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Role Of BipA As A Modulator Of Gene Expression And Ribosome Assembly In Escherichia Coli

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ROLE OF BIPA AS A MODULATOR OF GENE EXPRESSION AND RIBOSOME ASSEMBLY IN *ESCHERICHIA COLI*

by

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This dissertation, submitted by Promisree Choudhury in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

(Chairperson)

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

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Date: May 8, 2014

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table Page

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To my beloved grandparents,

my mom Sadhana and dad Shyamal!

ABSTRACT

In *Escherichia coli,* ribosomes are the basic protein-synthesizing cellular machines. Hence, regulation of ribosome synthesis and assembly as well as regulation of protein translation are very important for bacterial cells. However, the cellular mechanisms of both these processes are not yet completely understood and thus both are active fields of research. It has been suggested that the BipA protein is involved in the regulation of both these processes. The research described here focused on understanding the cellular function(s) of BipA in regulating protein synthesis and ribosome assembly.

BipA is a member of the elongation factor family of translational GTPases and shares protein domain homology to EF-G, EF-Tu and LepA. BipA is not essential for growth but has been proposed to be involved in the regulation of a variety of cellular processes which include protein synthesis, motility, capsule formation, antibiotic resistance, symbiosis, low temperature growth, and pathogenicity. These findings led us to the hypothesis that BipA is involved in regulating expression of target genes, presumably at the translational level. However, BipA also associates with the ribosome and the GTPase activity of BipA is induced in the presence of fully formed ribosomes and high levels of GTP. Additionally the cold-sensitive phenotype of a *bipA* mutant is similar to mutants of ribosome assembly factors. These characteristics of BipA supported an alternate hypothesis that BipA is involved in regulating ribosome assembly and/or biogenesis.

To test our first hypothesis we investigated the role of BipA as a regulator of translation by monitoring the effect of *bipA* deletion on the expression of different genes of the RcsBCD

pathway. The RcsBCD pathway regulates the expression of genes involved in the synthesis of flagella and capsule along with other genes and is regulated via either the RcsA-RcsB complex or via RcsB. Our results suggest that BipA regulates the expression of multiple genes of the Rcs pathway possibly by affecting RcsB expression.

To test our second hypothesis we determined the role of BipA in ribosome assembly, for which we compared the phenotypes of a *bipA* mutant to the phenotypes of a known ribosome assembly factor, DeaD. We also analyzed ribosome profiles and rRNA processing in strains lacking *bipA* and compared that to an isogenic wild-type. Our results suggest that BipA is involved in ribosome assembly, particularly in the biogenesis of the 50S ribosomal subunit.

Our results provide support for both of the starting hypotheses: expression studies indicate that deletion of *bipA* alters expression of multiple genes, and ribosomal profiling demonstrates dependence on BipA for proper assembly. It is possible that BipA facilitates these processes independent of one another, or that interference of one function indirectly leads to disruption of the other. While our results do not define the direct function of BipA, they expand our existing understanding of the protein and highlight the extent of cellular processes affected by BipA.

CHAPTER I

INTRODUCTION

Bacterial cells utilize about 90% of their total cellular energy for protein synthesis (Park et al., 2009), a large portion of which accounts for synthesizing the protein-manufacturing apparatus, the ribosome (Dethlefsen et al., 2007). Ribosomes are ribonucleoprotein complexes responsible for the decoding of mRNA to synthesize proteins. In an actively growing bacterial cell, ribosomes can constitute about 40% of the total dry mass of the cell (Wilson et al., 2007). Unlike the eukaryotic ribosome, which sediment, as an 80S particle, the prokaryotic ribosome sediments as a 70S particle, and is an assemblage of two subunits, the 50S large subunit and the 30S or small subunit. In *Escherichia coli*, the 50S subunit is composed of two types of ribosomal RNAs (rRNA), the 5S (120 nt) and 23S (2,904 nt) rRNA, along with 34 different structural ribosomal proteins (r-proteins), L1 through L36. Each of these proteins is present in single copy with the exception of protein L12 and its N-terminally acetylated derivative L7, which together are present in two copies. The 30S ribosomal subunit is made up of only one type of ribosomal RNA, the 16S (1,542 nt), and 21 ribosomal proteins, S1-S21 (Kaczanowska et al., 2007).

Ribosome Biogenesis

Biogenesis of the bacterial ribosome from its constituent rRNAs and r-proteins is a complicated process and it poses a challenge for cells to coordinate the synthesis of the rRNAs and r-proteins in a well-orchestrated manner to form the functional ribosomal particle. Ribosome biogenesis starts with synthesis of the three ribosomal RNAs, 5S, 16S and 23S, which

are transcribed from seven *rrn* operons, *rrn* A, B, C, D, E, G and H (Kaczanowska et al., 2007). Each of these operons has two promoters, P1 and P2 (Figure 1A). The 16S rRNA gene is located downstream of the P2 promoter followed by a spacer region that consists of tRNA genes. This tRNA spacer is then followed by the 23S and 5S rRNA genes. All seven *E. coli rrn* operons consist of only one copy each of the 16S and 23S rRNA genes, but the numbers of the 5S rRNA and tRNA genes varies. The three rRNAs (23S, 16S and 5S) are synthesized from a single transcript from the same precursor RNA molecule known as the 30S primary transcript (Figure 1B) (Davies et al., 2010). This primary transcript is processed by several endonucleases, most importantly by RNase III, which releases the precursor 23S and precursor 16S rRNAs from the transcript, thus separating the rRNA transcripts from the t-RNAs. The pre-23S rRNA contains three to seven additional nucleotides at its 5′ end and seven to nine additional nucleotides at its 3′ end, compared to the mature 23S rRNA sequence. The pre-16S rRNA, which is also called the 17S rRNA, contains 115 and 33 additional nucleotides at its 5′ and 3′ends respectively (Charollais et al., 2003). The precursor 16S rRNA is further processed by RNases including RNase E and RNase G to form the mature 16S rRNA molecule, while the precursor 23S rRNA is cleaved further by RNase III along with RNase T to form the mature 23S rRNA sequence (Davies et al., 2010).

Modifications of rRNAs predominantly occur post transcriptionally and they mainly consist of methylations and pseudouridylations. Some of these modifications are added to the naked rRNA while some are added after the binding of the r-proteins (Kaczanowska et al., 2007). Little is known about the biological functions of these modifications, but because most of these modifications are located in regions of the rRNA which are involved in the proper structure or functioning of the ribosomes (Brimacombe et al., 1993), it has been suggested that these

modified nucleotides are essential for proper association of the ribosomal subunits during ribosome biogenesis, peptide group transfer during translation and/or facilitating tRNA binding (Green et al., 1996).

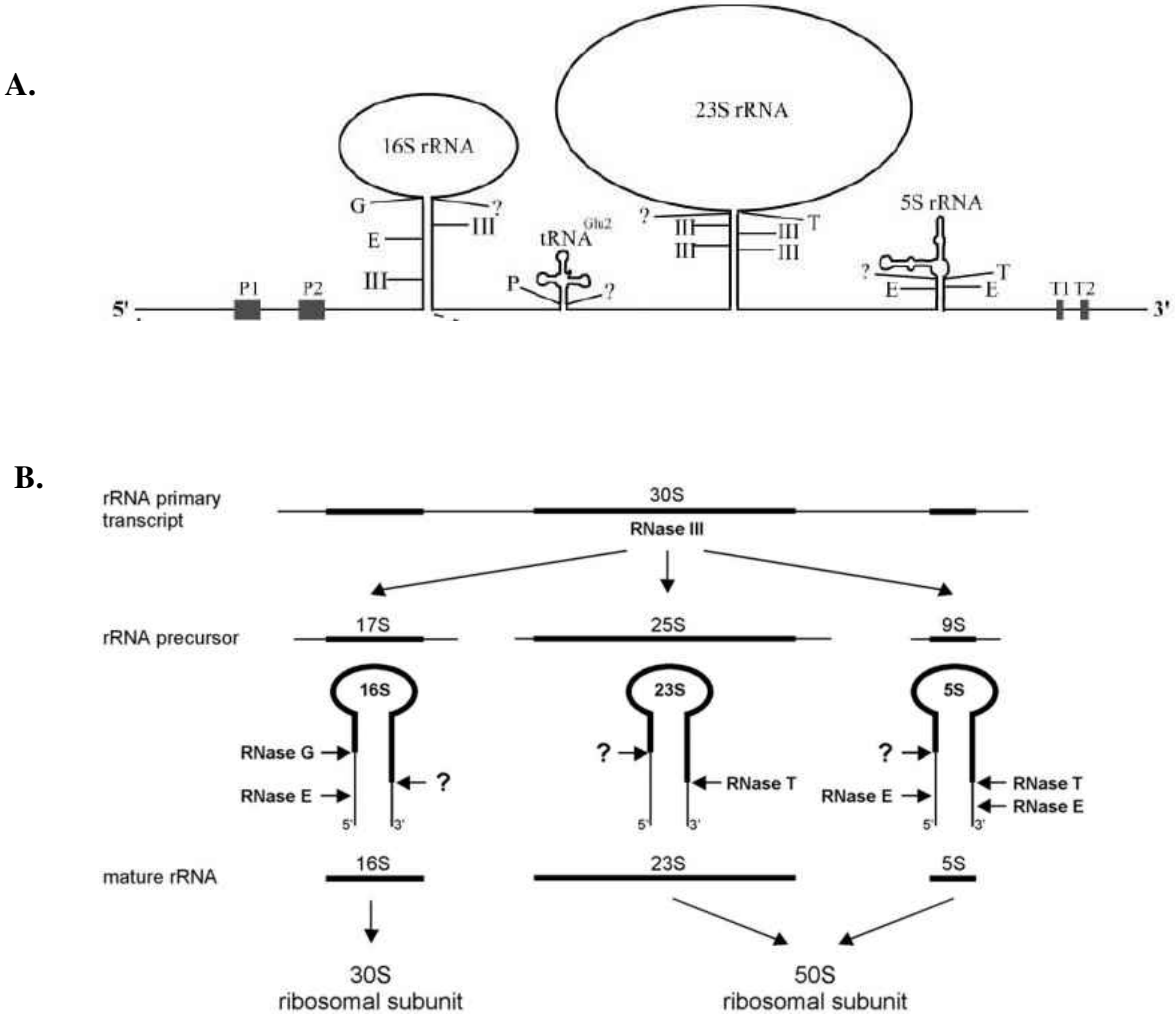


Figure 1: Schematic representation of the *rrnB* operon and RNaseIII processing of the 30S transcript.
(A) *rrnB* operon showing processing sites of the different RNases, P1 and P2 promoters, 16S and 23S rRNAs and tRNAs are indicated [Reprinted with permission from Microbiol. Mol. Biol. Rev., 2007, 71: 477-494, Kaczanowska et al.].
(B) RNase III processing of the 30S transcript. RNase III cleaves the 30S primary transcript to produce the 17S, 25S and 9S rRNAs which are further processed by other RNAses to form the mature 16S, 23S and 5S rRNA respectively. The 16S rRNA is assembled into the small ribosomal subunit whereas the 23S and 5S rRNAs are assembled into the large ribosomal subunit [Reprinted with permission from Molecular Microbiology, 2010, 78: 506–518 Davies et al.].

The ribosomal structural proteins begin binding to the rRNAs before rRNA transcription is completed, but final assembly does not occur until transcription is complete. Indeed, some of these proteins require post-transcriptionally modified rRNA nucleotides in order to bind (Kaczanowska et al., 2007). Binding of the r-proteins takes place in a sequential manner by which certain r-proteins bind directly to the rRNA and serve as the primary proteins that coordinate binding of the secondary r-proteins (Figure 2). Binding of the secondary r-proteins facilitates binding of the tertiary r-proteins to complete the assembly of the ribosomes. The *in vivo* assembly of the bacterial ribosome takes 2-3 minutes at 37°C, but reconstitution of the ribosome from its various components is much more complicated to achieve *in vitro* (Kaczanowska et al., 2007). It has been postulated that *in vivo* assembly of the ribosomal subunits utilizes accessory ribosome binding factors which play important roles in the assembly process but have not been fully identified and characterized (Britton et al., 2009).

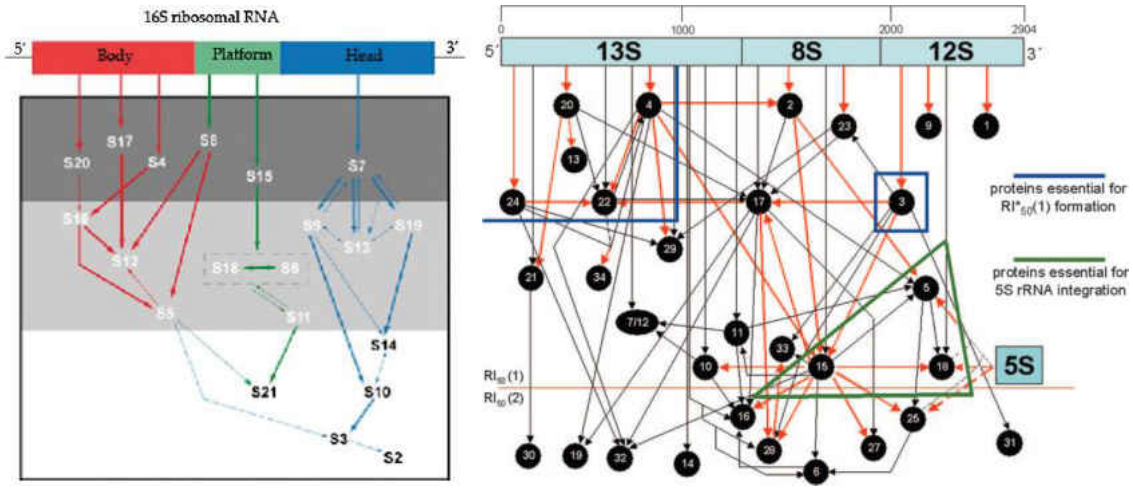


Figure 2: Assembly maps of the 30S and 50S ribosomal subunits: 30S assembly map (left) [Reprinted with permission from RNA, 2010, 16: 1990-2001, Xu et al.] and 50S assembly map (right) [Reprinted with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Protein Synthesis and Ribosome Structure: Translating the Genome, 2006, Chapter 3: 85-143, Nierhaus et al.] depicting the binding order of the primary, secondary and tertiary ribosomal proteins to the rRNA during ribosome biogenesis.

Ribosome-Associated Accessory Factors

Biochemical analyses of premature ribosomal complexes in yeast have revealed as many as 200 additional factors that participate in ribosome assembly (Woolford et al., 2013). On the other hand, fewer numbers of these accessory assembly factors have been shown to be directly involved in ribosome assembly in prokaryotes (Wilson et al., 2007). Most of these accessory ribosome assembly factors, which include RNA chaperones, rRNA modifying enzymes, RNA helicases and ribosome-associated ATPases/GTPases, have been suggested to function late in the assembly process and may perform overlapping functions. Mutations in the genes encoding most of these accessory factors result in defects in ribosome assembly and also impair maturation of the rRNAs (Charollais et al., 2003, 2004). Apart from Era and EngA, most of these auxiliary proteins are dispensable for cell viability and cells do not usually manifest a phenotype when grown under optimal conditions in the absence of these proteins (Wilson et al., 2010).

RNA chaperones: Ribosomal RNA is prone to form random base pairs with any complementary sequence. These incorrectly folded RNAs form very stable secondary structures and generate kinetically trapped intermediates that take time and energy to resolve into their native and functional form. RNA chaperones destabilize these unwanted RNA-RNA interactions, thus freeing the RNA from kinetic traps and assisting the correct folding process. Examples of proteins that have been suggested to have RNA chaperoning activity important for ribosome assembly include IF1and RimN (Kaczanowska et al., 2007).

rRNA modifying enzymes: In addition to the four nucleotides, cellular RNA contains more than 100 post-transcriptional modifications (Cantara et al., 2007). The two major types of rRNA modifications are methylation and pseudouridylation. In *E.coli* ribosomes there are 24

methylations, 10 in the 16S rRNA and 14 in the 23S rRNA, and 11 pseudouridylations, 1 in the 16S rRNA and 10 in the 23S rRNA (Kaczanowska et al., 2007). Most of these modifications are present in the decoding region of the 16S rRNA or in the peptidyl transfer center of the 23S rRNA (Brimacombe et al., 1993) which has led to the suggestion that these modifications are important for providing structural flexibility, for increasing hydrogen bond forming potential, and/or for improving base stacking ability of the rRNAs (Decatur et al., 2002). It is important to note that the ability of the pseudouridines to form an additional hydrogen bond imparts increased structural stability to the rRNAs. In spite of all these hypotheses, the exact function(s) of these modifications are still not understood completely. With the exception of some of these modifications, such as the methylation at G2251 in the 23S rRNA of yeast mitochondrial ribosomes, which is involved in the proper assembly of yeast mitochondrial ribosomes, or certain rRNA methylations which confer antibiotic resistance (Green et al., 1997), most of these modifications are dispensable for cell viability or ribosome function.

Pseudouridines (Ψ), or the C5-glycosidic isomer of uridines (Figure 3), are the most common and evolutionarily conserved modifications present on rRNA (Hamma et al., 2006). The enzymes that catalyze the isomerization of uridines to pseudouridines are known as pseudouridine synthases.

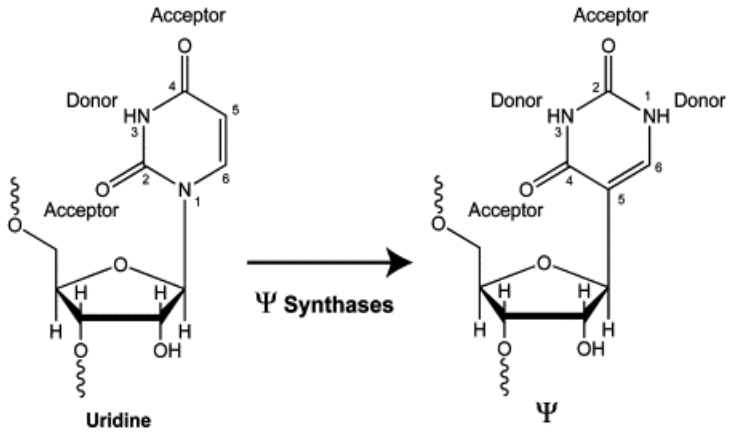


Figure 3: Pseudouridylation: Isomerization of uridine to pseudouridine in which the C-N glycosidic bond at C-1 position of uridine is isomerized to a C-C glycosidic bond at the C-5 position of pseudouridine (Ψ) by the pseudouridine synthase enzymes [Reprinted with permission from Chemistry and Biology, 2006,13: 1125–1135, Hamma et al.]

E. coli has a total of 11 pseudouridine synthases, grouped into five families. The TruA, TruB and TruD families of pseudouridine synthases mediate pseudouridylations in tRNA. The RsuA family is comprised of RsuA, RluB, RluE, and RluF, of which RsuA modifies a single uridine residue in the 16S rRNA and the other three modify uridine residues in the 23S rRNA. The RluA family consists of enzymes RluA, RluC, RluD and TruC. RluC and RluD modify three uridine residues each in the 23S rRNA, RluA modifies one uridine residue in the 23S rRNA and one residue in tRNA, and TruC modifies a single residue in tRNA (Hamma et al., 2006). Most of these pseudouridine synthases are dispensable for cell growth; however deletion of *rluA*, *rluD*, or *truB* manifests certain phenotypes (Raychaudhuri et al., 1998, 1999, Gutgsell et al., 2000). An *rluD* null mutation causes severe growth defects (Gutgsell et al., 2005). *truB* and *rluA* mutants, on the other hand, do not exhibit any growth defect when grown as pure cultures in rich media, but show a selective growth disadvantage when grown in a mixed culture in competition with wild-type strains (Gutgsell et al., 2000, Raychaudhuri et al., 1999).

RNA helicases: RNA molecules have an extremely high tendency to form intra-molecular interactions. Some of these interactions are required for the maturation of the rRNA or for its proper functioning. However, other inter- or intra-molecular RNA interactions can prove to be harmful for the cell (Fuller-Pace, F. V. 1994). RNA molecules thus require assistance from different proteins and/or other nucleic acids to aid in their maturation process. The RNA helicases are a class of RNA binding proteins that prevent single-stranded RNA from forming unnecessary interactions with other single-stranded RNA molecules or binding with other proteins. RNA helicases also assist in the proper folding and arrangement of long RNA molecules into larger macromolecular complexes such as the ribosome (Iost et al., 2006). The DEAD box family represents an important group of RNA helicases suggested to be involved in the process of ribosome biogenesis both in prokaryotes and eukaryotes (Iost et al., 2006, Rocak et al., 2004).

DEAD Box RNA helicases

RNA helicases of the DEAD box family are regarded as important mediators of RNA metabolism. This group of proteins is present in all eukaryotes and most prokaryotes. The name of this protein family is derived from the amino acid sequence D-E-A-D (Asp–Glu–Ala–Asp) (Wilson et al., 2007). The DEAD box family in *E. coli* consists of five members, DeaD, SrmB, DbpA, RhlB and RhlE (Table 1) (Iost et al., 2006). All these proteins are able to dissociate short RNA duplexes in an ATP-dependent manner and, with the exception of DbpA, the four other RNA helicases show no substrate specificity. Apart from RhlB, the DEAD box proteins are ribosome-associated and deletion of at least two of these (*srmB* or *deaD/csdA*) results in a cold-sensitive phenotype. Moreover, SrmB and DeaD are involved in assembly of the large ribosomal subunit at low temperature (Charollais et al., 2003, 2004)). However, over-expression

of either DeaD or SrmB does not suppress the cold-sensitive growth defect of each other suggesting that the function of both is required for correct ribosome assembly (Charollais et al., 2004). RhlB and RhlE have also been implicated as components of the multi-protein RNA degradosome in *E. coli* and *Pseudomonas syringae* respectively (Carpousis, A.J., 2007). Although *in vitro* analyses have revealed that the function of RhlB, RhlE and DeaD can be interchanged in the degradosome, functions of the *E. coli* DeaD box RNA helicases are not interchangeable *in vivo* (Iost et al., 2006).

Helicase	Functions	Helicase Activity	Phenotype of deletion mutant	ATPase activity
DbpA	ribosome biogenesis	Yes	No phenotype	Yes
RhlB	mRNA decay	Yes	No phenotype	Yes
RhlE	unknown	Yes	No phenotype	Yes
SrmB	ribosome assembly	Yes	Cold-sensitive	Yes
CsdA/DeaD	ribosome assembly; mRNA decay	Yes	Cold-sensitive	Yes

Table 1: The *E.coli* DEAD box RNA helicase family: This family consists of five proteins, DbpA, RhlB, RhlE, SrmB and CsdA/DeaD. These proteins are involved in cellular functions including RNA metabolism and ribosome biogenesis. All of these proteins exhibit ATP-dependent helicase activity and deletion of two of the five proteins leads to a cold-sensitive phenotype [Reprinted with permission from Nucleic Acids Research, 2006, 34: 4189-4197, Iost et al.].

DeaD protein: The DeaD protein, also known as CsdA or cold-shock DEAD-box protein A, is an RNA helicase that is involved in the biogenesis of the 50S ribosomal subunit (Charollais et al., 2004). *deaD* mutants are growth defective at cold temperature, accumulate unprocessed 23S rRNA and manifest altered ribosome profiles, with decreased proportions of 70S ribosomes and accumulation of precursors of the 50S subunit (Charollais et al., 2004). The cold-sensitive

growth defect of a *deaD* mutant is alleviated by overexpression of CspA or RNase R and is exacerbated by *rhlE* deletion (Awano et al., 2007). Expression of DeaD is induced by cold shock (Jones et al., 1996) and this protein is involved in the degradation of RNA during low temperature growth (Awano et al., 2007) as well as in unwinding mRNA secondary structures in the translation initiation region, thus facilitating translation of target genes (Butland et al., 2007). DeaD is a ribosome-associated ATPase and it interacts with several r-proteins as well as a pre-50S ribosomal particle (Charollais et al., 2004). In addition to its involvement in ribosome assembly and RNA metabolism, DeaD also affect the formation of coccobacillus morphology in *E. coli* cells at low temperature by an unknown mechanism (Pierce et al., 2011).

Ribosome-associated GTPases: The ribosome-associated GTPases (RA-GTPases) constitute a major component of the accessory ribosome assembly factors. These proteins were initially considered to be members of the Ras superfamily of GTPases, but now they are classified as a separate superfamily of GTPases due to the presence of distinctive protein domains that are absent in the small monomeric GTPases of eukaryotes (Britton, R.A., 2009). These additional protein domains have been suggested to be essential in mediating interactions with rRNAs, r-proteins or both.

Bacterial GTPases are divided into two major classes. The first class is designated as the TRAFAC (translation factors) and includes enzymes involved in translation including initiation, elongation and release factors, and also enzymes involved in signal transduction, cell motility and intracellular transport. The second class of GTPases, called SIMIBI (signal recognition particle, MinD and BioD) consists of the SRP (Signal Recognition Particle) GTPases and MinD-like ATPases, which participate in chromosome partitioning, membrane transport, and protein localization, and other enzymes with kinase and phosphate transferase activities. All the RA-

GTPases belong to the TRAFAC class and they share extensive homology in protein structure between the prokaryotic and eukaryotic world (Leipe et al., 2002). Mutations in genes encoding these GTPases exhibit pleiotropic phenotypes that suggest important relations between the ribosome assembly process and other metabolic processes such as cell cycle, stress responses, cell growth and nutrient availability (Britton, R.A., 2009). With the exception of Era and ObgE, the RA-GTPases are dispensable for growth (Gollop et al., 1991, Hwang et al., 2006). Mutants of some of these ribosome-associated GTPases exhibit a cold-sensitive growth defect when grown at lower temperature (Hwang et al., 2006) and also a defective ribosome assembly (Jiang et al., 2006). The ribosome assembly defects are often characterized as defective biogenesis of the individual 50S and 30S subunits, rather than coupling of the mature subunits. These mutants are sometimes referred to as the "SAD" mutants, or Subunit Assembly Defective mutants and they accumulate ribosome assembly intermediates that show incomplete processing of the rRNAs and altered expression of certain r-proteins (Guthrie et al., 1969).

