UNIVERSITY OF SOUTHAMPTON

STUDIES ON THE GLOBAL REGULATOR BIPA

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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Microorganisms use diverse mechanisms to control the expression of their genes and many of these processes are essential for cell survival. This dissertation concerns a novel type of regulatory protein, termed BipA, which is found in many bacteria including harmless commensals such as *Escherichia coli* K-12, symbionts such as *Sinorhizobium meliloti*, and pathogens such as *Salmonella* and enteropathogenic and enterohaemorrhagic *E. coli*. BipA regulates a range of cellular processes that influence both the survival and virulence of microorganisms. In contrast to other global regulatory proteins, however, it functions as a GTPase that interacts with 70S ribosomes, suggesting that it may influence the translation of one or more target mRNA transcripts.

It has previously been proposed that BipA directly regulates the translation of another global regulatory protein known as Fis. However, a phenotypic comparison *bipA* and *fis* null mutants of *E. coli* revealed that, while flagella-mediated cell motility was impaired in both mutants, the *fis* mutant successfully formed colonies at temperatures below 30°C whereas the *bipA* null mutant did not. These observations indicate the involvement of BipA in processes that are not mediated through Fis. They also suggest that BipA is positioned higher up in the regulatory hierarchy than Fis. In addition to comparing *bipA* and *fis* phenotypes, the effects of ectopic expression of Fis were studied. Aberrant Fis expression was shown to be deleterious to cell growth, blocking cell division and hence causing filamentation in *E. coli*.

During the course of this study results were obtained that were inconsistent with the findings presented for the control of Fis expression by BipA. Re-examination of some previously reported data indicates it is unsafe to conclude that BipA directly controls the expression of Fis. In view of these circumstances, a search for additional protein targets that are directly or indirectly regulated by BipA was initiated. Proteomic analysis of lag phase cells from a *bipA* null mutant of *Salmonella enterica* serovar Typhimurium and its parent strain uncovered candidate proteins whose expression appears to be influenced by BipA, including SipC, a component associated with the SPI-1 type three secretion system, which is important for virulence in this pathogen.

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CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Overview

All living organisms need to maintain and reproduce their functional systems to survive and to be perpetuated. Collectively, these processes constitute growth, which can be considered at the level of the individual cell or at the population level (Helmstetter *et al.*, 1979;Madigan *et al.*, 1997;Monod, 1949). A microbe's ability to grow and reproduce by cell division is one of its most important functions because a single bacterium of any species has a finite lifespan (Fredriksson and Nyström, 2006) therefore, cell division is essential to the maintenance of any bacterial species.

Despite their small size and relative genetic and structural simplicity, the mechanism by which bacteria reproduce (binary fission) is far from simple (Bramhill, 1997;Goehring *et al.*, 2005;Slater and Schaechter, 1974). In order to reproduce, a bacterial cell must duplicate its contents using numerous synthetic reactions (including replication of the bacterial chromosome). Then, segregation of its genome and cell contents must occur before it can proceed to divide and synthesize a new cell wall (Bartosik and Jagura - Burdzy, 2005;Bramhill, 1997;Cooper, 1996;Helmstetter, 1996). Bacteria use a number of different systems and proteins to perform these tasks (Goehring *et al.*, 2005;Kaguni, 2006;Leonard and Grimwade, 2005;Nielsen *et al.*, 2006) and these systems are carefully co-ordinated (Dewar and Dorazi, 2000). This co-ordination is essential to bacterial replication because an inappropriate level and/or activity of even a single component of the replication process, such as an enzyme, can have harmful consequences and prevent successful cell division (Bramhill, 1997;Kruse *et al.*, 2003;Weart *et al.*, 2005).

Understanding bacterial cell division is not only of fundamental interest, it may also facilitate the development of novel potent antimicrobial agents, which prevent cell division. For example, Läppchen *et al.* (2005) have already designed a novel antimicrobial agent to FtsZ, a protein that is known to play a key role in bacterial cell division, and have shown it to have no inhibitory effect on its eukaryotic homologue tubulin (Läppchen *et al.*, 2005). Further, such knowledge may be used to gain new insights into the evolution of the mechanisms of mitosis and meiosis that occur in higher organisms. However, before examining the molecular mechanisms which an individual bacterial cell uses to divide and reproduce the growth of bacterial populations will be discussed.

1.2 The growth of bacterial cultures

The growth of any bacterial culture proceeds as a succession of phases which are characterized by variations of the growth rate (Figure 1.1). These phases are:

Lag phase: growth rate null;

Acceleration phase: growth rate increases;

Exponential phase: growth rate constant;

Retardation phase: growth rate decreases;

Stationary phase: growth rate null;

Phase of decline: growth rate negative.

This is a general picture of growth in bacterial cultures but under certain conditions any one or several of these phases may be absent (Monod, 1949).

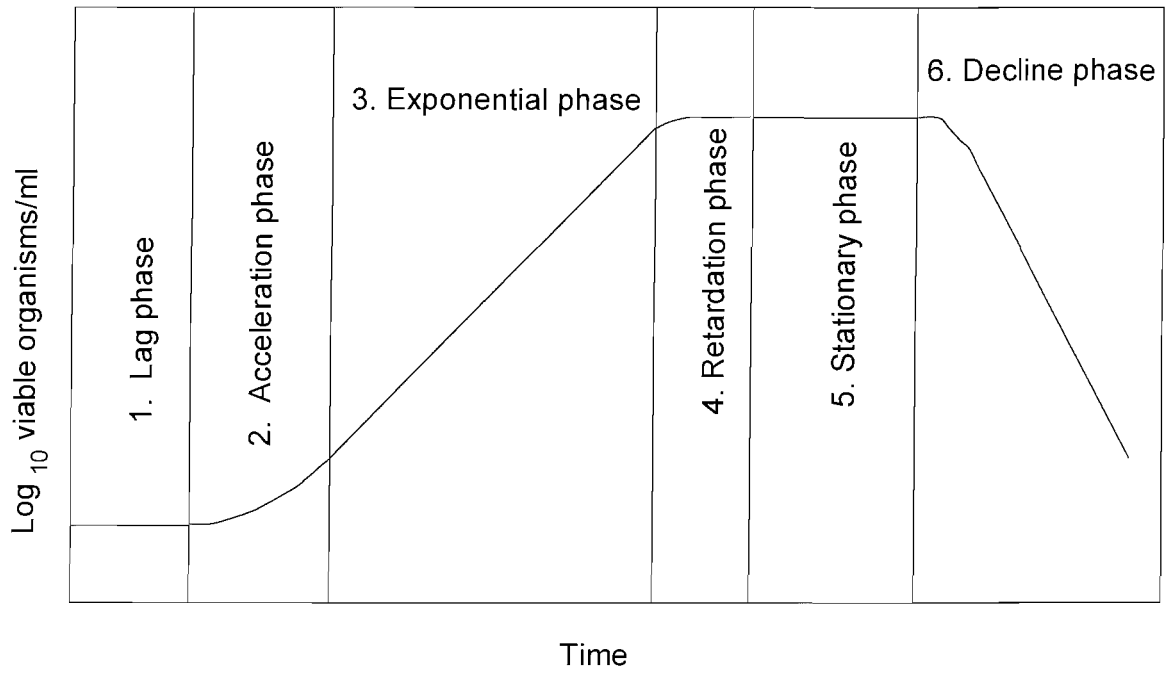


Figure 1.1. The growth of bacterial cultures; adapted from Monod (1949).

The lag and acceleration phases are periods of adjustment that allow the newly inoculated cells to adapt to the new nutrient conditions. These phases vary in length but tend to be longer if stationary phase cells are sub-cultured and may be completely absent if exponential phase cells are inoculated into fresh media of the same type (Monod, 1949).

The exponential phase occurs when the number of cells in a culture doubles per unit time period. It is a steady state where the growth rate is constant. The growth rate of a bacterial culture in exponential phase represents the overall velocity of the series of reactions which are used to synthesize cell substances. Most of these reactions will be enzymatic, so their rate will vary with the concentration of metabolites present (which depend on the culture medium composition), enzyme concentration and temperature (Monod, 1949).

The retardation phase is the result of the population reaching the limit of the culture conditions in terms of space and available nutrients. As the available nutrients begin to limit the culture's growth its growth rate falls. When the cells reach the stationary phase and there is no net increase in cell number, gene expression is altered so as to favour cell survival in nutrient-limited conditions. After remaining in the stationary phase for a prolonged period, the cells may enter a phase of decline and begin to die (Monod, 1949).

The next topic for consideration is the growth of an individual bacterial cell. This section will start by defining the processes a bacterial cell must perform in order to divide by discussing the bacterial cell cycle.

1.3 The bacterial cell cycle

The prokaryotic division cycle can be explained using the I + C + D rule (Helmstetter *et al.*, 1979). It was designed as a model for a hypothetical cell that contained a single non-replicating chromosome. I was defined as the time required for the cell to achieve the capacity to initiate chromosome replication (the interinitiation time – when the cell is primed for chromosome replication). This can vary with growth rate depending on the situation the cell finds itself in (available nutrients etc). Most of the I period requires protein synthesis, which occurs at a higher rate in rapidly growing cells. C was the time it takes to replicate the cell's chromosome and D was the time between chromosome replication and cell division. The C and D periods remain more constant in length at different growth rates. This rule states that a cell starts its duplication by carrying out all the necessary biosynthetic activities for chromosome replication, and once that is finished (after I minutes) chromosome replication occurs, after which the cell divides (C + D minutes later) (Cooper, 1979;Helmstetter, 1996). These concepts are shown schematically in Figure 1.2.

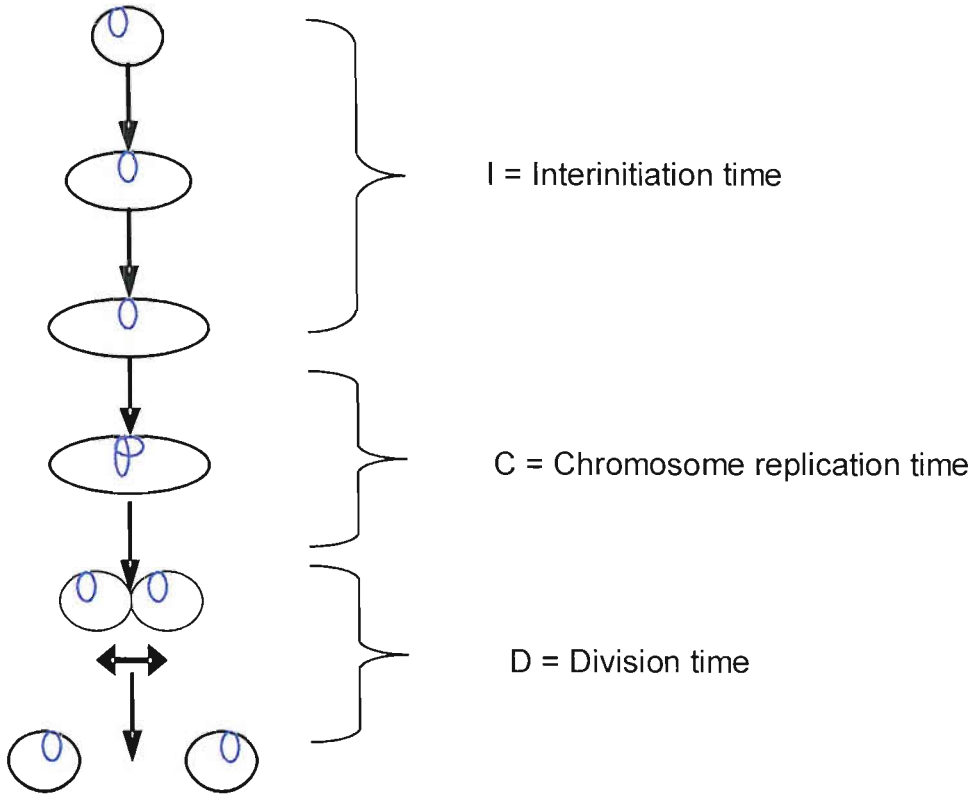


Figure 1.2. Binary fission and the bacterial cell cycle as described in the ICD model (Helmstetter *et al.*, 1979). The ovals represent individual cells and the blue small ovals represent their genomes.

Prokaryotic cells of the same strain growing at different rates may appear to show different cell division cycles, but the I + C + D rule can be used to model the division cycle for both slow and fast growing cells. Slow-growing cells clearly show the interinitiation time, chromosome replication time and division time. This is due to the length of the interinitiation time exceeding that of the chromosome replication time and division time combined which may be the result of nutrient and space limitations in stationary phase cells, or the adjustment of cellular metabolism in lag phase cells. Whatever the cause, slow growth rates mean that only one round of DNA replication occurs per cell at any given point, and in some cases, in cells growing with a doubling time greater than 60 minutes, there is a lag analogous to the G1 period observed in eukaryotic cells (Cooper, 1979;Helmstetter, 1996). Rapidly growing prokaryotic cells however, do not show this lag period. These are cells growing in optimum conditions with plenty of available nutrients and space which facilitate the speeding up of the biosynthetic reactions that occur in the interinitiation time, thereby reducing its length. The interinitiation time becomes shorter than the chromosome replication time which means, in cells with a doubling time of less than 40 minutes, a new round of DNA synthesis will start before the previous round of DNA replication has been completed. This results in multiple replication forks per chromosome and daughter cells in which chromosome replication has already begun (Cooper, 1979;Helmstetter, 1996).

The molecular mechanisms a bacterium uses to perform and regulate the events of the bacterial cell cycle and ultimately sucessfully divide will be discussed in the following passages.

1.4 Duplication of the cell contents and initiation of chromosome replication

The first hurdle to a dividing cell is to double its cellular contents and to initiate chromosome replication (this is undertaken in the interinitiation time (Helmstetter, 1996)). The expression level of the key initiator of DNA replication, DnaA, is of great importance to initiation, as is the nucleotide bound to it (as recent studies have indicated (McGarry *et al.*, 2004;Nishida *et al.*, 2002)). DnaA-ATP can initiate chromosomal DNA replication (McGarry *et al.*, 2004) through its interaction with the origin of chromosomal replication (*oriC*) causing DNA unwinding in an AT-rich region within *oriC* which triggers the commencement of replication of the bacterial chromosome. Other proteins help with this interaction. Additionally, the structure of *oriC* is key to subsequent reactions required for DNA replication, such as the positioning of the DnaA primosome. In *E. coli*, chromosome replication is initiated at the fixed origin *oriC* and proceeds bidirectionally from there to the terminus, *terC* (Messer and Weigel, 1996). However, a bacterial cell must also have the ability to control the initiation of chromosomal replication to ensure that each daughter cell receives the single complete genome they require to form a fully functional cell (Kaguni, 2006;Messer and Weigel, 1996), the control of replication initiation is discussed in the following section.

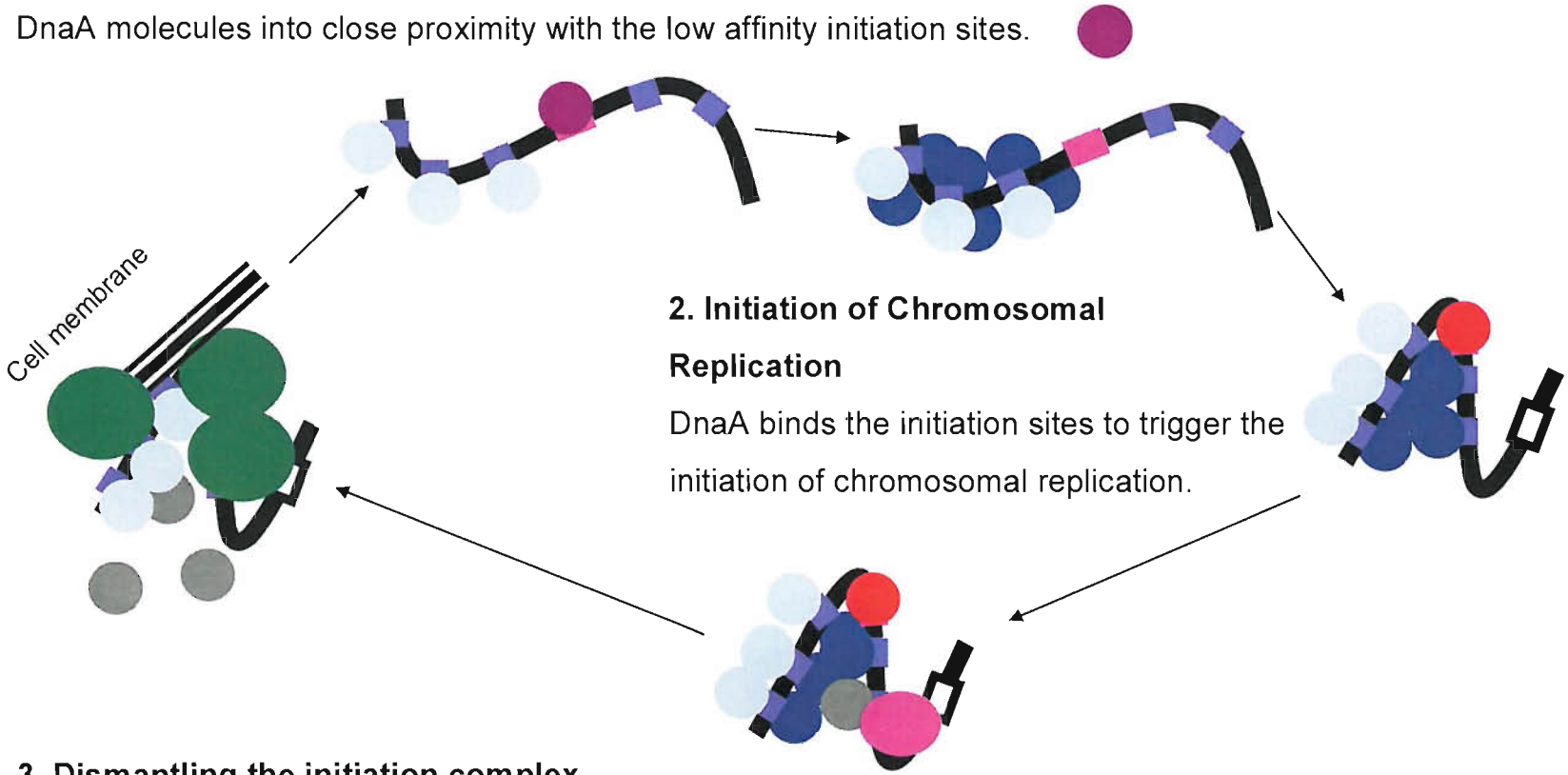
1.4.1 Control of replication initiation

Replication initiation is controlled at three levels; a) it is precisely timed with the bacterial cell cycle, b) each origin initiates only once per cell cycle and c) all the origins present in the cell initiate replication simultaneously (Messer and Weigel, 1996;Kaguni, 2006). Early studies on the division cycle of *E. coli* indicated that there is a relationship between chromosome replication and cell mass (Donachie, 1968). However, more recent studies revealed that the initiation mass increases with decreasing growth rate (Wold *et al.*, 1994) indicating that a factor or factors, other than mass itself, determines the timing of the initiation of chromosomal replication (Messer and Weigel, 1996;Kaguni, 2006).

Recently proposed models for the initiation of chromosome replication at *oriC,* reveal that it is a complicated process which requires the correct expression level and interaction with *oriC* of the key initiator of replication DnaA in its adenosine triphosphate bound form (DnaA-ATP). The intracellular DnaA-ATP level has been shown to oscillate over the cell cycle peaking around the time of initiation and decreasing rapidly post initiation (Kurokawa *et al.*, 1999). The initiation of chromosome replication at *oriC* also requires the action of the DNA bending proteins IHF and Fis, which alter the DNA topology of *oriC* to promote or prevent initiation (Ryan *et al.*, 2004;Wold *et al.*, 1996). In addition to this, negative regulatory systems are also necessary to prevent premature re-initiations of chromosome replication. The action of the replication apparatus (DNA polymerase III) is an important part of this, as is Dam methylation and sequestration of hemimethylated DNA by the action of the SeqA protein. These factors interact to form a cycle which is responsible for the initiation of chromosome replication. This is represented in Figure 1.3 (Kaguni, 2006;Leonard and Grimwade, 2005).

1. Building the initiation complex

DnaA levels are low, only high affinity DnaA sites are occupied and Fis bound to *oriC* prevents initiation. DnaA levels increase, further DnaA molecules bind to *oriC* and displace Fis. IHF then binds to *oriC* and alters its topology to bring DnaA molecules into close proximity with the low affinity initiation sites.



3. Dismantling the initiation complex

DNA polymerase inactivates bound DnaA. *oriC* is temporarily hemimethylated post initiation, SeqA binds to it and sequesters it to the cell membrane preventing re-initiation. Acidic phospholipids in the cell membrane cause DnaA to dissociate from *oriC*.

Figure 1.3. The cycle of initiation at *oriC*

Figure 1.3. The cycle of initiation at *oriC* adapted from Leonard and Grimwade (2005). The blue circles represent DnaA molecules, with the dark blue circles representing newly synthesized DnaA molecules and the light blue circles depicting DnaA molecules that may have been retained from the previous initiation cycle. The grey circles represent deactivated DnaA molecules and DnaA binding sites are represented as blue squares. The purple circles represent Fis molecules and the red circles represent IHF molecules. The Fis and IHF binding sites overlap and are shown as a pink rectangle. The pink circle represents DNA polymerase and the SeqA protein is depicted as green circles.

In the early interinitiation time, DnaA-ATP levels are low, which means only the high affinity DnaA binding sites (R1, R2 and R4) are occupied in *oriC*. As further DnaA-ATP is produced, its intracellular concentration increases, and as it does so, the number of DnaA-ATP molecules bound to *oriC* progressively increases. This continues until the DnaA-ATP molecules bound to *oriC* reach a level that interferes with the binding of Fis to *oriC* (Leonard and Grimwade, 2005). Fis is then displaced from *oriC* which relieves the initiation inhibition that Fis exerts (by altering the DNA topology into a non-permissive state) (Cassler *et al.*, 1995;Hiasa and Marians, 1994;Ryan *et al.*, 2004;Wold *et al.*, 1996). It should be noted, however, that the ability of Fis to inhibit initiation at *oriC* has been debated (Margulies and Kaguni, 1998).

The displacement of Fis from *oriC* allows the binding of IHF to *oriC*. IHF bound to *oriC* alters its DNA topology, bending it into an alternate configuration, which promotes the oligomerization of DnaA-ATP molecules. This places DnaA-ATP in close proximity to the low affinity DnaA binding initiation sites (I sites). This facilitates the binding of DnaA-ATP to the initiation sites. Binding of DnaA-ATP to the initiation sites triggers DNA strand separation at the AT-rich regions in *oriC*, opening up the double helix to allow the docking of the replication machinery, DnaB helicase and the polymerase holoenzyme, and thereby initiates chromosome replication (Leonard and Grimwade, 2005).

A cell must also be able to prevent premature re-initiations of chromosome replication to ensure the production of offspring with intact fully functional genomes. This means that once initiation is complete the initiation complex must be inactivated and disassembled. According to the current model, the immediate dismantling of the initiation complex may be aided by the movement of the helicase and polymerase, which displace IHF bound to *oriC*, causing a change in the conformation of the DNA which would remove DnaA-ATP molecules from close proximity to the initiation sites. However, to fully disassemble the complex and prevent premature re-initiations, negative

regulatory systems are required. Regulatory inactivation of DnaA (RIDA) is one such system, in which DNA polymerase III and the sliding clamp (with a Homologous to DnaA (Hda) cofactor) traverses *oriC*, inactivating *oriC* bound DnaA-ATP, through stimulating the ATPase activity of DnaA (Leonard and Grimwade, 2005).

Dam methylation is an essential component of another negative regulatory system for the control of the initiation of chromosomal replication. Methylation sites for the Dam methylase in *oriC* become temporarily hemimethylated upon replication. The DNA binding protein SeqA binds hemimethylated *oriC* and sequesters it to the cell membrane for a period known as the eclipse period. The mechanism then used to prevent premature re-initiations during the eclipse period is still unclear. However, it may involve SeqA modification of DNA topology, which prevents open complex formation (Torheim and Skarstad, 1999), or the negative regulation may be due to the movement of *oriC* to the membrane (because acidic phospholipids in the cell membrane may cause DnaA to dissociate from *oriC* and prevent their re-association (Makise *et al.*, 2002)). It is currently proposed that these negative regulatory mechanisms act in concert to reset *oriC* before the next round of initiation (Leonard and Grimwade, 2005).

In addition to these inhibitory mechanisms, replication of the *dnaA* gene represses the synthesis of new DnaA-ATP. Regulatory inactivation of DnaA will continue and cause the level of DnaA-ATP in the cell to fall. Further, as more of the DnaA binding chromosome is replicated, the DnaA binding to it will decrease the overall level of DnaA-ATP in the cell and finally, the replication of the DnaA binding site of the chromosome *datA*, which DnaA molecules bind to with very high affinity, will cause a further reduction in DnaA-ATP bound to *oriC*, allowing Fis to once again bind to the origin and repress initiation (Leonard and Grimwade, 2005).

Following successful initiation of chromosomal replication, the bacterial chromosome is replicated and replication is terminated in the terminus region *terC* (Hill, 1996;Messer and Weigel, 1996;Valjavec-Gratian *et al.*, 2005). After the termination of chromosomal replication the chromosomes are separated and segregated (Bartosik and Jagura - Burdzy, 2005;Nielsen *et al.*, 2006;Woldringh and Nanninga, 2006), the processes bacterial cells use to achieve this will be discussed next.

1.5 Bacterial chromosome segregation

Once a bacterial cell has successfully replicated its genome, its next challenge is to separate its chromosomes and segregate them. This must be carried out accurately to ensure that the daughter cells receive the single complete genome that they require to form fully functional cells after cell division (Bartosik and Jagura - Burdzy, 2005). The mechanism for bacterial chromosome segregation has not been fully elucidated and remains a subject of debate. However, a number of proteins and a *cis*-acting sequence have been found to be involved in the process (Bartosik and Jagura - Burdzy, 2005;Fekete and Chattoraj, 2005;Yamaichi and Niki, 2004).

1.5.1 Factors involved in chromosome segregation in *E. coli*

Faithful co-replicational segregation requires five distinct activities: DNA is pushed, directed, condensed, held and finally separated before cell division. The factors that have been discovered to be involved in bacterial chromosome segregation and their actions are summarised in Table 1.1 (Bartosik and Jagura - Burdzy, 2005).

Table 1.1. Factors involved in chromosome segregation in *E. coli* and their putative roles adapted from Bartosik and Jagura - Burdzy (2005).

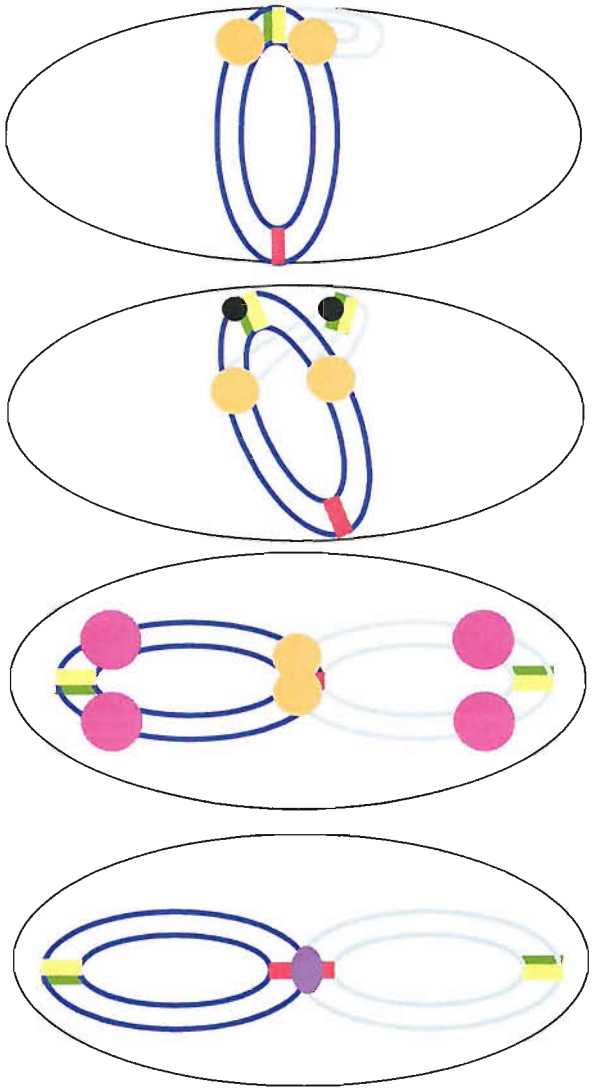
Name	Functions	Putative role	References
migS	A cis-acting, centromere like sequence which is essential for polar migration and the positioning of *oriC*.	Directing, holding	(Fekete and Chattoraj, 2005;Yamaichi and Niki, 2004)
SeqA	Replication initiation control, tethering the replication forks and DNA channelling during segregation	Directing	(Brendler *et al.*, 2000;Sawitzke and Austin, 2000)
MukB	Chromosome condensation/ recondensation, maintaining superhelicity	Condensing	(Holmes and Cozzarelli, 2000;Niki *et al.*, 1992)
HU and H-NS	Chromosome organization	Condensing	(Turn and Marko, 1998)
RNA polymerase	The driving force for bacterial chromosome segregation	Pushing	(Kruse *et al.*, 2006;Woldringh and Nanninga, 2006)
MreB	Directional chromosome movement and segregation	Directing, holding	(Gitai *et al.*, 2005;Jones *et al.*, 2001;Kruse *et al.*, 2003;Kruse and Gerdes, 2005;Margolin, 2005)
SetB	Chromosome segregation via interactions with MreB	Directing, holding	(Espeli *et al.*, 2003)
Min system	Regulation of division site placement	Directing	(Marston *et al.*, 1998;Shih *et al.*, 2003)
Tus	Replication termination, dimer resolution	Clearing	(Kamada *et al.*, 1996;Sahoo *et al.*, 1995)
XerCD recombinase and Topo IV	Dimer resolution, decatenation	Clearing	(Barre *et al.*, 2001)
FRS also called KOPES	*cis*-acting sequences	Clearing	(Bigot *et al.*, 2005;Ptacin *et*

	bound by FtsK to participate in dimer resolution, decatenation and translocation of DNA through the division septum		*al.*, 2006)
FtsK	Dimer resolution, decatenation, DNA translocation through the septum	Clearing	(Aussel *et al.*, 2002;Massey *et al.*, 2006;Yates *et al.*, 2006)

Many models have been proposed for bacterial chromosome segregation such as the factory model (extrusion - capture), the sister cohesion model and the bacterial mitosis model (Bartosik and Jagura - Burdzy, 2005;Margolin, 2005). The factory and sister cohesion models reflect a discrepancy in the data that exists regarding the timing of *oriC* separation. The factory model is based on the assumption that DNA movement and segregation is a consequence of the replication process and occurs concomitantly with it (Lemon and Grossman, 1998), while the sister cohesion model is based on the assumption that bacterial chromosome replication does not provide the driving force for segregation (Sunako *et al.*, 2001). However, the discrepancy in the data that exists regarding the timing of *oriC* separation may be the result of observations made using different experimental conditions and may reflect the use of a bimodal mechanism for chromosome segregation. In a bimodal mechanism for chromosome segregation, one part of the chromosome is segregated in a continuous manner and another part is subject to a delay prior to segregation.

In recent experiments, Neilsen *et al.,* 2006 followed 14 loci around the bacterial chromosome during segregation and observed that most markers were segregated in a continuous manner, but the terminus and a region immediately clockwise of the origin of replication was not. In contrast, these regions (which contain the proposed *E. coli* centromere, the migration site, *migS* (Fekete and Chattoraj, 2005;Nielsen *et al.*, 2006)) were subject to a much more extensive delay before segregation (Nielsen *et al.*, 2006). A bimodal mechanism for

chromosome segregation based on the observations of Neilsen *et al.*, 2006 seems highly plausible as it consolidates many of the existing theories regarding bacterial chromosome segregation by indicating the involvement of a sister loci cohesion period, the proposed *E. coli* centromere *migS* and the bacterial cytoskeletal protein MreB (Fekete and Chattoraj, 2005;Margolin, 2005;Nielsen *et al.*, 2006). In addition, such a mechanism would be consistent with the structural and physical aspects of bacterial chromosome segregation (Woldringh and Nanninga, 2006). Figure 1.4 summarises the mechanism of bacterial chromosome segregation.



At the initiation of chromosomal replication *oriC* is positioned at the mid cell and newly replicated DNA accumulates there. The *oriC* regions remain together prior to their separation for the sister cohesion period.

After the sister cohesion period the replicated origins are separated and moved outward, it is likely that *migS*, MreB and RNA polymerase are involved in this process. The rest of the bacterial chromosome is then segregated in a continuous manner following the path of the DNA arms.

Following its segregation the bacterial chromosome is condensed and organised by MukB and the Histone-like proteins HU and H-NS.

When replication is complete the terminus regions remain together, probably through the association of the FRS regions on the chromosome and the DNA translocase FtsK. FtsK activates dimer resolution to separate the chromosomes and finally completes chromosome segregation by translocating the remaining DNA through the division septum

Figure 1.4. The mechanism of bacterial chromosome segregation.

Figure 1.4. The mechanism of bacterial chromosome segregation. The ovals represent bacterial cells, the bacterial chromosomes are depicted in blue, dark blue for the original chromosome and light blue for the newly synthesized DNA. The origin is depicted in yellow, *migS* was depicted in green and the terminus region of the chromosome is depicted in red. DNA polymerase is depicted as orange circles, the MreB protein is depicted as black circles and the chromosome condensation proteins MukB, HU and H-NS were depicted as pink circles. The DNA translocase protein FtsK is depicted as a purple oval.

However, in preparation for cell division a newly formed cell must double all of its contents and prior to cell division all these contents must be distributed between the two daughter cells to ensure that both cells have all the components they need to survive and reproduce (Cooper, 1996). With this in mind the segregation of the other cellular components will now be briefly discussed.

1.5.2 Segregation of the other cellular components

To examine how the cell contents are distributed between the daughter cells, the cell itself must be divided into its three separate components: the cytoplasm, the genome and the cell surface (Cooper, 1996). The cell cytoplasm is segregated randomly into each of the daughter cells (Cooper, 1996). The segregation pattern of the cell surface is more complex. The side wall material in *E. coli* cells is segregated in a dispersive manner, i.e. each daughter cell gets 50% new glycan in their side walls. But the cell poles behave differently, once formed they are stable, and show a conservative segregation pattern. This means that each new daughter cell receives, one new cell pole (created during the closure of the division septum) and one old pole from the original parent cell (Bramhill, 1997;Cooper, 1996). Following the successful segregation of the cell contents the cell must divide, the molecular mechanism of bacterial cell division will be discussed next.

1.6 Cell division

Bacterial cell division requires the cooperative action of a large number of proteins to successfully constrict the cell centre and form a new cell wall to divide the daughter cells (Bramhill, 1997). However, bacterial cell division must be performed accurately to ensure that fully functional daughter cells are formed. The mechanism of bacterial cell division has yet to be fully elucidated, although, a number of cell division genes in *E. coli* have been identified (Bramhill, 1997). These studies isolated conditional lethal mutants that formed long non-septate filaments at restrictive temperature. This approach relied on the ability of the bacteria to continue to elongate the cylindrical portion of the cell, without cell division. The designation chosen for these mutations was *fts* (Filament forming Temperature Sensitive) (Bramhill, 1997).

The identification and characterisation of these genes has facilitated the elucidation of many of the key mechanistic elements of bacterial cell division (Bramhill, 1997;Goehring and Beckwith, 2005). These include: a mechanism for defining the division site, a mechanism for the formation and stabilisation of the division septum (at the division site) and a mechanism for the recruitment and assembly of a multiprotein complex at the division septum, which includes all the enzymes necessary for the separation of the cell to form two daughter cells (Bramhill, 1997;Goehring and Beckwith, 2005).

1.6.1 The definition of the division site (Z-ring localisation)

Two factors are important to cell division site placement, the Mini cell producing (Min) system and Nucleoid occlusion. Both these systems interfere with the formation of the Z-ring which is thought to define the division site. The nucleoid occlusion model proposes that the presence of the chromosome mass, in the centre of the cell, exerts an inhibitory effect on cell division. Then, when chromosome segregation occurs, a chromosome free zone is created at the cell centre that allows formation of the septum, as shown in Figure 1.5 (Bartosik and Jagura - Burdzy, 2005). Relatively little is known about the mechanism of nucleoid occlusion in comparison to the well studied Min system. However, recent experiments identified the first nucleoid occlusion factor, SlmA. The SlmA protein was shown to have the ability to bind both the nucleoid and to polymers of FtsZ which form the division septum (Z-ring). In consideration of this, it was proposed that SlmA mediates nucleoid occlusion by binding the nucleoid and interfering with septum formation in its vicinity (Bernhardt, 2005).

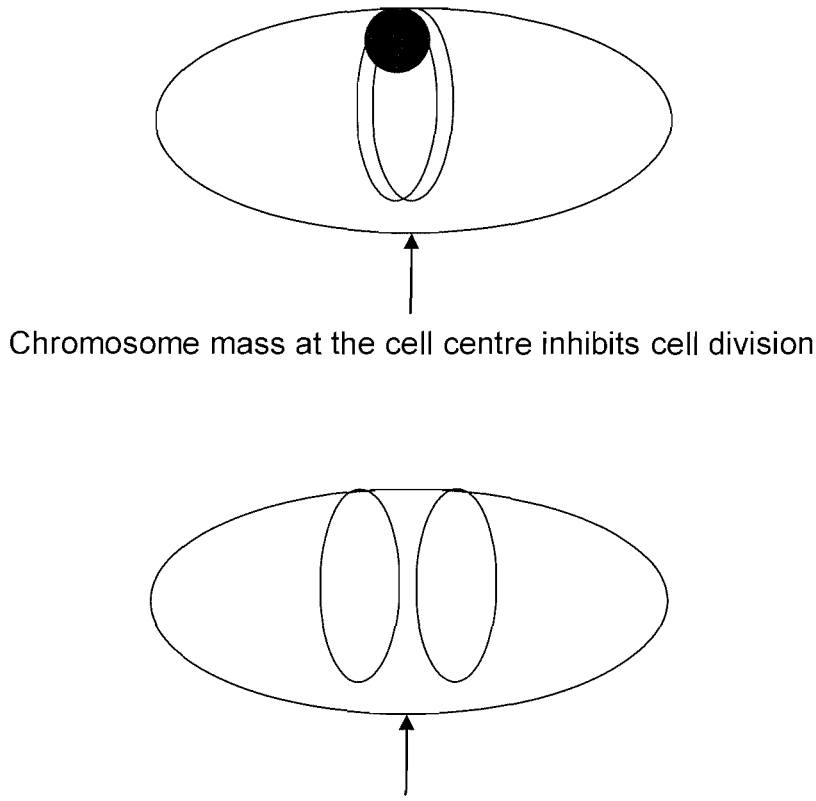


Figure 1.5. The nucleoid occlusion model for Z-ring localisation adapted from Bartosik and Jagura - Burdzy (2005). The large ovals represent cells, while the smaller ones represent chromosomes and the black circle represents the replication machinery.

1.6.2 The Min system

The Min system uses the three Min proteins: MinC, MinD and MinE, to prevent mini cell formation. Mini cells are formed by asymmetric cell divisions (at the cell poles), which produce small non-viable daughter cells, which do not contain chromosomes. MinC overexpression causes filamentous growth because MinC inhibits the formation of the FtsZ ring by preventing its polymerisation on the cell membrane (Bartosik and Jagura - Burdzy, 2005;Huang *et al.*, 2003).

MinD is a Walker type ATPase, which in the presence of ATP has the ability to bind both MinC (a division inhibitor) and the membrane. The MinCD complex forms a polar cap at one end of the cell; a MinE ring forms at the edge of the MinCD polar zone. This ring progresses towards the pole and ejects MinCD from the membrane by activating the MinD ATPase which leads to ADP release and the dissociation of the complex (MinCD and the membrane). After MinD nucleotide exchange, the MinCD complex assembles at the opposite cell pole. The MinE ring forms and the cycle progresses; by this mechanism the three Min proteins oscillate. The result of this oscillation is that the highest concentration of the division inhibitor MinC is localised to the cell poles, and its lowest concentration is at the cell centre (where the Z-ring is formed). This is represented in Figure 1.6 (Bartosik and Jagura - Burdzy, 2005;Huang *et al.*, 2003).

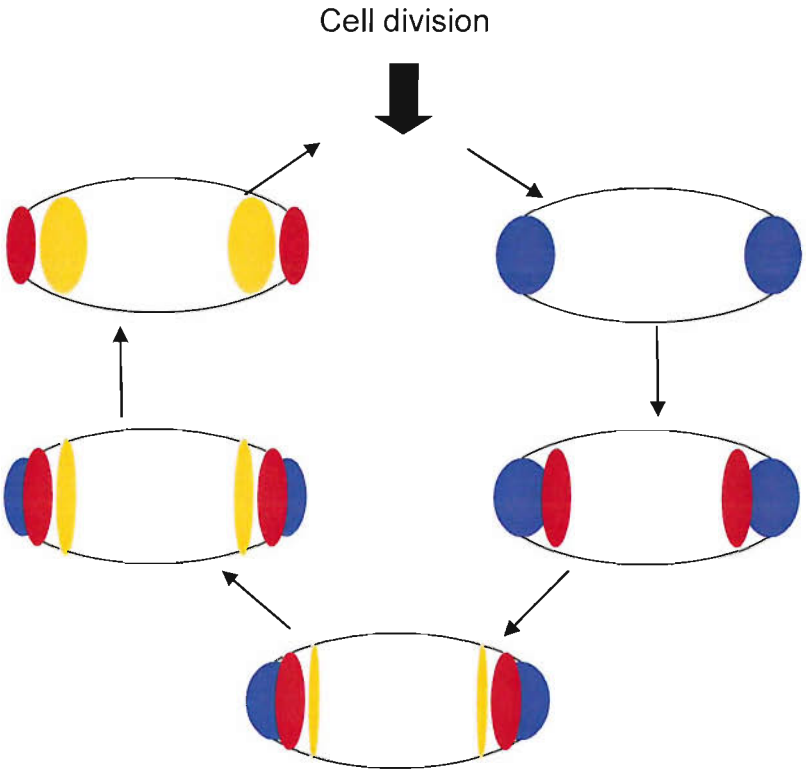


Figure 1.6. The Min system adapted from Bartosik and Jagura - Burdzy (2005). The blue circles represent the MinCD complex, the red ovals represent the MinE ring and the yellow ovals represent MinC.

The main role of the Min system is to prevent cell division at the nucleoid free cell poles, rather than directly defining the site of Z-ring formation (Kerr *et al.*, 2006). The combined activity of both nucleoid occlusion and the Min system are required for the definition of the division site (Bartosik and Jagura - Burdzy, 2005;Goehring and Beckwith, 2005;Huang *et al.*, 2003).

Once a bacterium has defined the site at which it will divide it starts the process of cell division. A summary of the process of cell division is presented in Figure 1.7.

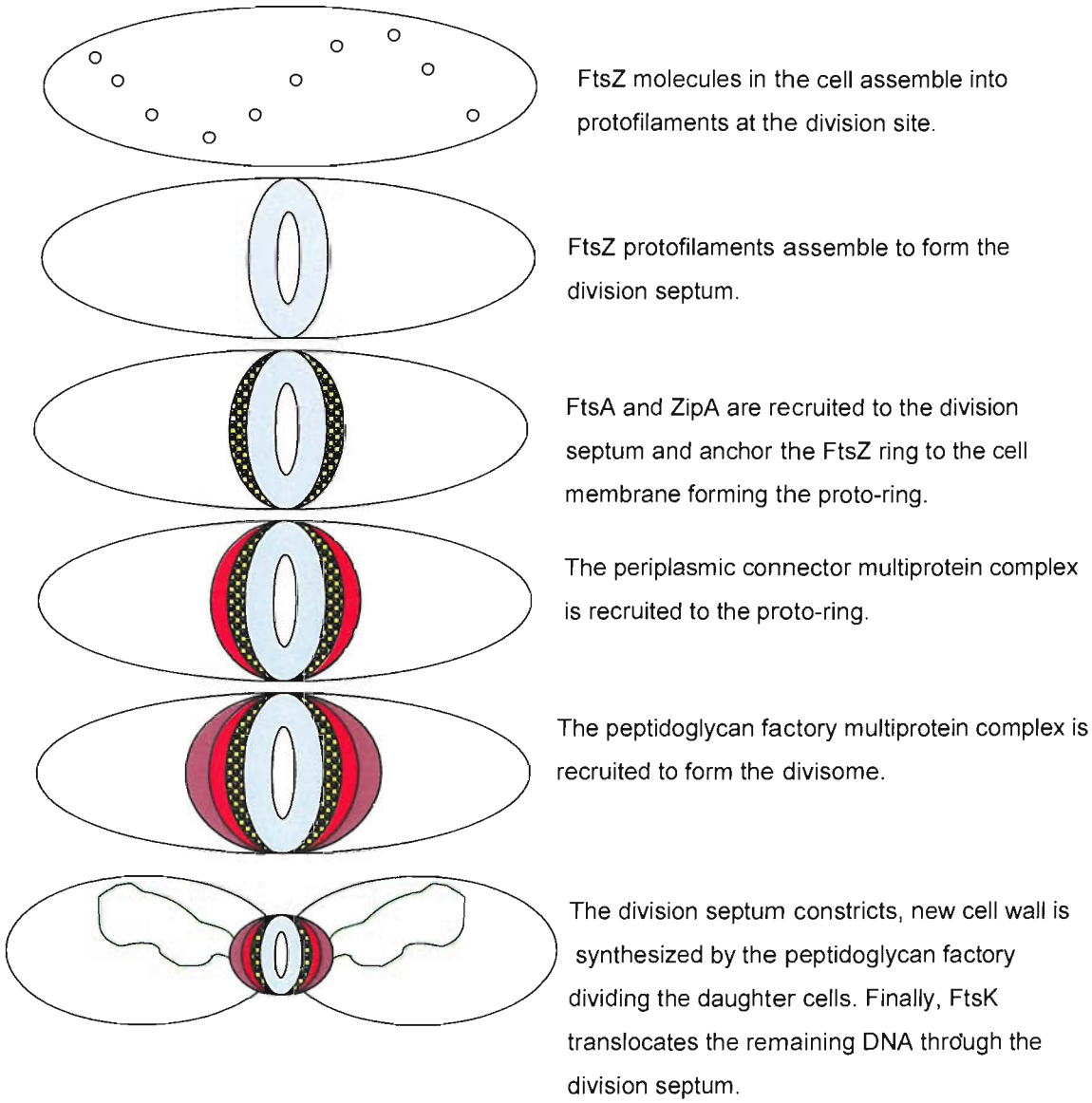


Figure 1.7. Cell division in *E. coli* (Bramhill, 1997;Goehring and Beckwith, 2005;Vicente and Rico, 2006). FtsZ molecules are depicted in blue, ZipA and FtsA are depicted in yellow and black. The periplasmic connector multiprotein complex is represented in red and the peptidoglycan factory multiprotein complex is depicted in purple. Bacterial DNA is depicted in green.

The first step in the process of bacterial cell division is the construction of the structure (the division septum) that will separate the cell to yield two daughter cells, this will be discussed next.

1.6.3 The formation and stabilisation of the division septum

Essential to the formation (and function) of the division septum is, the most important cell division gene, *ftsZ* (Bramhill, 1997;Goehring and Beckwith, 2005). The product of this gene, FtsZ, forms a ring at the cell centre, just prior to cell division to create the division septum (Amos *et al.*, 2004;Bramhill, 1997). There are approximately between 5,000 and 20,000 FtsZ molecules per cell in *E. coli*. This is enough to circle the mid point of the cell 10 to 40 times. This intracellular FtsZ level (enough to form a cytoskeleton) is crucial for normal cell division; a reduction prevents division, while a small increase causes increased division events, resulting in a mini cell phenotype. A large excess of FtsZ however prevents division, resulting in a filamentous phenotype (Bramhill, 1997;Lutkenhaus and Mukherjee, 1996).

A substantial amount of evidence points towards a role for the FtsZ ring in cell constriction (Bramhill, 1997;Esue *et al.*, 2005;Osawa and Erickson, 2006;Wang and Lutkenhaus, 1996), although the mechanism has not been fully elucidated. Recent studies have proposed that cell division occurs by a two-part mechanism. First the FtsZ ring (division septum) forms and is anchored to the membrane, where it may generate the cell constriction force through self interactions. Second, the downstream division proteins remodel the peptidoglycan cell wall (Osawa and Erickson, 2006;Esue *et al.*, 2005). However, the FtsZ ring may have more than one role in the process of cell division; Aarsman *et al.* (2005) proposed that the division septum is involved in the switch from cylindrical to polar peptidoglycan synthesis.

The FtsZ ring that forms the division septum must be stabilised and tethered to the cell membrane in order for it to be able to constrict. Two proteins have been shown to tether the FtsZ ring to the cell membrane FtsA and ZipA (Bramhill, 1997;Hale and de Boer, 1999;Pichoff and Lutkenhaus, 2005). These proteins are very important to cell division as when both FtsA and ZipA are inactivated, the FtsZ ring cannot form (Bramhill, 1997;Hale and de Boer, 1999;Pichoff and Lutkenhaus, 2005). However, it has been proposed that in addition to tethering the FtsZ ring to the membrane, the interaction of ZipA with FtsZ may influence the structure of the FtsZ ring itself and therefore may serve more than one purpose (Hale *et al.*, 2000).

1.6.4 The mechanism for the recruitment and assembly of a multiprotein complex at the division septum

After construction and stabilisation of the division septum, the enzymes necessary for cell separation and the formation of the two daughter cells must be recruited to the division septum. These components, known as the late division proteins, are essential for the final stages of cell division, such as chromosome segregation and cell wall synthesis. However, many of the late division proteins are currently of unknown function (Bramhill, 1997;Goehring and Beckwith, 2005). It was previously thought that the recruitment of the division proteins to the mid cell occurred according to a linear hierarchy (Bramhill, 1997). However, recent studies have revealed that the late division proteins do not necessarily associate sequentially with the division septum, the late division proteins that associate with each other may do so in the cytoplasm prior to the formation of the division septum and be recruited as a multiprotein complex. The exception to this was FtsN which required both the late and early components to associate (Goehring and Beckwith, 2005;Goehring *et al.*,

2005;Goehring *et al.*, 2006). This unique selectivity of FtsN led to the consideration that it may perform a regulatory role, to ensure that cell division does not go ahead until the multiprotein complex, located at the division septum (the divisome), is complete (Goehring *et al.*, 2005). These studies revealed that three multiprotein complexes form and associate to form the divisome, 1) the proto-ring (FtsZ, ZipA and FtsA), 2) the periplasmic connector (see Table 1.2 for proteins), and 3) the peptidoglycan factory (see Table 1.3 for proteins) (Goehring and Beckwith, 2005;Goehring *et al.*, 2006;Vicente and Rico, 2006).

Table 1.2. Proteins that make up the periplasmic connector multiprotein complex (Goehring and Beckwith, 2005;Goehring *et al.*, 2006;Vicente and Rico, 2006).

Protein name	Function
FtsK	Chromosome segregation
FtsQ	Unknown
FtsB	Unknown
FtsL	Unknown

Table 1.3. Proteins that make up the peptidoglycan factory multiprotein complex (Goehring and Beckwith, 2005;Goehring *et al.,* 2006;Vicente and Rico, 2006).

Protein name	Function
FtsW	Unknown
FtsI also known as PBP3	Division specific transpeptidase
FtsN	Unknown
AmiC	Amidase required for cell separation

1.6.5 Other proteins that influence cell division

Chaperonin proteins are also important to the process of cell division as imbalances in a number of chaperonin proteins block cell division. Mutants of *dnaK* divide poorly, and expression of an ATPase mutant of the DnaK protein blocks cell division. The sensitivity of cell division to the concentration chaperonin proteins, may be due to their influence on FtsZ levels, or chaperonins may play an additional role in cell division. There is some evidence for this, as recently Weart *et al.* (2005) found that the ClpX chaperone regulates FtsZ ring assembly in *Bacillus subtilis*. It was speculated that it may function as a general regulator of FtsZ ring formation in bacteria (Bramhill, 1997;Weart *et al.*, 2005).

1.6.6 Control of the expression of the cell division genes

The transcription of the cell division genes, including *ftsZ*, is tightly regulated by so-called gearbox promoters which ensure that the overall level of all the proteins is in balance with the total cell mass. FtsZ levels fluctuate periodically through the cell cycle by approximately 50%. Although this is not a major regulatory mechanism, it gives measurable differences in the cell's ability to divide. The *sdiA* gene encodes a transcriptional activator which acts on a promoter upstream of *ftsQ,* leading to increased FtsZ levels. The *sdiA* gene in turn responds to external signals secreted by bacteria. The *sdiA* mediated activation of *ftsQAZ* expression occurs at low cell densities. As the cellular levels of ppGpp (which is involved in the stringent response) increase, it affects cell division by increasing the transcription of *ftsZ* and *ftsA* through RNA polymerase interactions (Bramhill, 1997).

1.7 Cell division and cell shape

Different bacteria have different cell shapes which have been used both to classify and to describe bacterial species (Cabeen and Jacobs-Wagner, 2005). It is currently thought that the cytoskeleton and the cell walls of a bacterial cell are responsible for cell shape (Cabeen and Jacobs-Wagner, 2005). However, an efficient and reliable cell division system is also important to the maintenance of cell shape, as defects in cell division often alter cell shape (Bramhill, 1997;Cabeen and Jacobs-Wagner, 2005;Huang *et al.*, 2003).

In rod-shaped bacteria, such as *E. coli* and *Salmonella*, an interruption in cell division often results in cellular filamentation. Environmental stresses and shocks that induce filamentation include: mild temperature stress, pH or oxidative stress and exposure to high-hydrostatic pressure environments (Brandi *et al.*, 1989;Ishii *et al.*, 2004;Mattick *et al.*, 2003b). The main intracellular causes of filamentation are disruption of DNA synthesis or segregation, disruption of cell division or, DNA damage mediated induction of the SOS response which controls the cell division inhibitor SulA (Frandsen and D'Ari, 1993). The causes of filamentation are summarised in Figure 1.8.

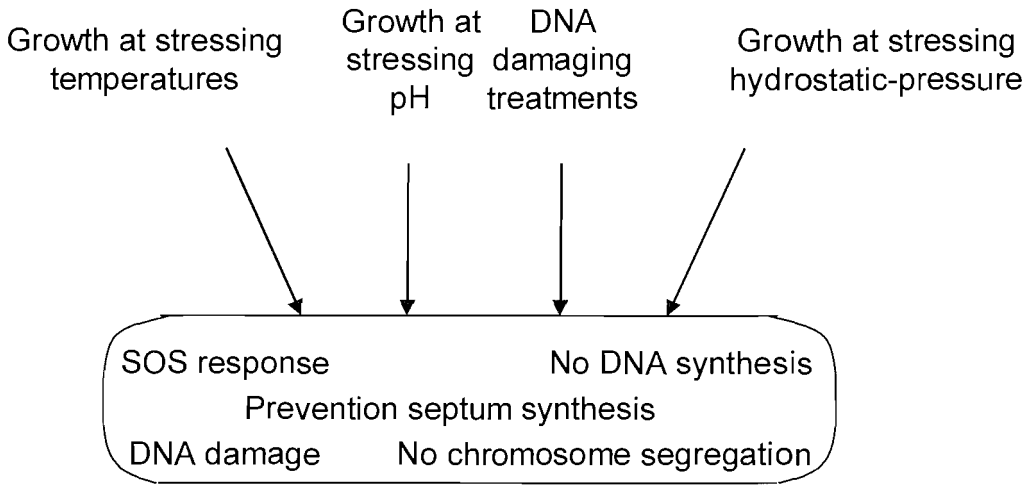


Figure 1.8. The intracellular and extracellular causes of filamentation.

However, the root cause of filamentation is usually a reduction in the expression of the essential division genes (Bi and Lutkenhaus, 1993;Delgado *et al.*, 2001;Gutherie and Wickner, 1990;Ito *et al.*, 2006;Vinella and D'Ari, 1994). Therefore, overexpression of the essential division genes often suppresses filamentation (Gutherie and Wickner, 1990;Jeong and Lee, 2003;Vinella and D'Ari, 1994). *Escherichia coli* possess mechanisms for altering the expression of their cell division genes in response to environmental stimuli (Figures 1.9 and 1.10) (Bi and Lutkenhaus, 1993;Sitnikov *et al.*, 1996).

