Investigation of the function of *yihA*

INVESTIGATION OF THE FUNCTION OF *YIHA*, AN ESSENTIAL GENE OF UNKNOWN FUNCTION IN *ESCHERICHIA COLI*

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ABSTRACT

Genomic data has led to the realization that nearly one third of the bacterial genes discovered have unknown functions. Promising drug targets are no doubt among the conserved and essential genes, but drug development awaits an understanding of the basic physiological roles of the cryptic bacterial proteins they encode. The pressing need for novel antibiotics mandates investigation of these essential bacterial genes of unknown function. For a subset of such genes, it is becoming increasingly apparent that they may play critical roles in ribosome assembly or function in bacteria. One such essential gene, encoding the putative GTPase YihA in *Escherichia coli* has been highlighted as a target of particular importance for study (Galperin M.Y. and E.V. Koonin. (2004). 'Conserved hypothetical' proteins: prioritization of targets for experimental study. Nucleic Acids Res **32**(18): 5452-63.). To study *yihA*'s function, a strain with *yihA* under P_{BAD} control was generated and used to examine the impact of the loss of the gene product on ribosome assembly. Depletion of the YihA protein was found to cause accumulation of 30S and 50S subunits at the expense of 70S ribosomes, and the formation of long filamentous cells apparently incapable of dividing. Chemical genetic interactions were identified through chemical synthetic lethality screening with antibiotics which target the ribosomal A-site, and antibiotics which affect the bacterial cell wall. Finally, investigation of the importance of several patches of basic residues of YihA through mutagenesis and *in vivo* complementation shows the protein to be tolerant of mutation to non-catalytic residues. The inability of mutants with changes to amino acids important for nucleotide binding to complement represents the strongest evidence to date that the YihA protein relies on GTP

binding and hydrolysis as part of its essential function *in vivo*. The findings of this study support a role for YihA in the poorly understood process of ribosome assembly *in vivo*, and represent important progress towards realization of this essential process as a source of novel antibiotic targets.

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PREFACE

Antibiotics for sensitization screening were provided by Chand Mangat. Some primers, and the pBS-*araBAD-Flank-Kan* plasmid used in this study were generously provided by Tracey Campbell. The solid culture plates for temperature sensitization screening were kindly scanned by Amrita Bharat. All other work reported herein was performed by David Comartin.

CHAPTER 1 – BACKGROUND INFORMATION

1.1 The study of Genes of Unknown Function in Bacteria

Antibiotic resistant bacterial pathogens pose a serious threat to patients in a clinical setting, as well as the public at large. In 2005, an estimated 94,360 patients suffered from invasive methicillin-resistant *Staphylococcus aureus* (MRSA) infections leading to an estimated death toll of 18,650 deaths following exposure to MRSA in the United States (Klevens, Morrison *et al.* 2007). The ability of bacteria to develop mechanisms of resistance mandates a continued effort to discover novel anti-bacterial chemistry with which to combat clinically relevant pathogens (Brown and Wright 2005; D'Costa, McGrann *et al.* 2006; Projan and Bradford 2007). With the successful sequencing of bacterial genomes has come the realization that a surprising breadth of crucial bacterial physiology remains to be understood (Blattner, Plunkett *et al.* 1997; Hinton 1997; Tatusov, Natale *et al.* 2001; Tatusov, Fedorova *et al.* 2003). Bacterial genes with unknown functions represent approximately one quarter of the conserved gene families identified thus far, and a further sixteen percent of gene families have only general function predictions ascribed (Tatusov, Koonin *et al.* 1997; Tatusov, Galperin *et al.* 2000; Tatusov, Fedorova *et al.* 2003). Genes encoding proteins with unknown functions in the best studied model bacterium, *Escherichia coli*, are named beginning with the letter 'y', and these so called '*y*-genes' represent an immense but important challenge for researchers (Hinton 1997; Tatusov, Koonin *et al.* 1997; Tatusov, Galperin *et al.* 2000; Tatusov, Fedorova *et al.* 2003). Of particular interest are essential *y*-genes with putative orthologs in a wide variety of bacterial species, which represent potential targets

for the development of truly novel antibacterial chemistry (Arigoni, Talabot *et al.* 1998; Galperin and Koonin 2004; Brown and Wright 2005). In order to pursue target based drug discovery, it is important that the function of the target protein be understood, allowing for assays to be developed which report on the critical *in vivo* functions of the protein (Brown and Wright 2005). Research that serves to illuminate the roles of essential bacterial proteins of unknown function can be regarded as one of the most important endeavors in microbiology today, both for the insights to be gained into bacterial physiology, and for the greatly needed targets that will arise for future drug discovery efforts (Arigoni, Talabot *et al.* 1998; Galperin and Koonin 2004; Brown and Wright 2005).