The ribosome assembly GTPases functions as molecular switches that switch between a GDP-bound state and a GTP-bound state. In most cases it has been found that these enzymes associate with the ribosome in their GTP-bound or ON state (Karbstein, K., 2007). Hydrolysis of the bound GTP leads to the GDP-bound or OFF state of the GTPases and they can no longer bind to the ribosome. These ribosome assembly GTPases have been speculated to act late in the ribosome assembly process (Jiang et al., 2006, Campbell et al., 2008) but their exact contribution in the complex process of ribosome assembly or in modulating any of the other cellular processes still remains under investigation (Britton, R.A., 2009).

The RcsBCD Pathway

The Rcs phosphorelay pathway was first identified by its role in the transcriptional regulation of genes involved in the synthesis of capsular polysaccharides in *E. coli* and was given the name Rcs or Regulator of capsule synthesis (Gottesman et al., 1985). This system has proved to be a complex example of a two-component signal transduction pathway present in bacterial cells. As the name implies, any two-component signal transduction system consists of two major components. The first is a sensor kinase which is required for sensing environmental signals and transmitting the signal in the form of transferring a phosphate group to the response regulator. The response regulator, which is the second component of this system, is generally a DNA-binding protein whose activity in regulation of gene transcription is controlled by its phosphorylation status. The Rcs phosphorelay system is a multicomponent system that plays a critical role in regulating the expression of multiple target genes (Majdalani et al., 2005).

The Phosphorelay Cascade

The sensor kinase of the Rcs phosphorelay system is RcsC, which is capable of autophosphorylation at a conserved histidine residue. The N-terminus of RcsC spans the inner membrane with a significant portion present as a periplasmic domain while the C- terminal cytoplasmic region consists of two major domains (Figure 4). The cytoplasmic domains of RcsC include a sensor kinase domain and an additional domain that resembles a receiver domain (Majdalani et al., 2005). The sensor kinase domain of RcsC undergoes autophosphorylation at the conserved histidine residue in response to an environmental stimulus such as osmotic shock. The phosphate group from the histidine residue in the sensor kinase domain of RcsC is then transferred to an aspartate residue in the receiver domain of the same protein. Then the phosphate group is transferred to a conserved histidine residue on a second inner membrane

protein, RcsD. Finally, RcsD transfers the phosphate group to an aspartate residue on the receiver domain of RcsB, the response regulator, thus activating RcsB. RcsB acts as a transcriptional regulator for the downstream genes of the pathway and can function either as a homodimer or as a heterodimer with RcsA, an auxiliary protein. Regulation of this pathway can take place via either the RcsA-dependent branch (through RcsA-RcsB heterodimer) or the RcsA-independent branch (through RcsB homodimer) (Majdalani et al., 2005).

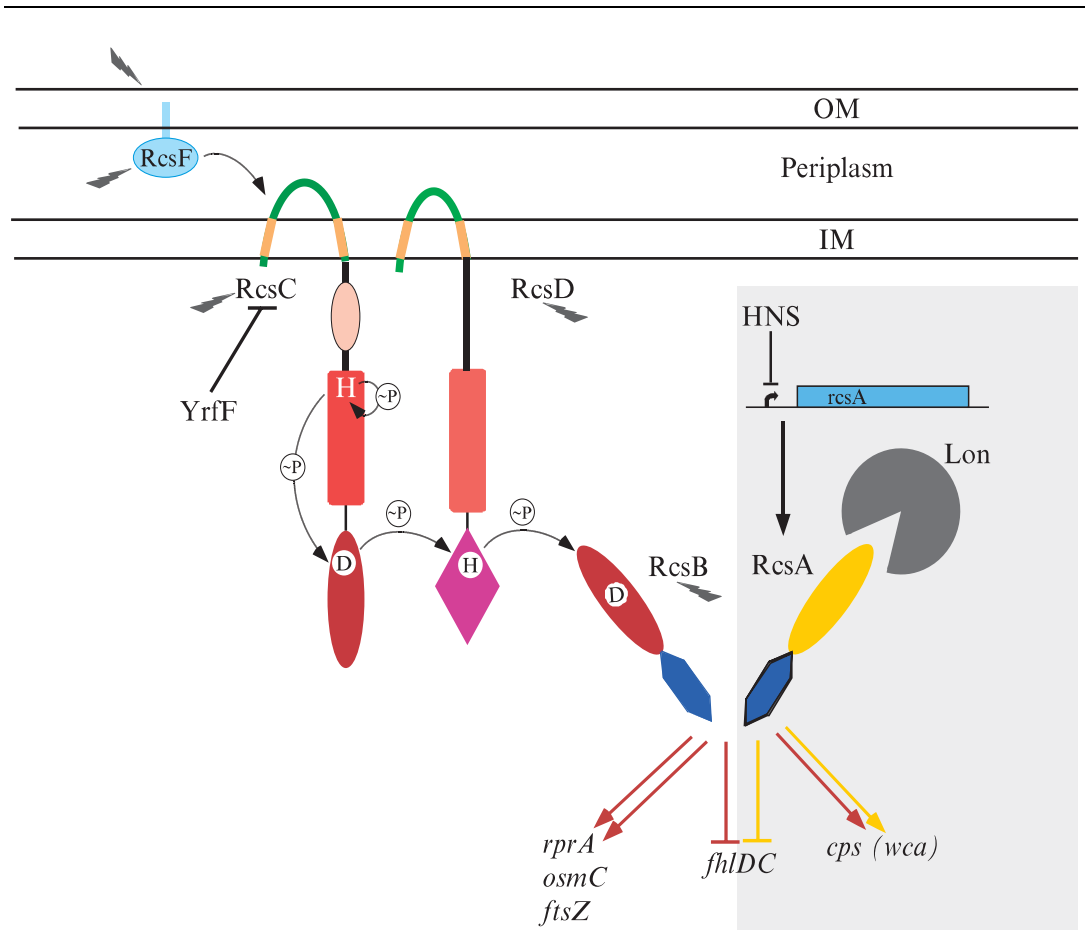


Figure 4: The RcsBCD phosphorelay cascade: Insult to the cell membrane is sensed by the RcsF protein which activates the sensor kinase RcsC via phosphorylation. RcsC subsequently activates RcsD leading to the activation of the response regulator RcsB. Once activated, RcsB can function either alone or in association with the auxiliary protein RcsA to regulate transcription of downstream genes of the pathway [Reprinted with permission from Methods in Enzymology, 2007, 423: 349-362 Majdalani et al.]

RcsA-Dependent and RcsA-Independent Branches

The RcsB response regulator of the RcsBCD pathway becomes activated when a phosphate group is transferred to its conserved aspartate residue by RcsD. The RcsB protein displays two modes of regulatory activities. First is the RcsA-dependent mode, in which RcsB interacts with RcsA and functions as a heterodimer. The second mode is the RcsA-independent mode in which RcsB functions as a homodimer. Both RcsB and RcsA are members of the LuxR family of transcriptional regulators with DNA-binding helix-turn-helix motifs in their C-terminal domains (Wehland et al., 1999). It has been suggested that the RcsB homodimer regulates transcription by interacting with the RNA polymerase and this binding stabilizes RcsB. The DNA binding site of the RcsB homodimer is just upstream of the -35 binding site of RNA polymerase and is different from the RcsA-RcsB binding site. Binding of the RcsA auxiliary protein is also suggested to stabilize RcsB, and this heterodimer complex binds DNA approximately 50-100 nucleotides upstream of the -35 binding site of RNA polymerase (Majdalani et al., 2005, Wehland et al., 2000).

Targets of the RcsBCD Pathway

RcsA-dependent targets

Capsule synthesis: The Rcs proteins were originally identified by their role in the positive regulation of the *cps* genes that are necessary for the synthesis of the colanic acid capsular polysaccharide in *E. coli* (Majdalani et al., 2005). Expression of the capsule synthesis genes is controlled by a complex network of regulators including RcsA and RcsB which function as positive regulators. The *cps* genes can be activated by two different pathways. Insult to the membrane such as desiccation or osmotic shock impacts the levels of the membrane-bound protein, MdoH, which is involved in the biosynthesis of membrane-derived oligosaccharides,

MDOs (Ebel et al., 1997). Differences in the levels of MDOs in response to environmental stimuli act as a signal for the sensor kinase, RcsC, which relays the signal as described above to activate RcsB and triggers synthesis of the *cps* genes. The second pathway that leads to the activation of the *cps* genes involves the other positive regulator of the pathway, RcsA. RcsA is an unstable protein and is degraded by the ATP-dependent protease, Lon (Ebel et al., 1997). Mutations in the Lon protease stabilize RcsA and result in increased synthesis of the capsular polysaccharide and formation of mucoid colonies. However, *cps* expression cannot be activated by RcsA in the absence of RcsB, suggesting that RcsA functions as an auxiliary protein that partner with RcsB to activate capsule synthesis gene expression (Stout et al., 1991)

RcsA can also activate its own expression as evidenced by a 100-fold increase in the expression of an *rcsA-lacZ* transcriptional fusion in the presence of the RcsA protein (Ebel et al., 1999). Additionally, the histone-like protein H-NS acts as a negative regulator of *rcsA* transcription, and *hns* mutants exhibit a mucoid phenotype due to over-production of capsular polysaccharide (Sledjeski et al., 1995).

Flagella synthesis: In *E. coli*, synthesis of the flagella, motility, and chemotaxis require more than 50 genes that are expressed in a hierarchical fashion (Chilcott et al., 2000). The Class I genes include the transcriptional activators *flhD* and *flhC* which are organized into an operon and are required for the expression of the Class II genes. Genes in Class II encode proteins that are required for the structure and assembly of the hook and basal body as well as the transcriptional regulators FlgM and σ^{28}. FlgM is an anti-sigma factor that binds to and inactivates the sigma factor σ^{28}/σ^{F}. Synthesis of the flagellar export components and the basal body by the Class II genes results in export of FlgM, releasing the sigma factor, σ^{28}, which can then transcribe the Class III genes. The Class III genes consist of the flagellin gene *fliC* and the genes required for

chemotaxis. Thus activation of the flagella synthesis genes and motility are dependent on the expression of the master regulator *flhDC* (Chilcott et al., 2000).

The promoter of the *flhDC* master operon serves as an important check-point at which the decision to initiate or inhibit flagella synthesis takes place. Therefore this promoter is controlled by a number of global regulators including H-NS and the CAP-cAMP complex. Other regulators of *flhDC* expression include positive regulation by the QseCB two-component system (Sperandio et al., 2002) and negative regulation by the LysR-type regulator LrhA (Lehnen, et al., 2002). Environmental conditions such as high temperature, high inorganic salt concentration and low pH are some of the other factors that can affect flagellin synthesis and motility (Shi et al., 1993, Soutourina et al., 2003). In addition to these, expression of *flhDC* also is regulated by the RcsBCD phosphorelay system. This regulation requires the co-factor RcsA and the response regulator RcsB, which functions as a negative regulator for flagella synthesis and motility (Francez-Charlot et al., 2003, Fredericks et al., 2006). The *flhDC* operon is the only known negatively regulated target of the RcsBCD phosphorelay cascade.

RcsA-independent targets

The RcsA protein binds to and stabilizes RcsB, and the RcsA-RcsB heterodimer can function both as a positive (*cps*) and a negative (*flhDC*) regulator (Majdalani et al., 2005). But the RcsB protein also can function as a homodimer. The promoters that are not dependent upon the RcsA protein and only require the RcsB protein for activation are known as the RcsA-independent promoters. Some of the known RcsA-independent targets include *ftsZ*, *osmC* and *rprA*.

The FtsZ protein is critical for the process of cell division in almost all bacteria, making it an important target that is subject to a variety of regulatory signals. FtsZ is a GTPase that is

structurally homologous to eukaryotic tubulins and it polymerizes to form a contractile ring at the site of cell division (Vaughan et al., 2004). FtsZ expression levels in the *ftsZ84* mutant are very low and these mutants are growth-defective and cannot form colonies when grown in LB agar media with no added NaCl. Overexpression of the RcsB protein activates *ftsZ* expression and is able to restore colony forming ability in the *ftsZ84* mutant strain (Gervais et al., 1992). Thus, RcsB functions as a positive regulator for *ftsZ* expression (Carballès et al., 1999).

OsmC is a non-essential envelope protein. Expression of the *osmC* gene is induced by osmotic shock and is growth phase dependent (Gutierrez et al., 1991). The *osmC* gene is transcribed from two overlapping promoters that are regulated by several factors including H-NS and the stationary phase sigma factor, σ^S. In vitro studies have indicated that RcsB is a positive regulator of *osmC* expression and facilitates RNA polymerase binding to at least one of the promoters of the *osmC* gene. Overexpression of RcsB significantly increased the *osmCp1* promoter activity during exponential phase of growth but had no effect on the *osmCp2* activity in either the exponential or stationary growth phase (Davalos-Garcia et al., 2001).

The small regulatory RNA, *rprA* (RpoS regulator RNA A), is essential for translational regulation of the stationary phase sigma factor, σ^S. The upstream region of the *rpoS* mRNA forms a double-stranded hair-pin structure that prevents access of the ribosome to the Shine-Dalgarno sequence in the translation start site, thus inhibiting translation of the *rpoS* mRNA. RprA base-pairs with the inhibitory region of the hair-pin structure in *rpoS* mRNA, preventing formation of the inhibitory structure and facilitating *rpoS* translation. Mutation in the *rprA* gene inhibits activation of RpoS after an osmotic shock suggesting that *rprA* is necessary for wild-type production of RpoS when cells are subjected to osmotic shock (Majdalani et al., 2001). Majdalani and co-workers (2002) reported that the promoter activity of *rprA* is significantly

decreased in an *rcsB* null mutant and the promoter activity was restored by complementing the null mutation with a multicopy plasmid expressing RcsB. RcsA on the other hand had a very low stimulatory effect on *rprA* expression. These data suggested that RcsB, but not RcsA, is required for *rprA* expression (Majdalani et al., 2002).

H-NS

The "Histone-like nucleoid structuring protein" (H-NS) is a nucleoid-associated multifunctional protein involved in gene regulation and alteration of DNA topology (Dame et al., 2000). H-NS is a global transcriptional regulator that modulates the expression of a large number of genes, mostly by acting as a repressor of transcription. The expression of about 5% of the genes or the accumulation of their proteins was altered in an *hns* mutant strain of *E. coli* suggesting that *hns* regulates the expression of a large number of genes (Danchin et al., 2001).

In most cases H-NS acts as a repressor of transcription, and mutation in *hns* leads to increased synthesis of the target gene products. One such negatively regulated target of H-NS is *rcsA* (Sledjeski et al., 1995). The negatively regulated targets of H-NS are also influenced by a variety of environmental factors such as temperature, pH, osmolarity, anaerobiosis or growth phase. In contrast, H-NS acts as a positive regulator of motility and flagella biogenesis as mutations in *hns* result in reduced expression of *flhD* and *fliA* (Bertin et al., 1994). Additionally, H-NS is a dual regulator of capsule synthesis genes. At 37°C, H-NS positively regulates the expression of the group 2 capsule gene clusters in *E. coli,* but at 20°C H-NS functions as a transcriptional repressor (Rowe et al., 2000). The mechanisms of H-NS-mediated transcriptional regulation are still elusive and several models have been proposed by different groups of researchers (Atlung et al., 1997).

BipA

Identification and characterization: The BipA/TypA protein was first identified in 1993 as a product of the o591 gene which has 38.4% to 45.3% similarity in amino acid sequence in the amino-terminal end with the elongation factor-G (EF-G) protein from different bacterial species (Plunkett et al., 1993). In 1995, Freestone and co-workers identified this protein as one of the phosphorylated proteins present in the enteropathogenic *E. coli* strain, MAR001 (Freestone et al., 1995). The same group demonstrated in 1998 that this protein was phosphorylated on a tyrosine residue in the MAR001 strain and thus the protein was named TypA or Tyrosine phosphorylated protein A (Freestone et al., 1998). This phosphorylation of the TypA protein was absent in the non-pathogenic K-12 strain of *E. coli,* implicating that phosphorylation of BipA might be significant in pathogenesis. The protein was re-named BipA or BPI-inducible protein A in 1995 when Qi and co-workers reported that expression of this protein was induced in *Salmonella typhimurium* in response to a cationic antimicrobial protein, BPI (bactericidal/permeability-increasing protein), released by neutrophils in response to gram negative bacterial infection (Qi et al., 1995). *bipA* has since been found in a wide variety of bacterial species including plant, animal and human pathogenic bacteria such as *Agrobacterium tumefaciens*, *Shigella flexneri*, *Klebsiella pneumoniae*, and *Yersinia pestis,* in plant symbionts such as *Sinorhizobium meliloti* and *Bradyrhizobium japonicum,* and in insect symbionts such as *Buchnera aphidicola* and *Photorhabdus luminescens* (Margus et al., 2007). A study by Margus and co-workers (2007) illustrated that *bipA* was present in at least one copy in 165 bacterial genomes, especially in genomes larger than 1.5Mb while genomes smaller than this usually lacked this gene (Margus et al., 2007). Function of BipA appears to be conserved throughout most of the bacterial world as the *bipA* gene from *Sinorhizobium meliloti* was able to complement the cold-sensitive phenotype

of an *E. coli* K-12 strain and vice versa, suggesting a conserved role for this protein (Kiss et al., 2004).

Structure and homology: BipA is a 67 kDa protein that is homologous to the elongation factor family of GTPases which includes EF-G, EF-Tu, EF-Ts and LepA (Margus et al., 2007, Finn et al, 2008). BipA has three domains (Figure 5): the amino-terminal domain (domain I) consists of a GTP-binding elongation factor signature motif whereas the carboxy terminus (BipA_C) is the ribosome binding domain (Kiss et al., 2004). Except EF-Tu, all members of the family have five domains and they share homology in domains I and II. Domain III of BipA is homologous to domain III of EF-G and LepA. EF-G has a unique domain IV not present in the other members of the family but the C-terminal domain of EF-G (EF-G_C) is present in BipA and LepA as the fourth domain in these two proteins (Finn et al., 2008). EF-G, BipA and LepA also have unique C-terminal domains.

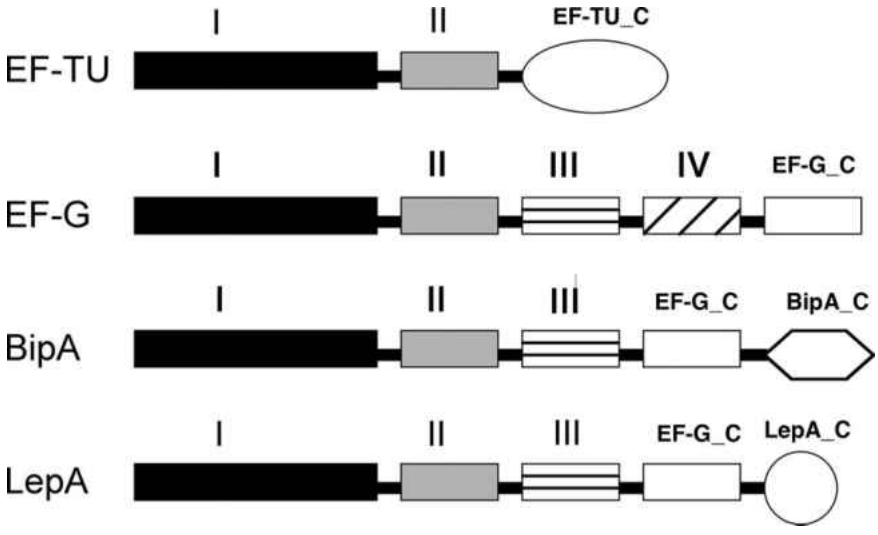


Figure 5: Schematic representation of the domain structure of members of the elongation factor family of GTPases [As depicted in the Pfam database, Finn et al., 2008]. Domain I and II are conserved among all four proteins, whereas Domain III is homologous in EF-G, BipA and LepA. The C-terminal domain of EF-G (EF-G_C) is present as a fourth domain in BipA and LepA. EF-G has a unique domain IV which is absent in the other members. EF-Tu, BipA and LepA have unique C-terminal domains, EF-Tu_C, BipA_C and LepA_C respectively.

BipA is unique among this family of proteins as this is the only protein that has been reported to play a role in diverse cellular pathways. While EF-G and EF-Tu are required for protein translation, LepA has been reported to be involved in the back-translocation of ribosomes, perhaps allowing an opportunity to correct binding errors in tRNA (Qin et al., 2006). While EF-Tu and EF-G are required for cell viability, BipA and LepA are both dispensable for growth (deLivron et al., 2008).

Association with the ribosome: In *Salmonella typhimurium*, BipA exhibits two distinct ribosome binding modes. During growth at 37°C, BipA associates with the 70S ribosomes but under conditions of stress such as stringent response or during growth at high or low temperatures, the protein binds to the 30S ribosomal subunits. BipA associates with the ribosome in its GTP-bound state and dissociates from the ribosome when the bound GTP is hydrolyzed to GDP. The GTPase activity of BipA is enhanced in the presence of 70S ribosomes and cellular levels of GTP, and the alarmone ppGpp influenced the association of BipA with the ribosomes (deLivron et al., 2008).

Functions of BipA

BipA shares extensive structural homology to the elongation factor family of GTPases but unlike the other members of this family, BipA has been suggested to play a role in regulating diverse cellular processes in different bacteria. *bipA* was identified under a wide range of physiological conditions by various genetic screens in both pathogenic and non-pathogenic strains of *E. coli* and *Salmonella,* which suggested a putative function of the protein as a global regulator. The following describe the various conditions under which BipA was identified:

(a) *Regulation of protein synthesis*: Studies investigating the role of BipA with EPEC (Enteropathogenic *E. coli*) strain, MAR001, used two-dimensional gel electrophoresis to reveal that inactivation of *bipA* resulted in an altered total protein profile during exponential growth and carbon starvation (Freestone et al., 1998). The expression profiles of at least 12 proteins (or their isoforms) were altered in a *bipA* mutant in comparison to its wild-type counterpart. During exponential growth conditions, expression of the global regulator protein H-NS was moderately increased in a *bipA* mutant but this increase in expression was more prominent during glucose starvation. Two other proteins whose expression was increased in a *bipA* mutant during glucose starvation were the isoforms of proteins Csp15 (carbon starvation-inducible protein) and UspA (universal stress protein A).

(b) *Resistance to host defense*: As mentioned earlier, BipA was one of the six proteins whose expression was induced by the BPI protein in *Salmonella typhimurium* strain SL1344 (Qi et al., 1995). Later, Farris and co-workers (1998) demonstrated that a *bipA* mutant of the EPEC strain MAR001 was hypersensitive to very low concentrations of BPI, thus suggesting that BipA mediates resistance to this cationic antimicrobial human defense protein (Farris et al., 1998). Furthermore, addition of formate to the growth media prior to the addition of the P2 peptide from BPI protein protected stationary phase *Salmonella* and *E. coli* cells from BPI, and this formate-mediated protection required BipA (Barker et al., 2000).

(c) *Pathogenesis*: When HeLa cells were infected with wild-type and *bipA* mutants of EPEC, the Δ*bipA* strains were unable to form cytoskeletal rearrangements (Farris et al., 1998) and failed to form micro-colonies (Grant et al., 2003). These *bipA* mutants also failed to form attaching and effacing lesions during infection of host cells. BipA was also found to be a positive regulator of *espC,* a member of the IgA serine protease family of auto-transporters, and also several gene

clusters of the LEE pathogenicity island in both EPEC and EHEC (Enterohemorrhagic *E. coli*) through transcriptional control of the LEE encoded regulator, Ler (Grant et al., 2003). These observations again suggested a role for BipA as a key regulator of several virulence-associated factors in pathogenic strains of *E. coli*.

(d) *Resistance to antibiotics*: In order to assess the genetic basis of intrinsic multidrug resistance in bacteria, Duo and co-workers (2008) performed a genetic screening using *E. coli* as a model. Out of the 4000 transposon insertion mutants that were screened for increased or decreased resistance to the antibiotic chloramphenicol, six mutants were identified to be more sensitive and one of these mutants had an insertional inactivation in the *bipA* gene. This observation suggested BipA to be associated with drug resistance (Duo et al., 2008).

(e) *Flagella-mediated motility*: BipA acts as a negative regulator of flagella-mediated cellular motility in EPEC as *bipA* mutants of this strain secrete large amounts of the protein flagellin and were hyper-flagellated (Farris et al., 1998). These mutants were also hyper-motile when tested on motility agar media (Grant et al., 2003) suggesting that BipA is a negative regulator of cell motility.

(f) *Capsule synthesis*: BipA was also implicated as a regulator of capsule synthesis in K5 strains of *E. coli.* As revealed by Rowe and co-workers (2000), both H-NS and BipA were required for maximal transcription of group 2 capsule genes in pathogenic strains of *E. coli* at 37°C. However at 20°C, both H-NS and BipA acted as transcriptional repressors for the group 2 capsule genes, thus suggesting a dual regulatory function for these two proteins in capsule synthesis. The mechanism of regulation is not clear and the authors suggested that BipA's role in regulation of capsule synthesis was more indirect (Rowe et al., 2000).