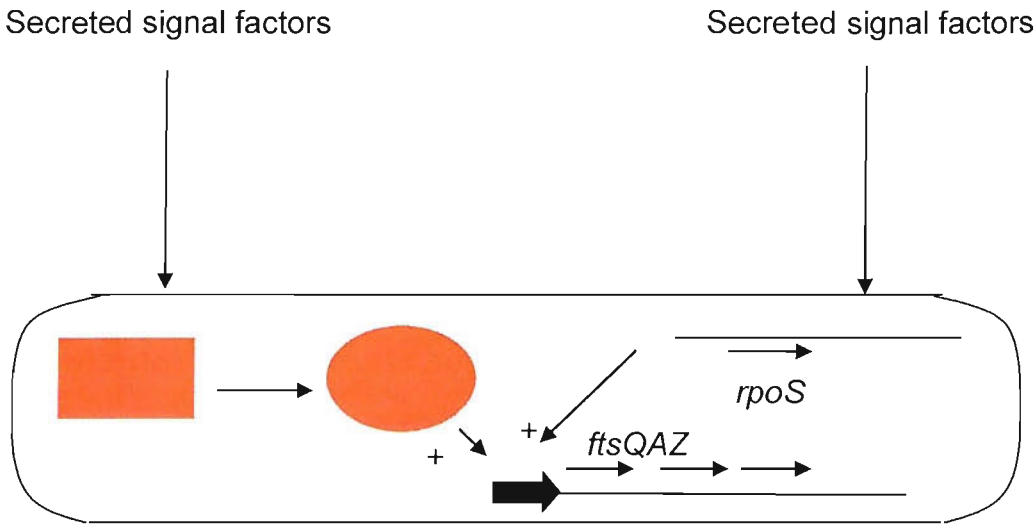


Figure 1.9. Quorum sensing influences the expression of the essential cell division genes, adapted from Sitnikov *et al.* (1996). The factor responsive SdiA protein is depicted as an orange square in its inactive state and an oval in its active state.

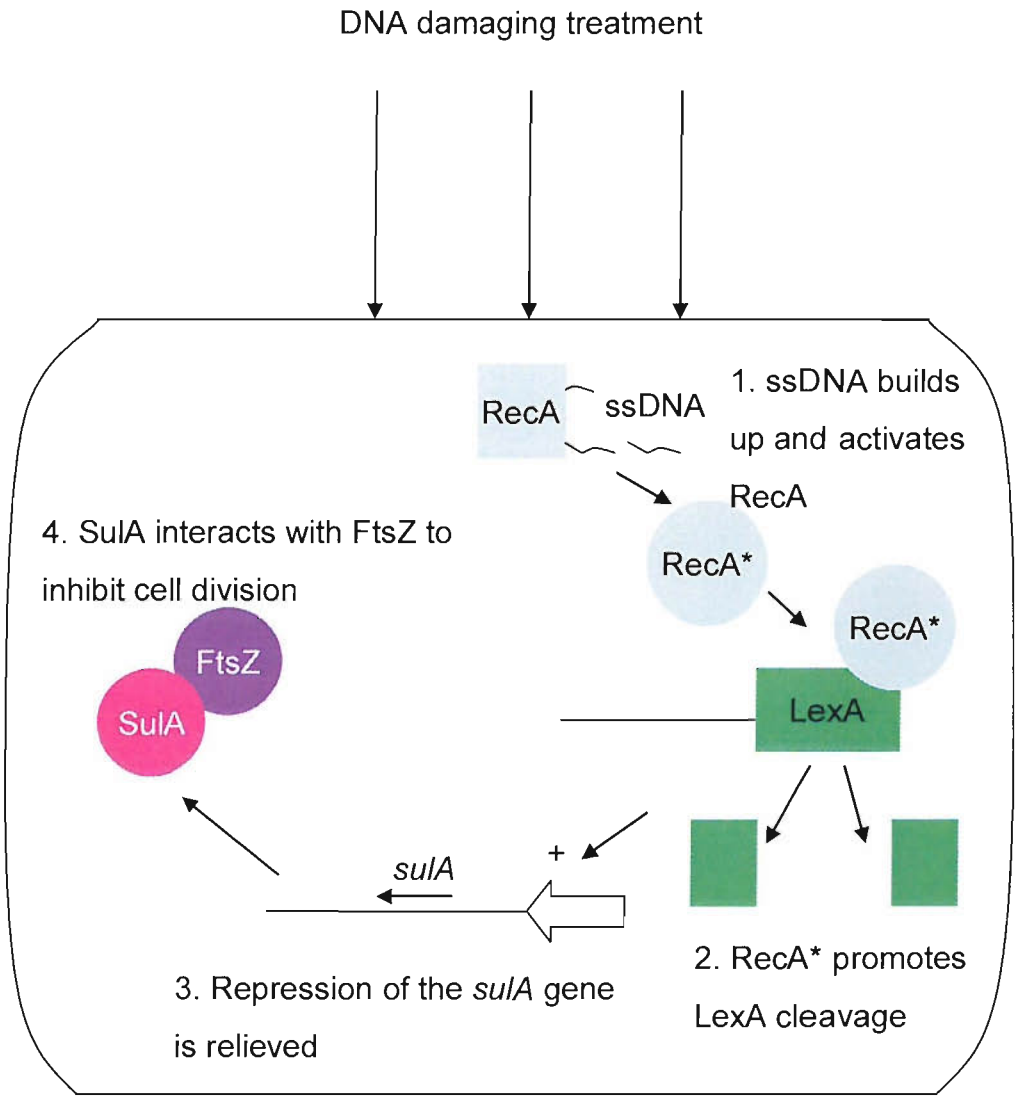


Figure 1.10. Bacteria produce the cell division inhibitor SulA in response to DNA damage. RecA is depicted as a blue square in its inactive form and an oval in its active state. The green rectangle and green squares depict LexA and its cleavage products respectively. SulA and FtsZ molecules are depicted in pink and purple respectively (Bi and Lutkenhaus, 1993;Walker, 1996).

Bacterial cells have the ability to increase or decrease their growth rate (as mentioned previously) to ensure their survival as the environmental conditions, which they find themselves in, change. Bacteria achieve this by altering the time it takes for them to double their cellular contents (as discussed previously) to either shorten or lengthen their interinitiation time (Helmstetter *et al.,* 1979;Helmstetter, 1996;Monod, 1949). The length of the interinitiation time is altered by changing the rate of protein synthesis in the cell, because proteins are the largest constituent of any bacterial cell. Bacterial cells alter their rate of cellular protein synthesis by changing the number of ribosomes (which are responsible for all cellular protein synthesis) per cell, this is achieved through the growth rate dependent regulation of ribosome synthesis (Dennis *et al.,* 2004) which is discussed next.

1.8 Bacterial growth and ribosome synthesis

To prepare for and support rapid cell growth, much of the cell mass must be devoted to the production of ribosomes in order to support the high rate of protein synthesis that must occur under such conditions (i.e. conditions that shorten the interinitiation time) (Cashel *et al.*, 1996;Dennis *et al.*, 2004). In rapidly growing bacteria ribosomes can account for up to 50% of the cellular dry mass (Zengel and Lindahl, 1994). However, ribosomes are structurally complex multi-component organelles consisting of three ribosomal ribonucleic acids, 5S RNA, 16S RNA and 23S RNA, and at least fifty ribosomal proteins (Cashel *et al.*, 1996;Zengel and Lindahl, 1994) and their production is energy expensive. Inappropriate over production of ribosomes is deleterious to cells (diverting the cells' limited resources to the production of unnecessary ribosomes reduces the cells' overall growth rate) (Stevenson and Schmidt, 1998). Bacteria avoid this situation by subjecting the production of ribosomal components to a complex regulatory system which couples ribosome production to growth rate (Cashel *et al.*, 1996).

1.8.1 Control of ribosome synthesis in *E. coli*

The global regulatory system that controls ribosome synthesis in *E. coli* directly controls the rate of ribosomal RNA synthesis from the seven ribosomal RNA operons contained in the *E. coli* genome. The rate of ribosomal protein synthesis is negatively regulated by free ribosomal proteins which reduce both the translation rate and the stability of the messenger RNAs for ribosomal proteins. The concentration of free ribosomal protein is dependent on the level of free ribosomal RNA available for it to associate with which means that the control of the production of ribosomal RNA indirectly controls the production of

ribosomal proteins and therefore regulates ribosome synthesis as a whole (Dennis *et al.*, 2004;Keener and Nomura, 1996;Lindahl and Zengel, 1986).

E. coli uses a number of regulatory mechanisms to ensure that ribosome synthesis is adjusted according to the cellular growth rate, and the interplay of these mechanisms ensure that the control of rRNA transcription is very robust. A change made to one aspect of one system will often induce a compensatory change to restore the correct level of rRNA transcription and ribosome synthesis (Gaal and Gourse, 1990;Jinks - Robertson *et al.*, 1983;Nomura *et al.*, 1984;Paul *et al.*, 2004b). Extensive research has led to the formation of many hypotheses for the control ribosome synthesis. However, certain mechanistic details remain unclear despite the discovery of many of the key regulatory mechanisms used to control ribosomal RNA synthesis (Cashel *et al.*, 1996;Dennis *et al.*, 2004;Gralla, 2005;Keener and Nomura, 1996;Zhang X *et al.*, 2006).

1.8.2 Ribosomal RNA promoter regions

Ribosomal RNA promoter regions show many unique features which are important to their regulation. Each of the seven ribosomal operons in *E. coli* are transcribed from two promoters, Ribosomal RNA promoter 1 (*rrn* P1) and Ribosomal RNA promoter 2 (*rrn* P2). *rrn* P1 promoters are stronger than *rrn* P2 promoters at moderate to rapid growth rates but both *rrn* P1 and *rrn* P2 have been shown to be regulated by similar mechanisms. However, *rrn* P1 is regulated to a greater extent than *rrn* P2 which means that in exponential phase cells, due to greater stimulation, most transcripts originate from *rrn* P1 and in stationary phase cells, due to a lesser extent of repression, most transcripts originate from *rrn* P2 (Paul *et al.*, 2004b).

The core ribosomal RNA promoters show consensus, or near-consensus, -10 and -35 hexamers for recognition by the sigma 70 subunit of the RNA polymerase holoenzyme. However, these promoters also contain a non-consensus 16 base pair spacer region and a CG-rich discriminator sequence located immediately downstream of the -10 hexamer. These unique features result in the formation of unstable short-lived transcription complexes, which is key to their regulation (Gralla, 2005;Paul *et al.*, 2004b).

1.8.3 Ribosomal RNA P1 promoters

rrn P1 promoters show specific features which add to their strength and are integral to their regulation. The organisation of these promoters is illustrated in Figure 1.11. The key features are: the three to five Fis binding sites, and the UP element (Paul *et al.*, 2004b). These features will be discussed in the following sections.

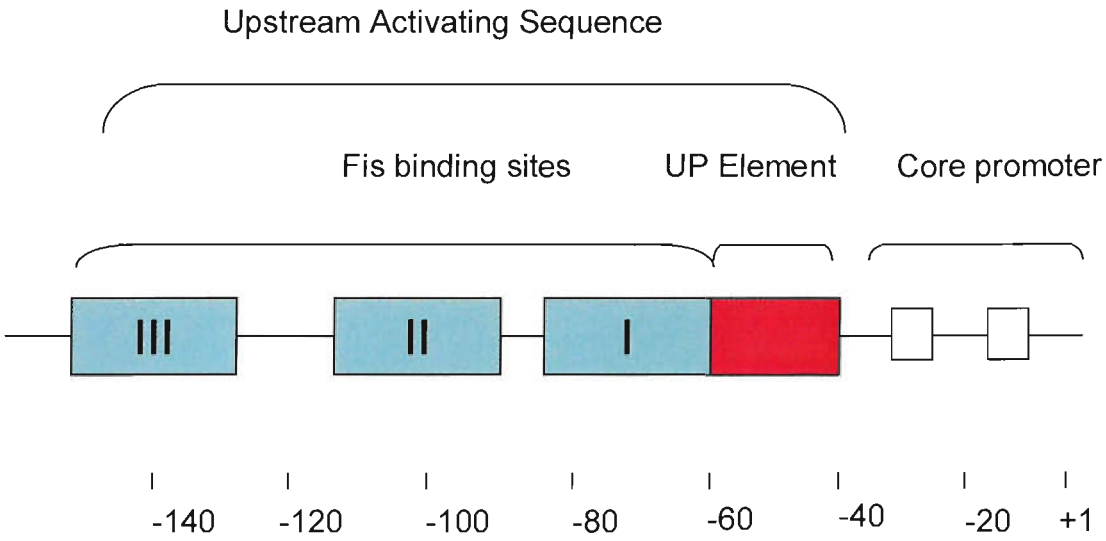


Figure 1.11. Organisation of the *E. coli rrnB* P1 promoter. The upstream activating sequence (UAS) includes the UP element, shown in red, and three Fis binding sites, shown in light blue. The core promoter contains the -10 and -35 recognition hexamers for RNA polymerase, depicted as squares (Bokal *et al.,* 1995;Paul *et al.,* 2004b).

1.8.4 UP element

The AT-rich UP element greatly increases the strength of ribosomal RNA promoters because it interacts with the alpha subunit of the RNA polymerase holoenzyme in a manner that stabilises the promoter RNA polymerase complex and stimulates transcription (Paul *et al.*, 2004b).

1.8.5 Fis binding sites

The transcriptional activator protein Fis binds to the three to five Fis binding sites upstream of ribosomal RNA promoters and stimulates transcription (Paul *et al.*, 2004b;Richins and Chen, 2001;Ross *et al.*, 1990). The Fis mediated activation is due to the stabilisation of the transcription complex and the promotion of the initial steps in the transcription initiation pathway (Bokal *et al.*, 1995;Bokal *et al.*, 1997;Paul *et al.*, 2004b;Zhi *et al.*, 2003). However, binding sites for H-NS overlap the Fis binding sites which means that Fis and H-NS compete for DNA binding in this region. Unlike Fis, H-NS inhibits transcription from ribosomal RNA promoters by inducing the formation of dead-end complexes between RNA polymerase and the promoter, which cannot perform elongation (Afflerbach *et al.*, 1998;Afflerbach *et al.*, 1999;Gralla, 2005;Paul *et al.*, 2004b).

1.8.6 The mechanism of control of ribosomal RNA promoters in *E. coli*

The currently known regulators that control ribosomal RNA transcription, and thereby ribosome synthesis, are listed in Table 1.4. These regulators consist of: two nucleotide regulators (iNTP and ppGpp) which act on RNA polymerase, in conjunction with the RNA polymerase binding co-regulatory protein DksA, to increase or decrease the activity of ribosomal RNA promoters; and two regulatory proteins Fis and H-NS, which bind upstream of ribosomal RNA promoters and either stimulate (Fis) or inhibit (H-NS) transcription from ribosomal RNA promoters (Gralla, 2005;Paul *et al.*, 2004b).

Table 1.4. Key regulators used in the control of ribosomal RNA transcription in *E. coli*.

Regulator	Type of regulator	Action in the regulation of rRNA transcription
iNTP	Stimulatory nucleotide regulator	When present, in conjunction with DksA, it acts on RNA polymerase to stimulate transcription from rRNA promoters (Gralla, 2005;Paul *et al.*, 2004b).
ppGpp	Inhibitory nucleotide regulator	When present, in conjunction with DksA, it acts on RNA polymerase to inhibit transcription from rRNA promoters (Gralla, 2005;Nomura *et al.*, 1984).
DksA	Nucleotide co-regulatory protein	Stimulates or inhibits rRNA transcription by binding to RNA polymerase in conjunction with the relevant nucleotide regulator (iNTP or ppGpp) (Gralla, 2005;Paul *et al.*, 2004b;Paul *et al.*, 2004a).
Fis	Stimulatory protein	When present, Fis binds to the Fis binding sites present upstream of rRNA promoters and interacts with RNA polymerase stabilising its binding to rRNA promoters and stimulates transcription (Bokal *et al.*, 1997;Gralla, 2005;Ross *et al.*, 1990;Zhi *et al.*, 2003).
H-NS	Inhibitory protein	When present, H-NS binds to sites upstream of rRNA promoters (overlapping the Fis binding sites) and inhibits transcription by locking the promoter-bound RNA polymerase into an inactive complex (Afflerbach *et al.*, 1998;Gralla, 2005).

The action of these regulators is coordinated by changes in their cellular levels, which are growth phase dependent as illustrated in Figure 1.12 (Paul *et al.*, 2004b).

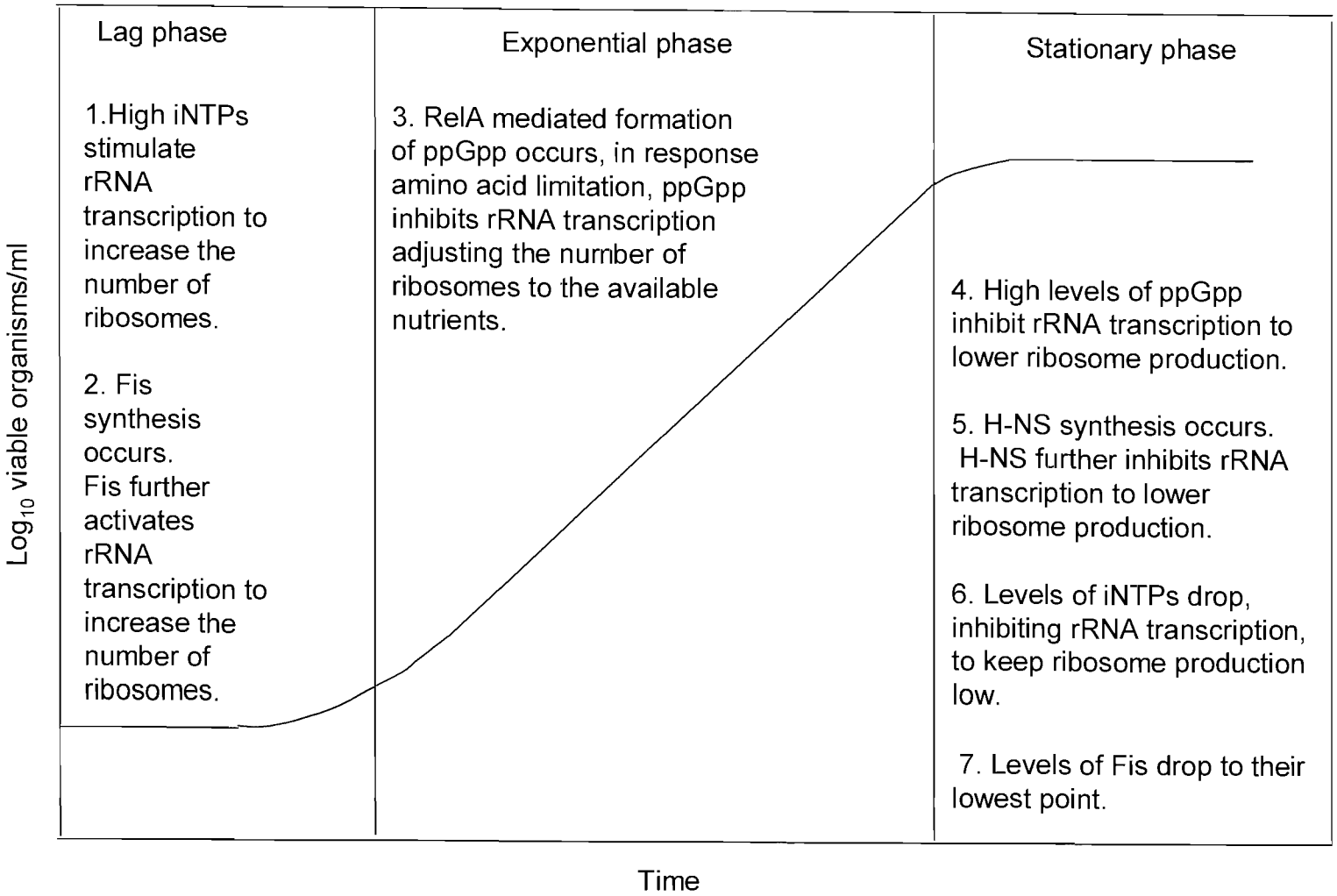


Figure 1.12. Control of ribosome synthesis adapted from Paul *et al.* (2004b).

The control of ribosome synthesis in each phase of growth (as illustrated in Figure 1.12) will now be discussed in further detail, by discussing each growth phase in turn.

1.8.7 Lag phase

In the lag phase of growth, after inoculation into fresh media, the level of free initiating nucleotide triphosphates (ATP and GTP for ribosomal P1 promoters) will be high. This means that the initiating nucleotide triphosphates (iNTPs) can bind to the RNA polymerase holoenzyme (in close proximity to its active site) in conjunction with the RNA polymerase binding protein DksA (Nickels and Hochschild, 2004;Paul *et al.*, 2004a). This ensures that the requirement of ribosomal RNA promoters for high concentrations of initiating nucleotide triphosphates is satisfied, thereby relieving this constraint and facilitating maximal activity. The specific action of initiating nucleotide triphosphates in stimulating transcription from ribosomal RNA promoters has not yet been fully elucidated, but it may involve a transient stabilisation of the RNA polymerase promoter complex which promotes transcription initiation, or the presence of the iNTP may induce a conformational change in the complex, converting it to a complex capable of transcription initiation (Gralla, 2005;Paul *et al.*, 2004b).

In the lag phase the level of the transcriptional activator protein Fis increases from less than 100 to over 50,000 copies per cell (before the first cell division) (Ball *et al.*, 1992;Osuna *et al.*, 1995). Recent experiments have demonstrated the importance of DksA and the initiating nucleotide triphosphate (CTP) in transcription from the Fis promoter (Mallik *et al.*, 2006). Considering this, it is tempting to speculate that the high levels of free initiating nucleotide triphosphates in the lag phase induce Fis synthesis. When it is present, Fis binds to the Fis binding sites upstream of ribosomal RNA promoters and

stimulates transcription through RNA polymerase recruitment and aiding the initial steps of transcription initiation (Bokal *et al.*, 1995;Bokal *et al.*, 1997;Paul *et al.*, 2004b;Richins and Chen, 2001;Ross *et al.*, 1990;Zhi *et al.*, 2003). The actions of iNTPs and Fis increase ribosome synthesis in the lag phase to facilitate faster growth and take advantage of the nutrient rich conditions. However, Fis synthesis is largely shut off as the cells enter exponential growth, and Fis levels drop as a function of cell division (Ball *et al.*, 1992;Osuna *et al.*, 1995). Therefore, as cells enter exponential growth the Fis induced stimulation of ribosomal promoters is removed, and the initiating nucleotide triphosphate level remains constant which means that another regulator must take control of ribosome synthesis (Gralla, 2005;Paul *et al.*, 2004b).

1.8.8 Exponential phase

During the exponential phase of growth ribosome synthesis is controlled by guanosine 5'-diphosphate 3'- diphosphate (ppGpp) and the stringent response (Gralla, 2005;Paul *et al.*, 2004b). The stringent response is provoked when the cell faces amino acid starvation, which causes an increase in the amount of uncharged tRNAs present in the cell. This means the cell does not contain sufficient amino acylated tRNAs to meet the rate of protein synthesis, which can result in the presence of a "hungry codon" in the translating ribosome. This "hungry codon" can be non-enzymatically bound to a cognate uncharged tRNA at the acceptor site (although binding is weak in comparison to binding of the cognate charged tRNA at this site), which prevents translation and causes the ribosome to stall in an inactive state. In the exponential phase the stringent response is primarily mediated by the RelA protein. The RelA protein detects stalled ribosomes via their 3' extrusion of mRNA, where upon it transfers the β,γ - phosphates of ATP to GDP or GTP to yield guanosine 5'-triphosphate 3'-diphosphate (pppGpp) and guanosine 5'-diphosphate 3'- diphosphate (ppGpp).

RelA is then released from the ribosome and it can "hop" to the next stalled ribosome where the cycle is repeated (Figure 1.13). The resulting high levels of pppGpp and ppGpp, collectively referred to as (p)ppGpp, alter gene expression by repressing genes associated with the translational apparatus (including ribosomal RNA transcription) and stimulating the expression of genes encoding metabolic enzymes, particularly those involved in amino acid biosynthesis (Wendrich *et al.*, 2002).

ppGpp alters gene expression at various promoters through binding to the RNA polymerase holoenzyme in the secondary channel close to its active site (Artsimovitch *et al.*, 2004). However, its effect is significantly amplified by the binding of the RNA polymerase binding protein DksA (Paul *et al.*, 2004a), which is believed to stabilise the RNA polymerase ppGpp complex (Figure 1.13) (Peredina *et al.*, 2004). It is further proposed that ppGpp and DksA use this mechanism to regulate all genes within the ppGpp responsive regulon and that the sequence of the promoter region itself determines both the regulatory outcome (inhibition or stimulation) and the extent of the effect (Artsimovitch *et al.*, 2004;Paul *et al.*, 2004b;Peredina *et al.*, 2004). In support of this, it has been shown that ppGpp and DksA directly stimulate transcription from amino acid biosynthetic promoters (Paul *et al.*, 2005).

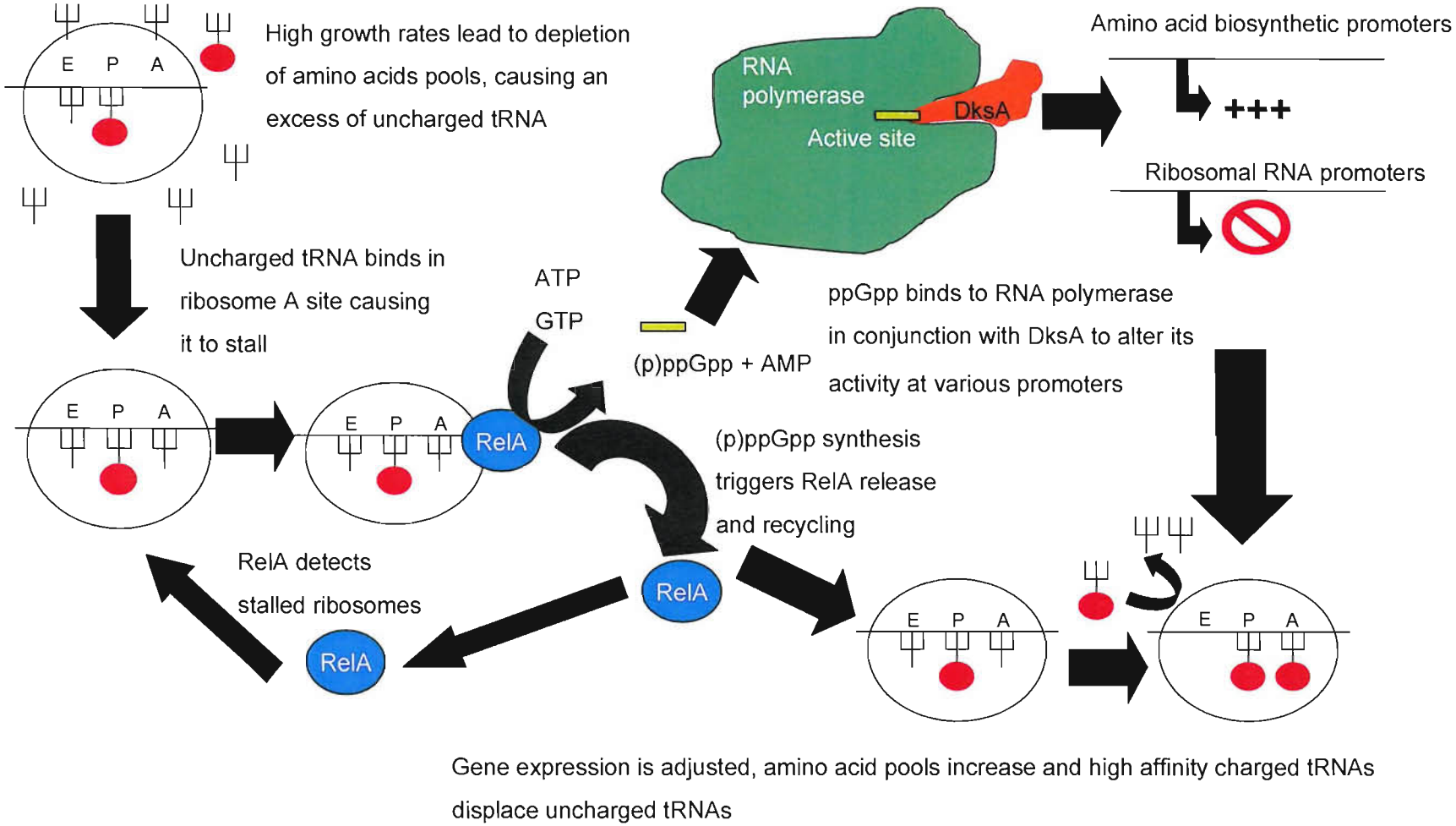


Figure 1.13. Stringent control of ribosomal RNA operons in exponential phase

Figure 1.13. Stringent control of ribosomal RNA operons in exponential phase based on diagrams presented by Wendrich *et al.* (2002) and Gralla (2005). Ribosomes are represented by white circles, their A, P and E sites are indicated by the corresponding letters. Three pronged forks represent transfer RNAs (tRNAs), charged tRNAs have a red circle (depicting an amino acid) bound to them. The RelA protein is depicted as a light blue circle, ppGpp is depicted as a yellow rectangle, DksA is shown in orange and RNA polymerase is depicted in green.

However, as cells enter stationary phase the nucleoid-associated protein, which inhibits rRNA synthesis, H-NS is synthesized (Afflerbach *et al.*, 1998;Gralla, 2005;Paul *et al.*, 2004b). This means that the regulatory systems controlling ribosome synthesis change once again, this will be discussed next.

1.8.9 Stationary phase

As stated previously, high levels of H-NS repress ribosomal RNA transcription by binding upstream of the ribosomal RNA promoters and locking them into dead-end complexes. This action significantly contributes to the shut-off of ribosomal RNA transcription as cells enter the stationary phase (Afflerbach *et al.*, 1998;Gralla, 2005;Paul *et al.*, 2004b). The stringent response induced by the formation of ppGpp further inhibits the transcription of ribosomal RNA promoters at slow growth rates. The level of ppGpp is modulated by the SpoT protein which can degrade (p)ppGpp or synthesise it. At high growth rates the former process prevails. These and other observations have led to the proposal that the rate of translation of the *spoT* mRNA determines the conformation and ultimately the activity of the protein produced, with low growth rates facilitating the formation of the unstable (p)ppGpp synthetase (Zhang *et al.*, 2006).

As the cell goes further into stationary phase (p)ppGpp levels fall. However, the initiating nucleotide levels drop to very low levels, which efficiently represses transcription from ribosomal RNA promoters and prevents excessive ribosome synthesis (Gralla, 2005;Paul *et al.*, 2004b).

1.8.10 Other mechanisms important for the control of ribosome synthesis

Ribosome synthesis is also controlled at the level of transcription elongation by a mechanism known as antitermination (Paul *et al.*, 2004b). Antitermination is essential to ensure that the long untranslated ribosomal RNA operons are both fully and efficiently transcribed. The mechanism of ribosomal RNA antitermination allows RNA polymerase to read through potential rho-dependent termination signals present in ribosomal RNA operons. This is achieved through the interaction of RNA polymerase and various protein factors (including certain ribosomal proteins) at an anti-terminator site which results in the construction of an altered transcriptional complex which displays altered terminator recognition and transcription elongation properties (Paul *et al.*, 2004b;Torres *et al.*, 2004).

Both ribosomal RNA leader regions and spacer regions contain anti-terminator sites, the features of which include; *boxA*, *boxB* and *boxC* sequences in leader regions, and *boxA* and *boxB* sequences in spacer regions. These *cis*-acting sequences interact with trans-acting protein factors such as the N utilization substance proteins (NusA, NusB, NusE, NusG) and ribosomal protein S4 to modify the transcriptional complex (Torres *et al.*, 2004). It has been found that the *boxA* sequence is essential to antitermination. Therefore, it maybe responsible for the initial recruitment of protein factors to the antitermination site, through its binding to NusB and stabilisation of the complex by NusE, that ultimately creates the modified transcriptional complex which facilitates the transcription of ribosomal RNA operons (Greive *et al.*, 2005;Torres *et al.*, 2004).

However, the main focus of this study is the novel ribosome binding global regulatory protein, BipA, which was recently shown to control the expression of the transcription factor Fis. Fis regulates a number of important processes in the cell (Nilsson *et al.*, 1990;Zhi *et al.*, 2003;Merickel *et al.*, 2002;Ross *et al.*,

1990;Wold *et al.*, 1996), therefore, the structure, regulation and action of Fis will be discussed next.

1.9 Factor for inversion stimulation (Fis)

Fis is an important global regulatory protein in enteric bacteria (Merickel *et al.*, 2002) because it is integral to the regulation of many cellular processes that are essential to the growth, and ultimately the survival of microorganisms. The processes that involve Fis include ribosome synthesis, initiation of DNA replication and phage-mediated DNA recombination reactions (Huber *et al.*, 1985;Johnson and Simon, 1985;Johnson *et al.*, 1986;Nilsson *et al.*, 1990;Ross *et al.*, 1990;Ryan *et al.*, 2004). However, Fis was initially identified due to its ability to stimulate DNA inversion reactions (Huber *et al.*, 1985;Johnson and Simon, 1985;Johnson *et al.*, 1986;Kahmann *et al.*, 1985).

1.9.1 The structure of Fis

Fis is a 11.2 kDa site-specific DNA binding protein and naturally its structure is integral to its cellular activity. Early studies revealed that there are two functional regions in Fis, a C-terminal DNA binding region and a N-terminal region that is required for DNA inversion reactions (Osuna *et al.*, 1991) (Figure 1.14).

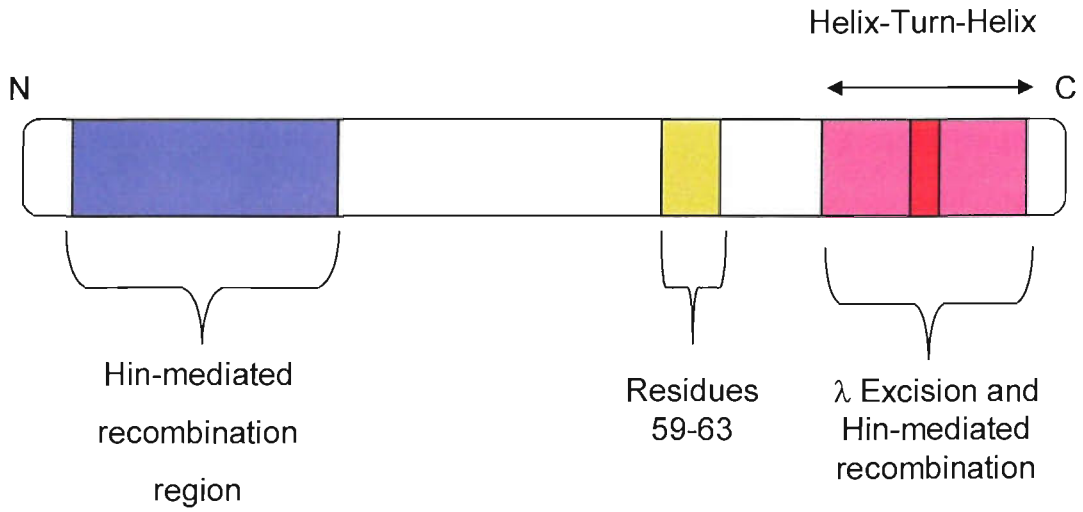


Figure 1.14. Functional organisation of Fis adapted from Osuna *et al.* (1991). The N and C termini are represented by N and C, the blue region represents the Hin mediated recombination region, while the pink and red regions depict the C-terminal DNA binding region.

A crystal structure by Kostrewa *et al.* (1991) described Fis as a compact globular homodimer, with each subunit containing four α-helices (A to D) separated by short β-turns (Figure 1.15). The C and D helices near the C-terminus correspond to the DNA binding helix-turn-helix (HTH) region of the protein. However, the 24 N-terminal amino acids were poorly resolved in this structure (Kostrewa *et al.*, 1991). Subsequently, it was established that they form flexible β-hairpin arms that extend from the N-termini of each subunit and interact with DNA recombinase (Safo *et al.*, 1997). The two α-helices (A and B) nearest the N-terminus make up the core dimerization region of the protein and this arrangement causes the two Fis monomers that make up the Fis homodimer to intertwine as subunits from each monomer are exchanged during dimerization (Cheng *et al.*, 2000;Merickel *et al.*, 2002;Topping *et al.*, 2004).

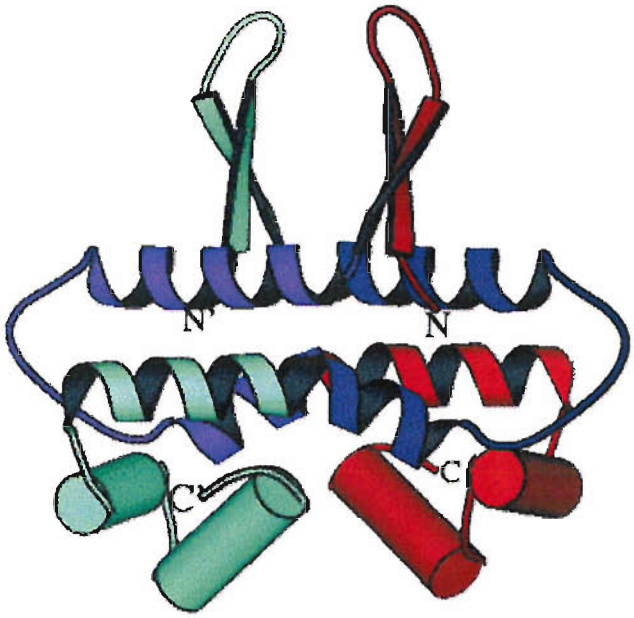


Figure 1.15. The structure of the Fis homodimer taken from Topping *et al.* (2004). The Ribbon diagram shows the structure of the intertwined Fis dimer. Cylinders represent the helix-turn-helix motifs responsible for the DNA binding of Fis. One monomer is coloured green (N-terminal β-strands, 10-25 residues, and C-terminal helices) and blue (central residues 26-57). The other monomer is coloured red and purple with the same boundaries.

1.9.2 Fis as a global regulatory protein

Fis is a global regulatory protein affecting multiple processes in *E. coli* and other enteric bacteria (Merickel *et al.*, 2002). Fis can act as a transcriptional activator or a repressor, but Fis-mediated regulation does not occur exclusively when Fis is at its highest concentration in the cell. Rather, it occurs at every stage of growth and even when cellular Fis levels are low (Hirsch and Elliott, 2005;Jackson *et al.*, 2004). In addition to the processes previously mentioned Fis also regulates key aspects of metabolism (González-Gil *et al.*, 1996;Jackson *et al.*, 2004;Kelly *et al.*, 2004;Yoon *et al.*, 2003), certain virulence processes (Falconi *et al.*, 2001;Kelly *et al.*, 2004;Lim *et al.*, 2006;Schecter *et al.*, 2003;Yoon *et al.*, 2003) for example positive control of the locus of enterocyte effacement gene cluster in enteropathogenic *E. coli* (Goldberg *et al.*, 2001), and DNA supercoiling (Keane and Dorman, 2003;Schneider *et al.*, 1997). Fis deficient mutants are viable, but they display a number of aberrant phenotypes including loss of Hin-mediated DNA inversion, cell filamentation, increased lag times and reduced growth rates in rich medium. Fis-constitutive mutants, however, show normal logarithmic growth, but exhibit reduced survival during the stationary phase (Osuna *et al.*, 1995).

1.9.3 Regulation of Fis expression

Fis expression levels vary dramatically according to the growth phase and in response to changing environmental conditions (Ball *et al.*, 1992). On the outgrowth from stationary phase (after dilution in fresh media) Fis increases from less than 100 to over 50,000 copies per cell before the first cell division. However, as cells enter exponential growth, Fis synthesis is largely shut off and its level drops as a function of cell division (Ball *et al.*, 1992).

The regulation of Fis expression involves several regulatory mechanisms (Dorman and Deighan, 2003;Ninnemann *et al.*, 1992;Schneider *et al.*, 1997). The Fis protein negatively regulates the *fis* operon *in vivo* and *vitro*, through autoregulation (Ball *et al.*, 1992) whereas Integration host factor positively regulates Fis expression (Mallik *et al.*, 2006). However, the expression of Fis is also regulated by the superhelical density of the DNA. Fis expression is maximal at high levels of negative supercoiling but deviations from the optimal supercoiling levels decrease the activity of the *fis* promoter (Schneider *et al.*, 2000). The *fis* operon is also subject to stringent control and is regulated by the level of free initiating nucleotide triphosphate for the operon (cytosine triphosphate, CTP) (Mallik *et al.*, 2004;Mallik *et al.*, 2006;Ninnemann *et al.*, 1992;Walker and Osuna, 2002).

Recent work has revealed that the *fis* P promoter is subject to growth phase dependent control and stringent control, in a manner which is analogous to the control of transcription from ribosomal RNA promoters (Figure 1.16). The level of free initiating nucleotide triphosphate (iNTP) activates (if the iNTP level is high) or represses (if the iNTP level is low) transcription from *fis* P, in conjunction with the RNA polymerase binding protein, DksA. However, stringent control further regulates the *fis* P promoter under conditions of amino acid starvation (Mallik *et al.*, 2006).

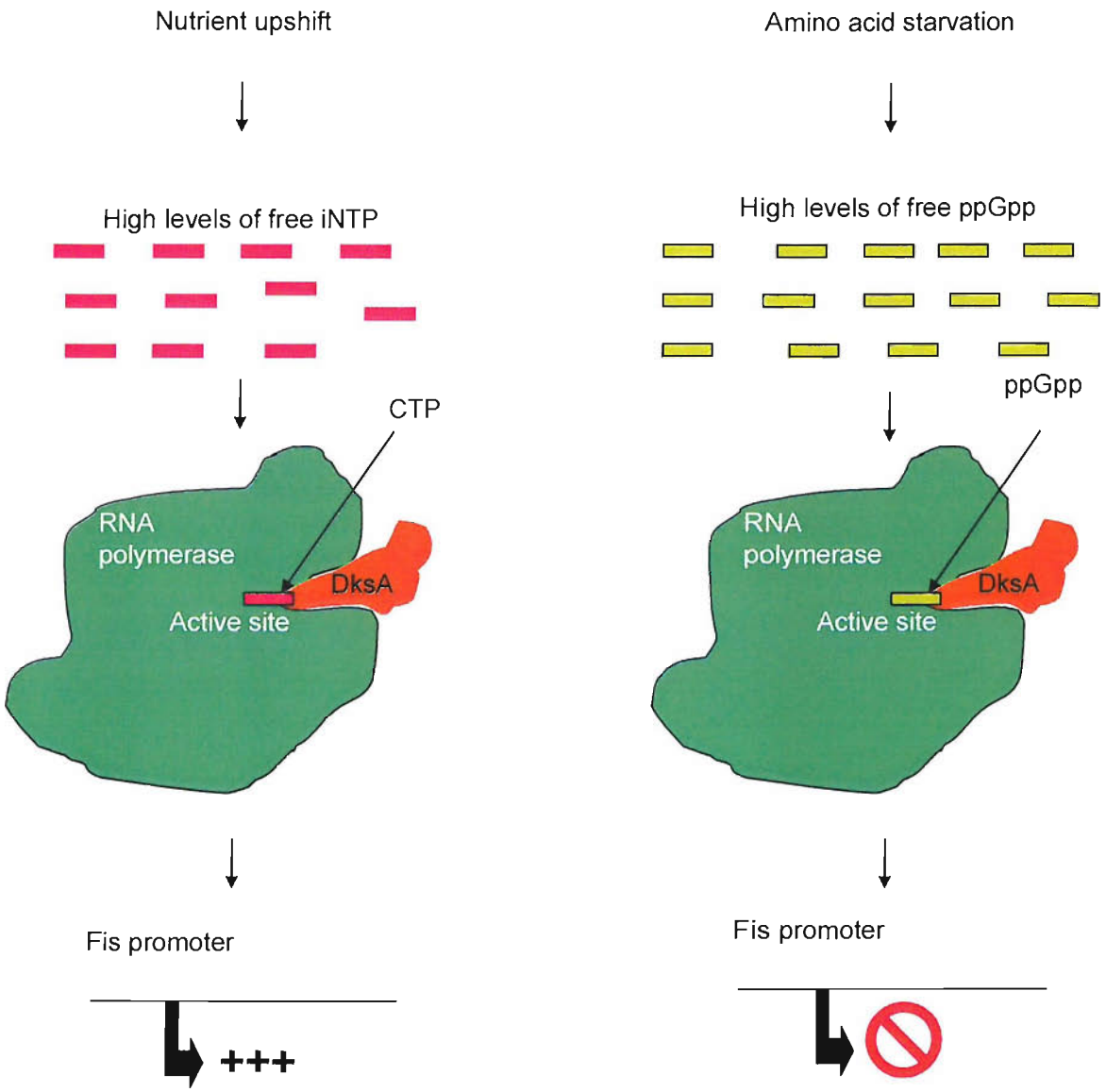


Figure 1.16. Control of Fis expression by the low molecular weight effectors CTP and ppGpp. CTP molecules are depicted as dark pink rectangles and the yellow rectangles depict ppGpp molecules. RNA polymerase is depicted in green and DksA is shown in orange.

Both the complexity and stringency of the regulation of Fis expression suggest that Fis itself is responsible (at least in part) for the growth rate dependent regulation of its target genes (Mallik *et al.*, 2006). This is indeed the case for many examples such as: the transcription of ribosomal RNA operons (Nilsson *et al.*, 1990;Ross *et al.*, 1990), the transcription of *rpoS* (which encodes the stationary phase sigma factor, σ^s, of RNA polymerase) (Hirsch and Elliott, 2005), and the transcription of the *ndh* gene (which encodes NADH dehydrogenase II) (Jackson *et al.*, 2004). It should also be considered that as a nucleoid-associated protein, Fis participates in the organisation of the bacterial nucleoid (Dorman and Deighan, 2003). Fis also has the ability to alter the supercoiling level of DNA (Schneider *et al.*, 1997) and its expression is tightly regulated by the level of DNA supercoiling (Schneider *et al.*, 2000). This has led to the proposal that Fis may sense and fine tune the level of DNA supercoiling (Dorman and Deighan, 2003).

1.9.4 The action of Fis at Fis-regulated promoter regions

The sequence of each individual promoter region will ultimately determine the regulatory interaction and action of Fis on transcription. However, the regulatory action of Fis has often been attributed to two of its properties: its ability to bind and bend DNA (Pan *et al.*, 1996) thereby altering the DNA topology in the promoter region (Opel *et al.*, 2004), and its ability to interact with the C-terminal domain RNA polymerase (Zhi *et al.*, 2003).

In this study it is aimed to gain further insight into the regulation of bacterial gene expression through examining the novel global regulatory protein, BipA, and its control of Fis expression. However in order to achieve this, the known mechanisms for the control of bacterial gene expression must be considered, therefore, the regulation of bacterial gene expression is the next topic for discussion.

1.10 Regulation of bacterial gene expression

Bacteria encounter a wide range of environments, which means that effective and efficient regulation of gene expression is essential to their survival. Bacteria regulate their gene expression to enable them to adjust their cellular systems and physiology to the environmental changes they encounter; whether that change is a reduction in available space or nutrients, the presence of a new carbon source or a change in the temperature, osmolarity or the pH of the environment. However, *in vivo* it is likely that more than one condition will change at a time, which means that regulatory events must be coordinated to ensure that the cell's response is appropriate for its conditions. This also means that regulation of gene expression can be considered globally as the effect of a stimulus on many genes in the cell, or specifically when considering the effect of a stimulus on a single operon. Bacterial cells have specific regulatory systems for the control of a particular operon, for example the *lac* or *ara* operons (Lewis, 2005;Schleif, 2000). In addition, they possess global regulatory systems which affect many genes in many operons, such as the SOS response (Janion, 2001). All mechanisms for the regulation of bacterial gene expression, however, act to influence the progression of the DNA to the active protein; this is illustrated in Figure 1.17 (Dale, 1998).

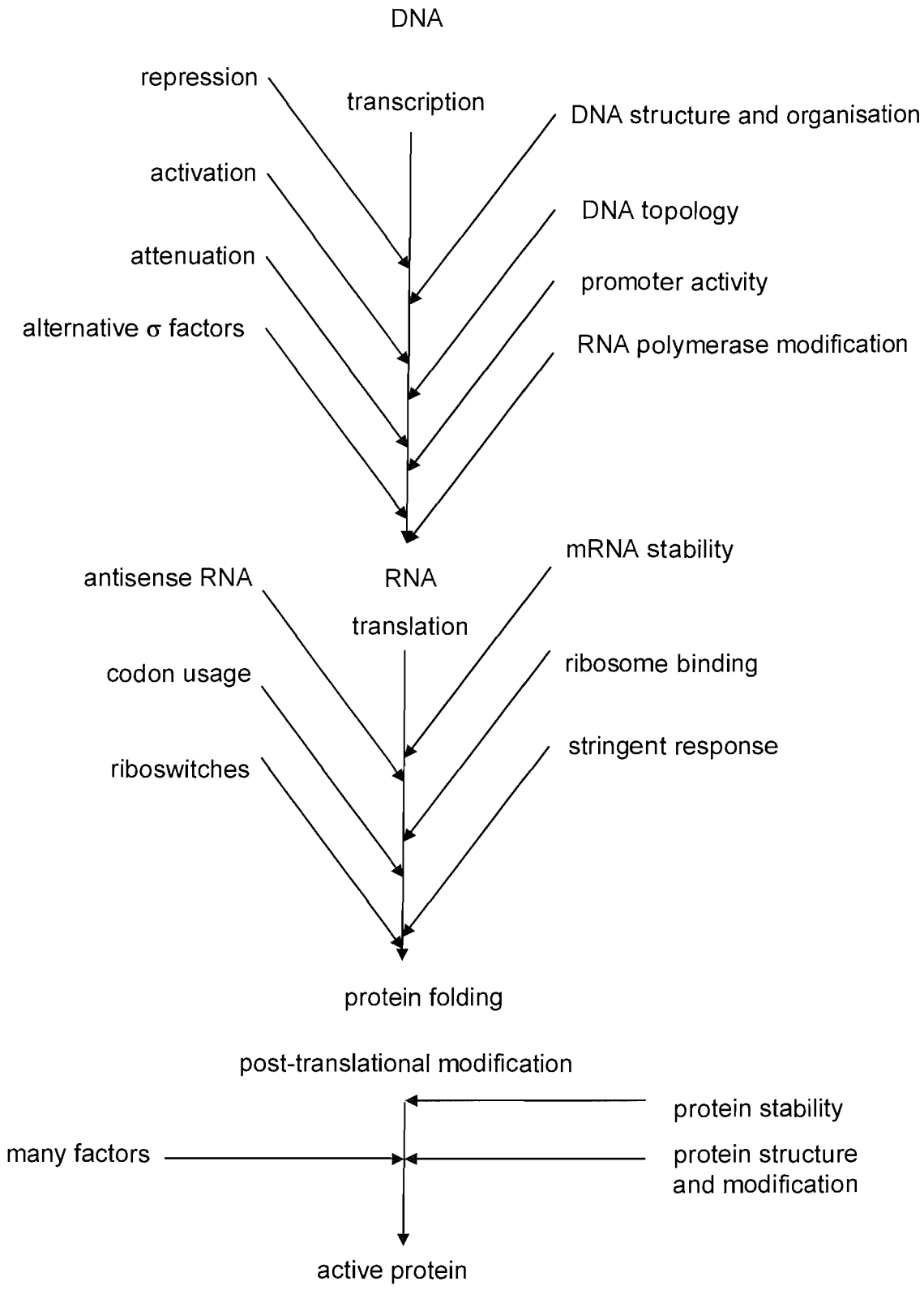


Figure 1.17. Factors that regulate bacterial gene expression, adapted from Dale (1998).

This study is primarily concerned with a global regulatory protein, BipA, that has all the hallmarks of a ribosome-binding GTPase (Farris *et al.*, 1998) and has also been shown to bind to ribosomes *in vitro* (Owens *et al.*, 2004). Accordingly, it is appropriate to consider the regulation of translation in some detail in the next section.

1.11 Regulation of bacterial gene translation

Recent findings have yielded new insights into the regulation of gene expression concerning translational control. In some cases they have revealed further details about factors that were previously observed to control translation for example mRNA stability (Kaberdin and Bläsi, 2006;Rasmussen *et al.*, 2005;Storz *et al.*, 2004), while in other cases, new mechanisms for the control of global regulatory systems have been uncovered that mediate a response to environmental conditions, e.g. the translational control of the stationary phase sigma factor σ^s or σ^{38} (Repoila *et al.*, 2003).This has revealed the importance of translational control to the overall control of gene expression. In many cases the synthesis of a protein is regulated at the level of translation initiation, and regulation at this level may be particularly important for the precise control of bacterial gene expression (Kozak, 1999).

1.11.1 The regulation of translation initiation - Ribosome binding and the Shine-Dalgarno sequence

The efficiency of the initiation of translation and the formation of the initiation complex greatly influence gene expression (Kozak, 1983;Londei, 1998). Both the 5' untranslated region of a prokaryotic messenger RNA and the sequences surrounding its start codon contain determinants that influence this efficiency. One of these determinants is the Shine-Dalgarno (S-D) sequence (Chang *et al.*, 2006;Kozak, 1983;Londei, 1998;Stenström and Isaksson, 2002). In 1974 Shine and Dalgarno sequenced the 3' terminal end of the prokaryotic 16S rRNA and found that it contained the sequence 5'accuccuua3' and they observed that part of this sequence was complementary to a purine rich sequence 5'ggaggu3' which was present, in full or in part, in various different coliphage mRNA species. This purine rich sequence, which was later known as the Shine-Dalgarno sequence, was always located at a similar position in the mRNA 5' of the start codon. It was postulated that, as the 3' terminal residues of the 16S rRNA are not involved in any base paired structure, that they may interact with the purine rich Shine-Dalgarno sequence, base pairing with it to promote binding of the mRNA to the ribosome and therefore the initiation of translation (Shine and Dalgarno, 1974). The ability of the anti-Shine-Dalgarno sequence on the 3' terminal end of the 16S rRNA to base pair with the S-D sequence on a mRNA was later confirmed when 16S rRNA-mRNA complexes were isolated (Figure 1.18) (Steitz and Jakes, 1975).

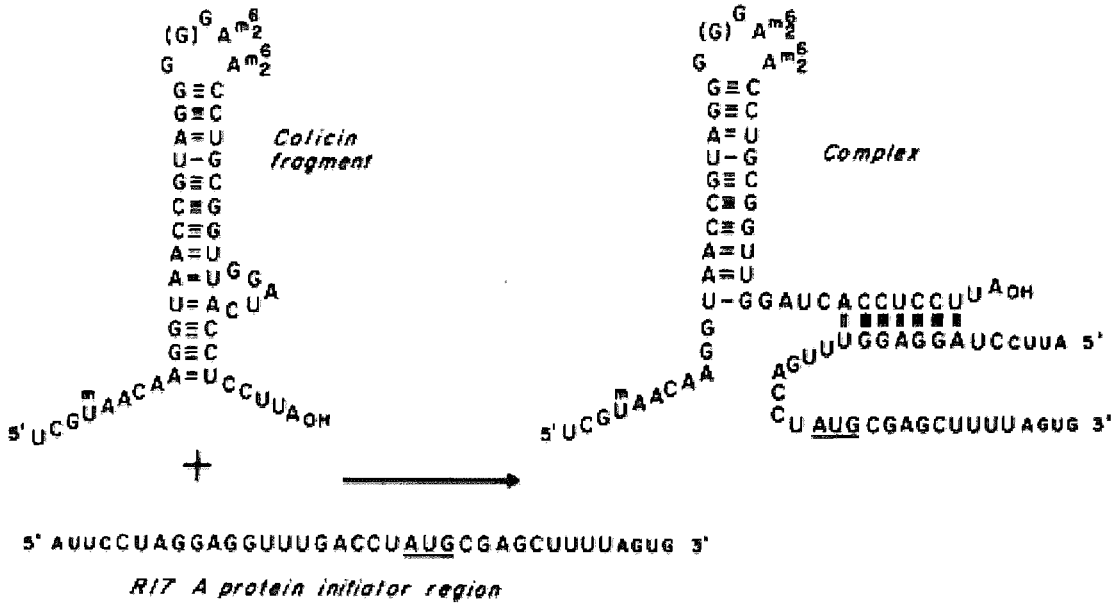


Figure 1.18. A postulated hydrogen bonding between the 16S ribosomal RNA and the translation initiation region of the R17 bacteriophage protein A RNA taken from Steitz and Jakes (1975).

In the time since its discovery, the purine rich Shine-Dalgarno sequence has been found (5' of the start codon) in all but a few exceptional genes in *E. coli* (Shultzaberger *et al.*, 2001). Convincing *in vivo* evidence for the role of the Shine-Dalgarno sequence and the complementary anti-Shine-Dalgarno region in translation has been obtained from experiments in which the anti-Shine-Dalgarno region on the 16S rRNA was mutated. Mutation dramatically altered the expression of many cellular proteins and in many cases the difference in a protein's expression correlated with the change in the level complementarity of their Shine-Dalgarno sequences to the new mutant anti-Shine-Dalgarno (anti-S-D) sequence (Jacob *et al.*, 1987). In addition, another research group demonstrated that by mutating the anti-Shine-Dalgarno region on the 16S rRNA to increase its complementarity to the Shine-Dalgarno region on an mRNA, it is possible to create a subpopulation of mutant ribosomes which enhance the translation of the selected mRNA (Hui and de Boer, 1987). The importance of the interaction of the S-D sequence with the anti-S-D sequence (on the 3' terminal end of the 16S rRNA) to the translation of an mRNA was also demonstrated using ribosomes that were pre-incubated with a pentanucleotide that was complementary to the 3' end of the 16S rRNA. This pre-incubation blocked the initiation of protein synthesis *in vitro*, confirming the requirement of available anti-S-D sequence for the initiation of translation (Eckhardt and Lührmann, 1979).

A Shine-Dalgarno sequence usually located between the seventh and twelfth base upstream of the start codon is a common feature of prokaryotic mRNAs (Chang *et al.*, 2006;Kozak, 1983). The efficiency of the Shine-Dalgarno sequence anti-Shine-Dalgarno region interaction is affected by three parameters; the length of complementarity, the distance between Shine-Dalgarno sequence and the start codon and secondary structure that surrounds the Shine-Dalgarno sequence and may mask it (Kozak, 1983).