1.2 YihA

The *E. coli* gene *yihA* was listed among the top ten interesting genes of unknown function in a recent effort to prioritize essential bacterial proteins as targets of study (Galperin and Koonin 2004). The *yihA* gene was first discovered in 1982, but remained unstudied until after being confirmed as an essential gene (Joyce and Grindley 1982; Arigoni, Talabot *et al.* 1998; Dassain, Leroy *et al.* 1999; Mushegian 2005). The 210 amino acid sequence of YihA (NCBI Accession NP_418301) includes residues conserved among guanine nucleotide triphosphate hydrolyzing enzymes (GTPases), specifically members of the translation-factor related family of P-loop GTPases (Dassain, Leroy *et al.* 1999; Leipe, Wolf *et al.* 2002). The purified recombinant YihA from *E. coli* was found to have negligible hydrolytic activity, though binding to guanosine 5' triphosphate (GTP)

and guanosine 5' diphosphate (GDP) were found to occur with dissociation constants (K_d) of $2.7x10^{-5}$ M and $3\ x10^{-6}$ M respectively (Lehoux, Mazzulla *et al.* 2003). Crystal structures of the *Bacillus subtilis* ortholog YsxC crystallized with the non-hydrolysable GTP analog guanosine-5'-(β,γ)-imidotriphosphate (GMPPNP), GDP, and without nucleotide have been solved (Das, Sedelnikova *et al.* 2004; Ruzheinikov, Das *et al.* 2004). Comparison of the three states indicates that two regions of the protein have different conformations when different nucleotides are bound; and these two regions correspond to the switch I (SWI) and switch II (SWII) regions which are found to undergo rearrangements during hydrolysis in other GTPases (Sprang 1997; Leipe, Wolf *et al.* 2002; Ruzheinikov, Das *et al.* 2004; Caldon and March 2003). For YsxC, structural data has shown that Asn33 (Asn36 for YihA) is positioned to interact with β-γ phosphate bridging oxygen, however the position of a water molecule in the active site could not be resolved and limited insight into catalytic mechanism was possible beyond that which had been inferred by homology (Ruzheinikov, Das *et al.* 2004). Aside from the nucleotide binding G-domains, no known protein folds or motifs have been identified within YihA, though a preponderance of positively charged residues has been noted, and the suggestion made that the protein may interact with rRNA (Ruzheinikov, Das *et al.* 2004). In particular, a patch of conserved basic residues has been identified adjacent to G3/Switch II region of the protein that are notably conserved in *Bacillus subtilis and Homo sapiens* and suggested as important in rRNA/GAP binding (Ruzheinikov, Das *et al.* 2004).

Evidence gathered during initial efforts to determine the function of YihA *in vivo* supported a role for the protein in cell division (Dassain, Leroy *et al.* 1999). Reductions

of cellular levels of YihA lead to *E. coli* which have multiple chromosomes and are significantly elongated into filaments presumably due to failure to undergo septation and normal cell division (Dassain, Leroy *et al.* 1999). The over-expression of several known cell-division genes, *ftsQ, ftsI, ftsA,* and *ftsZ* was reported to suppress the phenotype of YihA-depletion, further bolstering its hypothesized role in cell division (Dassain, Leroy *et al.* 1999). Finally, it is worth noting that YihA was included in a large scale search for protein-protein interactions among 857 tagged non-membrane proteins in *E. coli,* but no interacting proteins were identified (Butland, Peregrin-Alvarez *et al.* 2005).