(g) *Growth at low temperature*: In *E. coli*, BipA is also required for growth at low temperature. A *bipA* null mutant manifests a cold-sensitive growth phenotype when grown at 20°C (Pfennig et al., 2001). This cold-sensitive phenotype is seen in both *E. coli* strain K-12 and in the EPEC strain E2348/69, indicating that phosphorylation status of BipA is irrelevant for this function (Grant et al., 2003). This finding also demonstrated that the function of BipA is not restricted to bacterial pathogenesis.

The cold-sensitive phenotype of a *bipA* mutant can be alleviated by deletion of *rluC,* which encodes the enzyme pseudouridine synthase RluC, which modifies three uridine residues in the 23S rRNA of the large ribosomal subunit. This suppression of the cold-sensitive phenotype was specific for *rluC* as deletion of the other pseudouridine synthase coding genes*, rluA, rluB, rluE* or *rluF* did not have any effect on cold sensitivity. *rluC* deletion not only alleviated the cold-sensitive growth defect of a *bipA* mutant, but also partially rescued the capsule synthesis defect, suggesting that the absence of the three pseudouridine residues on the 50S ribosomal subunit enables bacterial cells to grow at low temperature and synthesize capsule independently of BipA (Krishnan et al., 2008).

Summary and Hypotheses

BipA is a ribosome associated GTPase that has been implicated as a regulator of various cellular processes such as motility, capsule synthesis, growth at low temperature, antibiotic resistance and pathogenesis. However the mechanism by which BipA affects these various processes remains elusive. A summary of observations regarding BipA includes the following:

1. BipA is structurally homologous to the translational GTPases, EF-G, EF-Tu and LepA.
2. BipA mutants are hyper-flagellated and hypo-capsulated.

3. BipA associates with the ribosome and this ribosome-association is stimulated in the presence of ribosomes and GTP.
4. BipA mutants exhibit a cold-sensitive phenotype similar to mutants of other ribosome assembly factors.

These observations have led to two hypotheses for BipA function. BipA's homology to the translational GTPases and the variety of cellular pathways affected by *bipA* mutants leads to the hypothesis that BipA is a translational GTPase. However, the cold-sensitive phenotype of a *bipA* mutant and its association with the bacterial ribosome, are very similar to the characteristics of ribosome assembly factors, proposing an alternative hypothesis that BipA is involved in ribosome assembly. The major goal of this project was to clarify the function of BipA.

To study BipA's function as a translational regulator and/or a ribosome assembly factor, we took the following approaches:

1. Since *bipA* mutants are hyper-flagellated and hypo-capsulated, and the genes for flagella (*flhDC, fliA*) and capsule (*cpsB*) synthesis and regulation are regulated by the RcsA-RcsB transcriptional regulator of the RcsBCD pathway, we examined whether BipA is involved in the regulation of genes of the RcsBCD pathway.
2. Because *bipA* mutants exhibit a cold-sensitive phenotype similar to mutants of other ribosome assembly factors, we compared additional phenotypes of a *bipA* mutant to those of a known ribosome assembly factor, DeaD. Additionally, we investigated the role of BipA in ribosome assembly by analyzing ribosome profiles and rRNA processing of a *bipA* mutant to investigate any defects in ribosome assembly.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and Media

All bacterial strains used were derivatives of *E. coli* K-12 and are listed in appendix A. Strains MG1655 and TB28 were used as wild-type strains for most of the experiments and the mutants were their isogenic counterparts. Bacterial cells were grown in Luria Bertani (LB) liquid media or on LB agar plates (Silhavy et al., 1984). Kanamycin (50 mg/l) or ampicillin (125 mg/l) were added to LB media and used as a selective media when required. M9 minimal media (Silhavy et al., 1984) was used to select against the donor strain, S17-1 (*λpir*)/pJMSB8, after conjugation to resolve the *res-npt-res* (kanamycin) cassette (Kristensen et al., 1995).

Strain Construction Techniques

Transduction

Preparation of P1 phage lysate:

The donor strain was grown overnight in 5 ml LB broth with continuous shaking at 37°C. The following day, 50 µl of the overnight culture was inoculated into 5 ml of LB broth containing 0.2% glucose and 5 mM $CaCl_2$ and incubated at 37°C with continuous shaking for 45-60 minutes or until growth was barely visible. 100 µl of $P1_{vir}$ phage lysate was added to the growing culture and growth was continued at 37°C for another 2-3 hours or until the culture became clear. Chloroform (100 µl) was added to the tube and the culture was vortexed vigorously to lyse any remaining cells. The mixture was then centrifuged at 1200 x *g* for five

minutes to pellet cellular debris and the supernatant was gently collected from the top and transferred to sterile screw-capped tubes. 100 µl of chloroform was added to the tubes, and samples were vortexed and stored at 4°C.

P1 transduction:

The recipient strain was grown overnight at 37°C in 5 ml of LB broth with continuous shaking. The following day, the overnight culture was centrifuged and the pellet was resuspended in 2.5 ml of 10 mM $MgSO_4$, 5 mM $CaCl_2$. 100 µl of the resuspended bacterial cells were mixed with 100 µl of the appropriate P1 phage lysate and the mix was incubated at 30°C for 30 minutes with no shaking. 100 µl of cells or 100 µl of phage lysate were also incubated at 30°C for 30 minutes without shaking to serve as controls. After 30 minutes incubation, 1 ml of LB containing 10 mM sodium citrate was added to each of the tubes and incubation was continued at 37°C for 45-60 minutes without shaking. Following incubation, the cultures were centrifuged, the supernatant was discarded and the pellet was vortexed for 10 seconds. 100 µl of 1M sodium citrate was added to the pellet and samples were vortexed again. Cells were plated on LB agar media with appropriate antibiotics and incubated overnight at 37°C to select for the desired transductant. Gene deletion was detected and confirmed using PCR (discussed later).

Conjugation

The donor (S17-1 *λpir*/pJMSB8) (Kristensen et al., 1995) and recipient strains were grown overnight at 37°C with continuous shaking. While the recipient strain was grown in LB broth, the donor strain was grown in LB media containing ampicillin to maintain the pJMSB8 plasmid. The following day the cultures were diluted 1:100 in their respective media and grown to mid-log phase. 100 µl of the recipient was mixed with 400 µl of the donor in an eppendorf tube and centrifuged at about 5000 x *g* for five minutes. The pellet was re-suspended in 50 µl

LB broth and the mixture was seeded on the center of an LB agar plate. The plates were incubated at 37°C for 6-8 hours, without inverting the plates. 1 ml of LB broth was added to the plates following incubation and the cells were harvested by scraping. The harvested cells were serially diluted to 10^{-7} and 50 µl each of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated on minimal media to select against the donor strain. The plates were incubated overnight at 37°C. Colonies growing on the minimal media were patched onto LB agar media and onto media containing the appropriate antibiotics (kanamycin and ampicillin) to screen for strains that had lost the ampicillin plasmid and the kanamycin resistance gene.

Transformation

Preparation of Competent Cells:

Overnight cultures of recipient strains were grown in 5 ml of LB broth at 37°C with aeration. The following day, the overnight culture was inoculated into fresh LB media at a 1:100 dilution and grown to mid-log phase. Cultures were centrifuged at approximately 3000 x *g* at 4°C for 10 minutes and the pellet was re-suspended in an equal volume of ice-cold sterile 10% glycerol. The cells were centrifuged for two more times (3000 x *g*, 4°C, 10 minutes) reducing the volume of glycerol by half the first time and by $1/10^{th}$ for the second wash. Finally the pellet was resuspended in 1/200 times the initial culture volume in ice-cold 10% glycerol, aliquoted into 50 µl portions and stored at -80°C for future use.

Plasmid isolation:

Plasmid DNA was isolated from five ml bacterial cultures grown overnight using the Qiaprep Miniprep plasmid isolation kit (Qiagen) following manufacturer's instructions. The bacterial cells were pelleted by centrifugation at about 18, 000 x *g* for five minutes and the pellets were resuspended in 250 µl buffer P1 (supplied with the kit) and transferred to an

eppendorf tube. 250 µl buffer P2 (supplied with the kit) was added to the tube and samples were mixed thoroughly by inverting the tube four-six times. Next, 350 µl buffer N3 (supplied with the kit) was added to the same tube and samples were again mixed immediately and thoroughly. The samples were centrifuged for 10 minutes at about 18,000 x *g* and the supernatant was applied to the center of a QIAprep spin column (supplied with the kit) by pipetting. The column (with the sample) was centrifuged at 18,000 x *g* for 30-60 seconds and the flow-through was discarded. Following centrifugation the column was washed with 0.5 ml of buffer PB (supplied with the kit) by centrifuging again for 30-60 seconds and the flow-through was discarded. Next the column was washed with 0.75 ml buffer PE (supplied with the kit) once by centrifuging for 30-60 seconds and then centrifuging again for one minute to remove any residual wash buffer. Following washing, the column was placed in a clean 1.5 ml eppendorf tube. Plasmid DNA was eluted by adding 50 µl buffer EB (supplied with the kit) to the center of the column, letting it stand for one minute and then centrifuging for one minute. The eluent containing the plasmid DNA was stored at -20°C for future use.

Electroporation:

The electro-competent recipient cells were thawed on ice. Plasmid DNA was added to the recipient cells at ratios of 1:1000 and 1:10,000 and the mixture was transferred very gently to the bottom of electroporation cuvettes, making sure no bubbles were formed. The voltage of the electroporator (Biorad *E. coli* Pulser) was set at 2.5 volts and the cells were pulsed as directed by the manufacturer. 1 ml of LB broth was immediately added to the pulsed cells in the cuvettes, and samples were transferred to eppendorf tubes and incubated at 37°C for 60 minutes with no shaking. 50 µl of cells were plated onto selective media and incubated overnight at 37°C.

Following growth, a few of the colonies were re-streaked on selective media and incubated at 37°C overnight to confirm antibiotic resistance.

β-Galactosidase Assays

β-galactosidase assays were performed as described (Miller, 1992). Overnight cultures of the strains to be assayed were grown at 37°C in LB with aeration. The following day the strains were sub-cultured in 5 ml LB at a 1:100 dilution and grown to mid-log phase. Cultures were immersed in ice for 20 minutes to inhibit further growth and culture density was measured by absorbance at 600 nm. Next, 0.2 and 0.5 ml of bacterial culture were transferred into two separate tubes and Z buffer (0.06 M Na_2HPO_4.7H2O, 0.04 M NaH_2PO_4.H2O, 0.01 M KCl, 0.001 M $MgSO_4$.7H2O, 0.05 M β-mercaptoethanol, pH 7.0) was added to each of the tubes to a final volume of 1 ml. 1 ml of Z buffer without any bacterial cells was used as a negative control. 70 µl chloroform and 35 µl 0.1% SDS was added to each of the assay mixtures; samples were vortexed and incubated at 28°C for five minutes. 200 µl of ONPG (ortho-Nitrophenyl-β-galactoside) was added to each of the reaction tubes and the reaction was allowed to proceed until yellow color was detectable. After sufficient yellow color had developed, the reaction time for each of the reactions was noted and the reactions were quenched by addition of 0.5 ml of 1 M Na_2CO_3. The samples were centrifuged briefly at about 1000 x *g* for five minutes to remove any cellular debris. The yellow supernatant from each of the tubes was collected and the OD was measured at 420 nm. The enzyme activity was calculated in Miller units using the following equation:

$$1 \text{ Miller unit} = 1{,}000 \times OD_{420} / (T \times V \times OD_{600})$$

T = time (in min) of reaction, V = volume (in ml) of culture used, OD_{420} = absorbance of the reaction product at 420 nm, OD_{600} = absorbance of the bacterial culture at 600 nm.

Statistical significance was calculated by a one-way ANNOVA and Dunnett's test. The *p* values were set at $p < 0.01$.

DNA extraction for PCR

200 µl of overnight culture of bacterial strains to be used for PCR was mixed with 800 µl of sterile water in eppendorf tubes. The mixture was boiled for 10 minutes and centrifuged at 18,000 x *g* for four minutes. The supernatant containing bacterial DNA was transferred to a fresh tube and stored at -20°C.

Polymerase Chain Reaction

Most PCR reactions were performed using a 2400 Gene Amp Thermo Cycler (Perkin Elmer). Amplification reactions to detect gene deletions were performed using Epicenter's Failsafe PCR kit. The kit consisted of a variety of premixes and an enzyme mix (DNA polymerase). The premixes contained buffered salt solution with all four dNTPs, various amounts of $MgCl_2$, and FailSafe PCR Enhancer and are efficient for amplifying fragments up to 20 kb in size. The premix that gave best results for a particular template/primer pair combination was used for further amplification reactions.

Primer annealing temperature was determined based on the melting temperatures (Tm), of the primer pair as determined by sequence and the PCR reactions were carried out for 25 cycles. Following PCR, results were confirmed by 1% agarose gel electrophoresis.

Bacterial growth curves

Growth curve analysis was performed using a Bioscreen C Microbiology Reader from Labsystem. Overnight cultures of strains to be analyzed were grown at 37°C or 20°C (for cold-sensitivity assays) in LB media. For chloramphenicol and NaCl sensitivity assays, LB media

was supplemented with chloramphenicol or NaCl (as discussed below). The following day the overnight cultures were diluted to optical density (OD_{600}) of 0.02. 300 µl of culture was added in triplicate to wells of a honeycomb plate. The OD_{600} of each strain vs time elapsed was measured and plotted in a graphical form using excel. For monitoring cold-temperature growth, overnight cultures were grown at 37°C, diluted and transferred to the honeycomb well plate as described earlier. The growth temperature was set at 20°C in the Bioscreen C Reader, with continuous shaking and OD_{600} was monitored at regular intervals of 30 minutes.

Chloramphenicol sensitivity assay

Cultures of the experimental strains were grown overnight at 37° C in LB media. The following day, cultures were diluted in LB media or LB media containing 2 µg/ml chloramphenicol to an OD_{600} of 0.02, and 300 µl of each of the cultures was added (in triplicate) to a honeycomb plate. Growth was monitored at 37°C, with continuous shaking for 15 hours using the Bioscreen C Microbiology Reader. Growth curves were generated by plotting OD_{600} vs time of growth for each of the bacterial strains in media with or without chloramphenicol.

NaCl sensitivity assay

Overnight cultures of the experimental strains were grown in LB media at 37°C and subcultured the following day to OD_{600} of 0.02 in LB media either with or without 1 M added NaCl. 300 µl of each of the cultures was transferred to honeycomb well plates in triplicate. Cultures were grown for for 35 hours at 37°C with continuous shaking in the Bioscreen C growth monitoring system. Results were represented graphically by plotting OD_{600} against total time of experiment.

Motility Assay

Overnight cultures of bacterial strains grown in Tryptone broth (TB) media (1% tryptone and 0.5% sodium chloride) at 37°C were diluted the following day in TB media and OD_{600} was measured to calculate the bacterial cell number. Sterile filter paper disks were placed on the center of TB agar plates and 5 µl of each of the cultures containing approximately the same number of bacteria was seeded on to the surface of the disks. The plates were incubated at 34°C. The culture was allowed to swarm until it reached the edge of the plates and the diameter of the swarming was recorded every four hours. The experiment was continued for 22 hours. The results were represented graphically by plotting swarm diameter (in cm) against time of experiment (in hours).

Sucrose Gradient Centrifugation

Ribosome extracts were collected from bacterial cells and polysome profiling was performed as described by Charollais and co-workers (2003).

Isolation of ribosomes:

Bacterial cells were grown in 25 ml of rich media overnight at 37°C or at 20°C with continuous shaking, subcultured at 1:100 dilution and grown to an OD_{600} of 0.5-0.7. Just before harvesting, chloramphenicol was added to the growing bacterial cultures at a final concentration of 100 µg/ml to inhibit growth and to stall the ribosomes on the mRNA to prevent further translation. The cells were cooled rapidly by immersing in ice for 20-30 minutes and collected by centrifugation at 3000 x *g* for 10 minutes at 4°C. Any residual media was completely removed from the tubes and the pellet was frozen overnight at -20°C. The following day the frozen pellets were thawed on ice, resuspended in 200 µl of freshly prepared 1X buffer A (10 mM Tris-HCl pH 7.5, 60 mM KCl, 10 mM $MgCl_2$) containing 0.5 mg/ml lysozyme and

transferred to eppendorf tubes. The samples were again frozen overnight at -20°C and thawed on ice the next day. The samples were treated with 150 µl buffer A containing 0.5% Brij 58, 0.5% Deoxycholate and 0.1 unit/µl RQ1 DNase (RNase-free DNase, Promega) and incubated on ice for an additional 20 minutes or until loss of viscosity was observed. Then the samples were centrifuged at about 18,000 x *g* for 10 minutes at 4°C to remove cellular debris. Additional centrifugations were performed under the same conditions until a clear supernatant was obtained. The supernatant was transferred to fresh eppendorf tubes and the extract was frozen in a dry ice ethanol bath and stored at -80°C or loaded immediately on sucrose gradients.

Preparation of sucrose gradients:

Linear 10-40% (w/v) sucrose gradients (30 ml) were prepared using a two-chamber device and stored at 4°C until used. The sucrose solutions were made in buffer B (10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$ and 1 mM DTT).

Centrifugation and data analyses:

Ribosome extracts were loaded onto the sucrose gradients and centrifuged at 22,000 rpm for 19 hours at 4°C using a SW28 rotor (Beckman). Following centrifugation, the tubes were held steady and upright using a clamp stand and a tiny hole was introduced on the bottom of the tube using a fine needle. 300 µl fractions containing separated samples were collected from the tubes from bottom to top. Absorbance of the collected fractions was measured at 260 nm to detect rRNA using a spectrophotometer (Biotek, Inc) and Gene 5.2 software. A_{260} values of each fraction were plotted and represented graphically using Microsoft Excel.

Quantitative Real-Time Polymerase Chain Reaction

RNA isolation:

For qRT-PCR analyses, bacterial cells were grown at either 37°C (to be used as control) or cold-shocked for 30 minutes at 15°C in order to induce expression of the cold shock genes. Whole cell RNA was isolated from these cultures using Trizol reagent (Life Technologies) following the protocol supplied with the reagent. Briefly, cells grown to an OD_{600} of 0.5-0.6 and were collected by centrifuging at about 3000 x *g* for five minutes. The pellet was resuspended in 1 ml of Trizol by pipetting and incubated at room temperature for five minutes. 200 µl of chloroform was added to each of the tubes; samples were shaken vigorously for 15 seconds and incubated at room temperature for three minutes. The samples were centrifuged at 12,000 x *g* for 15 minutes at 4°C. Following separation, the colorless aqueous phase was gently transferred to a fresh eppendorf tube without collecting any of the residues from the interphase or the organic phase. 500 µl of isopropyl alcohol was added to the aqueous phase, along with 2 µl of glycogen solution (20 mg/ml, Amresco), samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x *g* for 10 minutes at 4°C. Following separation the supernatant was discarded and the RNA pellet was washed with 1 ml 70% ethyl alcohol. The mixture was gently vortexed and centrifuged at 7,500 x *g* for five minutes at 4°C. The pellet was air dried for 30 minutes, suspended in pre-warmed RNase-free water or RNA secure reagent (Life Technologies) and incubated at 65-70°C for 10 minutes. If RNA was to be utilized for qRT-PCR, the samples were treated with RNase-free DNase to remove DNA contamination. cDNA was synthesized from the RNA using Superscript III reverse transcriptase. For all other downstream applications the RNA was stored at -80°C for future use.

DNase Treatment of RNA:

The isolated RNA was treated with RNase free DNase (Promega) to remove traces of residual DNA following the protocol supplied with the DNase. About 1 µl (volume adjusted based on concentration) of RNA was mixed with 1 µl DNase and 1 µl 10X reaction buffer (supplied with the DNase). Water (RNase-free) was added to the mixture to adjust the volume to 10 µl. The reaction mixture was incubated at 37°C for 30 minutes. 1 µl DNase stop solution was added to the mixture to terminate the reaction and samples were incubated at 65°C for 10 minutes to deactivate the DNase. The RNA was stored at -80°C or used immediately for making cDNA.

cDNA synthesis:

RNA was converted to cDNA using the Superscript III first strand synthesis system (Invitrogen) following manufacturer's instructions. RNA was mixed with random hexamers and dNTPs and incubated at 65°C for five minutes and cooled on ice for about a minute. Then the cDNA synthesis mix (10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase OUT, Superscript III reverse transcriptase) was added to each RNA/primer mixture, gently mixed by centrifugation and samples incubated at 25°C for 10 minutes and 50°C for 50 minutes. Finally the reactions were terminated at 85°C for five minutes and allowed to cool on ice. 1 µl RNase H was added to each reaction tube and samples incubated at 37°C for 20 minutes to degrade the RNA in the RNA-DNA hybrid. The cDNA was either stored at -80°C or used immediately for qRT-PCR.

qRT-PCR:

1 µl cDNA was mixed with 0.5 µl forward primer, 0.5 µl reverse primer, 8 µl water (nuclease-free) and 10 µl Sybr green (Biorad). The mixture was centrifuged briefly at 1000 x *g*

for two minutes at room temperature and qRT-PCR was carried out using MyiQ2 Two Color Real-Time PCR Detection System (Biorad).

For qRT-PCR experiments, the *bipA* cDNA was amplified using primers *bipA*pc1 (5′-CTGGACGTTGAAGAACAGCA-3′) and *bipA*pc2 (5′-GGCTGAAGGTGGAGTACAGC-3′). *deaD* expression was monitored using primers *deaD*pc3 (5′-GCTGGATCTTCGAAACTCTGG-3′) and *deaD*pc4 (5′-CATATCGCCAACATCACGAC-3′). All reactions were normalized to the expression of *hcaT* (used as internal control) (Zhou et al., 2011) using primers *hcaT*pc1 (5′-ACTTTCCGCCGTTG TAGTG-3′) and *hcaT*pc2 (5′- CGCCTGTAAACGGATGACTT-3′). All primers were annealed at 59.5°C (primer annealing temperature was determined from Tm of the primer pair). Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001).

Northern blot analysis

Agarose gel electrophoresis:

0.25 g agarose was boiled in 21.75 ml water (nuclease free) and allowed to cool. 2.5 ml 10X MOPS [3-(N-morpholino) propanesulfonic acid] buffer, 0.75 ml formaldehyde and 4 µl ethidium bromide (Amresco, 10 mg/ml) were added to the mixture which was then carefully poured into the gel apparatus and allowed to set. 250 ml running buffer (225 ml water, 25 ml 10X MOPS) was added to gel apparatus and the gel was pre-soaked in the running buffer for 10-15 minutes before loading the samples. The RNA samples were mixed with equal volume of sample buffer (50 µl formamide, 17.5 µl formaldehyde, 10 µl 10X MOPS and 22.5 µl nuclease free water) and heated at 65-70°C for 10 minutes. The denatured RNA samples were mixed with ½ the sample volume of loading dye (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol), loaded onto the gel and the gel was electrophoresed at 110 volts for 40 minutes. After completion of electrophoresis, the gel was visualized with UV light to check for RNA. The gel

was soaked in nuclease-free water for at least 20 minutes, trimmed and washed with 10X SSC (saline sodium citrate; 0.3 M sodium citrate, pH 7.0, 3 M NaCl) buffer for at least 20 minutes.

Transfer:

The nylon membrane (MSI, Micron Separations Incorporation) was cut to the exact size of the gel and labeled with a pencil. Five pieces of filter paper were cut to the exact size of the gel and a filter paper wick was prepared, long enough to drape over both edges of the support and into the transfer buffer. The nylon membrane was soaked in nuclease-free water and then washed with 10X SSC for at least 15 minutes. The transfer tray was prepared by draping the support with the filter paper wick (pre-soaked in 10X SSC) and filling the tray with 10X SSC. The gel was placed on the wick, taking care no air bubbles were trapped between the gel and the wick. The membrane was carefully placed on top of the gel so that the labeled side was in contact with the gel and air bubbles were gently removed without damaging the gel or the membrane. One piece of the filter paper was soaked in 10X SSC and placed on top of the membrane followed by the remaining four pieces. Strips of parafilm were used to cover the wick surrounding the gel to prevent drying of the wick. A 2-3 inches stack of absorbent paper towels was placed on top of the filter papers, followed by a plexiglass and a small weight. The transfer process was continued overnight.

The following day the towels and the filter papers were carefully removed without disturbing the membrane. The membrane was gently removed using forceps, washed with 2X SSC for about 10 minutes to remove traces of agarose and air-dried. The transferred RNA was cross-linked to the nylon membrane by exposing both sides of the membrane to UV light for five minutes in a UV stratalinker (Stratagene). The membrane was stored in a ziplock bag at -20°C or used immediately for the hybridization reaction.

Probe preparation: 16S rRNA processing was monitored using a 5′-biotin-tagged 16S mature (16S-M) (5′-TACTCACCCGTCCGCCACTC-3′) probe (Midland Oligos) and the biotin tag was detected using Immun Star chemiluminescent substrate (Biorad). Processing of the 23S rRNA was monitored using 5′-γ P^{32} dATP labelled 23S mature (23S-M) (5′-AAGGTTAAGCCTCAC GGTTC-3′) and 23S upstream (23S-U) (5′-CGCTTAACCTCACAAC-3′) probes (IDT DNA).

The 5′ ends of the 23S-M and 23S-U probes were labelled with γ P^{32}-dATP using the following protocol:

In a 0.5 ml eppendorf tube, 6.4 µl water was mixed with 1 µl 10X NEB buffer (New England Biolabs), 0.4 µl (40 pmol) probe, 2 µl γ P^{32} dATP (Ultratide/Isoblue γ P^{32} dATP, MP biomedicals, Specific Activity 6000 Ci/mmol, 10uCi/ul) and 0.3 µl T4 PNK (Polynucleotide kinase, New England Biolabs). The mixture was incubated at 37°C for 30-60 minutes. Following incubation, 40 µl TE buffer was added to 10 µl of the reaction mixture. 50 µl of the total mixture was then added to Micro BioSpin columns (Biorad) and purified according to manufacturer's instructions. The purified probes were stored at -20°C till future use.