The number of complementary nucleotides in the Shine-Dalgarno (S-D) sequence varies between individual mRNAs but a typical S-D sequence is 4 to 5 nucleotides in length (Kozak, 2005). Changing the number of complementary nucleotides in the Shine-Dalgarno sequence of an mRNA often alters the expression of the protein in question. It is clear that reducing the number of complementary nucleotides in the S-D sequence of an mRNA often impairs its translation. However, the relationship between the number of complementary nucleotides and the efficiency of translation initiation is not a simple one; it is complicated by the fact there is a most favoured region of interaction with the anti-Shine-Dalgarno sequence on the 16S rRNA 5'cucc3', a short 3 base pair complementarity to this region may confer a high level of expression, while a 3 base pair complementarity to the nucleotides either side of this sequence will result in a low level of expression (Kozak, 1983). While examining the role of the Shine-Dalgarno sequence in translation, it was found that increasing the number of complementary nucleotides from 5 to 8 resulted in an increase in initiation efficiency of roughly four fold. This result is consistent with a simple kinetic model of mRNA ribosome binding (Ringquist *et al.*, 1992). However, this is not always the case; sometimes increasing the length of the Shine-Dalgarno sequence and therefore the number complementary nucleotides causes little or no increase in translation (Kozak, 2005). In fact, the presence of a highly complementary Shine-Dalgarno sequence is not beneficial to the rate of translation. Excessively stable binding of a Shine-Dalgarno sequence to its anti-Shine-Dalgarno region present on the 16S rRNA (due to the presence of an extended Shine-Dalgarno region) reduces gene expression, possibly due to ribosome stalling at the initiation step of translation which may be caused by the redundant stability of the ribosome-message binding (Komarova *et al.*, 2002;Kozak, 2005). Although, the presence of an extended Shine-Dalgarno sequence is beneficial to the translation of mRNAs that start with a codon other than AUG or in which the initiation site is masked by secondary structure (Kozak, 2005).

The S-D sequence is an important part of the mechanism for the selection of the initiator region in an mRNA by 30S ribosomal subunit. It is thought to act kinetically to increase the probability of mRNA binding to the 30S subunit and increase the precision of translation by bringing the start codon into close proximity to the ribosomal peptidyl tRNA binding site (hence the optimal spacing required for S-D sequences), as illustrated in Figure 1.19 (Calogero *et al.*, 1988;Chen *et al.*, 1994;Ringquist *et al.*, 1992). Data regarding the timing of formation of S-D anti-S-D interactions support this model; S-D anti-S-D interactions have been detected in conditions which precluded binding to the AUG start codon (Kozak, 1999). Recent studies have determined that S-D anti-S-D interactions stabilise the ribosome:mRNA complex prior to the formation of the first peptide bond (Uemura *et al.*, 2007).The S-D anti-S-D interactions may anchor the mRNA to the 30S subunit and promote ribosome entry while the mRNA is unfolded, to prevent the formation of secondary structure that may hinder the initiation of translation, by blocking access to the AUG start codon (Kozak, 1999). The formation of the first peptide bond, however, destabilises the S-D anti-S-D complex which may be important to ribosome movement in the first translocation step (Uemura *et al.*, 2007).

In recent work, the increase in translation caused by a Shine-Dalgarno sequence located upstream of an initiation codon was confirmed. However, it was also revealed that a Shine-Dalgarno sequence located downstream of an initiation codon (in the early coding region of the gene) decreases the translation and hence the expression of the gene. In the *E. coli* K-12 genome, Shine-Dalgarno or Shine-Dalgarno like sequences are avoided in the early coding regions of genes. The work led to the consideration that Shine-Dalgarno sequences may serve to direct translation, a conclusion which is in accordance with the current model for the role of the Shine-Dalgarno sequence in translation (Jin *et al.*, 2006).

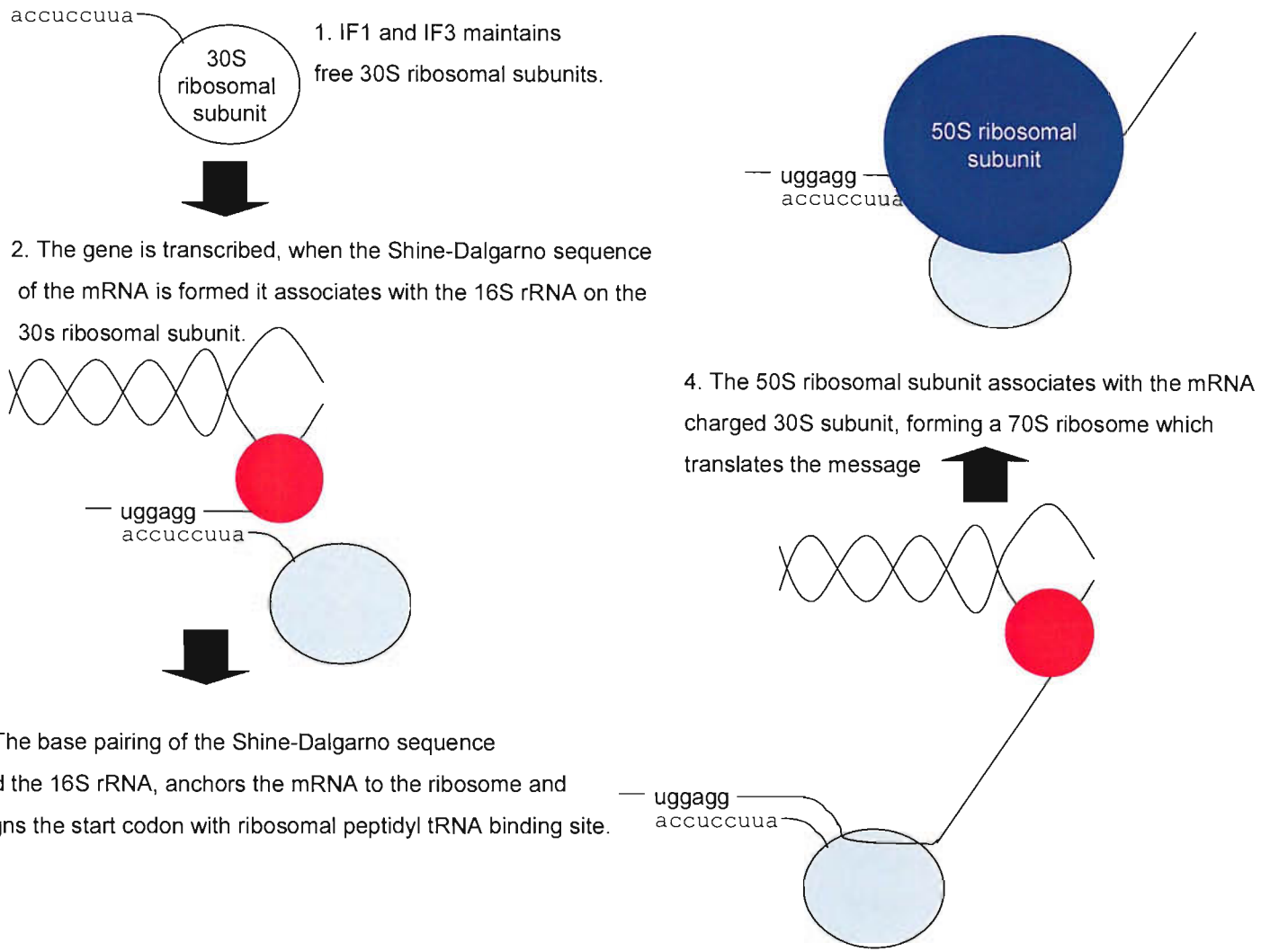


Figure 1.19. The role of the Shine-Dalgarno sequence in the initiation of translation.

Figure 1.19. The role of the Shine-Dalgarno sequence in the initiation of translation. The light blue circles depict 30S ribosomal subunits while the larger dark blue circles depict 50S ribosomal subunits. The curved double helix represents the DNA and the red circle represents RNA polymerase. The Shine-Dalgarno sequence on the mRNA and the anti-Shine-Dalgarno sequence on the 16S rRNA are displayed. The straight line represents the mRNA.

The Shine-Dalgarno sequence is not essential to the process of translation initiation for every mRNA. Adenine and uracil rich sequences that form no stable secondary structure may not require a S-D sequence to initiate translation (Kozak, 2005). In recent research, increasing numbers of non-Shine-Dalgarno led and leaderless genes have been discovered, which has led to the realisation that, ribosome binding through the association of primarily the Shine-Dalgarno region and the anti-Shine-Dalgarno region of the 16S rRNA, may not be a universal mechanism. Therefore there must be a mechanism for ribosome binding and translation initiation that does not involve a Shine-Dalgarno region (Chang *et al.*, 2006;Moll *et al.*, 2002). It is tempting to speculate that the presence of various types of mRNAs such as Shine-Dalgarno-led, non-Shine-Dalgarno led, long leadered non-Shine-Dalgarno led, leaderless and novel mRNAs, as proposed in Chang *et al.* (2006), might further facilitate the regulation of gene expression in prokaryotes.

1.11.2 Other Sequences that may affect the initiation of translation

In prokaryotes the start codon is very important in determining the efficiency of translation initiation. AUG is the most commonly used and the most efficient start codon, but prokaryotes can also use GUG and UUG codons for initiation (O'Donnell and Janssen, 2001;Ringquist *et al.*, 1992). It has been found that the initiation codon is an important determinant of ribosome binding strength and translation efficiency, both in the presence and absence of a 5' untranslated region and Shine-Dalgarno sequence (O'Donnell and Janssen, 2001). However, the AUG start codon is particularly important for efficient expression of leaderless mRNAs, because leaderless mRNAs are only expressed if they contain an AUG or GUG start codon, and those starting with GUG are expressed at very low levels. The presence of a Shine-Dalgarno sequence in

the 5' untranslated region restores expression of mRNAs with alternative start codons (GUG and UUG) (O'Donnell and Janssen, 2001;Ringquist *et al.*, 1992). Therefore the combination of features within the mRNA, such as the start codon and Shine-Dalgarno sequence, may act in concert to regulate gene expression (Ma *et al.*, 2002).

Other areas of sequence have been tested for involvement in the initiation of translation such as the sequence that separates the Shine-Dalgarno sequence from the start codon, which if mutated can sometimes reduce translation of the mRNA, or adenine and uracil rich codons downstream of the start codon which are associated with higher levels of translation. However, these regions have not been found to play a defined role in translation initiation. It may be that the stimulatory effect of upstream or downstream adenine and uracil rich sequences may be the result of a reduction in secondary structure surrounding the initiation site, which facilitates efficient translation (Kozak, 2005). Although certain guanine rich codons have been shown to inhibit translation at certain positions in the vicinity of the start site, these codons have been found to be deleterious to translation for unknown reasons (Kozak, 2005).

1.11.3 Other mechanisms for the regulation of translation initiation

Prokaryotes possess specific regulatory mechanisms to regulate the efficiency of translation initiation. In many cases this gives them a fast response when adjusting cellular gene expression to adapt to a change in the environmental conditions. The various mechanisms (summarised in Table 1.5) include, the formation of alternate base-paired secondary structures in the mRNA, or between the mRNA and a small regulatory RNA, or by utilising mRNA binding

proteins (Kaberdin and Bläsi, 2006;Rasmussen *et al.*, 2005;Romby and Springer, 2003;Winkler *et al.*, 2003).

Table 1.5. Other mechanisms for the regulation of translation initiation

Regulatory mechanism for translation initiation	Example	References
Translational coupling	The *atp* (ATP synthase) operon of *E. coli* is translationally coupled. The coupling mechanism of *atp*HA involves, ribosomes that reach stop codon of *atp*H disrupting the stable secondary structure present in the translation initiation region of *atp*A, these ribosomes then reinitiate to translate *atp*A.	(Rex *et al.*, 1994)
Alternate secondary structure formation due to mRNA properties	Thermo-responsive RNA mediated translational control of the heat shock response sigma factor (σ^{32}) encoded by *rpoH*. At 30°C the *E. coli rpoH* mRNA translation initiation region is occluded by secondary structure which is destabilised during heat shock allowing *rpoH* translation.	(Kaberdin and Bläsi, 2006)
Alternate secondary structure formation	The *ompA* (outer membrane protein A) mRNA interacts with	(Rasmussen *et al.*, 2005)

through base pairing with a small regulatory RNA	the 78 nucleotide small regulatory RNA called SraD to cause rapid *ompA* mRNA decay on entry of stationary phase. SraD expression is induced on the transition from exponential phase to stationary phase, when present the SraD RNA (in conjunction with the RNA chaperone protein Hfq) binds to *ompA* 5'UTR and prevents it binding to ribosomes, this results in mRNA decay.	
Alternate secondary structure formation through base pairing with low molecular weight effectors (riboswitches)	The conserved mRNA S-box is an S-adenosylmethionine (SAM) binding domain. Binding of SAM an S-box causes an allosteric modification in the mRNA secondary structure which alters gene expression.	(Winkler *et al.*, 2003)
Translational repression by an mRNA binding protein	Threonyl-tRNA synthetase represses the translation of its own mRNA by binding to it and preventing its binding to ribosomes.	(Romby and Springer, 2003)

Now it is appropriate to discuss the novel global regulatory protein which is the focus of this study, BipA.

1.12 Bactericidal/permeability-increasing protein inducible protein A (BipA): a ribosome binding global regulatory protein

1.12.1 The discovery of BipA

BipA is a novel global regulatory protein that is thought to regulate translation (Farris *et al.*, 1998;Grant *et al.*, 2003;Owens *et al.*, 2004). It was discovered while investigating the response of *Salmonella* Typhimurium cells to cell surface damage caused by a (host derived) antimicrobial protein. The phagocytic cells that a microbe encounters within a host, possess a range of antimicrobial proteins including: bactenecins, cathepsin G, defensins, lysozyme and bactericidal/permeability-increasing protein (BPI). Many of these proteins bind with high affinity to lipopolysaccharide molecules, found on the surface of gram negative bacteria, and increase the permeability of the outer membrane, giving the antimicrobial proteins access to their targets within the bacterial cell. A microbe's response to the antimicrobial proteins may ultimately determine their survival and persistence (Qi *et al.*, 1995).

The response of *S.* Typhimurium cells to cell surface damage was examined by comparing the protein expression pattern of BPI-treated *S.* Typhimurium cells to the expression pattern of untreated *S.* Typhimurium control cells. Two-dimensional gel electrophoresis and N-terminal microsequencing were used to identify the proteins that were induced or repressed following exposure to BPI. A novel protein was found to be induced over sevenfold by BPI treatment and hence was named bactericidal/permeability-increasing protein inducible protein A (BipA). Sequence analysis of BipA suggested that it belonged to the GTPase superfamily which interacts with ribosomes (Qi *et al.*, 1995). It was subsequently also identified by Freestone *et al.* (1998) while examining tyrosine

phosphoproteins in *E. coli*. Therefore, it has also been named tyrosine phosphoprotein A (TypA) (Freestone *et al.*, 1998).

1.12.2 Key features of BipA

BipA has a number of features that excite research interest. These are summarised in Table 1.6. The consideration of these features in conjunction with the pleiotropic effects of *bipA* disruption *in vivo*, led to the hypothesis that BipA exerts its regulatory effect by modulating the translation of one or more proteins in the cell. Of particular importance to this hypothesis are the findings that the *bipA* sequence is homologous to the ribosome binding GTPase superfamily, and BipA's ability to bind to 70S ribosomes (Farris *et al.*, 1998;Owens *et al.*, 2004).

Table 1.6 Key features of BipA.

BipA feature	Regulatory implications	References
BipA is a tyrosine phosphoprotein	Involvement in cellular signalling networks.	(Farris *et al.*, 1998;Freestone *et al.*, 1998)
BipA is capable of autophosphorylation	Involvement in cellular signalling networks. Regulation of the activity of BipA.	(Farris *et al.*, 1998)
BipA is homologous to the ribosome binding GTPase superfamily	Involvement in translation.	(Farris *et al.*, 1998)
BipA has GTPase and ATPase activity	Involvement in translation. Regulation of the activity of BipA.	(Farris *et al.*, 1998;Owens *et al.*, 2004)
BipA binds to 70S ribosomes	Involvement in translation.	(Owens *et al.*, 2004)

1.12.3 Sequence analysis of BipA

Sequence analysis was carried out on the enteropathogenic *E. coli* (EPEC) BipA homologue. The results revealed that the open reading frame coded for a 591 residue protein, which displayed substantial sequence similarity to the members of the ribosome binding GTPase superfamily, with particular homology to Elongation factor G (which participates in the final stage of the elongation cycle in translation (Voet and Voet, 1995)), as illustrated in Figure 1.20. The strongest sequence identity was found in the amino-terminal third of the protein, which included regions that define a guanine nucleotide-binding pocket. But stretches of conservation were found throughout the BipA sequence. It was also found that BipA shared significant sequence similarity with domain IV of Elongation factor G, which is believed to interact with ribosomes (Farris *et al.*, 1998).

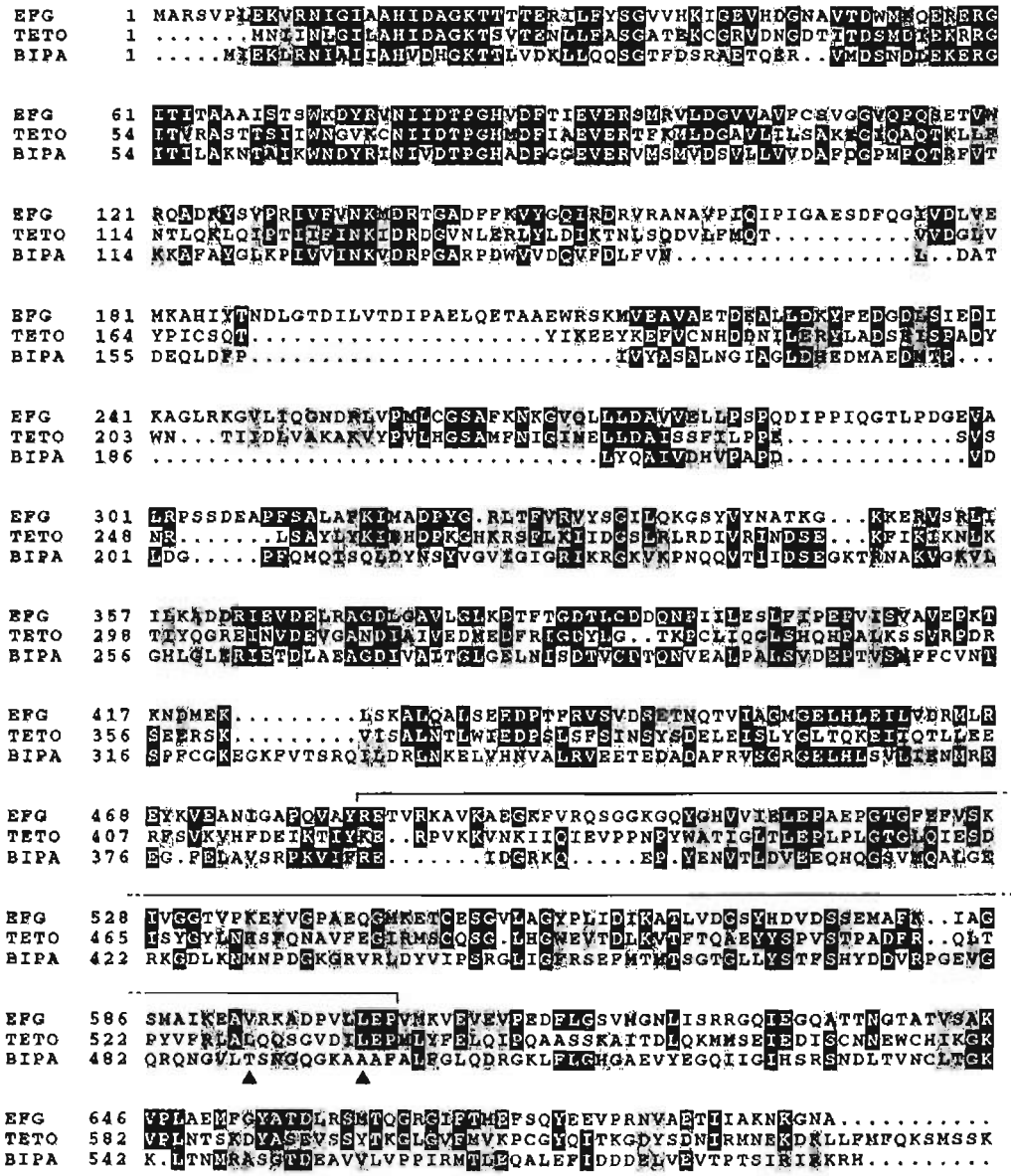


Figure 1.20 The predicted sequence of the enteropathogenic *E. coli* BipA protein and its alignment with elongation factor G (EF-G) and the Tet (O) tetracycline resistance protein taken from Farris *et al.* (1998). The conserved regions are indicated by shaded areas, the highly conserved areas are indicated by dark shading. The bracketed area indicates the residues that interact with the small ribosomal subunit.

The ribosome binding GTPase protein family contains soluble ribosomal factors and intracellular signalling proteins. They share a common structural motif that binds guanine nucleotides (such as guanine triphosphate), and catalyses the hydrolysis of guanine triphosphate (Voet and Voet, 1995). Sequence analysis also revealed that *bipA* homologues are widely distributed in bacteria and are also found in certain eukaryotes (Figure 1.21).

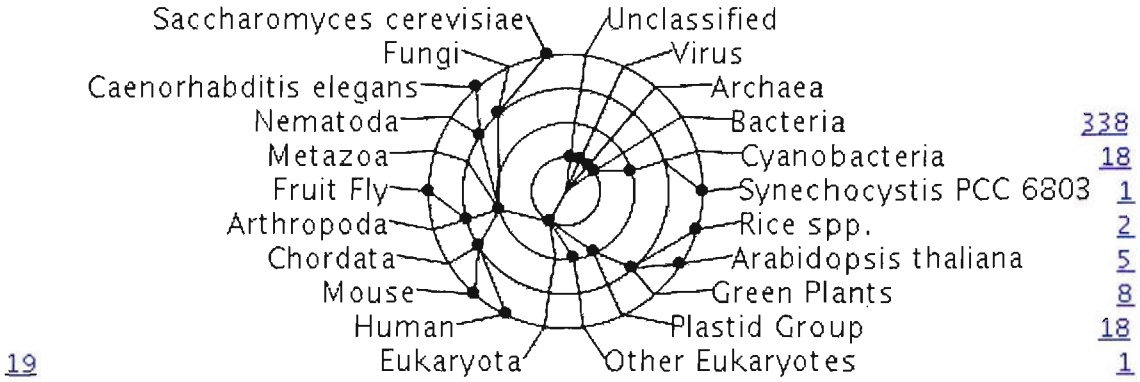


Figure 1.21. BipA homologues are widely distributed in bacteria and are present in certain eukaryotes (European bioinformatics institute). This taxonomy display shows the taxonomic range of *bipA* and the number of *bipA* homologues associated with each lineage. The blue numbers indicate the number of *bipA* homologues in each phylum. The taxonomy-tree root is at the centre of the circular display. The outermost circle contains selected model organisms and the inner circles display the nodes of the taxonomy-tree. The redial lines led to the description for each node. The nodes are either true taxonomic nodes or artificial nodes (Unclassified, Other Eukaryotes and the Plastid group) created for this display. Although some attempt has been made to group the nodes the position of the node on the inner circle has no significance.

To date, homologues of BipA have been experimentally characterised, at least in part, from enteric bacteria such as *Salmonella* Typhimurium, enteropathogenic *E. coli* and *E. coli* K-12 (Barker *et al.*, 2000;Farris *et al.*, 1998;Grant *et al.*, 2003;Pfennig and Flower, 2001;Qi *et al.*, 1995). In addition, in recent work the *bipA* homologue, *typA* gene, of the microbial plant symbiont *Sinorhizobium meliloti* was characterised and shown to regulate both housekeeping functions and symbiosis (Kiss *et al.*, 2004), demonstrating the widespread distribution of the *bipA* gene and its regulatory actions.

1.12.4 BipA interacts with 70S ribosomes

Gel filtration experiments were used to determine if BipA binds to 70S ribosomes. It was found that when BipA was passed through the column in isolation it eluted late due to its small size. However when 70S ribosomes and BipA were passed through the column together, the BipA elution pattern changed and it eluted earlier, with a substantial portion co-eluting with the ribosomes. This work suggests that BipA binds to 70S ribosomes (Figure 1.22). Control experiments showed that a non-ribosome binding protein (phosphofructokinase) did not co-elute under the same conditions (Grant *et al.*, 2003;Owens *et al.*, 2004). This data supports the hypothesis that BipA modulates the translation of its regulatory targets.

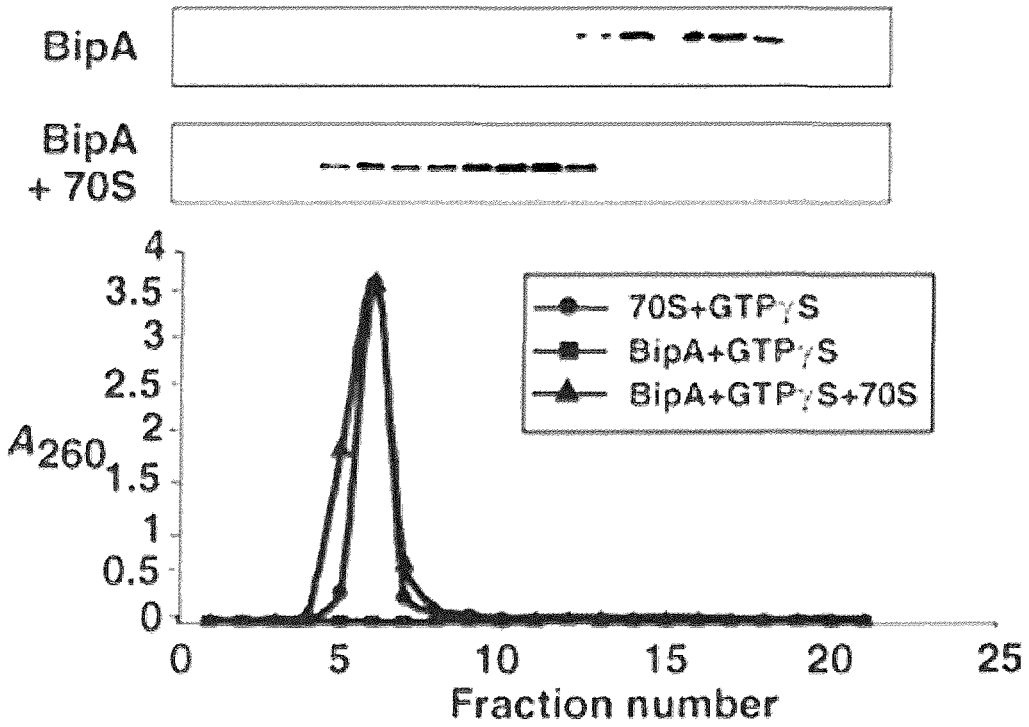


Figure 1.22. BipA binds to ribosomes shown by gel filtration, taken from Owens *et al.* (2004). Components were passed down a sephacryl S300 column (singly or in combination) the fractions eluted that contained BipA or EF-G were detected by SDS-PAGE and immuno-blotting. Absorbance at 260 nm was used to identify 70S ribosome containing fractions.

These studies also revealed that the addition of Elongation factor G (EF-G) to 70S ribosomes and BipA, blocked the binding of BipA to the ribosome. This meant that when the gel filtration experiments were performed on EF-G, 70S ribosomes and BipA together, BipA eluted late while EF-G eluted with the ribosomes. This suggests that EF-G and BipA bind to the same site, or to overlapping sites on the 70S ribosome (Owens *et al.*, 2004).

Further support for the hypothesis that BipA modulates the translation of its regulatory targets came from the examination of BipA's GTPase activity in the presence of 70S ribosomes. These studies revealed that BipA had a very low basal level of GTPase activity, but this was stimulated by the addition of intact 70S ribosomes (Owens *et al.*, 2004). It was also found that the 70S ribosome stimulated GTPase activity of BipA was inhibited by high guanine diphosphate to guanine triphosphate (GDP:GTP) ratios. However, the 70S ribosome stimulated GTPase activity of EF-G was not affected by this. In addition, the 70S ribosome stimulated GTPase activities of BipA and EF-G were found to be similarly inhibited by the translation inhibitors thiostrepton and α-sarcin. Although the antibiotic fusidic acid inhibited the 70S ribosome stimulated GTPase activity EF-G it did not affect the 70S ribosome stimulated GTPase activity of BipA. This work revealed many similarities between EF-G and BipA and led to the consideration that BipA interacts with ribosomes in a similar manner to Elongation factor G (Owens *et al.*, 2004).

1.12.5 BipA is a global regulatory protein

The construction and characterisation of *bipA* null mutants revealed that BipA regulates multiple diverse cellular processes, ranging from cell motility to attaching and effacing lesion formation (Farris *et al.*, 1998;Grant *et al.*, 2003), as illustrated in Figure 1.23. The processes regulated by BipA can be divided

into two groups: those that affect the growth and survival of the bacterium and those that affect bacterial virulence (it should be noted, however, that these groups will not be mutually exclusive).

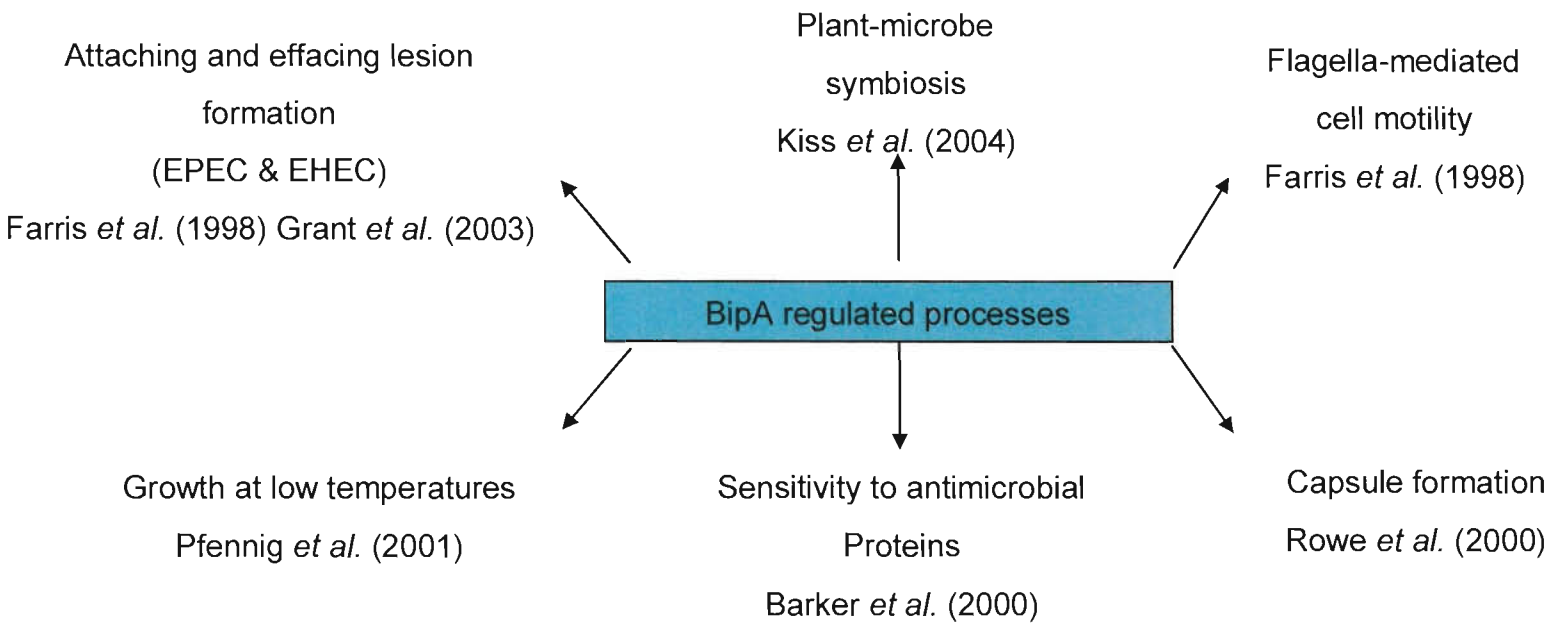


Figure 1.23. BipA regulated cellular processes. This diagram summarises some of the processes which have been previously found to be regulated by BipA. The processes are arranged around the central caption with the appropriate references.

1.12.6 BipA regulates bacterial growth and survival

BipA has been reported to regulate bacterial growth and survival by affecting, growth at low temperatures, cell motility, the translation of the global regulatory protein Fis and, in certain nitrogen fixing bacteria, the establishment of symbiosis (Farris *et al.*, 1998;Grant *et al.*, 2001;Kiss *et al.*, 2004;Owens *et al.*, 2004;Pfennig and Flower, 2001).

It has been previously observed that *bipA* null mutants of *E. coli* K-12 and enteropathogenic *E. coli* display cold-sensitivity unless BipA expression is restored by the introduction of plasmid-borne BipA (Grant *et al.*, 2001;Pfennig and Flower, 2001). Therefore, BipA is essential for growth at low temperatures in *E. coli* K-12 and enteropathogenic *E. coli*. However, *bipA* null mutants of *Salmonella* Typhimurium or *Salmonella* Enteritidis were not found to be cold-sensitive (A. Grant Ph.D. thesis (2001), University of Southampton), indicating that BipA may not affect growth at low temperatures in *Salmonella* strains. BipA has been also found to be involved in lipopolysaccharide core biosynthesis in *E. coli* K-12 (Moller *et al.*, 2003).

BipA regulates flagella mediated cell motility. However, BipA affects flagellar mediated cell motility differently in different strains of *E. coli*. A *bipA* null mutant of the enteropathogenic *E. coli* strain, MAR001*bipA::cat*, indicates that BipA negativity regulates flagella mediated cell motility. Thus, MAR001*bipA::cat* displayed hypermotility and released a large amount of flagellin to the culture medium. These results suggest that BipA appears to negatively regulate the expression or export flagellin (Farris *et al.*, 1998). However, when the motility of an *E. coli* K-12 *bipA* null mutant strain (MG1655*ΔbipA*) was tested, it was found that the mutant cells showed reduced motility, when compared to the wild type parent strain, indicating that BipA positively regulates cell motility in *E. coli* K-12 (A. Grant; Ph.D. thesis (2001), University of Southampton).

BipA has been reported to positively regulate the expression, of the global regulatory protein, Fis, which controls processes that are essential to cell growth, such as ribosome synthesis. Owens *et al.* (2004) observed that *fis* messenger RNA transcripts were abundant in *bipA* null mutant cells although very little Fis protein was present. The wild type level of Fis expression was restored in *bipA* null mutant cells by the introduction of plasmid-borne BipA. This demonstrated that BipA positively regulates the expression of Fis and implied that BipA regulates Fis translation (Owens *et al.*, 2004).

In the soil bacterium *Sinorhizobium meliloti* BipA also regulates certain housekeeping functions such as growth at low temperatures and certain stress responses. However in addition to this, it performs a host-plant dependent role in the establishment of symbiosis (Kiss *et al.*, 2004).

1.12.7 BipA regulates specific bacterial virulence determinants

BipA regulates bacterial virulence by affecting the sensitivity of microbes to certain bactericidal proteins produced by phagocytic cells in the host immune response. Additionally, it is involved in the expression of the group two capsules (which aid the bacterium in resisting phagocytosis) and the ability of the microbe to colonise within host (Barker *et al.*, 2000;Farris *et al.*, 1998;Grant *et al.*, 2003).

The importance of BipA for resistance to the antimicrobial protein BPI has been demonstrated using *bipA* null mutants of *Salmonella* Typhimurium and enteropathogenic *E. coli*. The enteropathogenic *E. coli bipA* null mutant was found to be over two log orders more sensitive than the parent strain to 1 μg of BPI (Farris *et al.*, 1998). The *Salmonella* Typhimurium *bipA* null mutant was used to demonstrate that BipA was required for formate-mediated protection

from BPI (Barker *et al.*, 2000). However, in similar assays, examining the sensitivities of the enteropathogenic *E. coli bipA* null mutant and its wild type parent strain to, the antimicrobial protein, protamine, the *bipA* null mutant showed only a modest decrease in cell viability in comparison to its parent. This suggests that the protective action of BipA shows some specificity (Farris *et al.*, 1998).

BipA regulates the expression of the *E. coli* group 2 capsules (K antigens). K antigens are expressed on the majority of extra-intestinal isolates of *E. coli* that are associated with invasive disease, with particular capsules being associated with certain diseases. Expression of the K antigens is temperature dependant because capsules are only expressed at temperatures above 20°C. BipA was found to repress K antigen transcription at 20°C and enhance its transcription at 37°C (Rowe *et al.*, 2000).

BipA has been shown to be of regulatory importance to the ability of enteropathogenic *E. coli* (EPEC) to interact with host cells. This interaction is important for the microbe's adherence and colonisation of its host's gut, and results in the formation of the attaching and effacing lesion. The interaction involves the removal of microvilli and condensation of polymerised actin beneath the adherent bacteria, forming so-called pedestals on the host cell surface, as shown in Figure 1.24 (Knutton *et al.*, 1989;Rosenshine *et al.*, 1992;Rosenshine *et al.*, 1996).

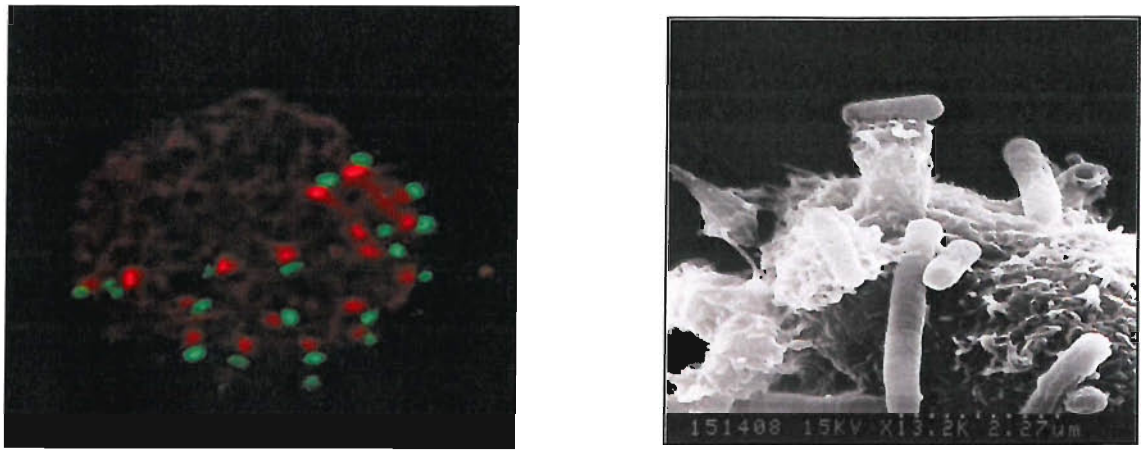


Figure 1.24. Hyperformation of pseudopods by enteropathogenic *E. coli* overexpressing BipA, taken from Farris *et al.* (1998). The EPEC (MAR001) *bipA::cat* mutant was transformed with a BipA over expressing plasmid pBipA incubated with host derived cells (HeLa cells) then examined by confocal microscopy, shown on the left, or by scanning electron microscopy shown on the right. For the confocal microscopy the EPEC cells were tagged with green fluorescent protein and actin was labelled with rhodamine-conjugated phalloidin.

Attaching and effacing lesion formation is essential to the pathogenesis of both enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) (Grant *et al.*, 2003). It is encoded in on one of the most extensively studied pathogenicity islands, the locus of enterocyte effacement (LEE). This cluster of genes specifies a type III secretion system (including *E. coli* secretion proteins, Esc, Secretion of *E. coli* proteins, Sep, and Chaperone for *E. coli* secreted proteins, Ces), certain secreted target proteins (*E. coli* secreted proteins, Esp, and the translocated intimin receptor, Tir) and an outer membrane protein, intimin (Elliot *et al.*, 2000). In EPEC maximal expression of the LEE encoded proteins requires the plasmid encoded regulator (Per). The LEE encoded regulator (Ler) is part of the Per-mediated regulatory cascade that stimulates the expression of the genes encoded by the LEE (Gomez - Duarte and Kaper, 1995).

The effect of depletion and overexpression of BipA on the formation of actin-rich pedestals on cultured HeLa cells was investigated. Cells were infected with parental EPEC, the *bipA::cat* mutant or a plasmid complemented *bipA::cat* mutant. As expected, the parental EPEC triggered microvilli destruction and actin accumulation beneath the adherent bacteria. But under identical conditions, the *bipA::cat* mutant failed to trigger cytoskeletal rearrangements, although the bacteria adhered to the host cells. However, infection of HeLa cells with the *bipA::cat* mutant over expressing BipA (due to transformation with *bipA* on a high copy number plasmid) not only restored actin accumulation, but also induced the production of numerous EPEC-associated pseudopods; this is shown in Figure 1.24 (Farris *et al.*, 1998). This led to the conclusion that BipA is necessary for EPEC-directed reorganization of the cytoskeleton in host cells (Farris *et al.*, 1998).

Further study into the effect of BipA depletion on attaching and effacing lesion formation was carried out by Grant *et al.* (2003). Another enteropathogenic *E. coli* (EPEC) *bipA* null mutant strain was constructed using the well

characterised EPEC strain E2348/69 and was named AG2. This mutant displayed phenotypic characteristics similar to those previously reported for the MAR001 EPEC *bipA* null mutant (Farris *et al.*, 1998;Grant *et al.*, 2003). As AG2 was defective in its ability to produce attaching and effacing lesions, the levels of several key LEE-encoded proteins were compared in the presence and absence of BipA (Grant *et al.*, 2003).

Immunoblots demonstrated that the levels of EspB and intimin were reduced in the *bipA* null mutant (AG2) while EspA, EspD and Tir were undetectable, relative to wild type cells (Figure 1.25). However, the introduction of a plasmid-borne copy of *bipA* into AG2 restored the expression of all the proteins to approximately wild type levels (Grant *et al.*, 2003).

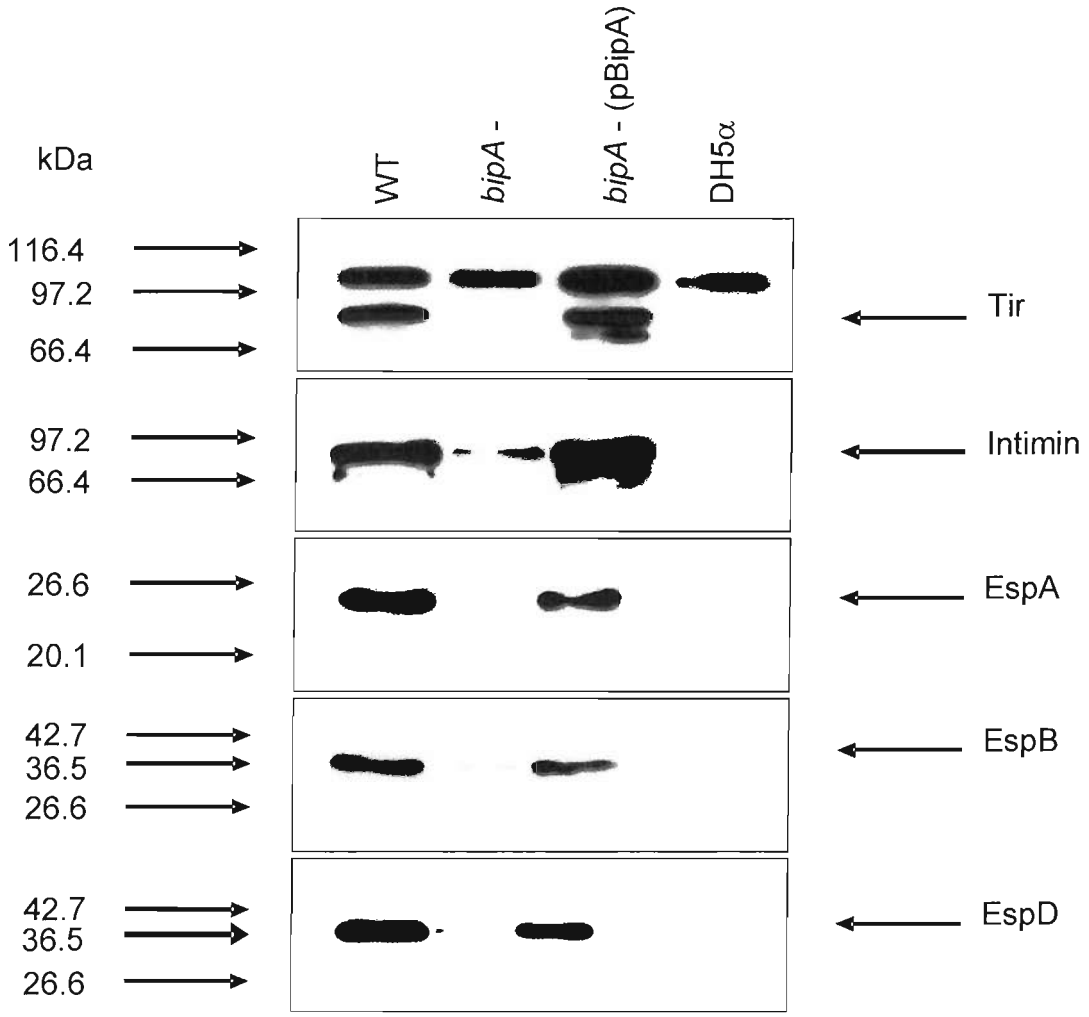


Figure 1.25. Immunoblots of protein samples from E2348/69 (wild type, lane 1), AG2 (E2348/69*bipA::cat* lane 2), AG2(pAJG4) (E2348/69*bipA::cat*(pBipA) lane 3) and DH5α *E. coli* K-12 control strain (lane 4), taken from Grant *et al.* (2003).

To determine whether BipA regulated the translation or transcription of these proteins, the abundance of the relevant mRNA transcripts for the proteins were compared, in the presence and absence of BipA (Grant *et al.*, 2003). It was found that in the absence of BipA the transcription of the following genes was reduced *escT*, *escC* and *escV*, *map/orf19*, *tir*, and *escD*. While *escG*, which is divergently transcribed from the LEE1 operon, was only marginally decreased in the absence of BipA. This was supported by an EPEC derivative carrying an *espD::lacZ* transcriptional fusion; β - galactosidase expressed from the fusion was down regulated five-fold in a *bipA* negative background. This led to the conclusion that BipA is required for the expression of all five major operons of the LEE, and that its effect is exerted at the transcriptional level (Grant *et al.*, 2003).

Elliot *et al.,* (2000) found that the LEE encoded regulator (Ler) is essential for the expression of multiple LEE located genes in both EPEC and EHEC. The genes regulated by Ler include those encoding the type III secretion pathway, the *E. coli* secreted proteins, the Translocated intimin receptor and Intimin. Therefore, Ler is very important to the process of attaching and effacing lesion formation (Elliot *et al.*, 2000).

The Ler deletion mutants of EPEC and EHEC examined by Elliot *et al.,* (2000) showed some similar phenotypic characteristics to *bipA* null mutant cells. They displayed altered adherence to epithelial cells and expressed novel fimbriae (Elliot *et al.*, 2000). This suggested that BipA might operate by inducing the transcription of the LEE1 operon (which contains the *ler* gene). Ler would then up-regulate the rest of the LEE located genes. Grant *et al.* (2003) tested this by constructing a plasmid that expressed a full-length Ler protein tagged with a c-Myc epitope at its C-terminus (pAJG10). Expression of the Ler protein (encoded on pAJG10) restored the expression of intimin in *bipA* null mutant cells, suggesting that heterologous expression of Ler complements the *bipA* null mutant phenotype. To determine whether BipA modulated Ler transcription,

transcription mapping was carried out. *ler* mRNA transcripts were readily detectable in samples from wild type cells. However, they could not be detected in the *bipA* null mutant. Similar results were found in EHEC (Grant *et al.*, 2003).

In EPEC, the products of the *per* operon are required for efficient transcription of *ler*. The *per* operon is located on the EAF plasmid (Gomez - Duarte and Kaper, 1995). Therefore, the hypothesis that *per* is required for BipA's control of Ler expression was tested. It was found that the Per regulator can compensate for the absence of BipA, but BipA does not control *ler* transcription through the Per regulator. Grant *et al.* (2003) also found that BipA did not regulate flagella mediated cell motility through the Ler regulator. The introduction of the *ler::myc* fusion plasmid into *bipA* null mutant cells did not complement the hypermotile phenotype (Grant *et al.*, 2003).

1.12.8 Findings regarding BipA's regulation of *ler* transcription

BipA's unique structure (it shows substantial sequence identity to ribosome-binding GTPases, and lacks any discernible DNA-binding motifs) means that it is unlikely that BipA's regulation of Ler transcription is direct. This indicates that BipA may regulate the translation of another regulatory protein (such as a transcription factor) which then directly affects Ler transcription (Grant *et al.*, 2003). Several possible candidate regulatory proteins have been eliminated previously including, H-NS and IHF (Grant *et al.*, 2003). However, when Goldberg *et al.* (2001) found that *fis* null mutants failed to induce the aggregation of actin necessary to form the attaching and effacing lesion, and concluded that Fis regulates the LEE gene cluster. It was decided to examine Fis expression in *bipA* null mutants and their wild type parent strains (Owens *et al.*, 2004).

It was found that BipA positively regulates the expression of the global regulatory protein Fis. Owens *et al.* (2004) observed that *fis* mRNA transcripts were abundant in *bipA* null mutant cells although very little Fis protein was present, and found that the introduction of plasmid-borne BipA restored Fis expression in *bipA* null mutant cells. In addition, when ectopic Fis expression was induced in a *bipA* null EPEC mutant it was found that it restored the cells ability to express the EspA and EspD (Owens *et al.*, 2004). These findings demonstrated that BipA positively regulates the expression of Fis, and implied that BipA regulates Fis translation (Owens *et al.*, 2004).

Other findings that supported BipA's positive regulation of Fis were: that both *fis* and *bipA* null mutants display altered the rates of plasmid supercoiling after transfer into fresh media (Owens *et al.*, 2004) and that BipA expression was found to be growth rate dependent in a manner similar to Fis, with maximal expression in early exponential phase (Owens *et al.*, 2004).

1.12.9 BipA's effect on Fis

Further to the discovery of BipA's positive regulation of Fis expression, the effect of constitutive production of BipA on Fis expression was examined, using a strain in which *bipA* transcription is tightly regulated by L-arabinose (Grant *et al.*, 2001). Constitutive production of BipA resulted in the level of Fis expression remaining high as cells progressed through the exponential phase of growth, and the protein was still readily detectable three hours after nutrient up shift (when the protein was undetectable from cells in which BipA expression was repressed) (Owens *et al.*, 2004). These results obtained by Owens *et al.* (2004) indicated that BipA is required for Fis synthesis and thereby modulates the expression of its downstream targets, and that constitutive BipA expression delays the onset of the growth phase dependent decrease in Fis levels.

1.12.10 BipA positively regulates the translation of Fis

Two lines of evidence were used to determine that BipA regulates the translation of Fis. The first set of data concerned the level of *fis* transcription in the presence or absence of BipA. This data was obtained using a construct which contained *lacZ* under the control of the *fis* promoter region. It was found that BipA does not effect Fis transcription (Owens *et al.*, 2004). The second set of data concerned the number of *fis* mRNA transcripts, and the amount of Fis protein produced, in the presence or absence of BipA. It was observed that *fis* mRNA transcripts were abundant in *bipA* null mutant cells, although only a small amount of Fis protein was present under the same conditions. This indicated that BipA's regulation of Fis occurs at the level of translation (Owens *et al.*, 2004). The conclusion that BipA regulates the translation of Fis led to the examination of the *fis* mRNA sequence for unusual features that might indicate a BipA response element. It was found that the 5' untranslated region (UTR) shows an exceptionally high degree of complementarity with the 3' end of 16S ribosomal RNA. Typically, the ribosome binding sites of *E. coli* mRNAs display 3 to 6 nucleotides which are complementary to the 3' end of 16S rRNA (Shultzaberger *et al.*, 2001). However, 12 of the 18 nucleotides immediately preceding the *fis* start codon were complementary to 16S rRNA. The anti Shine-Dalgarno segment of 16S rRNA was also positioned differently relative to the start codon when *fis* mRNA was compared with other transcripts (Owens *et al.*, 2004) (Figure 1.26).

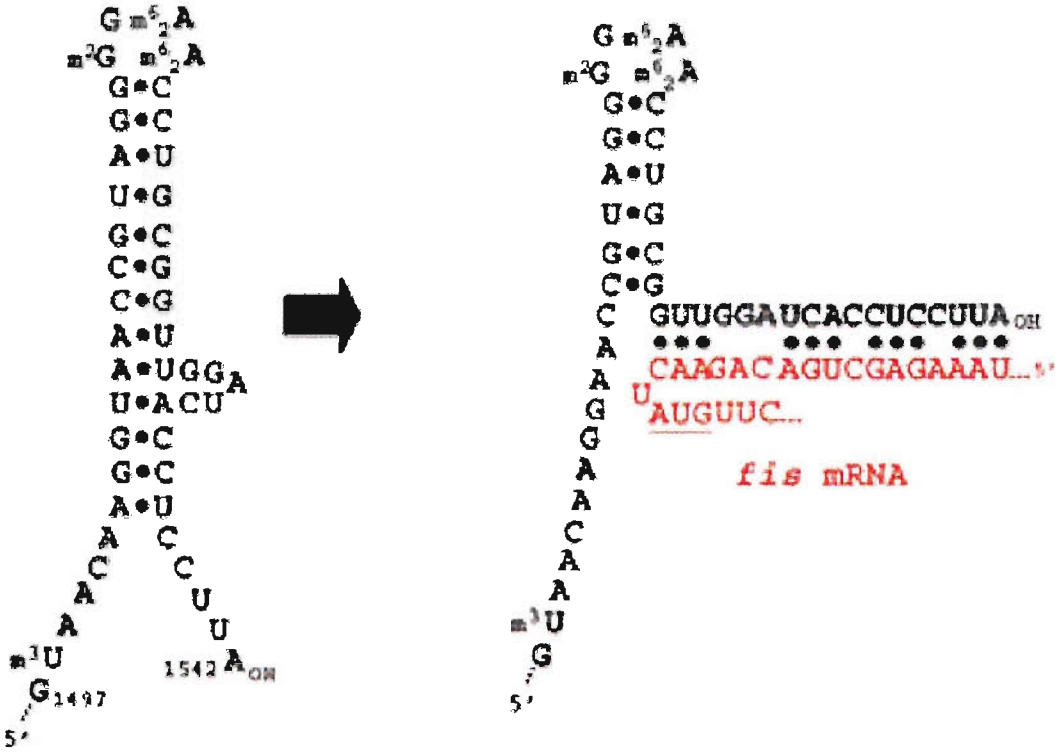


Figure 1.26. The current model for BipA's regulation of Fis expression taken from Owens *et al.* (2004). The extended complementarity between the translation initiation region of the *fis* mRNA (shown in red) and the 3' end of the 16S ribosomal RNA (shown in black).

Owens *et al.* (2004) studied the role of the extended translation initiation region in the BipA-mediated regulation of *fis* by creating mutant derivatives of the *fis* promoter region fused to the structural gene for *lacZ*. The levels of β - galactosidase expressed *in vivo* in the presence or absence of BipA were then compared. BipA enhanced the expression of a hybrid mRNA carrying the wild type *fis* translation initiation region and the mutant derivatives specified by plasmids pJGP2, pJGP3 and pJGP5. However, BipA had no effect on transcription when the native translation initiation region for *lacZ* was present (pJGP6) which indicated that the 5' untranslated region of *fis* contains a BipA-response element. The hybrid mRNA specified by pJGP4 showed only a marginal increase in expression in the presence of BipA, which suggests that the presence of complementary nucleotides at positions -2, -3 and -4 of *fis* mRNA, relative to the start codon, are important for BipA regulation. This is the current model for the regulation of Fis expression by BipA (Owens *et al.*, 2004).

1.13 Aims and objectives

Previous work indicates that BipA is a global regulator in *E. coli*, controlling multiple cellular processes including the formation of attaching and effacing lesions in enteropathogenic *E. coli*. Grant *et al.* (2003) found that BipA positively regulates the transcription of the LEE (responsible for attaching and effacing lesion formation) indirectly through Ler.

- The initial aim of the project was to identify the regulator that BipA controls (at the translational level) which in turn positively regulates the transcription of Ler, Factor X.

However, shortly into the project, Owens *et al.* (2004) reported that BipA positively regulated, the nucleoid associated protein, Fis which positively regulates the LEE gene cluster by activating Ler transcription (Goldberg *et al.*, 2001;Owens *et al.*, 2004). The report of the regulation of Fis by BipA produced amended aims:

- To further examine the regulation of Fis by BipA, by comparing the phenotypic characteristics of both *fis* and *bipA* null mutants (such as comparative motilities and cold sensitivities) to determine whether other BipA regulated processes may also involve Fis.

- To identify further targets (direct or indirect) of BipA.

