3: Studies on the regulation of the *bipA* gene

3.1 Introduction

BipA appears to be a global regulator that has influence over a number of cellular processes that are metabolically expensive for the cell (e.g. flagella-mediated motility, capsule synthesis and type III secretion) (Farris *et al.*, 1998; Rowe *et al.*, 2000; Grant *et al.*, 2003). As yet, the mechanism(s) by which BipA exerts its regulatory effect is unknown, nor is it known how the *bipA* gene itself is regulated. It would seem plausible that a gene encoding a factor that regulates multiple energy-intensive processes would be under tight regulation itself, and would perhaps respond to a number of specific stimuli. This would prevent the unnecessary production of downstream products at times when they are not required. Identifying these stimuli, and thus the regulator(s) of *bipA*, is therefore of some significance. Additionally, understanding when BipA is normally expressed and what influences its control, should focus efforts to identify further genes and processes governed by this protein.

The *bipA* gene is located at 88 minutes on the *E. coli* K-12 chromosome, and shares an intergenic regulatory region with the divergent *glnA* gene, which encodes glutamate synthase. Within this integenic region there are three *bipA* promoters that are known to drive *bipA* transcription (D. Studholme, unpublished data), and prior to this study, the conditions that suited activation of these promoters were unknown. As BipA was linked to the regulation of a number of cellular processes, many of which are expensive in terms of energy and resource requirements, it was possible that *bipA* transcription responded to changes in nutritional condition of the growth medium. To test this theory, the activity of the *bipA* promoters was monitored under different nutritional conditions using a similar *lacZ*-based reporter gene system as described by Simons *et al.* (1987). Specifically, a series of DNA fragments carrying portions of the *bipA* regulatory region were inserted into the reporter plasmid pRS415, and thereby fused to a promoterless *lacZ* gene. It was then possible to assess the contributions of specific regulatory elements to *bipA* gene expression under a range of conditions, by measuring β-galactosidase activity (See Chapter 2, section 2.7) (Figure 3.1).

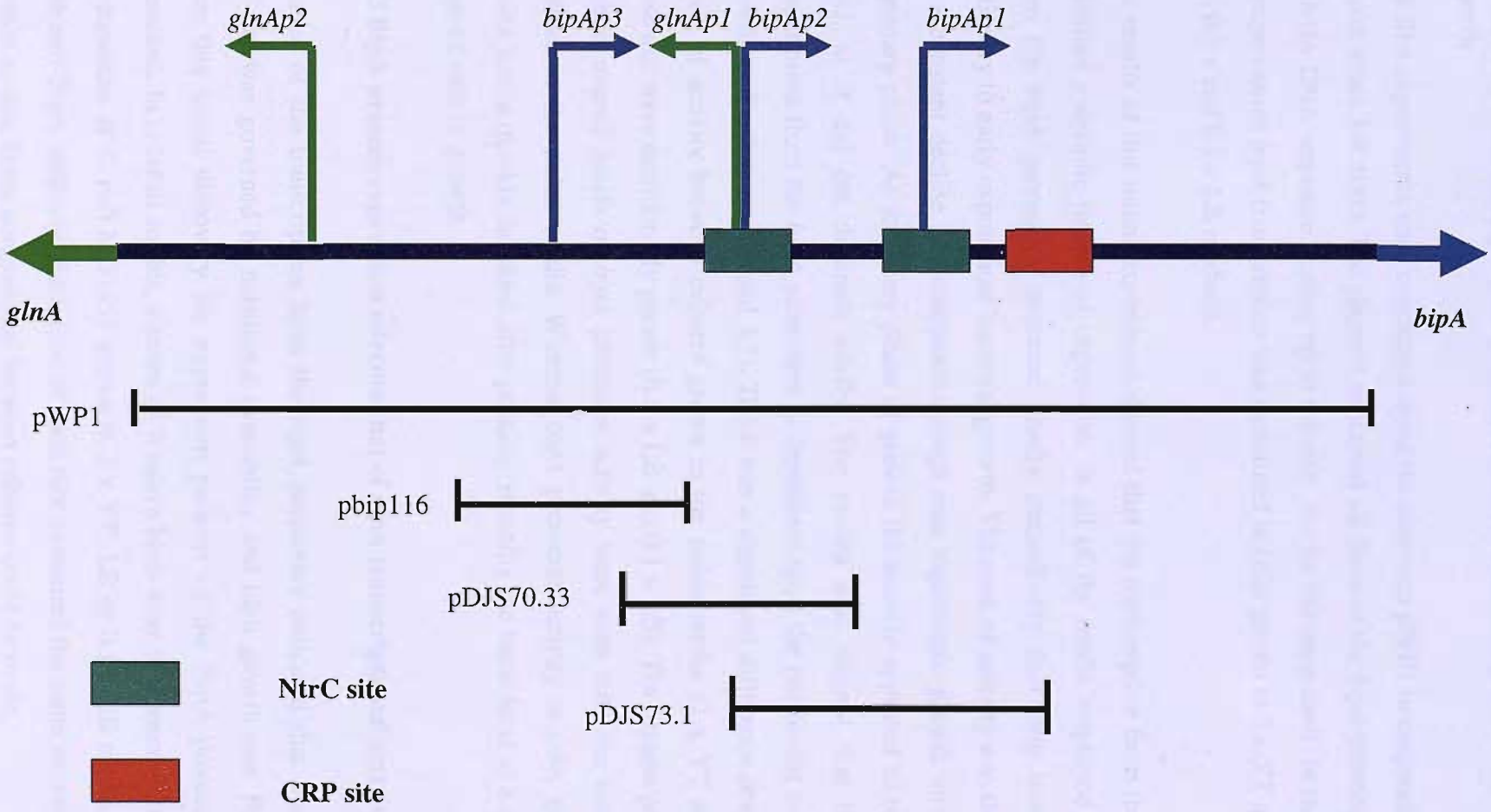


Figure 3.1 The intergenic regulatory region of the *bipA* and *glnA* genes, showing the locations of the fragments present in each of the tested constructs.

3.2 Activity from the *bipA* gene promoters shows a distinct pattern throughout growth

The first experiments were conducted using the construct pWP1 in conjunction with the *E. coli* strain MC1000. The plasmid contained all three of the *bipA* promoters together with the DNA sequence leading up to the start site for the gene itself. In the first series of experiments *bipA* transcription was monitored in cells grown in 2 x YT medium, and 1 x, 0.5 x and 0.1 x LB medium.

The results of this initial experiment showed that the transcription from the *bipA* gene exhibited a definite pattern of expression. In all of the media employed the activity from the *bipA* promoters increased briefly immediately following transition from stationary to early exponential bacterial growth. The peak of activity was then followed by a constant decline that continues through mid logarithmic growth and through to stationary phase. At stationary phase of growth the activity appeared to reach a basal level, as it did not diminish totally. The results also suggest that the level of transcription from the *bipA* promoters is dependent upon the nutritional content of the growth media (Figures 3.2 and 3.3). There was a significant difference observed in the levels of activity between cultures grown in the richer media (2 x YT and LB) and those that were nutritionally poorer (0.5 x LB and 0.1 x LB). The biggest peaks and the highest overall levels of *bipA* promoter activity were seen with the faster growing cultures in the richer media. Whereas *bipA* promoter activity in cells grown in the poorer media quickly subsided after peaking, reaching the basal level at a much earlier stage of culture growth.

3.3 BipA protein expression mirrors that of *bipA* transcriptional activity

Studies of the transcription from the *bipA* promoters indicated that *bipA* promoter activity was governed by nutritional availability and high growth rate. Following on from this initial discovery the expression patterns of the BipA protein were then examined. In order to do this, a series of Western blots were performed with whole-cell preparations of *E. coli* MG1655 grown in 2 x YT, LB or 0.1 x LB media, challenged with anti-BipA antibody. Each lane of each blot contained the same amount of protein sample, so that direct comparisons between cultures could be made.

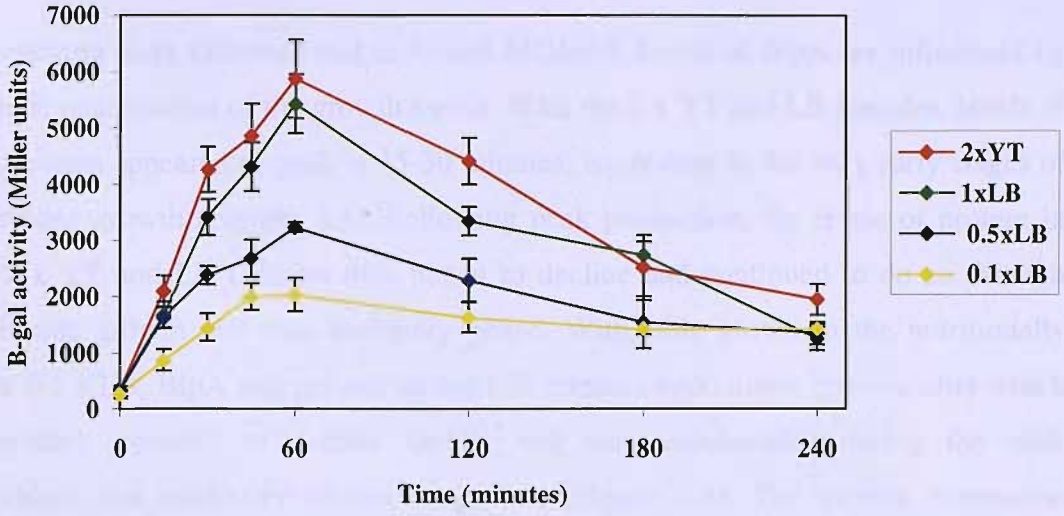


Figure 3.2 Comparison of β-galactosidase activity from pWP1 in MC1000, grown in media of decreasing nutritional content. Cells were grown overnight before being diluted 1:100 into fresh medium, grown to A_{600} of 1.0, before further dilution again in 1:100 into fresh medium. Samples were then taken at time intervals of 0, 15, 30, 60, 120, 180, 240 and 300 minutes and assayed for β-galactosidase activity. As a control, a culture of MC1000 bearing the pRS415 reporter plasmid alone, i.e. with no DNA insert, was grown in LB. The background β-galactosidase activity was subtracted prior to plotting the data. Error bars are plotted as standard deviation. 2xYT compared to 1xLB, p=ns, t-test. Points 2-5 1xLB/2xYT compared to 0.5xLB and 0.1xLB, $p<0.05$, t-test. All other points p=ns, t-test.

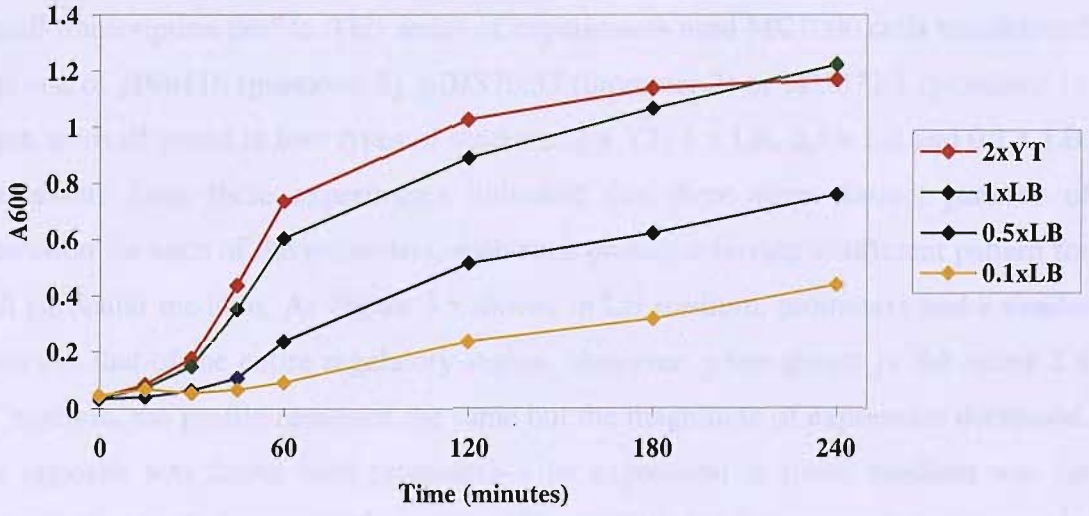


Figure 3.3 Growth curves of MC1000 (pWP1) during β-galactosidase assays in the four types of media tested.

The resulting blots indicated that in *E. coli* MG1655, levels of BipA are influenced by the nutritional content of the growth media. With the 2 x YT and LB samples, levels of BipA protein appeared to peak at 15-30 minutes, equivalent to the very early stages of logarithmic growth (Figures 3.4). Following peak production, the levels of protein in both 2 x YT and LB cultures then began to decline and continued to do so through logarithmic growth and into stationary phase. With cells grown in the nutritionally poorer 0.1 x LB, BipA was present up until 30 minutes into culture growth, after which the protein appeared to decline rapidly and was undetectable during the mid-logarithmic and stationary phases of growth (Figure 3.4). The protein expression profiles of the cultures from each media exhibited remarkably close similarities to patterns of *bipA* transcription in the same media type. In rapidly growing cells both mRNA and protein production were maximal in the early logarithmic phase of growth and declined to a minimal level by stationary phase.

3.4 The three promoters of the *bipA* gene show different individual patterns of activity throughout culture growth

The next step was to investigate the activities of the promoters for *bipA*, to determine the extent to which each influenced expression, and to find out if they each reflected the overall transcription profile. This series of experiments used MC1000 cells transformed with one of pBip116 (promoter 3), pDJS70.33 (promoter 2) or pDJS73.1 (promoter 1), which were all tested in four types of medium: 2 x YT, 1 x LB, 0.5 x LB and 0.1 x LB. The results from these experiments indicated that there were distinct patterns of expression for each of the promoters, with each promoter having a different pattern for each particular medium. As Figure 3.5 shows, in LB medium, promoter1 had a similar pattern to that of the entire regulatory region. However, when grown in the richer 2 x YT medium, the profile remained the same but the magnitude of expression decreased. The opposite was found with promoter3 – its expression in richer medium was far stronger than in the weaker LB medium (Figure 3.9). With promoter2 the change in transcription with each medium type was less dramatic. Indeed β-galactosidase expression was consistently low with no definitive peak of expression, suggesting that

it was the weakest of the three promoters, or was not expressed under the conditions tested (Figure 3.7).

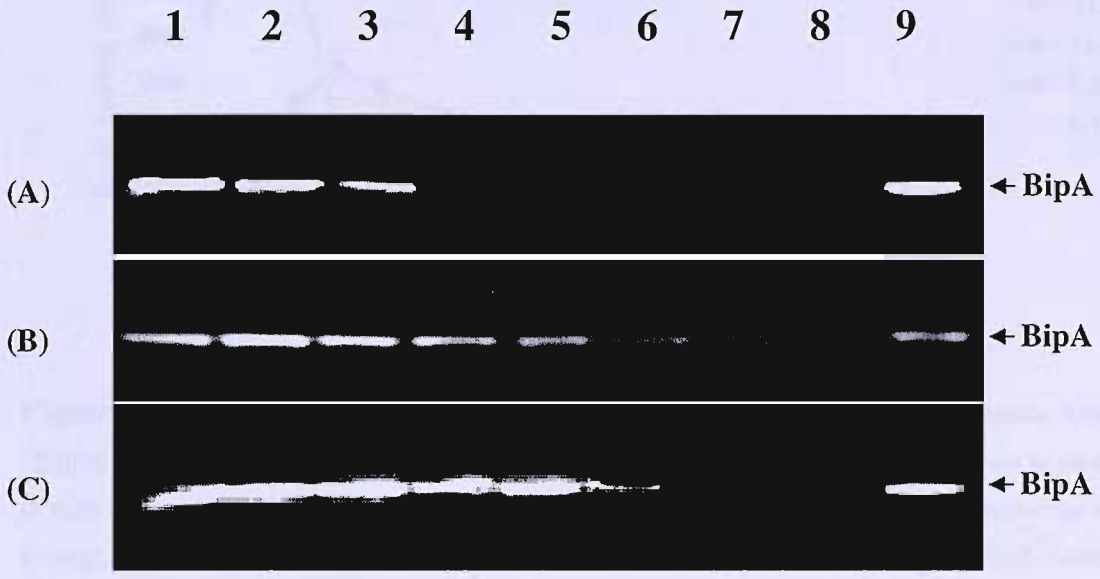


Figure 3.4 BipA protein expression pattern throughout culture growth

An immunoblot showing the levels of BipA protein throughout the growth of *E. coli* K-12 grown in 0.1x LB medium. The immunoblots show similar patterns of protein levels to that of *bipA* gene promoter activity. Bacterial cultures were grown in 0.1 x LB (A), 1 x LB (B) and 2 x YT (C) media with samples being taken at 0, 15, 30, 45, 60, 120, 180 and 240 minutes (lanes 1-7 respectively). Cells were harvested by centrifugation and whole cell extracts prepared in 2x final sample buffer. Extracts were then resolved by PAGE using a 4-12 % NuPAGE gel. Samples were then transferred to nitrocellulose and probed with antibodies specific for BipA. Bands were visualised using the Supersignal chemiluminescence substrate.

Lane 1: *E. coli* K-12 sample t = 0 mins **Lane 2:** *E. coli* K-12 sample t = 15 mins
Lane 3: *E. coli* K-12 sample t = 30 mins **Lane 4:** *E. coli* K-12 sample t = 60 mins
Lane 5: *E. coli* K-12 sample t = 120 mins **Lane 6:** *E. coli* K-12 sample t = 180 mins
Lane 7: *E. coli* K-12 sample t = 240 mins **Lane 8:** Δ*bipA E. coli* t = 60 mins
Lane 9: Δ*bipA E. coli* K-12 (pAJG4) t = 60 mins

Equivalent amounts of protein from each sample were loaded.
Exposure times for each blot (relative to LB blot) (A) 2.5x, (B) 1x and (C) 1.5x.