Hybridization:

Sonicated herring sperm DNA (0.5 mg/ml) was denatured at 100°C for 10 minutes and added to Church buffer (1 mM EDTA, 1% BSA, 0.5 M sodium phosphate monobasic, 7% SDS, at pH 7.2) to a final DNA concentration of 100 µg/ml to prepare the pre-hybridization solution. The membrane was placed in a 50 ml Falcon tube and 5 ml of the pre-hybridization solution was added to the tube. The denatured DNA was pre-hybridized to the membrane for two hours at 65°C with continuous shaking. The hybridization solution was prepared by adding the desired probe to Church buffer at a final concentration 100 ng/ml (probe was pre-heated at 100°C for 10 minutes before adding to the buffer). After pre-hybridization, 5 ml of the hybridization solution

was added to the tube and the hybridization reaction was continued overnight at 60°C (for 16S-M and 23S-M probes) or at 45°C (for 23S-U probe) with continuous shaking. Following hybridization, membranes were washed twice with wash buffer I (6X SSC) at 30°C for 30 minutes and then twice with wash buffer II (3X SSC, 0.1% SDS) for 20 minutes at 56°C (for 16S-M probe), 58°C (for 23S-M probe) and 42°C (for 23S-U probe) (Charollais et al., 2004). For the biotinylated probe, membranes were allowed to dry and the biotin tag was detected using a biotin detection kit (Thermo Scientific) and Immun Star chemiluminiscent substrate (Biorad). For radio-labelled probes, the membranes were wrapped with saran wrap and exposed to a Phosphor screen (Amersham Biosciences) for signal development. For re-probing, membranes were boiled for one minute in 40 ml of stripping buffer (0.1% SDS, 0.05X SSC) and re-hybridized with the second probe overnight.

Signal development for biotin-tagged probes:

The membrane was washed with 1X wash/blocking buffer (supplied with the kit) for five minutes at room temperature with moderate shaking. Then the membrane was washed with the blocking solution (1% w/v blocking reagent dissolved in 1X wash/blocking buffer) for 30 minutes at room temperature. Next, the Streptavidin-AP conjugate (supplied with the kit) was added to the membrane and the reaction was continued for 30 minutes at room temperature. Following this step the membrane was washed twice (15 minutes each wash) using the wash/blocking buffer. The chemiluminiscent Immun Star substrate (Biorad) was next added to the membranes and the reaction was continued for 10-20 minutes at room temperature. The signals were monitored and band intensities were quantified using the Quantity One imaging software.

Signal development for radio-labelled probes:

Following washing, the membranes were exposed to a Phosphor screen by placing inside a cassette (Amersham Biosciences) for about an hour and signals were visualized using a Typhoon scanner (Amersham Biosciences). Band intensities were quantified using Image J software (inspired by NIH Image). Before re-probing, membranes were scanned to check for loss of previous signal.

CHAPTER IIIA

RESULTS

Role of BipA and RluC in Regulating Genes of the RcsBCD Pathway

BipA previously has been reported to be involved in the regulation of multiple cellular pathways. Mutants of *bipA* are hyper-flagellated and hyper-motile (Farris et al., 1998) and show decreased capsule synthesis (Rowe et al., 2000). Since the RcsBCD pathway regulates the principal transcriptional regulator of the bacterial flagellum, *flhDC*, the sigma factor for regulating the transcription of genes involved in motility and flagella synthesis, *fliA,* and also the capsular polysaccharide colanic acid synthesizing gene, *cpsB*, we hypothesized that BipA might be involved in regulating the expression of genes in this pathway. Additionally, because deletion of the pseudouridine synthase coding gene *rluC* alleviated the cold-sensitive growth defect and partially suppressed the capsule synthesizing defect of a *bipA* mutant (Krishnan et al., 2008), we investigated whether *rluC* deletion or a *bipA rluC* double deletion altered expression of the genes of the RcsBCD pathway. We used *lacZ* reporter fusions to multiple genes within the pathway and measured β-galactosidase activity of the fusions as an indicator of expression levels.

RcsA-Independent Branch

Role of BipA and RluC in *rprA* Expression

Upon activation by an environmental stimulus such as periplasmic stress, the RcsBCD pathway regulates the downstream genes of both the RcsA-dependent or RcsA-independent branch (Majdalani et al., 2005). In the RcsA-independent branch, the RcsB response regulator

functions independently without binding to the auxiliary protein, RcsA (Majdalani et al., 2007). The RcsB protein positively regulates the expression of several genes including *rprA* (Majdalani et al., 2002), *osmC* (Davalos-Garcia et al., 2001) and *ftsZ* (Carballès et al., 1999). Altered expression of the genes of the RcsA-independent pathway by Δ*bipA* and/or Δ*rluC* would suggest a function of these proteins in regulating the expression of RcsB or genes upstream in the pathway that regulate RcsB. However, if Δ*bipA* and Δ*rluC* do not affect the expression(s) of genes of the RcsA-independent pathway, that may suggest a role for these two proteins in the regulation of RcsA or its upstream regulators.

To investigate the role of BipA and RluC in the regulation of the RcsA-independent branch, an *rprA-lacZ* transcriptional fusion (Castanié-Cornet et al., 2006) was utilized. The *bipA* and/or *rluC* gene(s) were deleted from the strain GEB658 (Castanié-Cornet et al., 2006) by insertion of a kanamycin insertion cassette to construct the single and double deletion mutants of GEB658, which harbors the *rprA-lacZ* reporter fusion. Strains were grown as described in materials and methods and β-galactosidase activity of the four strains was measured (Figure 6). The expression of the *rprA-lacZ* fusion was significantly decreased in the *bipA* mutant, *rluC* mutant and in the *bipA rluC* double mutant compared to the GEB658 (WT) strain. The expression of the *rprA-lacZ* fusion was 150 Miller units in the wild-type strain and was reduced to 105 Miller units in a *bipA* mutant, 61 Miller units in an *rluC* mutant and 35 Miller units in a *bipA rluC* double mutant. These data suggest that both BipA and RluC positively regulate the expression of *rprA* and deletion of both these genes has an additive effect in decreasing *rprA* expression. Since RcsB positively regulates *rprA* expression, it is possible that BipA and RluC regulate *rprA* expression through regulation of RcsB or its upstream regulators.

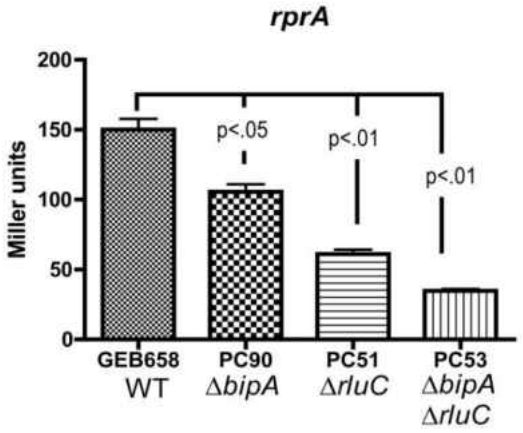


Figure 6: BipA and RluC positively regulate *rprA* expression: Expression of *rprA* was analyzed in WT (GEB658), Δ*bipA* (PC90), Δ*rluC* (PC51) and Δ*bipA*Δ*rluC* (PC53) strains. Strains were grown and β-galactosidase assays were performed as described in materials and methods. Experiments were conducted at least three times and statistical analyses were performed by a one-way ANNOVA and Dunnett's post hoc test. Statistical significance was set at $p < 0.01$.

RcsA-Dependent Branch

Role of BipA and RluC in *fliA* Expression

In the RcsA-dependent branch of the RcsBCD pathway, the RcsA-RcsB heterodimer negatively regulates the promoters of the genes involved in flagella biosynthesis and cell motility (Fredericks et al., 2006). Additionally, transcription of a number of genes involved in motility and flagella synthesis are regulated by FliA (σ^{28}) (Ohnishi et al., 1990). BipA on the other hand was implicated to be a negative regulator of flagella synthesis and motility in EPEC, EHEC and K12 strains of *E. coli* as *bipA* mutants of these strains were hyper-flagellated and hyper-motile (Farris et al., 1998, Grant et al., 2003). Also, previous results from our lab suggest that BipA is involved in the positive regulation of colanic acid synthesis as transcription of the *cpsB* gene was decreased in a Δ*bipA* mutant (Krishnan et al., 2008). Because both *fliA* and *cpsB* are regulated by the RcsA-RcsB heterodimer, we investigated whether Δ*bipA* and/or Δ*rluC* might alter expression of *fliA*.

To analyze the role of BipA and RluC in regulating *fliA* expression, expression levels of a *fliA-lacZ* transcriptional fusion were measured (Figure 7). Deletion of *bipA* increased *fliA* expression from 1140 Miller units to 1727 Miller units, whereas deletion of *rluC* had no significant effect. *fliA* expression in a *bipA rluC* double mutant strain was elevated to almost similar levels (1588 Miller units) as in a *bipA* mutant and *fliA* xpression in an *rluC* mutant (865 Miller units) was not significantly different from the wild-type. These data suggest that BipA negatively regulates *fliA* transcription while RluC has no effect on *fliA* transcription.

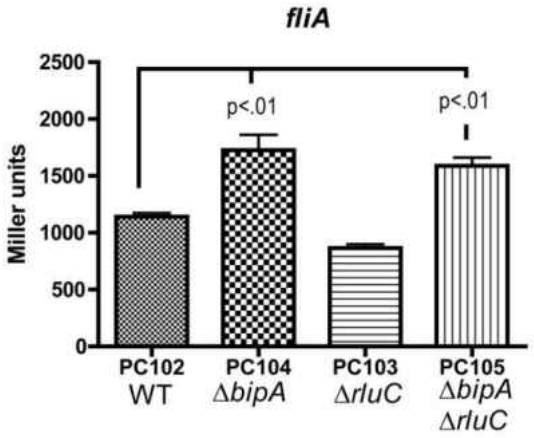


Figure 7: BipA negatively regulates *fliA* expression: The pXL11 plasmid harboring the *fliA-lacZ* transcription fusion was introduced into WT (TB28), Δ*bipA*, Δ*rluC* and Δ*bipA* Δ*rluC* strains to construct strains PC102, PC104, PC103 and PC105 respectively. Strains were grown and β-galactosidase assays were performed as described in materials and methods. All experiments were conducted at least three times and statistical analyses were performed by a one-way ANNOVA and Dunnett's post hoc test. Statistical significance was set at $p < 0.01$.

Role of BipA and RluC in H-NS Expression

The global regulator protein, H-NS, regulates the expression of multiple genes of the RcsBCD pathway. H-NS functions as a transcriptional activator for *flhD* and *fliA* (Bertin et al., 1993), as a transcriptional repressor for *rcsA* expression (Sledjeski et al., 1995), and as a dual regulator for capsule synthesizing genes, by acting as a transcriptional activator and repressor of the group 2 capsule gene clusters at 37°C and 20°C respectively (Rowe et al., 2000). Because

our studies suggested that BipA and RluC are involved in regulation of multiple genes of the RcsBCD pathway, we sought to determine whether either or both of these proteins had a regulatory effect on H-NS expression. An *hns-lacZ* translational fusion (Desch et al., 1993) was introduced into a Δ*bipA*, Δ*rluC* or Δ*bipA* Δ*rluC* strain and β-galactosidase activity was measured (Figure 8). H-NS expression in a *bipA* mutant was reduced from 1607 to about 617 Miller units. This reduction was more severe in an *rluC* and *bipA rluC* mutants (39 and 34 Miller units respectively). These data suggest that both BipA and RluC positively regulate H-NS expression.

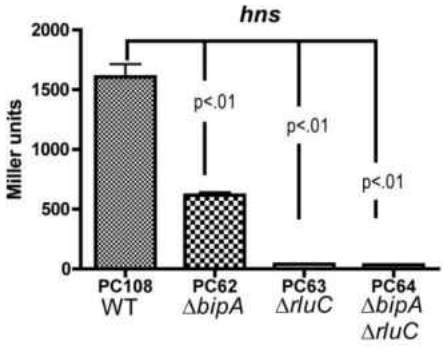


Figure 8: BipA and RluC positively regulate H-NS expression: The *hns-lacZ* translational fusion was movedfrom strain KNS4 into WT (TB28), Δ*bipA*, Δ*rluC* and Δ*bipA* Δ*rluC* backgrounds, to construct strains PC108, PC62, PC63 and PC64 respectively. Strains were grown as described in materials and methods and β-galactosidase activity was measured. All experiments were conducted at least three times and statistical analyses were performed by a one-way ANNOVA and Dunnett's post hoc test. Statistical significance was set at $p < 0.01$.

Summary

To summarize, our data suggest that BipA and RluC are involved in the regulation of multiple genes of the RcsBCD pathway with BipA affecting positive regulation of *rprA* and H-NS and negative regulation of *fliA*. RluC on the other hand appears to be a positive regulator of *rprA* and H-NS expression but has no effect on *fliA* expression. Additionally, BipA and RluC appear to work in concert for *rprA* regulation, but for regulation of *fliA* or H-NS, BipA functions independent of RluC.

CHAPTER III B

RESULTS

Role of BipA in Ribosome Biogenesis

BipA is a ribosome binding GTPase and the GTPase activity of the protein is triggered in the presence of fully formed ribosomes and GTP (deLivron et al., 2008). Mutants of *bipA* exhibit a cold-sensitive phenotype when grown at 20°C (Pfenning et al., 2001) similar to other ribosome assembly factors. However, deletion of *rluC* alleviates the cold-sensitive growth defect of a *bipA* mutant suggesting that the absence of the three pseudouridine residues added by RluC on 23S rRNA structurally modify the ribosomes by an unknown mechanism allowing bacterial cells to function independent of BipA (Krishnan et al., 2008). Taken together these observations suggest a plausible role of BipA in modulating the ribosome structure. We hypothesized that BipA is involved in the biogenesis and/or assembly of the bacterial ribosome and we utilized two approaches to test our hypothesis.

Approach 1: To investigate the role of BipA in ribosome biogenesis, we first compared the phenotypes of a *bipA* mutant to other ribosome assembly factors such as DeaD (50S subunit biogenesis factor) (Charollais et al., 2004), RsgA (postulated 30S subunit biogenesis factor) (Campbell et al., 2008) and RluC (postulated 50S subunit biogenesis factor) (Jiang et al., 2007). We constructed a series of single and double gene deletion mutants such as Δ*bipA,* Δ*deaD* and Δ*bipA* Δ*deaD* and compared their phenotypes. We tested for cold sensitivity, motility and

chloramphenicol sensitivity as these are some of the conditions under which *bipA* mutants have been reported to have observable phenotypes.

Deletion of *bipA* and *deaD* have a Synergistic Effect on Cold Sensitivity

DeaD is a ribosome biogenesis factor and mutants lacking *deaD* display a cold-sensitive phenotype (Charollais et al., 2004). We investigated whether deletion of *deaD* had any effect on the cold sensitivity of a *bipA* mutant. Our rationale was that, exacerbation of cold sensitivity of a *bipA* mutant by *deaD* deletion could indicate involvement of BipA in ribosome biogenesis and/or assembly. Therefore WT, Δ*bipA,* Δ*deaD,* and Δ*bipA* Δ*deaD* strains were grown at 37°C and at 20°C and their growth behavior was monitored (Figure 9). At 37°C, all strains exhibited similar growth characteristics. At 20°C, both Δ*bipA* and Δ*deaD* strains displayed a cold-sensitive phenotype (as expected) although Δ*deaD* grew even more slowly than Δ*bipA*. However, deletion of both *bipA* and *deaD* resulted in an exacerbated phenotype. The *bipA deaD* double mutant displayed a longer lag phase and delayed entry into stationary phase compared to the wild-type or the individual mutants. These data suggest that, similar to DeaD, BipA might be involved in ribosome assembly and/or biogenesis.

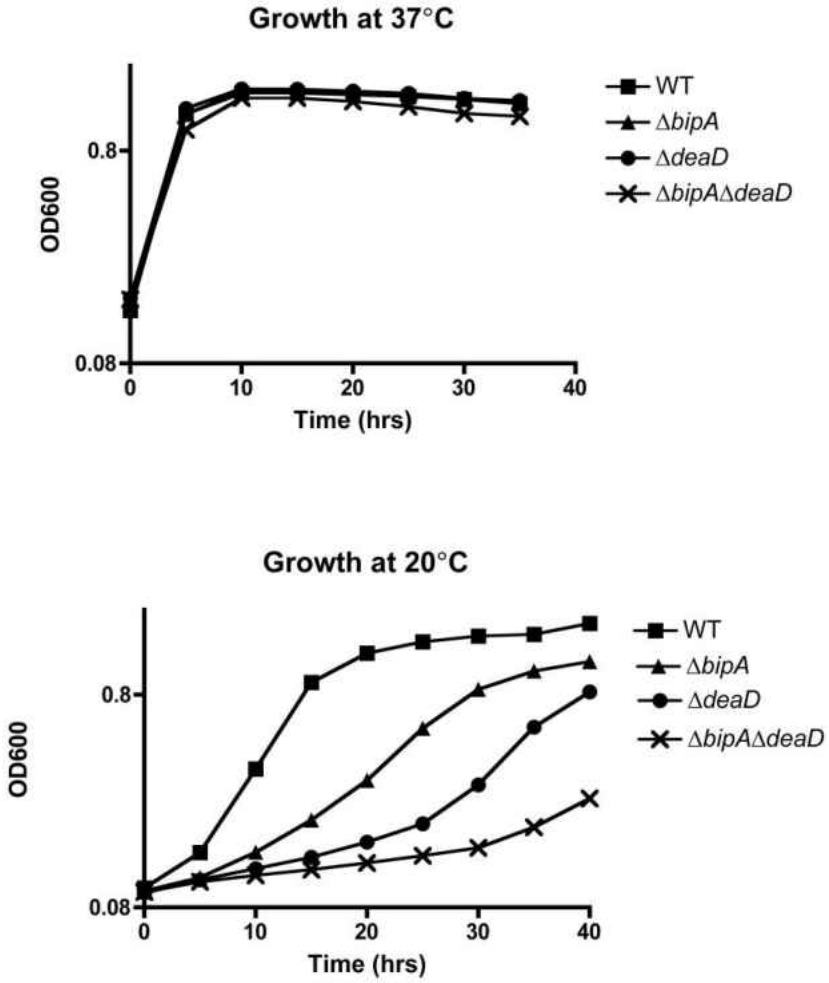


Figure 9: Deletion of *bipA* and *deaD* have a synergistic effect on cold sensitivity: Growth analyses were performed with wild-type (MG1655), Δ*bipA* (PC28), Δ*deaD* (PC95) and Δ*bipA* Δ*deaD* (PC96) strains grown at optimal temperature (37°C) or at low temperature (20°C) using a Bioscreen C Microbiology Reader as described in materials and methods. The experiment was done at least three times with three biological replicates.

Deletion of *bipA* and *deaD* have a Synergistic Effect on Chloramphenicol Sensitivity

Genetic screening by random transposon mutagenesis to identify mutants with increased or decreased resistance to the antibiotic chloramphenicol suggested that mutation in the *bipA* gene leads to increased susceptibility to the antibiotic in comparison to wild type strains (Duo et al., 2008). Because deletion of *deaD* had a synergistic effect on the cold-sensitive phenotype of a *bipA* mutant, we determined whether *deaD* deletion had any effect on the chloramphenicol

sensitivity of a *bipA* mutant. We compared the growth patterns of Δ*bipA*, Δ*deaD,* and Δ*bipA* Δ*deaD* mutants to the wild-type strain in the presence and the absence of chloramphenicol (Figure 10). As noted by Duo and co workers (2008), the Δ*bipA* strain demonstrated slowed growth in the presence of chloramphenicol. We also observed slightly decreased growth of the *deaD* mutant in the presence of the antibiotic. Furthermore, the double mutant displayed a synergistic effect with a decreased growth rate compared to wild-type or either individual mutant. As with the cold sensitivity, the exacerbated phenotype suggests that the two genes are important for similar processes.

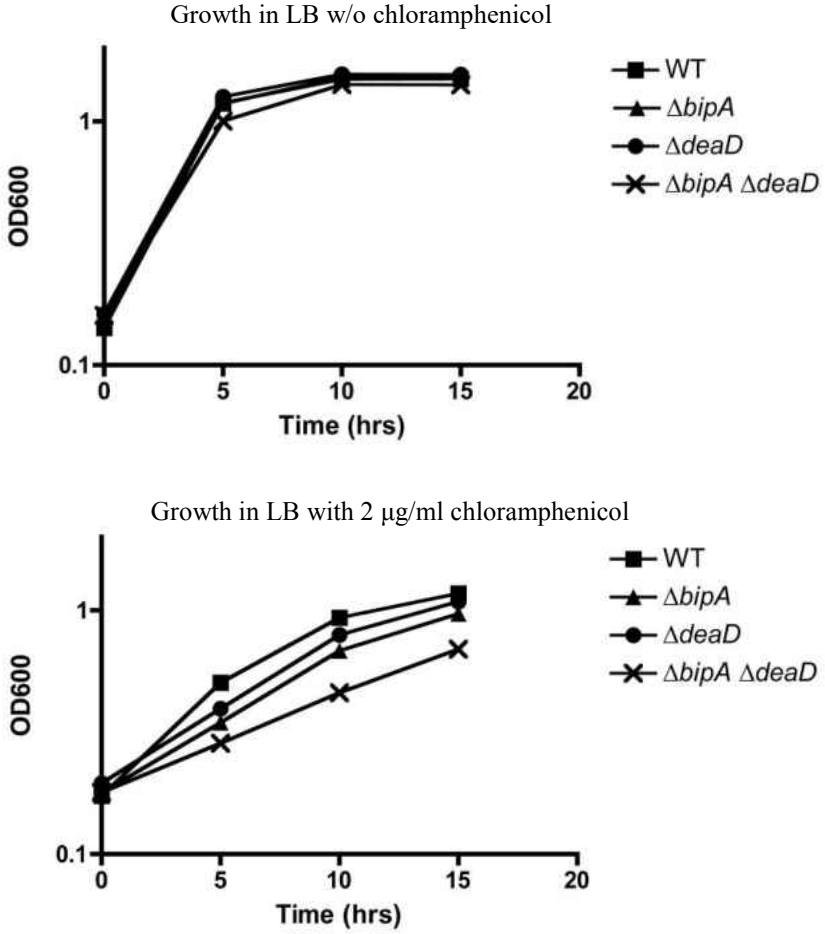


Figure 10: Deletion of *bipA* and *deaD* have a synergistic effect on chloramphenicol sensitivity: Wild-type, Δ*bipA*, Δ*deaD*, and Δ*bipA* Δ*deaD* strains were grown in LB media with or without chloramphenicol and growth was monitored using a Bioscreen C Microbiology Reader as described in materials and methods. Experiments were done at least three times with three biological replicates.

Deletion of *bipA* and *deaD* have a Synergistic Effect on Motility

In EPEC strain E2348/69, BipA was implicated to be involved in the negative regulation of flagell-mediated motility and flagellin biosynthesis, as *bipA* mutants were hyper-motile and secreted large amounts of flagellin into the culture media (Farris et al., 1998, Grant et al., 2003). To continue our investigation of the relationship between *bipA* and *deaD,* we investigated whether *deaD* deletion also affected motility (Figure 11). The swarming pattern of wild-type, Δ*bipA,* Δ*deaD* and Δ*bipA* Δ*deaD* strains on motility agar plates was monitored at regular intervals. In contrast to previous observations reported by other groups, we did not observe the hyper-motile behavior of a Δ*bipA* strain compared to the wild-type strain. Although we are unsure of the reason for this discrepancy, the difference in strain background could be the reason for the loss of hyper-motility of a *bipA* mutant as our experiments were performed with a non-pathogenic laboratory strain of *E. coli*, K-12/MG1655, whereas the hyper-motility and the hyper-flagellation phenotypes were observed with EPEC strain E2348/69. In this experiment, the Δ*deaD* strain also behaved similar to wild-type. However, the Δ*bipA* Δ*deaD* mutant displayed a reduced level of motility compared to the wild-type or either single mutant.

To confirm that the difference in motility of the four experimental strains was not due to a difference in growth rate, we monitored growth of wild-type, Δ*bipA,* Δ*deaD* and Δ*bipA* Δ*deaD* strains in tryptone broth at 34°C (Figure 12). We observed no difference in growth in the four strains confirming that the decreased motility of the Δ*bipA* Δ*deaD* was not due to decreased growth of the latter in this media.

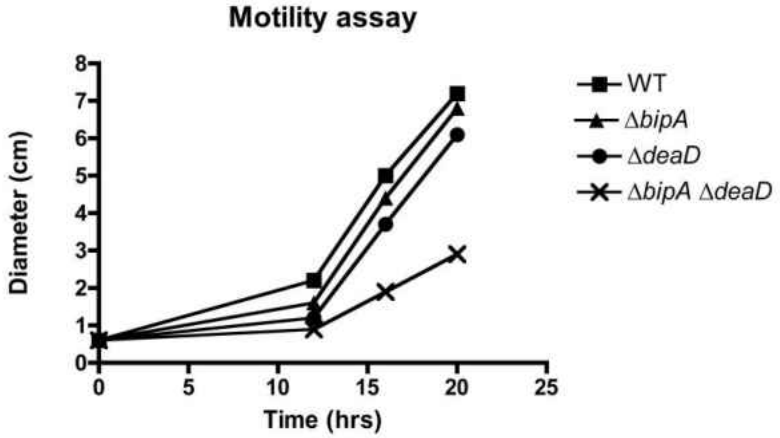


Figure 11: Deletion of *bipA* and *deaD* have a synergistic effect on motility: Motility assays were performed with a wild-type (MG1655), Δ*bipA,* Δ*deaD* and Δ*bipA* Δ*deaD* strains as described in materials and methods. Experiments were conducted at least three times to confirm results.