- To further elucidate the mechanism by which BipA regulates the translation of Fis.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials and Reagents

The following materials and reagents were extensively used:

Qiagen mini plasmid preparation kit
Qiagen Qiaprep Gel extraction kit
Sigma-Aldrich Gene elute midi plasmid preparation kit
Sigma-Aldrich Gene elute maxi plasmid preparation kit
Invitrogen 1 kb DNA ladder
New England Biolabs broad range protein marker
MultiMark multi-coloured standard (Invitrogen)
Promega Restriction Enzymes
Promega DNA polymerase I large (Klenow) fragment
Promega *Pfu* DNA polymerase
Promega T4 DNA Ligase
Promega Shrimp Alkaline Phosphatase (SAP)
Sigma-Aldrich BCA assay kit
Ethidium bromide (Sigma-Aldrich)
Isopropyl β-d-thiogalactopyranoside (IPTG, Melford)
5-Bromo-4-chloro-3-indolyl-β-d-galactoside (X-GAL, Melford)
ortho-nitrophenyl-β-d-galactopyranoside (ONPG, Sigma-Aldrich)

Unless otherwise stated all other reagents were acquired from Promega, New England Biolabs or Sigma-Aldrich.

2.1 Bacterial Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2 All bacterial strains were stored at -80°C in 25% glycerol (1:1 (v/v) culture:50% glycerol).

Table 2.1. Bacterial strains used in this study[a].

Strain	Relevant Genotype	Source or reference
Escherichia coli strains		
MG1655	*E. coli* K-12 λ⁻ F⁻ *ilvG rfb*-50 *rph*-1 OR:H48:K-	(Blattner *et al.*, 1997)
MG1655*ΔbipA*	*E. coli* K-12*ΔbipA* derivative (previously known as *ΔyihK*)	(Arigoni *et al.*, 1998;Guyer *et al.*, 1981)
E2348/69	Enteropathogenic *E. coli* 0127:H6 clinical isolate	(Levine *et al.*, 1985)
E2348/69*bipA::cat*	Enteropathogenic *E. coli bipA* null mutant. Cm^R	(Grant, 2001;Grant *et al.*, 2003)
CSH50	*E. coli* K-12 λ⁻ F⁻ *ara Δ(lac-pro) rps*L *thi fim*E::IS*1*	(Miller, 1972)
CSH50*fis::kan*	*E. coli* K-12*Δfis* derivative. Kan^R	(Koch *et al.*, 1988)
MG1655*Δlac*	*E. coli* K-12*Δlac* derivative	
MG1655*ΔbipAΔlac*	*E. coli* K-12*ΔbipAΔlac* derivative	(Grant *et al.*, 2003)
Salmonella enterica serovar Typhimurium strains		
LB5010	*Salmonella* Typhimurium LT2 restriction deficient mutant	(Bullas and Ryu, 1983)
SL1344	*Salmonella* Typhimurium *hisG*46	(Wray and Sojka, 1978)

[a] Kan^R = Kanamycin resistant

Cm^R = Chloramphenicol resistance

Table 2.2. Plasmids used in this study[b].

Plasmid	Relevant characteristics	Reference
pRJ807	Contains the *fis* coding region downstream of the *lacZ* 5' UTR, under the control of an IPTG inducible *tac* promoter. Ap^R Kan^R	(Osuna *et al.*, 1991)
pMH1	pRJ807 mutant. pRJ807 was cut at an *Nde*I site and the resultant sticky ends were filled in with DNA polymerase I to form blunt ends which were then ligated to form pMH1. Ap^R Kan^R	This study
pBAD*fis*	Contains the *fis* open reading frame downstream of the arabinose inducible pBAD promoter. Ap^R	(A. Kelly; Ph.D. thesis (2004), University of Dublin)
pAJG38	Contains the *bipA* open reading frame under the control of an arabinose inducible promoter pBAD. Ap^R	(Grant *et al.*, 2001)
pRS414	Contains the *lacZ* open reading frame. Ap^R	(Simons *et al.*, 1987)
pMH2	Contains the *fis* 5'untranslated region and *lacZ* ORF under the control of an arabinose inducible promoter pBAD. Ap^R	This study
pMH4	Contains the native *lacZ* 5'untranslated region bound to *lacZ* under the control of an arabinose inducible promoter pBAD. Ap^R	This study

[b] Ap^R = Ampicillin resistant

Kan^R = Kanamycin resistant

2.2 Culture conditions

Unless otherwise stated bacteria were grown aerobically (with shaking at 230 rpm) at 37°C in Luria-Bertani (LB) medium. Luria-Bertani media was made using 20.6g of LB EZmix per litre (Sigma-Aldrich). LB agar was made by supplementing LB with 1.2% (w/v) agar (Oxoid). Tryptone medium was used for motility plates (1% (w/v) Tryptone, 0.5% (w/v) NaCl, 0.01% (w/v) Thymine, 0.35% (w/v) Agar). All medium was sterilised at 121°C and 15 psi for 20 minutes. After cooling they were supplemented, where appropriate, with the relevant antibiotic(s) before inoculation.

Strains were revived by streaking on LB agar plates (if necessary with the appropriate antibiotic). Overnight cultures were set up, by inoculating 10 ml LB (if necessary containing the appropriate antibiotic) with a single colony taken from an LB agar plate. Cultures were grown at 37°C (with shaking at 230 rpm) overnight. The following day, the overnight culture was used to inoculate fresh pre-warmed LB medium. Unless otherwise stated, 1 ml of the overnight culture was diluted in 40 ml of fresh LB medium, then the cultures were grown at 37°C (with shaking at 230 rpm) to the appropriate optical density (600 nm) for experimentation.

When performing transformations, cells were recovered in SOC medium (2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.05% (w/v) NaCl, 1% (v/v) 250 mM KCl solution, to which 0.5% (v/v) of a 2 M MgCl solution (sterile) and 2% (v/v) of filter sterilised 1 M glucose solution was added prior to use).

2.3.1 Antibiotics

Antibiotic stock solutions were made up in analytical grade water, filter sterilised with 0.2 μm filters (Sartorius) and stored at ˉ 20°C. All stock solutions were used at a volume of 1 μl/ml. The concentrations of the stock solutions were:

Ampicillin	-	100 mg/ml
Kanamycin	-	50 mg/ml

2.3.2 IPTG (Isopropyl β-d-thiogalactopyranoside) and Arabinose

Stock solutions of IPTG and arabinose were made up in analytical grade water, filter sterilised with 0.2 μm filters (Sartorius) and stored at ˉ 20°C (IPTG was also stored in the dark). The concentrations of the stock solutions were:

IPTG	-	100 mM
Arabinose	-	2 M

2.3.3 X-GAL (5-Bromo-4-chloro-3-indolyl-β-d-galactoside)

X-GAL stock solution was made up in N,N-dimethylformamide (Sigma-Aldrich), stored at ˉ 20°C, and used at a volume of 1 μl/ml LB agar. The stock solution concentration was:

X-GAL	-	40 mM

2.3.4 Sodium azide

Sodium azide stock solution was made up in analytical grade water and stored at - 20°C. The concentration of the stock solution was:

Sodium azide - 100 mM

2.4 Monitoring bacterial growth

Growth of bacterial cultures was monitored spectrophotometrically with the optical density of the culture measured at a wavelength of 600 nm (OD_{600}). This was used both for routine measurement, and for the estimation of cell quantities for β - galactosidase assays. 0.1 to 1 ml of the culture was transferred to a cuvette (Fisher) and, where necessary, made up to a final volume of 1 ml with LB medium, capped (Parafilm) and shaken. The OD_{600} value was measured in a spectrophotometer against a blank cuvette containing LB broth and the result was adjusted for the dilution as necessary.

2.5 Motility assays

Motility experiments were carried out as described by Farris *et al.* (1998). The strains to be tested were stabbed into motility plates, and grown at 37°C for 12 hours.

2.6 Cold sensitivity assays

The cold sensitivity assays were carried out as detailed by Grant *et al.* (2001), by streaking the appropriate strains out to single colonies on duplicate LB agar plates, one of which was incubated at 37°C overnight while the other was incubated at 27°C overnight.

2.7 β - Galactosidase assays

β - Galactosidase assays were carried out essentially as described in Miller (1992). Overnight cultures were prepared for each of the strains to be assayed. These cultures were then inoculated into fresh media and grown for the required time. To prevent further growth, the cultures were immersed in ice-water for 10 minutes, after which the bacterial density was measured and the assay performed. The bacterial cell density was recorded by measuring the optical density (600 nm). Then 0.1 to 0.5 ml aliquots of culture were immediately added to 0.9 to 0.5 ml of the assay medium Z Buffer (depending on whether the culture has a high or low activity). Z Buffer consists of: 60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 50 mM β - mercaptoethanol.

100 μl of chloroform and 50 μl of 0.1% SDS solution were added to the assay mixtures and the tubes were vortexed for 10 seconds (to open the cells). The tubes were then placed in a 28°C water bath for 5 minutes. 0.2 ml of 4 mg/ml ONPG was added and after mixing the tubes were incubated in a 28°C water

bath while the colorimetric reaction took place. The time taken to develop sufficient yellow colour was recorded and then 0.5 ml of 1 M Na_2CO_3 was added to stop the reaction. The reaction mixture was placed in a microfuge tube and centrifuged for 2 minutes at 13,000 x *g*. The supernatant was transferred to a cuvette (Fisher) and its optical density at 420 nm was recorded. The amount of β - galactosidase activity was calculated as shown below and expressed in Miller units (Miller, 1992).

Enzyme units can be calculated as follows:

$$\frac{1000 \times OD_{420}}{t \times v \times OD_{600}} = \text{Units of } \beta \text{ - galactosidase}$$

OD_{420} read from the reaction mixture.

OD_{600} reflects the cell density before the assay.

t = the time of the reaction in minutes.

v = the volume of culture (in millilitres) used in the assay.

2.8 Bacterial viability assays

Bacterial viability was assessed by measuring the number of colony forming units for both the test strain MG1655(pRJ807) and the control strain MG1655(pMH1), as detailed by Madigan *et al.* (1997). MG1655(pRJ807) and MG1655(pMH1) cultures were grown to an OD_{600} of 0.5, induced with 2 mM IPTG and grown for a further 90 minutes. The cultures were serially diluted in ice-cold phosphate buffered saline (PBS, Sigma-Aldrich) and 100 μl of each dilution was then plated onto LB agar plates supplemented with the appropriate antibiotics. Following incubation overnight at 37°C, colonies on the plates were counted and the dilution factor was used to work out the number of colony forming units per millilitre of the original culture.

2.9 Transformation

Plasmid DNA was transformed into competent host cells by one of two methods – either plasmid DNA was transformed into cells made chemically competent by treatment with calcium chloride through a heat shock-triggered DNA uptake mechanism, or plasmid DNA was transformed into cells by electroporation, which uses a high voltage electro-shock treatment to trigger DNA uptake.

Negative controls were set up for each transformation to ensure that the colonies obtained were due to the inclusion of plasmid DNA and not the result of contamination or defective antibiotic selection plates.

2.9.1 Chemically competent calcium chloride method

Competent cells were prepared and transformed using calcium chloride as described in Sambrook (1989). Overnight cultures were prepared for each of the strains to be transformed. 1 ml of each overnight culture was transferred into fresh media and grown (at 37°C with shaking) to an optical density (600 nm) of 0.4. The cultures were transferred into ice-cold 50 ml polypropylene tubes, stored on ice for 10 minutes and then the cells were recovered by centrifugation at 4000 x *g* for 10 minutes at 4°C. The medium was decanted from the cell pellets which were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$). Following this, the cells were recovered by centrifugation, as detailed above. The cell pellets were resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ (per 50 ml of original culture). At this point the cells were competent and were used for transformation.

To perform the transformation, 200 μl of the competent cell suspension (for each of the plasmids to be introduced) was transferred into a sterile ice-cold microfuge tube using a chilled micropipette tip. The DNA was added to each tube (in a volume of 1 to 10 μl) and the contents were mixed gently. The tubes were then placed on ice for 30 minutes, before transfer to a preheated 42°C circulating water bath for 90 seconds. The tubes were then incubated on ice for 1 to 2 minutes. Following this 800 μl of SOC medium was added to each tube to revive the cells, and the tubes were incubated for 45 minutes at 37°C with shaking.

To isolate transformants, 200 μl of the transformed competent cells was spread onto LB agar plates containing the appropriate antibiotics, which were incubated at 37°C overnight. The next day the resultant transformants were counted, and single colonies were restreaked to purity (Sambrook *et al.*, 1989).

2.9.2 Electroporation transformation

A 50 ml overnight culture was prepared for each of the strains to be transformed. This was then diluted 5-fold into fresh medium with a final volume of 250 ml and grown (with shaking at 230 rpm) at 37°C to an optical density (600 nm) of 0.6. The culture was then chilled on ice for 5 minutes. All subsequent steps were carried out at 4°C, and all reagents were pre-chilled before use.

The cells were harvested by centrifugation for 10 minutes at 7400 x *g* in a pre-chilled 4°C rotor. The supernatant was removed and the pellet was resuspended in 5 ml of 10% (v/v) glycerol solution, then recovered by centrifugation at 9600 x *g* in a pre-chilled 4°C rotor. The supernatant was

removed and the pellet was resuspended in 0.75 ml 10% (v/v) glycerol solution. The cell suspension was then divided into 80 μl aliquots, in pre-chilled sterile 1.5 ml microfuge tubes, which were snap-frozen in liquid nitrogen and stored at -70°C.

To perform the transformation, electroporation cuvettes (0.1 cm gap, Web Scientific) and 200 μl pipette tips were pre-cooled at -20°C and the frozen cells were gently thawed on ice. Once the cells had thawed, 1 to 2 μl of DNA in sterile water was mixed with them before incubation on ice for a further minute. The pulse generator (Bio-Rad) was set to 1.25 kV and 200 Ω. The cell/DNA mixture was transferred to an ice-cold electroporation cuvette which was placed in the pulse generator chamber and an electroshock was delivered. Immediately afterwards, 1 ml of SOC medium was added to the cells, which were then transferred to a sterile 1.5 ml microfuge tube and incubated at 37°C for 1 hour with shaking to revive them.

To isolate transformants, 200 μl of the transformed cells were spread onto LB agar plates containing the appropriate antibiotics. The plates were then incubated at 37°C overnight. The following day the transformants were counted, and single colonies restreaked to purity.

2.10 Preparation of plasmid DNA

The type of plasmid DNA preparation carried out depended on the quantity and quality of plasmid required. Small scale mini preparations were used for initial screening of transformants, while larger scale preparations were used to generate larger amounts of higher quality plasmid DNA for sequencing, and molecular biology operations such as cloning.

The procedure used to prepare plasmid DNA was a modified alkaline lysis method in which plasmid containing bacterial cells were lysed and the proteins were denatured (SDS). The plasmid DNA was denatured (NaOH) and cellular RNA was degraded (RNase A). Neutralization of the lysis reaction with high salts caused the precipitation of the chromosomal DNA and proteins which were then removed by centrifugation. Neutralization also promoted the re-annealing of the plasmid DNA which was then isolated by adsorption onto a silica based column, washed and desalted then eluted in nuclease-free water (Birnboim and Doly, 1979;Vogelstein and Gillespie, 1979).

2.10.1 Plasmid mini preparation

Small-scale plasmid preparations were carried out using the Qiagen plasmid mini preparation kit and following the manufacturer's instructions. The DNA was eluted in 30 μl of nuclease-free water.

2.10.2 Plasmid midi preparation

Medium-scale plasmid preparations were carried out using the Sigma-Aldrich Gene elute plasmid midi preparation kit and following the manufacturer's instructions. The DNA was eluted in 1 ml of nuclease-free water.

2.10.3 Plasmid maxi preparation

Large-scale plasmid preparations were carried out using the Sigma-Aldrich Gene elute plasmid maxi preparation kit and following the manufacturer's instructions. The DNA was eluted in 3 ml of nuclease-free water.

2.10.4 Determination of DNA concentration

The concentration of DNA samples was determined spectrophotometrically by measuring the absorbance at 230 nm, of the DNA sample in question, using the NanoDrop (NanoDrop ND 1000 spectrophotometer). The instrument was blanked using 2 μl of sterile water then 2 μl of sample was loaded onto the pedestal and its absorbance was taken at 230 nm, which was used to derive the DNA concentration. The DNA purity was assessed by 260/280 ratio (which should be 1.8 for uncontaminated DNA) which was also determined spectrophotometrically by the NanoDrop.

2.10.5 DNA Sequencing

A final concentration of 1 to 2 μg of DNA was dried down in a vacuum concentrator and sent to MWG Biotech for automated sequencing. Appropriate sequencing primers were designed and ordered for the sequencing reactions. They were made by MWG as part of the service and used to perform the automated sequencing. The sequencing primers used are listed in Table 2.3.

Table 2.3. Primers used for sequencing in this study

Primer	Primer sequence 5' to 3'
pKK1	gcttatcatcgactgcacggt
pKK3	aagttcggcaagcgcatcacc
lac re3	cgtgcatctgccagtttga
pMH2 r4	ccgtcactgcgtctttta

2.11 DNA modifying enzymes, separation and visualisation

DNA modifying enzymes were purchased from Promega and used according to the manufacturer's instructions.

2.11.1 Agarose gel electrophoresis of DNA

Unless otherwise stated, both plasmid DNA and DNA fragments were resolved using horizontal 0.7% agarose-ethidium bromide gels prepared essentially as described by Sharp *et al.,* (1973). Agarose was made up in TAE buffer (0.484% (w/v) Tris base, 0.114% (v/v) glacial acetic acid and 0.04% (v/v) 0.25 M Na_2EDTA pH 8.0 supplemented with 0.6 µg/L of ethidium bromide). The gels were prepared by heating the agarose in TAE. Afterwards the gels were poured and left to set for 30 minutes.

DNA samples were prepared by mixing them with one volume of Orange G gel loading buffer (10 mM Tris HCl pH7.5, 50 mM Na_2EDTA, 10% (v/v) Glycerol, 0.4% (w/v) Orange G made up in TE buffer, TE buffer consists of 1% (v/v) 1 M Tris HCl pH8.0 and 0.04% (v/v) 0.25 M Na_2EDTA pH8.0). After sample loading electrophoresis was carried out in TAE buffer containing 0.6 µg ethidium bromide at 120 V for the required time. Separated fragments were visualised under UV light, using the Gene Genius Bioimaging system (Syngene). Fragment sizes were approximated by comparison with a 1 kb ladder, which was made up as follows; 5.6µl 1 kb ladder, 15 µl Orange G gel loading buffer and 79.4 µl nuclease-free water.

2.11.2 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were carried out using Promega *Pfu* polymerase according to the manufacturer's instructions. PCR reactions were performed in a 50 µl reaction volume and in 0.5 ml centrifuge tubes, using a PCR Sprint thermal cycler (Thermo Electron Corporation). PCR reaction mixtures consisted of: 1 x Promega *Pfu* polymerase reaction buffer, 200 µM of each dNTP, 10 pmol of each primer, 2.5 U of *Pfu* polymerase and less than 0.5 µg of template DNA. The thermal cycler conditions used were: 94°C for 1 minute for the initial denaturation of the DNA, then 35 cycles of the following, 94°C for 30 seconds, 64°C for 40 seconds, 72°C for 6 minutes, after which there was a 5 minute final extension at 72°C. The oligonucleotides used for amplification in these studies were synthesized by MWG Biotech. Table 2.4 lists these oligonucleotides.

Table 2.4. Primers used for amplification in this study.

Primer	Primer sequence 5' to 3'
pJGP1 Forward	aaaaaaaaaagtcgacacagaaataaagagctgacagaactatgttcgaa caacgcggccgcgtcgttttacaacgtcgtgactgg
pJGP1 Reverse	ccccccccccctgcagattattattttgacaccagacc
lacZ Fw	aaaaaaaaaagtcgacattgttaaagtgtgtcctttgtcgaatgttcg aacaacgcggccgcgtcgttttacaacgtcgtgactgg

2.11.3 Restriction enzyme digestion

Restriction enzymes were purchased from Promega and used according to the manufacturer's instructions; full details can been found in the Promega Protocols and Applications Guide (1996). The reaction mixtures consisted of 0.2 to 1.5 µg DNA, 1 X restriction enzyme buffer, 1 µg/µl BSA and nuclease-free water. After assembling the mixture and mixing, it was briefly spun and then the appropriate volume of restriction enzyme was added (which depended on the concentration of DNA to be digested). The reactions were incubated at the optimum temperature for 1 to 4 hours and the resultant fragments were analysed by agarose gel electrophoresis (Sharp *et al.*, 1973).

2.11.4 Shrimp Alkaline Phosphatase dephosphorylation

Shrimp Alkaline Phosphatase was purchased from Promega and used following the manufacturer's instructions. Reaction mixtures consisted of 1 X Shrimp Alkaline Phosphatase buffer, DNA and Shrimp Alkaline Phosphatase (1 unit/µg DNA) in a final volume of 40µl. Reactions were incubated at 37°C for 15 minutes, then further Shrimp Alkaline Phosphatase (1 unit/µg DNA) was added prior to incubation for a further 15 minutes at 37°C. The reactions were stopped by heating the mixture for 15 minutes at 65°C.

2.11.5 DNA polymerisation reactions

DNA polymerase I large (Klenow) fragment (Promega) was used for "filling in" reactions with unlabeled dNTPs. Reaction mixes containing: 1 to 4 µg DNA, 1 X Klenow buffer, 20 µg/ml BSA, 40 µM of each dNTP, nuclease-free water and 1 unit of Klenow fragment per microgram DNA were constructed. Reaction mixtures were then mixed, briefly spun and incubated at room temperature for 10 minutes. The reactions were stopped by heating them for 10 minutes at 75°C.

2.11.2 Ligation of DNA

T4 DNA ligase (Promega) was used to join strands of modified DNA using the method suggested in the Promega Protocols and Applications Guide (1996). Ligation reactions were typically set up with a 1:3 molar ratio of vector to insert DNA. Each reaction contained: vector and insert DNA, 1 X Ligase buffer, T4 DNA Ligase, and was made up to a total volume of 10 µl with nuclease-free water. The reactions were mixed, briefly spun, then either blunt ended ligation was carried out by incubation at 15°C to 20°C overnight, or sticky-ended ligation was carried out by incubation at 4°C overnight.

2.11.6 Isolation and Purification of DNA

After DNA modification the components in the reaction mixture were removed by one of two methods. The Qiagen Qiaprep Gel extraction kit was used to

purify or isolate DNA according to the manufacturer's instructions. Enzymatic DNA reactions were cleaned up using column purification and gel extraction was used to isolate and purify particular DNA fragments. DNA was typically eluted in 20 μl of nuclease-free water.

2.12 Protein preparation and analysis

2.12.1 Total cell protein preparation

1 ml of the culture was diluted into fresh media, in duplicate, at a 1 in 40 dilution. The cells were grown (with shaking at 230 rpm) at 37°C to an optical density (600 nm) of 0.5 whereupon one of the duplicate cultures was induced with IPTG or arabinose (2 mM, final concentration). Following the addition of inducer (if appropriate) the cultures were grown for a further 90 minutes before the cells from 10 ml of each culture were harvested by centrifugation in 15 ml sterile falcon tubes (Greiner Bio-One) at 4°C and 4,000 x *g* for 10 minutes. The supernatant was discarded and the pellet was resuspended in 0.5 ml Final Sample Buffer (10% (v/v) 0.625 M Tris-HCl, pH6.8, 20% (v/v) 0.35 M SDS, 10% (v/v) Glycerol, 5% (v/v) 2-mercaptoethanol, 4% (v/v) 7.47 mM Bromophenol Blue). The samples were then boiled for 5 minutes, prior to transfer to 1.5 ml centrifuge tubes and centrifugation at 13,000 x *g* for 5 minutes. Finally, the supernatants were transferred to fresh 1.5 ml sterile microfuge tubes and stored at -20°C until required for further analysis.

2.12.2 Protein preparations for Immuno-blotting

Cultures (1 ml of culture was diluted 1 in 40 into fresh media, unless otherwise stated) were grown to the appropriate optical density (600 nm), and induced (if appropriate) for the required time. The cells were then harvested by centrifugation at 4,500 x *g* for 10 minutes (at 4°C) and the supernatants were discarded. The pellets were washed with 5 ml ice-cold HEPES buffer (10 mM

HEPES pH 7.0 pre-cooled to 4°C) and re-centrifuged, at 4,500 x *g* for 10 minutes (at 4°C). The supernatants were again discarded and the pellets were resuspended in 1.6 ml ice-cold HEPES buffer prior to sonication (6 cycles of 15 microns; 15 sec on, 1 minute off, on ice). Finally the sonicates were centrifuged at 13,000 x *g* in a microcentrifuge, the supernatants transferred to fresh microfuge tubes and stored at -80°C.

The BCA assay (Lowry *et al.*, 1951) was used to determine protein concentrations of the samples. Following protein estimation, the samples were diluted 1 in 4 with 2 X Final Sample buffer (20% (v/v) 0.625M Tris HCl pH6.8, 40% (v/v) 0.35 M SDS, 20% (v/v) Glycerol, 10% (v/v) 2-mercaptoethanol, 8% (v/v) 7.47 mM Bromophenol Blue) and boiled for 5 minutes. Samples were stored at -20°C if they were not immediately used for analysis by SDS-PAGE (Laemmli, 1970).

2.13 Protein preparations for Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) analysis

2.13.1 Lag phase SL1344 samples

Cultures, in 25 ml of LB medium, were grown overnight with agitation at 37°C. The following day the cultures were diluted 1 in 40 into fresh pre-warmed LB medium and grown for a further 6 minutes (with agitation) at 37°C prior to harvesting by centrifugation 12,900 x *g* for 5 minutes at 37°C. The supernatant was discarded, the pellet was washed with 30 ml LB, and re-centrifuged (at 15,700 x *g* for 5 minutes at 37°C). The cells were frozen in liquid nitrogen, resuspended in 1 ml of ice-cold HEPES buffer (10 mM HEPES pH 7.0 pre-cooled to 4°C) and sonicated (6 cycles of 15 microns; 15 sec on, 1 minute off, on ice). The resulting lysates were transferred to fresh centrifuge tubes and snap-frozen in liquid nitrogen for storage at -80°C.

2.13.2 BCA assay for determination of protein concentration

Protein concentrations for the protein samples prepared were determined using the BCA assay kit purchased from Sigma-Aldrich (Lowry *et al.*, 1951). The measurements were used to equalise the amounts of protein loaded onto SDS-PAGE gels and to ensure that the protein concentrations of the protein samples prepared for proteomic analyses were sufficient. BCA assays were performed with microtitre plates as detailed in the manufacturer's instructions. A BSA standard curve was prepared that covered the range from 0 to 1.0 mg/ml BSA against which the test samples were read. The test samples were diluted suitably (if necessary) to achieve an accurate concentration and the value was

adjusted for the dilution in the subsequent interpretation of the results. The protein concentrations were determined spectrophotometrically at 570 nm.

2.14 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially by the method of Laemmli (1970), using standard 13% SDS-polyacrylamide gels. The gels were cast in the Bio-Rad mini protean III gel casting system and run in Bio-Rad mini protean III gel electrophoresis tanks. Following electrophoresis, the gels were stained to visualise the protein bands with Coomassie blue stain (0.25% (w/v) Coomassie Brilliant Blue, 45.4% (v/v) methanol and 9.2% (v/v) glacial acetic acid) for 20 minutes, prior to de-staining with 5% (v/v) methanol, 7.5% (v/v) glacial acetic acid. Gels were photographed using the Gene Genius Bioimaging system (Syngene) and the sizes of proteins, in the samples, was estimated by comparison with broad range protein marker standards (2 to 212 kDa) purchased from New England Biolabs. An additional lysozyme protein marker was created by diluting a 0.05 g/ml lysozyme solution 1 in 2 with final sample buffer and boiling the sample for 5 minutes.

2.15 Immuno-blotting

Protein samples to be immunoblotted were separated on a 13% SDS-polyacrylamide gel. Prior to protein transfer, a nitrocellulose membrane (Schleicher and Schuell) was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol). The transfer was carried out using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad), as detailed in the manufacturer's instructions. The transfer was carried out at 200 mA for 2.5 hours. Transfer of a pre-stained protein marker (MultiMark multi coloured standard, Invitrogen) from the SDS-polyacrylamide gel to the membrane was used to determine if the transfer was successful. After blotting, the membrane was washed in blocking buffer (10 mM Tris-HCl (pH 7.5), 1.875 mM Na_2EDTA (pH8.0), 133 mM NaCl, 2% (w/v) milk powder (Asda Handy pints), 0.5% (v/v) Triton X 100) at room temperature for 1.5 hours with gentle agitation. The blocking buffer was then removed and the primary antibody (diluted 1 in 500 with blocking buffer, Table 2.5) was incubated with the membrane overnight at 4°C. The following day, the antibody was removed and the membrane was washed three times with blocking buffer, prior to application of the secondary antibody (Immunopure Goat anti-rabbit IgG peroxidase conjugated, Pierce Biotechnology), diluted 1 in 160,000 with blocking buffer. Following incubation at room temperature with gentle agitation for one hour, the membrane was washed three times in Tris-saline buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl). The bound antibodies were then detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer's instructions. Luminescence was detected using X-ray film (Pierce) which was manually processed (using Developer and Fixer purchased from Photosol).

Densitometry was used to quantify expression differences shown on the immuno-blots, by scanning them with the Gene Genius Bioimaging system

(Syngene) then densitometry was performed using the Genesnap/Gene tools software (Syngene).

Table 2.5. Primary antibodies used in this study.

Antibody	Source
Anti Fis polyclonal	Professor C. Dorman, Trinity College Dublin
Anti DnaA polyclonal	Dr Konieczny, University of Gdańsk

2.16 Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) analysis

Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) analysis was used to compare the protein expression profiles of a *bipA* null mutant and its wild type parent strain. This technique involves digestion of the protein samples under investigation with Trypsin and labelling of the resultant peptides with amine-reactive isobaric tags (same mass, different tags). The labelled peptide mixtures are then combined and separated using Liquid Chromatograpy and finally subjected to analysis by Tandem Mass Spectrometry. Tandem mass spectrometry induces fragmentation of the peptides upon collision induced dissociation which causes the isobaric tags to release the reporter ions. The reporter ions and peptide fragments are detected simultaneously; the peak area ratios of the reporter ions are used to determine the relative abundance of the peptide within each sample while the peptide fragments are used to identify the protein from which they were derived (Choe *et al.*, 2005;Ross *et al.*, 2004). This approach offers a number of advantages over other methods for proteome analysis such as, indiscriminate labelling and the ability to directly compare up to four protein samples in one experiment (Choe *et al.*, 2005;Ross *et al.*, 2004;Wu *et al.*, 2006).

Samples for iTRAQ analysis were prepared and their protein concentrations measured as detailed previously. The samples (100 µg of each) were then labelled with iTRAQ tags (iTRAQ reagent kit, Applied Biosystems) according to the manufacturer's instructions and combined. The combined samples were then fractionated to reduce the complexity prior to analysis by tandem mass spectrometry, as follows.

2.16.1 Strong cation exchange fractionation

Labelled peptides were lyophilised in a vacuum concentrator and resuspended in 200 µl of Buffer A (10 mM H_3PO_4, 25% acetonitrile) prior to fractionation on a polysulfoethyl strong cation exchange 5 µm column (Phenomenex, 150 mm x 4.60 mm), using a Ultimate capillary/Nano LC system (Dionex corporation) with a constant flow rate of 0.2 ml/min and an injection volume of 200 µl. The fractionation was carried out over an 85 minute gradient between Buffer A and Buffer B. Buffer B consisted of 10 mM H_3PO_4, 25% acetonitrile, 500 mM KCl. The gradient consisted of 5% Buffer B for the first 20 minutes, 5% Buffer B to 40% Buffer B for the next 20 minutes, then 40% Buffer B to 100% Buffer B for 10 minutes, 100% Buffer B for 20 minutes then from 100% Buffer B to 5% Buffer B for the final 15 minutes. The chromatogram was monitored via a UV detector and Phenomenex 2 software (Phenomenex). Fractions, which were collected every minute, were dried down in a vacuum concentrator before mass spectrometric analysis.

2.16.2 Mass spectrometric analysis and data processing

Mass spectrometric analysis was carried out in the Centre for Proteomic Research (University of Southampton) essentially as detailed in Skipp *et al.* (2005) using a Q–Tof Global Ultima mass spectrometer (Waters) fitted with a NanolockSpray™ source to achieve better than 10 ppm mass accuracy for the precursor ions. All mass spectra were automatically processed MassLynx 4.0 (Waters) and searched against the NCBI non-redundant database using ProteinLynx 2.25 software (Waters) (Skipp *et al.*, 2005). The Mascot software package (Matrix Science) was used to normalise the peptide data and weight the protein quantitation wherever possible. The normalised data was then used

to determine the average correction factor and it was applied to the proteins which were not successfully identified and quantitated using Mascot.

2.17 Phase contrast microscopy

Overnight cultures were diluted 1 in 40 into fresh pre-warmed LB and grown to an optical density at 600 nm of 0.5. Where appropriate, the cultures were then induced with 2 mM IPTG for 90 minutes, prior to the addition of 5 mM sodium azide to immobilise the cells. 5 µl of each sample was placed on a microscope slide (BDH), covered with a cover slip (TAAB) and examined using a phase contrast microscope (Leica DM RBE) fitted with a digital camera (Hamamatsu, Orca CCD) for viewing and recording images. Cell images were recorded using both the X 40 objective and the X 60 objective. Cell length measurements were collected using ImageJ software. Data for a calibration slide was also recorded at the same magnification, size and resolution as the cell images, to provide a scale for the images.

CHAPTER 3

PHENOTYPIC COMPARISON OF FIS AND BIPA NULL MUTANTS

3 Phenotypic comparison of *fis* and *bipA* null mutants

3.1 Introduction

It has been established that BipA is a protein with global regulatory properties that is involved in processes that are essential to bacterial growth and also survival in various stress conditions (reviewed in chapter one) (Farris *et al.*, 1998;Grant *et al.*, 2003;Pfennig and Flower, 2001;Rowe *et al.*, 2000). However, many of BipA's characteristics are unlike those of the majority of global regulatory proteins. For example BipA displays significant sequence similarity to the ribosome binding GTPases such as EF-G (Farris *et al.*, 1998) and has been shown experimentally to bind to ribosomes. Its GTPase activity is also stimulated in the presence of ribosomes (Owens *et al.*, 2004). These characteristics led to the hypothesis that BipA regulates gene expression through a novel mechanism, most logically by modulating the rate of translation of one or more target genes. It was further hypothesized that the products of the putative target gene(s) might then interact with key cellular signalling mechanisms to bring about BipA's many *in vivo* effects.

Although BipA has been shown to regulate many cellular processes in *E. coli* evidence about its mechanism of action has proved elusive. Significant progress has been made, however, in elucidating the mechanism of BipA's control of the formation of attaching and effacing lesions in EPEC. Previous studies have established that these lesions require the expression of a cluster of genes termed the locus of enterocyte effacement (LEE), which is located at 82 min on the chromosome of EPEC (Elliott *et al.*, 1998;McDaniel *et al.*, 1995). The LEE, which encodes a type three secretion system and certain effector proteins that are injected into host cells through it, is controlled in part by a

regulatory protein termed Ler (for LEE encoded regulator) (Elliot *et al.*, 2000). The available evidence indicates that BipA indirectly controls the transcription Ler by changing the level of another regulator (Grant *et al.*, 2003). The second unidentified regulator, which was provisionally named Factor X, was proposed to be regulated by BipA at the translational level, in keeping with the known properties of BipA. It was reasoned that the identification of the putative Factor X would facilitate the elucidation of the regulatory mechanism used by BipA to regulate gene expression.

Shortly after the current project commenced a potential candidate for Factor X was put forward. The target gene in question was *fis* which encodes the global regulatory protein Fis (Owens *et al.*, 2004). In view of this work it was naturally of interest to compare the phenotypes of both *bipA* and *fis* null mutants. If disruption of the *fis* or *bipA* genes caused similar changes this might imply that both gene products were involved in the same cellular signalling pathway(s). However, it was also of interest to determine whether BipA regulated processes that were not controlled by Fis because this would shed light on BipA's regulatory hierarchy and put studies in a broader perspective. Accordingly, this chapter describes studies to compare the phenotypes of *bipA* and *fis* null mutants of *E. coli* K-12.

3.2 The motility of *fis* and *bipA* null mutants

Farris *et al.* (1998) established that a *bipA* null mutant of EPEC strain MAR001 (MAR001*bipA::cat*) displayed hypermotility, indicating that BipA negatively regulates cell motility in EPEC. This effect was associated with increased secretion of flagellin, the major structural protein of the flagellar filament, into the growth medium. However, when the motility of an *E. coli* K-12 *bipA* null mutant strain (MG1655Δ*bipA*) was tested, it was found that the mutant cells showed reduced motility relative to the wild type parent strain. Therefore, in contrast to EPEC, BipA appears to positively regulate cell motility in *E. coli* K-12 (A. Grant; Ph.D. thesis (2001), University of Southampton).

To extend these results, the motility of MG1655Δ*bipA* was compared with its wild type parent strain, MG1655, and also with the motility of an *E. coli* K-12 *fis* null mutant strain, CHS50*fis::kan*. Because the latter strain has a different genotype to MG1655, the motility of its parent strain, CSH50, was also examined. To achieve this, cells of the appropriate strains were stabbed into motility plates and grown overnight at 37°C. The motility of each strain was then assessed by measuring the diameters of the resultant colonies and comparing them. Figure 3.1 shows an example of the colonies sizes that result when CSH50 and CHS50*fis::kan* are tested in this way while Figure 3.2 summarizes the results.

Figure 3.1. Fis positively regulates motility in *E. coli* K-12. Bacterial cultures were stabbed onto motility agar and incubated at 37°C overnight. The strain on the left is CSH50*fis::kan* and on the right is CSH50.

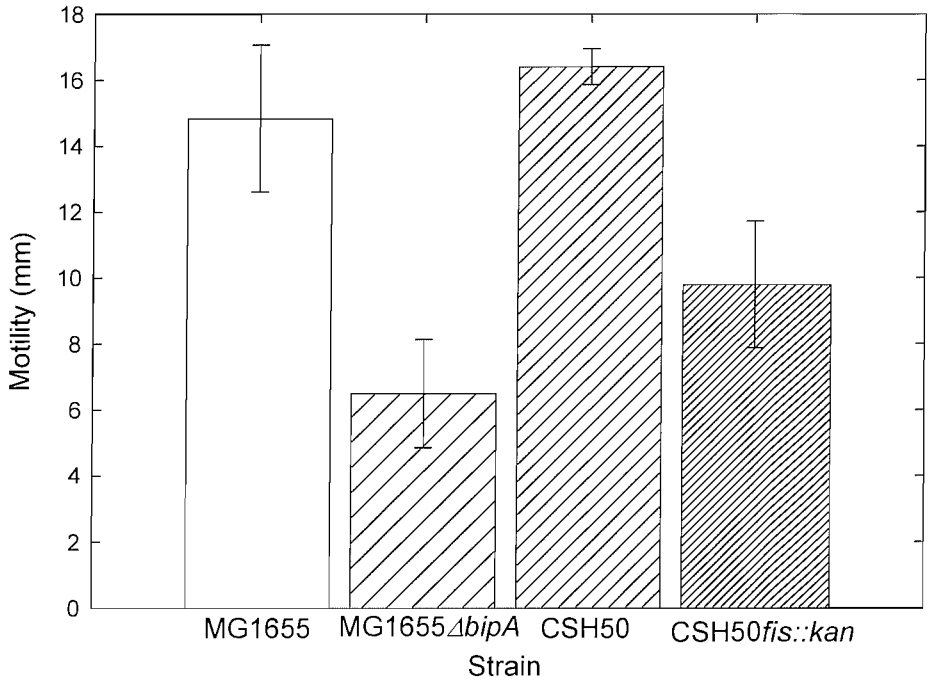


Figure 3.2. Both BipA and Fis positively regulate motility in *E. coli* K-12. Bacterial cultures were stabbed into motility agar and incubated at 37°C overnight. The strains examined were MG1655, MG1655*ΔbipA*, CSH50 and CSH50*fis::kan*.

The motility phenotype of a *bipA* null mutant of *E. coli* K-12 was confirmed - it showed reduced motility when compared to its wild type parent strain indicating that BipA positively regulates cell motility in *E. coli* K-12. It was also found that a *fis* null mutant strain of *E. coli* K-12 displayed reduced motility compared to its wild type parent strain, indicating that both Fis and BipA positively regulate motility in *E. coli* K-12.

3.3 The cold sensitivity of *fis* and *bipA* null mutants

Pfennig *et al.*, (2001) discovered that *bipA* is essential for growth at temperatures below 30°C in *E. coli* K-12 and Grant *et al.*, (2001) found that this is also the case for the EPEC strain E2348/69. Therefore, it was of interest to determine whether mutants defective in Fis also displayed this mutant phenotype. Accordingly, the cold sensitivity of both *fis* and *bipA* null mutants as well as their wild type parent strains was examined.

Each strain was streaked out to single colonies on duplicate LB agar plates. One set of plates was then incubated at 37°C overnight while the other set was incubated at 27°C overnight. The relative growth was then recorded qualitatively for each strain.

Figure 3.3. Fis is not essential for growth at low temperatures in *E. coli* K-12. Strains were streaked to single colonies on LB agar plates and incubated overnight at 37°C or 27°C respectively. The strains on each plate were CSH50 on the left, CSH50*fis::kan* on the right and MG1655Δ*bipA* at the bottom. The plate on the left was incubated at 37°C overnight and the plate on the right was incubated at 27°C overnight.

As expected, BipA was important for growth at the lower temperature in *E. coli* K-12 as the *bipA* null mutant failed to form single colonies when grown overnight at 27°C. However, *fis* null mutants successfully formed single colonies when grown overnight at 27°C and showed no significant difference in growth relative to their wild type parent strain. Therefore, Fis is not essential for growth at low temperatures, in contrast to BipA.

3.4 Discussion

The present study found that both *fis* and *bipA* null mutants of *E. coli* K-12 had reduced motility in comparison with their wild type parent strains. Thus, both BipA and Fis positively regulate flagella-mediated cell motility. This confirms previous work with other bacterial strains or species (Kelly *et al.*, 2004;Osuna *et al.*, 1995) and suggested that mutants of these proteins have very similar phenotypes. However, this evidence alone is insufficient to allow one to conclude that BipA and Fis act in the same pathway to regulate bacterial cell motility. Many different factors are now known to regulate the expression of the flagellar system (Chilcott and Huges, 2000). Thus, it would be entirely possible for different regulatory pathways to produce similar motility phenotypes. By the same token, one cannot determine from these results if BipA acts on Fis or *vice versa*. It was therefore necessary to examine other phenotypes of *bipA* and *fis* null mutants to see if there were differences.

One additional phenotype of *bipA* null mutants that is also readily assayed is lack of growth at temperatures below 30°C (Grant *et al.*, 2001;Pfennig and Flower, 2001). The present study indicates that, in contrast to BipA, Fis is not required for growth at relatively low temperatures. This result implies that BipA regulates cellular targets that are not controlled by Fis. Thus, it would seem that BipA is positioned at a higher level in the regulatory hierarchy relative to Fis, assuming that both components are involved in the same regulatory cascade(s).

Taken together, these differences suggest two possibilities: (i) that BipA is a master regulator that controlled additional targets in addition to Fis, or (ii) that the regulatory circuits controlled by the two proteins were quite different. In the second case, the similar phenotypes obtained in the mutant motility assays

would be coincidental and would be due to influences on different intermediate regulators. These possibilities are explored further in subsequent chapters.

CHAPTER 4

ABERRANT FIS EXPRESSION PREVENTS NORMAL CELL GROWTH IN E. COLI

4 Aberrant Fis expression prevents normal cell growth in *E. coli*

4.1 Introduction

As discussed previously, BipA is a protein with global regulatory properties (Farris *et al.*, 1998;Grant *et al.*, 2003;Owens *et al.*, 2004). Thus, the study of its mechanism of action may unveil a new dimension to the control of gene expression. The report that BipA regulates the translation of the *fis* mRNA (Owens *et al.*, 2004) prompted studies to explore BipA's control of Fis in greater detail. In particular, it would be of interest to know why the *fis* mRNA would need such stringent control mechanisms. This in turn might uncover other mRNAs that are directly regulated by BipA, as the results presented in chapter 3 indicate that BipA may regulate processes that are not controlled by Fis. This chapter describes some initial studies to address such questions.

4.2 Aberrant Fis expression prevents normal cell growth

One possible explanation for BipA-mediated control of Fis over and above the other control mechanisms that have been reported is that aberrant Fis expression may affect cell growth. The rationale for this hypothesis was that aberrant expression of the native *fis* mRNA in the absence of BipA would be deleterious to cells, because it would remain bound via its extended Shine-Dalgarno sequence (Owens *et al.*, 2004) to ribosomes in an inactive state. This model further predicts that *fis* mRNA molecules bearing different 5' untranslated regions should not become hyper-stably bound to ribosomes, and therefore should not be deleterious to cell growth.

To investigate the effect of aberrant expression of the *fis* mRNA the Fis expression plasmid pRJ807 was used as it bears a non-native 5' untranslated

region (UTR), specifically the construct contains the *fis* coding region but the 5' UTR of *lacZ*, under the control of an IPTG inducible *tac* promoter (Figure 4.1 (Osuna *et al.*, 1991)).

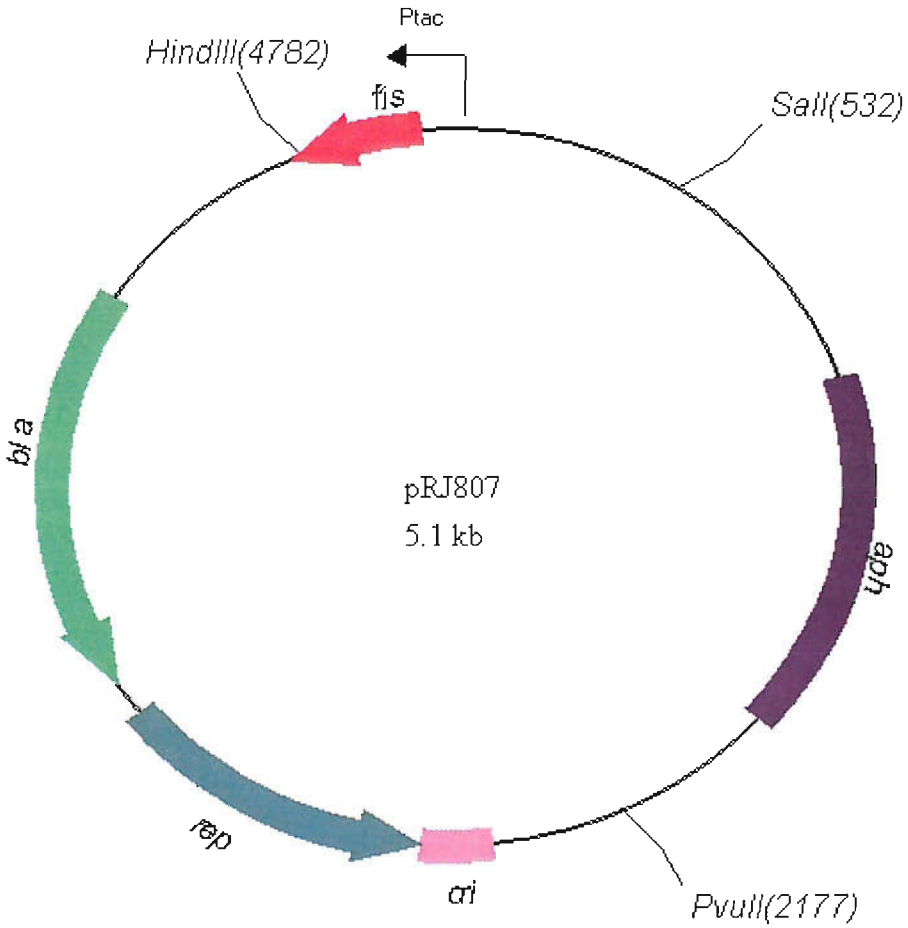


Figure 4.1. Schematic representation pRJ807. This plasmid contains the *fis* open reading frame bearing the non-native 5' untranslated region from *lacZ*, which is under the control of an IPTG inducible *tac* promoter (Osuna *et al.*, 1991).

Prior to carrying out any experiments it was necessary to verify both the structure of pRJ807 and its ability to express Fis. DNA sequencing using oligonucleotide primers that were complementary to the region upstream of the *tac* promoter were used to obtain sequence data for the region in question. The results confirmed that the plasmid contained an open reading frame coding for the wild type Fis protein (Osuna *et al.*, 1991) (Figure 4.2).

Figure 4.2. pRJ807 contains the *fis* open reading frame under the control of an IPTG inducible *tac* promoter (de Boer *et al.,* 1983;Osuna *et al.,* 1991). The sequence data obtained for pRJ807 was aligned with the P*tac*I sequence (de Boer *et al.,* 1983), depicted in red, and the sequence of the *E. coli* K-12 *fis* gene (Blattner *et al.,* 1997), depicted in blue. Sequences were matched using the Align software package (Pearson *et al.,* 1997). The start and stop codons of the *fis* gene are underlined. The *fis* sequence data was taken from the file gi|49175990 (Accession number: NC_000913).

To confirm that induced MG1655(pRJ807) and MG1655Δ*bipA*(pRJ807) cells produced a protein of the correct size for Fis, duplicate samples were grown to an optical density (600 nm) of 0.5. Then one sample of each strain was induced (with 2 mM IPTG) for 90 minutes. Protein samples were then prepared for the cultures and analysed by SDS-PAGE (Figure 4.3). A large band, at the predicted position for Fis was observed when pRJ807 was induced.

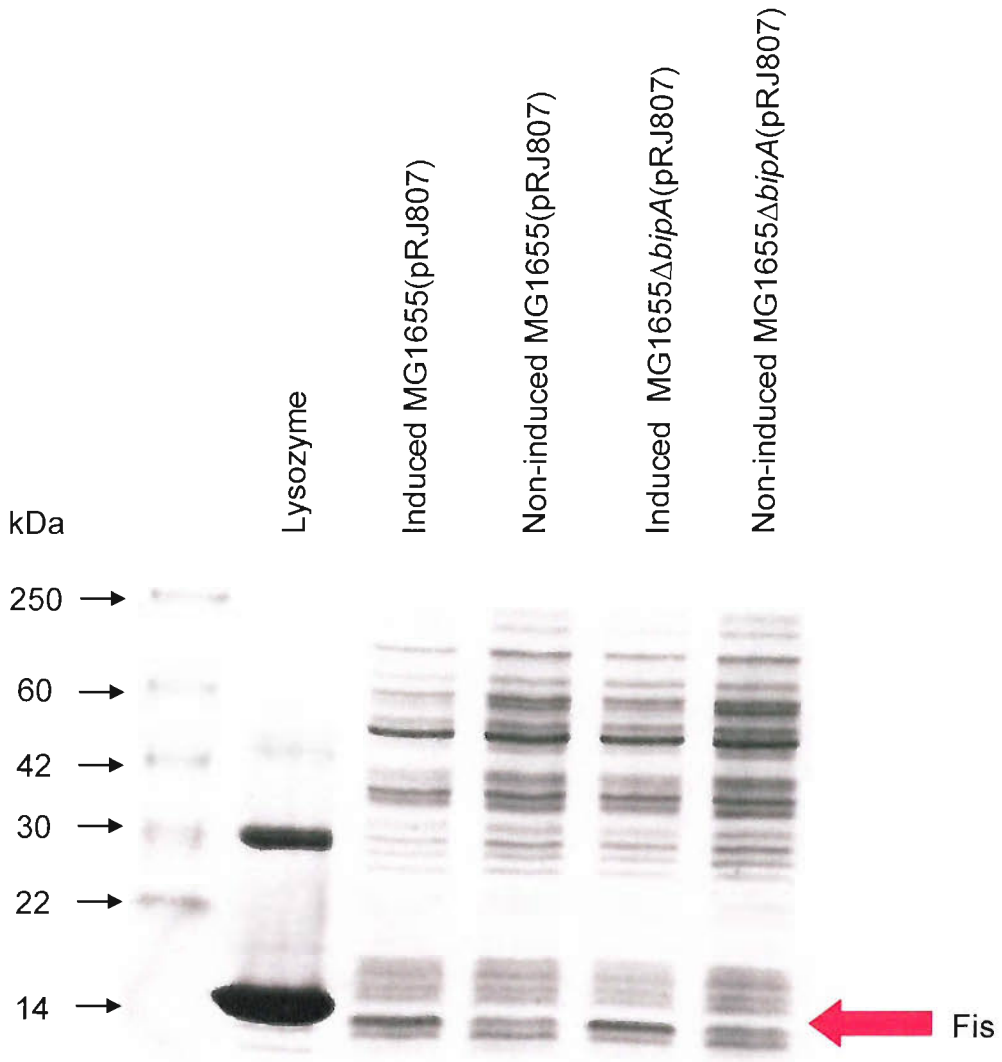


Figure 4.3. Exposure of MG1655(pRJ807) and MG1655Δ*bipA*(pRJ807) cells to IPTG induces aberrant Fis expression. Duplicate cultures of MG1655(pRJ807) and MG1655Δ*bipA*(pRJ807) were grown to an optical density (600 nm) of 0.5 and one sample from each strain was induced with 2 mM IPTG for 90 minutes. Protein samples were then prepared for the cultures and analysed by SDS-PAGE. The red arrow indicates the 11 kDa Fis protein band.

Following verification of pRJ807 the effect of aberrant expression of the *fis* mRNA was examined. This was approached by monitoring the growth of MG1655(pRJ807) and MG1655*ΔbipA*(pRJ807) with and without induction of Fis expression.

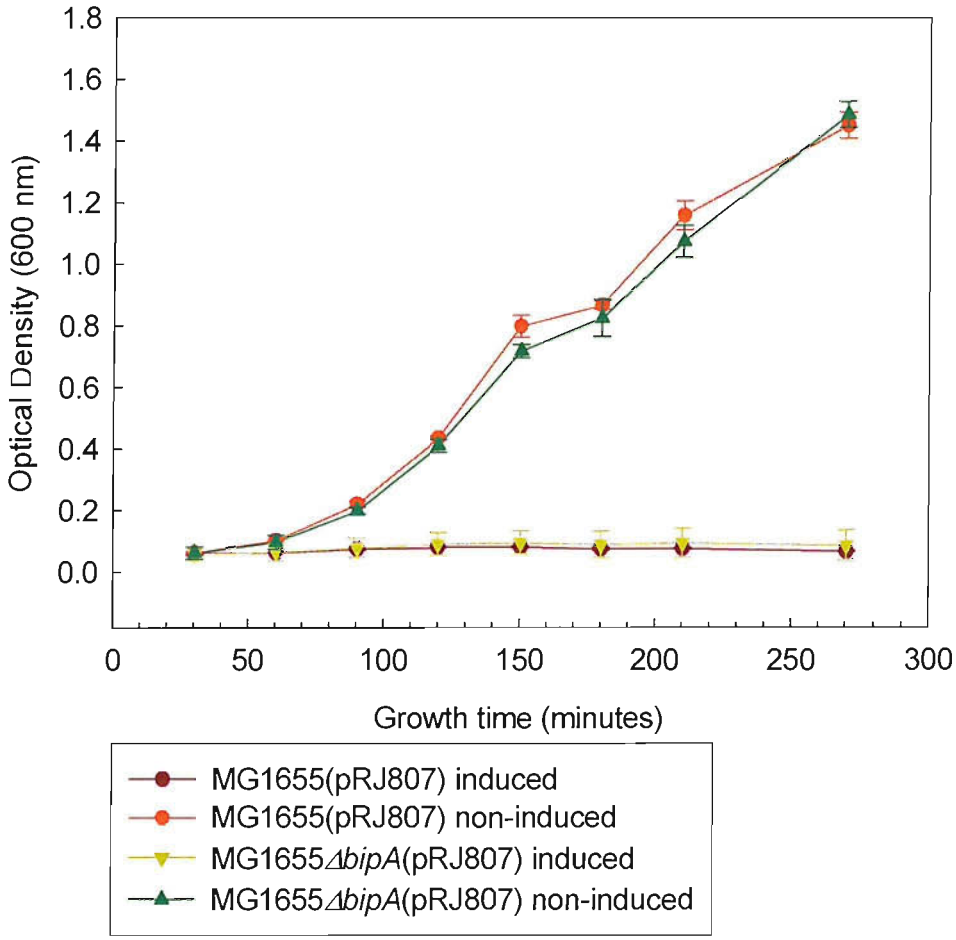


Figure 4.4. Aberrant Fis expression causes growth inhibition in MG1655 and MG1655Δ*bipA*. MG1655 and its *bipA* null mutant were transformed with the Fis expression plasmid pRJ807. The resultant strains were used to assess the impact of expressing Fis with a non-native 5' UTR. After inoculation Fis expression was induced in one sample from each strain with 2 mM IPTG, after which, the growth of the cultures was monitored. The results are the average of four replicates.

Induction of Fis expression from pRJ807 abolished the growth of MG1655(pRJ807) and MG1655Δ*bipA*(pRJ807) cells. In contrast, cell growth continued in non-induced cells, although the rate was comparatively slow relative to the non plasmid bearing parent strains.

4.3 The growth inhibition caused by aberrant Fis expression from pRJ807 is not lag-phase specific

It was considered that the growth inhibitory effect, described above, could be due to the induction of Fis expression in lag phase cells, rather than the result of the induction of Fis expression alone. To eliminate this possibility, the experiment was repeated using cells in the early exponential phase of growth (OD_{600} = 0.2). It was found that changing growth phase at induction of Fis expression did not prevent growth inhibition. However, the effect was a little delayed, because the cells continued to increase in optical density (at a similar rate to the non-induced cells) for half an hour post-induction.