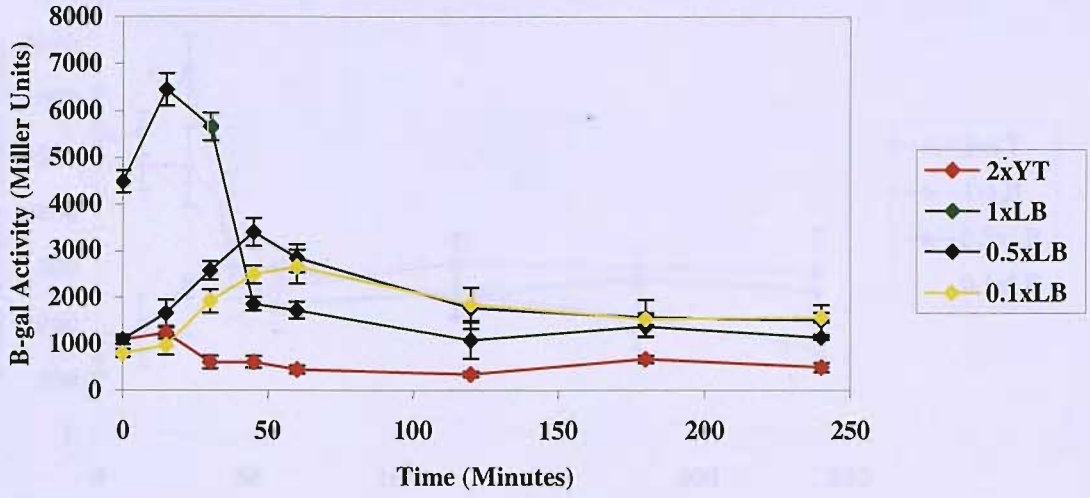


Figure 3.5 Expression profiles of *bipA* promoter1, as measured by β-galactosidase expression from the pDJS73.1 reporter plasmid, under different media conditions. Each condition tested was done in triplicate in each assay executed, with there being at least three assays completed. The graph shows the mean average values and error bars are plotted as standard deviation. For points 1-3 1xLB, $p<0.05$, t-test. All others p=ns, t-test.

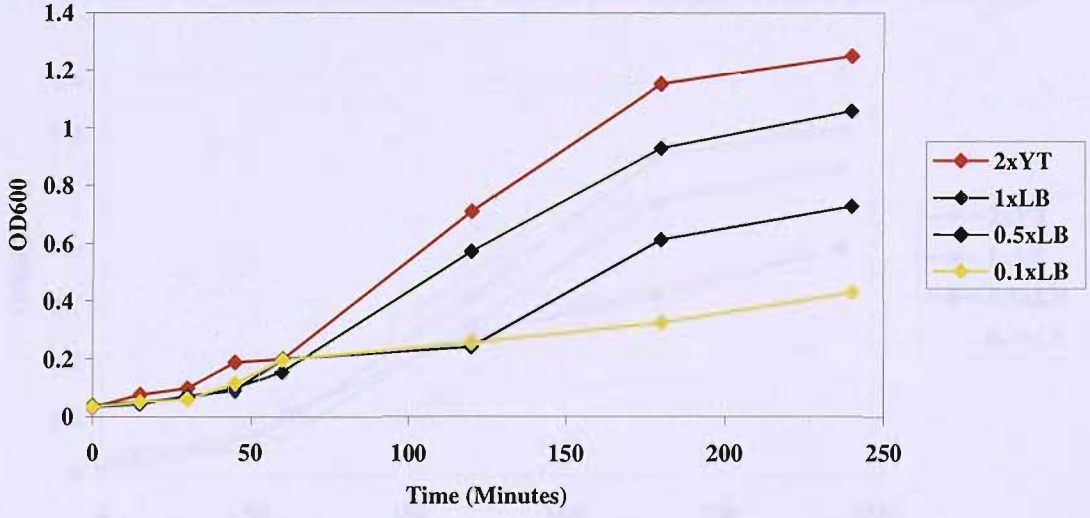


Figure 3.6 The growth rates of MC1000 (pDJS73.7) in the four types of media during the eperiment shown in Figure 3.7.

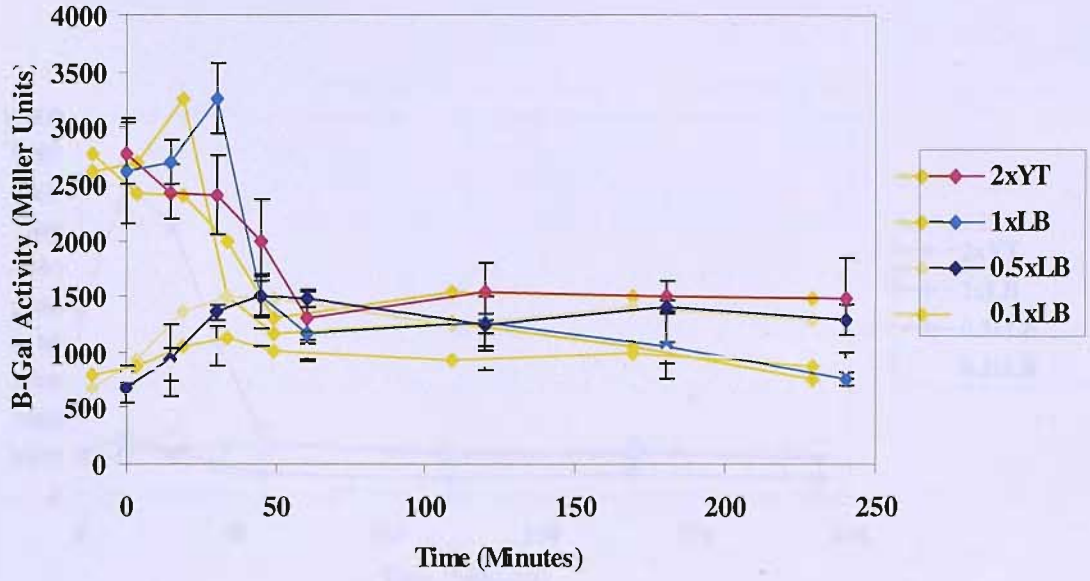


Figure 3.7 Transcription profiles of *bipA* promoter2, as measured by β-galactosidase expression from the pDJS70.33 reporter plasmid, under different media conditions. Each assay was carried out at least three times, with there being three test cultures per condition, per experiment. The graph shows the mean average values and error bars are plotted as standard deviation. 2xYT compared to 1xLB, p=ns, t-test. Points 1-3 1xLB/2xYT compared to 0.5xLB and 0.1xLB, $p<0.05$, t-test. All other points p=ns, t-test.

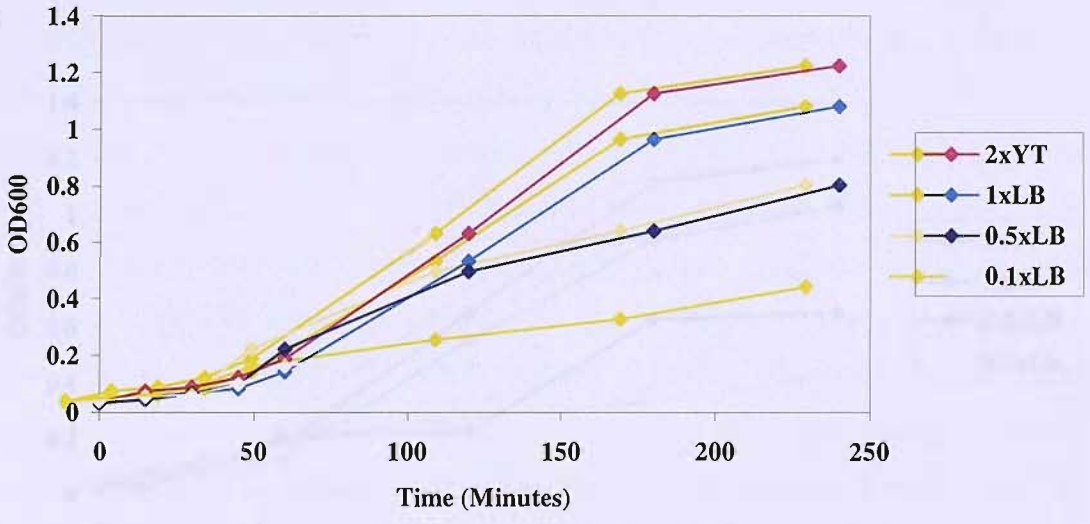


Figure 3.8 The growth rates of MC1000 (pDJS70.33) in the four types of media during the experiment shown in Figure 3.9.

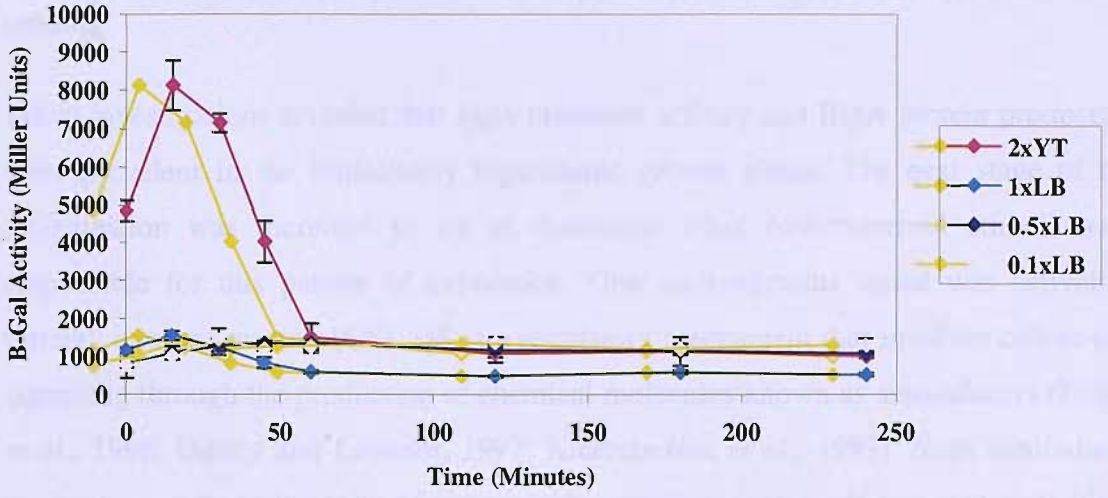


Figure 3.9 Transcription profiles of *bipA* promoter P3, as measured by β-galactosidase expression from the pBip116 reporter plasmid, under different media conditions. Each assay was carried out at least three times, with there being three test cultures per condition, per experiment. The graph shows the mean average values and error bars are plotted as standard deviation. For points 1-4, 2xYT compared to 1xLB, $p<0.05$, t-test. All others p=ns, t-test.

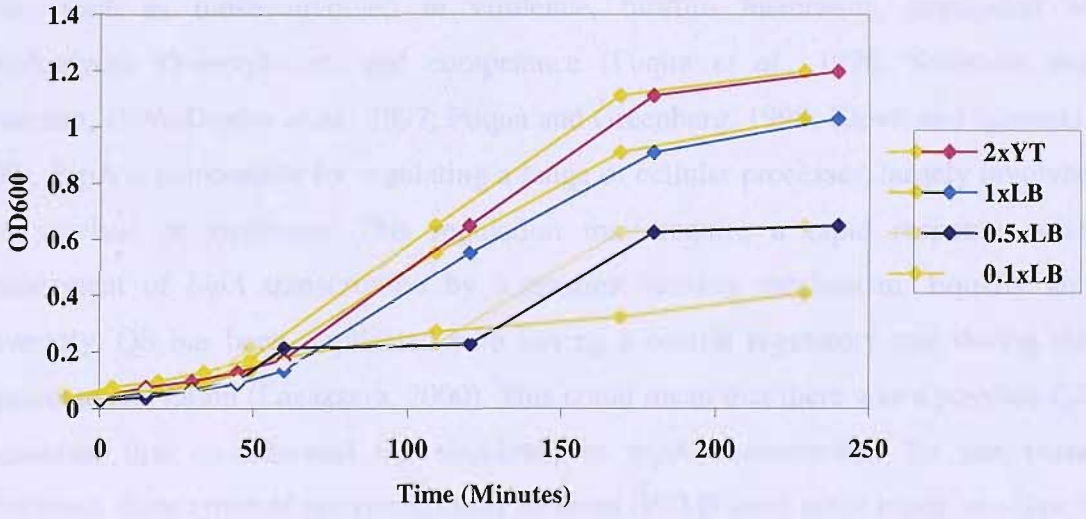


Figure 3.10 The growth rates of MC1000 (pBip116) in the four types of media during the experiment shown in Figure 3.11.

3.5 Expression of the *bipA* gene does not appear to be influenced by quorum sensing

Initial investigations revealed that *bipA* promoter activity and BipA protein production were prevalent in the initial/early logarithmic growth phase. The next stage of the investigation was therefore to try to determine what environmental stimuli were responsible for this pattern of expression. One such stimulus tested was activation through quorum sensing (QS). QS is a regulatory mechanism that involves cell-to-cell signalling through the production of chemical molecules known as autoinducers (Fuqua *et al.,* 1996; Dunny and Leonard, 1997; Kleerebezem *et al.,* 1997). Such autoinducer molecules can be in the form of amino acids, peptides, fatty acids and acyl derivatives of homoserine lactone, as is the case with the LuxS QS system. These autoinducer molecules are produced by Gram negative and Gram positive bacterial cells alike, and are often made in response to a variety of environmental stimuli. Furthermore, other bacterial cells of either the same or different species can interpret molecules of certain QS of one bacterium. This would allow an individual bacterium to 'sense' members of its own population, as well as other species of bacteria, in the local vicinity. Responses to autoinducer molecules often lead to the regulation of expression of a diverse range of genes, such as those involved in virulence, biofilm maturation, regulation of peptidoglycan O-acetylation, and competence (Fuqua *et al.,* 1996; Solomon and Grossman, 1996; Davies *et al.,* 1997; Fuqua and Greenberg, 1998; Kievit and Iglewski, 2000). BipA is responsible for regulating a range of cellular processes, largely involved with survival or virulence. This regulation may require a rapid response, with enhancement of *bipA* transcription by a quorum sensing mechanism. Equally and conversely, QS has been implicated with having a central regulatory role during the response to starvation (Lazazzera, 2000). This could mean that there was a possible QS mechanism that co-ordinated the shutdown of *bipA* transcription. To test these hypotheses, three types of pre-conditioned medium (PCM) were made using one-litre 1 x LB cultures of MC1000 grown to early- (0.3 OD_{600}) mid-exponential (0.6 OD_{600}) and stationary phase (1.0 OD_{600}) (Figure 3.11). Preparation of PCM was as outlined in Chapter 2, section 2.1.4. Following initial trials with 50:50 ratios of spent medium to fresh 2 x LB, the ratios were altered to try to ensure that over- dilution of any quorum

sensing factors in the final PCM did not occur. The negative results of these experiments suggested that QS did not feature in the regulation of *bipA* transcription.

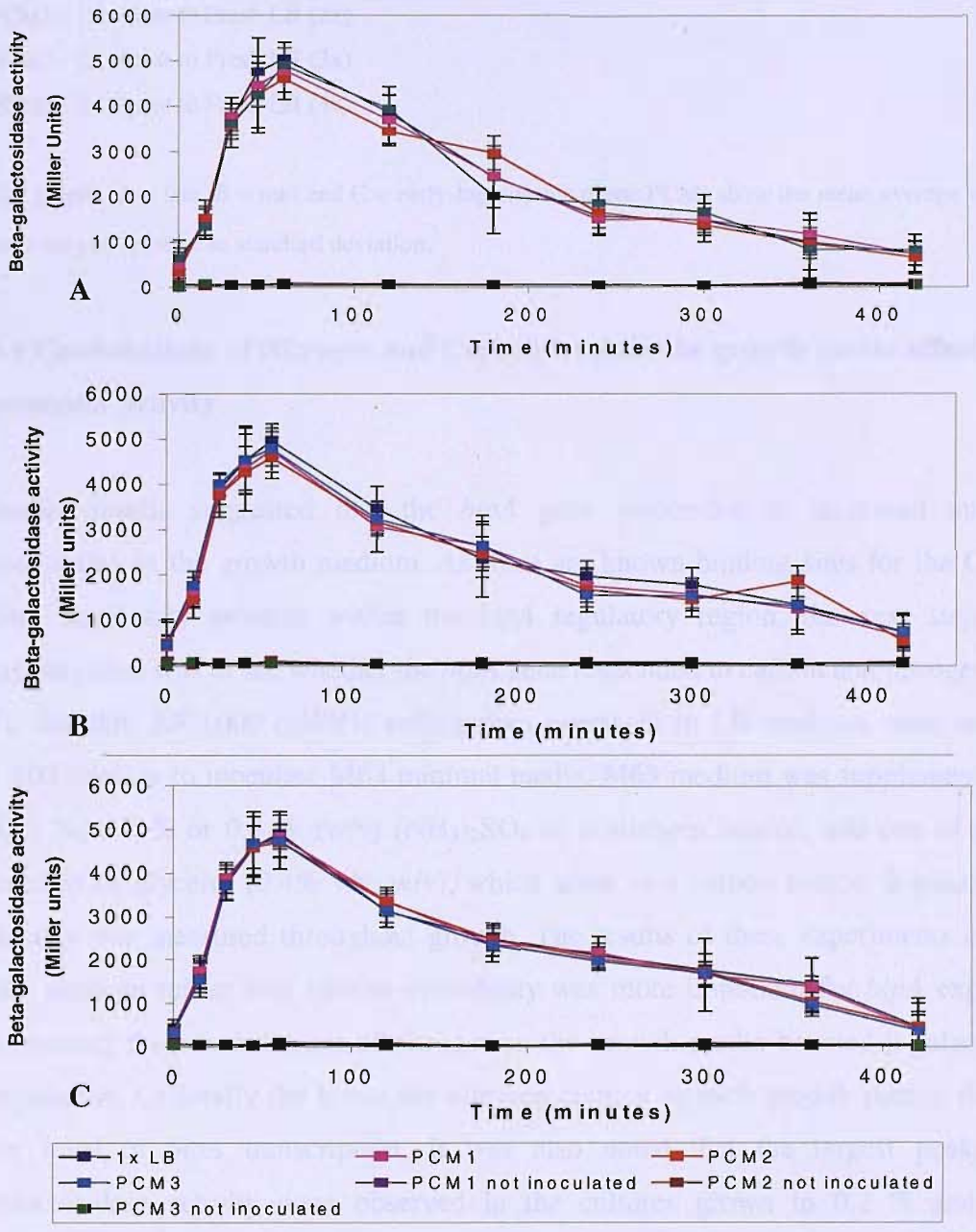


Figure 3.11

Quorum sensing has no effect on β-galactosidase expression from MC1000 (pWP1) cells tested in late, mid, early-logarithmic phase cell-derived preconditioned media. Preconditioned media (PCM) was prepared as described in Materials and Methods. Overnight cultures of MC1000 were diluted 1:100 into the pre-conditioned media or into normal LB medium (the same batch of LB that was used to create the PCM) and then grown, with agitation, at 37 °C. Samples were taken at the following times: 0, 15, 30, 60,

120, 180, 240, 300, 360 and 420 minutes. Each PCM was tested at least three times, with each test carried out in triplicate.