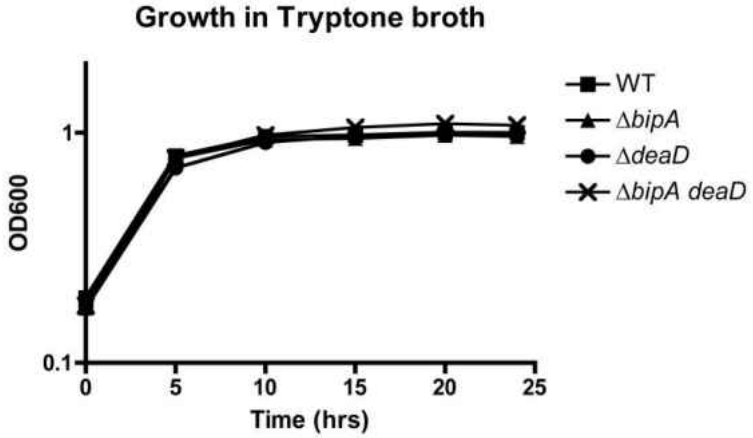


Figure 12: Growth in tryptone broth: WT, Δ*bipA,* Δ*deaD* and Δ*bipA* Δ*deaD* strains were grown overnight in tryptone broth at 37°C. The following day strains were subcultured (1:100) in the same media and grown at 34°C. Growth was measured using a Bioscreen C Microbiology Reader and OD_{600} of each of the strains vs time was measured and plotted as described in materials and methods.

Deletion of *rluC* does not Alleviate Cold Sensitivity of a Δ*deaD* Mutant

Previous research from our laboratory indicated that deletion of *rluC* alleviated the cold sensitivity of a *bipA* mutant when grown at 20°C (Krishnan et al., 2008). Since deletion of *deaD* exacerbated the cold sensitivity of a *bipA* mutant, suggesting closely related functions for BipA

and DeaD, we investigated the effect of *rluC* deletion on the cold-sensitive phenotype of a *deaD* mutant.

Growth was monitored of wild-type (MG1655), Δ*bipA* (PC28), Δ*rluC* (PC33), and Δ*bipA* Δ*rluC* (PC34) strains at 20°C (Figure 13). Our results replicated our previously published observation that deletion of *rluC* rescues the cold-sensitive phenotype of a *bipA* mutant. We then compared growth of a Δ*deaD* Δ*rluC* double mutant strain at 20°C to wild-type and the individual mutants (Figure 14). We found that deletion of *rluC* did not alleviate Δ*deaD* cold sensitivity, suggesting that the absence of the three pseudouridine residues added by RluC does not allow ribosome assembly to take place independent of DeaD.

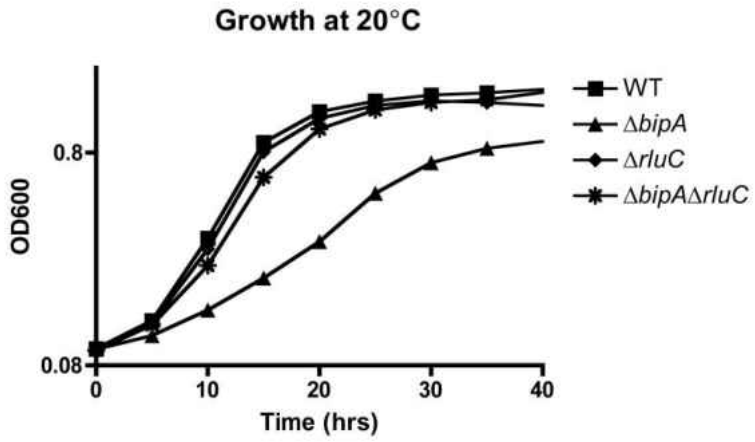


Figure 13: Deletion of *rluC* alleviates the cold sensitivity of a Δ*bipA* strain: Wild type (MG1655), Δ*bipA*, Δ*rluC* and Δ*bipA* Δ*rluC* strain were grown at 20°C and growth was monitored using a Bioscreen C Microbiology Reader as described in materials and methods. Experiments were repeated three times with three biological replicates.

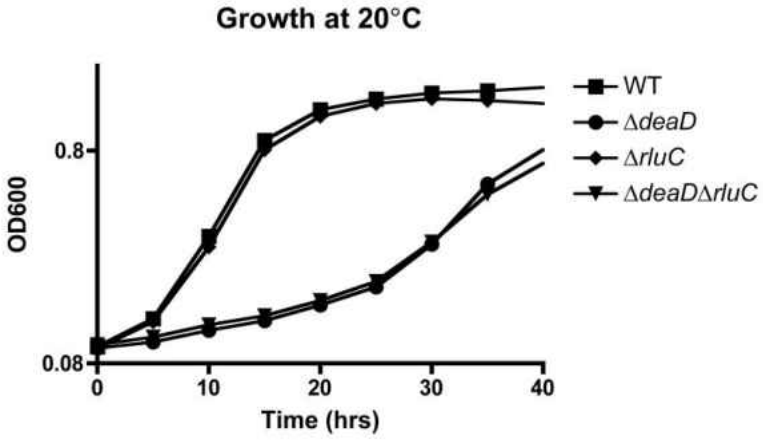


Figure 14: Deletion of *rluC* does not rescue Δ*deaD* cold sensitivity: Wild type, Δ*deaD*, Δ*rluC* and Δ*deaD* Δ*rluC* strain were grown at 20°C and growth was monitored using a Bioscreen C Microbiology Reader as described in materials and methods. Experiments were done three times with three biological replicates.

Deletion of *bipA* Increases Resistance to High Salt Concentration

Hase and co-workers (2009) reported that removal of the RsgA (Ribosome Small Subunit-Dependent GTPase A) GTPase confers resistance to *E. coli* cells against high salt stress (Hase et al., 2009). RsgA has been suggested to be involved in the maturation of the small ribosomal subunit (Campbell et al., 2008) and the GTPase activity of RsgA was highly enhanced in the presence of the small subunit (Daigle et al., 2004). Additionally, *rsgA* deletion mutants exhibit a slow-growth phenotype, accumulate increased proportions of 50S and 30S subunits (Campbell et al., 2008) and are defective in 16S rRNA processing (Himeno et al., 2004). Based on these observations, we sought to determine whether removal of *bipA* also conferred salt resistance. Wild-type and Δ*bipA* strains were grown in LB media with or without added NaCl (1M) and growth was monitored (Figure 15). We found that similar to an *rsgA* mutant, *bipA* mutants were resistant to high salt concentration. This data further supported our hypothesis and suggested the involvement of BipA in ribosome assembly

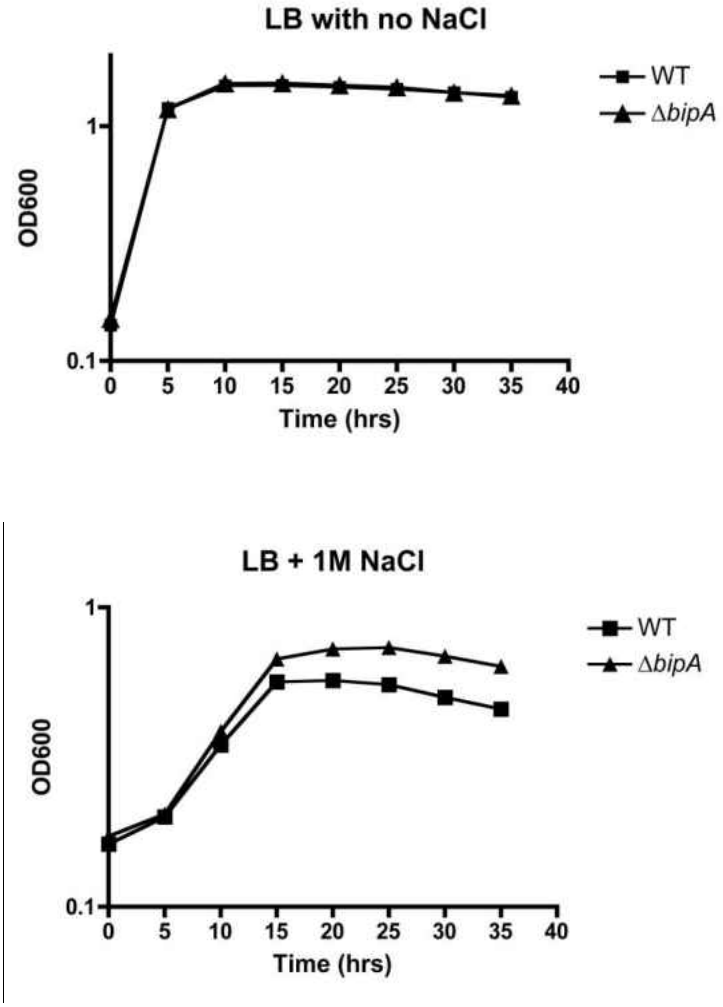


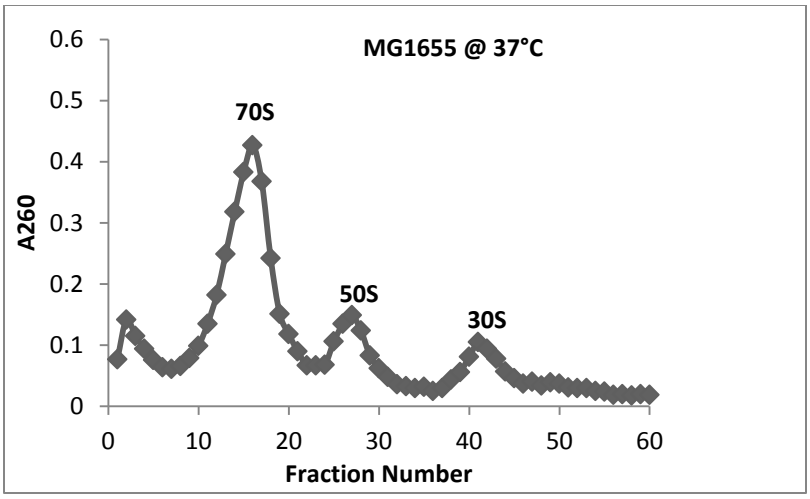
Figure 15: Mutants of *bipA* are resistant to salt stress: A wild-type and *bipA* deletion mutant were grown at 37°C in LB media without (up) or with (down) added NaCl (1M) and growth was monitored as described in materials and methods. Experiments were repeated at least three times with three biological replicates.

Approach 2: Our results so far corroborated our hypothesis that BipA is involved in the complex process of ribosome assembly/biogenesis because phenotypes of a *bipA* mutant were significantly exacerbated by deletion of *deaD,* implicating closely related functions for BipA and DeaD. Next we sought to determine if deletion of *bipA* results in a ribosome assembly defect similar to that observed in a *deaD* mutant. Since deletion of *rluC* or *deaD* alleviated or exacerbated the phenotypes of a *bipA* mutant respectively, we also investigated whether *rluC* or *deaD* deletion influenced the ribosome assembly process of a *bipA* mutant.

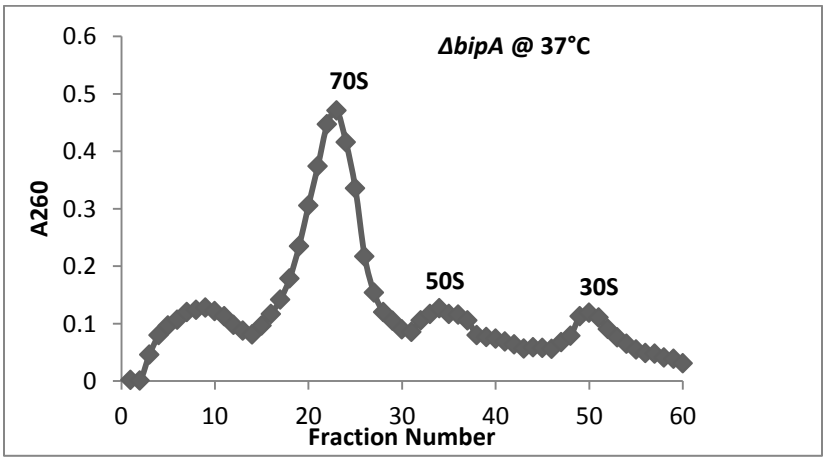
Deletion of *bipA* Results in Ribosome Assembly Defects

To analyze possible ribosome assembly defects in a *bipA* mutant, we compared the ribosome profiles of a wild-type and a *bipA* mutant grown at 37°C and at 20°C (Figure 16). Ribosomes were isolated from growing cells and separated on sucrose gradients. Spectrophotometric detection of the rRNA allowed us to determine relative ratios of 70S ribosomes, 50S subunits, and 30S subunits in each sample. The profiles of wild-type and the *bipA* mutant were similar when grown at 37°C (Figure 16 A and B), but when grown at 20°C the ribosome profile of a *bipA* mutant was significantly altered compared to wild-type and was characterized by a decreased proportion of 50S subunits, increased levels of 30S subunits and apparent accumulation of a minute proportion of precursor 50S particle fractionating at a slightly lower density than 50S (Figure 16 D). This observation was intriguing as mutants of ribosome assembly factors, particularly the 50S assembly factors such as SrmB or DeaD when grown at cold temperature, exhibit ribosomal defects (Charollais et al., 2003, 2004) very similar to what we observed. These data support our previous observations and hypothesis suggesting that BipA is involved in ribosome assembly as bacterial cells lacking *bipA* exhibit defective ribosome assembly when grown at 20°C.

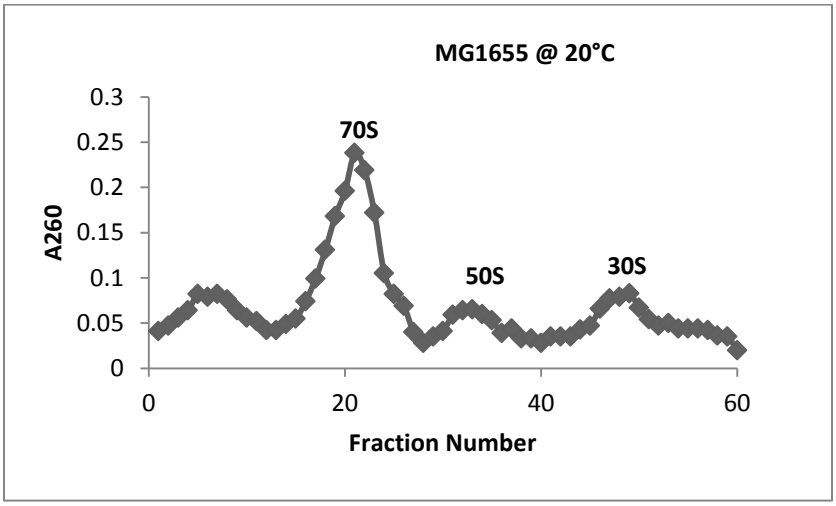
A.


MG1655 @ 37°C
70S
50S
30S
A260
Fraction Number
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60

B.


ΔbipA @ 37°C
70S
50S
30S
A260
Fraction Number
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60

C.


MG1655 @ 20°C
70S
50S
30S
A260
Fraction Number
0.3
0.25
0.2
0.15
0.1
0.05
0
0
20
40
60

.

Figure 16 cont.

D.

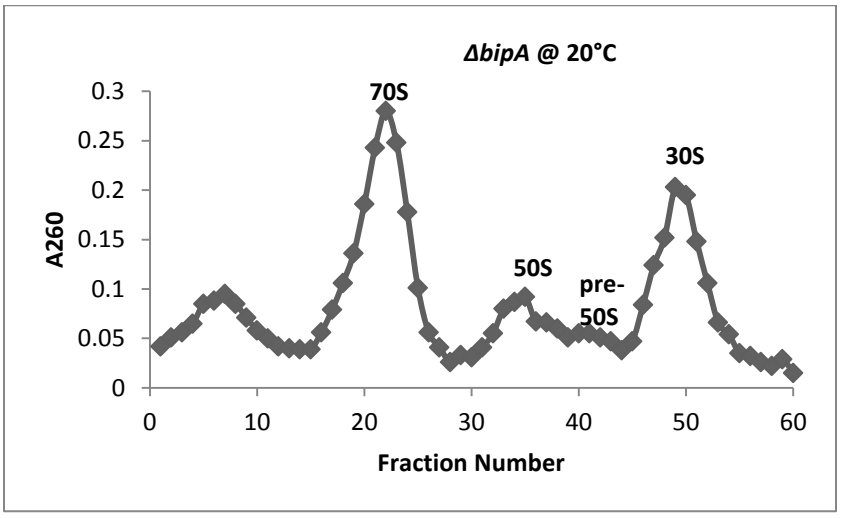
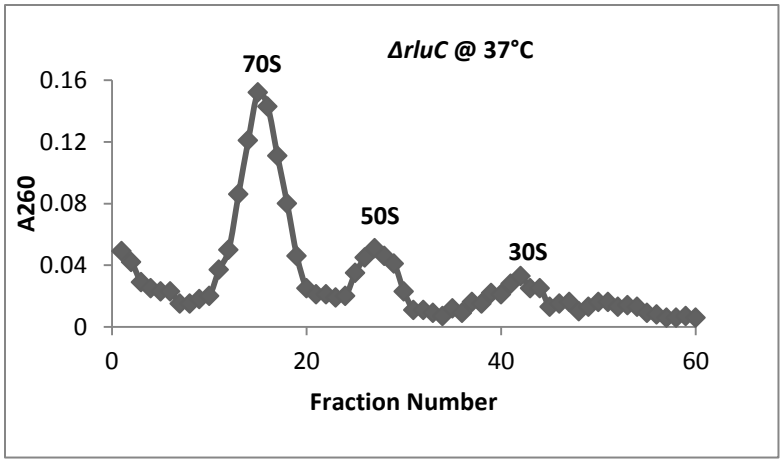


Figure 16: Deletion of *bipA* leads to ribosome assembly defects at 20°C: Comparison of ribosome profiles of wild-type and *bipA* mutant grown at 37°C (A and B) or 20°C (C and D). Experiments were performed as described previously in material and methods. Fractions were removed from the tubes such that early fractions were higher density than later fractions.

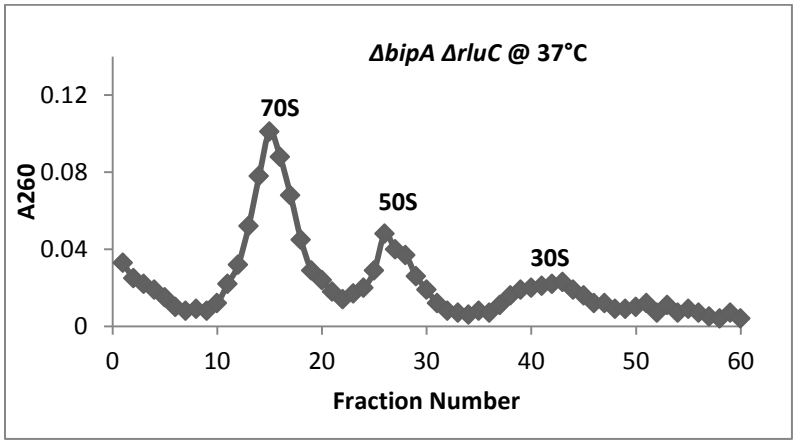
Deletion of *rluC* Alleviates the Ribosome Assembly Defects of a *bipA* Mutant

As mentioned previously deletion of *rluC* alleviates the cold-sensitivity and the capsule synthesizing defect of a Δ*bipA* mutant (Krishnan et al., 2008). Since Δ*bipA* strains exhibit defective ribosome assembly, we investigated whether the ribosomal defects of a *bipA* mutant were affected by *rluC* deletion. We compared the ribosome profiles of wild-type, Δ*bipA*, Δ*rluC* and Δ*bipA* Δ*rluC* strains grown at 37°C (Figure 17 A and B) or 20°C (Figure 17 C and D). No differences in the ribosome profiles of the different strains were observed at 37°C. However, at 20°C deletion of *rluC* alleviated the ribosome assembly defects of a *bipA* mutant (Figure 17D). The ribosome profile of a *bipA rluC* double mutant was similar to that of the wild-type (Compare figure 16 C and 17 D) suggesting that the *rluC* deletion enabled bacterial cells to assemble functional ribosomes independent of BipA. We observed no difference in the ribosome profile of the *rluC* mutant grown at either temperature (Figure 17 A and C).

A.


ΔrluC @ 37°C
70S
50S
30S
A260
0.16
0.12
0.08
0.04
0
0
20
40
60
Fraction Number

B.


ΔbipA ΔrluC @ 37°C
70S
50S
30S
A260
0.12
0.08
0.04
0
0
10
20
30
40
50
60
Fraction Number

C.

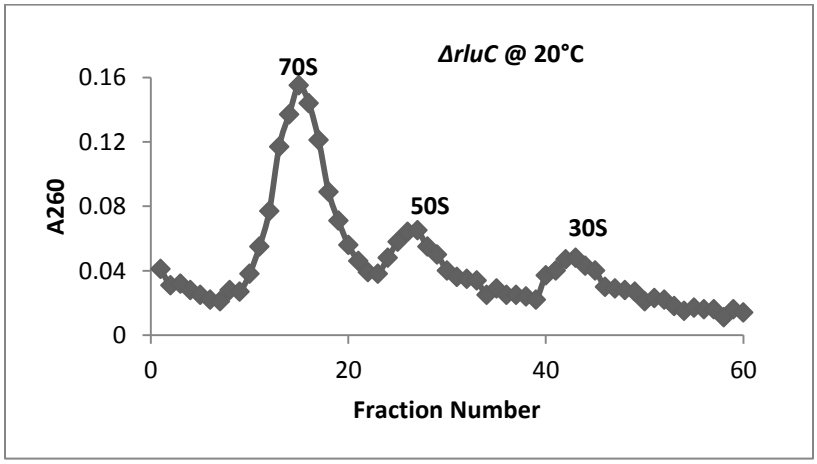

ΔrluC @ 20°C
70S
50S
30S
A260
0.16
0.12
0.08
0.04
0
0
20
40
60
Fraction Number

Figure 17 cont.

D.

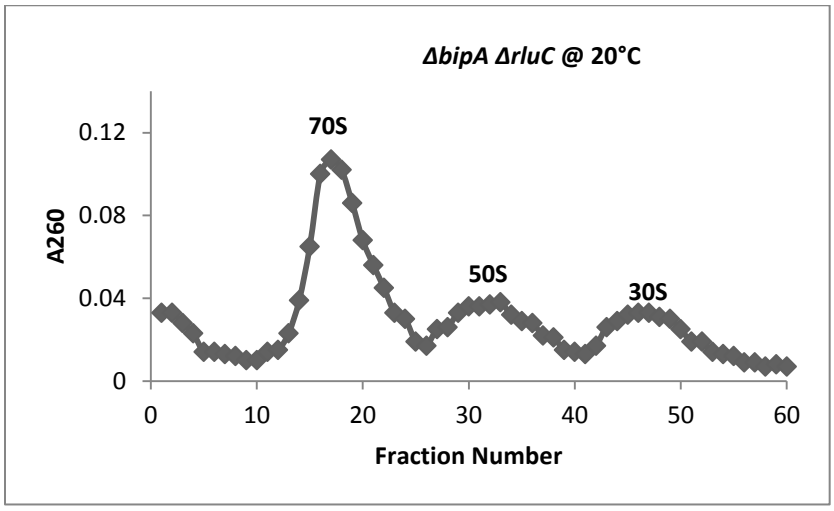


Figure 17: Deletion of *rluC* alleviates the ribosomal defects of a *bipA* mutant: Ribosome profile analyses of an *rluC* mutant and a *bipA rluC* double mutant grown at 37°C (A and B) or 20°C (C and D). Experiments were performed as described previously (Refer Figure 16 for ribosome profiles of wild-type and *bipA* mutant).

Deletion of *bipA* and *deaD* Affects Ribosome Assembly Defects Synergistically

As discussed previously the DeaD protein has been reported to be a 50S biogenesis factor and deletion of *deaD* in *E. coli* leads to ribosome biogenesis defects with decreased levels of 50S subunits, increased levels of 30S subunits and accumulation of a precursor 50S particle (Charollais et al., 2004). This precursor 50S particle was consisted of a precursor 23S rRNA (p23S) and the abundance of the late ribosome assembly proteins was relatively low in a *deaD* deletion mutant compared to a wild-type, suggesting the involvement of DeaD in the late stages of ribosome assembly (Charollais et al., 2004).

Analyses of growth of a wild-type, a *bipA* mutant, a *deaD* mutant and a *bipA deaD* double mutant at 20°C revealed that deletion of *deaD* exacerbated the growth defect of a Δ*bipA* mutant. Therefore we predicted that *deaD* deletion would also exacerbate the ribosome assembly defects of a *bipA* mutant (Figure 18). We generated ribosome profile of the *bipA deaD*

double mutant strain grown at 37°C (Figure 18 B) and 20°C (Figure 18 D) and compared that to ribosome profiles of the parent strain as well as the individual mutants (Compare figure 16 and figure 18).

The ribosome profile of the *deaD* mutant at 37°C was similar to wild-type and the *bipA* mutant grown at the same temperature (Compare figure 18 A and figure 16 A, B). However at 20°C, the ribosome profile of a Δ*deaD* mutant indicated defective ribosome assembly, characterized by decreased level of 50S subunits, increased level of 30S subunits and accumulation of a pre 50S particle (Figure 18 C). These results are consistent with those that have been previously reported (Charollais et al., 2008). The double mutant, Δ*bipA* Δ*deaD,* displayed even grater defects in ribosome profiles, with reduced 70S levels, increased 50S and 30S subunits, and accumulation of a precursor 50S particle. Exacerbation of the ribosomal assembly defects of a *bipA* mutant by deletion of *deaD* suggested that both these proteins are involved in ribosome assembly/biogenesis during low temperature growth.