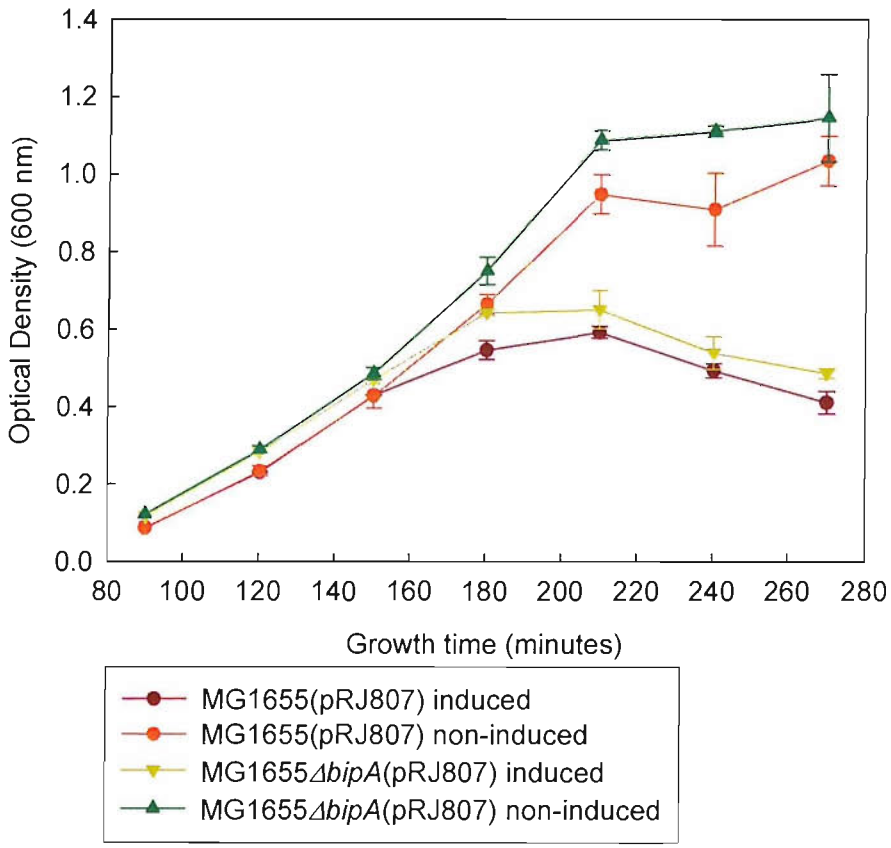


Figure 4.5. Inducing aberrant Fis expression in early log phase, rather than the lag phase does not prevent growth inhibition. MG1655 and MG1655Δ*bipA* were transformed with the Fis expression plasmid pRJ807 and grown to an optical density (600 nm) of 0.2 then Fis expression was induced in one of the duplicate samples with 2 mM IPTG. The growth of the cultures was monitored over time.

Another possible explanation for the observed inhibition of growth following induction of Fis synthesis from pRJ807 was an increased metabolic burden on cells due to the high level production of Fis. To test this possibility, the concentration of IPTG used to induce Fis expression was systematically varied. MG1655(pRJ807) and MG1655*ΔbipA*(pRJ807) were grown to an optical density (600 nm) of 0.2 and then induced with IPTG at a final concentration ranging from 2 mM to 0.1 mM, cell growth was then monitored spectrophotometrically (Figure 4.6). MG1655(pRJ807) was then used to continue this study. MG1655(pRJ807) cells were induced with IPTG at a final concentration ranging from 2 mM to 2 nM and cell growth was monitored (Figure 4.7). Additionally, the growth of strains bearing pRJ807 on solid media in the presence or absence of different concentrations of IPTG was semi-quantitatively monitored (Table 4.1).

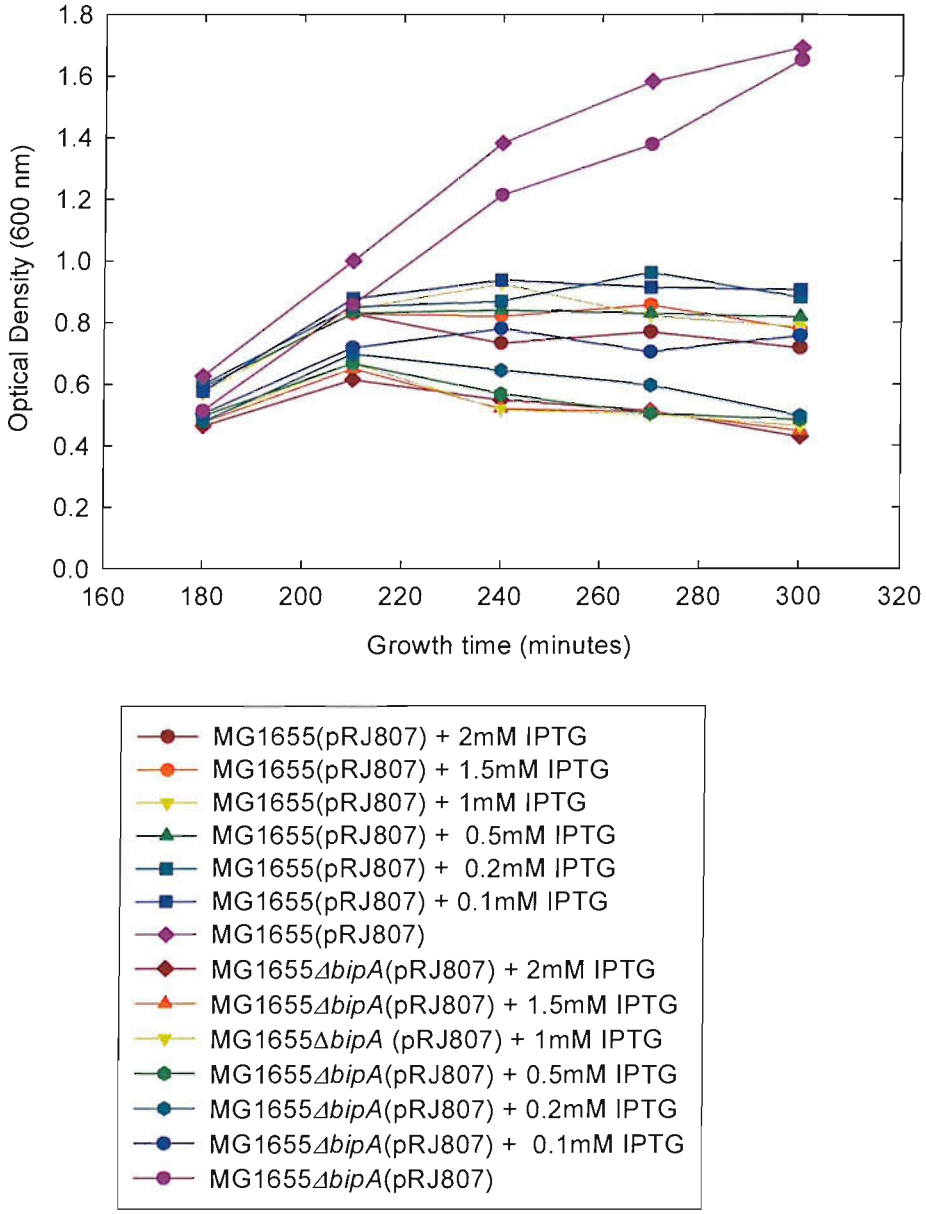


Figure 4.6. A 20-fold reduction in the final IPTG concentration used to induce Fis expression from pRJ807 does not alleviate growth inhibition. MG1655(pRJ807) and MG1655Δ*bipA*(pRJ807) were grown to an optical density (600 nm) of 0.2 and Fis expression was induced with final IPTG concentrations that ranged from 2 mM to 0.1 mM. The growth of the cells was monitored spectrophotometrically.

Table 4.1. Growth inhibition due to induction of Fis expression from pRJ807 in cells on solid media is proportional to IPTG concentration[c].

Strain	IPTG concentration (mM)	Growth rating
MG1655(pRJ807)	0	5
MG1655*ΔbipA*(pRJ807)	0	5
MG1655(pRJ807)	0.1	3
MG1655*ΔbipA*(pRJ807)	0.1	3
MG1655(pRJ807)	0.2	2
MG1655*ΔbipA*(pRJ807)	0.2	2
MG1655(pRJ807)	0.5	1
MG1655*ΔbipA*(pRJ807)	0.5	1

[c] Single colonies of MG1655(pRJ807) and MG1655*ΔbipA*(pRJ807) were streaked onto solid media containing various levels of IPTG, and grown overnight at 37°C. Then the resultant growth was evaluated and rated from 0 (no growth) to 10 (a lawn).

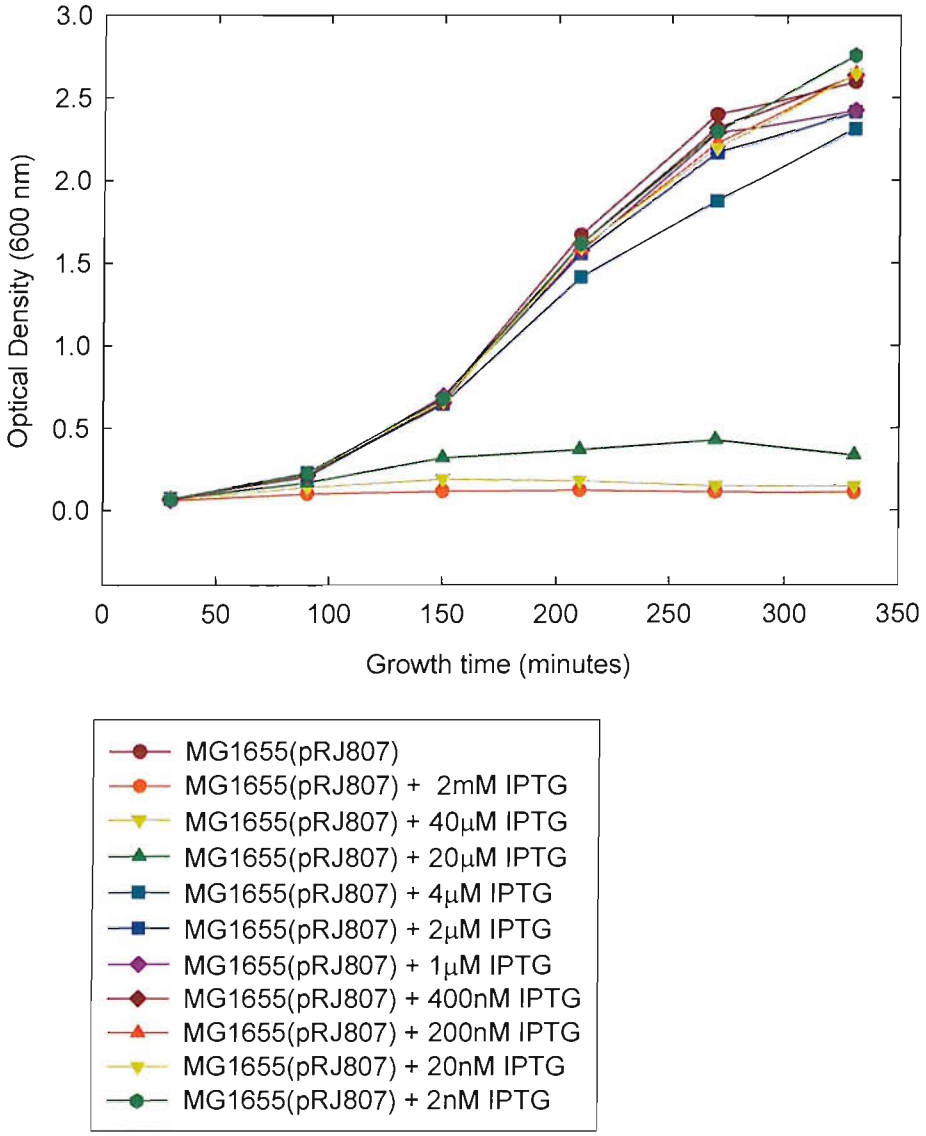


Figure 4.7. A 1000-fold reduction in the final IPTG concentration used to induce Fis expression from pRJ807 alleviates growth inhibition. MG1655(pRJ807) cells were induced at inoculation with final IPTG concentrations that ranged from 2 mM to 2 nM. The growth of the cells was monitored spectrophotometrically.

The minimum inhibitory concentration of IPTG that caused growth abolition in MG1655(pRJ807) was 20 μM (Figure 4.7). Growth inhibition also occurred on solid media in proportion to the amount of IPTG present (Table 4.1).

Collectively, these results show that growth abolition following induction of Fis expression was extremely potent and observable over a wide range of final IPTG concentrations.

4.4 The growth inhibition effect requires Fis expression from pRJ807

There are two possible causes for the growth inhibition observed when Fis expression is induced from pRJ807. First, it is possible that inhibition is not Fis expression specific but instead results from the expression of an mRNA with a non-native 5' untranslated region. Alternatively, growth inhibition may be due to the expression of the Fis protein.

To determine whether the growth inhibition depended on the expression of the Fis protein, a mutant form of pRJ807 was constructed. The derivative termed pMH1, was constructed by cutting pRJ807 with *Nde*I, introducing a frameshift mutation by "filling in" the sticky ends with DNA polymerase I and finally ligating the resultant blunt ends. This resulted in a construct with severely reduced levels of Fis expression. For further details see section 4.10.

Plasmid pMH1 was transformed into MG1655 and MG1655*ΔbipA*. It was found that pMH1 DNA transformed into MG1655 with a 10^4-fold (i.e. 7.13×10^4 CFU per μg DNA as opposed to 7 CFU per μg DNA, respectively) higher efficiency than pRJ807 DNA. It was also observed that strains containing pMH1 formed normal-sized colonies on solid media, in contrast to the same strains containing pRJ807.

To determine definitively whether Fis expression was required for growth inhibition by pRJ807, cell growth was monitored in liquid cultures.

MG1655(pRJ807), MG1655Δ*bipA*(pRJ807), MG1655(pMH1) and MG1655Δ*bipA*(pMH1) were cultured with and without induction and their growth was recorded over time.

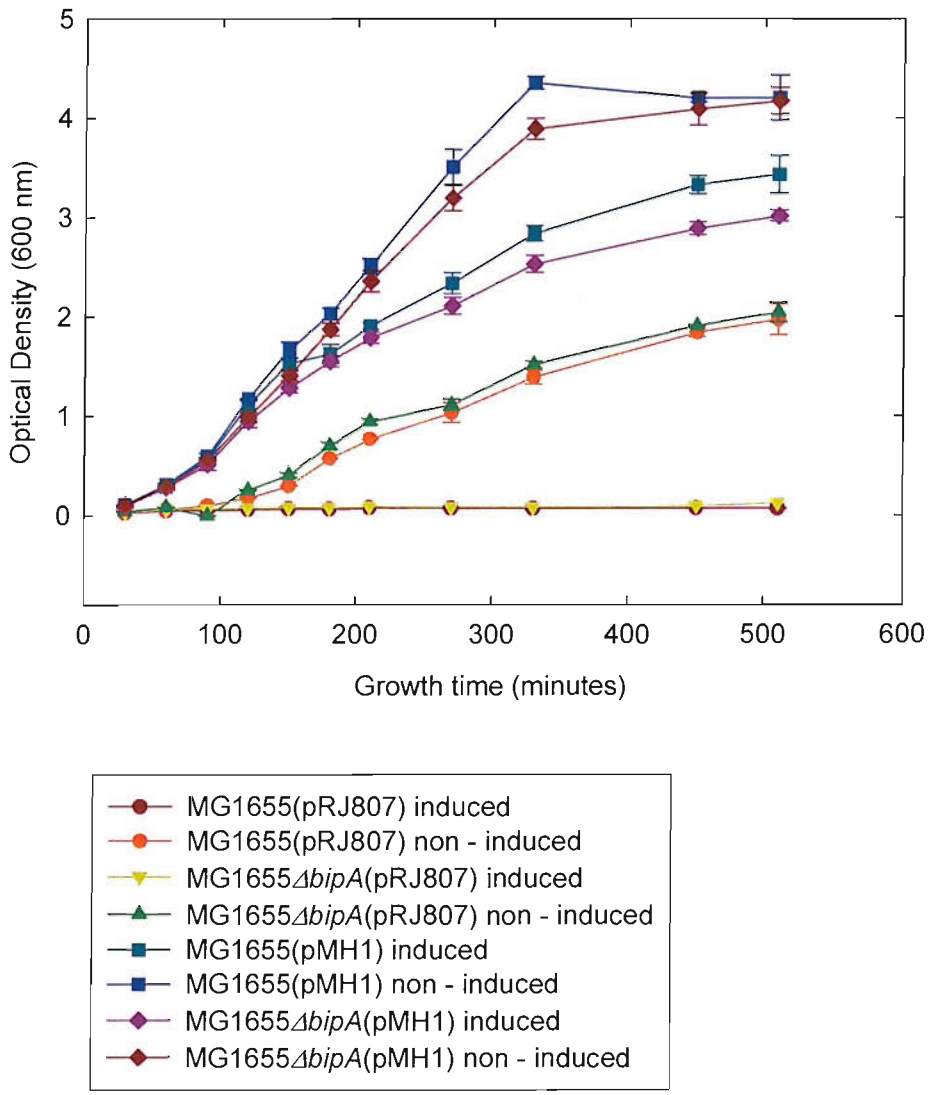


Figure 4.8. Growth inhibition requires Fis expression. MG1655 and MG1655*ΔbipA* cells containing pRJ807 or pMH1, were cultured with or without 2 mM IPTG and their growth was monitored over time.

As shown in Figure 4.8, strains containing pMH1 showed no growth defect after induction with IPTG, in contrast to strains containing pRJ807 which grew slowly without induction and even slower on induction. These results suggest that Fis expression from pRJ807 is required for the growth inhibition effect.

4.5 Aberrant Fis expression from pRJ807 alters the expression of genes that are not part of the Fis regulon

Growth inhibition following expression of Fis from pRJ807 could be due to an effect on a specific target or it might affect the expression of multiple genes. To distinguish between these alternatives the expression of the chromosomal *lacZ* gene, which does not appear to be part of the Fis regulon (Kelly *et al.*, 2004) was monitored following induction of Fis from pRJ807.

Strikingly, the induction of Fis expression totally abolished β - galactosidase expression in MG1655(pRJ807) and MG1655*ΔbipA*(pRJ807). In contrast, β - galactosidase expression in cultures containing the control plasmid pMH1 continued despite the induction of expression from pMH1 (Figure 4.9). This implies that the induction of aberrant Fis expression from pRJ807 decreases the expression of an unrelated gene in the cell.

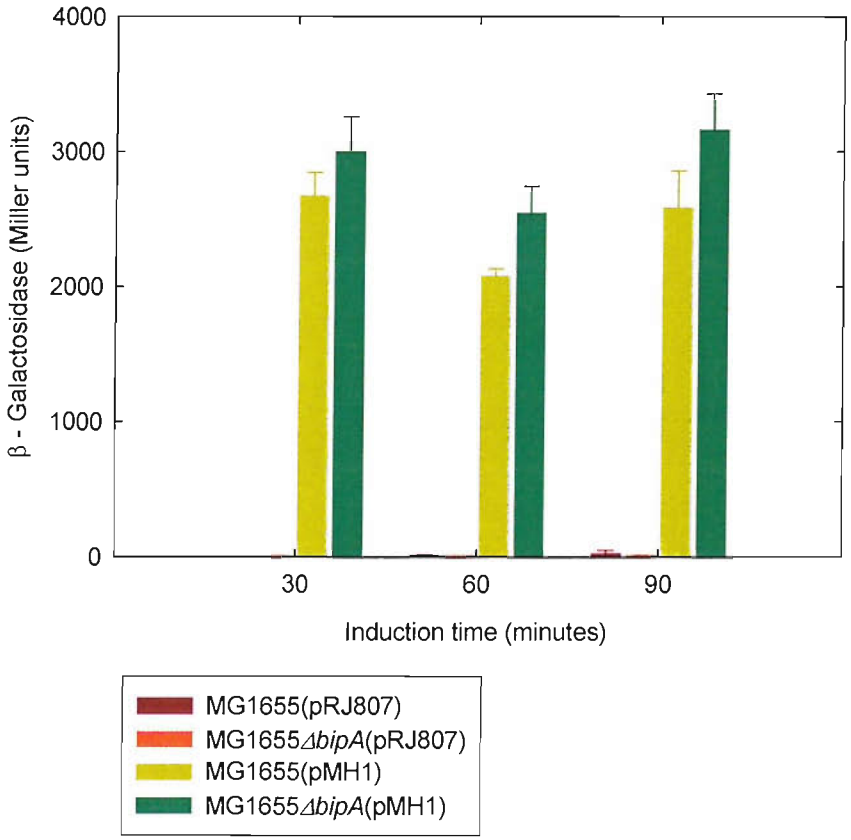


Figure 4.9. Aberrant Fis expression from pRJ807 abolishes β - galactosidase expression. MG1655 and MG1655Δ*bipA* cells containing pRJ807 or pMH1 were grown to an optical density (600 nm) of 0.5 then induced with 2 mM IPTG. β - galactosidase assays were then carried out at intervals to assess the level of this enzyme in the cells.

4.6 Aberrant Fis expression from pRJ807 reduces the number of colony forming units per ml of culture

The effect of inducing aberrant Fis expression from pRJ807 on the number of colony forming units (CFU) obtained was measured. MG1655(pRJ807) and MG1655(pMH1) cells were grown to an optical density (600 nm) of 0.5, induced with 2 mM IPTG and grown for a further 90 minutes. Samples were then plated on LB agar plates (with the appropriate antibiotics) and after overnight incubation, the number of CFU/ml was recorded (Figure 4.10).

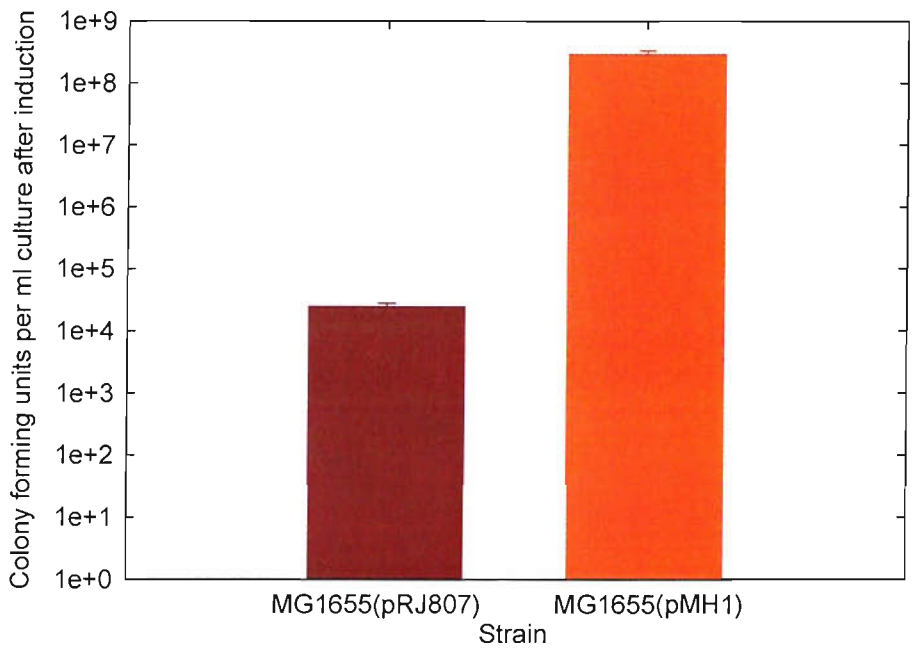


Figure 4.10. Aberrant Fis expression from pRJ807 causes a reduction in CFU/ml in *E. coli*. MG1655 cells containing pRJ807 or pMH1 were grown to an optical density (600 nm) of 0.5, induced with 2 mM IPTG for 90 minutes, and plated on LB agar plates (with the appropriate antibiotics). The number of CFU/ml was recorded after overnight incubation at 37°C (n = 3).

Induction of Fis expression from pRJ807 caused a 10,000-fold decrease in CFU per ml with MG1655(pRJ807) cells. In comparison, MG1655(pMH1) cells grew in the expected numbers. The dramatic drop in the number of colony forming units in response to aberrant Fis expression from pRJ807 might suggest that growth inhibition is a bactericidal effect. However, the cessation of cell division, without loss of viability would give a similar result (Mattick *et al.*, 2000;Mattick *et al.*, 2003b;Mattick *et al.*, 2003a). With this in mind, it was decided to determine whether aberrant Fis expression caused cell shape changes in *E. coli*.

4.7 Aberrant Fis expression causes filamentation

Disruptions to cell division often result in abnormal cell shape in *E. coli*. For example disruption of the *fts* genes in *E. coli* cause the mutant cells to elongate, as they are unable to divide, thereby forming filaments (Bramhill, 1997). In addition, deletion of the *min* genes also induces cell shape changes, resulting in mini cell formation (Huang *et al.,* 2003). These considerations prompted an examination of the shape of cells in which aberrant Fis expression was induced. Cultures of MG1655(pRJ807) and MG1655(pMH1) were grown to an optical density (600 nm) of 0.5 and induced with 2 mM IPTG. After a further 90 minutes of incubation at 37°C, samples were examined by phase contrast microscopy.

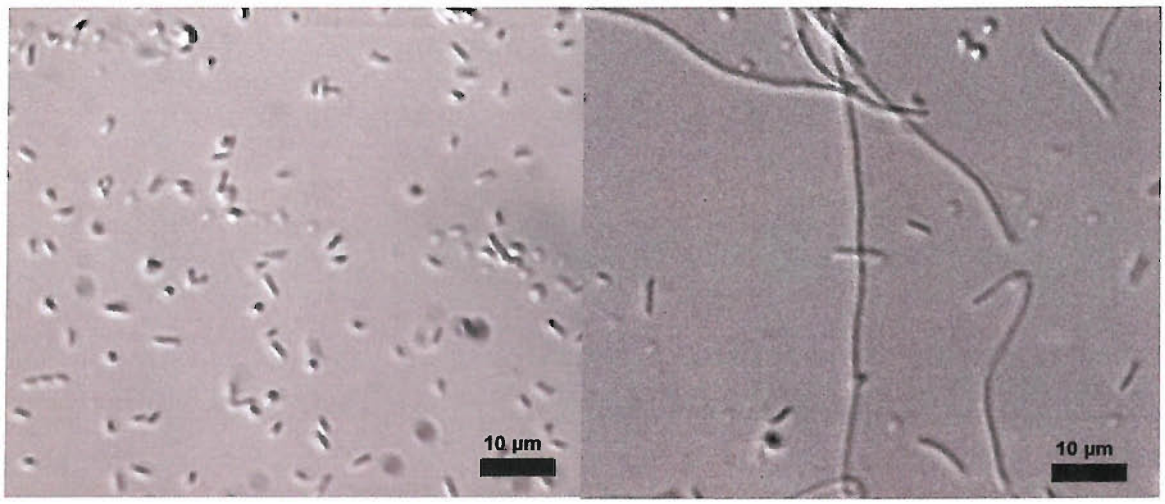


Figure 4.11. Aberrant Fis expression from pRJ807 causes filamentation. MG1655(pRJ807) and MG1655(pMH1) cultures grown to an optical density (600 nm) of 0.5, were induced with 2 mM IPTG for 90 minutes. Samples of each culture were examined by phase contrast microscopy using a X 40 objective. The strain on the left is MG1655(pMH1) and on the strain on the right is MG1655(pRJ807).

Aberrant expression of Fis from pRJ807 caused cells to elongate and hence to form long filaments. The average cell length of the MG1655(pMH1) cells was 2.2 microns (ranging from 1.7 microns to 3.7 microns). However, the average length of the MG1655(pRJ807) cells was 15.5 microns (although cell length varied greatly, ranging from 1.8 microns to 53.4 microns) (Figure 4.11). This indicates that the growth inhibition effect is not bactericidal.

Interestingly, Fis had been the focus of many research groups for many years yet this growth defect associated with aberrant Fis expression does not seem to have been reported previously.

4.8 Fis expression from pBAD*fis* does not cause growth inhibition

The results summarised in Figure 4.7 suggest that minimal induction of Fis expression from pRJ807 causes growth inhibition. To determine if the plasmid-mediated induction of Fis invariably caused growth inhibition another Fis expression vector, pBAD*fis* (A. Kelly; Ph.D. thesis (2004), University of Dublin), was tested. The structure of pBAD*fis*, shown in Figure 4.12, was checked using restriction mapping (data not shown). The Fis expression vector, pBAD*fis* contains the *Salmonella* Typhimurium *fis* open reading frame, which shares 98.3% sequence identity with the *E. coli* K-12 *fis* gene which is encoded by pRJ807 (data not shown). MG1655 and MG1655*ΔbipA* cells containing pBAD*fis* were incubated with and without inducer (2 mM L-arabinose) and their growth was monitored spectrophotometrically.

In contrast to previous findings, cultures that expressed Fis from pBAD*fis* did not show a significant difference in growth rate relative to control cultures (Figure 4.13).

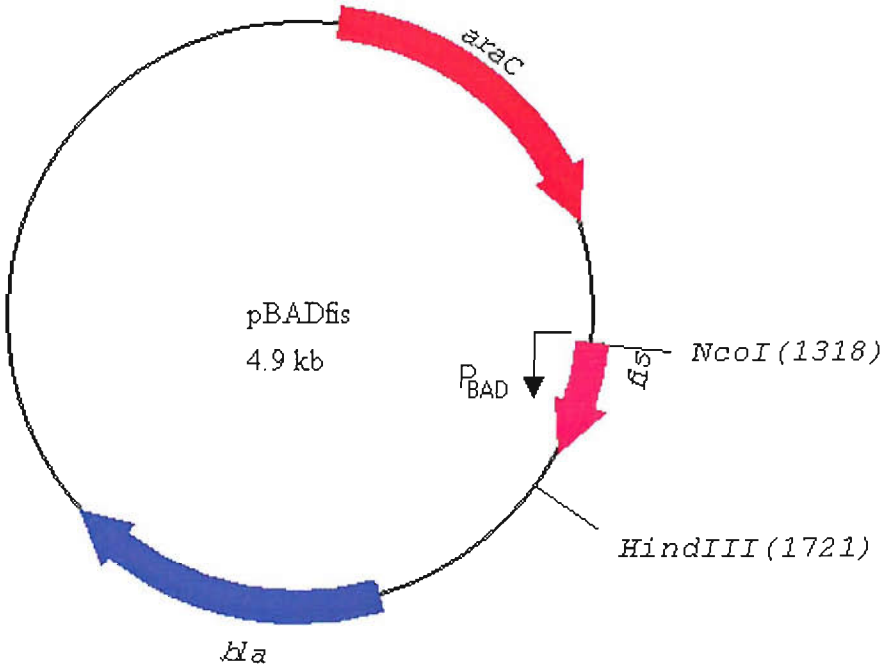


Figure 4.12. Schematic representation of pBAD*fis*. This construct contains the *fis* open reading frame under the control of an arabinose inducible P_{BAD} promoter (A. Kelly; Ph.D. thesis (2004), University of Dublin).

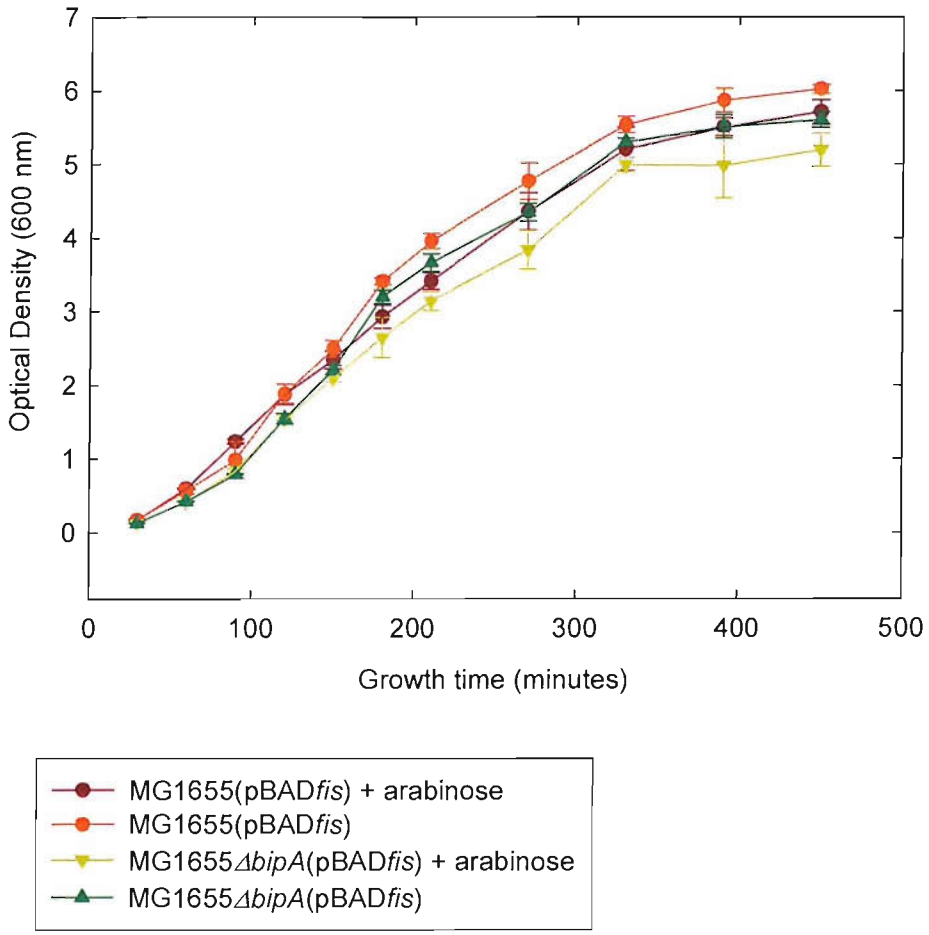


Figure 4.13. Expression of Fis from pBAD*fis* does not cause growth inhibition in *E. coli*. MG1655 and MG1655*ΔbipA* cells containing pBAD*fis* were cultured with and without inducer (2 mM arabinose) and their growth was monitored (n = 3).

The growth of MG1655(pBAD*fis*), MG1655(pRJ807) and MG1655(pMH1) cultures were also directly compared (Figure 4.14).

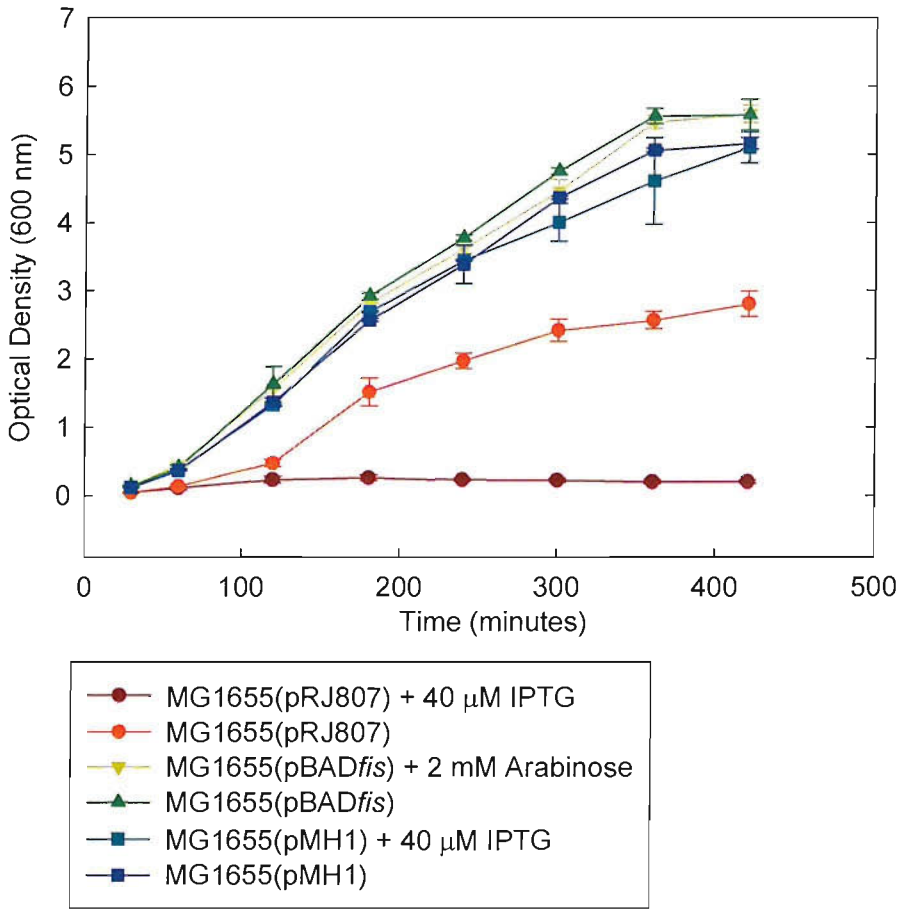


Figure 4.14. Growth inhibition is a pRJ807-specific effect. MG1655 cells containing pBAD*fis*, pRJ807 and pMH1 were cultured with and without inducer (2 mM arabinose for pBAD*fis*, and 40 μM IPTG for pRJ807 and pMH1 containing cultures) and their growth was monitored over time (n=4).

As expected, only MG1655(pRJ807) displayed growth inhibition when Fis expression was induced. MG1655(pBAD*fis*), with or without induction, showed no significant difference in its growth rate relative to the control strain MG1655 (pMH1). In addition, MG1655(pBAD*fis*) and MG1655(pMH1) cultures showed no significant difference in their final optical densities at the end of the experiment. However, MG1655(pRJ807) showed an extended lag time and a significantly lower overall growth rate when compared to MG1655(pBAD*fis*) and MG1655(pMH1) cultures (with or without induction). In addition, the final optical density achieved by the MG1655(pRJ807) cultures was significantly lower than that achieved by MG1655(pBAD*fis*) and MG1655(pMH1) cultures (with or without induction).

Both pBAD*fis* and pRJ807 lack the native Shine-Dalgarno region of the *fis* gene. However, while Fis expression from pRJ807 caused growth inhibition in *E. coli* (even when induced with a very low concentration of IPTG, 40 μM) Fis expression from pBAD*fis* did not. To further investigate the cause of this difference, the levels of Fis expressed from pBAD*fis* and pRJ807 were compared. Duplicate cultures of MG1655(pBAD*fis*) and MG1655(pRJ807) were grown to an optical density (600 nm) of 0.5, whereupon one culture of each strain was induced with IPTG or arabinose. The cultures were grown for a further 90 minutes, after which time protein samples were prepared from them and analysed by immuno-blotting using a Fis-specific antibody probe (Figure 4.15).

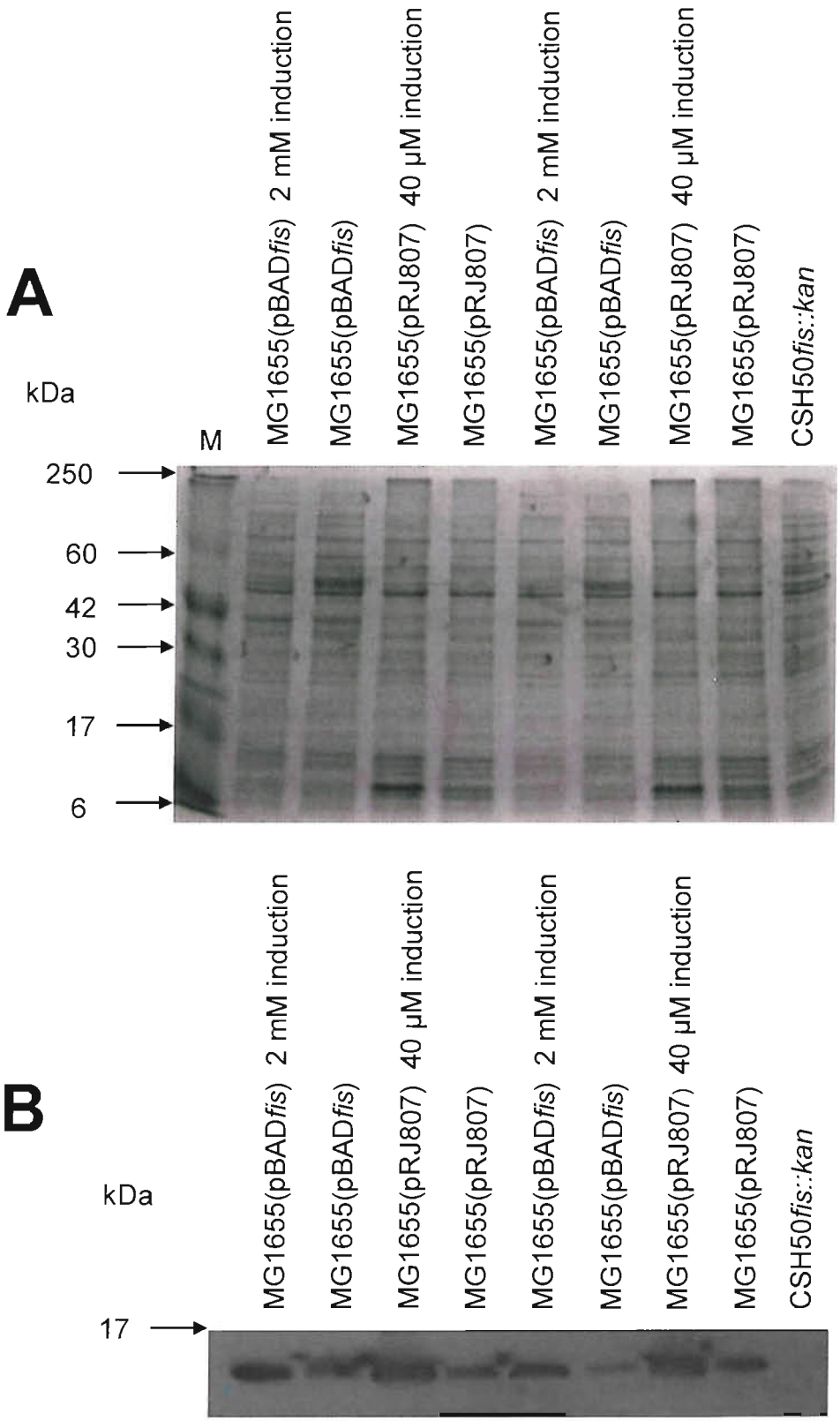


Figure 4.15. MG1655(pRJ807) cells induced with a sub-maximal amount of IPTG still produced a higher level of Fis than MG1655(pBAD*fis*) cells induced with 2 mM arabinose.

Figure 4.15. MG1655(pRJ807) cells induced with a sub-maximal amount of IPTG still produced a higher level of Fis than MG1655(pBAD*fis*) cells induced with 2 mM arabinose. Duplicate cultures of MG1655(pRJ807) and MG1655(pBAD*fis*) were grown to an optical density (600 nm) of 0.5, after which, one of each was induced with 40 μM IPTG (pRJ807) or 2 mM arabinose (pBAD*fis*). The cultures were then grown for a further 90 minutes, after which time protein samples were prepared. CSH50*fis::kan* cultures, grown to mid-exponential phase, were used to create a Fis negative control sample. The protein samples were analysed by immuno-blotting using a Fis-specific antibody. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the immuno-blot. The immuno-blot was scanned into a video image capture system (Gene Genius Bioimaging system, Syngene) then the intensities of the Fis-reactive protein bands was measured by performing densitometry with the Genesnap/Gene Tools software (Syngene).

It was found that cells containing pRJ807 or pBAD*fis* expressed a higher level of Fis when they were induced (Fis expressed increased 2-fold with a standard deviation of 0.1-fold from pRJ807, and 2-fold with a standard deviation of 0.7-fold from pBAD*fis*). However, cells containing pRJ807 expressed a higher level of Fis than cells containing pBAD*fis*. Non-induced cells containing pRJ807 expressed a 1.4-fold higher level of Fis, with a standard deviation (SD) of 0.6-fold, than non-induced cells containing pBAD*fis*. In addition, induced cells containing pRJ807 expressed a 1.5-fold higher level of Fis (with a SD of 0.07-fold) than induced cells containing pBAD*fis*.

Collectively, these results indicate that the high level of Fis expression from pRJ807 is integral to the growth inhibition that occurs when Fis expression is induced from pRJ807. It should be noted, however, that the measurement of Fis levels by densitometry is not highly accurate. Therefore, one should be cautious about drawing solid conclusions from this part of the study. However, these results led to the realisation that the high level of Fis expression from pRJ807 is integral to the growth inhibition that occurs when aberrant Fis expression is induced. It was then decided to compare Fis expression at its highest level in Lag phase cells (Ball *et al.,* 1992;Mallik *et al.,* 2006) to the level of Fis expressed from pRJ807.

Initially, it was attempted to investigate in greater detail the level of Fis protein produced by induced MG1655(pRJ807) cells. Protein samples were prepared from cells induced with a range of IPTG concentrations and immuno-blotted. Additionally, samples were analysed from cells harbouring pMH1. As shown in Figure 4.16 the level of Fis produced in cells treated with 40 μM and 2 mM IPTG were virtually the same (Fis expression was 1.05-fold higher with a standard deviation of 0.02-fold with 2 mM IPTG relative to 40 μM IPTG), suggesting that near-maximal induction could be achieved with the lower concentration. Additionally, densitometry measurements indicate that non-induced MG1655(pRJ807) cells express 1.9-fold (with a standard deviation of 0.4-fold) more Fis than MG1655(pMH1) cells.

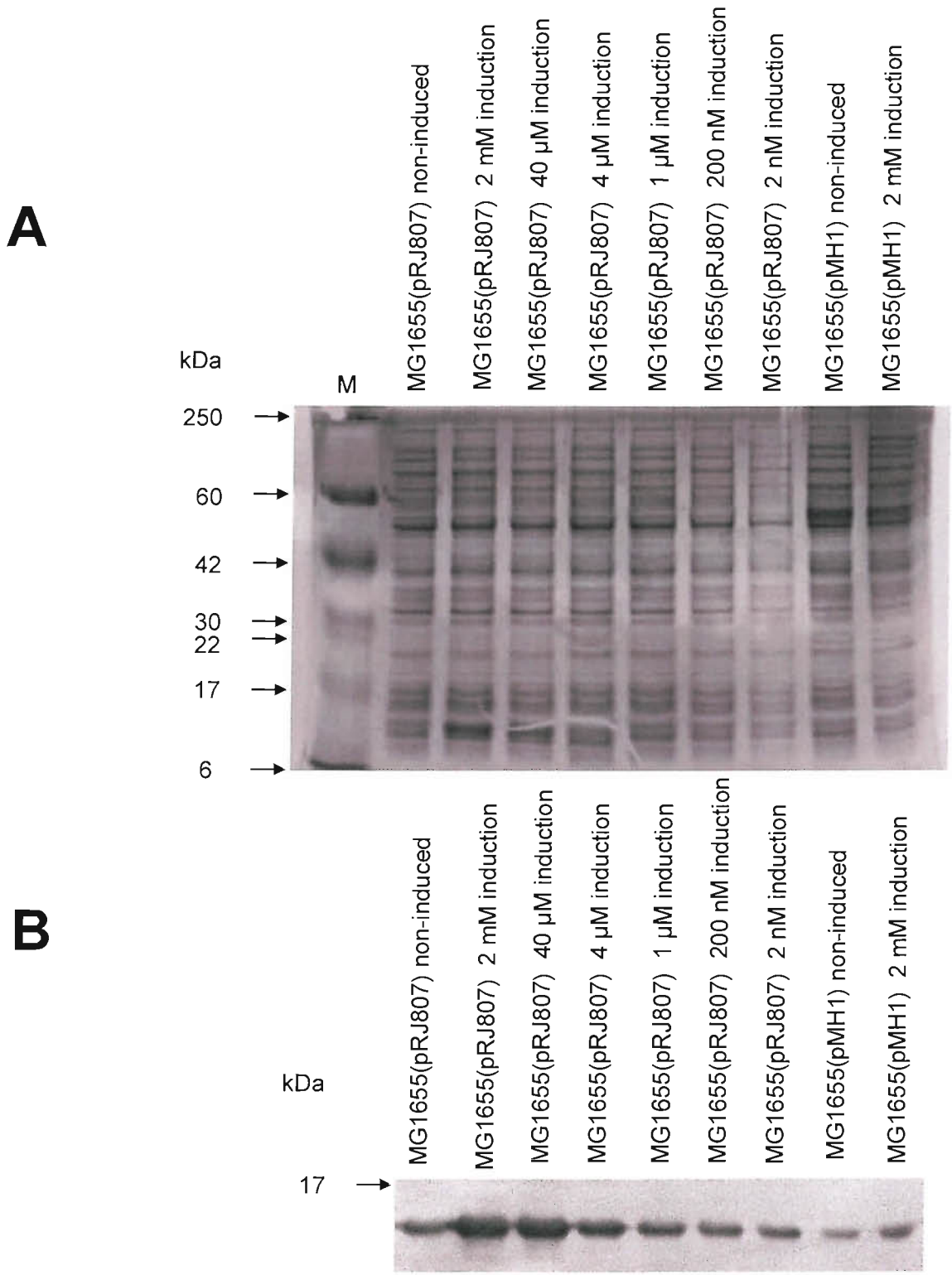


Figure 4.16. The level of Fis expression from pRJ807 which causes growth inhibition.

Figure 4.16. The level of Fis expression from pRJ807 which causes growth inhibition. MG1655(pRJ807) and MG1655(pMH1) cells were grown to an optical density (600 nm) of 0.5 and induced (if appropriate) with various concentrations of IPTG for 90 minutes, after which time protein samples were prepared and analysed by immuno-blotting using a Fis-specific antibody probe. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the immuno-blot. Immuno-blots were scanned into a video image capture system (Gene Genius Bioimaging system, Syngene) then the intensities of the Fis-reactive protein bands was measured by performing densitometry using the Genesnap/Gene Tools software (Syngene).

Having determined the level of Fis expression that triggers the inhibition of cell division in MG1655(pRJ807) cells, it was of interest to compare it to the level of Fis expressed normally in lag phase cells. Previous studies indicate the Fis levels are at their highest in such cells (Ball *et al.*, 1992;Mallik *et al.*, 2006). This was accomplished by preparing protein extracts from lag phase cells of MG1655 and MG1655Δ*bipA*. Overnight cultures were diluted fifty-fold into fresh pre-warmed LB medium, grown for 2 or 6 minutes, and protein samples were prepared. The samples were then analysed by immuno-blotting using a Fis-specific antibody probe (Figure 4.17).

The level of Fis expression in induced cultures of MG1655(pRJ807) was 6.25-fold higher (with a large SD of 1.7-fold) relative to that from an equivalent number of lag phase cells of MG1655, or 4.5-fold higher (with a SD of 0.3-fold) relative to an equivalent number of lag phase MG1655Δ*bipA* cells. It should be noted, however, that a significant proportion of the Fis protein found in MG1655(pRJ807) samples is contributed by the native *fis* gene located on the *E. coli* chromosome. Thus, the additional level of Fis due to expression from pRJ807 will actually be less than that observed relative to lag phase levels of Fis.

In non-induced cells of MG1655(pRJ807), Fis expression was still higher than in the equivalent plasmid-free cells in lag phase by 1.5-fold (with a standard deviation of 0.1-fold).

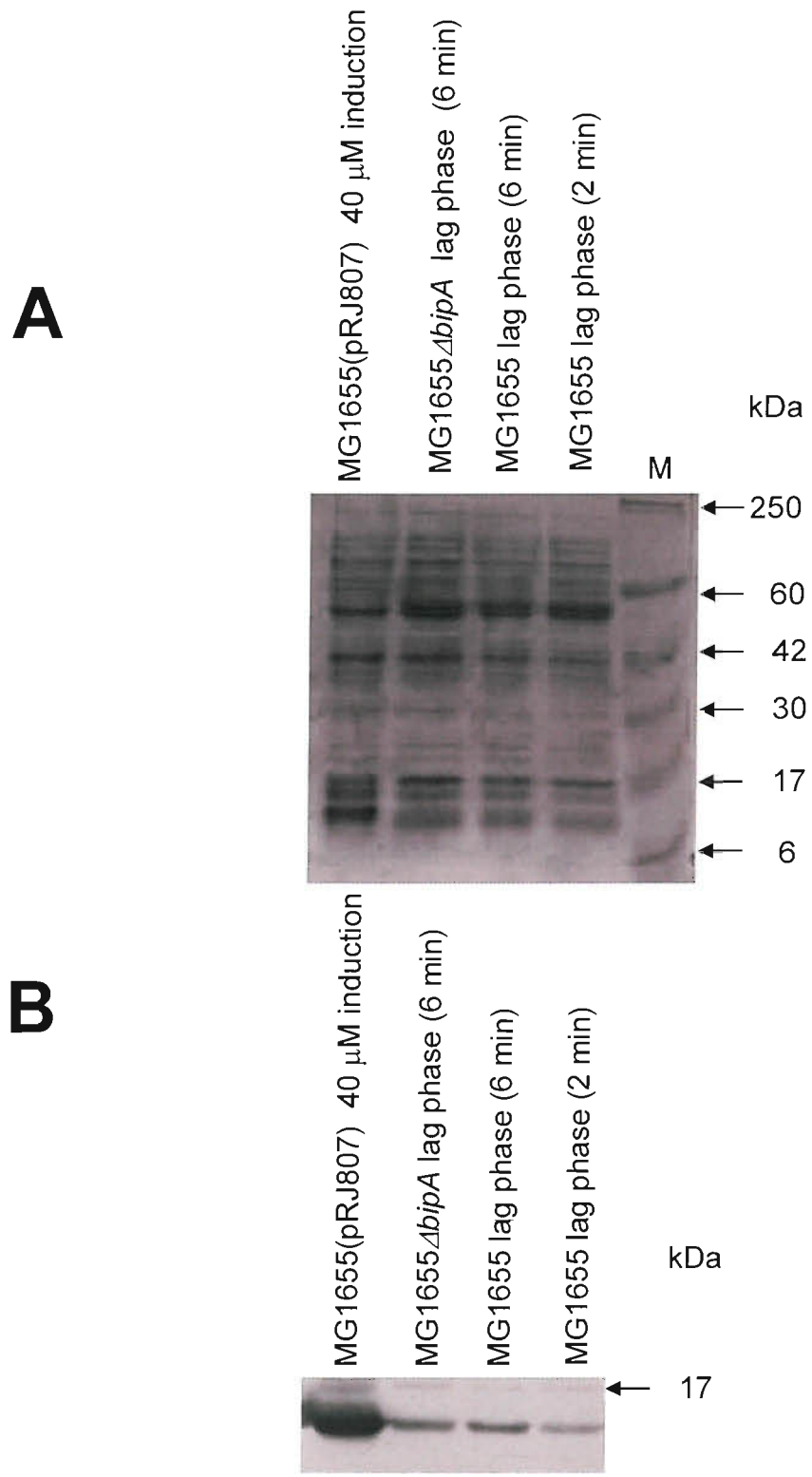


Figure 4.17. MG1655(pRJ807) cells induced with a sub-maximal amount of IPTG still produce a significantly higher level of Fis than lag phase MG1655.

Figure 4.17. MG1655(pRJ807) cells induced with a sub-maximal amount of IPTG still produce a significantly higher level of Fis than lag phase MG1655. MG1655(pRJ807) cells were grown to an optical density (600 nm) of 0.5 and induced with 40 μM IPTG then grown for a further 90 minutes, after which time protein samples were prepared. Lag phase protein samples were created by diluting overnight cultures of MG1655 and MG1655Δ*bipA* (1 in 50) in pre-warmed LB and growing the cultures for 2 or 6 minutes, then preparing protein samples from them. The protein samples were analysed by immuno-blotting using a Fis-specific antibody. Panel A shows the protein loaded for the western blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the corresponding immuno-blot. The Immuno-blot was scanned into a video image capture system (Gene Genius Bioimaging system, Syngene) then the intensities of the Fis-reactive protein bands were measured by performing densitometry with the Genesnap/Gene Tools software (Syngene).