Partway through the present study, data were published that offered insight into the function of *ysxC*, the *B. subtilis* ortholog of *yihA.* It was shown by Schaefer *et al.* that depletion of the YsxC protein led to accumulation of 30S subunits and precursors to the 50S subunit, and that the pre-50S subunit had reduced levels of L16, L27 and L36 large ribosomal subunit proteins (Schaefer, Uicker *et al.* 2006). The same group more recently demonstrated that YsxC co-purified with rRNA, was able to bind to 50S subunits in the presence of a non-hydrolyzable analogue of GTP, interacted with L1, L3, L6 and L10 as a complex, L7/12 and L23 and L27 as a complex, and was pulled down by over-expressed L1, L6, and L7/L12 large ribosomal subunit proteins (Wicker-Planquart, Foucher *et al.* 2008). The study provided the first evidence of interacting partners for YsxC, and established a probable role for YsxC functioning in ribosome function or assembly. The poorly understood process of *in vivo* ribosome assembly and the spatial separation of L1 from L7/L12, L3 and L6 leave the precise function of YsxC on the ribosome an open question (Wicker-Planquart, Foucher *et al.* 2008).

At the outset of this study in 2005, no evidence had been published as to the specific physiological function of YihA/YsxC, however the mounting evidence of roles in ribosome assembly for related GTPases suggested the possibility of ribosome assembly as the essential function of YihA (Caldon, Yoong *et al.* 2001; Leipe, Wolf *et al.* 2002; Caldon and March 2003; Brown 2005).

1.3 P-loop GTPases in Bacteria

Guanine nucleotide triphosphatases play a wide variety of roles in both prokaryotic and eukaryotic physiology (Sprang 1997; Caldon, Yoong *et al.* 2001; Caldon and March 2003; Bourne, Sanders *et al.* 1990; Bourne, Sanders *et al.* 1991; Bourne 1995). The GTPases in prokaryotes are typified by a three-state cycle; GTP bound ('active' or 'on'), GDP bound ('inactive' or 'off'), and a state in which no nucleotide is bound ('empty' or 'apo') (Bourne, Sanders *et al.* 1990; Bourne 1995; Sprang 1997; Caldon, Yoong *et al.* 2001; Caldon and March 2003). The transitions between these three states are coupled to interactions with proteins specific for each GTPase (Bourne, Sanders *et al.* 1990; Bourne 1995; Sprang 1997; Caldon, Yoong *et al.* 2001; Caldon and March 2003). GTPases in their active states typically interact with target proteins which act as GTPase-activating proteins (GAP), increasing the intrinsic rate of hydrolysis by the GTPase (Bourne, Sanders *et al.* 1990; Bourne 1995; Sprang 1997; Caldon, Yoong *et al.* 2001; Caldon and March 2003). The energy liberated during hydrolysis is typically used to drive a conformational change in the target protein, or in the case of kinetic proof reading by EF-Tu, the rate of hydrolysis is compared to the rate of dissociation of amino-acyl-tRNAs

from the ribosomal A-site (Thompson, Dix *et al.* 1986; Bourne 1995; Sprang 1997). Release of GDP to allow reconstitution of an active GTP-bound form is typically slow, and in many cases the rate of exchange is increased by co-factors called guanine nucleotide exchange factors (GEFs) or guanine nucleotide release proteins (GNRPs) (Bourne, Sanders *et al.* 1990; Bourne, Sanders *et al.* 1991). Cellular levels of GTP under normal conditions are significantly higher than GDP, meaning that release of GDP is followed by the rapid binding of GTP and that the empty forms of the enzymes are not known to play any physiologically important roles (Caldon and March 2003; Bourne, Sanders *et al.* 1990; Bourne, Sanders *et al.* 1991).

The most abundant structural classes of nucleotide triphosphatases in bacteria contain a characteristic phosphate-binding loop (P-loop), a core β-sheet and surrounding series of α–helices created by repeating α-helix-β-strand architecture (Leipe, Wolf *et al.* 2002; Caldon and March 2003; Brown 2005). These P-loop NTPases are identified by the presence of five conserved sequence elements called G-domains (G1-G5) (see Figure 1, Table 1) that include the previously described Walker A (G1) and Walker B (G3) motifs (Walker, Saraste *et al.* 1982; Sprang 1997; Leipe, Wolf *et al.* 2002; Caldon and March 2003).