A.

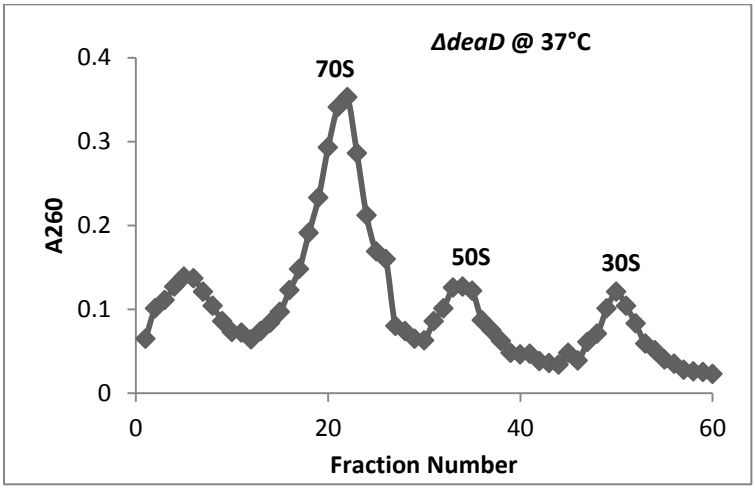
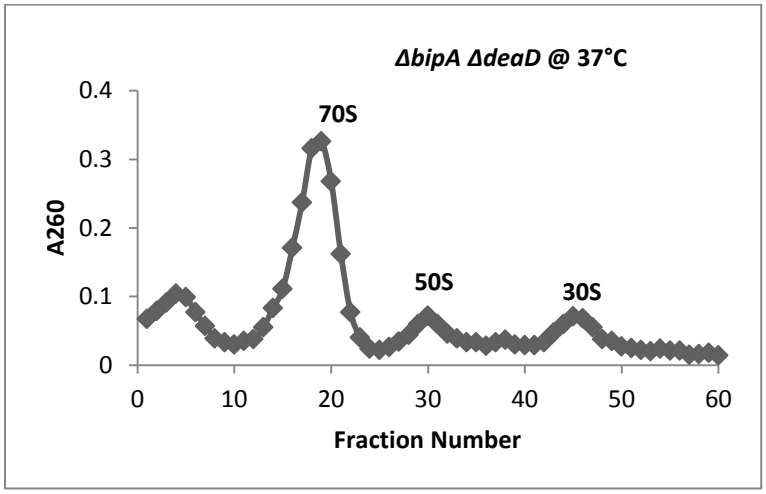
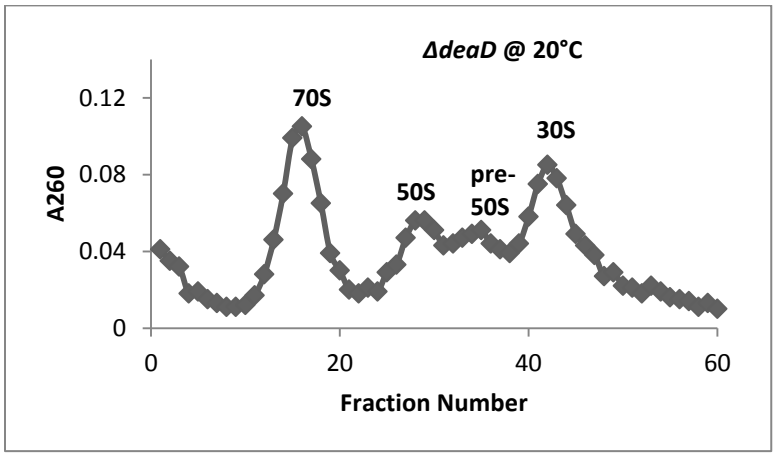


Figure 18 cont.

B.



C.



D.

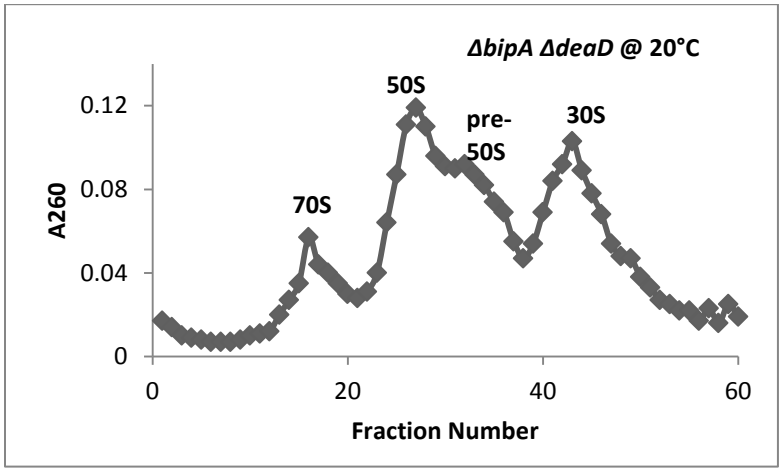


Figure 18: Deletion of *bipA* and *deaD* affects ribosome assembly defects synergistically: Ribosome profile analyses of a *deaD* mutant and a *bipA deaD* double mutant grown at 37°C or 20°C. Experiments were performed as previously described (Refer Figure 16 for ribosome profiles of wild-type and *bipA* mutant).

Deletion of *bipA* Results in a 23S rRNA Processing Defect

Defects in ribosome assembly are often accompanied by rRNA processing defects as processing of the rRNAs is accomplished in the polysomes (Srivastava et al., 1990). Mutants lacking ribosome assembly factors such as DeaD or SrmB exhibit RNase III processing defects of the 30S primary transcript resulting in accumulation of 23S and/or 16S rRNA precursors, p23S and/or p16S (17S) respectively (Charollais et al., 2003, 2004). Thus, impaired 50S subunit biogenesis can lead to accumulation of the p23S rRNA particle and sometimes the 17S rRNA, as an indirect consequence of the 50S biogenesis defect (Charollais et al., 2003).

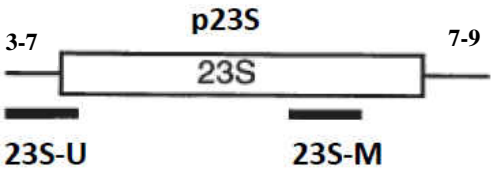
Wild-type bacteria grown under optimal growth conditions accumulate 17S rRNA because 17S rRNA does not require processing by RNases for maturation or assembly into fully formed ribosomes (Srivastava et al., 1989). The p23S rRNA that accumulates in bacterial cells lacking ribosome assembly factors is three or seven nucleotides longer at the 5′- end and seven to nine nucleotides longer at the 3′- end than the mature 23S rRNA (Charollais et al., 2003, 2004). Because our ribosome profile analyses suggested a defect in ribosome assembly in the Δ*bipA* strains grown at 20°C, we further characterized this defect by monitoring 23S and/or 16S rRNA processing.

To monitor the relative abundance of the p23S precursor rRNA compared to the mature 23S rRNA we performed Northern blot analyses of rRNA isolated from the relevant strains. We used a probe (23S-M) specific for the mature sequence of the 23S rRNA and another probe (23S-U) complimentary to the upstream sequence present in the p23S rRNA and calculated the abundance of precursor using the equation p23S/(p23S + 23S) (Figure 19).

Our results suggest that there is an increase in the p23S/(p23S + 23S) ratio (0.52) in the *bipA* mutant compared to a wild-type (0.31) (Figure 19 B). Even more p23S rRNA was

observed in the *deaD* mutant (0.62) (Figure 19D). This observation is consistent with our previous results because deletion of *deaD* leads to a more severe cold-sensitive phenotype than a *bipA* mutant (Figure 9) and the ribosome assembly defects of a *deaD* mutant are also more drastic than a *bipA* mutant (Compare Figure 16 D and Figure 18 C). However surprisingly and unlike our other observations, the p23S/(p23S + 23S) ratio in the *bipA deaD* double mutant was similar to that of the *bipA* mutant (0.54) (Figure 19 D).

We assessed the ability of *rluC* deletion to suppress the phenotype of Δ*bipA*. Again we found that deletion of *rluC* rescued the 23S rRNA processing defect of the *bipA* mutant as the p23S/(p23S + 23S) ratio was decreased in the *bipA rluC* double mutant (0.35) compared to the *bipA* mutant (0.52) and was restored to levels near wild-type. This observation is consistent with our previous results where *rluC* deletion alleviated the ribosome assembly defects of the *bipA* mutant. The p23S/(p23S + 23S) ratio for the *rluC* mutant alone was 0.42, slightly higher than wild-type suggesting that there is a slight 50S subunit biogenesis defect in a *rluC* mutant at 20°C. This observation was not surprising since RluC has been previously reported to be associated with a pre-50S ribosomal particle, suggesting that RluC might be involved in 50S subunit biogenesis (Jiang et al., 2007).

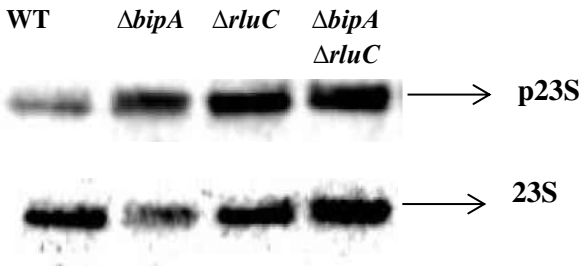


A.

B.

Strains	P/(P+M)
Wild-type	0.31
ΔbipA	0.52
ΔrluC	0.42
ΔbipAΔrluC	0.35
ΔdeaD	0.62
ΔbipAΔdeaD	0.54

C.



D.

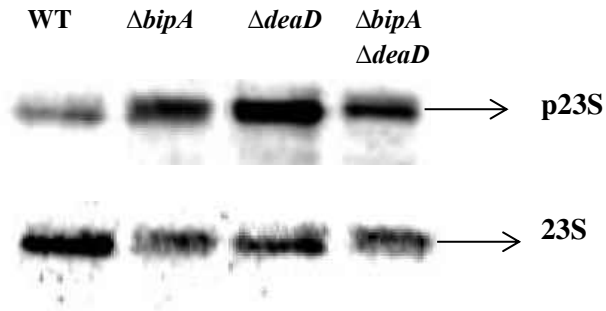


Figure 19: Deletion of *bipA* leads to 23S rRNA processing defect: Northern blot analyses were performed as described in materials and methods. (A) Schematic representation of the 23S rRNA showing the mature, upstream and downstream regions along with the annealing sites for the 23S-U and 23S-M probes (Charollais et al., 2003). Northern blot analysis results with: (B) Wild-type and Δ*bipA* rRNA, (C) Wild-type, Δ*bipA,* Δ*rluC* and Δ*bipA* Δ*rluC* rRNA and (D) Wild-type, Δ*bipA,* Δ*deaD* and Δ*bipA* Δ*deaD* rRNA.

A similar approach was used to examine the level of 17S rRNA in our mutants. In this case the 17S rRNA can be distinguished from the 16S rRNA with a single probe as the precursor is 115 nucleotides longer than the 16S rRNA at the 5′- end and 33 nucleotides longer at the 3′- end and they will migrate as distinct bands on agarose gels. We compared the accumulation of 17S rRNA in the Δ*bipA* strain grown at 20°C versus the wild-type strain grown under the same conditions (Figure 20 A). There was a slight increase in the 17S/(16S + 17S) ratio (0.30) in the *bipA* mutant compared to the wild-type (0.23). We again analyzed the effect of the double deletion, Δ*bipA* Δ*deaD*, as well as the capacity for Δ*rluC* to alleviate any effects of Δ*bipA*. The defect was slightly exacerbated by *deaD* deletion (0.38) (Figure 20 D). Additionally, deletion of *rluC* rescued the minor 16S rRNA processing defect of the *bipA* mutant as the 17S/(16S + 17S) ratio was decreased in the *bipA rluC* double mutant (0.18) compared to the *bipA* mutant (0.30) (Figure 20 C). This observation is again consistent with our previous results.

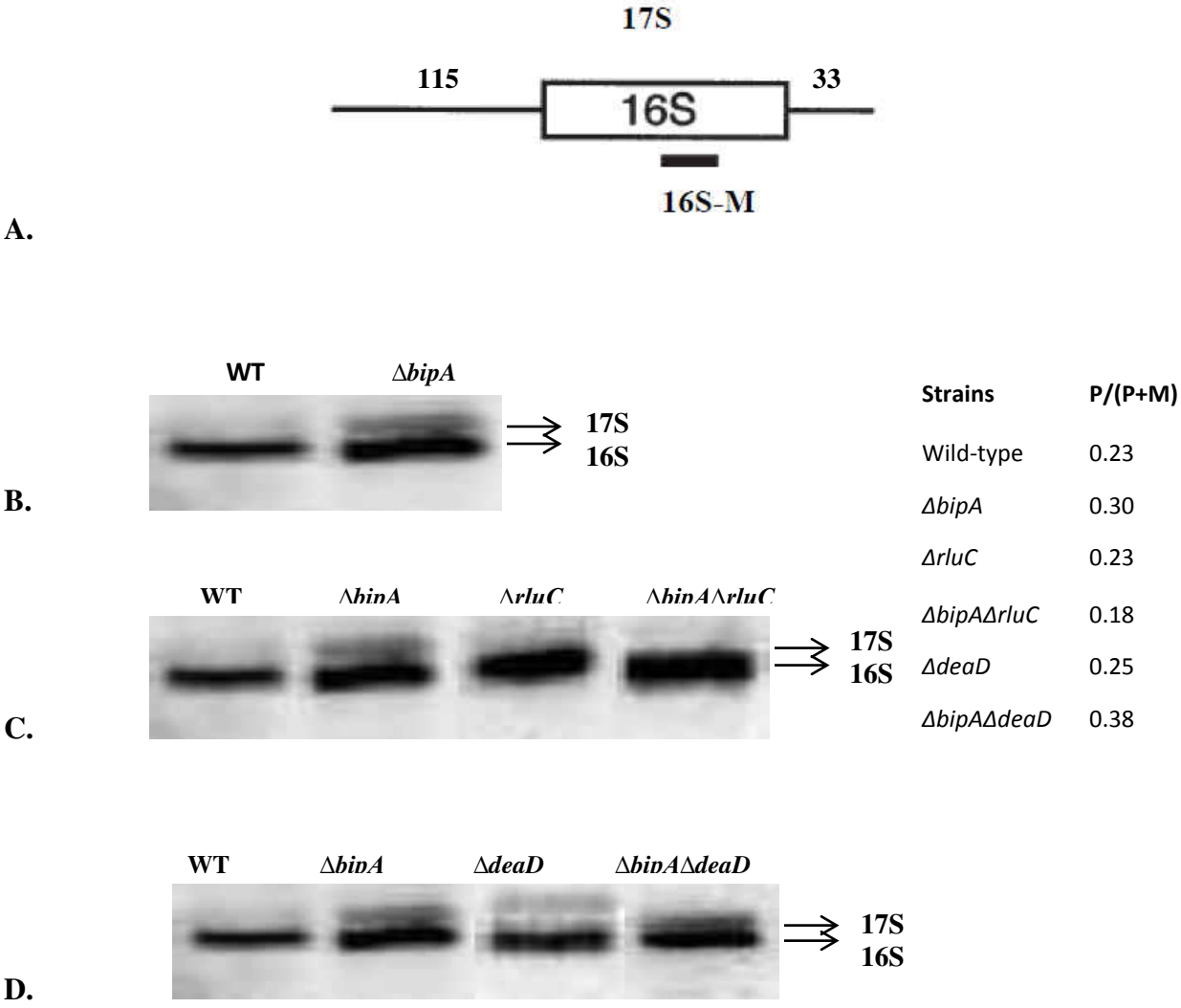
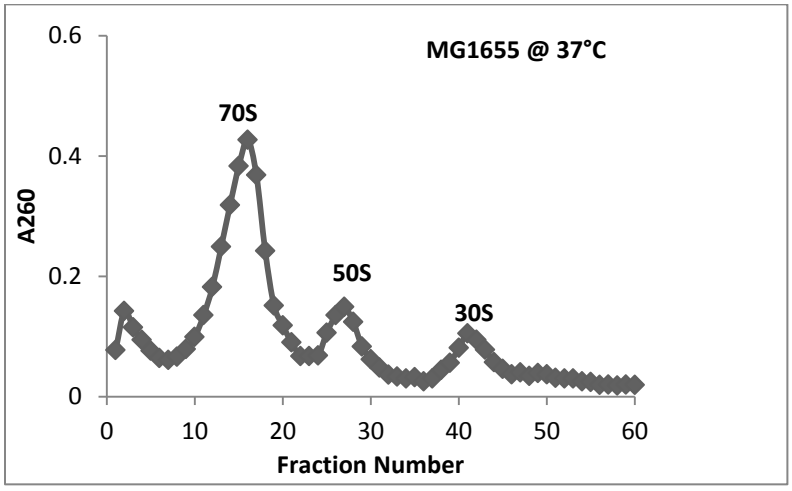


Figure 20: Deletion of *bipA* result in a slight 16S rRNA processing defect: Northern blot analyses were performed as described in materials and methods. (A) Schematic representation of the 16S rRNA and 17S showing the mature, upstream and downstream regions along with the annealing site for the 16S-M probe (Charollais et al., 2003). Northern blot analysis results with: (B) Wild-type and Δ*bipA* rRNA, (C) Wild-type, Δ*bipA,* Δ*rluC* and Δ*bipA* Δ*rluC* rRNA and (D) Wild-type, Δ*bipA,* Δ*deaD* and Δ*bipA* Δ*deaD* rRNA.

Deletion of *lepA* Does not Result in Defective Ribosome Assembly

LepA (also known as EF4) is a member of the elongation factor family of GTPases and shares protein domain homology to BipA (Finn et al., 2008). Since BipA and LepA are structurally homologous and Δ*bipA* strains demonstrated defective ribosome assembly, we investigated whether deletion of *lepA* also leads ribosomal defects at 20°C (Figure 21). Ribosome profiles of Δ*lepA* strains grown at 37°C and 20°C were compared to a wild-type. Our results demonstrate that the ribosome profile of a *lepA* mutant grown at either temperature appeared very similar to the ribosomal profile of the wild-type strain suggesting that unlike BipA, LepA is not involved in the process of ribosome assembly.

A.



B.

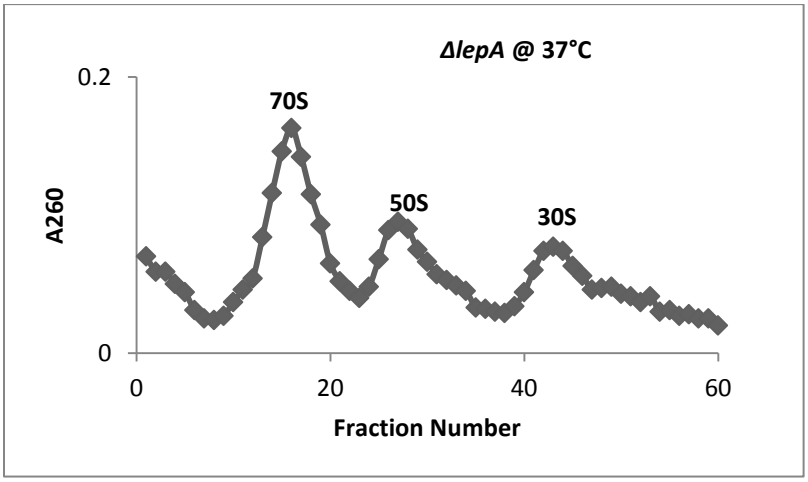
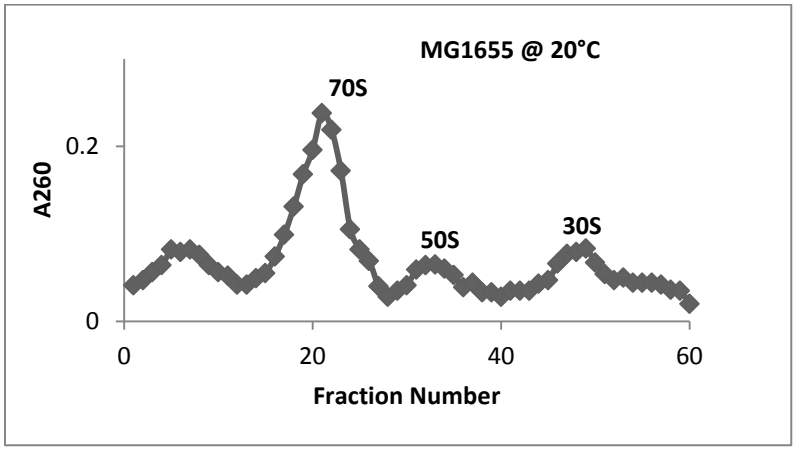


Figure 21 cont.

C.



D.

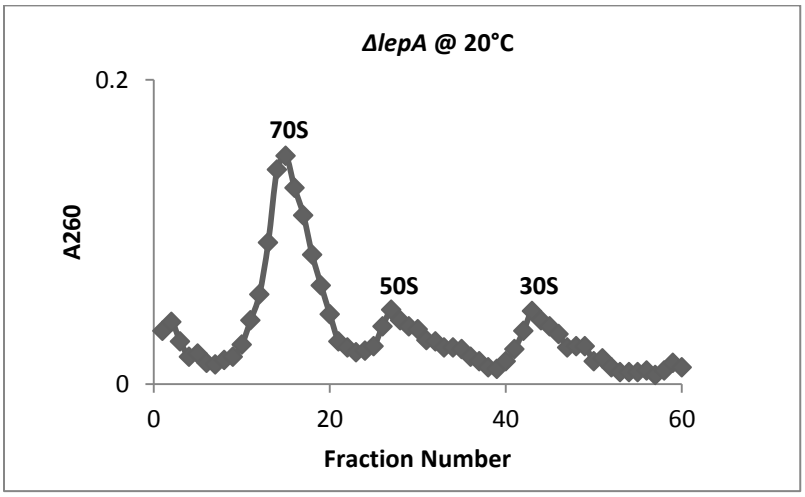


Figure 21: Deletion of *lepA* does not lead to ribosomal defects: Comparison of ribosome profiles of wild-type (MG1655) and *lepA* mutants grown at 37°C or 20°C. Ribosome profile analyses were performed as previously described.

bipA Expression is not Induced by Cold Shock

Cold-shock response: When exponentially growing bacterial cells are shifted from 37ºC to cold temperatures, such as 15ºC or below, they experience a transient inhibition of translation and protein synthesis. This phase of bacterial growth called the "acclimation phase" is characterized by a decrease in polysomes, accompanied by an increase in the number of free 70S, 50S and 30S ribosomal subunits (Thieringer et al., 1998). During this phase, the synthesis of normal cellular proteins or non-cold shock proteins drops drastically, temporarily inhibiting cell growth

(Yamanaka et al., 1999). This temporary growth inhibition acts as a signal for inducing the synthesis of cold shock proteins. It has been previously shown that the ribosome-binding cold shock proteins such as DeaD, RbfA and IF2 bind to the free ribosomal subunits and also to the free 70S ribosomes and convert the non-functional "cold-shocked" ribosomes into functional ribosomes, resuming protein synthesis and growth. During this phase called the "Cold Adapted" phase, the synthesis of the cold shock proteins gradually drops and the synthesis of the normal cellular proteins is again elevated allowing cell growth to resume (Yamanaka et al., 1999). Thus many of the known ribosome assembly factors such as DeaD and RbfA are also members of the cold shock family of proteins (Thieringer et al., 1998).

Because our results demonstrated a plausible involvement of BipA in bacterial ribosome assembly/biogenesis we investigated whether BipA was also a cold shock protein. The level of *bipA* expression was compared in wild-type cells grown at 37°C and 15°C by qRT-PCR (Figure 22). We found that *bipA* expression was not altered by cold shock, suggesting that, unlike DeaD, BipA is not a cold shock protein.

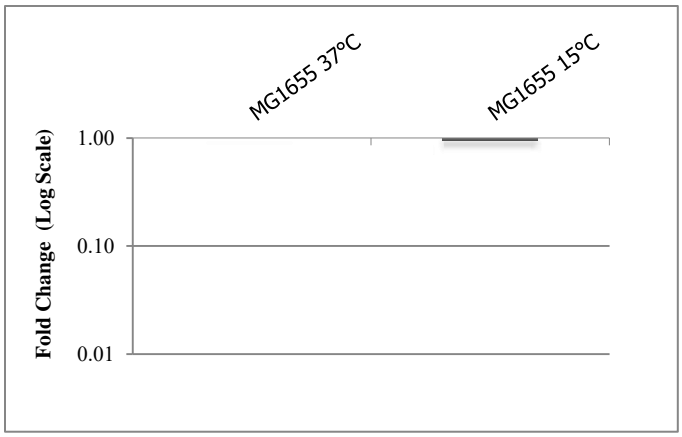
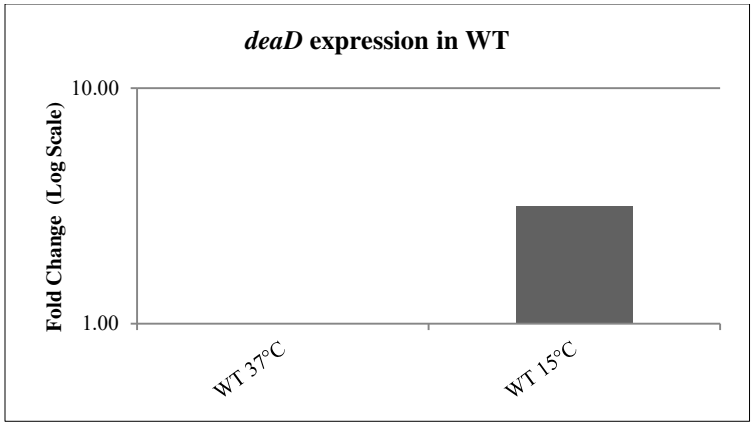


Figure 22: *bipA* expression is not induced during cold shock response: Comparison of *bipA* expression in wild-type (MG1655) grown at 37°C or cold shocked at 15°C. qRT-PCR analyses were performed as described in materials and methods. Experiments were performed at least three times with three biological replicates.