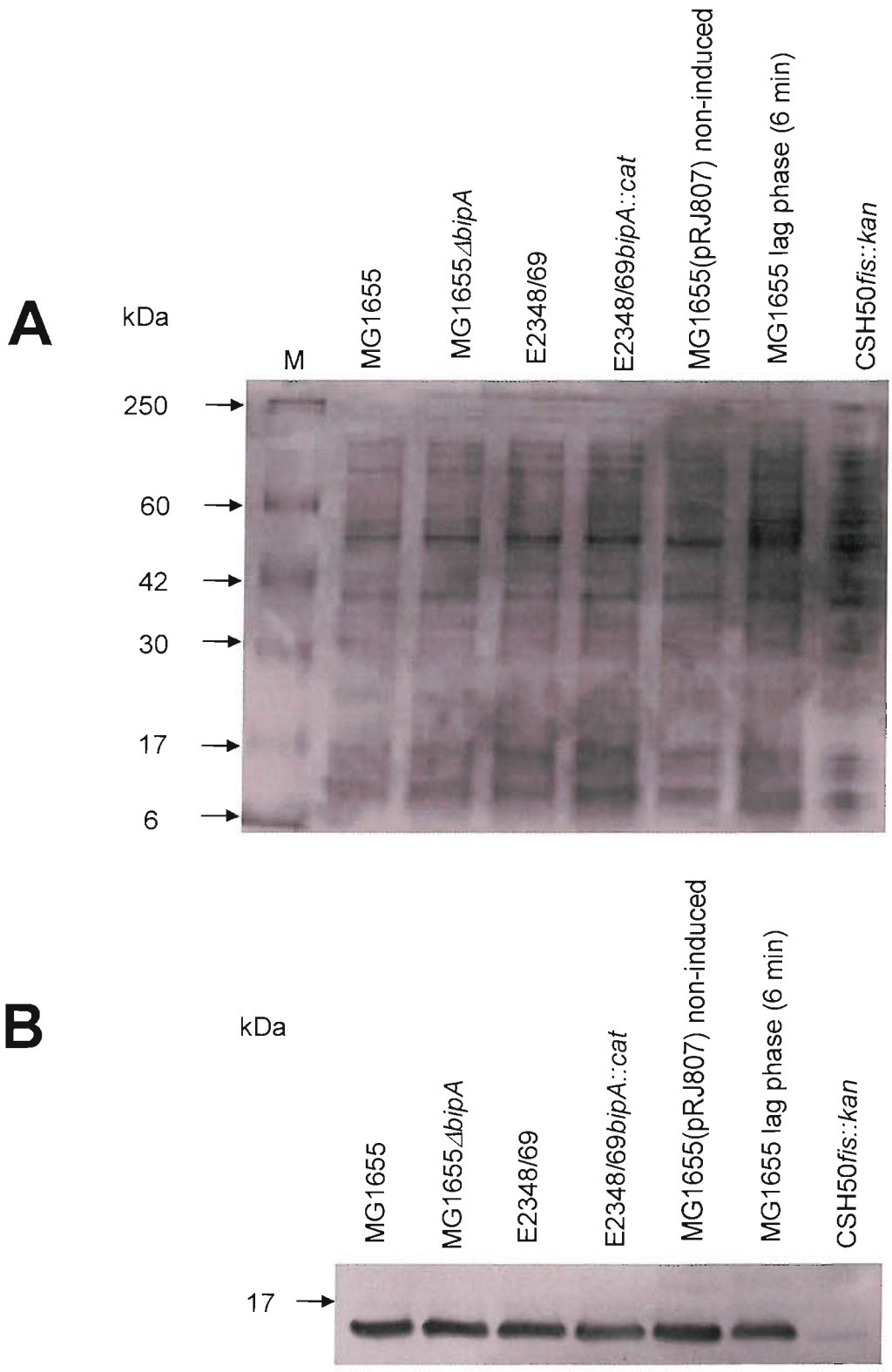


Figure 4.18. MG1655(pRJ807) cells produce a higher level of Fis than MG1655 cells in lag phase.

Figure 4.18. MG1655(pRJ807) cells produce a higher level of Fis than MG1655 cells in lag phase. MG1655(pRJ807) and CSH50*fis::kan* cultures were grown to an optical density (600 nm) of 0.5 and were then cultured for a further 90 minutes, after which time protein samples were prepared. Lag phase protein samples were created by diluting overnight cultures of MG1655 (1 in 50) in pre-warmed LB and growing the cells for 6 minutes, then preparing protein samples. The protein samples were then analysed by immuno-blotting using a Fis-specific antibody. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the immuno-blot. The Immuno-blot was scanned into a video image capture system (Gene Genius Bioimaging system, Syngene) then the intensities of the Fis-reactive protein bands were measured by performing densitometry with the Genesnap/Gene Tools software (Syngene).

4.9 Aberrant Fis expression from pRJ807 does not affect the level of DnaA expression in *E. coli*

According to the current model for initiation of chromosomal replication, proposed by Leonard and Grimwade (2005), the initiation of chromosome replication depends on the level of DnaA expression. At a threshold level, the number of DnaA molecules will be sufficient to displace Fis bound to *oriC*. Consideration of this model therefore led to the hypothesis that, the high level of Fis expressed from pRJ807 may mean that DnaA levels are inadequate to displace Fis binding at *oriC*. Alternatively, increased levels of Fis might affect the level of DnaA protein in the cell, which might also block replication. With this in mind, it was decided to compare the level of DnaA expression in cells aberrantly expressing Fis and in normal cells. It should be noted, however, that a block in DNA replication is not the same as a block in cell division which usually results from a reduction in the expression of the essential cell division genes (Delgado *et al.*, 2001;Gutherie and Wickner, 1990;Vinella and D'Ari, 1994).

Protein samples were prepared from MG1655(pRJ807), MG1655(pMH1) and MG1655 cells grown to an optical density (600 nm) of 0.5 and induced (where appropriate) with various concentrations of IPTG for 90 minutes. Additionally, protein samples from lag phase cells were prepared by diluting overnight cultures of MG1655 one in fifty into pre-warmed LB and growing the cells for 6 minutes prior to harvesting and preparing extracts. The protein samples were analysed by immuno-blotting using a DnaA-specific antibody probe (Figure 4.19).

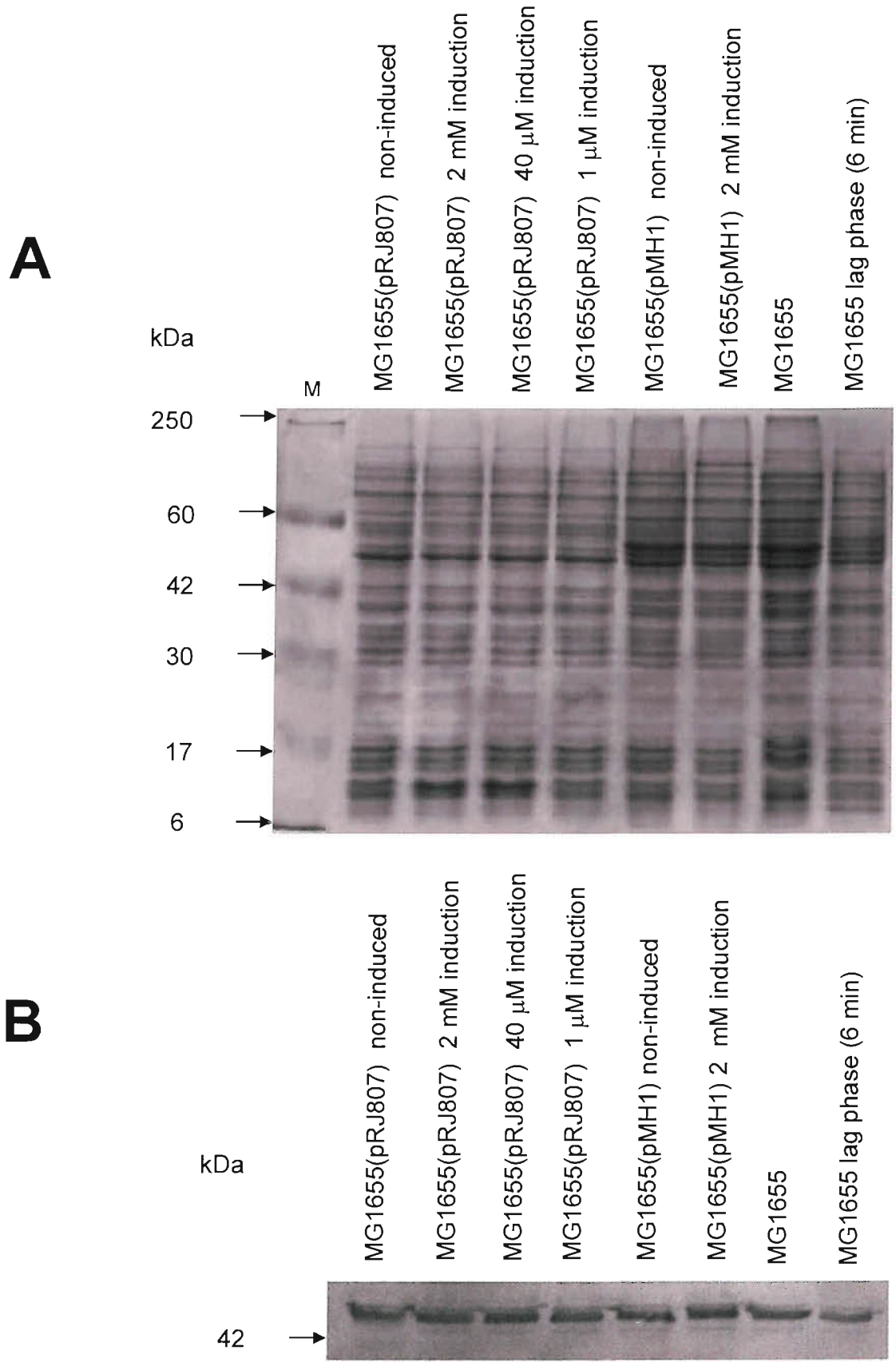


Figure 4.19. Fis expression from pRJ807 does not affect the level of DnaA expressed in *E. coli*.

Figure 4.19. Fis expression from pRJ807 does not affect the level of DnaA expressed in *E. coli*. MG1655(pRJ807), MG1655(pMH1) and MG1655 cells were grown to an optical density (600 nm) of 0.5 then induced (where appropriate) with various concentrations of IPTG for 90 minutes, after which time protein samples were prepared. Lag phase protein samples were prepared by diluting overnight cultures of MG1655 (1 in 50) in pre-warmed LB growing the cells for 6 minutes then preparing protein samples from them. The protein samples were then analysed by immuno-blotting using a DnaA-specific antibody. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the immuno-blot.

As indicated by Figure 4.19 there was no significant difference in the level of DnaA expression in cells aberrantly expressing Fis in comparison with normal cells. Thus, it is unlikely that the inhibitory effect of Fis overexpression is due to partial repression of DnaA synthesis.

4.10 Discussion

Previous studies have shown that null mutants of *fis* display cell filamentation whereas cells constitutively expressing this protein exhibit normal logarithmic growth (Osuna *et al.*, 1995). The present study extends these findings by showing that aberrant expression of Fis from pRJ807 causes growth inhibition in *E. coli*. This effect occurred regardless of whether cells were in the lag or log phase, or whether liquid or solid media were used. Further, growth inhibition required the expression of the Fis protein; as evidenced by the absence of the phenotype in cells carrying pMH1. Subsequent analysis revealed that the *Nde*I site that was mutated in pRJ807 was in fact in the origin of replication region of pRJ807 rather than in the *fis* gene. This is likely to reduce the copy number of the plasmid (Masai and Arai, 1988). In keeping with this, Figure 4.16 presents evidence indicating that Fis expression is significantly lower in cells containing pMH1. Thus, this plasmid can be used as an expression control for subsequent experiments.

Induction of Fis synthesis from pRJ807 led to the complete cessation of cell division as indicated by the lack of increase in optical density (600 nm) and reduction in CFU in plating experiments. This is in contrast to the results reported by Choe *et al.* (1999) who observed cells aberrantly Fis only had a 33% decrease in growth rate (in comparison to cells expressing a *fis* mutant). However, they used a different growth medium (minimal M9 media, supplemented with glycerol (Choe *et al.*, 1999)).

To further characterise the phenomenon, effects on the expression of a gene that is apparently unrelated to those of the *fis* regulon (*lacZ*) were examined. The striking reduction in β-galactosidase observed on Fis induction underscores the dramatic changes in cell physiology that occur under these conditions. However, SDS-PAGE studies of gene expression after induction of Fis expression from pRJ807 (data not shown) indicate that not all proteins

show reduced expression in response to Fis production. Indeed, some proteins increase in expression in response to Fis expression. This indicates that growth inhibition is unlikely to be due to an abolition of protein synthesis. This conclusion is supported by the finding that Fis expression from pRJ807 causes cells to form filaments. Filamentation occurs when cells are able to duplicate their cell contents (which requires protein synthesis) but are unable to divide. The changes in cellular gene expression caused by Fis expression may be the result of Fis-mediated activation or repression of specific target genes. In this respect, Fis is a transcriptional regulator of many genes (Falconi *et al.*, 2001;Goldberg *et al.*, 2001;González-Gil *et al.*, 1996;Hirsch and Elliott, 2005;Jackson *et al.*, 2004;Kelly *et al.*, 2004), including those for ribosomal RNAs (Nilsson *et al.*, 1990). In nutrient-rich conditions, Fis serves as an activator of these genes. However, ribosome over-production is deleterious to cells and causes a reduction in growth rate (Stevenson and Schmidt, 1998). Therefore, the growth inhibitory effect might be the result of Fis causing the over production of ribosomes; e.g. by stimulating the transcription of ribosomal RNA operons which might in turn deplete the amount of RNA polymerase available for expression of the essential cell division genes thereby preventing cell division (Bramhill, 1997). In future work, both the level of ribosome production and the level of expression of the essential cell division proteins such as FtsZ should be examined in cells aberrantly expressing Fis from pRJ807.

The discovery that aberrant Fis expression causes cell filamentation is interesting. Filamentation is usually transient which means that while the cell is exposed to the environmental stress that is causing it to form filaments, it will continue to grow but fail to divide. However, when the stress factor is removed the filamentous cells will divide yielding a multitude of individual cells (Frandsen and D'Ari, 1993;Mattick *et al.*, 2000;Mattick *et al.*, 2003b;Mattick *et al.*, 2003a). Therefore filamentation is not a bactericidal effect. The transient filamentation that occurs in response to nutritional upshift is of particular interest in view of the present findings (Kepes and D'Ari, 1987;Sloan and Urban, 1976). Transient filamentation on nutrient upshift involves the essential cell division protein FtsZ although the signal the cell uses to delay cell division

is poorly characterised; it could be a modified protein, an RNA or internal metabolite pools. With this in mind it is interesting to consider that Fis expression has been recently shown to be regulated by the level of free initiating nucleotide triphosphate (Mallik *et al.*, 2004;Mallik *et al.*, 2006), which induces Fis expression on nutritional upshift, causing Fis to reach its highest intracellular level before the first cell division (Ball *et al.*, 1992). It is tempting to speculate that the high initiating nucleotide triphosphate levels that occur on nutrient upshift and that upregulate Fis expression (Mallik *et al.*, 2004;Mallik *et al.*, 2006) may provide the signal for the transient filamentation that occurs in response to nutrient upshift (Kepes and D'Ari, 1987). Thus, the high level of Fis expression (Ball *et al.*, 1992) that results from this upregulation may be a part of the mechanism that delays cell division during transient filamentation.

4.10.1 Possible models for aberrant expression of Fis-mediated filamentation

Although it is clear that prolonged extensive filament formation (like that observed here) is detrimental to cell growth, under certain conditions, such as the outgrowth from stationary phase following nutrient upshift, it may benefit the cell to have a mechanism for delaying cell division to allow it to adapt to its new conditions (for example, by increasing its ribosome content to facilitate the high rate translation which is required at high growth rates (Cashel *et al.*, 1996;Dennis *et al.*, 2004)). As high levels of Fis expression have been shown here to cause filamentation in *E. coli*, it can be speculated that the high levels of Fis expression induced on nutrient upshift (which peak prior to the first cell division) may act to delay cell division, while the cell is primed for rapid exponential growth. The potential mechanisms for Fis-mediated filamentation are illustrated in Figure 4.20.

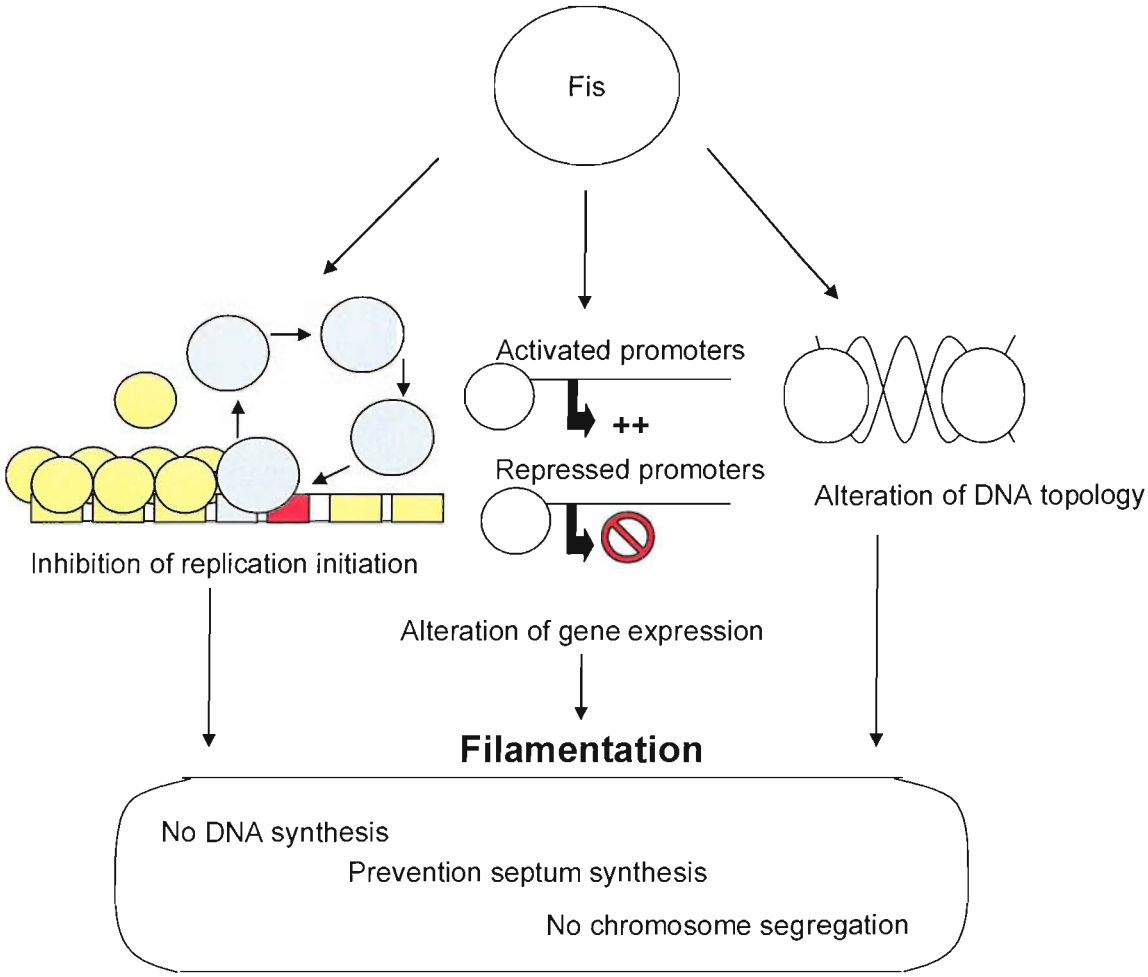


Figure 4.20. Potential models for Fis-mediated filamentation. Light blue circles represent Fis molecules while the light blue squares show Fis binding sites. Yellow circles and squares depict DnaA molecules and their binding sites, respectively, and the red square represents the Integration host factor binding site in *oriC*.

There are three properties of Fis that could be used to induce filamentation: Fis may inhibit the initiation of chromosomal DNA replication (Cassler *et al.,* 1995;Hiasa and Marians, 1994;Ryan *et al.,* 2004;Wold *et al.,* 1996) to disrupt the cell cycle and cell division, or it may directly regulate the transcription of its target genes (Falconi *et al.,* 2001;Goldberg *et al.,* 2001;Hirsch and Elliott, 2005;Jackson *et al.,* 2004;Kelly *et al.,* 2004) to reduce the amount of free RNA polymerase and thereby disrupt the transcription of essential cell division genes (Bramhill, 1997), or it may alter the level of supercoiling of the chromosome (Azam *et al.,* 1999;Pan *et al.,* 1996;Schneider *et al.,* 1997).

Before considering these explanations it was important to rule out other possible factors, e.g. some plasmid-specific effect. Therefore, the capacity of another Fis expression vector to trigger growth inhibition was tested. In contrast to the results obtained with pRJ807, the Fis expression construct pBAD*fis* did not induce growth inhibition in *E. coli*. Further investigation revealed that pRJ807 produces a significantly larger amount of Fis than pBAD*fis*, with or without induction. The data indicates that the growth abolition caused by Fis expression from pRJ807 is associated with the high level of Fis that it produces.

Additionally, the level of Fis expression associated with growth inhibition was significantly higher than the highest level of Fis expression observed in normal cells in lag phase. For instance, the level of Fis expressed from pRJ807 induced with 40 μM IPTG was significantly higher than the highest level found in lag phase MG1655 cells. Furthermore, Fis expression in MG1655(pRJ807) without induction was greater than the level of Fis expressed in lag phase MG1655 cells.

Although a block in cell division is not the same as a block in DNA replication, to explore the reasons for growth inhibition in more detail, the level of DnaA protein in inhibited and non-inhibited cells was compared. The rationale for this experiment was that initiation of chromosome replication depends on the

level of DnaA expression and binding to *oriC* being high enough to displace Fis bound to *oriC* (Kaguni, 2006;Leonard and Grimwade, 2005).

The level of DnaA expressed in growth inhibited and non-inhibited cells was not significantly different. Therefore, increased expression of Fis does not appear to influence DnaA levels. However, a limitation of this experiment is the fact that the intracellular level of DnaA is carefully regulated by multiple mechanisms that might override the effect of increased Fis concentration (Kaguni, 2006;Leonard and Grimwade, 2005). Regardless of this possibility, however, it is clear that under the experimental conditions used, Fis does not alter the amount of DnaA in the cell. This result makes the alternative hypothesis – that the level of Fis is sufficiently high so as to prevent its displacement from *oriC* by DnaA – more attractive. However, testing this idea is outside the scope of the present project.

In conclusion, the work carried out here lends support to studies already in the literature that implicate Fis in chromosome replication (Cassler *et al.*, 1995;Hiasa and Marians, 1994;Ryan *et al.*, 2004;Wold *et al.*, 1996). It is noteworthy, however, that similar results and indeed similar levels of Fis were found regardless of whether the strain expressed BipA. This discrepancy is explored further in the following chapters.

CHAPTER 5

REPORTER GENE CONSTRUCTS pJGP1 AND pJGP6 DO NOT DISPLAY THE EXPECTED STRUCTURES OR PROPERTIES

5 Reporter gene constructs pJGP1 and pJGP6 do not display the expected structures or properties

5.1 Introduction

Many gene products are difficult or impossible to assay directly, e.g. because the component in question is produced in amounts that are below the limit of experimental detection or, in the case of enzymes, due to the non-availability of suitable substrates. For these reasons, a range of reporter gene systems have been developed, which allow gene expression to be monitored by measuring the level or activity of an easily assayable enzyme such as β-galactosidase (Silhavy and Beckwith, 1985). Reporter gene systems involve the genetic fusion of the regulatory elements of the gene under investigation with a gene encoding a reporter protein/enzyme. Commonly, the open reading frame of the reporter gene, together with the Shine-Dalgarno sequence, is placed immediately downstream of the promoter region of the gene in question so as to produce a transcriptional fusion. Such constructs will reflect the amount of transcription from the promoter under different conditions. Alternatively, the open reading frame of the reporter gene can be placed an appropriate distance from the Shine-Dalgarno sequence of the gene of interest and in the appropriate orientation to produce a translational fusion. In this case, the reporter gene will reflect the degree of translation of the gene in question under various conditions. Although certain reporter gene constructs sometimes produce anomalous results (Forsberg *et al.*, 1994), their extensive use over the years provides good evidence of the general reliability of this experimental approach.

The finding that BipA regulates Fis expression (Owens *et al.*, 2004) raised the question of whether control was at the transcriptional or post transcriptional

level. In an attempt to address this question, the *fis* messenger RNA was examined for unique features that might indicate a BipA response element. It was found that the ribosome binding site in the 5' untranslated region of the *fis* mRNA has an exceptionally high degree of complementarity to the 3' end of the 16S ribosomal RNA. In contrast to the ribosome binding sites of typical *E. coli* mRNAs which typically display 3 to 6 nucleotides of complementarity to the 3' end of 16S ribosomal RNA (Shultzaberger *et al.*, 2001), the Shine-Dalgarno region of the *fis* mRNA displays 12 nucleotides of complementarity to the 3' end of the 16S ribosomal RNA. It was also noted that the anti-Shine-Dalgarno segment of 16S ribosomal RNA was also positioned differently relative to the start codon when *fis* mRNA was compared with other transcripts (Owens *et al.*, 2004).

Consideration of these findings led to the hypothesis that the high level of complementarity between the *fis* Shine-Dalgarno region and the anti-Shine-Dalgarno segment of the 16S ribosomal RNA may cause the *fis* message to become hyper-stably associated with the ribosome, thereby preventing translation. It was further proposed that BipA may act to destabilise this interaction and hence allow the translation of the *fis* message. To test this hypothesis a series of plasmids was constructed in which the *fis* 5' untranslated region, or mutant derivatives of it, were fused to the *lacZ* coding sequence. pJGP1 had a wild type *fis* S-D sequence whereas pJGP2 had a mutant S-D sequence in which 3 nucleotides were mutated to reduce the S-D/anti-S-D complementarity to 9 base pairs. pJGP3 had a mutant S-D sequence in which 2 nucleotides were mutated to increase the S-D/anti-S-D complementarity to 14 base pairs but pJGP4 contains a mutant S-D sequence in which 6 nucleotides were mutated to decrease the S-D/anti-S-D complementarity to 6 base pairs. Additionally, pJGP5 had a mutant *fis* S-D sequence in which 5 nucleotides were mutated to increase the S-D/anti-S-D complementarity to 16 base pairs.

The constructs were then placed in a BipA-positive or -negative background and assayed to determine the level of β-galactosidase expressed from them *in vivo*. It was found that BipA enhanced the expression of the hybrid mRNA carrying the wild type *fis* 5' untranslated region and the mutant derivatives specified by plasmids pJGP2, pJGP3 and pJGP5. Further, it was also reported that BipA had no effect on the expression of a hybrid mRNA that contained the native 5' untranslated region of *lacZ* bound to the *lacZ* gene (pJGP6). The results presented in the paper by Owens *et al.* (2004) indicated that BipA regulates *fis* expression at the level of translation and that the extended S-D sequence of the *fis* mRNA is required for this regulatory effect.

Subsequent studies with strains carrying the pJGP series of plasmids produced apparently aberrant results (M. Hodey, unpublished observations). Accordingly, it was deemed necessary to investigate the structures and properties of two representative plasmid constructs, pJGP1 and pJGP6, in greater detail. This chapter describes the results of this investigation.

5.2 pJGP1 and pJGP6 do not display the expected structures

As an initial check, the structures of plasmids pJGP1 and pJGP6 were re-analysed by restriction mapping. Figures 5.1 and 5.2 show the construction scheme and structures of pJGP1 and pJGP6 described in Owens *et al.* (2004).

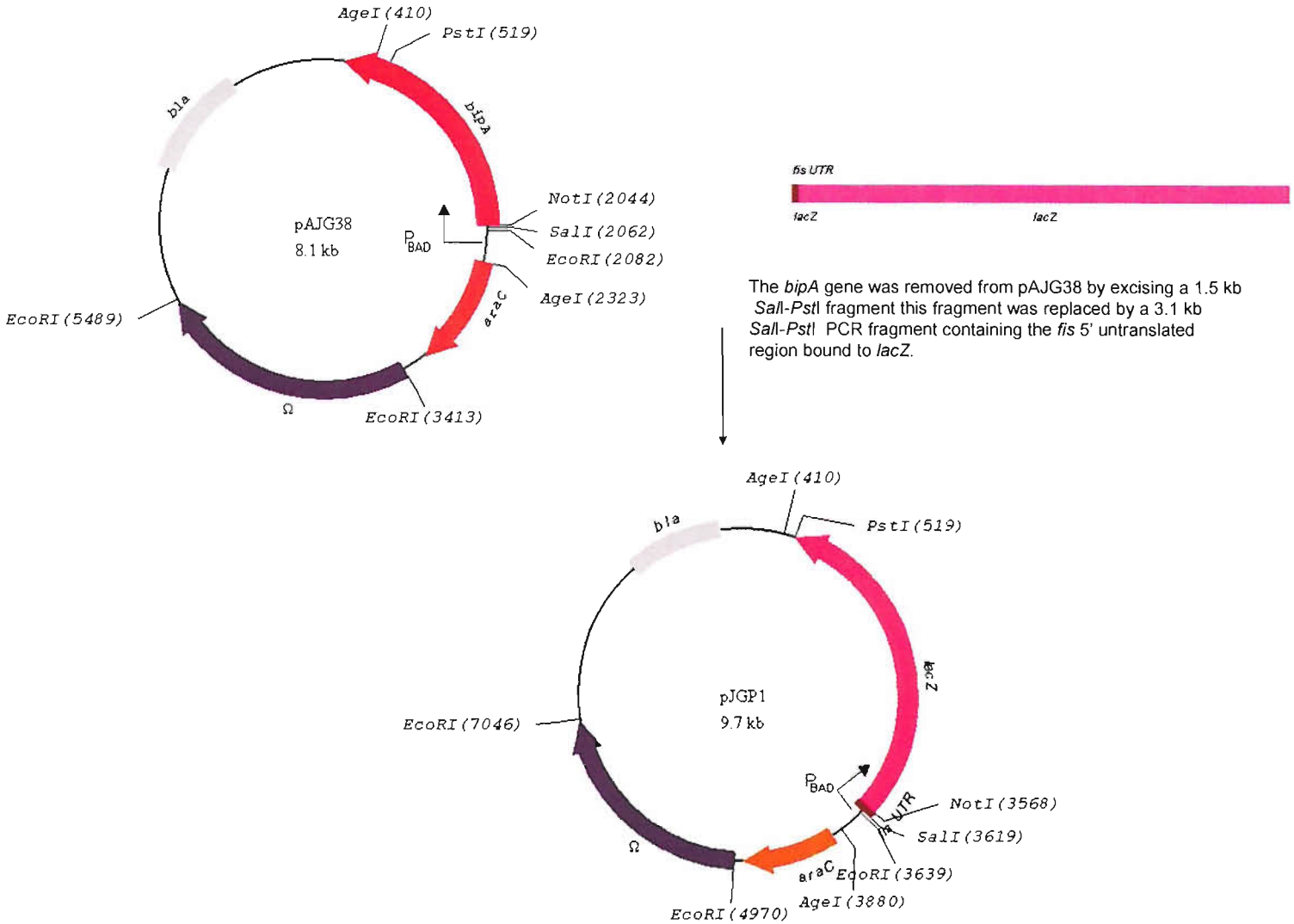


Figure 5.1. The construction of pJGP1 (Owens *et al.*, 2004).

Figure 5.1. The construction of pJGP1 (Owens *et al.,* 2004). The parent plasmid pAJG38 (Grant *et al.,* 2001) was *Pst*I and *Sal*I cut and the 6576 bp vector backbone was gel-extracted. PCR, using pRS414 (Simons *et al.,* 1987) as a template, was used to create a fragment containing the *fis* 5' untranslated region (including the native S-D sequence) bound to the *lacZ* coding sequence. The amplified fragment was trimmed with *Pst*I and *Sal*I and ligated into the pAJG38-derived vector backbone to make pJGP1. Ω indicates the presence of an omega fragment, which contains transcriptional and translational stop signals, as well as a streptomycin/spectinomycin resistance gene (Prentki and Krisch, 1984). The restriction sites that were important for the construction and subsequent restriction mapping of pJGP1 are shown.

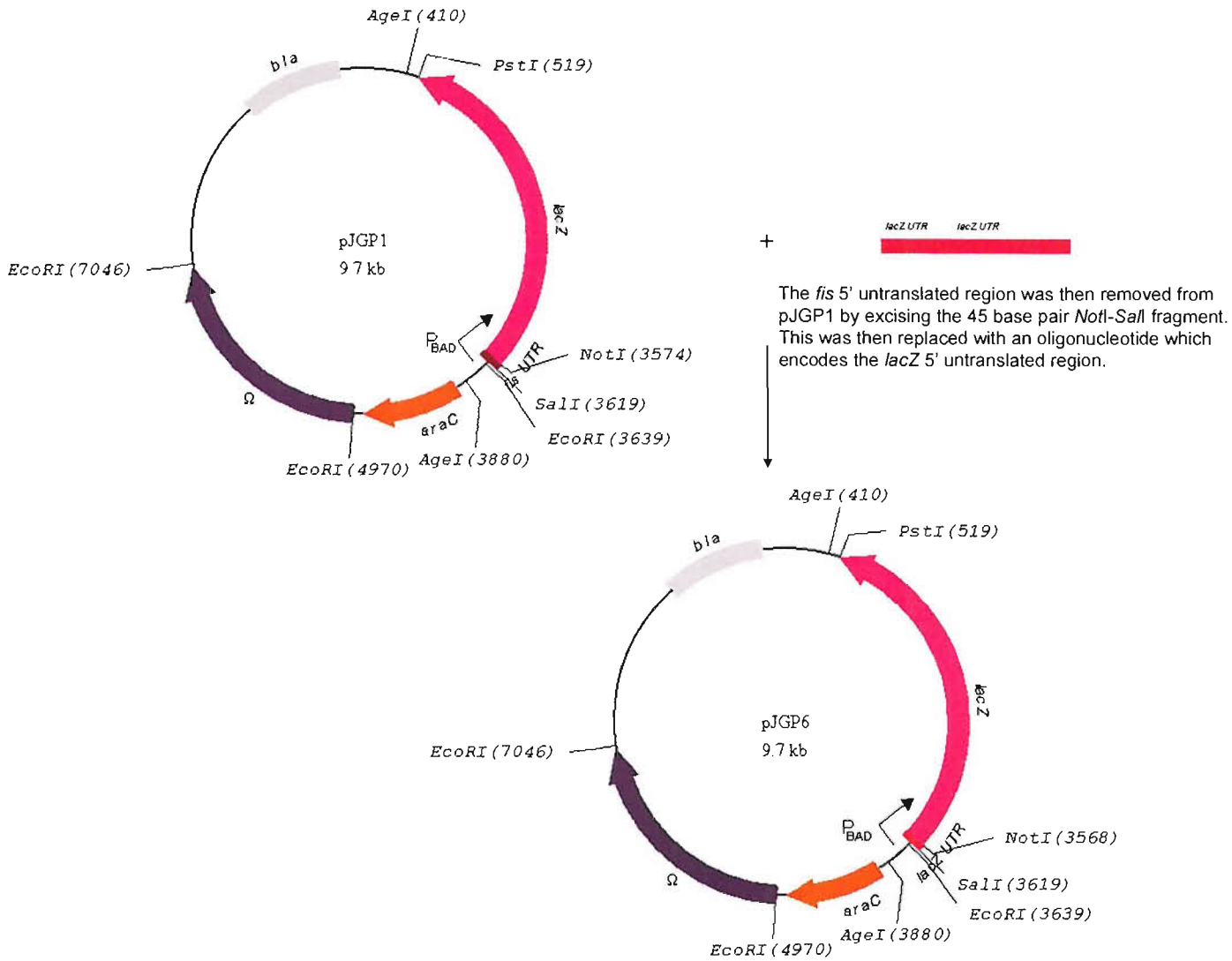


Figure 5.2. The construction of pJGP6 (Owens *et al.*, 2004).

Figure 5.2. The construction of pJGP6 (Owens *et al.,* 2004). A 45 base pair double-stranded DNA fragment containing the S-D sequence of *lacZ* was created by annealing oligonucleotides purchased from MWG Biotech. The synthetic fragment, which had sticky ends that were complementary to *Not*I and *Sal*I cut DNA, was ligated into *Not*I and *Sal*I cut pJGP1 DNA (a 9.7 kb fragment that had previously been gel purified). The resulting construct was designated pJGP6. The restriction sites that were important for the creation and subsequent restriction mapping of pJGP6 are shown.

Plasmid DNA for pJGP1, pJGP6 and their parent plasmid pAJG38, was digested with various restriction enzymes (*Age*I, *Eco*RI, *Pst*I, *Not*I and *Sal*I, *Hinc*II, *Dra*I and *Hin*fI) and the resultant DNA fragments were analysed by agarose gel electrophoresis. The sizes of the fragments obtained were compared with the fragments predicted for the constructs, which were deduced from the structures described in Owens *et al.* (2004) (pJGP1 and pJGP6) and Grant *et al.* (2001) (for pAJG38).

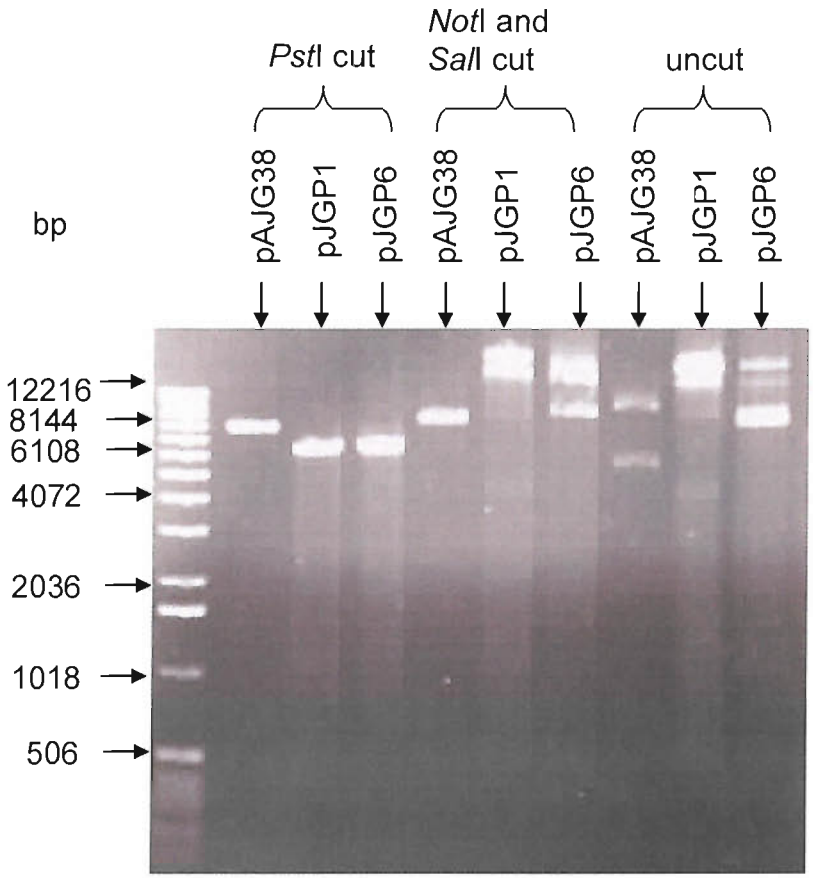


Figure 5.3. In contrast to their parent plasmid pAJG38 pJGP1 and pJGP6 do not form the expected fragments when digested with *Pst*I or *Not*I and *Sal*I. pJGP1, pJGP6 and pAJG38 were prepared and digested with *Pst*I or *Not*I and *Sal*I, the resultant fragments were resolved using agarose gel electrophoresis. The predicted and observed fragmentation patterns for pAJG38, pJGP1 and pJGP6 digested with *Pst*I is given in Table 5.1. The leftmost lane shows molecular size markers.

Table 5.1. Comparison of the predicted and observed DNA fragments formed when pAJG38, pJGP1 and pJGP6 were digested with *Pst*I[d].

pAJG38	pAJG38	pJGP1	pJGP1	pJGP6	pJGP6
Predicted fragments (bp)	Observed fragments (bp)	Predicted fragments (bp)	Observed fragments (bp)	Predicted fragments (bp)	Observed fragments (bp)
8119	~ 8100	9676	~6100	9676	~6100

[d] pJGP1, pJGP6 and pAJG38 were prepared and digested with *Pst*I the resultant fragments were then resolved using agarose gel electrophoresis. Predicted and observed fragment sizes were determined and compared.

In contrast to their parent plasmid pAJG38 which was successfully digested with *Not*I and *Sal*I to form the expected 8101 base pair fragment (Figure 5.3) pJGP1 and pJGP6 did not digest with *Not*I and *Sal*I.

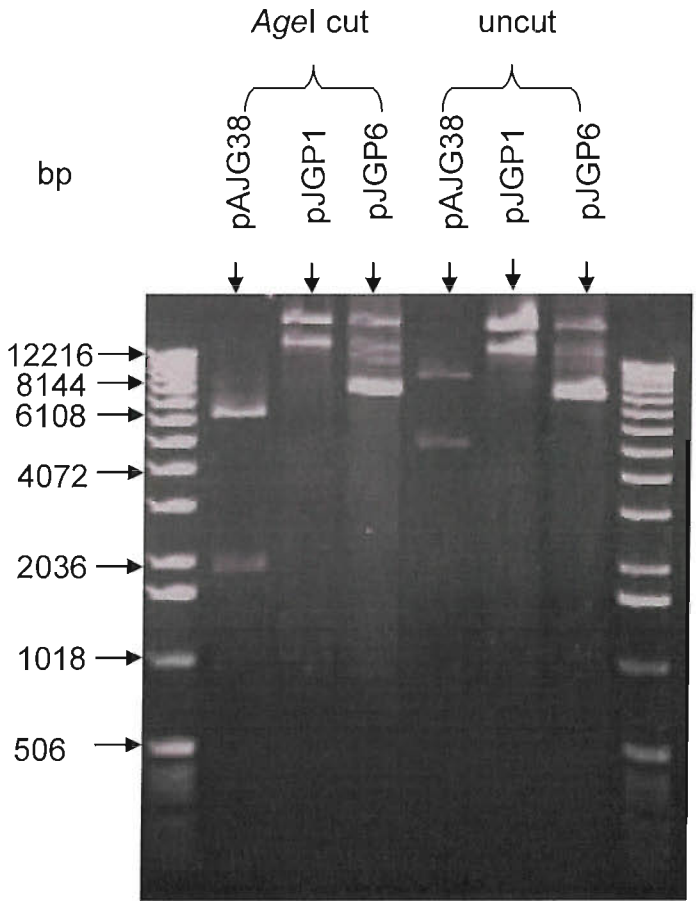


Figure 5.4. pJGP1 and pJGP6 do not form the expected fragments when digested with *Age*I in contrast to their parent plasmid pAJG38. pJGP1, pJGP6 and pAJG38 were prepared and digested with *Age*I and the resultant fragments were resolved using agarose gel electrophoresis. The outermost lanes show molecular size markers.

Although pAJG38 digested with *Age*I to form the expected DNA fragments (6206 base pairs and 1913 base pairs) pJGP1 and pJGP6 failed to digest with *Age*I.

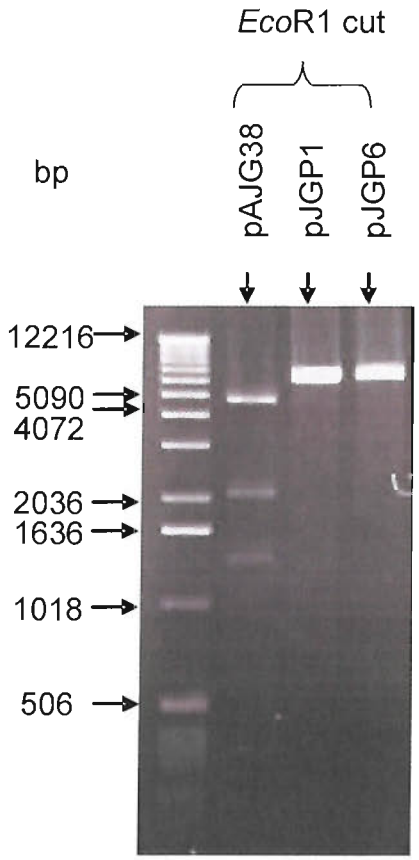


Figure 5.5. pJGP1 and pJGP6 do not form the expected fragments when digested with *Eco*R1 in contrast to their parent plasmid pAJG38. Samples of pJGP1, pJGP6 and pAJG38 DNA were digested with *Eco*R1 and the resultant fragments were resolved using agarose gel electrophoresis. The predicted and observed distribution of *Eco*RI sites is given in Table 5.2. The leftmost lane contains DNA marker standards.

Table 5.2. Comparison of the predicted and observed *Eco*R1 sites for pAJG38, pJGP1 and pJGP6[e].

pAJG38 Predicted fragments (bp)	pAJG38 Observed fragments (bp)	pJGP1 Predicted fragments (bp)	pJGP1 Observed fragments (bp)	pJGP6 Predicted fragments (bp)	pJGP6 Observed fragments (bp)
4712	~ 4700	6269	~6100	6269	~6100
2076	~ 2000	2076		2076	
1331	~ 1300	1331		1331	

[e] pJGP1, pJGP6 and pAJG38 were prepared and digested with *Eco*R1 the resultant fragments were then resolved using agarose gel electrophoresis. Predicted and observed fragment sizes were determined and compared.

Multiple batches of pJGP1 and pJGP6 DNA all failed to produce the fragments predicted for their structure (detailed in Owens *et al.* (2004)) when digested with *Age*I, *Eco*RI, *Pst*I, *Not*I or *Sal*I restriction enzymes. In contrast the parent plasmid pAJG38 consistently formed the fragments predicted for its structure (detailed in Grant *et al.* (2001)) under the same digestion conditions. In view of these results, it was decided to use frequent cutting restriction enzymes to restriction map pJGP1 and pAJG38 in the hope that the results will reveal the how related pJGP1 and pAJG38 are to each other.

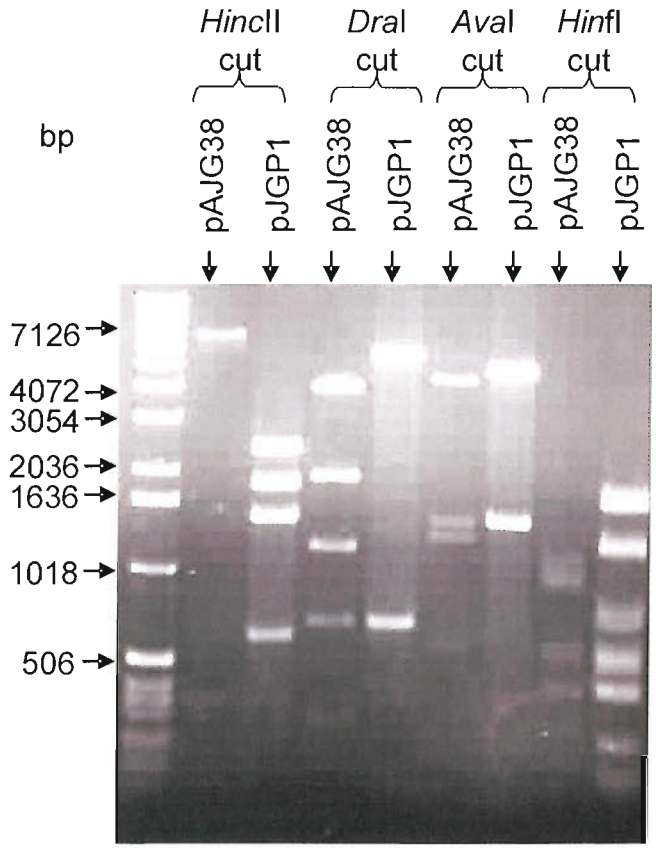


Figure 5.6. In contrast to its parent plasmid pAJG38 pJGP1 does not form the expected fragments when digested with *Hinc*II, *Dra*I, *Ava*I or *Hin*fI. pJGP1 and pAJG38 were prepared and digested with *Hinc*II, *Dra*I, *Ava*I or *Hin*fI, and the resultant fragments were resolved using agarose gel electrophoresis. The predicted and observed DNA fragments for pAJG38 and pJGP1 digested with *Hinc*II, *Dra*I, *Ava*I or *Hin*fI are given in Tables 5.3 to 5.6. The left hand lane contains DNA marker standards.

Table 5.3. Comparison of the observed and predicted DNA fragments for pAJG38 and pJGP1 digested with *Hinc*II[f].

pAJG38	pAJG38	pJGP1	pJGP1
Predicted (fragments bp)	Observed (fragments bp)	Predicted (fragments bp)	Observed (fragments bp)
7145	~ 7126	6578	~ 2500
342	~ 344	1828	~ 1800
332		624	~ 1900
		463	~ 1400
			~ 600

[f] pJGP1 and pAJG38 were prepared and digested with *Hinc*II and the resultant fragments were then resolved using agarose gel electrophoresis. Predicted and observed fragment sizes were determined and compared. Fragments that were too small to be resolved (less than 300 base pairs long) were not included in the table.

Table 5.4. Comparison of the predicted and observed DNA fragments formed when pJGP1 and its parent plasmid pAJG38 were digested with *Dra*I[g].

pAJG38	pAJG38	pJGP1	pJGP1
Predicted (fragments bp)	Observed (fragments bp)	Predicted (fragments bp)	Observed (fragments bp)
4008	~ 4000	5565	~ 6108
1942	~ 2000	1942	~ 700
1198	~ 1200	1198	
692	~ 700	692	

[g] pJGP1 and pAJG38 were prepared and digested with *Dra*I the resultant fragments were then resolved using agarose gel electrophoresis. Predicted and observed fragment sizes were determined and compared. Fragments that were too small to be resolved (less than 300 base pairs long) were not included in the table.

Table 5.5. Comparison of the observed and predicted fragments formed when pAJG38 and pJGP1 were digested with *Aval*[h].

pAJG38 Predicted (fragments bp)	pAJG38 Observed (fragments bp)	pJGP1 Predicted (fragments bp)	pJGP1 Observed (fragments bp)
4435	~ 4500	4014	~ 5090
1443	~ 1500	1459	~ 1500
1298	~ 1300	1443	
584	~ 600	1298	
		584	

[h] pJGP1 and pAJG38 were prepared and digested with *Aval* and the resultant fragments were then resolved using agarose gel electrophoresis. Predicted and observed fragment sizes were determined and compared. Fragments that were too small to be resolved (less than 300 base pairs long) were not included in the table.

Table 5.6. Comparison of the predicted and observed DNA fragments formed when pJGP1 and its parent plasmid pAJG38 were restriction digested with *Hin*fI[i].

pAJG38 Predicted (fragments bp)	pAJG38 Observed (fragments bp)	pJGP1 Predicted (fragments bp)	pJGP1 Observed (fragments bp)
1160	1200 - 900	1608	~ 1700
1075		1160	~ 1200
966		1075	~ 700
596	600 - 500	966	550 - 500
557		596	~ 396
517		567	
402	400 - 350	562	
396		557	
383		517	
		396	
		383	

[i] pJGP1 and pAJG38 were prepared and digested with *Hin*fI and the resultant fragments were then resolved using agarose gel electrophoresis. Predicted and observed fragment sizes were determined and compared. Fragments that were too small to be resolved (less than 300 base pairs long) were not included in the table.

Again, pJGP1 DNA consistently failed to yield the restriction fragment patterns predicted for its structure (detailed in Owens *et al.* (2004)) when cut with any of the frequent-cutting restriction enzymes used in this study. In contrast, its parent plasmid, pAJG38, consistently formed the fragments predicted for its structure (as detailed by Grant *et al.* (2001)) when cut with any of the restriction enzymes used in this study. Notably, the fragments formed by pJGP1 showed very little similarity to those formed by pAJG38, indicating that pJGP1 is not related to pAJG38. Collectively, these results led to the conclusion that pJGP1 and pJGP6 do not display the expected structures as described in Owens *et al.* (2004).

5.3 pJGP1 and pJGP6 do not display the expected properties

It was next decided to determine whether pJGP1 and pJGP6 displayed key predicted properties i.e. induction of β - galactosidase expression following addition of L-arabinose, due to the presence of the arabinose-inducible P_{BAD} promoter located upstream of the *lacZ* gene (Owens *et al.*, 2004). This was investigated by transforming pJGP1 and pJGP6 into the naturally *lacZ* negative *Salmonella enterica* serovar Typhimurium strain LB5010, and culturing the resultant derivatives in the presence or absence of arabinose. After 2 hours of growth, the levels of β - galactosidase expression in the induced and non-induced cultures was compared.

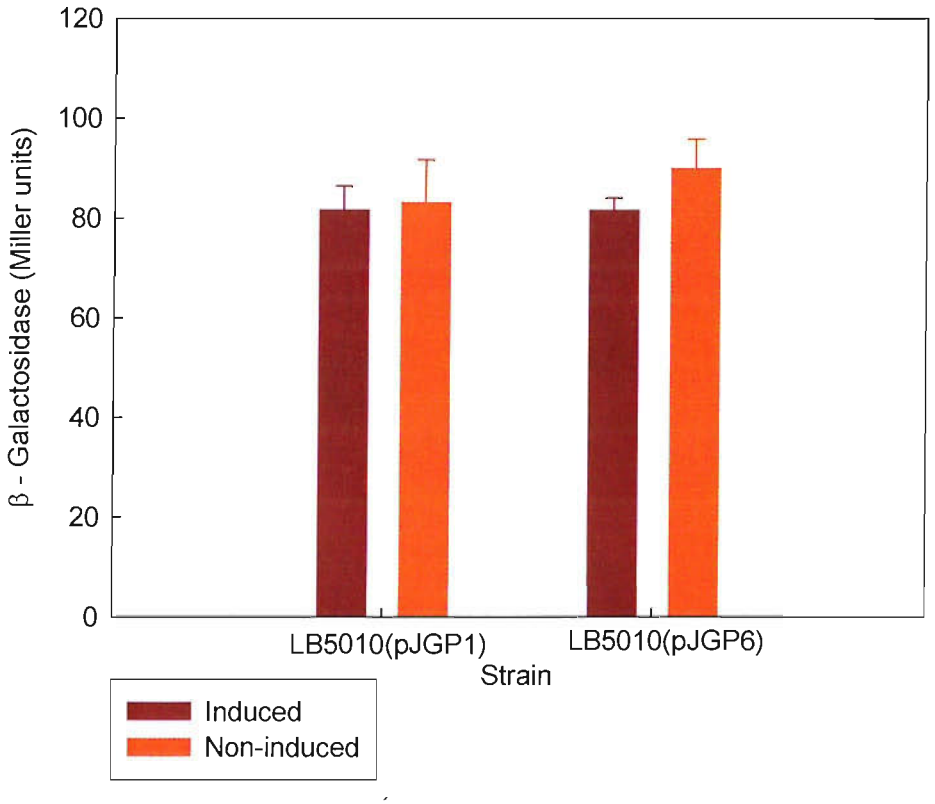


Figure 5.7. Exposure of LB5010(pJGP1) and LB5010(pJGP6) to arabinose fails to induce β - galactosidase expression. LB5010(pJGP1) and LB5010(pJGP6) were grown with and without induction (with 2 mM arabinose) and after 2 hours of growth the cultures β - galactosidase expression was measured using β - galactosidase assay. These results are the average of three assays.

It was found that there was no significant difference in the level of β - galactosidase expression from pJGP1 and pJGP6 in the presence or absence of arabinose. Therefore, pJGP1 and pJGP6 did not display a critical expected property, at least in *S. enterica* serovar Typhimurium.

5.4 Construction of pJGP1 and pJGP6 equivalent plasmids

Because it was found that the pJGP1 and pJGP6 constructs used in the present study did not show the expected structures or properties, it was decided to re-construct them. This would allow re-testing of the hypothesis that BipA regulates the expression of the *fis* gene at the translational level, via a sequence motif associated with the S-D region.

The constructs pMH2 and pMH4, which have structures equivalent to those predicted for pJGP1 and pJGP6 respectively, were created as illustrated in Figures 5.8 and 5.9. Using pRS414 plasmid DNA as a template and the primers pJGP1 Forward and pJGP1 Reverse, PCR was used to create a DNA fragment in which the *fis* 5' untranslated region and start codon was fused in-frame with the *lacZ* coding region from the 9th codon onwards. The fragment was then trimmed with *Pst*I and *Sal*I and ligated with the largest fragment arising from a similar digestion of pAJG38 DNA (Figure 5.8).

To construct a plasmid equivalent to pJGP6 (Owens *et al.*, 2004). pRS414 DNA was used as a template and the primers *lacZ* Fw and pJGP1 Reverse were used to amplify via PCR a DNA fragment in which the *lacZ* 5' untranslated region and start codon was fused in-frame with *lacZ* from the 9th codon onwards. As with the other construct, the fragment was trimmed with *Pst*I and *Sal*I and ligated with the largest fragment arising from a similar digestion of pAJG38 DNA (Figure 5.9).

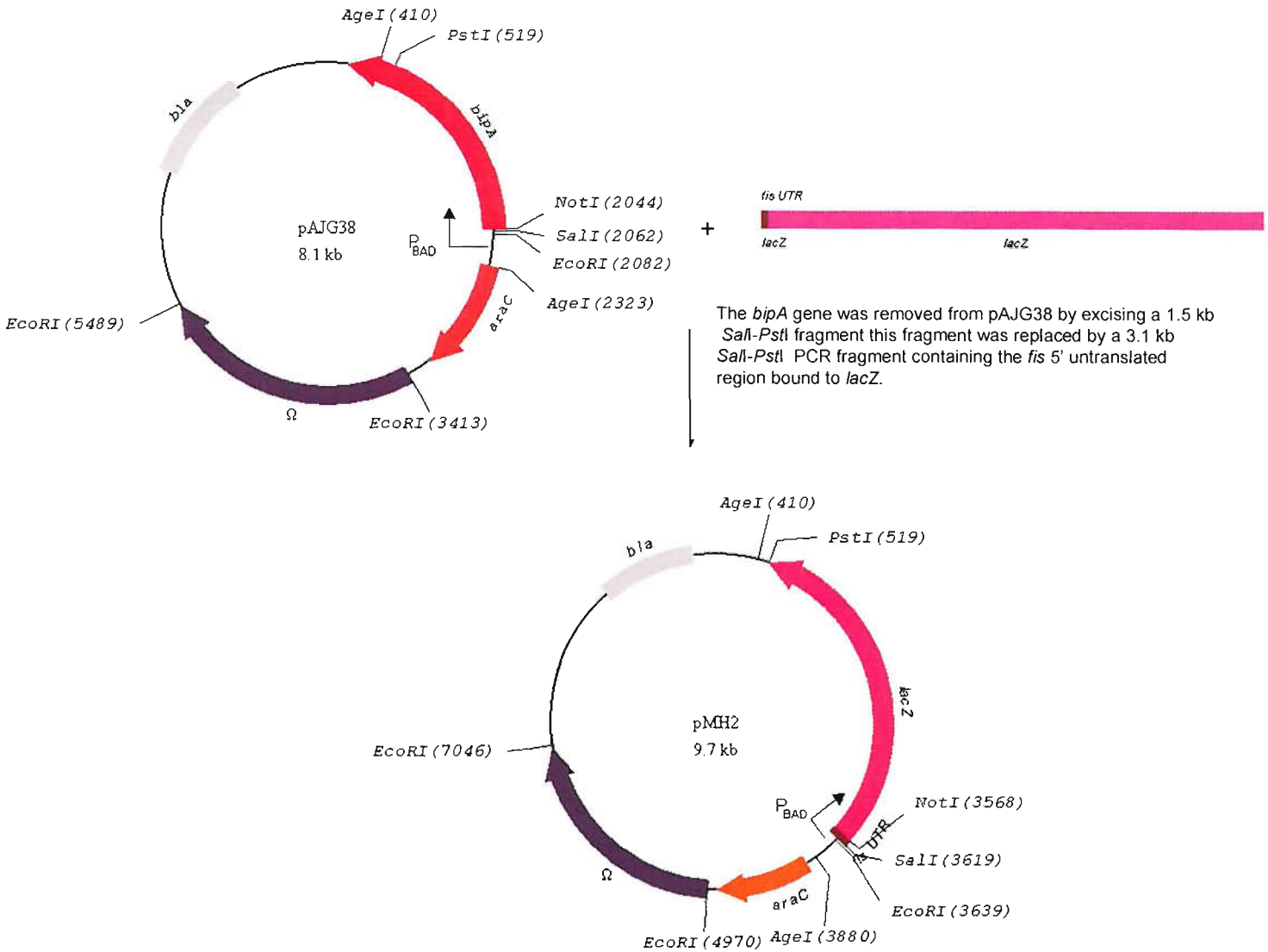


Figure 5.8. The construction of pMH2.