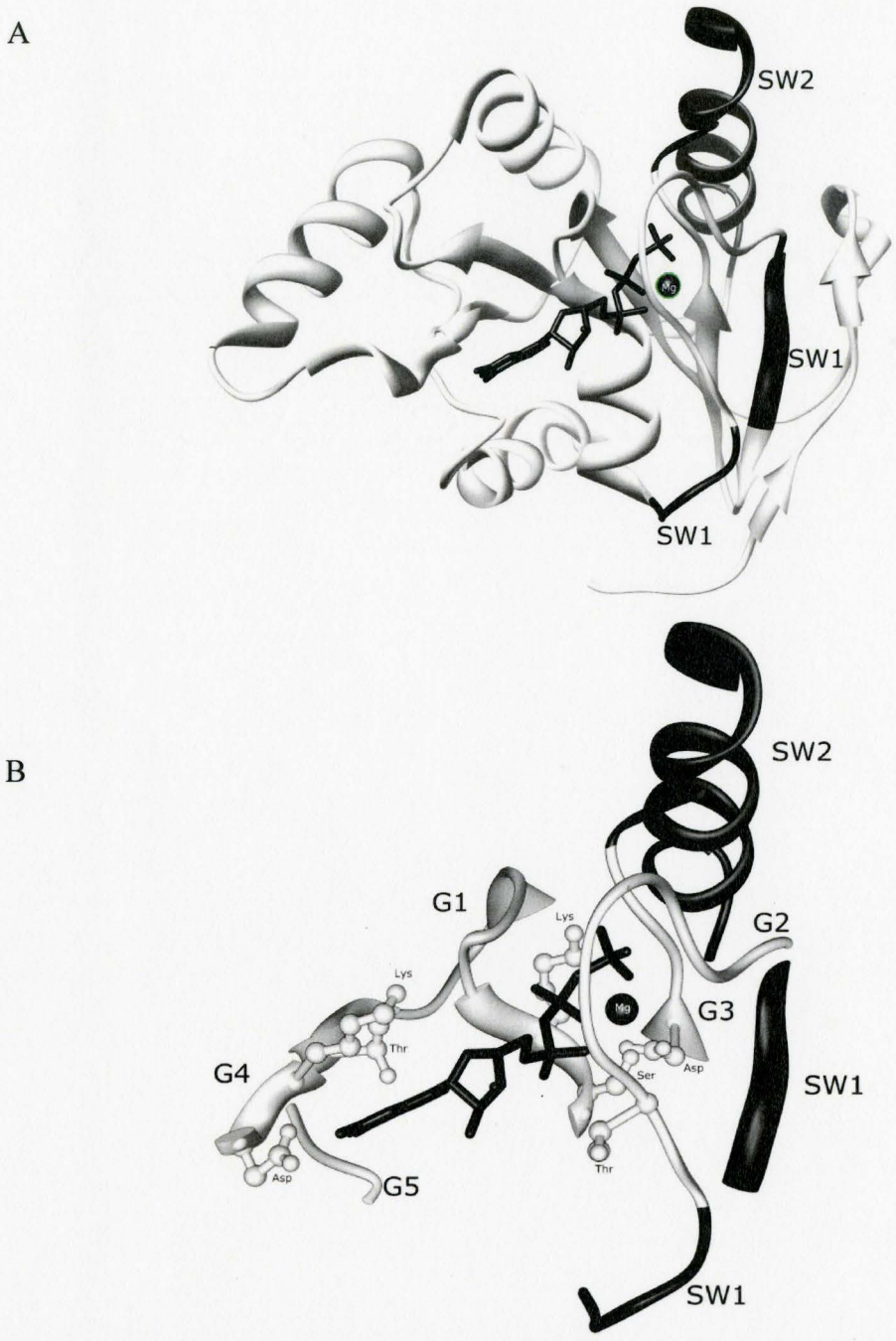


Figure 1.1 Structure of YsxC Bound to GMP-PNP. (A) A ribbon diagram of the YsxC bound to the non-hydrolyzable GTP analogue GMP-PNP and magnesium (black) (RCSB PDB entry 1SVW) (Berman, Westbrook *et al.* 2000) (Ruzheinikov, Das *et al.* 2004;Bourne, Sanders *et al.* 1991; Das, Sedelnikova *et al.* 2004). The two switch regions are labelled and coloured black (Sprang 1997; Ruzheinikov, Das *et al.* 2004). **B.** The active site and G-domains of YsxC with active-site consensus residues of each G-domain visible and labeled is shown (Sprang 1997). Models were prepared using UCSF Chimera software (Sprang 1997; Pettersen, Goddard *et al.* 2004).