Deletion of *bipA* Does not Affect *deaD* Expression

Deletion of *deaD* exacerbated the phenotypes and the ribosomal defects of a *bipA* mutant leading to the speculation that BipA and DeaD might be involved in the same physiological pathway. If BipA and DeaD were members of the same pathway, we hypothesized that deletion of *bipA* should influence *deaD* expression. We monitored *deaD* expression level in a *bipA* mutant grown at 37°C or cold-shocked at 15°C and compared it to the wild-type (Figure 23). As expected since DeaD is a cold-shock protein (Jones et al., 1996), expression of *deaD* was induced more than three-folds in both the wild-type and *bipA* mutant by cold-shock. However, *deaD* expression in a *bipA* mutant was increased to a similar level as in the wild-type, suggesting that expression of *deaD* is independent of BipA regulation (Figure 23).



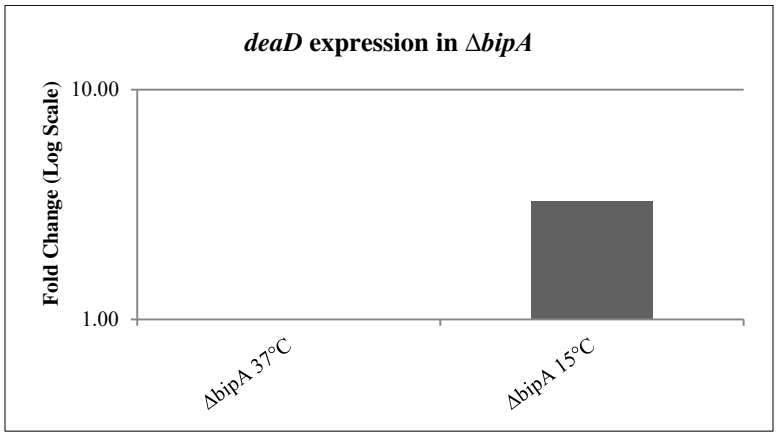


Figure 23 cont.

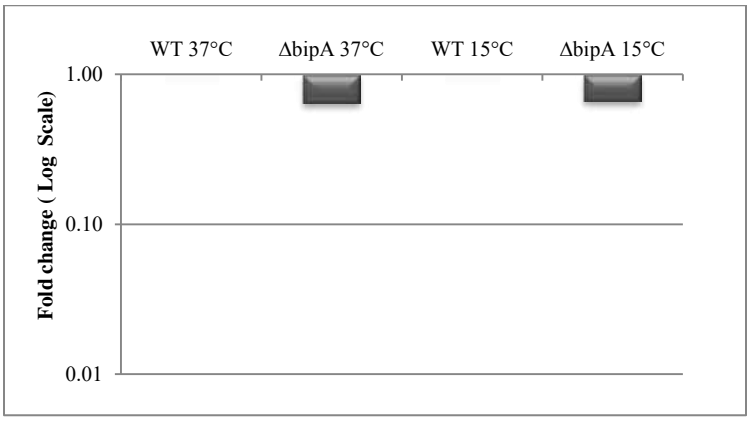


Figure 23: Deletion of *bipA* does not induce *deaD* expression after cold shock: Comparison of *deaD* expression in a wild-type and Δ*bipA* mutant grown at 37°C or cold shocked at 15°C. qRT-PCR analyses were performed as described in materials and methods. Experiments were performed at least three times with three biological replicates.

Taken together our results demonstrate that deletion of *deaD* exacerbates the cold-sensitivity, motility, and chloramphenicol sensitivity of a *bipA* mutant. Deletion of *bipA* also results in a 50S subunit biogenesis defect which is characterized by an increased proportion of the 30S subunit, decreased proportion of the 50S subunit and accumulation of a small proportion of a precursor 50S particle. The 50S biogenesis defect of a *bipA* mutant is exacerbated by *deaD* deletion, alleviated by *rluC* deletion, and is characterized by a 23S rRNA processing defect. The 23S rRNA processing defect of a *bipA* mutant result in the accumulation of a p23S rRNA particle but the proportion of this p23S rRNA in a *bipA* mutant is lower than in a *deaD* mutant. We also found a slight increase in the 17S rRNA in a *bipA* mutant which is probably due to an indirect consequence of the 50S subunit biogenesis defect.

Our results also suggest that *bipA* expression is not induced by cold shock, unlike *deaD*, and regulation of *deaD* expression during a cold shock response is independent of BipA. We also demonstrate that LepA, in spite of the similarity in protein domain structure with BipA, is not involved in ribosome assembly.

CHAPTER IV

DISCUSSION

BipA is a ribosome-associated GTPase with unknown function. Deletion of *bipA* results in pleiotropic phenotypes suggesting that BipA may be involved in the regulation of numerous cellular processes (deLivron et al., 2008). However, the precise role of BipA still needs to be elucidated.

BipA is a member of the elongation factor family of GTPases which includes the translational GTPases, EF-G, EF-Tu and LepA (EF4). A comparison of the protein domain structure of EF-G, EF-Tu, LepA and BipA reveals that the latter shares distinct regions of homology to the other members of the family (Finn et al., 2008). Freestone and co-workers (1998) proposed BipA to be important for regulation of bacterial protein synthesis (Freestone et al., 1998). These observations funneled the speculation that BipA has a unique role in translational regulation.

However, an alternate hypothesis implicates BipA in the complex process of ribosome assembly. deLivron and Robinson demonstrated that in *Salmonella enterica* BipA associates with the 70S ribosome under normal cellular conditions, but under conditions of stress, BipA was found with the 30S subunit. BipA bound to the 70S ribosomes only in the GTP bound state and the GTPase activity of BipA was stimulated in the presence of ribosomes and GTP (deLivron et al., 2008). These characteristics of BipA are similar to classic ribosome assembly GTPases. Additionally, similar to mutants of other ribosome assembly factors, mutants of *bipA*

manifest a cold-sensitive phenotype (Pfenning et al., 2001), and this cold-sensitivity was alleviated by removal of all the three pseudouridine residues on the 23S rRNA of the 50S subunit that are added by the pseudouridine synthase, RluC (Krishnan et al., 2008). This correlation between the pseudouridylation status of the 23S rRNA and BipA function, as well as the phenotypes of a *bipA* mutant, suggested involvement of BipA in ribosome assembly. We followed two different approaches to investigate the cellular function of BipA in the regulation of translation and ribosome assembly/biogenesis.

BipA and the RcsBCD Pathway

As mentioned previously, *E. coli bipA* mutants are hypo-capsulated (Rowe et al., 2000) and hyper-flagellated (Farris et al., 1998). Because transcriptional regulation of the genes for synthesis of the bacterial capsule and flagella are mediated by the RcsB response regulator and the RcsA auxiliary protein of the RcsBCD pathway (Majdalani et al., 2005), we hypothesized that BipA might be a regulator of one or more genes of this pathway. Additionally, we investigated the effect of *rluC* deletion on expression of genes of this pathway as *rluC* deletion was able to suppress the cold-sensitivity and capsule synthesizing defect of a *bipA* mutant (Krishnan et al., 2008). We therefore predicted that Δ*rluC* would reverse additional phenotypes of Δ*bipA*.

RcsB, the principal regulator of the RcsBCD pathway, functions via either the RcsA-independent branch or the RcsA-dependent branch (Majdalani et al., 2005). To analyze BipA's role in regulation of the genes of the RcsBCD pathway, we first investigated whether BipA was involved in RcsA-independent regulation. Since *rprA* expression is regulated by RcsB, independent of RcsA (RcsA-independent branch) (Majdalani et al., 2002), we used an *rprA-lacZ* reporter fusion (Castanié-Cornet et al., 2006) to monitor the expression of *rprA* in a *ΔbipA* strain

and compared it to the wild-type. Our data suggest that BipA was involved in the positive regulation of *rprA* as the expression of an *rprA-lacZ* fusion was decreased in a *ΔbipA* strain. These data supported the hypothesis that BipA regulates gene expression of the RcsBCD pathway by regulating the expression of RcsB or its upstream regulators.

The RcsB-RcsA heterodimer is a negative regulator of the FlhDC transcriptional regulator which positively regulates the expression of the *fliA* sigma factor (Francez-Charlot et al., 2003). If BipA is a positive regulator of RcsB, we would expect that deletion of *bipA* would result in decreased levels of RcsB and increased levels of FlhDC and FliA. Our results support this hypothesis as expression of the *fliA-lacZ* transcriptional fusion was increased upon deletion of *bipA*.

The global regulator protein H-NS acts as a positive and/or negative regulator of multiple genes of the RcsBCD pathway which include *rcsA* (Sledjeski et al., 1995), *flhD, fliA* (Bertin et al., 1994) and *cpsB* (Rowe et al., 2000). In addition to the negative regulation by the RcsB-RcsA heterodimer (Francez-Charlot et al., 2003), the *flhD* and *fliA* genes are positively regulated by H-NS (Bertin et al., 1994). Since our data suggested that BipA is involved in the negative regulation of *fliA*, we investigated the role of BipA in regulating H-NS expression by using an *hns-lacZ* translational fusion. Our data suggested that BipA is a positive regulator of H-NS as H-NS expression was down-regulated in a *ΔbipA* mutant. This observation further suggested that BipA's regulation of the genes of the RcsBCD pathway, especially *fliA*, is not mediated via H-NS because positive regulation of H-NS by BipA would have otherwise led to increased *fliA* expression. Our results suggest that *fliA* expression is negatively regulated by BipA.

Deletion of *rluC* alleviated the cold-sensitive growth defect and the capsule synthesizing defect of a *bipA* mutant (Krishnan et al., 2008). Therefore, we monitored the effect of *rluC*

deletion and *bipA rluC* double deletion on *rprA*, *fliA* and H-NS expression. We found that *rluC* deletion exacerbated the negative regulatory effect of a *bipA* mutant, as deletion of *rluC* decreased *rprA* expression in a *bipA* mutant by almost three-fold. However, deletion of *rluC* did not alter *fliA* or H-NS expression in a *bipA* mutant suggesting that deletion of *rluC* does not always alter BipA function. Our results demonstrated that BipA and RluC work in concert to regulate the expression of *rprA* but BipA's regulation of *fliA* and H-NS is independent of RluC.

Taken together, these data support our first hypothesis and suggest that BipA is involved in the regulation of multiple genes of the RcsBCD pathway and this regulation may be mediated via RcsB or its upstream regulators but not via RcsA or H-NS. Since no data are available to suggest that BipA binds DNA, it is not clear whether BipA is a transcriptional regulator. Indeed, BipA associates with the ribosome and this association requires GTP hydrolysis. It is possible that BipA regulates the translational efficiency of target genes by an unknown and likely novel mechanism. However, further experiments using translational *lacZ* fusions to the RcsB protein or any of its upstream regulators such as RcsC and/or RcsD is required to reach a definitive conclusion.

BipA and Ribosome Assembly

BipA is a ribosome binding GTPase, and the GTPase activity of BipA is stimulated in the presence of ribosomes (deLivron et al., 2008). Additionally, the cold-sensitive phenotype of a *bipA* mutant is similar to mutants of other ribosome assembly factors such as SrmB and DeaD (Charollais et al., 2003, 2004). We therefore hypothesized that BipA is involved in ribosome assembly.

Ribosome assembly or biogenesis defects are characterized by increased or decreased proportions of individual subunits, decreased levels of fully formed ribosomes, accumulation of

premature subunits, accumulation of precursor rRNAs, and/or altered expression levels of r-proteins. To address the hypothesis that BipA is a ribosome assembly factor we took two approaches. First we compared the phenotypes of a Δ*bipA* mutant to those of known ribosome assembly factors such as DeaD and RsgA. Second, we analyzed the ribosome profiles of mutant strains and compared to the parent strain. We also examined rRNA processing patterns in the wild type and different mutants to detect any defects.

We compared the cold sensitivity, motility, and chloramphenicol sensitivity of a Δ*bipA,* Δ*deaD* and a Δ*bipA* Δ*deaD* double mutant to that of wild-type *E. coli* cells. In all cases we observed a similar pattern in which deletion of both *bipA* and *deaD* resulted in an intensification of the individual phenotypes, suggesting that BipA and DeaD may be involved in the same cellular pathway, although they likely affect different steps.

A study involving RsgA, which is a ribosome small subunit binding GTPase, suggested that removal of *rsgA* conferred resistance to high salt stress in *E. coli* and the salt shock restored proper ribosome assembly as well as corrected the 16S rRNA maturation defects in *rsgA* mutants, rendering the process of ribosome biogenesis independent of RsgA (Hase et al., 2009). Intrigued by this observation, we investigated whether deletion of *bipA* also conferred salt resistance to *E. coli* cells. Our results demonstrate that, similar to an *rsgA* mutant, a *bipA* mutant was also resistant to high concentrations (1M) of sodium chloride. This evidence further supported our hypothesis that BipA may be involved in ribosome biogenesis.

DeaD is a ribosome assembly factor (Charollais et al., 2004) and the expression of *deaD* is induced when cells are subjected to cold-shock (Jones et al., 1996). Since BipA and DeaD had multiple characteristics in common we investigated whether BipA expression was also induced when exponentially growing bacterial cells were shifted from 37°C to 15°C or "cold-shocked".

We compared *bipA* expression in wild-type cells grown at 37°C versus 15°C by qRT-PCR. Our data suggest that BipA is not a cold-shock protein as expression of *bipA* was not induced when cells were cold-shocked at 15°C. We also investigated if BipA was involved in *deaD* regulation by comparing *deaD* expression in a *bipA* mutant and comparing it to an isogenic wild-type strain cold-shocked at 15°C. The absence of BipA did not alter *deaD* expression under these conditions. These data suggest that even though BipA and DeaD may contribute to the same cellular functions, the regulation of the two genes is independent of one another.

To more definitively test the hypothesis that BipA is a ribosome assembly factor, we compared the ribosome profiles of a *bipA* mutant, a *deaD* mutant and a *bipA deaD* double mutant to that of the parent strain grown at both optimum temperature (37°C) and cold temperature (20°C). Our results demonstrated that a *bipA* mutant grown at 20°C contained an increased proportion of the 30S subunit, decreased proportion of the 50S subunit, and accumulation of a presumed 50S precursor. When both *bipA* and *deaD* were deleted, the ribosome profile displayed even greater defects. However, the ribosome assembly defects of the Δ*bipA* Δ*deaD* strain no longer existed when this strain was grown for longer periods and allowed to enter into stationary phase, suggesting that bacterial cells lacking both BipA and DeaD are able to overcome the ribosome assembly defects, but the process takes more time. This finding also provides an explanation of the extremely long lag phase of the *bipA deaD* double mutant. Taken together, these observations suggest that BipA was necessary for proper and/or efficient assembly of ribosomes during low temperature growth and in the absence of both BipA and DeaD, assembly of ribosomes is negatively affected.

Because we have observed that deletion of *rluC* suppresses phenotypes of Δ*bipA,* we sought to determine whether deletion of *rluC* also corrected the ribosome assembly defects of a

bipA mutant. To our gratification, our results demonstrated that the ribosome assembly defect of a *bipA* mutant was alleviated by *rluC* deletion. These data support and extend our previous findings that BipA function is required only when rRNA is fully pseudouridinylated. We do not know the basis for this requirement.

BipA and LepA belong to the same family of translational GTPases that also includes EF-G and EF-Tu (Finn et al., 2008). However, unlike EF-G and EF-Tu, BipA and LepA are dispensable for growth. Additionally, BipA and LepA are almost entirely homologous in their protein domain architecture, with the exception of their unique C-terminal domains (Finn et al., 2008). However, unlike Δ*bipA*, *lepA* mutants are not cold-sensitive. Since *bipA* mutants exhibit defective ribosome assembly, we sought to determine whether LepA was also involved in ribosome assembly. Surprisingly, our data suggested that unlike Δ*bipA* mutants, Δ*lepA* mutants were not defective in ribosome assembly and the ribosome profile of the latter was very similar to that of a wild-type grown at either 37°C or 20°C. This finding suggested that even though BipA and LepA belong to the same family of translational GTPases and the two proteins are structurally similar, they are involved in different cellular functions.

The ribosome assembly factor DeaD is also an RNA helicase that is involved in the assembly of the 50S ribosomal subunit at 20°C and deletion of *deaD* results in defective ribosome assembly characterized by a decreased proportion of 50S subunit, increased 30S subunit, and accumulation of a pre-50S ribosomal particle. Processing of the 23S rRNA is also impaired in a *deaD* mutant and the relative abundance of r-proteins that are required during the late stages of ribosome assembly are decreased in a *deaD* mutant suggesting that DeaD is involved in the late stages of ribosome assembly (Charollais et al., 2004). Our findings demonstrate that cold-sensitivity, motility, chloramphenicol sensitivity, and ribosome assembly

defects are all exaggerated when both *deaD* and *bipA* are deleted, suggesting the two proteins function synergistically.

To determine whether deletion of *bipA* resulted in a 23S and/or 16S rRNA processing defect we examined these rRNAs. It is important to mention that a wild-type strain of *E. coli* such as MG1655, when grown at low temperature such as 20°C, exhibits a slight ribosome assembly defect of the 50S subunit (Jiang et al., 2007). This defect was visible in our ribosome profile analysis of MG1655 strain grown at 20°C, as there was a slightly lower proportion of the 50S subunit compared to the 30S subunit. Therefore it is not surprising that wild-type bacteria grown at 20°C will also accumulate small amounts of precursor 23S rRNA. Additionally, Srivastava et al., (1989) suggested that the 17S precursor of 16S rRNA can assemble into fully formed ribosomes and does not require processing at the 5′- and 3′- ends (Srivastava et al., 1989, 1990). We determined the ratio of precursor rRNA/(precursor + mature rRNA) in the different mutant strains in comparison to an isogenic wild-type strain (MG1655) and investigated whether a *bipA* mutant accumulated precursor 23S and/or 16S rRNA when grown at 20°C.

Our results demonstrated that the *bipA* mutant accumulated a relative proportion of precursor 23S rRNA which was higher than the wild-type strain but less than a *deaD* mutant. This observation was not surprising as the cold-sensitive phenotype and the ribosome assembly defects of a *bipA* mutant were less severe than a *deaD* mutant as well. These data supported our previous results from the ribosome profiles and further bolstered our hypothesis that BipA is involved in ribosome assembly. Analysis of the 16S rRNA revealed that the 17S/(16S + 17S) ratio in a *bipA* mutant was slightly higher in a *bipA* mutant than in the wild-type strain. We predict that this slight accumulation of the 17S rRNA was an indirect consequence of the 50S biogenesis defect and was not biologically significant. This phenomenon has been previously

observed. SrmB is a 50S biogenesis factor and deletion of *srmB* results in accumulation of precursor 23S rRNA along with precursor 16S rRNA, which is an indirect consequence of defective 50S biogenesis (Charollais et al., 2003). Additionally, deletion of *rluC* was able to alleviate both the 23S rRNA processing defect and the slight 16S rRNA processing defect of the *bipA* mutant. Also, deletion of both *bipA* and *deaD* exacerbated the 16S rRNA processing defect in the double mutant. However, deletion of both *bipA* and *deaD* did not enhance the 23S rRNA processing defect in this mutant and we are not entirely sure of the reason for this discrepancy.

The results presented here support our second hypothesis that BipA is a ribosome assembly factor that is involved in the biogenesis of the 50S ribosomal subunit. Growth at low temperature accentuates the need for BipA, a characteristic common to many ribosome assembly factors. However, we do not know exactly which step of ribosome biogenesis is influenced by BipA and further experiments are needed to confirm this.

BipA is not essential for bacterial survival and mutants of *bipA* grow similar to a wild-type at 37°C, suggesting that bacterial cells require BipA only under specific conditions. Since BipA functions have been mostly identified during different kinds of stress response such as stringent response (deLivron et al., 2008), aberrant temperature (deLivron et al., 2008, Krishnan et al., 2008), antibiotic resistance (Duo et al., 2008), synthesis of capsule (Rowe et al., 2000), chemotaxis and regulation of virulence (Farris et al., 1998, Grant et al., 2003), we propose a mechanism for the cellular function of BipA. When bacterial cells are subjected to stress, BipA functions to modulate ribosome biogenesis and/or assembly which improves the translational efficiency and/or accuracy of genes involved in stress regulation, allowing the bacteria to cope with stress. However, the absence of the three pseudouridine residues added by RluC on the 23S rRNA, results in one of three possible outcomes; cells can either become independent of BipA,

and regain functionality; their functional defects can be exacerbated; or they may remain unaltered. With the limited amount of information about the mechanism of action of pseudouridines in modulating ribosome structure and/or function, it is difficult to predict the exact mechanism by which the absence of the three pseudouridines on 23S rRNA allow bacterial cells to function independent of BipA. It is possible that the lack of these three pseudouridines structurally modifies the ribosome which allows ribosome biogenesis by an alternate pathway that does not require BipA. However, it is also possible that BipA modulates translational efficiency and ribosome biogenesis by two completely unrelated mechanisms. The precise functions of BipA in the regulation of these two cellular processes remain under investigation.

The mechanism of how BipA function is triggered during stress response is still elusive. However, it is known that the stress response alarmone, ppGpp (guanosine-3',5'-bisdiphosphate) influences the ribosome association properties of BipA in a concentration dependent manner (deLivron et al., 2008). During a stringent reponse, when bacterial cells are starved for amino acids and carbon, cellular levels of GTP fall while ppGpp levels increase. Increased levels of ppGpp inhibited the ribosome binding and GTP hydrolyzing properties of BipA (deLivron et al., 2008) suggesting that BipA function is influenced by cellular levels of GTP and ppGpp, and increased concentration of ppGpp during stressed conditions might stimulate BipA function.

This is not the first time that a ribosome assembly GTPase has been implicated in the regulation of stress response. In *E. coli*, Era and ObgE are two such ribosome assembly GTPases that have been suggested to be involved in regulation of stress response (Verstraeten et al., 2011). The RbgA protein of *B. subtilis* is another example of a ribosome assembly GTPase which is required during growth under stressed conditions (Britton et al., 2009).

Overall our data enhances the existing knowledge of the cellular function of the BipA protein and suggests that BipA is involved in the modulation of protein synthesis, especially in the regulation of the expression of stress response genes and also in ribosome assembly, particularly in the biogenesis of the large ribosomal subunit.

Appendix

List of *E. coli* Strains

Strains	Genotype	Reference
GEB658	MC4100 *ara*$^{+}$ *rprA142::lacZ*	Castanié-Cornet et al., 2006
JW5531	*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *λ,*$^{-}$ *ΔdeaD774::kan,* Δ*(rhaD-rhaB)568, hsdR514*	Baba et al., 2006
KK30	TB28 Δ*bipA<>res*	Krishnan et al., 2008
KK33	TB28 Δ*bipA<>res* Δ*rluC::res-npt-res*	Krishnan et al., 2008
KNS4	MC4100*(hns-lacZ)hyb2, kanR*	Dersch et al., 1993
MG1655	*rph-1 ilvG rfb-50*	Guyer et al., 1981
S17-1*λpir/* pJMSB8	Tp^{r} Sm^{r} *recA thi hsdRM*$^{+}$ RP4::2-Tc::Mu::Km Tn*7* *λpir*	Kristensen et al., 1995
TB28	MG1655 Δ*lacZYA <>frt*	Bernhardt et al., 2004
PC28	MG1655 Δ*bipA<>res-npt-res*	This study
PC30	MG1655 Δ*bipA<>res*	This study
PC33	MG1655 Δ*rluC<>res-npt-res*	This study
PC34	PC30 Δ*rluC<>res-npt-res*	This study
PC51	GEB658 Δ*rluC<>res-npt-res*	This study
PC53	PC54 Δ*rluC<>res-npt-res*	This study
PC54	GEB658 Δ*bipA<>res*	This study
PC55	PC33 Δ*rluC<>res*	This study

Strains	Genotype	Reference
PC57	TB28 Δ*rluC<>res-npt-res*	This study
PC60	TB28 Δ*bipA* Δ*rluC<>res*	This study
PC61	PC57 Δ*rluC<>res*	This study
PC62	KK30 *(hns-lacZ)hyb2, kanR*	This study
PC63	PC61 *(hns-lacZ)hyb2, kanR*	This study
PC64	PC60 *(hns-lacZ)hyb2, kanR*	This study
PC95	MG1655 Δ*deaD::kan*	This study
PC96	PC30 Δ*deaD::kan*	This study
PC97	PC55 Δ*deaD::kan*	This study
PC99	TB28 Δ*bipA<>res-npt-res*	This study
PC100	PC99 Δ*bipA<>res*	This study
PC101	PC100 Δ*rluC<>res-npt-res*	This study
PC102	TB28/pXL11	This study
PC103	PC57/pXL11	This study
PC104	PC99/pXL11	This study
PC105	PC101/pXL11	This study
PC133	MG1655 Δ*lepA::kan*	This study

List of *E. coli* plasmids

Plasmid Name	Genotype	Reference
pXL11	*fliA-lacZ* promoter fusion in pRS528	Pruss et al., 2001

REFERENCES

1. **Anderson, J.K., Smith, T.G., and Hoover, T.R.** (2010). Sense and sensibility: flagellum-mediated gene regulation. Trends Microbiol. *18*, 30–37.
2. **Atlung, T., and Ingmer, H.** (1997). H-NS: a modulator of environmentally regulated gene expression. Mol. Microbiol. *24*, 7–17.
3. **Awano, N., Xu, C., Ke, H., Inoue, K., Inouye, M., and Phadtare, S.** (2007). Complementation analysis of the cold-sensitive phenotype of the *Escherichia coli csdA* deletion strain. J. Bacteriol. *189*, 5808–5815.
4. **Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H.** (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. *2*, 2006.0008.
5. **Barker, H.C., Kinsella, N., Jaspe, A., Friedrich, T., and O'Connor, C.D.** (2000). Formate protects stationary-phase *Escherichia coli* and *Salmonella* cells from killing by a cationic antimicrobial peptide. Mol. Microbiol. *35*, 1518–1529**.**
6. **Bernhardt, T.G., and de Boer, P.A.J.** (2004). Screening for synthetic lethal mutants in *Escherichia coli* and identification of EnvC (YibP) as a periplasmic septal ring factor with murein hydrolase activity. Mol. Microbiol. *52*, 1255–1269.