Figure 5.8. The construction of pMH2. The parent plasmid pAJG38 (Grant *et al.,* 2001) was cut with *Pst*I and *Sal*I and the DNA fragments were dephosphorylated (to prevent re-ligation) then column cleaned. PCR, using pRS414 (Simons *et al.,* 1987) as a template, was used to create a fragment containing the *fis* 5' untranslated region (including the native S-D sequence) bound to the *lacZ* coding sequence. The amplified fragment was trimmed with *Pst*I and *Sal*I and ligated into the pAJG38-derived vector backbone to make (the pJGP1 equivalent plasmid) pMH2. Ω indicates the presence of an omega fragment, which contains transcriptional and translational stop signals, as well as a streptomycin/spectinomycin resistance gene (Prentki and Krisch, 1984). The restriction sites that were important for the construction and subsequent restriction mapping of pMH2 are shown.

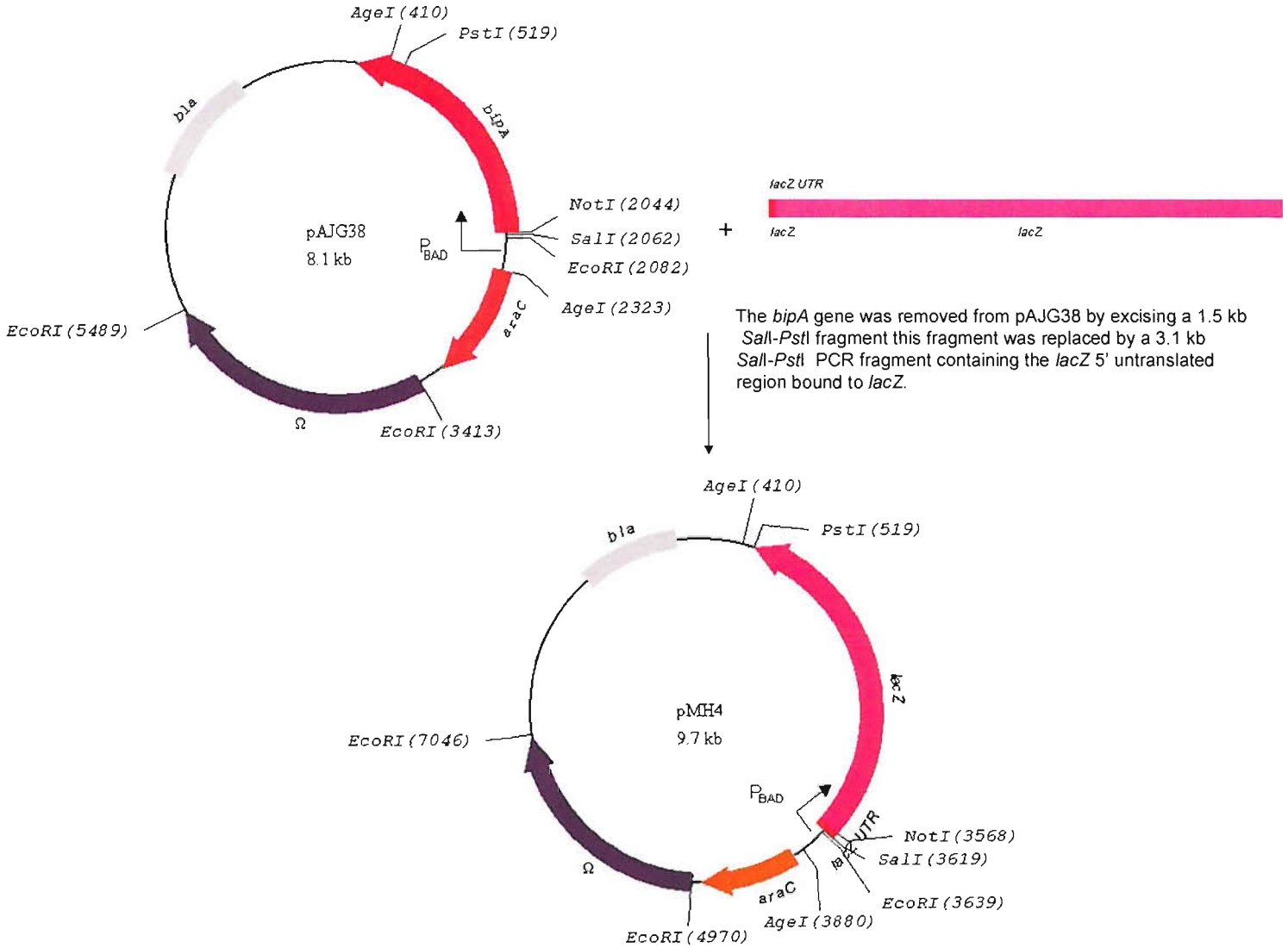


Figure 5.9. The construction of pMH4.

Figure 5.9. The construction of pMH4. The parent plasmid pAJG38 (Grant *et al.,* 2001) was digested with *Pst*I and *Sal*I and the DNA fragments were dephosphorylated (to prevent re-ligation) then column cleaned. pRS414 (Simons *et al.,* 1987) was used as a template for PCR to create a DNA fragment containing the *lacZ* 5' untranslated region (including the native S-D sequence) bound to the *lacZ* coding sequence. The amplified fragment was trimmed with *Pst*I and *Sal*I and ligated into the pAJG38-derived vector backbone to make (the pJGP6 equivalent plasmid) pMH4. Ω indicates the presence of an omega fragment (Prentki and Krisch, 1984). The restriction sites that were important for the creation and subsequent restriction mapping of pMH4 are shown.

In both cases, the ligated DNA was introduced by transformation into MG1655Δ*lac* cells, prior to the selection of transformants on LB agar plates containing both ampicillin and Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The inclusion of Xgal in the selective media meant that colonies expressing β - galactosidase could be readily identified. Plasmid DNA was prepared from representative blue colonies and restriction mapping was used to identify candidates with the predicted structures. Restriction mapping for the pMH2 candidate plasmids is shown in Figure 5.10 and Tables 5.7 to 5.9, and restriction mapping for the pMH4 candidate plasmids is shown in Figure 5.13 and Table 5.10. The candidate plasmids were then sequenced using the *lac* re3 and pMH2 r4 primers to confirm that the structure of the relevant region was correct. Sequence data for pMH2 is shown in Figures 5.11 and 5.12. Sequence data for pMH4 is shown in Figures 5.14 and 5.15. The resulting plasmids, pMH2 and pMH4, have structures identical to the ones predicted for pJGP1 and pJGP6, respectively. To avoid confusion, however, they will be referred to here using the pMH nomenclature, to distinguish them from the plasmids described in the study by Owens *et al.* (2004).

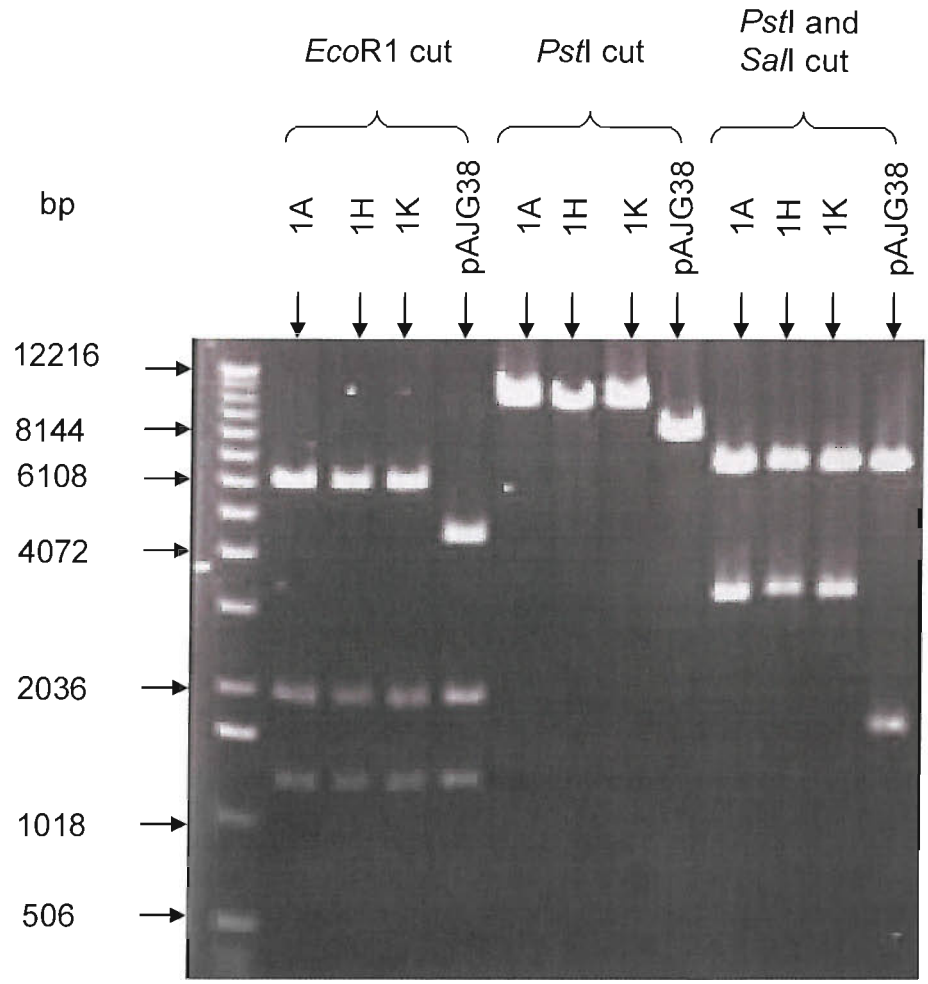


Figure 5.10. The pMH2 candidate plasmids display the expected structure when digested with *Eco*R1, *Pst*I, and double digested with *Pst*I and *Sal*I . The plasmid DNA candidates for pMH2 were named 1A, 1H and 1K. The pMH2 candidate plasmids and their parent plasmid pAJG38 were restriction digested with *Eco*R1, *Pst*I, and double digested with *Pst*I and *Sal*I. The resultant fragments were analysed by agarose gel electrophoresis. The predicted fragments for pMH2 and pAJG38, and observed fragments for the pMH2 candidate plasmids and pAJG38 are compared in Tables 5.7 to 5.9. The leftmost lane contains DNA marker standards.

Table 5.7. Comparison of the predicted fragments for pAJG38 and pMH2 digested with *Eco*R1, with the observed fragments formed by pAJG38 and the pMH2 candidate plasmids (1A, 1H and 1K) digested with *Eco*R1[j].

pAJG38	pAJG38	pMH2	1A	1H	1K
Predicted fragments (bp)	Observed fragments (bp)	Predicted fragments (bp)	Observed fragments (bp)	Observed fragments (bp)	Observed fragments (bp)
4712	~ 4700	6269	~6100	~6100	~6100
2076	~ 2000	2076	~2000	~2000	~2000
1331	~ 1300	1331	~1300	~1300	~1300

[j] The pMH2 candidate plasmids and pAJG38 were prepared and digested with *Eco*R1 and the resultant fragments were then resolved using agarose gel electrophoresis. Predicted fragments for pMH2 and pAJG38 were compared to the observed fragments sizes for pAJG38 and the pMH2 candidate plasmids (1A, 1H and 1K).

Table 5.8. Comparison of the predicted fragments for pMH2 and pAJG38 digested with *Pst*I, with the observed fragments formed by the pMH2 candidate plasmids (1A, 1H and 1K) and pAJG38 digested with *Pst*I[k].

pAJG38	pAJG38	pMH2	1A	1H	1K
Predicted fragments (bp)	Observed fragments (bp)	Predicted fragments (bp)	Observed fragments (bp)	Observed fragments (bp)	Observed fragments (bp)
8119	~ 8100	9676	~9600	~9600	~9600

[k] The pMH2 candidate plasmids and pAJG38 were prepared and digested with *Pst*I. The resultant fragments were then resolved using agarose gel electrophoresis. Predicted fragments for pMH2 and pAJG38 were compared to the observed fragments for pAJG38 and the pMH2 candidate plasmids (1A, 1H and 1K).

Table 5.9. Comparison of the predicted fragments for pAJG38 and pMH2 digested with *Pst*I and *Sal*I, with the observed fragments formed by pAJG38 and the pMH2 candidate plasmids (1A, 1H and 1K) digested with *Pst*I and *Sal*I[I].

pAJG38	pAJG38	pMH2	1A	1H	1K
Predicted fragments (bp)	Observed fragments (bp)	Predicted fragments (bp)	Observed fragments (bp)	Observed fragments (bp)	Observed fragments (bp)
6576	~6600	6576	~6600	~6600	~6600
1543	~1600	3100	~3100	~3100	~3100

[I] The pMH2 candidate plasmids and pAJG38 were prepared and digested with *Pst*I and *Sal*I and the resultant fragments were then resolved using agarose gel electrophoresis. Predicted fragments for pMH2 and pAJG38 were compared with the fragments observed for pAJG38 and the pMH2 candidate plasmids (1A, IH and 1K).

Figure 5.11. pMH2 contains the *fis* 5' untranslated region (including the S-D sequence) bound to the *lacZ* coding sequence from the 9th codon onwards.

Figure 5.11. pMH2 contains the *fis* 5' untranslated region (including the S-D sequence) bound to the *lacZ* coding sequence from the 9th codon onwards. The pMH2 candidate plasmid named 1A was sequenced and shown to have the correct structure, thereafter it was re-named pMH2. The Sequence Manipulation Suite 2 software package was used to reverse complement and restriction digest the sequence data. The Align software package (Pearson *et al.,* 1997) was used to align the reverse complement of the sequence data for pMH2 (depicted in black) with the reverse complement of the *E. coli* K-12 (Blattner *et al.,* 1997) *lacZ* coding sequence (gi:49175990, accession number NC_000913) and the sequence of the forward PCR primer used to create the inserted fragment pJGP1 Fw. The *fis* 5' untranslated region (including the S-D sequence) was obtained from the *E. coli* K-12 genome (Blattner *et al.,* 1997) (gi:49175990, accession number NC_000913). The *lacZ* coding sequence is depicted in red as is the numbers which refer to the position of the aligned region. The sequence of the forward PCR primer (including the S-D sequence) is depicted in purple. The start codon is depicted in blue and underlined. The *Not*I restriction site is underlined.

Figure 5.12. The pMH2 region downstream of the *fis* 5' untranslated region was derived from its parent plasmid pAJG38. The Sequence Manipulation Suite 2 software package was used to simulate digestion the sequence data. The Align software package (Pearson *et al.*, 1997) was used to align sequence data for pMH2 with the sequence of its parent plasmid pAJG38 (Grant *et al.*, 2001). The pAJG38 sequence is depicted in pink and the pink numbers above the sequence refer to the position of the aligned region within the plasmid sequence. The sequence data for pMH2 is depicted in black. The *Sal*I restriction site is underlined.

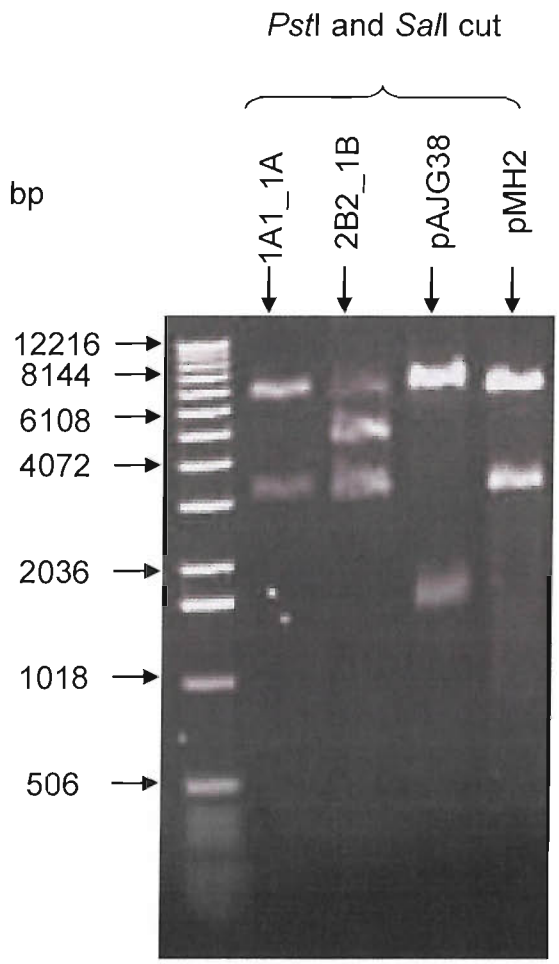


Figure 5.13. The pMH4 candidate plasmid 1A1_1A displays the expected structure when double digested with *PstI* and *SalI*. The plasmid DNA candidates for pMH4 were named 1A1_1A and 2B2_1B. The pMH4 candidate plasmids, pAJG38 and pMH2 were restriction digested with *PstI* and *SalI*. The resultant fragments were analysed by agarose gel electrophoresis. The predicted fragments for pMH2, pMH4 and pAJG38 and observed fragments for the pMH4 candidate plasmids, pMH2 and pAJG38 are compared in Table 5.10. The leftmost lane contains DNA marker standards.

Table 5.10. Comparison of the predicted fragments for pMH2, pMH4 and pAJG38 restriction digested with *Pst*I and *Sal*I with the observed fragments for the pMH4 candidates, pMH2 and pAJG38[m].

pAJG38	pAJG38	pMH2 and pMH4	pMH2	1A1_1A	2B2_1B
Predicted fragments (bp)	Observed fragments (bp)	Predicted fragments (bp)	Observed fragments (bp)	Observed fragments (bp)	Observed fragments (bp)
6576	~6600	6576	~6600	~6600	~6600
1543	~1600	3100	~3100	~3100	~5000
					~3100

[m] The pMH4 candidate plasmids, pMH2 and pAJG38 were prepared and digested with *Pst*I and *Sal*I and the resultant fragments were then resolved using agarose gel electrophoresis. Predicted fragments for pMH4, pMH2 and pAJG38 were compared with the fragments observed for pMH2, pAJG38 and the pMH4 candidate plasmids (1A1_1A and 2B2_1B).

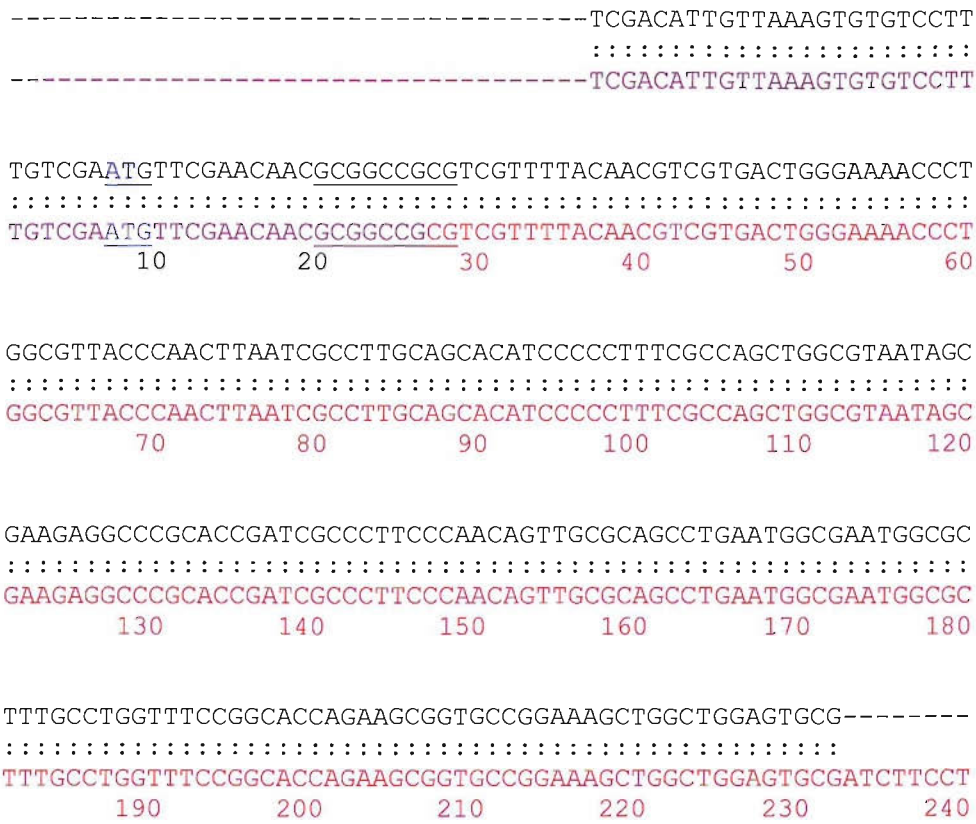


Figure 5.14. pMH4 contains the *lacZ* 5' untranslated region (including the S-D sequence) bound to the *lacZ* coding sequence from the 9th codon onwards. The pMH4 candidate plasmid 1A1_1A was sequenced and shown to have the correct structure, thereafter it was re-named pMH4. The Sequence Manipulation Suite 2 software package was used to reverse complement and restriction digest the sequence data. The Align software package (Pearson *et al.,* 1997) was used to align the reverse complement of the sequence data for pMH4 (depicted in black) with the reverse complement of the *E. coli* K-12 (Blattner *et al.,* 1997) *lacZ* coding sequence (gi:49175990, accession number NC_000913) and the sequence of the forward PCR primer used to create the inserted fragment, *lacZ* Fw. The *lacZ* coding sequence is depicted in red as is the numbers which refer to the position of the aligned region. The sequence of the forward PCR primer *lacZ* Fw (including the S-D sequence) is depicted in purple. The sequence of the *lacZ* 5' untranslated region (including the S-D sequence) was obtained for *E. coli* K-12 (Blattner *et al.,* 1997) (gi:49175990, accession number NC_000913). The start codon is depicted in blue and underlined. The *Not*I restriction site is underlined.

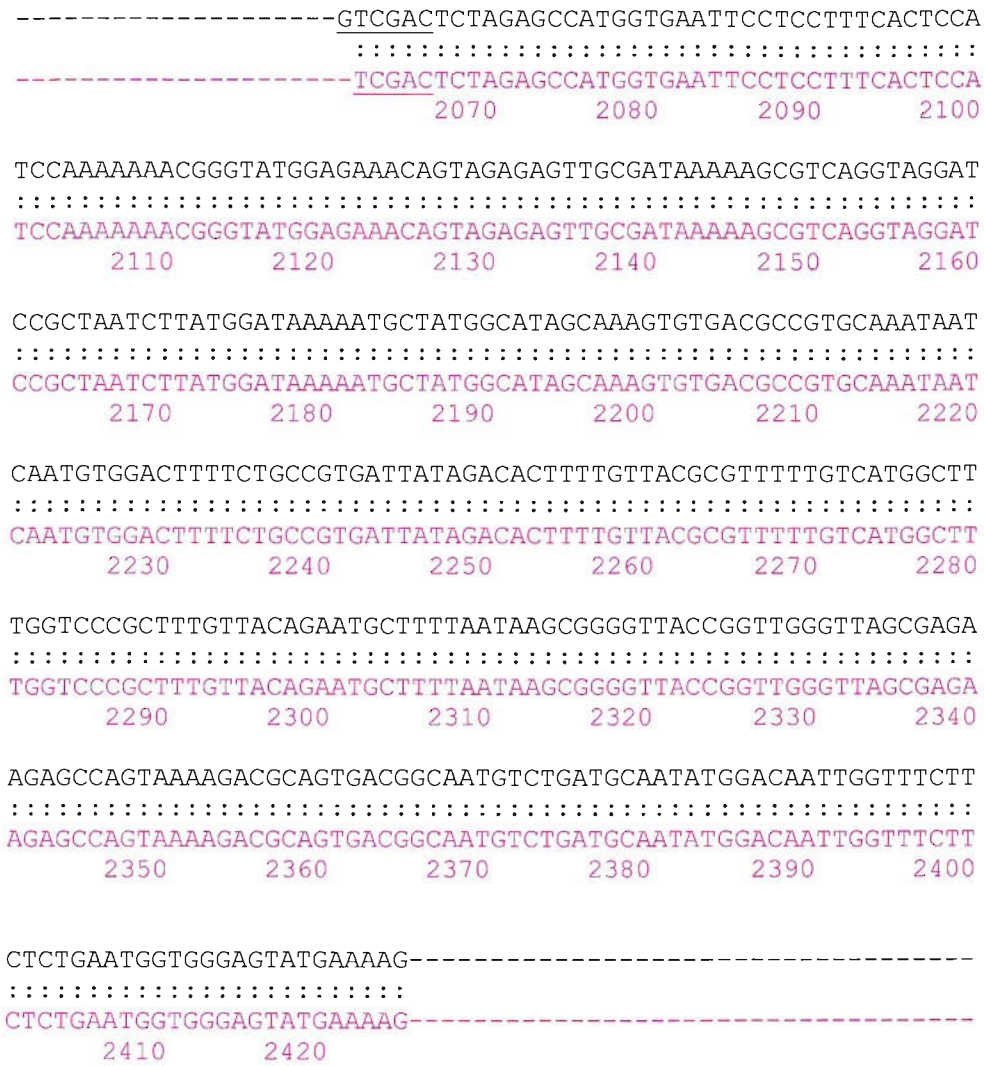


Figure 5.15. The region downstream of the *lacZ* 5' untranslated region of pMH4 was derived from its parent plasmid pAJG38. The Sequence Manipulation Suite 2 software package was used to simulate digestion the sequence data. The Align software package (Pearson *et al.,* 1997) was used to align sequence data for pMH4 with the sequence of its parent plasmid pAJG38 (Grant *et al.,* 2001). The pAJG38 sequence is depicted in pink and the pink numbers below the sequence refer to the position of the aligned region within the plasmid sequence. The sequence data for pMH4 is depicted in black. The *Sal*I restriction site is underlined.

5.5 The *fis* 5' untranslated region does not confer responsiveness to BipA

Following the production of pMH2 and pMH4 it was finally possible to test the model proposing that BipA controls Fis expression at the translational level via the unusual Shine-Dalgarno sequence present in *fis* mRNA (Owens *et al.*, 2004). pMH2 and pMH4 were introduced by transformation into *E. coli* MG1655Δ*lac* and MG1655Δ*lac*Δ*bipA*. Duplicate cultures of the resultant strains were grown to an optical density (600 nm) of 0.5, whereupon one sample was treated with L-arabinose (2 mM, final concentration), while the other was treated with an equivalent volume of sterile water. After a further 90 minutes of incubation, β - galactosidase assays were carried out to determine the level of β - galactosidase activity in each culture.

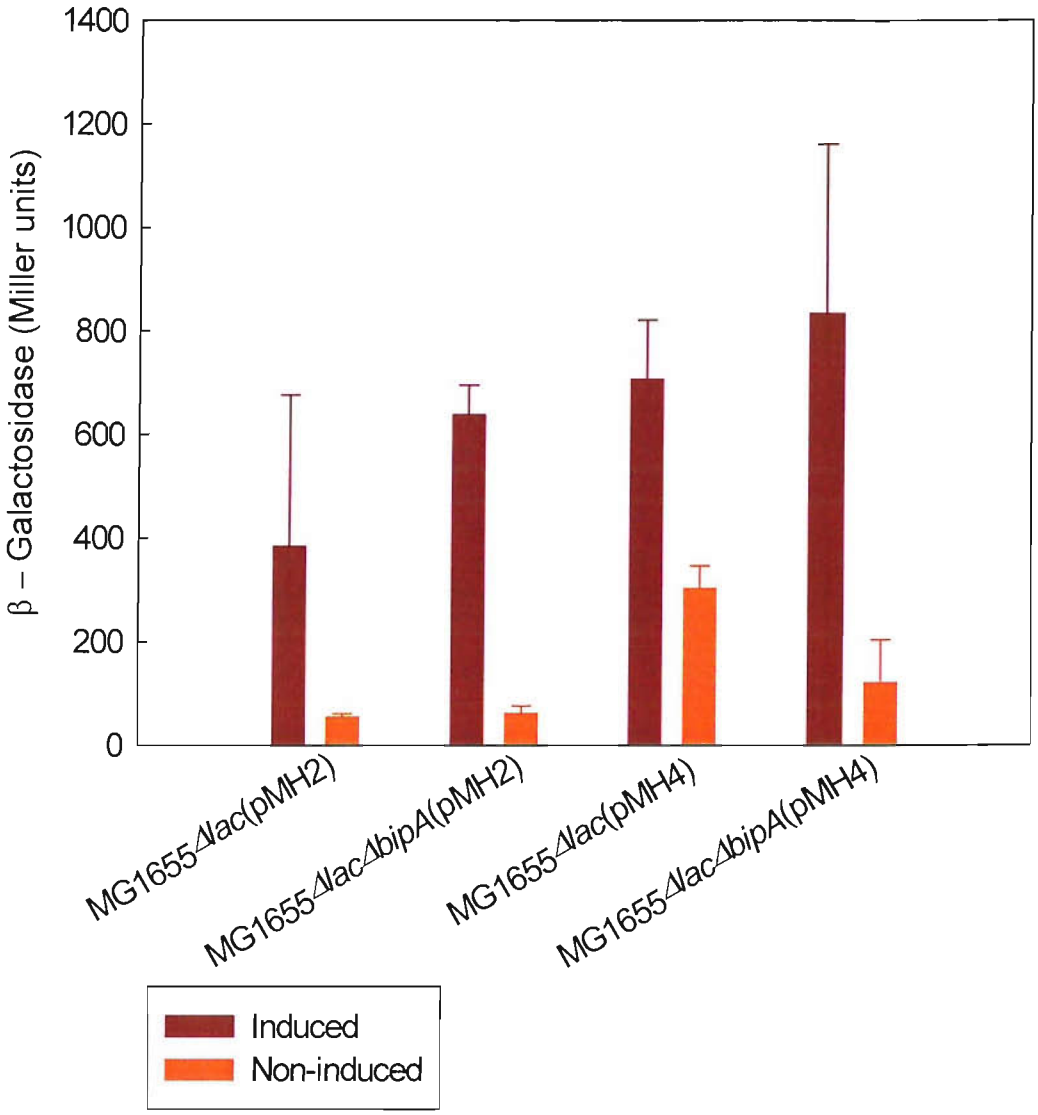


Figure 5.16. The *fis* 5' untranslated region does not contain a BipA response element. MG1655Δ*lac* and MG1655Δ*lac*Δ*bipA* cells containing pMH2 and pMH4 were grown to an OD_{600} of 0.5 then half the cultures were induced (with 2 mM arabinose) and the cultures were grown for a further 90 minutes, after which, β - galactosidase assays were carried out to measure the level of β - galactosidase expression in each of the cultures.

It was found that there was no significant difference between the level of β - galactosidase expressed from cells bearing pMH2 or the control construct pMH4, in the presence or absence of BipA (Figure 5.16). This indicates that the *fis* 5' untranslated region does not confer responsiveness to BipA. In other words the region in question does not appear to contain a putative BipA response element.

5.6 Aberrant expression of the *fis* 5' untranslated region does not affect cell growth in *E. coli*

The model for the mode of action of BipA proposed by Owens *et al.* (2004) suggests that the high level of complementarity between the S-D region of the *fis* transcript and the 3' end of the 16S ribosomal RNA causes the ribosome to lock on to the 5' end of the mRNA in the absence of BipA. A possible corollary of this model is that overexpression of the *fis* 5' untranslated region in the absence of BipA might lead to sequestration of ribosomes on this mRNA, thereby causing the cessation of cell growth.

To examine this possibility the 5' untranslated region of *fis* mRNA was overexpressed in the presence and absence of BipA to see if this affected cell growth. MG1655Δ*lac* and MG1655Δ*lac*Δ*bipA* cells harbouring pMH2 or pMH4 were cultured in the presence or absence of inducer (2 mM arabinose) and their growth was monitored over time.

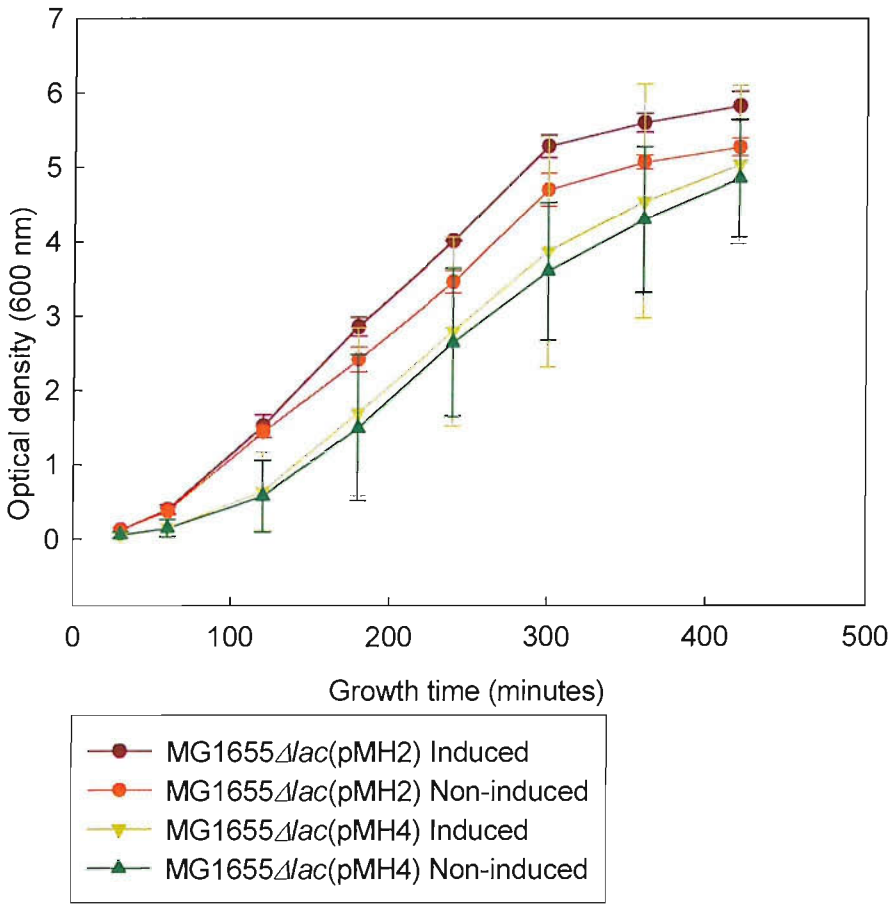


Figure 5.17. Overexpression of the *fis* 5' untranslated region does not affect cell growth in *E. coli*. MG1655Δ*lac* cells containing pMH2 and pMH4 were grown with and without induction (with 2 mM arabinose, at inoculation) and their growth was monitored over time. The results shown are the mean of three replicates.

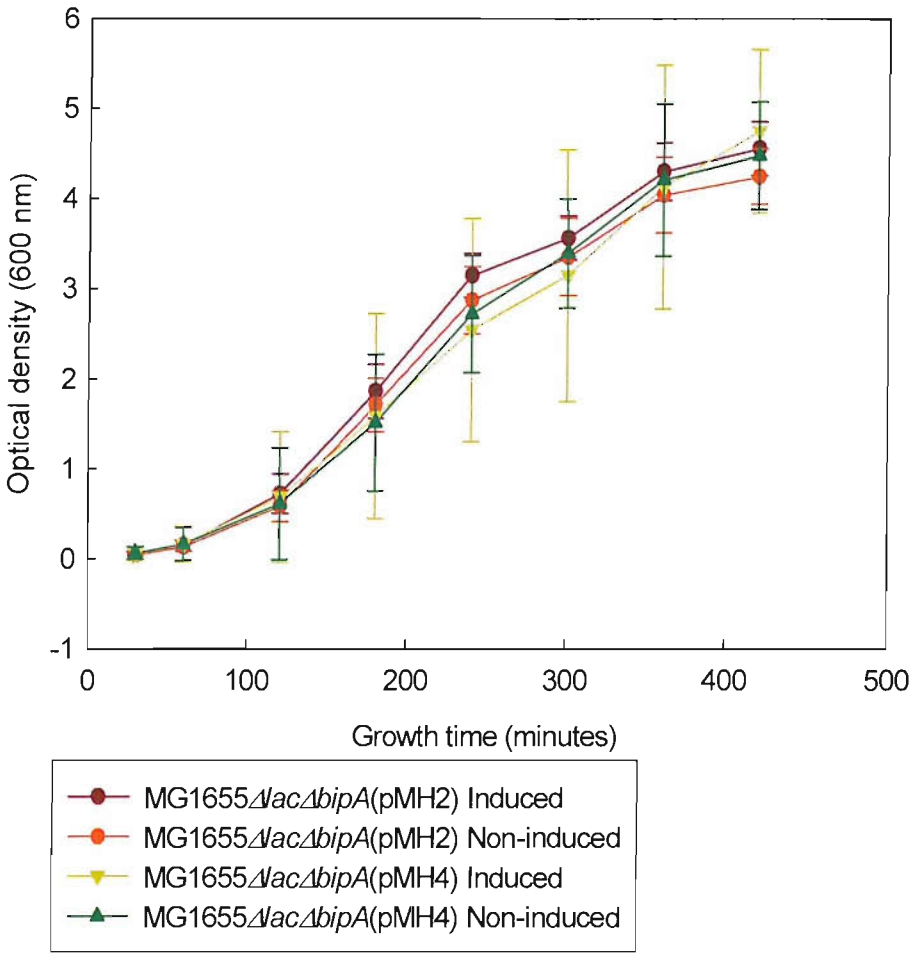


Figure 5.18. *E. coli* cell growth is not affected by overexpression of the *fis* 5' untranslated region. MG1655Δ*lac*Δ*bipA* harbouring pMH2 and pMH4 were grown with and without induction (with 2 mM arabinose, at inoculation) and their growth was monitored over time. The results shown are the mean of three replicates.

In contrast to a possible prediction of the model, cells overexpressing the *fis* 5' untranslated region showed no significant retardation in growth rate in the absence of BipA relative to cells that had BipA. Therefore, the aberrant expression of the *fis* 5' untranslated region does not affect cell growth in the absence of BipA.

5.7 Discussion

Examination of the plasmids pJGP1 and pJGP6 revealed that they failed to display the structures or critical functions reported for them by Owens *et al.* (2004).

Restriction mapping of pJGP1, pJGP6 and their parent plasmid pAJG38 revealed that the first two constructs failed to yield the expected DNA fragments when digested with restriction enzymes with recognition sites of ≥6bp (i.e. *Age*I, *Eco*RI, *Pst*I and double digested with *Not*I and *Sal*I). In contrast, pAJG38 reproducibly gave the expected fragmentation pattern under the same experimental conditions. Importantly, the DNA fragmentation patterns of pJGP1 and pJGP6 did not resemble that of their parent plasmid pAJG38 from which their vector backbone and therefore a significant amount of their sequence should have been derived (Owens *et al.*, 2004).

To further compare pJGP1 to its parent plasmid pAJG38 several frequent cutting restriction enzymes were used to map both plasmids (*Hinc*II, *Dra*I, *Ava*I and *Hin*fI) in the hope that this would give some indication of the extent to which to pJGP1 and pAJG38 are related. However, the two plasmids failed to show significant similarity and it was again observed that pJGP1 failed to yield the fragments predicted for its structure. These results indicate that pJGP1 and pJGP6 do not have the structures detailed for them in the paper by Owens *et al.* (2004).

In view of the above discrepancies it was decided to examine critical properties of pJGP1 and pJGP6; specifically, whether the addition of L-arabinose to cells bearing them induced β - galactosidase expression. Cultures containing pJGP1 and pJGP6 showed no significant difference in β - galactosidase expression when grown in the presence or absence of L-arabinose. Thus, they do not perform as described by Owens *et al.* (2004).

To explore the reasons behind the anomalous results in more detail, it was decided to re-construct pJGP1 and pJGP6. The structures of the re-constructed candidate plasmids were examined by restriction mapping and by DNA sequencing to ensure that they had the correct structures. The new constructs were named pMH2 and pMH4, respectively. These constructs were then tested to see if high-level expression of β - galactosidase in pMH2 was dependent on the presence of BipA. In contrast to the results reported by Owens *et al.* (2004) there was no significant difference in the levels of reporter enzyme expression irrespective of whether BipA was present or not.

As a further test, the possibility that overexpression of the *fis* 5' untranslated region in the absence of BipA would lead to growth inhibition was examined. Again, the results were not consistent with the hypothesis put forward in the paper by Owens *et al.* (2004). It was found that a *bipA* null mutant strain aberrantly expressing the 5' untranslated region of *fis* mRNA showed no significant difference in growth rate relative to cells that did not express this transcript at significant levels. Thus, this data also does not support the current model for the mechanism of action of BipA.

Collectively the results presented in this chapter are in conflict with data reported by Owens *et al.* (2004), and strongly suggest that that the current model needs major revision.

CHAPTER 6

FIS EXPRESSION LEVELS SHOW NO SIGNIFICANT DIFFERENCE IN WILD TYPE OR BIPA NULL MUTANT STRAINS

6 Fis expression levels show no significant difference in wild type or *bipA* null mutant strains

6.1 Introduction

Previous studies by several groups have collectively established that BipA has global regulatory properties; it regulates cellular processes as diverse as the expression of the LEE (in EPEC), flagella-mediated cell motility (various *E. coli* and *Salmonella* strains), synthesis of group 2 capsules (*E. coli* K5), synthesis of the core moiety of LPS (*E. coli* K-12), growth at temperatures below 30°C (*E. coli* strains and *Sinorhizobium meliloti*), and adaptation to low pH and SDS-mediated stress and microbe-plant symbiosis (*S. meliloti*) (Farris *et al.*, 1998;Grant *et al.*, 2003;Kiss *et al.*, 2004;Moller *et al.*, 2003;Pfennig and Flower, 2001;Rowe *et al.*, 2000). As stated previously, however, BipA is not a typical regulatory protein. Its structure and ability to interact with ribosomes suggests that it may influence the translation of one or more key effector proteins in the cell (Farris *et al.*, 1998;Owens *et al.*, 2004).

The discovery of a direct regulatory target would greatly facilitate studies to elucidate BipA's mechanism of action. In their studies on BipA-mediated control of the LEE (Grant *et al.*, 2003) ruled out a number of potential regulatory targets, including H-NS, IHF and Per. In the absence of definitive evidence, it was proposed to designate the direct target for BipA as 'Factor X'.

Shortly after commencing the present study, Owens *et al.* (2004) proposed that the global regulatory protein Fis was Factor X. The evidence for this conclusion was (i) the fact that Fis had been previously shown to upregulate the formation of the attaching and effacing lesion through increasing the expression of the LEE encoded regulator (Ler) (Goldberg *et al.*, 2001); (ii) the similar phenotypes of *bipA* and *fis* mutants and (iii) immuno-blotting studies, which showed that the

level of Fis protein, but not *fis* mRNA, was severely reduced in *E. coli* cells lacking BipA. It was also shown that BipA expression correlated with that of Fis insofar as both were highly expressed in the lag phase of growth but declined drastically as cells progressed through exponential phase into the stationary phase. These findings led to the proposal that BipA enhances the translation rate of the *fis* mRNA and hence increases the net level of Fis protein in cells. It was further postulated that Fis may act to increase the transcription of the gene for the LEE-encoded regulator, *ler* thereby increasing the expression of the LEE and certain other genes associated with pathogenicity. Although not explicitly stated, this model could also be applied to other bacterial cells that used Fis as a global regulator of gene expression (Owens *et al.*, 2004;Rosenshine *et al.*, 1992;Rosenshine *et al.*, 1996).

During the course of the present study discrepancies arose that cast doubt on the immuno-blotting results presented by Owens *et al.* (2004) that showed that Fis expression was significantly reduced in *bipA* null mutant cells. Accordingly, it was deemed important to re-investigate these studies in more detail. This chapter describes the results of immuno-blotting studies on wild type and *bipA* null mutant cells using a polyclonal antibody that is specific for the Fis protein.

6.2 Fis expression levels show no significant difference in wild type or *bipA* null mutant strains

To thoroughly examine whether BipA enhances the expression of Fis, protein samples were prepared from *bipA* null mutants and their parent strains. The samples were prepared from cells at different phases of growth to ensure that the physiological state of the cells did not account for any difference with the results reported by Owens *et al.* (2004). Specifically, protein samples were prepared from *E. coli* MG1655 and EPEC E2348/69 cells with optical densities (600 nm) of 0.2, 0.5, 0.8 (data not shown),1.5, from the lag phase of growth and finally from the late stationary phase of growth, which was prepared after 15 hours growth (data not shown). The *fis* null mutant strain CSH50*fis::kan* was used as a *fis* negative control. CSH50*fis::kan* cultures were grown to an OD_{600} of 0.5 then grown further for 90 minutes, prior to the preparation of protein samples. All of the samples were then analysed by immuno-blotting using a Fis-specific polyclonal antibody (kindly supplied by Professor Charles Dorman, Trinity College Dublin).

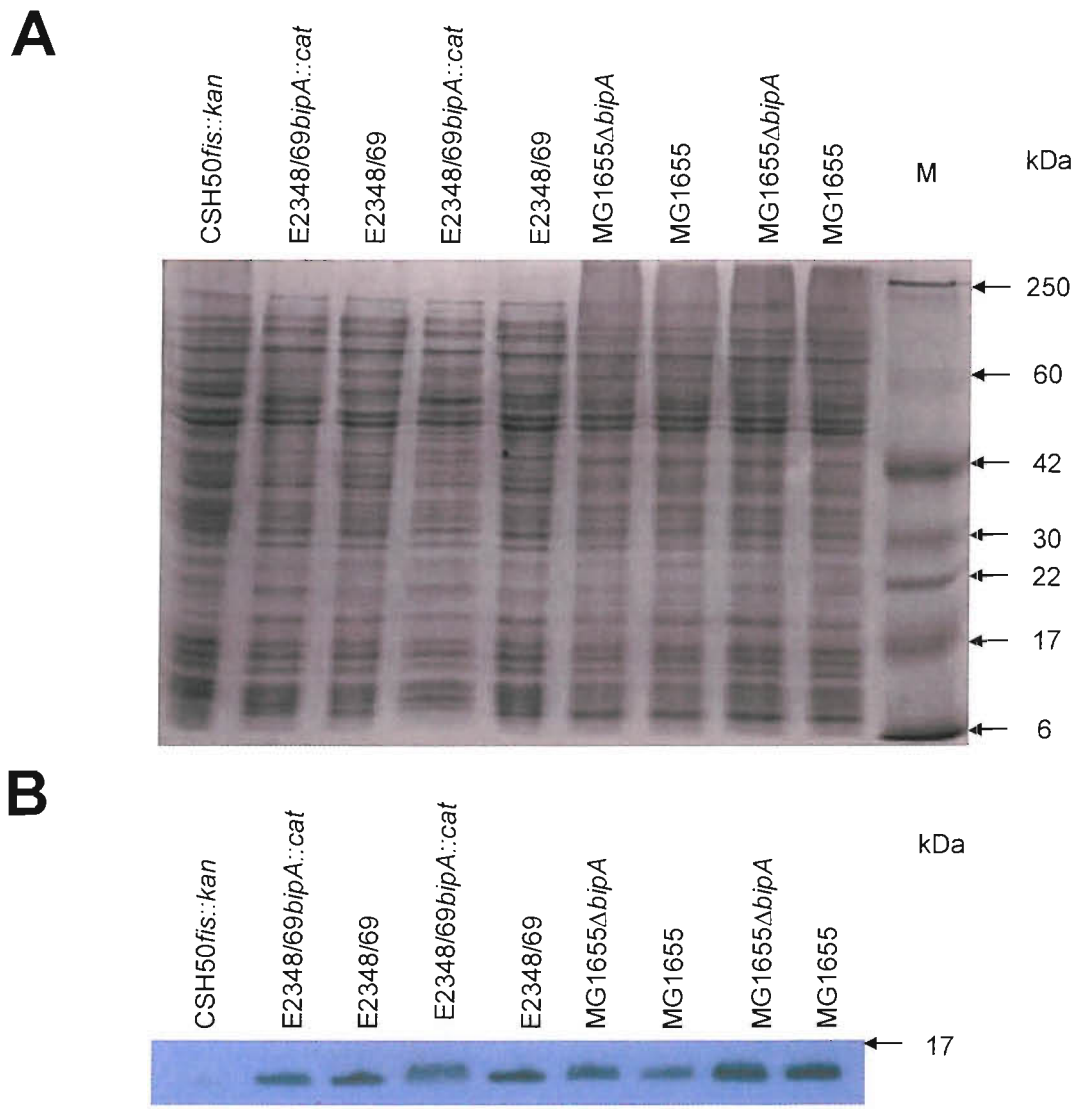


Figure 6.1. Wild type or *bipA* null mutant strains in the lag phase of growth show no significant difference in Fis expression levels.

Figure 6.1. Wild type or *bipA* null mutant strains in the lag phase of growth show no significant difference in Fis expression levels. Protein samples from lag phase cells were prepared by inoculating overnight cultures of MG1655, MG1655Δ*bipA*, E2348/69 and E2348/69*bipA::cat* one in fifty in pre-warmed LB medium and growing the cultures for 6 minutes. The cells were then harvested and protein samples prepared from them. As a negative control, CSH50*fis::kan* cells were grown to an optical density (600 nm) of 0.5 and then grown further for 90 minutes prior to preparation of protein samples. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. They were then probed with a Fis-specific antibody as described in Chapter 2. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the corresponding immuno-blot.

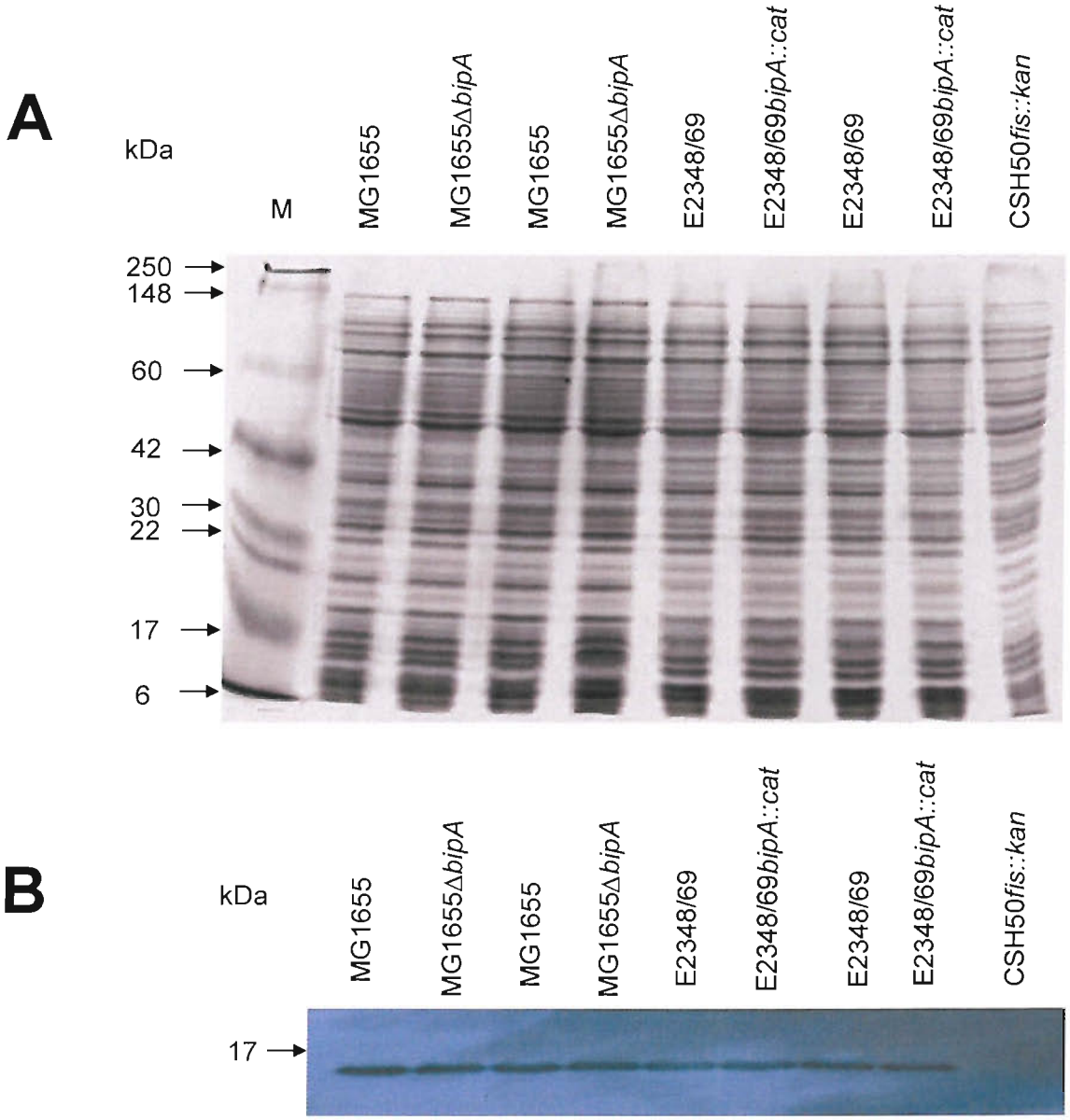


Figure 6.2. Wild type or *bipA* null mutant strains in the early exponential phase of growth show no significant difference in Fis expression levels.

Figure 6.2. Wild type or *bipA* null mutant strains in the early exponential phase of growth show no significant difference in Fis expression levels. Protein samples from early exponential phase were prepared by growing MG1655, MG1655Δ*bipA*, E2348/69 and E2348/69*bipA::cat* cultures to an optical density (600 nm) of 0.2. Then the cells were harvested and protein samples were prepared from them. As a negative control, CSH50*fis::kan* cells were grown to an optical density (600 nm) of 0. 5 and then grown further for 90 minutes prior to preparation of protein samples. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. They were then probed with a Fis-specific antibody as described in Chapter 2. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the corresponding immuno-blot.

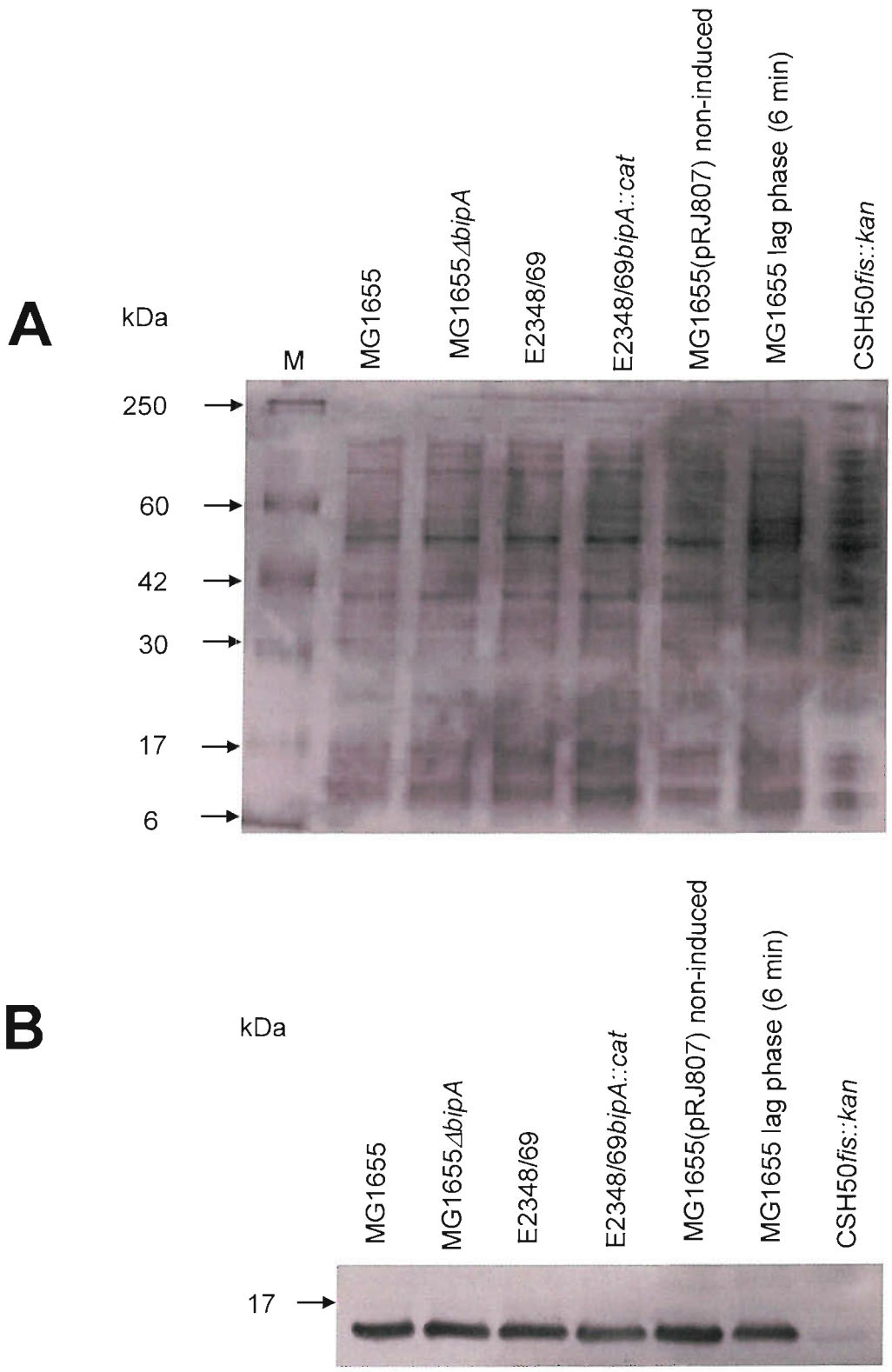


Figure 6.3. Wild type or *bipA* null mutant strains in the exponential phase of growth show no significant difference in Fis expression levels.