PCM1= 1:1 Spent to Fresh LB (2x)
PCM2= 2:1 Spent to Fresh LB (3x)
PCM3= 3:1 Spent to Fresh LB (4x)

The graphs (A = late, B = mid and C = early-logarithmic phase PCM) show the mean average values and error bars are plotted as standard deviation.

3.6 Combinations of Nitrogen and Carbon levels in the growth media affect *bipA* promoter activity

Earlier results suggested that the *bipA* gene responded to increased nutritional availability in the growth medium. As there are known binding sites for the CRP and NtrC regulatory proteins within the *bipA* regulatory region, the next step of the investigation was to see whether the *bipA* gene responded to carbon and nitrogen levels. To test this, MC1000 (pWP1) cells grown overnight in LB medium, were used in a 1:100 dilution to inoculate M63 minimal media. M63 medium was supplemented with 0.05 %, 0.2 % or 0.4 % (w/v) $(NH_4)_2SO_4$ as a nitrogen source, and one of glucose, fructose or glycerol (0.4% v/v, w/v), which acted as a carbon source. β-galactosidase activity was measured throughout growth. The results of these experiments indicated that nitrogen rather than carbon availability was more important for *bipA* expression. Increasing the concentration of nitrogen in the growth media boosted β-galactosidase expression. Generally the lower the nitrogen content in each growth media, the lower the level of *bipA* transcription. It was also noted that the largest peaks of β-galactosidase activity were observed in the cultures grown in 0.2 % and 0.4 % $(NH_4)_2SO_4$ (excess) with additional glucose. Interestingly, levels of β-galactosidase expression from cells grown under these two conditions were also significantly different from the levels of β-galactosidase expression recorded frpm 0.05 % $(NH_4)_2SO_4$ (+ glucose) grown cells. This was not the case for cultures grown under conditions of increasing nitrogen levels and supplemented with either fructose or

glycerol. Despite these observations and possible trends, generally, there were no statistically significant differences in expression between the culture types (Figure 3.12)

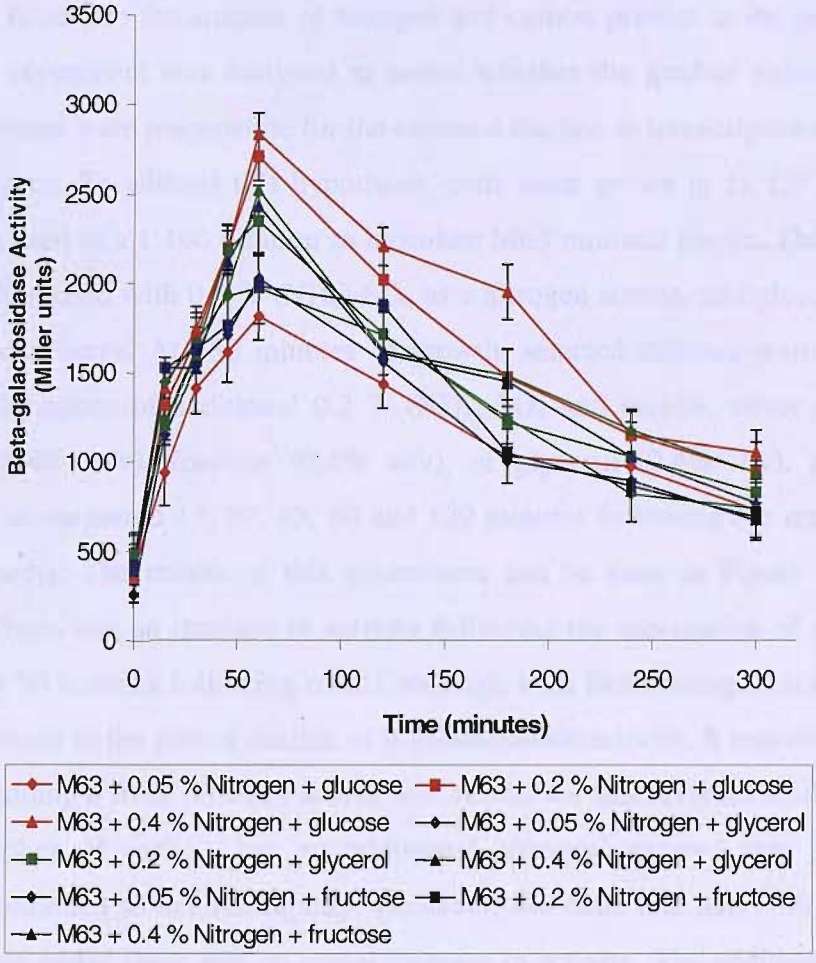


Figure 3.12 Comparison of *bipA* transcription in MC1000 (pWP1), grown in conditions of varying carbon and nitrogen levels. MC1000 (pWP1) cells were grown overnight in LB and subcultured into M63 medium by 1:100 dilution. Each assay was carried out at least three times, with there being three test cultures per condition, per experiment. The graph shows the mean average values and error bars are plotted as standard deviation. For points 4-6, M63 +0.05% nitrogen (+ glucose) compared to M63 + 0.2% and 0.4% (+ glucose), $p<0.05$, t-test. At all points, differences between 0.05%, 0.2% and 0.4% nitrogen (+ fructose), p=ns, t-test. At all points, differences between 0.05%, 0.2% and 0.4% nitrogen (+ glycerol), p=ns, t-test. At points 5 to 7, M63 + 0.4% (+ glucose) compared to M63 + 0.4% (+ glycerol/fructose), $p<0.05$, t-test. All other comparisons p=ns, t-test.

3.7 Recharging growth media with nitrogen and glucose can affect the decline in transcriptional activity from the *bipA* gene.

The previous experiment discovered that the magnitude of transcription of the *bipA* gene was related to the amount of nitrogen and carbon present in the growth medium. The next experiment was designed to assess whether the gradual exhaustion of these energy sources were responsible for the eventual decline in transcriptional activity from the *bipA* gene. To address this hypothesis, cells were grown in 1x LB overnight, and were then used in a 1:100 dilution to inoculate M63 minimal media. The M63 medium was supplemented with 0.2 % $(NH_4)_2SO_4$ as a nitrogen source, and glucose (0.4% w/v) as a carbon source. At 180 minutes of growth, selected cultures were supplemented with combinations of additional 0.2 % $(NH_4)_2SO_4$ and carbon, either in the form of glucose (0.4% w/v), fructose (0.4% w/v), or glycerol (0.4% v/v). β-galactosidase activity was measured 15, 30, 45, 60 and 120 minutes following the recharging of the growth media. The results of this experiment can be seen in Figure 3.13. With all cultures, there was an increase in activity following the inoculation of cells into fresh media. At 30 minutes following media recharge with fresh nitrogen and carbon, there was a decrease in the rate of decline of β-galactosidase activity. It was observed that the effect of adding a fresh nitrogen source was crucial for this reversal. Cells that received fresh supplies of carbon, but no additional nitrogen, showed that β-galactosidase activity continued to decline rapidly. However, the cells that had both fresh nitrogen and glucose added there was an actual increase in activity. The addition of glycerol or fructose with nitrogen did provoke a response, however the secondary peaks from cells in these cultures were less dramatic than the effect of glucose. Importantly, despite addition of fresh sources of nitrogen and carbon, any secondary peaks were less than the first peaks of activity. Furthermore, fresh nutrient supply was not enough to induce *bipA* transcription in a culture that was approaching stationary phase. This implied that there were other factors that were important for maintaining/slowing down *bipA* transcription, other than the impact of nitrogen and carbon exhaustion.

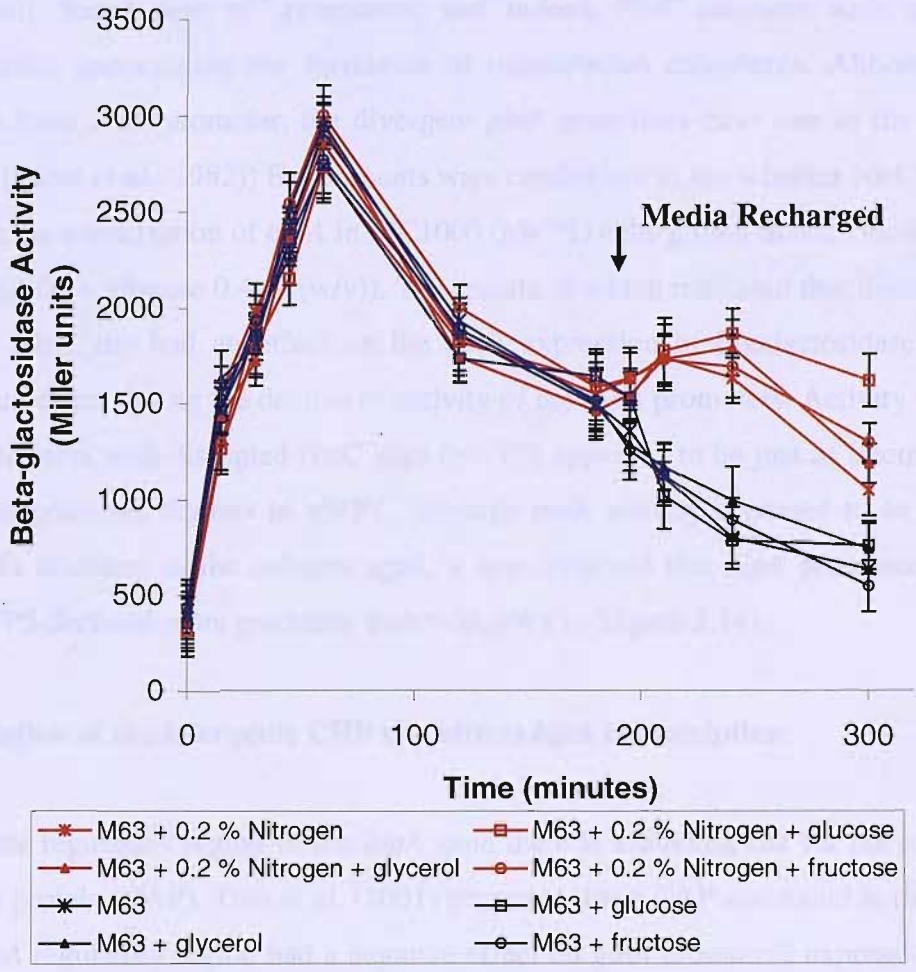


Figure 3.13 The effect of recharging M63 growth media on the transcriptional activity of the *bipA* gene. MC1000 (pWP1) cells were grown to stationary phase (OD_{600} 1.0) in 1x LB and used to inoculate fresh M63 minimal medium + glucose (0.4% w/v), by 1:100 dilution. Following 180 minutes of growth, selected cultures were then supplemented with additional 0.2 % $(NH_4)_2SO_4$, and either glucose, fructose (0.4% w/v) or glycerol (0.4% v/v). Further β-galactosidase readings were then taken at 15, 30, 45, 60 and 120 minutes. Each assay was carried out at least three times, with there being three test cultures per condition, per experiment.

For points 9 to 11, cultures recharged with nitrogen (+/- carbon source) compared to no recharge or recharge with carbon alone, $p<0.05$, t-test. All other comparisons, p = ns, t-test.

The graph shows the mean average values and error bars are plotted as standard deviation.

3.8 Mutation of the first NtrC-binding site affects *bipA* activity

The NtrC molecule is an enhancer-binding transcription activator, which is activated through phosphorylation under conditions when nitrogen is limited. NtrC enhancer sites are usually found near σ^{54} promoters, and indeed, NtrC interacts with σ^{54} RNA polymerases, encouraging the formation of transcription complexes. Although *bipA* does not have a σ^{54} promoter, the divergent *glnA* gene does have one in the form of *glnAp2*. (Pahel *et al.,* 1982)) Experiments were carried out to see whether NtrC had any effect on the transcription of *bipA* in MC1000 (pWP1) cells grown in M63 medium (0.2 % $(NH_4)_2SO_4$ + glucose 0.4 % (w/v)). The results of which indicated that disruption of the first NtrC site had an effect on the peak expression of β-galactosidase, but no significant effect during the decline of activity of the *bipA* promoters. Activity from the *bipA* promoters with disrupted NtrC sites (pWP5) appeared to be just as intense as the wild type promoter regions in pWP1, although peak activity appeared to be reached sooner. In addition, as the cultures aged, it was observed that *bipA* promoter activity with pWP5 declined more gradually than with pWP1. (Figure 3.14).

3.9 Mutation of the intergenic CRP site affects *bipA* transcription

Within the regulatory region of the *bipA* gene there is a binding site for the catabolite activator protein (CAP). Tian *et al.* (2001) proposed that a CAP site found in the shared *glnA-bipA* regulatory region had a negative effect on *glnA* promoter2 expression. With this CAP-binding site lying between the three promoters and the initiation site for the *bipA* gene, it was of interest to test whether it had any effect on transcription. To this end *bipA* promoter activity was analysed in MC1000 (pWP2), a construct made by S. Payot. pWP2 contains the intergenic region between *glnA* and *bipA* but has point-mutations in the CAP-binding site. Cells were grown to stationary phase (OD_{600} 1.0) in 1x LB and used to inoculate fresh M63 minimal medium + glucose (0.4% w/v) or glycerol (0.4 % v/v), in a 1:100 dilution. Interestingly, although the pWP2 cells expressed a peak activity of equal intensity and at the same time as the pWP1 cells, the rate of decline of activity was slightly, yet significantly greater. Furthermore, the type of carbon source had no bearing on the rate of decline, for pWP2 bearing cells grown in the presence of either glycerol or glucose displayed similar patterns (Figure 3.15).

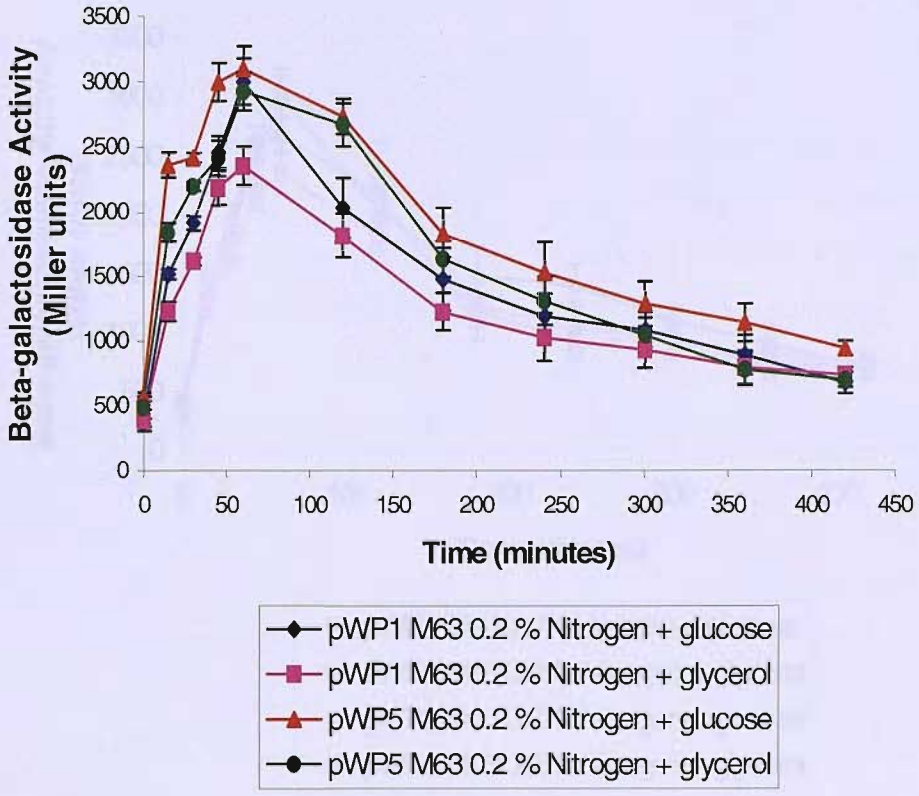


Figure 3.14 MC1000 (pWP1) transcription was compared to that of MC1000 (pWP5). The pWP5 plasmid was exactly the same as pWP1, except that there was a mutation in the NtrC-binding site, closest to the *bipA* gene. Both strains were grown to stationary phase (OD_{600} 1.0) in 1x LB and used to inoculate fresh M63 medium (0.2 % $(NH4)_2SO4$ + 0.4 % glucose (w/v) or 0.4 % glycerol (v/v)), by 1:100 dilution. The assay was carried out at least three times, with there being three test cultures per condition, per experiment.

The graph shows the mean average values and error bars are plotted as standard deviation.
Comparing pWP5 and pWP1 activity from glucose containing cultures: $p<0.05$ for points 2, 3, 4 and 6. All other points p=ns, t-test. Comparing pWP5 and pWP1 activity from glycerol containing cultures: $p<0.05$ for points 2, 3, 5, 6 and 7. All other points p=ns, t-test.