7. **Bertin, P., Terao, E., Lee, E.H., Lejeune, P., Colson, C., Danchin, A., and Collatz, E.** (1994). The H-NS protein is involved in the biogenesis of flagella in *Escherichia coli*. J. Bacteriol. *176*, 5537–5540.
8. **Brandi, A., Pon, C.L., and Gualerzi, C.O.** (1994). Interaction of the main cold shock protein CS7.4 (CspA) of *Escherichia coli* with the promoter region of *hns*. Biochimie *76*, 1090–1098.
9. **Brill, J.A., Quinlan-Walshe, C., and Gottesman, S.** (1988). Fine-structure mapping and identification of two regulators of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. *170*, 2599–2611.
10. **Brimacombe, R., Mitchell, P., Osswald, M., Stade, K., and Bochkariov, D.** (1993). Clustering of modified nucleotides at the functional center of bacterial ribosomal RNA. FASEB J. *7*, 161–167.
11. **Britton, R.A.** (2009). Role of GTPases in bacterial ribosome assembly. Annu. Rev. Microbiol. *63*, 155–176.
12. **Butland, G., Krogan, N.J., Xu, J., Yang, W.-H., Aoki, H., Li, J.S., Krogan, N., Menendez, J., Cagney, G., Kiani, G.C., Jessulat M. G., Datta, N., Ivanov, I., Abouhaidar, M. G., Emili, A., Greenblatt, J., Ganoza, M. C., and Golshani, A**. (2007). Investigating the in vivo activity of the DeaD protein using protein-protein interactions and the translational activity of structured chloramphenicol acetyltransferase mRNAs. J. Cell. Biochem. *100*, 642–652.
13. **Campbell, T.L., and Brown, E.D.** (2008). Genetic interaction screens with ordered overexpression and deletion clone sets implicate the *Escherichia coli* GTPase YjeQ in late ribosome biogenesis. J. Bacteriol. *190*, 2537–2545.

14. **Campbell, T.L., Henderson, J., Heinrichs, D.E., and Brown, E.D.** (2006). The *yjeQ* gene is required for virulence of *Staphylococcus aureus*. Infect. Immun. *74*, 4918–4921.

15. **Cantara, W.A., Crain, P.F., Rozenski, J., McCloskey, J.A., Harris, K.A., Zhang, X., Vendeix, F.A.P., Fabris, D., and Agris, P.F.** (2011). The RNA modification database, RNAMDB: 2011 update. Nucleic Acids Res. *39*, D195–201.

16. **Carballès, F., Bertrand, C., Bouché, J.P., and Cam, K.** (1999). Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. Mol. Microbiol. *34*, 442–450.

17. **Carpousis, A.J.** (2007). The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. Annu. Rev. Microbiol. *61*, 71–87.

18. **Castanié-Cornet, M.-P., Cam, K., and Jacq, A.** (2006). RcsF is an outer membrane lipoprotein involved in the RcsCDB phosphorelay signaling pathway in *Escherichia coli*. J. Bacteriol. *188*, 4264–4270.

19. **Charollais, J., Pflieger, D., Vinh, J., Dreyfus, M., and Iost, I**. (2003). The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli.* Mol. Microbiol. *48*, 1253–1265.

20. **Charollais, J., Dreyfus, M., and Iost, I.** (2004). CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. Nucleic Acids Res. *32*, 2751–2759.

21. **Chilcott, G.S., and Hughes, K.T.** (2000). Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar *typhimurium* and *Escherichia coli*. Microbiol. Mol. Biol. Rev. *64*, 694–708.

22. **Comartin, D.J., and Brown, E.D.** (2006). Non-ribosomal factors in ribosome subunit assembly are emerging targets for new antibacterial drugs. Curr. Opin. Pharmacol. *6*, 453–458.

23. **Daigle, D.M., and Brown, E.D.** (2004). Studies of the interaction of *Escherichia coli* YjeQ with the ribosome in vitro. J. Bacteriol. *186*, 1381–1387.

24. **Dame, R.T., Wyman, C., and Goosen, N.** (2000). H-NS mediated compaction of DNA visualized by atomic force microscopy. Nucleic Acids Res. *28*, 3504–3510.

25. **Davalos-Garcia, M., Conter, A., Toesca, I., Gutierrez, C., and Cam, K.** (2001). Regulation of *osmC* gene expression by the two-component system *rcsB-rcsC* in *Escherichia coli*. J. Bacteriol. *183*, 5870–5876.

26. **Davies, B.W., Köhrer, C., Jacob, A.I., Simmons, L.A., Zhu, J., Aleman, L.M., Rajbhandary, U.L., and Walker, G.C**. (2010). Role of *Escherichia coli* YbeY, a highly conserved protein, in rRNA processing. Mol. Microbiol. *78*, 506–518.

27. **Decatur, W.A., and Fournier, M.J.** (2002). rRNA modifications and ribosome function. Trends Biochem. Sci. *27*, 344–351.

28. **Del Campo, M., Kaya, Y., and Ofengand, J.** (2001). Identification and site of action of the remaining four putative pseudouridine synthases in *Escherichia coli*. RNA *7*, 1603–1615.

29. **deLivron, M.A., and Robinson, V.L.** (2008). *Salmonella enterica* serovar *Typhimurium* BipA exhibits two distinct ribosome binding modes. J. Bacteriol. *190*, 5944–5952.

30. **Dersch, P., Schmidt, K., and Bremer, E.** (1993). Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation. Mol. Microbiol. *8*, 875–889.

31. **Dethlefsen, L., and Schmidt, T.M.** (2007). Performance of the translational apparatus varies with the ecological strategies of bacteria. J. Bacteriol. *189*, 3237–3245.

32. **Duo, M., Hou, S., and Ren, D.** (2008). Identifying *Escherichia coli* genes involved in intrinsic multidrug resistance. Appl. Microbiol. Biotechnol. *81*, 731–741.

33. **Ebel, W., and Trempy, J.E.** (1999). *Escherichia coli* RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions to activate its own expression. J. Bacteriol. *181*, 577–584.

34. **Ebel, W., Vaughn, G.J., Peters, H.K., and Trempy, J.E.** (1997). Inactivation of *mdoH* leads to increased expression of colanic acid capsular polysaccharide in *Escherichia coli.* J. Bacteriol. *179*, 6858–6861.

35. **Falconi, M., Brandi, A., La Teana, A., Gualerzi, C.O., and Pon, C.L.** (1996). Antagonistic involvement of FIS and H-NS proteins in the transcriptional control of *hns* expression. Mol. Microbiol. *19*, 965–975.

36. **Farris, M., Grant, A., Richardson, T.B., and O'Connor, C.D.** (1998). BipA: a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. Mol. Microbiol. *28*, 265–279.

37. **Finn, R.D., Tate, J., Mistry, J., Coggill, P.C., Sammut, S.J., Hotz, H.-R., Ceric, G., Forslund, K., Eddy, S.R., Sonnhammer, E. L., and Bateman, A.** (2008). The Pfam protein families database. Nucleic Acids Res. *36*, D281–288.

38. **Francez-Charlot, A., Laugel, B., Van Gemert, A., Dubarry, N., Wiorowski, F., Castanié-Cornet, M.-P., Gutierrez, C., and Cam, K.** (2003). RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. Mol. Microbiol. *49*, 823–832.

39. **Fredericks, C.E., Shibata, S., Aizawa, S.-I., Reimann, S.A., and Wolfe, A.J.** (2006). Acetyl phosphate-sensitive regulation of flagellar biogenesis and capsular biosynthesis depends on the Rcs phosphorelay. Mol. Microbiol. *61*, 734–747.

40. **Freestone, P., Grant, S., Toth, I., and Norris, V.** (1995). Identification of phosphoproteins in *Escherichia coli*. Mol. Microbiol. *15*, 573–580.

41. **Freestone, P., Trinei, M., Clarke, S.C., Nyström, T., and Norris, V.** (1998). Tyrosine phosphorylation in *Escherichia coli.* J. Mol. Biol. *279*, 1045–1051.

42. **Fuller-Pace, F.V.** (1994). RNA helicases: modulators of RNA structure. Trends Cell Biol. *4*, 271–274.

43. **Gervais, F.G., Phoenix, P., and Drapeau, G.R.** (1992). The *rcsB* gene, a positive regulator of colanic acid biosynthesis in *Escherichia coli*, is also an activator of *ftsZ* expression. J. Bacteriol. *174*, 3964–3971.

44. **Gollop, N., and March, P.E.** (1991). A GTP-binding protein (Era) has an essential role in growth rate and cell cycle control in *Escherichia coli*. J. Bacteriol. *173*, 2265–2270.

45. **Gottesman, S., Trisler, P., and Torres-Cabassa, A.** (1985). Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J. Bacteriol. *162*, 1111–1119.

46. **Grant, A.J., Farris, M., Alefounder, P., Williams, P.H., Woodward, M.J., and O'Connor, C.D.** (2003). Co-ordination of pathogenicity island expression by the BipA GTPase in enteropathogenic *Escherichia coli* (EPEC). Mol. Microbiol. *48*, 507–521.

47. **Green, R., and Noller, H.F.** (1996). In vitro complementation analysis localizes 23S rRNA posttranscriptional modifications that are required for *Escherichia coli* 50S ribosomal subunit assembly and function. RNA *2*, 1011–1021.

48. **Gutgsell, N., Englund, N., Niu, L., Kaya, Y., Lane, B.G., and Ofengand, J.** (2000). Deletion of the *Escherichia coli* pseudouridine synthase gene *truB* blocks formation of pseudouridine 55 in tRNA in vivo, does not affect exponential growth, but confers a strong selective disadvantage in competition with wild-type cells. RNA *6*, 1870–1881.

49. **Gutgsell, N.S., Deutscher, M.P., and Ofengand, J.** (2005). The pseudouridine synthase RluD is required for normal ribosome assembly and function in *Escherichia coli*. RNA *11*, 1141–1152.

50. **Guthrie, C., Nashimoto, H., and Nomura, M.** (1969). Structure and function of *E. coli* ribosomes. 8. Cold-sensitive mutants defective in ribosome assembly. Proc. Natl. Acad. Sci. USA. *63*, 384–391.

51. **Gutierrez, C., and Devedjian, J.C.** (1991). Osmotic induction of gene *osmC* expression in *Escherichia coli* K12. J. Mol. Biol. *220*, 959–973.

52. **Guyer, M.S., Reed, R.R., Steitz, J.A., and Low, K.B.** (1981). Identification of a sex-factor-affinity site in *E. coli* as γδ. Cold Spring Harb. Symp. Quant. Biol. *45*, 135–140.

53. **Hamma, T., and Ferré-D'Amaré, A.R.** (2006). Pseudouridine synthases. Chem. Biol. *13*, 1125–1135.

54. **Hase, Y., Yokoyama, S., Muto, A., and Himeno, H.** (2009). Removal of a ribosome small subunit-dependent GTPase confers salt resistance on *Escherichia coli* cells. RNA. *15*, 1766–1774.

55. **Himeno, H., Hanawa-Suetsugu, K., Kimura, T., Takagi, K., Sugiyama, W., Shirata, S., Mikami, T., Odagiri, F., Osanai, Y., Watanabe, D., Goto, S., Kalachnyuk, L., Ushida, C., and Muto, A.** (2004). A novel GTPase activated by the small subunit of ribosome. Nucleic Acids Res. *32*, 5303–5309.

56. **Hommais, F., Krin, E., Laurent-Winter, C., Soutourina, O., Malpertuy, A., Le Caer, J.P., Danchin, A., and Bertin, P.** (2001). Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. Mol. Microbiol. *40*, 20–36.

57. **Huang, L., Ku, J., Pookanjanatavip, M., Gu, X., Wang, D., Greene, P.J., and Santi, D.V.** (1998). Identification of two *Escherichia coli* pseudouridine synthases that show multisite specificity for 23S RNA. Biochemistry *37*, 15951–15957.

58. **Hwang, J., and Inouye, M.** (2006). The tandem GTPase, Der, is essential for the biogenesis of 50S ribosomal subunits in *Escherichia coli.* Mol. Microbiol. *61*, 1660–1672.

59. **Iost, I., and Dreyfus, M.** (2006). DEAD-box RNA helicases in *Escherichia coli*. Nucleic Acids Res. *34*, 4189–4197.

60. **Jain, C.** (2008). The *E. coli* RhlE RNA helicase regulates the function of related RNA helicases during ribosome assembly. RNA *14*, 381–389.

61. **Jiang, M., Datta, K., Walker, A., Strahler, J., Bagamasbad, P., Andrews, P.C., and Maddock, J.R.** (2006). The *Escherichia coli* GTPase CgtAE is involved in late steps of large ribosome assembly. J. Bacteriol. *188*, 6757–6770.

62. **Jiang, M., Sullivan, S.M., Walker, A.K., Strahler, J.R., Andrews, P.C., and Maddock, J.R.** (2007). Identification of novel *Escherichia coli* ribosome-associated proteins using isobaric tags and multidimensional protein identification techniques. J. Bacteriol. *189*, 3434–3444.

63. **Jones, P.G., Mitta, M., Kim, Y., Jiang, W., and Inouye, M.** (1996). Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. *93*, 76–80.

64. **Kaczanowska, M., and Rydén-Aulin, M.** (2007). Ribosome biogenesis and the translation process in *Escherichia coli*. Microbiol. Mol. Biol. Rev. *71*, 477–494.

65. **Karbstein, K.** (2007). Role of GTPases in ribosome assembly. Biopolymers. *87*, 1–11.

66. **King, T.C., Sirdeshmukh, R., and Schlessinger, D.** (1984). RNase III cleavage is obligate for maturation but not for function of *Escherichia coli* pre-23S rRNA. Proc. Natl. Acad. Sci. USA. *81*, 185–188.

67. **Kiss, E., Huguet, T., Poinsot, V., and Batut, J.** (2004). The *typA* gene is required for stress adaptation as well as for symbiosis of *Sinorhizobium meliloti* 1021 with certain *Medicago truncatula* lines. Mol. Plant Microbe Interact. *17*, 235–244.

68. **Krishnan, K., and Flower, A.M.** (2008). Suppression of Δ*bipA* phenotypes in *Escherichia coli* by abolishment of pseudouridylation at specific sites on the 23S rRNA. J. Bacteriol. *190*, 7675–7683.

69. **Kristensen, C.S., Eberl, L., Sanchez-Romero, J.M., Givskov, M., Molin, S., and Lorenzo, V.D.** (1995). Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. J. Bacteriol. *177*, 52–58.

70. **Linder, P.** (2003). Yeast RNA helicases of the DEAD-box family involved in translation initiation. Biol. Cell *95*, 157–167.

71. **Livak, K.J., and Schmittgen, T.D.** (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

72. **Lehnen, D., Blumer, C., Polen, T., Wackwitz, B., Wendisch, V.F., and Unden, G.** (2002). LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in *Escherichia coli*. Mol. Microbiol. *45*, 521–532.

73. **Leipe, D.D., Wolf, Y.I., Koonin, E.V., and Aravind, L.** (2002). Classification and evolution of P-loop GTPases and related ATPases. J. Mol. Biol. *317*, 41–72.

74. **Maguire, B.A**. (2009). Inhibition of bacterial ribosome assembly: a suitable drug target? Microbiol. Mol. Biol. Rev. *73*, 22–35.

75. **Majdalani, N., and Gottesman, S.** (2005). The Rcs phosphorelay: a complex signal transduction system. Annu. Rev. Microbiol. *59*, 379–405.

76. **Majdalani, N., and Gottesman, S.** (2007). Genetic dissection of signaling through the Rcs phosphorelay. Methods Enzymol. *423*, 349–362.

77. **Majdalani, N., Chen, S., Murrow, J., St John, K., and Gottesman, S.** (2001). Regulation of RpoS by a novel small RNA: the characterization of RprA. Mol. Microbiol. *39*, 1382–1394.

78. **Majdalani, N., Hernandez, D., and Gottesman, S.** (2002). Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. Mol. Microbiol. *46*, 813–826.

79. **Margus, T., Remm, M., and Tenson, T.** (2007). Phylogenetic distribution of translational GTPases in bacteria. BMC Genomics *8*, 1471-2164.

80. **Miller, J.H.** (1992). A short course in bacterial genetics: A laboratory manual and handbook for *Escherichia Coli* and related bacteria. Cold Spring Harbor Laboratory, N.Y.

81. **Møller, A.K., Leatham, M.P., Conway, T., Nuijten, P.J.M., de Haan, L.A.M., Krogfelt, K.A., and Cohen, P.S.** (2003). An *Escherichia coli* MG1655 lipopolysaccharide deep-rough core mutant grows and survives in mouse cecal mucus but fails to colonize the mouse large intestine. Infect. Immun. *71*, 2142–2152.

82. **Nierhaus, K.H., and Lafontaine, D. L.** (2004). Protein synthesis and ribosome structure: Translating the genome. Ribosome assembly. p. 85-143. John Wiley & Sons, Inc., N. Y.

83. **Nomura, M., Gourse, R., and Baughman, G.** (1984). Regulation of the synthesis of ribosomes and ribosomal components. Annu. Rev. Biochem. *53*, 75–117.

84. **Ohnishi, K., Kutsukake, K., Suzuki, H., and Iino, T.** (1990). Gene *fliA* encodes an alternative sigma factor specific for flagellar operons in *Salmonella typhimurium*. Mol. Gen. Genet. *221*, 139–147.

85. **Park, Y.-J., Song, E.-S., Noh, T.-H., Kim, H., Yang, K.-S., Hahn, J.-H., Kang, H.-W., and Lee, B.-M.** (2009). Virulence analysis and gene expression profiling of the pigment-deficient mutant of *Xanthomonas oryzae* pathovar *oryzae*. FEMS Microbiol. Lett. *301*, 149–155.

86. **Paul, B.J., Ross, W., Gaal, T., and Gourse, R.L.** (2004). rRNA transcription in *Escherichia coli*. Annu. Rev. Genet. *38*, 749–770.

87. **Pfennig, P.L., and Flower, A.M.** (2001). BipA is required for growth of *Escherichia coli* K12 at low temperature. Mol. Genet. Genomics *266*, 313–317.

88. **Pierce, A., Gillette, D., Jones, P.G.** (2011). *Escherichia coli* cold shock protein CsdA effects an increase in septation and the resultant formation of coccobacilli at low temperature. Arch. Microbiol. *193*, 373–384.

89. **Plunkett, G., Burland, V., Daniels, D.L., and Blattner, F.R.** (1993). Analysis of the *Escherichia coli* genome. III. DNA sequence of the region from 87.2 to 89.2 minutes. Nucleic Acids Res. *21*, 3391–3398.

90. **Prüß, B.M., Liu, X., Hendrickson, W., and Matsumura, P.** (2001). FlhD/FlhC-regulated promoters analyzed by gene array and *lacZ* gene fusions. FEMS Microbiol. Lett. *197*, 91–97.

91. **Qi, S.Y., Li, Y., Szyroki, A., Giles, I.G., Moir, A., and O'Connor, C.D.** (1995). *Salmonella typhimurium* responses to a bactericidal protein from human neutrophils. Mol. Microbiol. *17*, 523–531.

92. **Qin, Y., Polacek, N., Vesper, O., Staub, E., Einfeldt, E., Wilson, D.N., and Nierhaus, K.H.** (2006). The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. Cell *127*, 721–733.

93. **Raychaudhuri, S., Conrad, J., Hall, B.G., and Ofengand, J.** (1998). A pseudouridine synthase required for the formation of two universally conserved pseudouridines in ribosomal RNA is essential for normal growth of *Escherichia coli.* RNA *4*, 1407–1417.

94. **Raychaudhuri, S., Niu, L., Conrad, J., Lane, B.G., and Ofengand, J.** (1999). Functional effect of deletion and mutation of the *Escherichia coli* ribosomal RNA and tRNA pseudouridine synthase RluA. J. Biol. Chem. *274*, 18880–18886.

95. **Rocak, S., and Linder, P.** (2004). DEAD-box proteins: the driving forces behind RNA metabolism. Nat. Rev. Mol. Cell Biol. *5*, 232–241.

96. **Rowe, S., Hodson, N., Griffiths, G., and Roberts, I.S.** (2000). Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic *E. coli*. J. Bacteriol. *182*, 2741–2745.

97. **Scott, K., Diggle, M.A., and Clarke, S.C.** (2003). TypA is a virulence regulator and is present in many pathogenic bacteria. Br. J. Biomed. Sci. *60*, 168–170.

98. **Shi, W., Li, C., Louise, C.J., and Adler, J.** (1993). Mechanism of adverse conditions causing lack of flagella in *Escherichia coli*. J. Bacteriol. *175*, 2236–2240.

99. **Silhavy, T.J., Berman, M.L., Enquist, L.W., and Laboratory, C.S.H.** (1984). Experiments with gene fusions. Cold Spring Harbor Laboratory, N.Y.

100. **Sledjeski, D., and Gottesman, S.** (1995). A small RNA acts as an antisilencer of the H-NS-silenced *rcsA* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA. *92*, 2003–2007.

101. **Soutourina, O.A., and Bertin, P.N.** (2003). Regulation cascade of flagellar expression in Gram-negative bacteria. FEMS Microbiol. Rev. *27*, 505–523.

102. **Sperandio, V., Torres, A.G., and Kaper, J.B.** (2002). Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol. Microbiol. *43*, 809–821.

103. **Srivastava, A.K., and Schlessinger, D.** (1989). Processing pathway of *Escherichia coli* 16S precursor rRNA. Nucleic Acids Res. *17*, 1649–1663.

104. **Srivastava, A.K., and Schlessinger, D.** (1990). Mechanism and regulation of bacterial ribosomal RNA processing. Annu. Rev. Microbiol. *44*, 105–129.

105. **Stout, V., and Gottesman, S.** (1990). RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. *172*, 659–669.

106. **Stout, V., Torres-Cabassa, A., Maurizi, M.R., Gutnick, D., and Gottesman, S.** (1991). RcsA, an unstable positive regulator of capsular polysaccharide synthesis. J. Bacteriol. *173*, 1738–1747.

107. **Thieringer, H.A., Jones, P.G., and Inouye, M.** (1998). Cold shock and adaptation. Bioessays *20*, 49–57.

108. **Tupper, A.E., Owen-Hughes, T.A., Ussery, D.W., Santos, D.S., Ferguson, D.J., Sidebotham, J.M., Hinton, J.C., and Higgins, C.F.** (1994). The chromatin-associated protein H-NS alters DNA topology in vitro. EMBO J. *13*, 258–268.

109. **Vaughan, S., Wickstead, B., Gull, K., and Addinall, S.G.** (2004). Molecular evolution of FtsZ protein sequences encoded within the genomes of archaea, bacteria, and eukaryota. J. Mol. Evol. *58*, 19–29.

110. **Verstraeten, N., Fauvart, M., Versées, W., and Michiels, J.** (2011). The universally conserved prokaryotic GTPases. Microbiol. Mol. Biol. Rev. *75*, 507–542.

111. **Wehland, M., Kiecker, C., Coplin, D.L., Kelm, O., Saenger, W., and Bernhard, F.** (1999). Identification of an RcsA/RcsB recognition motif in the promoters of exopolysaccharide biosynthetic operons from *Erwinia amylovora* and *Pantoea stewartii* subspecies *stewartii*. J. Biol. Chem. *274*, 3300–3307.

112. **Wehland, M., and Bernhard, F.** (2000). The RcsAB box. Characterization of a new operator essential for the regulation of exopolysaccharide biosynthesis in enteric bacteria. J. Biol. Chem. *275*, 7013–7020.

113. **Williamson, J.R.** (2008). Biophysical studies of bacterial ribosome assembly. Curr. Opin. Struct. Biol. *18*, 299–304.

114. **Wilson, D.N., and Nierhaus, K.H.** (2007). The weird and wonderful world of bacterial ribosome regulation. Crit. Rev. Biochem. Mol. Biol. *42*, 187–219.

115. **Woolford, J.L., and Baserga, S.J.** (2013). Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. Genetics *195*, 643–681.

116. **Xia, B., Ke, H., Shinde, U., and Inouye, M.** (2003). The role of RbfA in 16S rRNA processing and cell growth at low temperature in *Escherichia coli*. J. Mol. Biol. *332*, 575–584.

117. **Xu, Z., and Culver, G.M.** (2010). Differential assembly of 16S rRNA domains during 30S subunit formation. RNA *16*, 1990-2001.

118. **Yamanaka, K.** (1999). Cold shock response in *Escherichia coli*. J. Mol. Microbiol. Biotechnol. *1*, 193–202.

119. **Zhou, K., Zhou, L., Lim, Q.E., Zou, R., Stephanopoulos, G., and Too, H.-P.** (2011). Novel reference genes for quantifying transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR. BMC Mol. Biol. *12*, 1471-2199.