Figure 6.3. Wild type or *bipA* null mutant strains in the exponential phase of growth show no significant difference in Fis expression levels. Exponential phase protein samples were prepared by growing MG1655, MG1655Δ*bipA*, E2348/69 and E2348/69*bipA::cat* cells to an optical density (600 nm) of 0.5 then preparing protein samples from them. As a negative control, CSH50*fis::kan* cultures were grown to an optical density (600 nm) of 0.5 then grown further for 90 minutes prior to preparation of protein samples. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. They were then probed with a Fis-specific antibody as described in Chapter 2. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the corresponding immuno-blot.

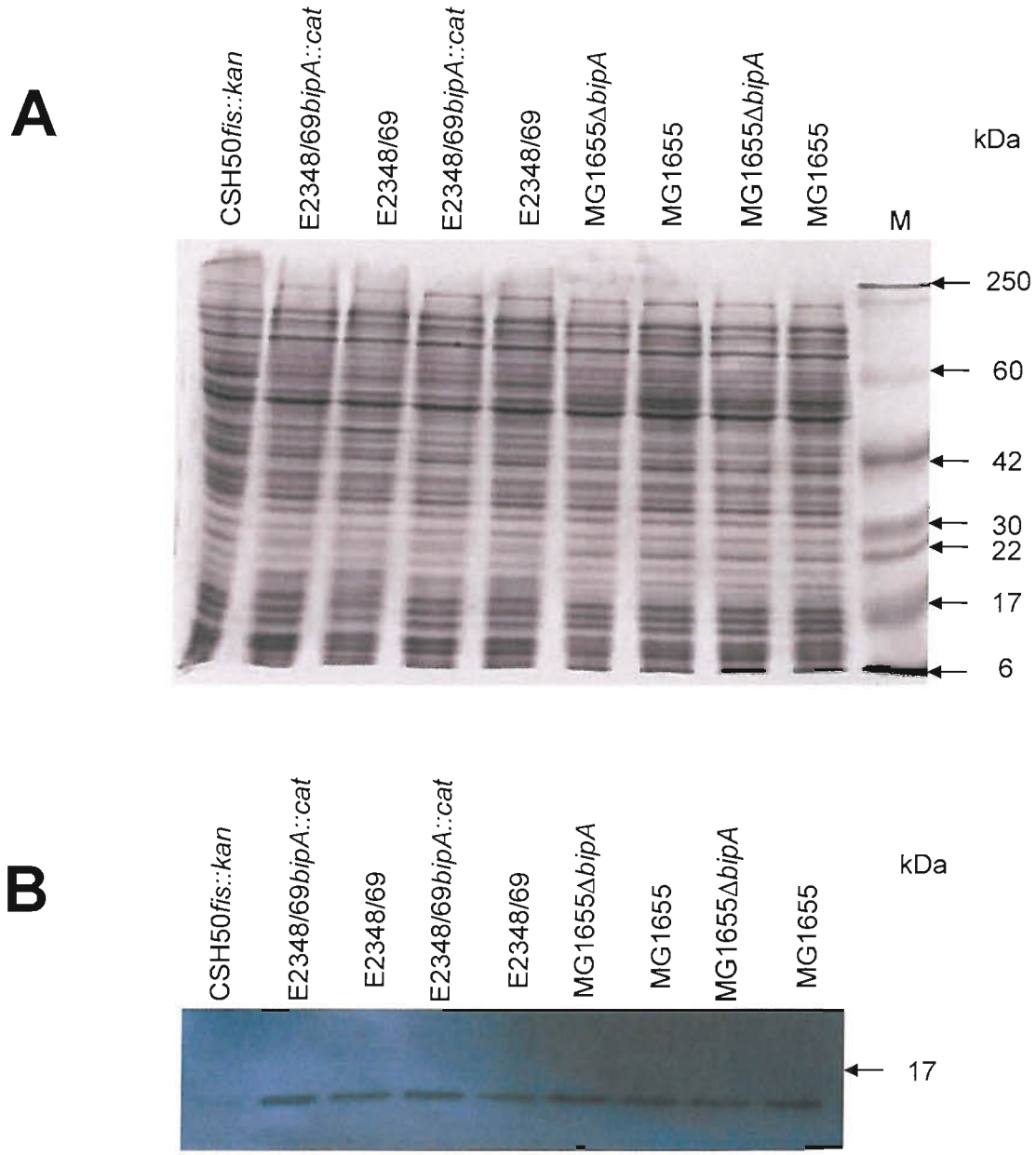


Figure 6.4 Wild type or *bipA* null mutant strains in the early stationary phase of growth show no significant difference in Fis expression levels.

Figure 6.4 Wild type or *bipA* null mutant strains in the early stationary phase of growth show no significant difference in Fis expression levels. Protein samples from early stationary phase were prepared by growing MG1655, MG1655Δ*bipA*, E2348/69 and E2348/69*bipA::cat* cells to an optical density (600 nm) of 1.5. The cells were then harvested and protein samples prepared from them. As a negative control, CSH50*fis::kan* cells were grown to an optical density (600 nm) of 0.5 and then grown further for 90 minutes prior to preparation of protein samples. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. They were then probed with a Fis-specific antibody as described in Chapter 2. Panel A shows the protein loaded for the immuno-blot on a Coomassie Blue stained SDS-PAGE gel. Panel B shows the corresponding immuno-blot.

As expected, the immuno-blots revealed that a single band, which migrated at approximately 11 kDa, cross-reacted with the Fis-specific antisera. This is consistent with the predicted molecular weight of the Fis protein and previous immuno-blotting experiments reported by other groups (Ball and Johnson, 1991;Osuna *et al.*, 1995;Richins *et al.*, 1997). However, as indicated in Figures 6.1 to 6.4, there was no significant difference between the levels of Fis protein in *bipA* null mutants and their parent strains regardless of the phase of growth. It should be noted, however, that after 15 hours of growth Fis was not detected in the *bipA* null mutants or in the parent strains (data not shown). This was not unexpected as previous investigators have shown that Fis levels drop to less than 100 copies per cell in the stationary phase (Ball *et al.*, 1992). Both *E. coli* K-12 and the EPEC strain E2348/69 gave similar results – it was not possible to distinguish any difference in the levels of Fis expression in parent and *bipA* null mutant cells.

In all cases, the *fis* negative control samples prepared from CSH50*fis::kan* cells did not yield a cross-reacting band on immuno-blotting confirming that the primary antibody used was Fis specific. It is therefore concluded that, at least under the conditions used in the present study, the presence or absence of BipA does not influence the level of the Fis protein in *E. coli*.

6.3 Discussion

The above results strongly suggest that BipA does not control the expression of Fis. This conclusion is supported by evidence that is consistent across the growth phase and for both *E. coli* K-12 and EPEC. The results obtained contradict those reported by Owens *et al.* (2004), where Fis expression was shown to be significantly reduced in *bipA* null mutant strains. At the time of writing, the reason for this discrepancy is difficult to pin point. However, several obvious sources of error include poor antibody specificity, use of incorrect strains, mistakes when loading samples into the SDS-PAGE gels and/or growth conditions that were not reproduced in the present study. The first possibility, use of an antibody with poor specificity for Fis, is unlikely as Owens *et al.* (2004) also reported a single cross-reacting band of the correct molecular weight and showed that it was not present in samples prepared from a *fis* null mutant. While the use of incorrect strains cannot be discounted, the mistake would have had to have occurred multiple times with multiple strains, including *bipA* null mutants of *E. coli* K-12, EPEC and additionally *S. enterica* (G. Pritchard; Ph.D. thesis (2006), University of Southampton). Thus, it is difficult to see how so many strains could have been repeatedly mixed up. Another possible source of error might have been uneven protein loadings. In the present study the protein concentration of each sample was measured using BCA assay so that equal amounts could be used to load SDS-PAGE gels. Further, replicate gels were stained with Coomassie Blue to verify the protein levels loaded (see Figures 6.1 to 6.4), thus helping to ensure that only evenly-loaded gels were immuno-blotted. While there is always the possibility of operator error, the consistent results obtained here from successive repeats indicates that the results of this study are reliable and reproducible.

Whatever the reason for the discrepancy, the results reported in this chapter strongly suggest that it is unsafe to conclude that BipA is required for the full expression of the Fis global regulatory protein. This in turn means that a direct

target for BipA (i.e. translationally regulated by BipA – Factor X) remains to be identified.

CHAPTER 7

CONTINUING THE SEARCH FOR BIPA REGULATED GENES: COMPARATIVE PROTEOMIC ANALYSIS OF LAG PHASE CELLS OF SL1344 AND SL1344ΔBIPA

7 Continuing the search for BipA regulated genes: Comparative proteomic analysis of lag phase cells of SL1344 and SL1344Δ*bipA*

7.1 Introduction

The data presented in chapters five and six of this thesis have led to the conclusion that, contrary to the data presented by Owens *et al.* (2004), Fis is not the BipA regulated protein that positively regulates *ler* transcription (i.e. Factor X) and hence the Locus for Enterocyte Effacement (Grant *et al.*, 2003;Owens *et al.*, 2004). This means that the regulatory target (or targets) of BipA that mediate its cellular effects remain to be determined. It was therefore decided to continue the search for BipA regulated genes to facilitate the elucidation of the mechanism of action of this novel ribosome binding global regulatory protein.

Recent studies have revealed that the expression of BipA is growth phase regulated and that it peaks in the lag phase, after which its level falls dramatically (over 25-fold as cultures progress toward stationary phase) (Owens *et al.*, 2004). This data led to the consideration that BipA expression may be regulated in a manner that ensures that its peak level of expression coincides with the expression of its regulatory target(s). It was decided, therefore, to use a proteomic approach to continue the search for the regulatory target or targets of BipA in protein samples prepared from lag phase cultures of a *bipA* null mutant and its parent strain. For the purposes of this study it was decided to use a *bipA* deletion mutant of *S.* Typhimurium SL1344 and its parent strain. However, it is likely that many of the results obtained with this bacterial pathogen can be extrapolated to *E. coli* in view of the generally high degree of DNA sequence conservation between the two bacterial species.

As a pre-requisite the growth of the two strains was measured over time to determine the length of lag phase under the experimental conditions used, and to decide when to harvest the cells for proteomic analysis. Protein extracts from the cells were then analysed using the iTRAQ strategy (Ross *et al.*, 2004). This non-gel based approach to proteomics provides quantitative data by measuring the relative amounts of specific reporter ions that are released from peptides tagged with isobaric reagents. Further details are given in chapter Two.

7.2 The lag phase in SL1344 and SL1344Δ*bipA* cells

To determine the extent of the lag phase in SL1344 and SL1344Δ*bipA* cells, overnight cultures were inoculated (at a 1 in 50 dilution) into pre-warmed LB medium and grown with shaking at 37°C. Cell growth was then monitored by measuring the optical density (600 nm) of the cultures every three minutes.

For both cultures the optical density increased only gradually for the first twelve minutes after inoculation. The growth rate then increased exponentially as the cells entered the acceleration phase of growth. It was therefore decided to harvest the SL1344 and SL1344Δ*bipA* cells after 6 minutes of growth to ensure that the cultures were in the lag phase.

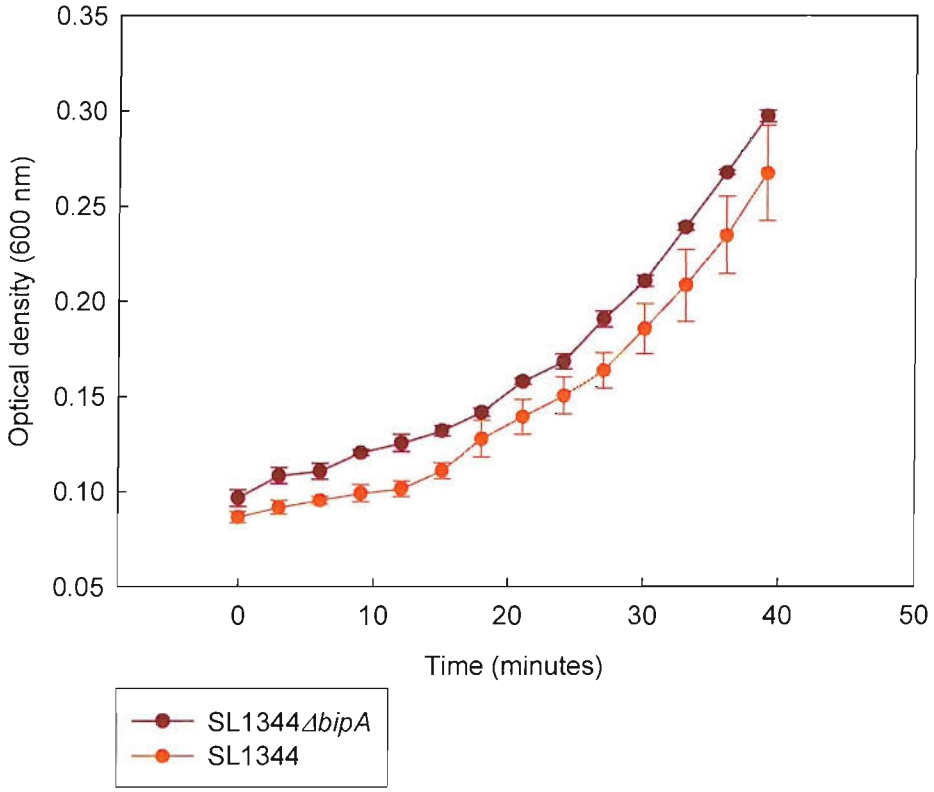


Figure 7.1. Determination of the lag phase for SL1344 and SL1344Δ*bipA* cultures. Overnight cultures of SL1344 and SL1344Δ*bipA* were diluted one in fifty in pre-warmed LB medium and grown at 37°C with shaking. The growth of the cultures was monitored every three minutes by measuring the optical density (600 nm). The results shown are the mean averages of three replicate experiments.

7.3 Lag phase SL1344 and SL1344Δ*bipA* protein samples

Protein samples prepared from lag phase cells of SL1344 and SL1344Δ*bipA* were examined by SDS-PAGE to compare the expression patterns and verify the results of the BCA assays used for measuring protein concentrations.

No significant differences were detected between the two samples (Figure 7.2) although this may be due to the relative insensitivity of SDS-PAGE. It was therefore decided to use iTRAQ analysis to further compare the expression patterns.

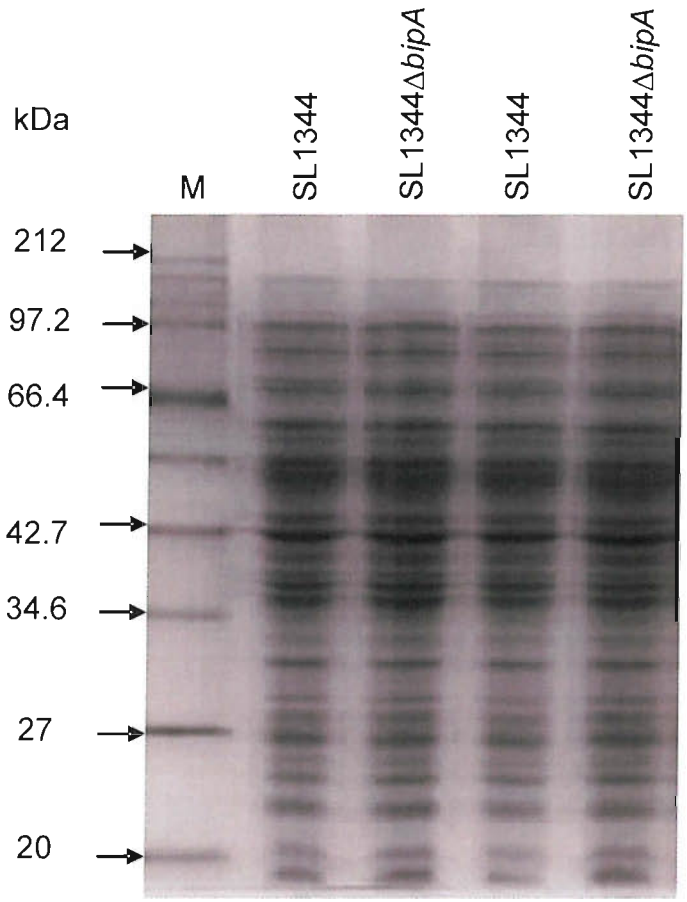


Figure 7.2. SDS-PAGE analysis of lag phase SL1344 and SL1344Δ*bipA* protein samples. Protein samples were prepared by diluting overnight cultures of SL1344 and SL1344Δ*bipA* one in fifty into pre-warmed LB medium, growing the cells for 6 minutes, harvesting the cells and then centrifugation and sonication. The protein samples were then analysed by SDS-PAGE. The figure shows the protein expression profiles obtained from two batches of SL1344 and SL1344Δ*bipA* cells.

7.4 iTRAQ analysis of SL1344 and SL1344Δ*bipA* lag phase protein samples

The lag phase protein samples were reduced, alkylated and treated with trypsin in order to produce tryptic peptides. The latter were then labelled with isobaric tags, combined and subjected to strong cation exchange fractionation to reduce the complexity of the sample. Finally, tandem mass spectrophotometry was carried out using a Q–Tof Global Ultima instrument (Waters) fitted with a NanolockSpray™ source, to achieve better than 10 ppm mass accuracy for the precursor ions.

Table 7.1. Proteins identified from SL1344 and SL1344Δ*bipA* cells in lag phase which are involved in cell envelope biogenesis[no].

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Soluble lytic murein transglycosylase	75.0	NP_463438	*slt*	5	0.99	0.95	0.28
Putative hydrogenase membrane component precursor	37.5	NP_460044	*ompA*	3	1.15	1.14	0.15
Putative transport protein	81.6	NP_459804	*ybiO*	2	1.44	1.38	0.73

[n] Peptides were only deemed to have been unambiguously identified if at least three consecutive y ions gave the correct sequence and if the intact peptide ion was within 50ppm of the predicted mass, this was calculated automatically using the MassLynx 4.0 software (Waters).

[o] The Mascot software (Matrix Science) was used to median normalise the peptide data and weight the protein quantitation wherever possible. The

normalised data was then used to determine the average correction factor and this was applied to the proteins which were not successfully identified and quantitated using the Mascot software.

Table 7.2. Cell motility proteins identified from lag phase SL1344 and SL1344Δ*bipA* cells.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Methyl accepting chemotaxis protein II	59.6	NP_460876	*cheM*	1	1.76	1.69	0.29
Chemotaxis docking protein	18.0	NP_460877	*cheW*	2	1.28	1.23	0.24
Flagellar biosynthesis protein	52.5	NP_461698	*fljB*	1	2.04	1.96	0.40
Putative fimbrial usher protein	88.3	NP_463428	*stjB*	1	1.21	1.16	0.21
Putative methyl accepting chemotaxis protein	58.9	NP_462067	1254675	1	1.20	1.15	0.30

Table 7.3. Cold shock involved protein identified from SL1344 and SL1344Δ*bipA* cells in lag phase.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Major cold shock protein	7.4	NP_462550	*cspA*	1	1.23	1.18	0.30

Table 7.4. Protein which functions in conjugative transfer identified from lag phase SL1344 and SL1344Δ*bipA* cells.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Putative solute DNA competence effector	25.4	NP_460802	*proQ*	1	1.08	1.04	0.04

Table 7.5. Proteins involved in DNA transactions identified from SL1344 and SL1344Δ*bipA* cells in lag phase.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
DNA polymerase III subunits gamma and tau	70.6	NP_459479	*dnaX*	2	1.21	1.16	0.22
DNA binding protein HLP II	15.5	NP_460710	*hns*	2	1.23	1.18	0.16
DNA binding protein HU alpha	9.5	NP_463039	*hupA*	2	1.64	1.69	0.39
DNA binding protein HU beta	9.2	NP_459447	*hupB*	4	1.21	1.33	0.12
Integration host factor alpha subunit	11.4	NP_460305	*himA*	2	1.05	1.16	0.26
Excinuclease ABC subunit B	76.1	NP_459776	*uvrB*	2	1.07	1.03	0.25
Transcription elongation factor NusA	55.4	NP_462200	*nusA*	2	0.93	0.9	0.33

Table 7.6. Metabolic proteins identified from lag phase SL1344 and SL1344Δ*bipA* cells.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Transaldolase	35.1	NP_459012	*talB*	3	2.04	1.96	0.40
Riboflavin synthase subunit beta	16.0	NP_459412	*ribH*	1	1.50	1.44	0.34
Triosephosphate isomerase	26.9	NP_462962	*tpiA*	1	1.24	1.19	0.25
Phosphoglycerate kinase	41.1	NP_461985	*pgK*	3	1.33	1.28	0.23
Pyruvate kinase	50.6	NP_460343	*pykF*	4	1.62	1.55	0.57
Phosphoglyceromutase	28.5	NP_459751	*gpmA*	1	1.08	1.04	0.05
NAD synthetase	30.5	NP_460276	*nadE*	2	1.28	1.23	0.24
ATP synthase subunit A	55.1	NP_462766	*atpA*	3	1.23	1.22	0.25
Malate dehydrogenase	32.5	NP_462269	*mbh*	1	1.23	1.18	0.07
PEP protein phosphotransferase	63.3	NP_461367	*ptsI*	1	1.16	1.11	0.01
isocitrate dehydrogenase	45.8	NP_460208	*icdA*	2	1.33	1.28	0.04
Purine nucleoside phosphorylase	26.0	NP_463426	*deoD*	1	1.27	1.22	0.42
Oxidoreductase	31.4	NP_462072	*yghA*	1	1.11	1.06	0.47
Glycerol kinase	56.0	NP_462967	*glpK*	7	1.28	1.47	0.34
Cystathionine gamma synthase	5.1	NP_462526	1255148	1	1.27	1.22	0.30
Galactose 1 phosphate uridylyltransferase	39.7	NP_459754	*galT*	1	1.16	1.11	0.53
Pyruvate dehydrogenase E1 component	99.5	NP_459157	*aceE*	3	1.22	1.17	0.18
Carbamoyl phosphate synthase large subunit	118.1	NP_459072	*carB*	5	1.08	1.04	0.30
Glyceraldehyde 3 phosphate dehydrogenase	35.6	NP_460256	*gapA*	2	1.38	1.32	0.45
Dihydrolipoamide acetyltransferase	66.1	NP_459158	*aceF*	4	1.14	1.09	0.17
Dihydrolipoamide dehydrogenase	50.6	NP_459159	*lpdA*	3	0.98	0.94	0.10
Aconitate hydratase	97.4	NP_460671	*acnA*	2	1.45	1.39	0.23
sn glycerol 3 phosphate dehydrogenase	56.9	NP_462428	*glpD*	5	1.37	1.21	0.28
sn glycerol 3 phosphate	44.1	NP_461228	*glpC*	3	1.09	1.04	0.42

dehydrogenase K small subunit							
Putative glutamic dehydrogenase like protein	48.0	NP_460751	125331 4	1	1.18	1.13	0.21

Table 7.7. Protein folding factors identified from SL1344 and SL1344Δ*bipA* cells in lag phase.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Trigger factor	48.0	NP_459443	*tig*	3	1.47	1.13	0.68
Molecular chaperone DnaK	69.2	NP_459017	*dnaK*	2	1.55	1.49	0.18
Chaperonin GroEL	57.2	NP_463194	*groEL*	5	1.45	1.61	0.23
Co chaperonin GroES	10.3	NP_463193	*groES*	1	1.28	1.23	0.24
ATP dependent protease	95.4	NP_461591	*clpB*	5	1.32	1.48	0.30

Table 7.8. Protein involved in transcription identified from lag phase SL1344 and SL1344Δ*bipA* cells.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
DNA directed RNA polymerase beta subunit	15.5	NP_463023	*rpoC*	6	1.24	1.19	0.21

Table 7.9. Proteins which function in translation identified from SL1344 and SL1344Δ*bipA* cells in lag phase.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Elongation factor Ts	30.3	NP_459222	*tsf*	4	1.07	1.00	0.29
Elongation factor Tu	43.3	NP_463015	*tuf*	7	1.25	1.36	0.21
Leucyl tRNA synthetase	96.9	NP_459640	*leuS*	2	1.32	1.27	0.00
30S ribosomal protein S13	13.2	NP_462322	*rpsM*	1	0.86	0.82	0.16
50S ribosomal protein L7 L12	12.3	NP_463021	*rplL*	3	1.22	1.14	0.36
50S ribosomal protein L3	22.2	NP_462344	*rplC*	2	1.11	0.97	0.54
30S ribosomal protein S1	61.1	NP_459956	*rpsA*	1	0.97	0.93	0.20
50S ribosomal protein L28	9.0	NP_462628	*rpmB*	1	0.99	0.95	0.23
50S ribosomal protein L5	20.3	NP_462332	*rplE*	1	1.23	1.18	0.49
50S ribosomal protein L22	12.2	NP_462339	*rplV*	3	1.68	1.25	0.16
30S ribosomal protein S10	11.8	NP_462345	*rpsJ*	2	1.03	0.81	0.61
30S ribosomal protein S7	17.6	NP_462351	*rpsG*	1	0.94	0.9	1.02
50S ribosomal protein L2	29.8	NP_462341	*rplB*	2	1.02	0.98	0.20
50S ribosomal protein L9	15.8	NP_463255	*rplI*	2	1.40	1.34	0.48
50S ribosomal protein L10	17.8	NP_463020	*rplJ*	3	1.30	0.94	0.46
50S ribosomal protein L4	22.1	NP_462343	*rplD*	3	1.18	0.76	0.33
30S ribosomal protein S17	9.7	NP_462335	*rpsQ*	2	1.03	0.99	0.33

Table 7.10. Signal transduction proteins identified from lag phase SL1344 and SL1344Δ*bipA* cells.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Response regulator	25.6	NP_460201	*phoP*	1	1.10	1.05	0.40
Response regulator	26.4	NP_462724	*torR*	1	1.05	1.12	0.17

Table 7.11. Proteins of unknown function identified from SL1344 and SL1344Δ*bipA* cells in lag phase.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Putative cytoplasmic protein	32.1	NP_462497	*yhiR*	1	1.05	1.01	0.47
Putative regulatory protein	23.2	NP_459846	1252388	1	1.00	0.96	0.04
Putative formate acetyltransferase	14.3	NP_461581	*yfiD*	2	1.25	1.2	0.25
GTP binding protein EngA	54.9	NP_461454	*engA*	2	1.21	1.16	0.26
Putative inner membrane protein	28.7	NP_463362	1256029	1	1.44	1.38	0.38
Putative cytoplasmic protein	6.5	NP_460765	1253328	1	1.43	1.37	0.93
Putative thiol alkyl hydroperoxide reductase	22.3	NP_459397	1251921	2	1.25	1.2	0.35
Hyperosmotically inducible periplasmic protein	21.4	NP_463417	*osmY*	2	1.09	1.11	0.31
Putative permease	25.9	NP_459785	*ybhL*	1	1.44	1.47	0.14
Putative translation initiation inhibitor	13.6	NP_463318	*yjgF*	2	1.17	1.12	0.13

Table 7.12. Proteins which function in *Salmonella* virulence identified from lag phase SL1344 and SL1344Δ*bipA* cells.

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Number of peptides identified	Average ratio wild type to *bipA* null mutant samples	Normalised average ratio wild type to *bipA* null mutant samples	Standard Deviation
Translocation machinery component	43.0	NP_461805	*sipC*	2	2.96	2.84	0.85
Secreted effector protein	61.9	NP_460064	*sopB*	2	1.31	1.26	0.09

The proteins identified were grouped according to their cellular functions. The data suggests that several proteins may be down-regulated in lag phase cells of the *bipA* null mutant. However the fold-changes were marginal. One of the apparently down-regulated proteins is a putative transporter encoded by *ybiO* which functions in cell envelope biogenesis. However, the fold-change result has a large standard deviation which may indicate that it is not significant.

Five proteins that function in cell motility all appear to be expressed at a reduced level in the *bipA* null mutant. However, the small reduction in expression and large standard deviations associated with three of these proteins suggests that these results may not be statistically significant. The two other proteins (methyl accepting chemotaxis protein II, CheM, and the flagellar biosynthesis protein, FljB), show a greater reduction in expression in the *bipA* null mutant. Collectively, therefore, the flagellar proteins may require BipA for maximal expression. The major cold shock protein CspA also showed a small decrease in expression in the *bipA* null mutant strain but again there was a large standard deviation which means it is unlikely to be significant.

Seven of the identified proteins function in DNA transactions. Three of these showed no statistically significant difference in expression between the two strains. The other four proteins showed a small reduction in expression in the *bipA* null mutant but had large standard deviations, which cast doubt on the significance of these results.

Twenty six of the identified proteins function as metabolic enzymes. Of the twelve that were expressed at a reduced level in the *bipA* null mutant, most had large standard deviations once again indicating that these results can only be viewed as tentative. Only four of the identified metabolic enzymes showed a significant reduction in expression in the *bipA* null mutant strain (transaldolase, malate dehydrogenase, isocitrate dehydrogenase and aconitate hydratase). The first of these enzymes functions in the pentose phosphate pathway while the others are involved in the tricarboxylic acid cycle. The findings therefore suggest that the absence of BipA affects some key central metabolic processes, albeit in a minor way.

Four of the five proteins identified which are involved in protein folding were expressed at a reduced level in the *bipA* null mutant. However, the small reduction in expression one of these proteins showed does not appear to be significant. The three remaining proteins, however, showed a significant reduction in expression in the absence of *bipA*. One of these proteins, DnaK, is responsible for the folding of 5% to 10% of the total cytoplasmic proteins in *E. coli* at 30°C. 10% to 15% of the total cytoplasmic proteins utilise GroEL for folding in *E. coli* cells under normal growth conditions (Houry, 2001).

Seventeen of the identified proteins are involved in translation. However, only two of these showed a modest decrease in expression in the *bipA* null mutant. The proteins in question (leucyl tRNA synthetase, 50S Ribosomal protein L22) are believed to be growth rate regulated in a similar manner to the other identified components (Dennis *et al.*, 2004;Paul *et al.*, 2004b;Paul *et al.*, 2004a). Thus it is difficult to rationalise these preliminary results.

None of the proteins identified that are involved in signal transduction showed a significant difference in expression between the two strains. Interestingly, however, two proteins involved in *Salmonella* virulence were both expressed at a significantly reduced level in the *bipA* null mutant. These proteins, translocation machinery component SipC and secreted effector protein SopB, respectively are part of the *Salmonella* pathogenicity island 1 type III secretion apparatus and a component exported via this system. Thus, it might be of interest to explore the involvement of BipA in the expression of this virulence system in further detail.

Table 7.13. Proteins identified which are encoded by the 90kb *S.* Typhimurium virulence plasmid pSLT (Sanderson *et al.*, 1995).

Protein name	Molecular Weight (kDa)	NCBI accession code	Gene/ Gene ID	Function	Average ratio test to control samples	Standard Deviation
Putative outer membrane protein	30.6	NP_490506	*orf6*	Cell envelope biogenesis	0.86	0.04
Putative inner membrane protein	28.4	NP_490550	1256176	Cell envelope biogenesis	0.89	0.08
Putative outer membrane protein	33.0	NP_490502	*srgB*	Cell envelope biogenesis	0.73	0.08
Putative inner membrane protein	16.8	NP_490523	1256205	Cell envelope biogenesis	0.82	0.04
Putative outer membrane protein	5.1	NP_490499	1256232	Cell envelope biogenesis	1.01	0.11
Entry exclusion protein	18.7	NP_490586	*traS*	Conjugative transfer	0.89	0.04
DNA binding protein	12.9	NP_490562	*traY*	Conjugative transfer	1.15	0.09
Pilus assembly protein	13.8	NP_490574	*trbI*	Conjugative transfer	0.87	0.13
oriT nickase helicase	191.6	NP_490592	*traI*	Conjugative transfer	0.94	0.05
ATP binding protein	100.3	NP_490573	*traC*	Conjugative transfer	1.06	0.08
Mating pair stabilization and pilus assembly protein	102.6	NP_490585	*traG*	Conjugative transfer	1.05	0.06
Mating pair stabilization protein	65.7	NP_490579	*traN*	Conjugative transfer	0.91	0.08
Pilus assembly protein	50.3	NP_490567	*traB*	Conjugative transfer	1.15	0.22

Pilus assembly protein	50.4	NP_490584	*traH*	Conjugative transfer	1.07	0.12
Mating signal protein	14.2	NP_490560	*traM*	Conjugative transfer	1.13	0.21
Pilus assembly protein	23.4	NP_490578	*trbc*	Conjugative transfer	1.12	0.07
OrfF	11.8	NP_490577	1256146	Conjugative transfer	1.74	0.20
ATP-binding protein/DNA transporter	84.5	NP_490588	*traD*	Conjugative transfer	1.02	0.06
finP binding protein	21.0	NP_490594	*finO*	Conjugative transfer	0.98	0.14
Pilus assembly protein	23.7	NP_490575	*traW*	Conjugative transfer	0.98	0.06
Pilin subunit acetylation	27.0	NP_490593	*traX*	Conjugative transfer	1.15	0.25
Conjugative transfer protein	9.1	NP_490569	*trbD*	Conjugative transfer	0.88	0.13
Putative transposase	9.7	NP_490526	1256203	DNA transactions	1.03	0.15
Resolvase	28.6	NP_490521	*rsdB*	DNA transactions	1.01	0.06
Putative cytoplasmic protein	16.7	NP_490551	1256173	DNA transactions	1.31	0.16
DNA replication protein	37.4	NP_490513	*repA2*	DNA transactions	1.09	0.09
DNA polymerase IV	47.6	NP_490544	*samB*	DNA transactions	1.11	0.21
Alpha-helical coiled-coil protein	41.3	NP_490538	*tlpA*	DNA transactions	0.82	0.04
Putative integrase protein	40.1	NP_490532	1256195	DNA transactions	1.04	0.03
Putative resolvase	20.5	NP_490535	1256192	DNA transactions	1.19	0.08
Single-strand DNA binding protein	18.7	NP_490554	*ssbB*	DNA transactions	0.98	0.14
Putative integrase protein	63.6	NP_490534	*rlgA*	DNA transactions	1.43	0.18
DNA replication protein	33.9	NP_490498	*repA*	DNA transactions	0.89	0.03

Putative adenine-specific DNA methylase	25.1	NP_490548	1256178	DNA transactions	0.80	0.11
Putative thiol-disulfide isomerase or thioredoxin	24.1	NP_490503	*srgA*	Metabolism	1.38	0.15
Putative carbonic anhydrase	26.5	NP_490536	1256193	Metabolism	1.11	0.08
Type II secretion system protein	38.3	NP_490533	1256196	Secretion	1.06	0.05
Usher protein	86.2	NP_490509	*pefC*	Secretion	1.07	0.10
Chaperone	25.4	NP_490508	*pefD*	Secretion	1.00	0.21
Putative ParB-like nuclease	72.9	NP_490556	1256169	Signal transduction	1.21	0.34
Plasmid partition protein B	36.7	NP_490543	*parB*	Signal transduction	0.89	0.20
Putative regulatory protein	29.1	NP_490500	*srgC*	Signal transduction	1.01	0.04
Putative cytoplasmic protein	16.0	NP_490552	1256174	Unknown	1.31	0.06
Hypothetical protein PSLT032. 1N	19.1	NP_861530	1256208	Unknown	0.91	0.27
Putative cytoplasmic protein	19.1	NP_490537	1256191	Unknown	1.06	0.15
Putative cytoplasmic protein	11.2	NP_490515	1256215	Unknown	0.98	0.12
Putative cytoplasmic protein	35.2	NP_490541	1256185	Unknown	0.79	0.16
Hypothetical protein PSLT050. 2N	19.3	NP_861532	1256188	Unknown	1.17	0.06
Putative phospholipase D	15.7	NP_490494	1256237	Unknown	1.16	0.26
Hydrophilic protein	65.3	NP_490529	*spvB*	Virulence	1.06	0.01
Hydrophilic protein	24.7	NP_490527	*spvD*	Virulence	1.24	0.23
Hydrophilic protein	27.5	NP_490528	*spvC*	Virulence	0.86	0.03
Regulator of the *spv* operon	33.7	NP_490531	*spvR*	Virulence	1.05	0.21

The majority of the detected proteins encoded by the pSLT virulence plasmid were expressed at similar levels in the *bipA* null mutant and its parent strain. For example, the five identified proteins that function in cell envelope biogenesis showed no real expression differences, although, a putative outer membrane protein, SrgB, was expressed at a slightly lower level in the *bipA* null mutant. Seventeen of the identified pSLT-encoded proteins function in conjugative transfer, and in sixteen of these cases there was no significant difference in expression between the two strains. Only OrfF was shown to be expressed at a significantly higher level in the *bipA* null mutant.

Twelve pSLT encoded proteins were identified that function in DNA transactions. Once again, the majority of these proteins showed no significant difference in expression between the two strains. Only two of these proteins, a putative cytoplasmic protein and a putative integrase protein were expressed at a slightly higher level in the *bipA* null mutant, suggesting that they are mildly down-regulated by BipA in wild type cells. Two metabolic enzymes were identified that are encoded by pSLT. One of these enzymes, a putative thiol-disulfide isomerase, was expressed at a slightly higher level in the *bipA* null mutant. None of the other proteins specified by the pSLT plasmid that were identified displayed any noteworthy differences in expression.

7.5 Discussion

In view of the results described in chapters five and six, together with the pleiotropic phenotype of null mutants of *bipA*, it was of interest to search for new targets that are directly or indirectly controlled by BipA. Protein samples from lag phase cells of SL1344 and SL1344Δ*bipA* were analysed by iTRAQ. In all a total of 212 proteins were identified via detection of the peptides derived from them. Two of these proteins were previously designated as hypothetical because they had only been found by computer analysis of DNA sequence data. The fact that peptides derived from them were detected experimentally confirms their existence.

The majority of the identified proteins showed no significant difference in their expression level in the *bipA* null mutant in comparison to its parent strain. Certain proteins, however, were expressed at a significantly altered level in the *bipA* null mutant, although this data should be viewed as preliminary. Further experimentation must be carried out to monitor the level of transcription and translation of each of these potential target genes to confirm the tentative conclusions described here.

Despite the roles for BipA at the cell surface of *E. coli* cells previously described in capsule formation (Rowe *et al.*, 2000) and LPS biosynthesis (Moller *et al.*, 2003) there was generally no significant difference in the expression of the proteins involved in cell envelope biogenesis. However, only a small number of these proteins were identified in this study, which means that it remains possible that BipA may regulate cell envelope proteins not identified here.

In chapter 3 of this study the role of BipA in growth at low temperatures was discussed the and cold sensitivity of a *bipA* null mutant of *E. coli* K-12 was confirmed. This data was in agreement with previous studies which established

that *bipA* is essential for growth at low temperatures in *E. coli* K-12 and the enteropathogenic *E. coli* strain E2348/69 (Grant *et al.*, 2001;Pfennig and Flower, 2001). However, it has also been found that BipA was not essential for growth at low temperatures in *S.* Typhimurium or *S.* Enteritidis (A. Grant; Ph.D. thesis (2001), University of Southampton). In keeping with these findings, the cold responsive protein CspA, which aids adaptation and survival at low temperatures (Horton *et al.*, 2000) and is induced upon nutrient up shift at 37°C (Yamanaka and Inouye, 2001), was shown in this study to be expressed at similar levels in the *bipA* null mutant and the parent strain of *S.* Typhimurium.

The role of BipA in the regulation of cell motility was also discussed in chapter 3 of this study. It has been previously found that BipA negatively regulates cell motility in the enteropathogenic *E. coli* strain MAR001 (Farris *et al.*, 1998) whereas it positively regulates cell motility in *E. coli* K-12, *Salmonella* Typhimurium and *Salmonella* Enteritidis (A. Grant; Ph.D. thesis (2001), University of Southampton). In agreement with this data it was found that two proteins which are involved in cell motility show a significant reduction in expression in the *bipA* null mutant strain. The proteins in question are methyl accepting chemotaxis protein II (encoded by *cheM*) and the flagellar biosynthesis protein (encoded by *fljB*). FljB is a late gene transcribed from stage 3 promoters of the flagellar/chemotaxis systems.

The methyl accepting chemotaxis proteins are transmembrane proteins which act as receptors for specific attractant or repellent stimuli and in response to those stimuli mediate a response that changes the behaviour of the bacterial cell to promote either smooth swimming or tumbling (Alexander and Zhulin, 2007;Stock and Surette, 1996;Szurmant and Ordal, 2004). A reduction in the expression of a methyl accepting chemotaxis protein can reduce the chemotactic response of a microorganism (Slocum and Parkinson, 1985).

The flagellar biosynthesis protein, FljB, is part of the flagella regulatory network. The genes in the flagellar and chemotaxis regulon are divided into three temporal classes, early, middle and late genes and successful flagella synthesis requires the coordinated synthesis of all three sets of genes (Chilcott and Huges, 2000). The *fljB* is one of the late genes in the flagellar and chemotaxis regulon, which form the external filament (propeller) that is essential for cell motility (Chilcott and Huges, 2000).

A reduction in the expression of both these genes in the absence of BipA is likely to impair flagella-mediated cell motility. This is consistent with previous observations of BipA's positive regulation of cell motility in *Salmonella* Typhimurium (A. Grant; Ph.D. thesis (2001), University of Southampton). To examine the contribution of the reduction in methyl accepting chemotaxis protein II (encoded by *cheM*) to the impaired motility of *bipA* null mutant cells, cell motility could be measured on swam plates containing different attractants (including aspartate) to determine whether the defect shows specificity (Slocum and Parkinson, 1985). However, to check the contribution of the reduction in FljB to the impaired motility of *bipA* null mutant cells the expression levels of *fljB* mRNA could be examined. In addition, it may be of interest to measure the number of flagellar filaments per cell for *bipA* null mutants and their parent strains.

The present results also tentatively suggest that BipA regulates cellular metabolism by increasing the expression of certain metabolic enzymes. The expression of four metabolic enzymes (transaldolase, malate dehydrogenase, isocitrate dehydrogenase and aconitase hydratase) was impaired in the absence of BipA. The transaldolase enzyme maintains the balance of metabolites in the pentose phosphate pathway. The other metabolic enzymes, malate dehydrogenase, isocitrate dehydrogenase and aconitase hydratase all act in the tricarboxylic acid cycle. Clearly the efficient conversion of absorbed nutrients to energy is an essential cell function in all biological systems (Luo *et*

al., 2007) and is expected to be especially crucial in lag phase cells. Therefore, it may be of interest to carry out metabolomic studies on *bipA* null mutants and their parent strains (Luo *et al.*, 2007), to determine the effect of the deletion of *bipA* on metabolism and energy production.

This study also suggested that BipA may regulate the cell's ability to successfully fold proteins because the molecular chaperones DnaK, GroEL and the ATP-dependent protease encoded by *clpB* were found to be expressed at a lower level in the *bipA* null mutant. Protein folding is an important mechanism for the control of gene expression (chapter one, Figure 1.17) which is used in every organism that has been studied (Buchner, 2002;Dougan *et al.*, 2002;Mayer and Bukau, 2005). Molecular chaperones are required for growth under both normal and stress conditions (Bukau and Walker, 1989;Duguay and Silhavy, 2004;Mayer and Bukau, 2005). Therefore, it may be of interest to test the sensitivity of *bipA* null mutants and their parent strains to heat shock.

BipA has been previously shown to regulate virulence determinants in *Escherichia coli* (Farris *et al.*, 1998;Grant *et al.*, 2003;Rowe *et al.*, 2000). The data from this study indicates that BipA may also regulate virulence in *Salmonella* Typhimurium as the translocation machinery component SipC and the secreted effector protein SopB appear to be expressed at a lower level in the *bipA* null mutant. SipC and SopB are part of the type three secretion system, encoded on *Salmonella* pathogenicity island 1, which *Salmonella* cells use to promote their uptake into non-phagocytic eukaryotic cells and to initiate intestinal infection (Chang *et al.*, 2005;Hayward and Koronakis, 2002;Miki *et al.*, 2004).Therefore, future studies to test the ability of a SL1344Δ*bipA* to induce cytoskeletal rearrangements should be considered.

Somewhat disappointingly, none of the quantitative differences detected by iTRAQ analysis were dramatic. This is in marked contrast to the immuno-blotting results of Grant *et al.* (2003), obtained with *bipA* null mutants of

enteropathogenic *E. coli*, where it was shown that several major effector proteins encoded on the LEE were barely expressed in the absence of BipA. However, the results are in keeping with the milder phenotypes of BipA mutants of *Salmonella*. It should be noted that even a 1.5 fold difference in the abundance of certain protein components can have a major impact on the physiology of the cell in certain cases, for example, if the affected protein is situated at the top of a regulatory cascade. In conclusion the results presented in this chapter have yielded various candidates for targets of BipA that may be directly or indirectly regulated. Some of these candidates are involved in processes such as central metabolism and protein folding, which have hitherto not been thought to be regulated by BipA.

CHAPTER 8

DISCUSSION

8 Discussion

The control of bacterial gene expression, a subject that was once thought to be relatively simple (Beckwith, 1996;Jacob and Monod, 1961), is becoming extremely complex (Beckwith, 1996;Chang *et al.*, 2006;Drolet, 2006;Jacob and Monod, 1961;Kaberdin and Bläsi, 2006;Kiryu *et al.*, 2005;Kozak, 2005;Lewis, 2005;Rasmussen *et al.*, 2005;Rex *et al.*, 1994;Romby and Springer, 2003;Stock *et al.*, 2000;Stock *et al.*, 1989;Winkler *et al.*, 2003;Yanofsky, 2000). It has now been established that bacteria possess multiple mechanisms and factors (Figure 1.17), which they use to tailor their gene expression to the environmental conditions they encounter (as discussed in chapter one).

This study also concerns the control of bacterial gene expression, but its focus has been the novel regulatory factor, BipA, which since its discovery (Qi *et al.*, 1995) has been shown to be involved in a plethora of cellular processes such as: capsule formation (Rowe *et al.*, 2000), cell motility (Farris *et al.*, 1998), growth at low temperatures (Grant, 2001;Pfennig and Flower, 2001), sensitivity to antimicrobial proteins (Barker *et al.*, 2000), formation of the attaching and effacing lesion (Farris *et al.*, 1998;Grant *et al.*, 2003), various stress responses, and plant-microbe symbiosis (Kiss *et al.*, 2004) (as discussed in chapter one). The striking resemblance of BipA to translational GTPases such as EF-G, coupled with its ability to bind to 70S ribosomes (Owens *et al.*, 2004) strongly suggest that it is unlike the previously identified factors bacteria use to manipulate cellular gene expression (Figure 1.17).

A central aim of this study was to define a component that is directly regulated by BipA, which could be used to elucidate the regulatory mechanism that BipA uses to control diverse cellular processes. Shortly after this study commenced, Fis was identified as the BipA-controlled regulator which up regulates *ler*

transcription and a model proposing that BipA regulates the translation of Fis was put forward (Owens *et al.*, 2004). In view of this research, the initial primary aim of the present study was to further examine the effect of Fis and BipA on cellular gene expression. Accordingly, a phenotypic comparison of *fis* and *bipA* null mutants was carried out (as detailed in chapter three).

8.1 Phenotypic comparison of *fis* and *bipA* null mutants

In keeping with the model proposed by Owens *et al.* (2004), both *bipA* and *fis* null mutants displayed impaired cell motility; this has also been observed previously using other bacterial strains and species (Grant, 2001;Kelly *et al.*, 2004;Osuna *et al.*, 1995). This result suggested that *fis* and *bipA* may participate in the same regulatory cascade affecting cell motility. However, flagella-mediated cell motility is affected by a number of regulatory pathways (Chilcott and Huges, 2000;Szurmant and Ordal, 2004), so it was also possible that Fis and BipA act through different pathways but generate the same observable phenotype.

The phenotypic comparison of *bipA* and *fis* null mutants of *E. coli* was continued by examining their ability to grow at low temperatures. It was found that, although BipA was essential for growth at low temperatures Fis was not, which indicates that BipA regulates at least one process independently of Fis. This finding further suggests that BipA may be a master regulator that controls the expression of multiple genes and that its position in the regulatory hierarchy is above the Fis protein.

It was then considered that a detailed examination of the mode of action used by BipA to regulate Fis would provide insights into the types of mRNAs that are regulated by BipA. In view of the growth inhibition effect observed when cells

lacking BipA are grown at temperatures below 30°C, the effect of aberrant Fis expression on cell growth was examined (as detailed in chapter four).

8.2 Ectopic Fis expression from pRJ807 causes growth inhibition

It was found that the ectopic expression of Fis from pRJ807 caused a dramatic growth inhibition in *E. coli*. This effect was shown to occur consistently when Fis expression from pRJ807 was induced under various different experimental conditions, indicating that the effect was not the result of the experimental conditions chosen. Further studies established that the growth inhibition effect was dependent on Fis expression from pRJ807. To characterise the phenotype further, effects on gene expression and cell shape were examined. These studies revealed that the expression of a gene not regulated by Fis – *lacZ* – was almost totally blocked following ectopic expression of Fis. Further, cells in which pRJ807 was induced were unable to divide and hence became filamented.

It is worth noting that, growth inhibition caused by aberrant expression of Fis has not been previously reported, even though this protein has been the focus of many research groups for many years. Previous studies have established that Fis levels are maximal in lag phase cells (Ball *et al.*, 1992), in which cell division is transiently inhibited (Kepes and D'Ari, 1987;Sloan and Urban, 1976). It is therefore tempting to speculate that high levels of Fis are responsible for the transient block in cell division that occurs in lag phase cells.

One possible mechanism for Fis-mediated growth inhibition is occlusion of DnaA molecules from *oriC*. In other words, when the Fis concentration exceeds a threshold level, it becomes impossible for DnaA to displace it from *oriC*. Alternatively, Fis expression from pRJ807 might affect the level of DnaA

expression. It was therefore decided to examine the level of DnaA protein in cells aberrantly expressing Fis in comparison with non-induced cells.

The level of DnaA showed no significant difference in cells aberrantly expressing Fis in comparison with control cells. Therefore, the growth abolition caused by aberrant Fis expression from pRJ807 is not due to changes in the expression of the initiator of chromosomal replication (Kaguni, 2006;Leonard and Grimwade, 2005). However, the results obtained do not rule out the possibility that high levels of Fis synthesis occlude DnaA from *oriC*. This idea merits further testing as it might provide an explanation for the inhibition of growth observed during the lag phase.

8.3 The reporter gene constructs pJGP1 and pJGP6 do not display the expected structures or properties

Studies with strains carrying the pJGP series of plasmids (described in Owens *et al.,* 2004), produced apparently aberrant results which prompted the investigation of the structure and properties of two representative constructs from this series, pJGP1 and pJGP6 (as discussed in chapter five).

Restriction mapping was used to examine the structures of pJGP1 and pJGP6 in comparison to their parent plasmid pAJG38. It was consistently found that although pAJG38 yielded the fragments predicted for its structure (Grant *et al.*, 2001), pJGP1 and pJGP6 failed to do so. These initial results prompted a more detailed examination of the relationship between pJGP1 and the parent plasmid pAJG38. Restriction mapping of both pJGP1 and its parent plasmid pAJG38 with frequent cutting restriction enzymes failed to confirm that pJGP1 and its parent plasmid pAJG38 were related.

Several critical properties reported for pJGP1 and pJGP6 were then tested. According to Owens *et al.* (2004) an arabinose inducible pBAD promoter controls β-galactosidase expression from pJGP1 and pJGP6. With this in mind, pJGP1 and pJGP6 were transformed into a naturally *lacZ* negative strain (LB5010) and the response of the resultant strains to arabinose addition was assessed using β-galactosidase assays. In contrast to expectations, exposure to arabinose did not induce β-galactosidase expression from pJGP1 and pJGP6. Collectively, these results were in direct contradiction of the structure and properties described for pJGP1 and pJGP6 by Owens *et al.* (2004). One possible explanation was that the strains placed in the lab stock collection might have been mixed up. It was therefore decided to re-construct pJGP1 and pJGP6 and to use the new versions to repeat key experiments described in Owens *et al.* (2004).

The constructs pJGP1 and pJGP6 were re-made and named pMH2 and pMH4, respectively. The structures of the constructs were confirmed using restriction mapping and DNA sequencing. These constructs were then used to test the model put forward by Owens *et al.* (2004), which proposes that BipA enhances the translation of the *fis* mRNA through an interaction with the unusual *fis* 5' untranslated region. pMH2 and pMH4 were placed in a *lacZ* negative strain and its *bipA* null mutant; β-galactosidase expression in the resultant strains with and without induction with L-arabinose was then measured. It was found that the level of β-galactosidase expression of the cultures showed no significant difference in the presence or absence of *bipA*. This indicated that the presence of the *fis* 5' untranslated region bound to the *lacZ* gene (encoded by pMH2) did not make β-galactosidase expression from pMH2 responsive to BipA. In conclusion, the results suggest that the *fis* 5' untranslated region does not encode a BipA response element. This conclusion is a direct contradiction of the results reported by Owens *et al.* (2004).

The model put forward by Owens *et al.* (2004) also proposes that the high level of complementarity between the *fis* 5' untranslated region and the 16S ribosomal RNA causes the hyper-stable binding of the *fis* mRNA to the ribosome in the absence of BipA. If this is true then expression of the *fis* 5' untranslated region in the absence of BipA might cause the sequestration of many or all ribosomes on the mRNA which would inhibit cell growth. It was decided to use pMH2 and pMH4 to test this possibility. Parental and *bipA* null mutant strains carrying pMH2 or pMH4 were cultured with and without induction and their growth rates were compared. In contradiction to the proposed model, there was no significant growth inhibition when the 5' untranslated region of *fis* was expressed in the absence of BipA.

The results presented in chapter five of this thesis conflict with those presented by Owens *et al.* (2004). During other studies, further discrepancies arose that cast doubt on certain western blotting results presented by Owens *et al.* (2004) which meant that it was necessary to re-investigate the level of Fis expression in a *bipA* null mutant in comparison to its parent strain (as discussed in chapter six).

8.4 Fis expression levels show no significant difference in wild type or *bipA* null mutant strains

Immuno-blotting with a Fis-specific antibody was used to compare the level of Fis expression in a *bipA* null mutant and in its parent strain at various time points during the growth of the culture. The level of Fis expression in the *bipA* null mutant showed no significant difference to the level of Fis expression in the parent strain at any time point examined. This result conflicts with similar western blotting experiments described by Owens *et al.* (2004), which indicated that Fis expression was negligible in a *bipA* null mutant relative to its parent strain.

Taken together, the results in chapter five and six of this thesis strongly suggest that it is unsafe to conclude that BipA controls the expression of the global regulatory protein Fis. This implies that a direct target for BipA remains to be identified. Accordingly, further research focused on a search for additional targets of BipA. To achieve this aim, a comparative proteomic analysis of a *bipA* null mutant and its parent strain was undertaken. Cells were taken at the peak of BipA expression, in the lag phase of growth (Owens *et al.*, 2004).

8.5 Comparative proteomic analysis of lag phase cells of SL1344 and SL1344Δ*bipA*

A number of potential targets of BipA were identified in this study. Some of the identified targets are involved in processes that have previously been found to be regulated by BipA, e.g. flagella-mediated cell motility (Farris *et al.*, 1998;Grant *et al.*, 2003). However, the other potential targets that were identified, indicate new roles for BipA notably in central metabolism and in the control of protein folding. However, it should be emphasized that the data is preliminary; further studies will be needed to validate the results.

To establish if any of the potential target genes identified is directly regulated by BipA the level of transcription and translation of each of these genes must be monitored and compared in a *bipA* null mutant and its parent strain. After a direct regulatory target of BipA is identified then the novel mechanism BipA uses to regulate gene expression can be elucidated.

CHAPTER 9

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