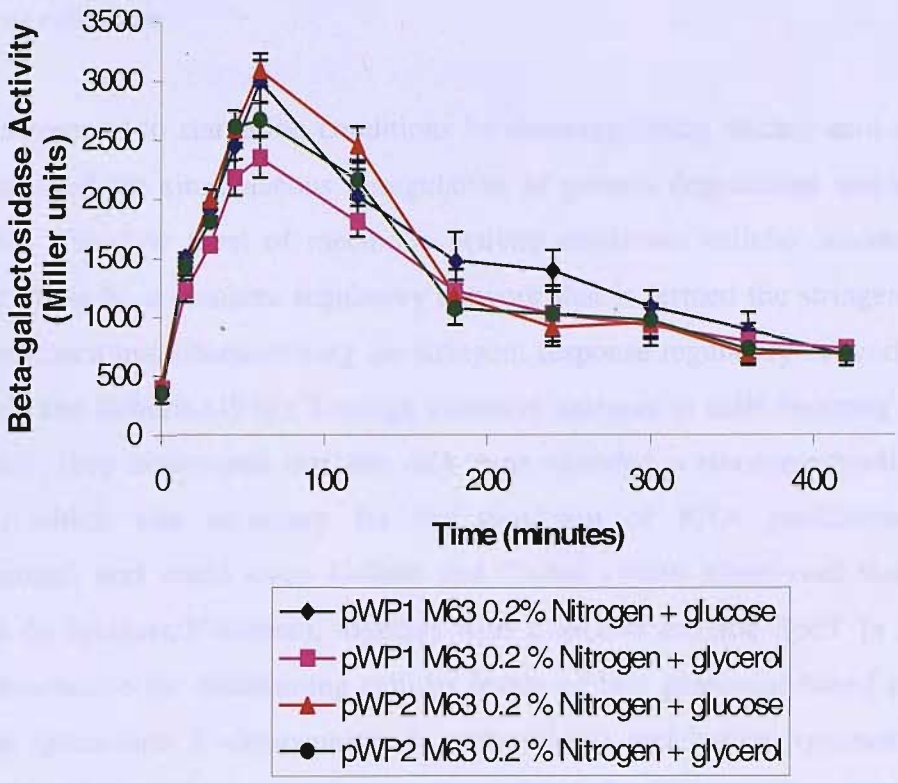


Figure 3.15 Comparison of levels of *bipA* transcription in cells bearing a mutated (pWP2) or wild-type (pWP1) CRP site in the *glnA-bipA* intergenic region. Both strains were grown to stationary phase (OD_{600} 1.0) in 1x LB and used to inoculate fresh M63 medium (0.2 % $(NH4)_2SO4$ + 0.4 % glucose (w/v) or 0.4 % glycerol (v/v)), by 1:100 dilution. The assay was carried out at least three times, with there being three test cultures per condition, per experiment.

The graph shows the mean average values and error bars are plotted as standard deviation.
Comparing pWP2 and pWP1 activity from glucose containing cultures: For points 6, 7, 8 and 9 $p<0.05$, t-test. All other points p=ns, t-test. Comparing pWP2 and pWP1 activity from glycerol containing cultures: All points p=ns, t-test.

3.10 Activity from the *bipA* promoters is also modulated by a component of the stringent response

Bacteria respond to starvation conditions by downregulating nucleic acid and protein synthesis, and the simultaneous upregulation of protein degradation and amino acid synthesis. This low level of metabolic activity conserves cellular resources, and is brought about by a complex regulatory network that is termed the stringent response. Initial research into characterising the stringent response regulatory network was done by Sands and Roberts (1952). Through mutation analyses in cells enduring amino acid starvation, they discovered that the *relA* gene encoded a ribosome-binding enzyme (RelA), which was necessary for the shutdown of RNA production. Another breakthrough was made when Gallant and Cashel (1969) discovered that the RelA enzyme (a synthase/3'-kinase), together with a second enzyme SpoT (a hydrolase), were responsible for maintaining cellular levels of two guanosine-based nucleotides, pppGpp (guanosine 3'-diphosphate, 5'-triphosphate) and ppGpp (guanosine 3', 5'-bispyrophosphate). The reaction catalysed by RelA/SpoT involved a phosphorylation step that converted intracellular GDP and GTP molecules to ppGpp and pppGpp respectively. Gallant and Cashel also reported that during starvation conditions, cells required a rapid accumulation of ppGpp and pppGpp molecules, prior to initiating metabolic shutdown (Gallant and Cashel, 1969, Cashel *et al.,* 1996). It was Haseltine and Bock (1973) who first proposed that increased ppGpp synthesis was linked to the cellular ratio of aminoacetylated tRNA to free tRNA. They predicted that during starvation conditions, there would be a bias towards the number of free tRNA molecules in the cell. This would increase the chances of free tRNA molecules being introduced to the A-sites of 70S ribosomes, which would stall protein synthesis. As a result of stalling an idling reaction within the ribosome would occur, stimulating the activity of ribosome-bound RelA protein. This model of activity has been backed up with more recent data, which also suggests that ppGpp has a similar role in a number of genetically diverse bacterial species (Avarbock *et al.,* 2000). The presence of high-levels of intracellular ppGpp leads to a rapid decline in the rate of transcription of rRNA and tRNA molecules, and as a consequence the growth rate of the cell. Previous results indicated that the *bipA* promoter activity appeared to be restricted to early

logarithmic phase and responded to growth-favourable conditions. Thus it was of interest to see if induction of the RelA protein, and shutdown of the cell metabolism, had any negative influence on *bipA* activity. As Figure 3.16 shows, induction of RelA via a plasmid-borne, IPTG-inducible copy of the *relA* gene, decreased the levels of transcription from the *bipA* promoters more rapidly than that of the non-induced cultures. In contrast, *bipA* activity was not disrupted by the induction of a mutated *relA* gene, which gave rise to an inactivated form of RelA. Furthermore, the effect on growth following induction can be seen in Figure 3.17, which shows that upon induction, growth in the *relA*-induced cultures is halted. This is the opposite of what is observed in the mutant *relA* and non-induced *relA* cultures, which are able to growth to stationary phase normally.

3.11 The global regulator Fis does not control BipA production

The global regulatory protein Fis is associated with activating genes that encode products needed during rapid growth, especially the rRNA promoters during nutritional upshift (Nilsson *et al.*, 1990; Nilsson *et al.*, 1992; Hirvonen *et al.*, 2001; Mushhelishvili and Travers, 2003) The protein binds to a degenerate consensus sequence (G/TNNYRNNA/TNNYRNNC/A) within the promoter region of the target gene, and enhances gene expression by aiding the interaction between the RNA polymerase and the promoter. The recently determined expression pattern of BipA suggested that the gene is most active during the early stages of logarithmic growth, at the same time the Fis protein is at its most abundant in the cell (Ball *et al.*, 1992; Ninnemann *et al.*, 1992). Interestingly, computer predictions suggested that there were eight weak Fis binding sites within the *bipA* regulatory region. (Delila program used, website: http://www.lecb.ncifcrf.gov/~toms/delilaserver.html) There was therefore the distinct possibility that as Fis was also highly prevalent at this stage of growth and is subject to stringent regulation itself, it may play a part in *bipA* regulation. To check whether this was the case, protein samples were taken from wild-type, *bipA*$^-$ and *fis*$^-$ cells grown in 1x LB medium to early logarithmic phase (OD_{600} = 0.25). These protein samples were separated by PAGE, then transferred onto nitrocellulose and probed with anti-BipA antibody (Figure 3.18).

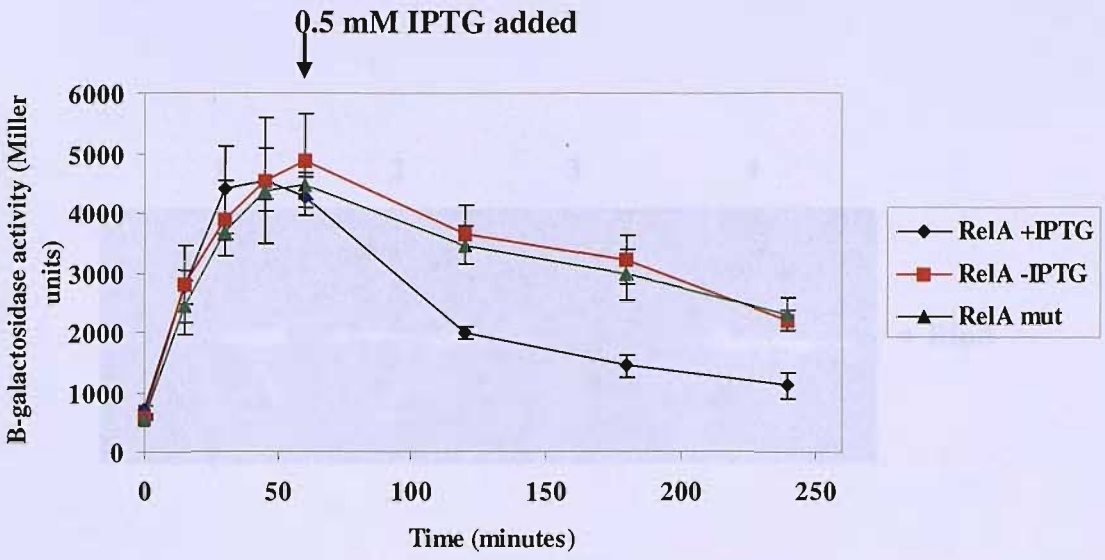


Figure 3.16 Effect of the *relA* gene on *bipA* transcription. Upon induction of a cloned *relA* gene via IPTG addition, *bipA* promoter activity drops dramatically, as opposed to what is seen with the non-induced and the inactive *relA* cultures. Each condition tested was done in triplicate in each assay executed, with there being at least three assays completed. *Escherichia coli* MC1000 (pWP1) were further transformed with one of two plasmids, pKT40 and pKT31 (K. Tedin). The plasmid pKT31 has an IPTG-inducible copy of *relA*, while pKT40 has an IPTG-inducible copy of a mutated and therefore inactive copy of *relA*. Cells were then assayed for β-galactosidase activity as previously, except that at t=60 minutes, IPTG (0.2 mM, final concentration) was added to the RelA and RelA mutant cultures.

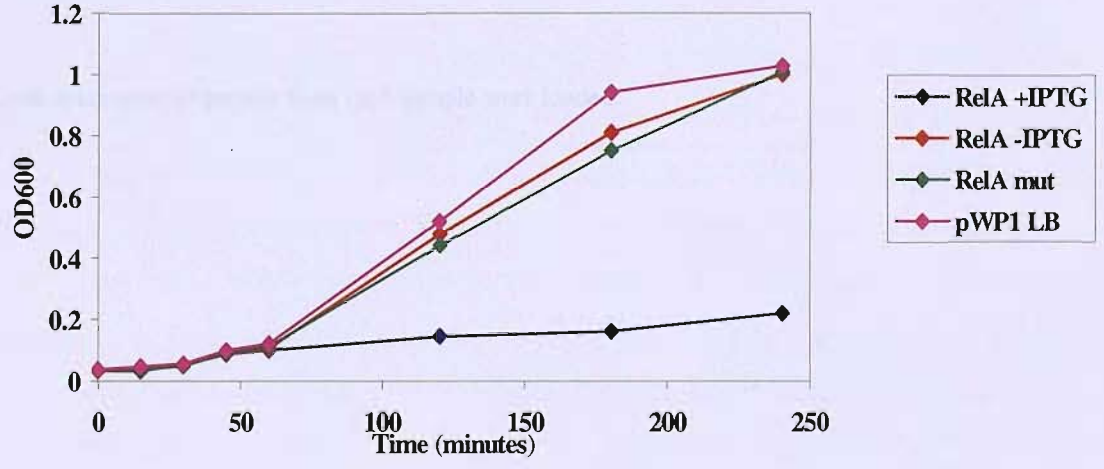


Figure 3.17 Effect of induction of *relA* on cell growth. Cultures were induced at t = 60 minutes, where appropriate, with a final concentration of 0.5 mM IPTG. Induction of the wild-type *relA* gene immediately prevented further culture growth. The K-12 control was a culture of MC1000 (pWP1) that had the IPTG added.

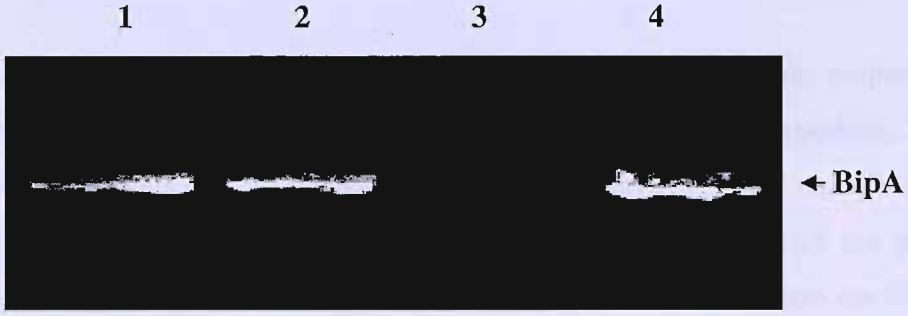


Figure 3.18 The presence or absence of the global regulator, Fis, does not appear to influence production of BipA protein. The results of an immunoblot of samples taken from cells grown in LB medium to an A_{600} 0.25. Cells were then harvested by centrifugation and whole cell lysate extracts prepared in 2x final sample buffer. Protein samples were separated by SDS-PAGE on a 13% polyacrylamide gel, then transferred onto nitrocellulose and probed with anti-BipA antibody.

Lane 1: CSH50 wild-type

Lane 2: CSH50 *fis::cat*

Lane 3: MG1655 Δ*bipA*

Lane 4: MG1655 Δ*bipA* (pAJG4) (see Table 2.2,Chapter 2)

Equivalent amounts of protein from each sample were loaded.

3.12 Summary and Discussion

In bacteria, gene regulatory proteins are generally controlled in response to environmental changes in order to generate a suitable response (Magasanik, 2000). Differences in the levels of their mRNAs following such environmental changes can be conveniently monitored via transcriptional fusions of the promoter(s) of the gene in question to a suitable reporter gene, e.g. *lacZ*. Although the measurements are indirect, they generally accurately reflect the activity of the native gene at the mRNA level. Previous studies have provided evidence that BipA is a regulatory protein of a large number of diverse cellular mechanisms (Grant *et al.,* 2003). Yet, prior to this study, very little was known about the regulation of the *bipA* gene itself, and in particular which factor(s) trigger activity from the *bipA* promoters, other than the initial discovery of BipA in *Salmonella* following BPI exposure (Qi *et al.,* 1995). The results in this chapter indicate that the *bipA* gene has, in fact, a distinct expression profile, one that is influenced by the nutritional strength of the growth media and is limited to early logarithmic phase of growth. Furthermore, *bipA* gene expression appears to be influenced by a number of regulatory components.

The experiments carried out so far in MC1000 *E. coli* K12 cells have yielded some interesting results, among which has been the discovery that the activity of the *bipA* promoters follows a consistent pattern. In each medium tested, be it nutritionally rich or poor, the pWP1 plasmid bearing all three *bipA* promoters registered a maximum peak of transcription just before early-logarithmic growth phase. Following this peak, *bipA* transcription declined as the cultures progressed through logarithmic growth. Promoter activity then appeared to reach a basal level by the time the cultures reached stationary phase. Interestingly the level of activity from the promoters is apparently influenced by the nutritional content of the growth media. In the media that was tested, cells grown in the nutritionally poor 0.5 x and 0.1 x LB displayed peak levels of promoter activity that were substantially inferior to equivalent cells grown in the relatively richer 1 x LB and 2 x YT media.

A similar expression pattern was witnessed with Western blots of the BipA protein from whole-cell samples taken during growth in different media. Indeed as the nutritional strength of the media improved, so did the level and duration of expression of BipA protein. These findings imply that the *bipA* gene responds to nutrient availability and is engaged under conditions suitable for rapid cell growth. As *bipA* transcription occurs immediately after nutrient upshift, it may be the case that BipA is involved in the early stages of a regulatory cascade. This could mean that there is a strong possibility that the primary target(s) for BipA, may also be prominent in early logarithmic growth.

3.12.1 The *bipA* promoters show individual characteristics when isolated

The transcription initiation regions of bacterial genes and operons can often contain multiple promoters that allow for multiple signals to affect expression (Opel *et al.*, 2001; Barnard *et al.*, 2004). In many cases the coupling of multiple promoters to a single gene or operon allows for increased flexibility in the adjustment of gene expression to particular growth conditions. Interestingly such multiple promoter arrangements can often be found in regulatory regions of genes with pleiotropic functions. For example, the regulation of expression from the *crp* gene. Out of the two tandem promoters that drive expression of the *crp* gene, only one requires DNA negative supercoiling and is subject to stringent control (Gonzalez-Gil *et al.*, 1998; Johansson *et al.*, 2000). Another example is the *cydAB* operon, which is involved in oxygen regulation, and has five tandem promoters. However only four of these promoters are known to respond to changes in oxygen availability (Govantes *et al.*, 2000).

Further investigations revealed that when isolated, the *bipA* promoters each have individual activity profiles, which are also influenced by the nutritional content of the growth media. In general, transcription from the first and third promoters is influenced by the nutritional content of the growth medium, while activity from the second promoter, despite changes to the growth media, appears to be less responsive and low-

level. This is of interest, as having three promoters of varying strength and recognising different stimuli, could provide the cell with a very tight but very responsive control over *bipA* expression. This very tight, highly responsive control could allow the cell to prioritise BipA production (and thus the engagement of the rest of the regulatory cascade) during times of stress or increased nutrient levels, in order to conserve cellular resources. Promoter interference can occur with tandem promoters when a high initiation frequency of an upstream promoter 'occludes' a downstream promoter (Adhya and Gottesman, 1982; Zhang and Bremer, 1996). It is conceivable that these various interactions between closely spaced promoters, especially when coupled to selective effects of transcription factors, have a potential for assembling regulatory circuits. However, quite how each of the three promoters interact with each other under different growth conditions, and whether variation in their interactions leads to different regulatory cascades has yet to be determined.

3.12.2 BipA is not influenced by Quorum-sensing

Prior to these experiments quorum sensing was a candidate for a form of stimulus for the *bipA* gene. This was because certain components known to be regulated by BipA, e.g. the LEE in EPEC, have also been shown to be regulated by quorum sensing (Sperandio *et al.*, 2001). Experiments carried out to determine whether the *bipA* gene in *E. coli* K12 cells responded to conditions that mimicked that of quorum sensing, indicated that the phenomenon did not influence *bipA* expression. Preconditioned medium derived from cultures harvested from early-, mid-logarithmic and stationary phase *E. coli* K12 cultures, failed to affect the levels of expression from constructs bearing the entire *bipA* regulatory region.

3.12.3 The influence of nitrogen and carbon availability on *bipA* promoter activity

Following the discovery that transcription of the *bipA* gene responded to nutritional upshift, further experiments were carried out to determine the effect of carbon source

and nitrogen levels. The results from initial experiments indicated that combinations of nitrogen and carbon had different effects on the peak transcriptional activity from the *bipA* gene. The results of these experiments also indicated that nitrogen rather than carbon availability was more important for *bipA* expression. Increasing the concentration of nitrogen in the growth media boosted β-galactosidase expression. The addition of a carbon source, particularly glucose did have a positive effect on transcription when added with high levels of nitrogen, although the statistical significance of the type of carbon source did vary.

This transcriptional behaviour may be due to the activities of the divergent gene, *glnA*. Interestingly, it was reported that the nitrogen and glucose levels in the growth medium influences the divergent *glnALG* operon promoters through the CRP and NtrC-binding sites (Tian *et al.*, 2001; Atkinson *et al.*, 2002). The *glnALG* operon encodes the most important enzyme of nitrogen assimilation, glutamine synthetase (Reitzer and Magasanik, 1986). Under nitrogen-deficient conditions, binding of NtrC-P to the two binding sites within the *bipA-glnA* intergenic regulatory region enhances transcription from the strongest of the *glnALG* promoters, *glnA*p2. This group simultaneously reported that activated CRP duly represses *glnA*p2 activity but allows very small amounts of *glnALG* expression by activating the very weak *glnA*p1 promoter. Thus the *glnA* promoters would be maximally engaged during situations that would be inappropriate for BipA production. It is possible then that *bipA* and *glnA* expression is mutually exclusive, and that both genes respond to the nitrogen and glucose levels available to the cell. If this was so, by adding fresh nitrogen and a carbon source to a late-logarithmic phase culture it was hypothesised that these conditions should favour *bipA* expression rather than *glnA*.

MC1000 (pWP1) cells grown in M63 medium which was then recharged with fresh 0.2 % $(NH4)_2SO4$ and a carbon source (glucose, fructose or glycerol (0.4% w/v, v/v)) was carried out. Addition of fresh nitrogen was crucial for restimulation of *bipA* transcription or a decrease in the rate of decline, as only when nitrogen was added were large secondary peaks of β-galactosidase activity observed. In contrast, when any of the carbon sources were added, but with no addition nitrogen, *bipA* transcription continued

to plummet. Interestingly the largest peaks were detected with cells that had the growth medium recharged with nitrogen and glucose. These cells yielded greater peaks of β-galactosidase activity than cells that had been recharged with nitrogen together with either of the other two carbon sources. These results confirmed that the addition of a new nitrogen source was vital for *bipA* expression, and that glucose was required for maximum effect. However, *bipA* expression continued to decline, albeit at a slower rate. This suggested that there were other factors that also negatively influenced *bipA* expression.

The importance of the first NtrC and CRP sites found in the *bipA* gene regulatory region was then tested. β-galactosidase levels of LB grown MC1000 pWP5 (bearing a disrupted NtrC-binding site in the *bipA* regulatory region) indicated that the transcription factor had an effect on peak *bipA* transcriptional activity. MC1000 (pWP2) cells (disrupted CRP site) expressed similar activity patterns to the MC1000 (pWP1) cells, although peak activity appeared to be extended. Following peak activity, the pWP2 bearing cells showed a more rapid rate of decline in activity than pWP1. Furthermore, with the pWP2 cells, the type of carbon source had no bearing on the rate of decline, for pWP2 bearing cells grown in the presence of either glycerol or glucose displayed similar expression patterns.

The simplest model to account for the effect of nitrogen and glucose on *bipA* transcription is that the NtrC and CAP-binding sites are used to flip between *bipA* and *glnA* activity. Such a regulatory relationship between *glnA* and *bipA*, based on available nitrogen and glucose would make economical sense. When the cell is plunged into an environment rich in nitrogen and glucose, the intracellular levels of active CRP and NtrC-P would be very low. With no repressors, activation of the *bipA* gene would ensue and the some of the energy intensive, BipA-regulated cellular processes would be engaged (e.g., pathogenicity in EPEC). As intracellular levels of nitrogen and carbon become depleted, the cell would need to slow down or switch off its metabolism and begin to engage genes required for survival under starvation conditions. Thus, with a drop in intracellular nitrogen levels, activation of NtrC molecules would ensue. These NtrC-P molecules would be available to positively regulate the *glnA*p2 promoter,

leading to further NtrC production. In doing so NtrC-P-binding would interfere with and limit *bipA* promoter activity. This would benefit the cell, as it would be seeking to limit energy and resource expenditure. Atkinson *et al.* (2002) have shown recently that if levels of available nitrogen reach a certain threshold, then activity from the *glnA* gene is itself limited by additional NtrC-P molecules binding to low affinity 'governor' sites found at -90, -65 and -45 bp from the *glnA* start codon. Furthermore, nitrogen assimilation is an energy intensive process itself, which is a possible drain on intracellular carbon levels. As the availability of carbon decreases, cAMP levels would increase, which would bind to CRP. Activated CRP would bind to the CRP site in the *bipA-glnA* intergenic regulatory region, which has been shown to down-regulate *glnA* expression 21-fold (Tian *et al.,* 2001). Thus the activity from both *bipA* and *glnA* promoters would both be hindered by CRP binding. In a recent report, Gosset *et al.* (2004) list a number of genes that are subject to CRP-dependent glucose repression, with many of these genes categorised according to the process they are associated with. Interestingly, genes associated with central metabolism, carbohydrate metabolism, amino acid metabolism and others related to stress responses are all repressed. Evidently, when the cell perceives a drop in nutritional availability, CRP is used to shutdown energy intensive processes in a co-ordinated manner. Part of this shutdown process could be the suppression of *bipA* transcription, thus prohibiting promotion of energy intensive processes such as virulence or flagella production.

3.12.4 The influence of RelA on *bipA* promoter activity

Reporter gene constructs with the CRP and NtrC binding sites eliminated still showed that transcription declined towards stationary phase of growth. This suggested that growth phase-related decline in *bipA* transcription was most likely due to an influence other than CRP and NtrC. The answer was contained within the results of experiments involving the induced expression of the product of the *relA* gene. These experiments showed that *bipA* transcription also responded to stringent control. Many stringently controlled and stationary phase-associated genes are regulated by ppGpp. This is through the regulating and enhancing the activity of the stringent sigma factor, σ^s, (Hirsch and Elliott, 2002; Jishage *et al.,* 2002; Nystrom, 2003) It is clear from the β-

galactosidase assay results from this chapter that ppGpp affects the transcription of *bipA*. However, whether this is through a mechanism that prevents *bipA* transcription inititation, mediated by σ^s is, as of yet, unknown. It is possible, however, that the *bipA* gene is affected by the stringent response and the activities of ppGpp indirectly.

In *E. coli*, an interesting situation occurs with the transcriptional regulation of the seven rRNA operons. Each operon has two promoters, and activity from the stronger of the two promoters, *rrn*P1 promoter, is regulated primarily at transcription initiation (Gourse *et al.,* 1996), by a number of factors including Fis, H-NS (Nilsson *et al.,* 1990; Ross *et al.,* 1990; Afflerbach *et al.,* 1998). Fis prevents unnecessary transcription from *rrn* P1 during starvation (Rochman *et al.,* 2002), but will stimulate rRNA transcription during nutritional upshift by recruiting RNA polymerase to the *rrn* P1 promoter, meanwhile H-NS is known to inhibit this latter process (Aiyar *et al.,* 2002; Dame *et al.,* 2002). However, activity from *rrn*P1 is also influenced by the availability of ATP, or in some cases GTP, and is inhibited by the presence of a third nucleotide, guanosine 5'-diphosphate 3'-diphosphate (ppGpp) (Cashel and Gallant, 1969; Gaal *et al.,* 1999; Barker *et al.,* 2001). It has been demonstrated that during transcription, the *rnn* P1 promoter forms unusually unstable open complexes with RNA polymerase that are particularly sensitive to low concentrations of the two former NTPs. In the presence of high concentrations of ATP/GTP efficient transcription is ensured, as the NTPs stabilise the RNA polymerase-promoter interaction (Gaal *et al.* 1997). It is regarded that the availability of NTP or the synthesis of ppGpp reflect the nutritional state and translational activity of the cell, with both molecules involved in two feedback loops that co-ordinate ribosome production (Murray *et al.,* 2003). High NTP levels could act as an indicator for increased translational capacity and that regulation by NTP sensing may be responsible for the growth rate related nature of the *rrn*P1 promoter (Gaal *et al.* 1997; Schneider *et al.,* 2002; Murray *et al.,* 2003). Although the mechanism that ppGpp uses in controlling *rrn* P1 activity is unclear, it is believed that ppGpp destabilises RNA polymerase interactions with *rrn* P1 (Barker *et al.,* 2001), and that the molecule acts within a negative feedback loop to halt ribosome production during periods of starvation or stationary phase of growth (Murray *et al.,* 2003).

An alternative proposal put forward to how ppGpp leads to the shutdown of ribosome production is that the synthesis ppGpp actively drains the cellular GTP pool, thereby frustrating *rrn* P1 and other GTP-dependent processes. Interestingly, ppGpp can influence intracellular GTP levels by inactivating IMP dehydrogenase, which is the first enzyme in the GMP synthesis pathway (Freese *et al.,* 1979). Furthermore, the production of ppGpp itself, from the conversion of GTP would also be a further drain on the level of cellular GTP. A mutation in the *relA* gene within *B. subtilis* results in an inhibition of sporulation following prolonged cell starvation. Freese and colleagues (1979) had shown that any effect RelA had on sporulation was in fact indirect. Sporulation occurred not through the regulatory effects of ppGpp but because the cellular levels of GTP dropped following extensive production of ppGpp. Since that initial finding a regulatory protein that is activated by low levels of GTP has been discovered in *B. subtilis.* CodY is a highly conserved protein in gram-positive bacteria and positively regulates a number of genes that are induced following transition of the culture from exponential growth to stationary phase and sporylation. CodY acts a transcriptional repressor of these genes when it is bound to a GTP molecule. Thus under conditions where GTP levels drop, such as slow growth or during stationary phase, CodY is unable to bind GTP and the stationary genes are derepressed. A model for CodY regulation involves a drop in GTP levels following GTP consumption by translation, ppGpp synthesis and IMP dehydrogenase shutdown (Cashel *et al.,* 1996; Ratmayake-Lecamwasam *et al.,* 2001).

Could this NTP sensing regulatory mechanism encompass BipA activity also? It is known that the regulatory activity of BipA depends upon its GTPase ability, which is heightened when the protein is associated with ribosomes (R. Owens, PhD thesis). In addition, R. Owens also determined that BipA has an approximately 3-fold higher affinity for GTP than GDP. As these affinities are sufficiently similar, it is probable that GDP greatly inhibits GTP hydrolysis by BipA, when in the presence of ribosomes. It is possible therefore that as BipA and other cellular GTP consuming processes continue to hydrolyse the cellular pool of GTP molecules, the resulting cellular build-up of GDP could gradually begin to inhibit activity from BipA and other susceptible GTPases. As BipA is associated with ribosomes, a drastic decrease in levels of intracellular GTP,

would lead to a cessation of rRNA production and the need for BipA would no longer remain. A potential feedback mechanism could be in place that allows the *bipA* gene to respond to halted rRNA production or low BipA protein activity. In the light of the findings of this chapter, further work that would determine the accuracy of this proposed feedback model for BipA would be worthwhile.

CHAPTER 4

Searching for further regulatory targets of BipA

CHAPTER 4: Searching for further regulatory targets of BipA

4.1 Introduction

Bacteria such as *E. coli* can be found in a number of environments, some more supportive of growth than others, and can experience rapid changes in growth conditions. Nutritional availability is a constant dilemma for bacteria and affects growth and, in the case of pathogens, their ability to carry out virulence. In addition, bacteria have to respond to a host of different stresses that they can encounter such as pH, oxidative stress and temperature. Previous studies have shown that the BipA protein is involved in *E. coli* and *Salmonella* responses to a number of cellular stresses such as oxidative stress (G. Howell, PhD thesis, 2004), cold shock (Grant *et al.,* 2001; Pfennig and Flower, 2001), and potentially to sudden decreases in pH (Fowler, unpublished observations). Furthermore, the present study has discovered that BipA is also produced in response to nutritional upshift. Like BipA, Fis is also accumulated during early logarithmic phases of growth, when there are high levels of nutrient available (Ball *et al.,* 1992; Azam *et al.,* 1999). Not only is Fis heavily involved in the regulation of genes associated with growth or metabolism, but it is also linked to the regulation of stress responses, in particular oxidative stress (Weinstein-Fischer *et al.,* 2000). Part of the response to nutritional availability and to oxidative stress is based on changes in DNA topology through the Fis-mediated control of Topoisomerase I enzyme activity. Interestingly, in *E. coli*, responses to other stresses such as acid, heat and cold shock also involve DNA topology alterations and activity from Topoisomerase I.

Given the similarities of expression patterns between BipA and Fis production there may be also a high degree of overlap between BipA and Fis, in terms of the genes or processes they regulate. This chapter describes the work done to verify any co-regulation between Fis and BipA, and to determine how intimate the regulatory relationship is between the two regulators.

4.2 *Escherichia coli* response to acid attack

The ability of *Escherichia coli* to resist acid is an essential quality as this enables the organism to survive the high levels of inorganic acid (H^+) present in the stomach and a combination of inorganic and organic acids (volatile fatty acids) encountered while passing through the lower gastro-intestinal tract. The definition of an acid resistance response (AR) is the ability of bacterial cells to survive a challenge with an acid of pH 2.5 or less. Previous studies have revealed that there are at least three major inducible AR mechanisms (AR1-3) that are employed by commensal *Escherichia coli* (Castanie-Cornet *et al.,* 1999). In addition *Salmonella* and *E. coli* cells also possess a log phase acid tolerance response (ATR) system (termed acid habituation in *E. coli*) (Goodson and Rowbury, 1989; Foster and Hall, 1992; Foster, 1999).

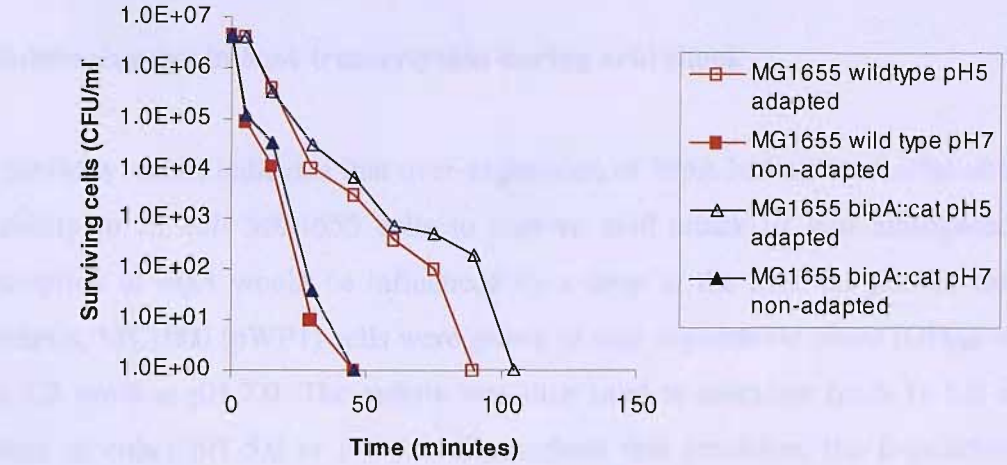
The first of these systems, AR1, is expressed during oxidative metabolism, regardless of growth pH, and will protect the cell at pH 2.5 in Luria-Bertani media. The regulatory mechanism of AR1 is poorly characterised, although it is believed that it is glucose repressed and that the stationary phase sigma factor, σ^s, is involved at some stage (Castanie-Cornet *et al.,* 1999). The other two systems are seen during fermentative metabolism, are activated by acid or by entry into the stationary phase of growth. The systems involve the activity of specific amino acid decarboxylases that rely upon exogenously supplied glutamate (AR2) (Lin *et al.,* 1995; Hersh *et al.,* 1996; De Biase *et al.,* 1999; Ma *et al.,* 2002; Ma *et al.,* 2003), or arginine (AR3) (Lin *et al.,* 1995; Castanie-Cornet *et al.,* 1999; Iyer *et al.,* 2003). Acid tolerance in *Salmonella* spp. or acid habituation in *E. coli* was first reported by Goodson and Rowbury in 1988. They discovered that when log-phase *E. coli* cells were pre-exposed to relatively mild (pH 5.0) acid conditions, for a short period of time, the bacteria had increased resistance to the effects of a second exposure to stronger, more lethal (pH 3.0-3.5) acid conditions. The results of subsequent analyses following these observations fail to agree as to the acidic strength required to induce total protection for *E. coli.* It was suggested that *E. coli* grown in enriched media at a pH ranging from anywhere between pH 4.0 to 6.0 conferred a small degree of protection whilst complete habituation was reported when cultures were pre-exposed to a pH of 5.0 to 5.5 (Rowbury, 1995). More recently

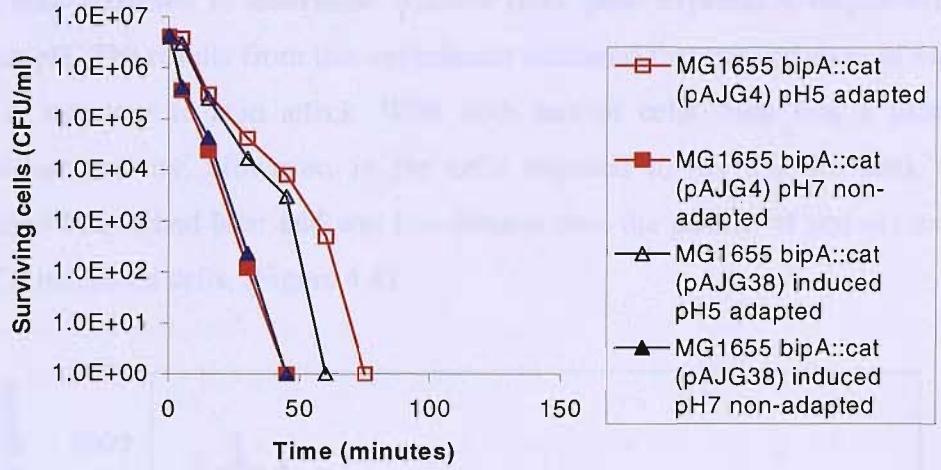
however, Paul and Hirshfield (2003) demonstrated that *E. coli* K-12 strains that were pre-exposed to pH 4.3 rich media were far more resistant to subsequent pH 3.0 exposure, than cells pre-exposed to pH 5.5 conditions. The regulation of acid habituation is not fully understood, although it is known that ATR in *E. coli* is different to that found in *Salmonella*. In *Salmonella* cells, the ATR is dependent on *rpoS* and *fur*, while in *E. coli* the response requires protein synthesis and is reliant upon the presence of the CysB protein, through an unknown mechanism. The unavailability of glucose, Fe^{3+}, Fe^{2+} and L-leucine does not hamper the *E. coli* ATR (Rowbury, 1997).

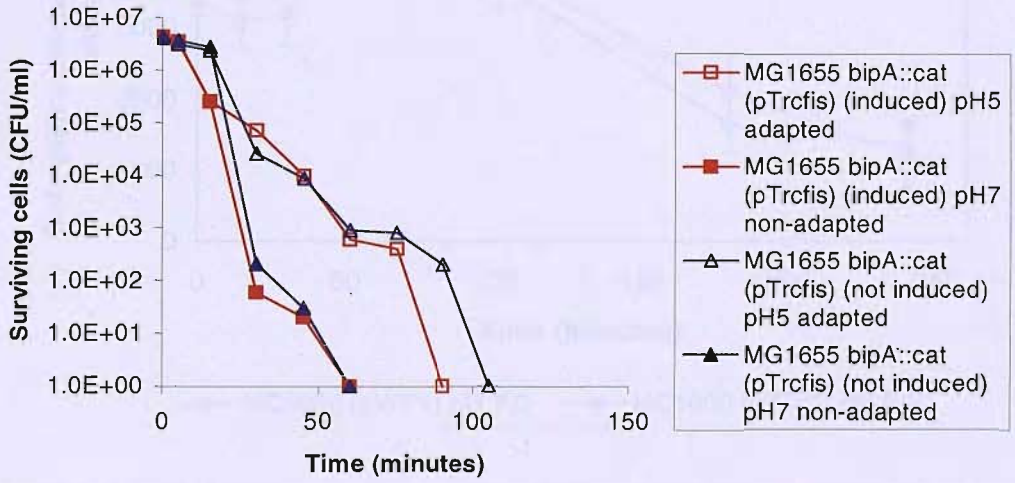
4.3 Overproduction of BipA appears to play a negative role in acid tolerance response (ATR), independent of Fis, in log-phase *E. coli* MG1655 cells

To determine if BipA influenced the ability of *E. coli* K-12 cells to engage the acid tolerance mechanism, cells were grown to mid-logarithmic phase of growth in LB medium at either pH 7.0 or pH 5.0. Samples were taken from the last two cultures at specified time points and were plated on LB agar plates to determine the number of viable cells. For the full method please refer to Chapter 2. It was found that a *bipA* null mutant of *E. coli* that had been adapted by incubation in LB medium at pH 5.0 displayed a marginally increased survival rate relative to its wild-type counterpart (Figure 4.1) (for identical conditions, between strains, p=ns, t-test).

Interestingly, adapted cells that were overexpressing BipA, through the induction of pAJG38 plasmid, appeared to be less able to survive at pH3, than the adapted wild-type cells of the first experiment ($p<0.05$, t-test). The results of the experiment suggest that overproduction of BipA protein may have an indirect negative effect on the regulation of acid tolerance in log-phase *E. coli* cells. Interestingly this could be in a Fis-independent manner (Figure 4.2). Adapted, non-induced cells of *E. coli* Δ*bipA::cat* (pTrc*fis*) survived as well as wild-type cells under pH3 conditions ($p<0.05$, t-test). Yet in the absence of BipA, Fis was able to confer some resistance to acid shock to adapted *E. coli* Δ*bipA::cat* (pTrc*fis*) cells in which Fis expression had been induced by the addition of 0.2 mM IPTG (Figure 4.3). p=ns, t-test (between wild-type and induced Δ*bipA::cat* (pTrc*fis*)).







Figures 4.1, 4.2 and 4.3 The effect of BipA and Fis proteins on the acid tolerance (habituation) response in mid logarithmic phase *E. coli* K-12 MG1655 strains. Bacteria were grown in 1x LB medium overnight before being used in the assay outlined in Chapter 2, section 2.6. 1 ml samples were taken, serially diluted and then plated, with the survivors counted the next day.

4.4 Subtle changes in *bipA* transcription during acid shock

The previous results indicated that over-expression of BipA had a detrimental effect on the ability of *E. coli* MG1655 cells to survive acid attack. It was anticipated that transcription of *bipA* would be influenced by a drop in the external pH. To test this hypothesis, MC1000 (pWP1) cells were grown to mid-logarithmic phase (OD_{600} = 0.6) in 1x LB broth at pH 7.0. The culture was then used to inoculate fresh 1x LB media adjusted to either pH 5.0 or pH 7.0. Throughout this procedure the β-galactosidase activity was measured to determine whether *bipA* gene expression responded to the change in pH. The results from this experiment indicated that transcription of *bipA* was altered in response to acid attack. With both sets of cells there was a peak of β-galactosidase activity. However, in the cells exposed to pH 5.0 the peak activity appeared to be reached later and was less intense than the pattern of activity exhibited by pH 7.0 incubated cells. (Figure 4.4).

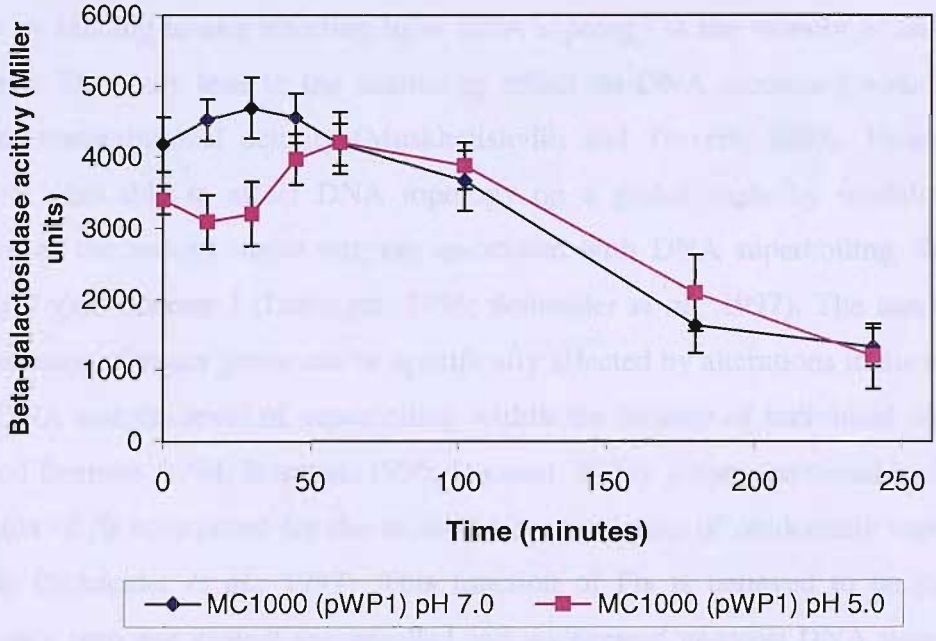


Figure 4.4 A graph illustrating the changes in *bipA* transcription patterns following inoculation of logarithmic phase MC1000 (pWP1) cells into fresh pH 5.0 or pH 7.0 1x LB media. For full details concerning the assay see Chapter 2. Aliquots of mid-logarithmic MC1000 (pWP1) cells were taken from a pH 7.0 LB culture and used to inoculate a second LB culture (kept at 37 °C), which was at either pH 5.0 or pH 7.0. Samples were taken for β-galactosidase analysis at the time points indicated. $p < 0.05$, t-test (for time points 1-4) p = sn, t-test (for time points 5-8).

4.5 BipA affects the Fis-mediated activation of Topoisomerase I

The topology of bacterial DNA is sensitive to certain environmental stimuli such as nutritional availability, osmolarity, oxidative stress and temperature (Goldstein and Drlica, 1984; Balke and Gralla, 1987; Friedman *et al.,* 1995). The level of negative DNA supercoiling is directly related to the energy levels (particularly the [ATP/ADP] ratio) of the cell. This is because the enzymatic activity of the negative supercoiling enzyme, DNA gyrase, is controlled by the intracellular [ATP/ADP] ratio (Higgins *et al.,* 1988; Hsieh *et al.,* 1991). Thus, during low levels of nutritional availability and therefore low energy levels, such as stationary phase growth, there is a decrease in negative supercoiling due to low activity of DNA gyrase. Conversely there is, in general, an increase in global negative DNA supercoiling following nutritional upshift or certain cellular stresses (Higgins *et al.,* 1988)

The Fis protein is classified as a histone-like protein, and is known to regulate genes directly by binding to and affecting local DNA topology in the vicinity of susceptible promoters. This may lead to the stabilising effect on DNA secondary structure that promotes transcriptional activity (Muskhelishvilli and Travers, 2003). However the protein is also able to affect DNA topology on a global scale by modulating the activities of the second major enzyme associated with DNA supercoiling, the DNA relaxing Topoisomerase I (Luttinger, 1995; Schneider *et al.,* 1997). The transcription and expression of many genes can be specifically affected by alterations in the topology of the DNA and the level of supercoiling within the locality of individual promoters (Free and Dorman, 1994, Dorman, 1996; Dorman, 2002). Upon nutritional upshift, the expression of *fis* is required for the increased accumulation of moderately supercoiled plasmids (Schneider *et al.,* 1997). This function of Fis is believed to be part of a homeostatic response against uncontrolled and widespread negative DNA supercoiling by DNA gyrase. This would allow for a diverse range of DNA topologies to occur on a local level, permitting fine-tuning of DNA-based cellular processes or for the correct timing for occupation by particular transcription factors at essential gene promoters (Schneider *et al.,* 1997). As BipA influences the levels of Fis protein, it was of some interest to see if BipA had a similar effect on global DNA topology in *E. coli* MG1655,

following nutrient upshift. A similar DNA topology experiment first outlined by Schneider *et al.* (1997) was conducted. Plasmid DNA was isolated from MG1655 (pTM918) cells and a *bipA*⁻ derivative at various time points during logarithmic growth in 2x YT medium. The degree of negative supercoiling of the plasmid DNA was then assessed to determine whether the presence of BipA had any discernable effect. The results indicated that the rate of negative supercoiling following nutrient upshift was higher in the MG1655 *bipA::cat* (pTM918) cells compared to the MG1655 wild type (pTM918) parental cells (Figure 4.5).

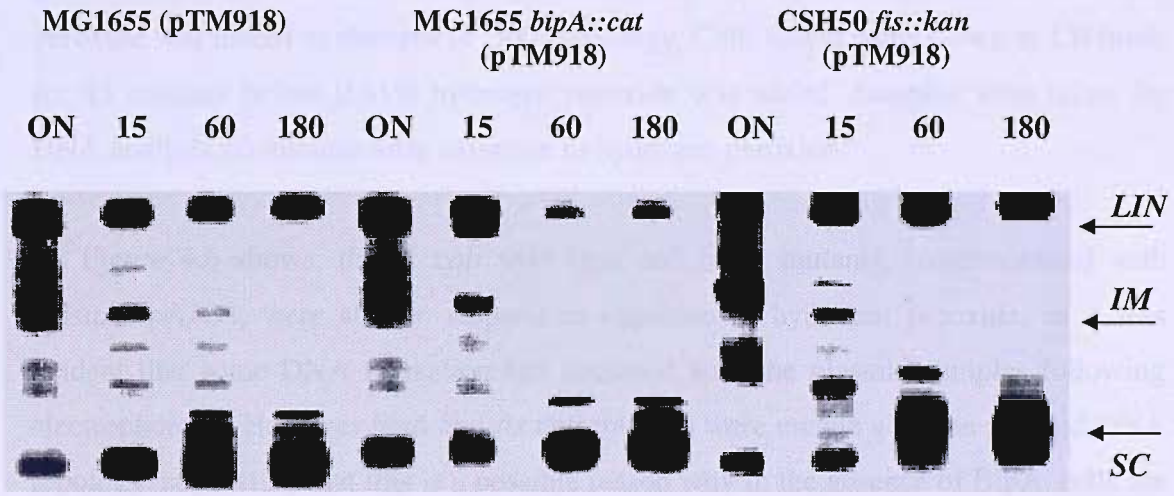


Figure 4.5 The kinetics of supercoiling of plasmid pTM918 in *E. coli* MG1655 wild type, MG1655 *bipA::cat* and CSH50 *fis::kan* cells during the growth phase. pTM918 plasmid DNA, which lacks any high affinity sites for Fis, was isolated at different intervals after subculturing the overnight cultures (ON) of each strain into fresh 2 x YT medium. Samples were loaded into a 1 % agarose gel. Electrophoresis was in the presence of 0.3 μg /ml chloroquine. Upon completion of electrophoresis, the gel was immersed in running buffer containing ethidium bromide until DNA bands could be detected and photographed.

LIN: linear plasmid

IM: subpopulation with intermediate superhelical density

SC: negatively supercoiled plasmid

4.6 DNA topology is altered in *E. coli* K-12 wild type and Δ*bipA* cells, following exposure to acid conditions and oxidative stress.

In addition to changes in nutrition, the Fis protein is able to modulate DNA topology in response to oxidative stress in a similar mechanism by increasing transcriptional activity from the gene that encodes for the Topoisomerase I enzyme, *topA*. Previous experiments had determined that *E. coli bipA*$^-$ mutants were hypersensitive to exposure to hydrogen peroxide (G. Howell, PhD thesis, 2004). As it was apparent that BipA, through Fis, was able to alter DNA topology following nutritional upshift, it was of great interest to see whether any hypersensitivity or a stress response to hydrogen peroxide was linked to changes in DNA topology. Cells tested were grown in LB broth for 45 minutes before 0.01% hydrogen peroxide was added. Samples were taken for DNA analysis 10 minutes after exposure to hydrogen peroxide.

As Figure 4.6 shows, the *E. coli* wild type and *bipA*$^-$ mutants, complemented with plasmid pAJG4, were able to respond to exposure to hydrogen peroxide, as it was evident that some DNA relaxation had occurred with the plasmid samples following electrophoresis. However *bipA* and *fis* null mutants were unable alter the plasmid DNA topology, suggesting that this is a possible reason why in the absence of BipA, cells are hypersensitive to oxidation.

The extent to which BipA exploited DNA topology changes was expanded further by investigating the effect BipA had on pTM918 extracted from cells exposed to acidic conditions. As explained in Chapter 2, cells were grown in 1 x LB (pH 7.0) to mid-exponential phase of growth before being used to inoculate a second volume of medium, at either pH 5.0 or pH 7.0. Cells were left to grow for 20 minutes before plasmid was extracted. As Figure 4.7 shows, the *E. coli bipA*$^-$ mutants expressed more supercoiled plasmid DNA than the wild type and mutants complemented with plasmid pAJG4. As acid tolerance/habituation is thought to require supercoiled DNA and uses factors such as CysB (Rowbury, 1996) for this purpose, the lack of BipA-Fis-mediated Topoisomerase I activity may allow *bipA*$^-$ mutants to be able to respond to the acid conditions more effectively than the wild type cells.

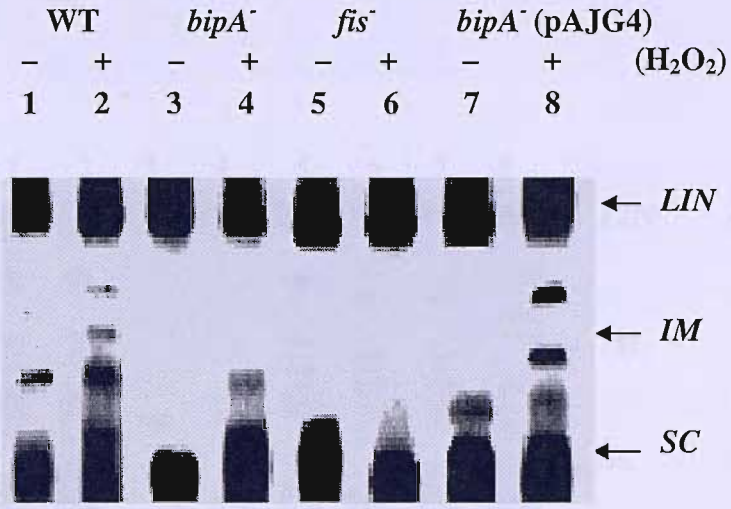


Figure 4.6 The kinetics of supercoiling of the plasmid pTM918 in *E. coli* MG1655 wild type, MG1655 *bipA::cat* and CSH50 *fis::kan* cells exposed to hydrogen peroxide during the growth phase. Samples of pTM918 plasmid DNA was isolated from cultures following 5 minutes exposure to hydrogen peroxide. Samples were loaded into a 1 % agarose gel. Electrophoresis was in the presence of 0.3 µg /ml chloroquine.

Lane 1: *E. coli* MG1655
Lane 2: *E. coli* MG1655 ($+H_2O_2$)
Lane 3: *E. coli* MG1655 Δ*bipA*
Lane 4: *E. coli* MG1655 Δ*bipA* ($+H_2O_2$)
Lane 5: *E. coli* CSH50 *fis::kan*
Lane 6: *E. coli* CSH50 *fis::kan* ($+H_2O_2$)
Lane 7: *E. coli* MG1655 Δ*bipA* (pAJG4)
Lane 8: *E. coli* MG1655 Δ*bipA* (pAJG4) ($+H_2O_2$)

LIN: linear plasmid
IM: subpopulation with intermediate superhelical density
SC: supercoiled plasmid

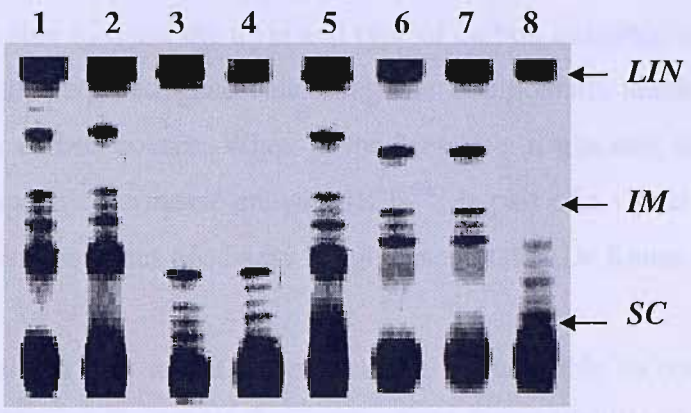


Figure 4.7 The kinetics of supercoiling of the plasmid pTM918 in *E. coli* MG1655 wild type, MG1655 *bipA::cat* and CSH50 *fis::kan* cells exposed to acidic conditions during the growth phase. Samples of pTM918 plasmid DNA was isolated from cultures following 20 minutes exposure to pH 7.0 or pH 5.0. Samples were loaded into a 1 % agarose gel. Electrophoresis was in the presence of 0.3 μg /ml chloroquine.

Lane 1: *E. coli* MG1655 (pH 7.0)
Lane 2: *E. coli* MG1655 (pH 5.0)
Lane 3: *E. coli* MG1655 Δ*bipA* (pH 7.0)
Lane 4: *E. coli* MG1655 Δ*bipA* (pH 5.0)
Lane 5: *E. coli* CSH50 *fis::kan* (pH 7.0)
Lane 6: *E. coli* CSH50 *fis::kan* (pH 5.0)
Lane 7: *E. coli* MG1655 Δ*bipA* (pAJG4) (pH 7.0)
Lane 8: *E. coli* MG1655 Δ*bipA* (pAJG4) (pH 5.0)

LIN: linear plasmid
IM: subpopulation with intermediate superhelical density
SC: supercoiled plasmid

4.7 BipA affects expression of *ptsG* mRNA

E. coli is able to sense the level and type of carbon available in the growth medium and activate the required genes encoding the components needed for the uptake of the particular carbon source. When in the presence of glucose, the product of the *ptsG*, a glucose-specific permease enzyme $IICB^{Glc}$, is part of a signal transduction system that switches on the genes necessary for glucose uptake (De Reuse and Danchin, 1991).

Importantly, it was recently reported that Fis was able to control *ptsG* transcriptional activity (Shin *et al.,* 2003). Depending upon the availability of glucose, Fis can repress or activate *ptsG* by forming nucleoprotein complexes with the repressor Mlc (high glucose) or the activator cAMP-CRP (low glucose) respectively (Shin *et al.,* 2003). To test the ability of BipA to influence $IICB^{Glc}$ levels, the level of *ptsG* mRNA was assessed in wild-type and BipA-deficient cells by primer extension analysis using ptsG1 primer (see Chapter 2 for details). For wild-type *E. coli* the levels of *ptsG* mRNA was highest in samples grown in the presence of glucose. However, the converse was the case for cells deficient in Fis or BipA. Levels of *ptsG* mRNA appeared to be higher in the *fis::kan* cell grown with glucose than in the Δ*bipA* glucose sample, but neither were as abundant as in the wild-type. Without the addition of glucose to the growth media, levels of *ptsG* mRNA were comparable in these mutants. The results suggest that the BipA protein influences the level of *ptsG* mRNA produced in response to glucose (Figure 4.8).

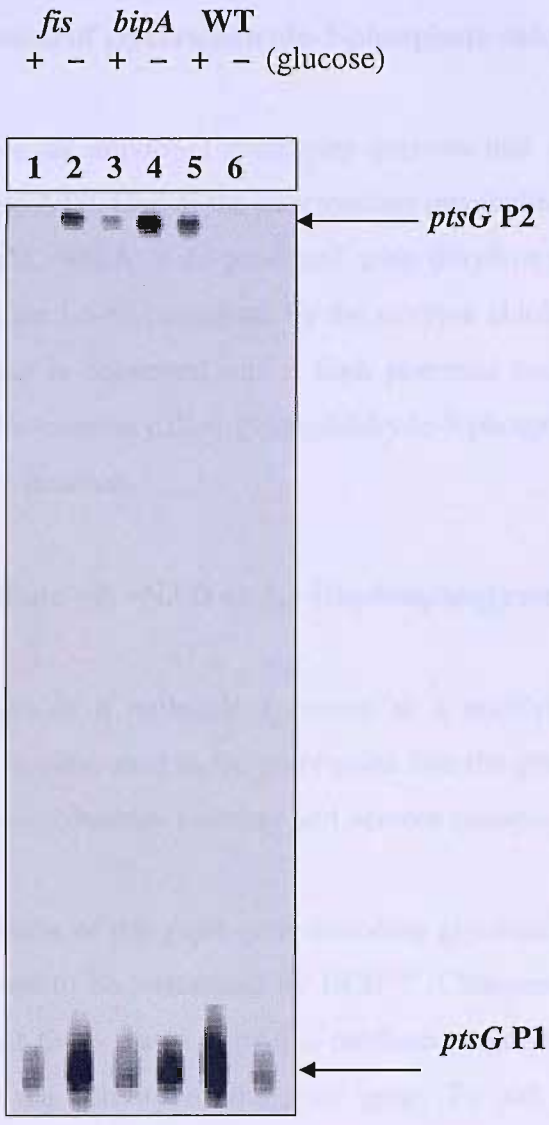


Figure 4.8 Primer extension analysis of the *ptsG* gene in *E. coli* K-12 Δ*bipA* cells. Total mRNA extracts were taken from mid logarithmic phase cultures (OD_{600} = 0.45). All mRNA samples were quantified, with exactly the same amounts from each sample being compared (10 µg). Reactions were performed as described in Chapter 2.

Lane 1: *E. coli* CSH50 *fis::kan* (+ 1.0 % glucose)

Lane 2: *E. coli* CSH50 *fis::kan* (no glucose)

Lane 3: *E. coli* MG1655 Δ*bipA* (+ 1.0 % glucose)

Lane 4: *E. coli* MG1655 Δ*bipA* (no glucose)

Lane 5: *E. coli* MG1655 (+ 1.0 % glucose)

Lane 6: *E. coli* MG1655 (no glucose)

4.8 BipA controls expression of glyceraldehyde-3-phosphate dehydrogenase

The glycolytic pathway is an important multi-step process that is used to degrade glucose in order to generate ATP. One of the intermediate metabolites of the pathway is glyceraldehyde-3-phosphate, which is co-produced with dihydroxyacetone phosphate from the splitting of fructose 1,6-bisphosphate by the enzyme aldolase. From there the glyceraldehyde-3-phosphate is converted into a high potential compound called 1,3-bisphosphoglycerate by the enzyme called glyceraldehyde-3-phosphate dehydrogenase by the following reversible reaction.

Glyceraldehyde-3-phosphate $+P_i$ +NAD $\Leftrightarrow$ 1,3-Bisphosphoglycerate +NADH $+H^+$

Glyceraldehyde-3-phosphate is a molecule common to a number of various sugar conversion pathways and is often used as the entry point into the glycolysis pathway by these sugars, e.g. fructose-1-phosphate pathway and pentose phosphate pathway.

Interestingly, the transcription of the *gapA* gene encoding glyceraldehyde-3-phosphate dehydrogenase was reported to be influenced by $IICB^{Glc}$ (Charpentier *et al.*, 1998). It was therefore possible that there was a BipA/Fis-mediated regulatory mechanism for *gapA* that could act via the activity of the *ptsG* gene. To aid in confirming this hypothesis, an activity assay was used to compare the levels of glyceraldehyde-3-phosphate dehydrogenase activity in *E. coli* K-12 wild-type and Δ*bipA* cells. The results indicated that there was a clear difference in the rate of activity between the strains. The rate of activity in the wild-type was almost twice that of the *bipA* and *fis* mutants. Wild-type levels were virtually restored in the mutant with addition of the pAJG4 plasmid. Similarly, the induction of the pTrcfis plasmid with 0.2 mM IPTG restored activity levels in the *E. coli* Δ*bipA* (pTrcfis) cells. This result implies that BipA does influence activity of glyceraldehyde-3-phosphate dehydrogenase, through a Fis-mediated mechanism (Figure 4.9).

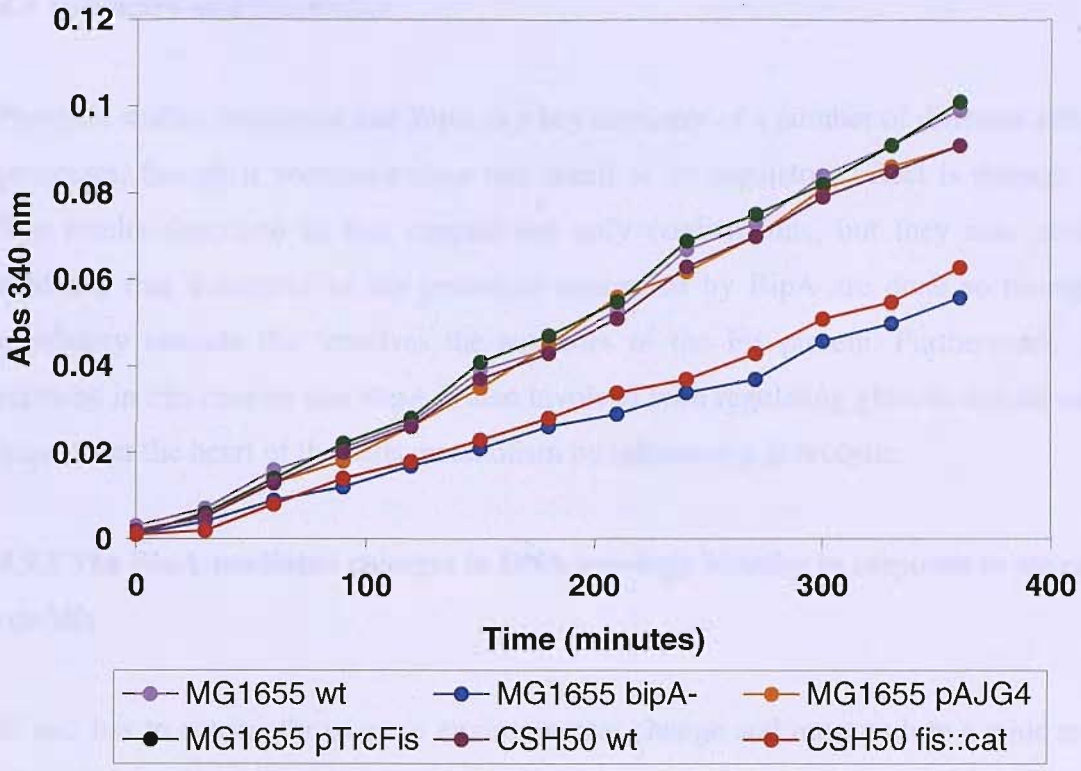


Figure 4.9 A graph showing the effect of BipA and Fis on the activity of glyceraldehyde-3-dehydrogenase. Bacterial strains were grown in 1 x LB medium to an OD_{600} = 0.25. Cells were harvested by centrifugation and whole cell protein collected following sonication. Equivalent amounts of protein were then added to sodium pyrophosphate/sodium arsenate buffer, See Chapter 2, section 2.9, for more details concerning the assay. From point 2 onwards, MG1655 wt, CSH50 wt, MG1655 (pAJG4) and MG1655 (pTrcFis) compared to MG1655 Δ*bipA* and CSH50 *fis::cat* $p<0.05$, t-test.

4.9 Summary and Discussion

Previous studies suggested that BipA is a key regulator of a number of different cellular processes, though it becoming clear that much of its regulatory effect is through Fis. The results described in this chapter not only confirm this, but they also provide evidence that a number of the processes controlled by BipA are done so through a regulatory cascade that involves the activities of the Fis protein. Furthermore, it is reported in this chapter that BipA is also involved with regulating glucose uptake and is required at the heart of the cells metabolism by influencing glycolysis.

4.9.1 The BipA-mediated changes in DNA topology kinetics in response to nutrient upshift

E. coli has to constantly adapt to environmental change and must endure a wide range of variation in nutrient supply. In order to activate the genes necessary for the appropriate response to these changes, a number of different strategies are employed, one of which is the controlled change in global DNA supercoiling (Dorman, 1996, Dorman, 2002). The energy charge of a cell is used to describe the amount of metabolically available energy for the cell, and can be defined as the cellular ratio of [ATP/ADP]. This balance of ATP to ADP is homeostatically maintained throughout logarithmic growth, where the ratio value is ~0.85. This value drops when the cell approaches stationary phase (Jensen *et al.,* 1995).

Many studies have shown that the level of global negative supercoiling is controlled by the cellular energy charge, as the enzymatic activity of the negative supercoiling enzyme, DNA gyrase, is controlled by the intracellular [ATP/ADP] ratio (Higgins *et al.,* 1988; Hsieh *et al.,* 1991). The other main cellular DNA topoisomerase, Topoisomerase I (relaxes negative superhelicity) is also key to this DNA topology-based regulatory mechanism. Controlling the activity of these two topoisomerases is Fis (Schneider *et al.,* 1999; Weinstein-Fischer *et al.,* 2000). The transcription of *fis* is itself partly regulated by changes in DNA topology, with transcription increasing under negative supercoiling conditions (Schneider *et al.,* 1997; Schneider *et al.,* 2000). The

results from the previous chapters prompted the hypothesis that BipA was required for a 'fast track' translation of *fis* mRNAs after *bipA* responding to nutritional upshift. Thus the effect on DNA topology following nutritional upshift, in the presence and absence of BipA, was assessed. It was found that in BipA$^-$ as with Fis$^-$ *E. coli* cells, the level of DNA negative supercoiling was affected. These findings suggested that a BipA regulatory cascade could incorporate changes in DNA topology. This was of some interest as some of the known BipA-regulated processes, particularly stress response genes are influenced by DNA topology.

4.9.2 BipA and the *E. coli* response to acid and oxidative stresses

In a previous study it was reported that BipA had an effect on the ability of EPEC MAR001 cells to form an acid tolerance response to extreme acid environments (Fowler, PhD thesis). This study expanded upon this original proposal, by investigating the involvement of BipA in the acid tolerance response of *E. coli* K-12 cells, in order to begin to understand how BipA is able influence the *E. coli* acid tolerance mechanism. Initial experiments compared the ability of *E. coli* K-12 wild-type cells and *bipA*$^-$ mutant cells to exert an acid tolerance response to pH3 acid conditions. The results showed that *bipA*- cells survived for marginally longer at pH 3.0 than the wild-type cells of *E. coli*. Over-production of BipA in adapted, induced *E. coli* Δ*bipA::cat* (pAJG38) cells was detrimental to their survival at pH3. This suggested that over-expression of BipA has an overall negative effect on the regulation of an acid tolerance response. Similar experiments were carried out to examine the ability of *bipA*- (pTrc*fis*) cells to mount an acid tolerance response. Induction of the *fis* gene also appeared to have a less negative effect on the survival rates of the cells, suggesting that regulation of the acid tolerance mechanism by BipA was either independent of Fis or is counteracted by mechanism that senses over-production of Fis. It is possible that Fis could be a positive regulator of one of the many other different acid resistance systems that can be found in *E. coli*. Over-expression of Fis in *bipA*- (pTrc*fis*) cells may be switching on a system that is not normally engaged by pH5 to pH3 shock, and that this alternative system may be assisting these cells to survive pH3 conditions. This could

explain why there would appear to be a perceived lack of BipA-Fis cooperation during acid shock.

Unfortunately the regulatory circuitry of the acid tolerance response is not very well understood, although a number of chemicals and molecular components common to other acid response mechanisms such as fur, glucose, Fe^{3+}, Fe^{2+} and L-leucine are all known to not play a part in the response by *E. coli* (Rowbury, 1997). However, it is appreciated that during acid tolerance, regulation of the response requires protein synthesis and involves DNA topology alterations. Furthermore it was reported that the *E. coli* acid tolerance is very dependent upon the activities of CysB, a DNA-bending protein that increases negative supercoiling in DNA (Rowbury, 1997). This led to speculation that the promoter regions of some of the genes critical for the acid tolerance response may require localised negative supercoiling by CysB in order to modulate transcriptional activity (Rowbury, 1997). Crucially, this study has determined that in concert with Fis and Topoisomerase I, BipA has control over DNA supercoiling, which could explain why BipA would have an overall negative effect on acid tolerance induction. A situation can be envisaged where the cell would want to quickly reset the activity of the acid tolerance genes in order to divert its energies to repair, recovery and perhaps growth, once the threat to the cell posed by the acid attack has been removed. To do this the negative supercoiling that initially triggered the acid tolerance response genes would have to be relaxed, perhaps through the Topoisomerase I enzymes under the measured control of Fis and BipA. However, in the *bipA* mutant cells, the absence of BipA and would lead to low levels of Topoisomerase I activity, which may be insufficient to counter the effect of the negative supercoiling enzymes. This could leave these cells at a distinct disadvantage compared to the wild-type cells, as the mutants would not be able to reorganise the DNA superhelicity as effectively. This inability to reach superhelical physiological levels following a stress may render the cell incapable of reconfiguring its metabolism allowing it to recover and respond to the next stimulus.

Acid tolerance experiments were carried out to compare the survival rates of *E. coli* wild-type, *bipA*$^-$ and trans-complemented cells of *E. coli*. It was discovered that, as before, following pre-incubation at pH 5.0, the *bipA*$^-$ cells perished before the wild-type

cells. However, following pH 5.0 pre-incubation, the *bipA*⁻ (pAJG38) cells (i.e. transcomplemented), which were maximally induced with 0.1 % arabinose, survived less well than the cells from the other cultures. The effect that exposure to acid had on the expression of *bipA* was then measured using MC1000 (pWP1) cells. Measurements of β-galactosidase activity were taken at specific time-points throughout the acid tolerance procedure, and activity was compared between the pH 7.0 1x LB cultures and the pH 5.0 1x LB cultures during the desensitisation process. Interestingly, it was observed that during preincubation, in pH 5.0 cultures, the peak of transcriptional activity for *bipA* was reached later and was maintained for slightly less time than in the pH 7.0 cultures.

As BipA is proposed to be produced so early in the growth phase, it is perceived to be near to the start of its large regulatory cascade. Assuming this point, such a small change in gene expression may be all that it takes to modulate a response to acid attack. Effector proteins, such as Fis, which may lie further down the cascade, could amplify this change in *bipA* expression tipping the cell towards making a suitable response to the threat. It is possible then that *bipA* expression above a certain threshold could put a stress on the cell, as highly elevated levels of BipA appear to limit the survival rates of cells that are undergoing acid stress. Additionally, the over-expression of BipA would lead to an increase in the activities of Topoisomerase I, possibly frustrating the activity of CysB. This could lead to the inefficient transcription of critical genes of the acid tolerance response mechanism, which specifically require localised, CysB-mediated negative supercoiling to occur within their regulatory regions.

An explanation for the late peak of activity may be found with BipA's regulatory links to the metabolic state of the cell. It is known that, in order to be engaged, acid tolerance requires cells to be in a logarithmic phase of growth and there to be high levels of protein production. This could rapidly exhaust the cell's resources leading to a second perilous event to contend with following on from the original stress. As results in this chapter suggest that BipA is directly involved in the uptake and utilisation of glucose and other aspects of the cellular metabolism that could be through Fis, it is possible that

following exposure to acid, the late peak in *bipA* transcription may be to provide more BipA protein.

Previous studies have shown that *E. coli* K-12 Δ*bipA* cells are unable to grow whilst incubated at low temperature (<30 °C) (Grant *et al.*, 2001; Pfennig and Flower, 2001). The *E. coli* response to cold shock involves an increase in the level of negative supercoiling of DNA, which is mediated by DNA gyrase and the histone-like proteins HU (Mizushima *et al.*, 1997) and Fis (via CspA (Brandi *et al.*, 1999)). One would anticipate that a similar BipA/Fis/Topoisomerase I-mediated response would occur when cells experience such stresses. The results of preliminary experiments suggest that Fis and one or more other regulatory components are linked to a BipA cold-shock response mechanism, as the reintroduction of an inducible copy of the *fis* gene failed to prevent temperature sensitivity in *E. coli* mutants (G. Pritchard, unpublished observations). The possibility that BipA, together with Fis, manipulates global superhelicity to control a number of genes needed for rapid response is an intriguing prospect, though clearly the BipA-mediated response circuit to cellular stress is far more complex than simply striking a balance between DNA gyrase and Topoisomerase I activity. Nonetheless, the results from this chapter have provided a suitable position from which further investigations can continue, so that the regulatory circuit that links BipA with the growing number of stress responses it is associated with can be completely unravelled.

4.9.3 BipA involvement in the uptake and utilisation of glucose

From earlier results in this study it was proposed that BipA was a key regulatory molecule that co-ordinated the cells response to increased nutritional availability. BipA's link with the rapid exploitation of nutritional upshift was reinforced by the discovery that it was intimately involved in the regulation of glucose uptake and utilisation. Similar to Fis, BipA was seen to modulate the levels of mRNA from the *ptsG* gene, which encodes the glucose transporter, $IICB^{Glc}$. This was followed by the discovery that the activity of glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved with a key stage during glycolysis, was also dependent upon the presence of

BipA. This raised the possibility that production of BipA is tied in with another feedback loop that is influenced by the availability of glucose. During growth with high levels of glucose available, a vacant CRP-binding site within the regulatory region of *bipA* does not hamper transcription. BipA is produced as a result and production of $IICB^{Glc}$ and glyceraldehyde-3-phosphate dehydrogenase is maintained through Fis. However as glucose levels fall, active CRP levels rise and, in combination with other factors, may force a decline in the rate of *bipA* transcription. Although perhaps not the main mechanism by which BipA production is halted, the deletion of a CRP binding site in the *bipA* promoter region results in extended transcription activity (See Chapter 3, section 3.9). Presumably CRP binding represses *bipA* thereby reducing $IICB^{Glc}$ and glyceraldehyde-3-phosphate dehydrogenase cellular levels, ultimately forcing a change in the metabolism of the cell.

Put into context, production of BipA would lead to a dramatic change in the cell's metabolism, which means that its production has to be controlled and permitted at times when the cell can afford to do so. The multiple elements that influence production of BipA is perhaps an in built mechanism that ensures there are sufficient resources available to the cell for it to maintain a prolonged state of metabolic activity, and to not let the cell be duped into producing vast amounts of expensive protein when they are not needed. BipA could therefore act as a link between the cell's metabolism together with its energy and resource levels and the co-operative initiation of energy-intensive cellular processes, such as those that are also mediated by Fis, such pathogenesis and motility.

CHAPTER 5

General Discussion

CHAPTER 5: General Discussion

5.1 Introduction

Previous studies detected a novel GTPase, which was subsequently termed BipA. It was soon realised that the protein was a key regulator of a number of important processes. Biochemical and structural analyses suggested that the BipA protein itself was quite unlike other regulatory proteins. Indeed, as previously described, BipA has strong sequence similarity to ribosome-binding GTPases, and is also activated for GTP hydrolysis in the presence of ribosomes (R. Owens, unpublished work). These characteristics suggested that BipA exerted its regulatory effect on its target genes in a totally novel manner by acting at the level of translational. It was therefore highly desirable to determine further the regulatory mechanism of this novel type of global regulator.

In view of the above background the chief aim of this study was to help provide a framework for characterising the BipA regulatory mechanism. The results described here confirm that BipA is indeed a novel type of global regulator, and further, that it has a possible intimate regulatory relationship with the global regulator Fis. What follows is a discussion of the main results of this study and a proposed biological rationale for BipA regulatory circuit and a new direction for future work into the mechanism.

5.2 BipA production is associated with growth phase and nutritional upshift

As BipA regulates a number of different cellular processes, it was anticipated that the protein acts high up in one or more regulatory cascades. This meant, of course, that the transcriptional expression of the target-or targets-that BipA acted directly upon most likely coincided with *bipA* expression. Thus it was of great importance to first determine the expression pattern of BipA in order to define the search parameters for a potential primary target for BipA. It was discovered that production of BipA was tied in with the onset of nutritional upshift, as it was witnessed that the *bipA* gene was most

active during the early logarithmic stage of growth. Indeed, *bipA* has three tandem promoters that respond to the growth rate and to increased nutritional availability. Although each promoter responds to different levels of nutrient, the net effect is that *bipA* transcription peaks during early-to mid-exponential phase, and declines rapidly thereafter. The factor or factors that are involved in the growth rate-associated activation of *bipA* remain unknown.

A possible mechanism for the activation of *bipA* transcription could involve alterations in the topology of the DNA within the vicinity of the *bipA* promoters. The transcriptional initiation of a large number of genes is affected by changes in DNA topology. Furthermore, it is known that changes in DNA topology are correlated with the energy status of the cell and that dramatic changes in global DNA topology occur during nutritional upshift. Further, examples of genes that are regulated by a supercoiling-induced realignment mechanism can be found in many processes, such as response to cold shock, osmotic shock, amino acid biosynthesis and carbon utilisation (Borowiec and Gralla, 1985; Jones *et al.,* 1987; Wang and Syvanen, 1992; Kusano *et al.,* 1996). It is possible that in the resting state for *bipA*, say when the ATP/ADP ratio is low (as with very late growth phase), the intergenic region between *glnA* and *bipA* would be less negatively supercoiled due to a decrease in DNA gyrase activity. This untwisting in the DNA upstream of *bipA* may position the three tandem *bipA* promoters in such a way that transcription is severely inefficient enabling only a basal level to occur, while at the same time possibly facilitating NtrC binding (where necessary) and enhancing *glnA* activity. During nutritional upshift, the negative supercoiling of the *glnA-bipA* intergenic region would increase, as there would be more ATP available to act as a cofactor for DNA gyrase. This tighter twisting of the DNA within the *glnA-bipA* intergenic region would reposition the *bipA* promoters so as to promote the binding of RNA polymerase. This may also be inhibitory for DNA-binding factors, such as NtrC and CRP, leading to an increase in the efficiency of *bipA* transcription, and the resulting in the explosion of transcriptional activity seen during growth in rich media.

This study also provides evidence that the NtrC and CRP binding sites within the *bipA* regulatory region contribute to repression of transcription of *bipA*. Increasing the availability of nitrogen and carbon, particularly glucose, were seen to have a positive impact on *bipA* transcription. Crucially, both NtrC and CRP binding have important roles during the transcription of the divergent gene *glnA*, which is involved in nitrogen assimilation (Reitzer and Magasanik, 1985; Reitzer and Schneider, 2001). It is possible that the NtrC and CRP-binding sites are used to switch between *bipA* and *glnA* activity or modulate activity according to the availability of critical nutrients. Thus under circumstances when the cell is in an environment rich in nitrogen and glucose, the intracellular levels of active CRP and NtrC would be very low. With the repositioning of the promoters due to relaxation of the DNA supercoiling and no interference from NtrC and CRP, maximum activation of the *bipA* gene would ensue. As growth continues and intracellular levels of nitrogen and carbon become depleted, the cell begins to slow down its metabolism and engages genes that are required for survival under starvation conditions. Activation of NtrC via phosphorylation increases with a drop in the level of intracellular nitrogen levels. These NtrC-P molecules would be available to positively regulate the *glnA*p2 promoter, leading to further NtrC production. Increased negative supercoiling of the promoter DNA and NtrC-P-binding would interfere with and limit *bipA* transcription. As the availability of carbon decreases, cAMP levels would increase, which would bind to and activate CRP. Activated CRP would bind to the CRP site in the *bipA-glnA* intergenic regulatory region, which has been shown to down regulate *glnA* expression by greater than 20-fold (Tian *et al.,* 2001). Thus the transcriptional activity from both *bipA* and *glnA* promoters would be blocked.

Further experimentation revealed that *bipA* transcription was negatively influenced by the stringent response and hence by ppGpp. It is known that the production of ppGpp from GTP would deplete the cellular GTP pool, thereby frustrating global transcription activities and other GTP-dependent processes. Interestingly, BipA has an approximately 3-fold higher affinity for GTP than GDP (R. Owens, unpublished work). As these affinities are sufficiently similar, it is probable that GDP greatly inhibits GTP hydrolysis by BipA, when in the presence of ribosomes. It is possible then that as cells

approach stationary phase, the combination of a decrease in availability of intracellular GTP, together with the effect of producing ppGpp would trigger a GTP sensing regulatory mechanism linked to BipA. This may feed back to *bipA*, reducing transcription and the rate production of BipA protein.

5.3 BipA's role in co-ordinating cellular metabolism and responses to stress

The discovery that BipA shares significant similarities to Fis, in terms of expression profile and regulatory targets has raised the possibility that the two proteins are part of an extensive regulatory circuit that influences a large number of different cellular processes. Although more is known about the numbers of regulatory targets of Fis than of BipA, there is a remarkable correlation in terms of the processes they govern in both *E. coli* and *S. typhimurium* judging by recent DNA microarray analysis (Kelly *et al.*, 2004). For instance, responses to nutritional upshift, cellular motility, oxidative stress EPEC pathogenesis and the cold shock response are all processes influenced by both proteins. To determine how intimate the relationship between Fis and BipA was, a series of experiments were carried out to assess the impact of each protein had on other key metabolic and stress response processes.

In this way, it was discovered that Fis and BipA are components of *E. coli* responses to acidic stress. Curiously, BipA had an overall negative influence on cells making an acid tolerance response. Cells that lacked a copy of the *bipA*$^{-}$ gene were more able to survive exposure to acid than their wild type counterparts, while the introduction of an inducible copy of *fis* in to the *bipA*$^{-}$ mutants restored and, in some cases, exacerbated this inability to tolerate acidic media. Overexpression of an inducible *bipA* gene also exaggerated the negative effect on *E. coli* survival in increasingly acidic media.

A possible explanation for the above effects came from an experiment designed to measure the effect of BipA on the global DNA topology. This indicated that, through Fis, expression of the GTPase prolonged the relaxation of DNA following nutrient upshift. Interestingly, it has been suggested that DNA topology, more specifically negatively supercoiled DNA, is an important factor during the regulation of the acid

tolerance response of *E. coli* (Rowbury, 1997). In keeping with this process, tests revealed that, following exposure to acid, the DNA topology in *bipA*$^+$ cells was generally relaxed, while the DNA in the mutants was more negatively supercoiled.

This effect on DNA topology is repeated in mutant and wild type cells exposed to the oxidative effects of H_2O_2 (Weinstein-Fischer *et al.,* 2000). Fis influences changes in DNA topology and is also involved in the regulation of the response to oxidative stress. However, in contrast to acidic stress, the response to oxidative stress requires relaxed rather than negatively supercoiled DNA (Weinstein-Fischer *et al.,* 2000). Thus, one would predict that BipA and Fis would act in concert to positively regulate a response to H_2O_2. Consistent with this idea, studies with *Salmonella* have shown that adaptation to H_2O_2-induced oxidative stress is impaired in *bipA* null mutants (G. Howell, PhD thesis, 2004) Interestingly induction of Fis did not confer the ability of *bipA* mutants to survive cold shock, indicating that this particular BipA-mediated response is more complicated, and requires the intervention of other regulatory components.

Following the earlier discovery that BipA was associated with nutritional upshift and that Fis was a key regulator of a number of metabolic pathways, it was of great interest to determine the extent to which BipA influenced the cell's central metabolism. Therefore, experiments were conducted to assess the involvement of BipA in the processes of glucose uptake and utilisation. This particular process was selected as Fis was previously reported to be a major regulatory influence (Gonzalez-Gil *et al.,* 1996; Shin *et al.,* 2003). It was discovered that similar to Fis, BipA was able to regulate the mRNA levels of *ptsG*, which encodes the glucose-specific permease enzyme IICB^{Glc}. Furthermore, it was later found that BipA was also required for the high level expression of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of the glycolytic pathway. Intriguingly, it is known that IICB^{Glc} is involved in the transcriptional regulation of *gapA*, the gene that encodes glyceraldehyde-3-phosphate dehydrogenase (Charpentier *et al.,* 1998). Thus it is most probable that positive regulation of *ptsG* by BipA, through or with Fis, results in a knock on positive effect with *gapA*.

In summary, these results lead to the proposal that BipA is a key regulatory molecule that, in partnership with Fis, is able to link the availability of glucose (and therefore the nutritional status of the cell) to the cell's ability to the regulate and co-ordinate responses to a variety of cellular stresses, pathogenicity, motility and other demanding cellular processes.

5.4 Future work:

Collectively, the results of previous studies in the O'Connor laboratory and from the available literature, together with the findings of this report allow for a fresh insight into the possible workings of the BipA regulatory cascade. The similarities between Fis and BipA, in terms of regulatory targets and gene expression indicate a close partnership between the two.

The evidence is in place that supports the notion that BipA is a novel form of regulator that makes an impact upon global gene activity through or with Fis. The potential for BipA to be involved in the regulation of/with another global regulator is an exciting prospect, especially as the regulation occurs at the level of translation.

However there are many questions that still need answering:

First, what is the identity of the critical component that triggers *bipA* transcription? We know that transcription is linked to nutritional upshift, but the exact mechanism remains unknown. As BipA is involved in processes that involve changes in DNA topology, it may be the case that a similar regulatory mechanism is in place for *bipA*. The increase in negative superhelicity of the DNA forming the *glnA-bipA* intergenic region could reposition the *bipA* promoters in such a way that transcription is boosted. Production of BipA coincides with the boosted transcription of *fis*, which is also triggered by increased negative DNA supercoiling following nutritional upshift. Furthermore, how is BipA protein produced so rapidly? Could there be a BipA-specific 'fast track' mechanism that allows for a controlled burst of BipA production to prepare the cell for Fis.

Second, at the other end of the spectrum, what is the precise mechanism that causes a decline in BipA production? It is anticipated that an important regulatory component such as BipA would have several regulatory mechanisms that prevent inappropriate BipA production. This study has touched upon a number of possible candidates, but further investigation is clearly required.

Third, what is the relationship between BipA and Fis?

The Fis protein is a key global regulator, and as discussed earlier, is involved in many cellular processes. As a consequence of its importance to the cell, the *fis* gene is heavily regulated at the level of transcription (Nasser *et al.,* 2002; Mallik *et al.,* 2004). It is known that transcription of the *fis* gene is strongly enhanced by increases in negative supercoiling (Schneider *et al.,* 2000). Furthermore, the DNA-bending protein IHF is also known to positively regulate transcription at the *fis* gene, which can stimulate levels of *fis* mRNA three- to fourfold, by binding to a specific site located at –116 bp relative to the *fis* start site (Pratt *et al.,* 1997; Schneider et al., 2000). Transcription of *fis* is also negatively autoregulated, with high levels of Fis protein leading to transcription repression (Ninnemann *et al.,* 1992). Maximal *fis* mRNA levels were reported to be six times higher in *E. coli fis*$^-$ mutant cells than in the isogenic *E. coli fis*$^+$ cells (Ball *et al.,* 1992). This autoregulation was attributed to the presence of six Fis binding sites within the *fis* promoter region, which could account for the inability of RNA polymerase to bind to the *fis* promoter when Fis protein is in abundance (Ball *et al.,* 1992). Interestingly, the CRP transcription regulator can modulate *fis* transcription by acting as either an enhancer or inhibitor of *fis* transcription. CRP enhances *fis* transcription when there are low levels of Fis protein, whereas together Fis and CRP synergistically repress *fis* transcription (Nasser *et al.,* 2001).

Transcription of *fis* is also controlled by growth rate through specific sequences that lie between the -35 and -10 of the *fis* promoter region (Ninnemann *et al.,* 1992; Walker *et al.,* 1999). In addition, the levels of intracellular pools of nucleotide triphosphates also appear to influence transcription initiation during growth phase-dependent activation

(Mallik *et al.,* 2004). Furthermore, it has been proposed that when cells are starved for amino acids, *fis* transcription is inhibited by a stringent control mechanism (Ninnemann *et al.,* 1992; Walker *et al.,* 2002; Mallik *et al.,* 2004). Although stringent control is linked to the -35 and -10 of the *fis* promoter region also, the precise regulatory mechanism is as of yet unknown and there is some controversy surrounding this proposal (Walker *et al.,* 1998). This is because *fis* mRNA levels in cells that lack the ability to produce ppGpp, a key component of the stringent response, are comparable to that of wild type levels (Ball *et al.,* 1992).

Does BipA play a part in Fis production, or are they extremely co-operative in terms of regulatory targets? As we know, BipA is a member of the ribosome-binding GTPase superfamily, and hydrolyses GTP in the presence of intact ribosomes (R. Owens, unpublished work). The addition of BipA to this lengthy list of regulatory mechanisms for *fis* could indicate a second layer of regulation at the translational level. It may be the case that Fis and BipA merely co-operate and that there is no formal regulatory link between the two proteins. However the possibility of both proteins belonging to an extended regulatory circuit invites further study. It would be of great interest to determine whether BipA does influence Fis production at the level of translation. Indeed, investigations have already begun with further experimentation revealing that BipA regulation of *fis* was at the post-transcriptional level, as *fis* mRNA levels were not depleted in *bipA*- strains of *E. coli* K-12 and transcription of *fis* was unaffected (M Hodey, unpublished results).

Fourth, recent DNA microarray analysis suggests that Fis is a vital factor for metabolism and virulence in *S. typhimurium* (Kelly *et al.,* 2004). Results from this study suggest that BipA also controls Fis production in *S. typhimurium.* It would be of some interest to determine the similarities between BipA:Fis co-operative regulation in *E. coli* and *S. typhimurium*, and would no doubt make for an interesting second study.

Finally a fifth point to make is that it must be the case that BipA directly regulates other factors in addition to Fis, as homologues of BipA can be found in many bacterial species, as well as in plants. In contrast, Fis is restricted to members of the

Enterobacteria family, although it is possible that in other species BipA has been modified and adapted for a similar use so that it can interact with a Fis equivalent in these other bacteria. Further research will no doubt reveal the identity of other molecules that belong to the BipA regulatory cascade.

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