Conserved G-Domain Residues of The P-loop GTPases

G-Domain	Synonyms	Consensus	Interactions
1	Walker A	**GxxxxGK(S/T)**	**K:** Hydrogen bonds with α, β phoshpates **S/T:** Coordinates Mg^{2+}
2	Switch I, Effector Loop	**T**	**T:** Mg^{2+} **Non-conserved Residues:** GAP interactions
3	Switch II, Walker B	**DxxG**	**D:** Mg^{2+} via a water molecule **G:** Backbone amide hydrogen bonds with γ-phosphate
4	N/A	**(N/T)KxD**	**T:** Guanine ring oxygen 6 **K:** Backbone methylene groups with guanine ring **K:** ε-amino group with ribose oxygen **D:** Side-Chain carboxyl oxygens hydrogen bond with guanine amides at positions 1 and 2
5	N/A	N/A	Amide backbone interactions with exocyclic oxygen from guanine.

Table 1. The conserved G-domain residues of P-loop GTPases and their relevant chemical interactions with guanine nucleotide triphosphates based on homology to other GTPases (Du, Black *et al.*) (Ruzheinikov, Das *et al.* 2004). An 'x' indicates any amino acid.

The roles of the five G-domains have been well documented. The G1, or 'P-loop' region interacts with the α and β phosphates through a conserved lysine residue whose ε-amino group interacts via hydrogen bonds with the oxygen atoms of the phosphate groups, and also contains a conserved serine or threonine residue that interacts with an active-site magnesium (Mg^{2+}) (Bourne, Sanders *et al.* 1991; Sprang 1997). The presence of an active site Mg^{2+} cation increases the affinity of the active site for GDP and GTP in Ras, and is found in the active site of all P-loop GTPases with bound nucleotide studied to date (Sprang 1997). The G2 region, known also as SWI, contains threonine residue that assists in Mg^{2+} positioning, but also contains conserved family-specific residues involved in protein-protein interactions with GAPs (Bourne, Sanders *et al.* 1991; Sprang 1997). The G3/SWII region has a conserved aspartic acid for coordinating the Mg^{2+} via a water

molecule, and a conserved glycine donates a hydrogen bond from its backbone amide to and oxygen from the γ-phosphate (Sprang 1997; Bourne, Sanders *et al.* 1991). The two switch regions show considerable changes in relative position between the GDP and GTP-analog bound crystal structures of GTPases, including YsxC (Bourne, Sanders *et al.* 1991; Sprang 1997; Ruzheinikov, Das *et al.* 2004). The G4 region meanwhile interacts with the guanine ring and ribose sugar through conserved lysine and aspartic acid residues (Sprang 1997). The final region, G5, is often overlooked despite important interactions between a backbone amide and the oxygen of the guanine ring, probably due to the lack of a consensus sequence for this region (Bourne, Sanders *et al.* 1991; Sprang 1997; Ruzheinikov, Das *et al.* 2004). Collectively, G4 and G5 are the source of guanine selectivity in GTPases (Bourne, Sanders *et al.* 1991; Sprang 1997).

The catalytic mechanism of small Ras-like GTPases involves a one step nucleophilic substitution reaction (S_N2) where a water molecule acts as a nucleophile (Webb and Eccleston 1981; Feuerstein, Goody *et al.* 1989; Sprang 1997).
The precise nature of the transition state remains controversial (Webb and Eccleston 1981; Feuerstein, Goody *et al.* 1989; Maegley, Admiraal *et al.* 1996; Scheffzek, Ahmadian *et al.* 1997; Du, Black *et al.* 2004; Li and Zhang 2004; Grigorenko, Nemukhin *et al.* 2007). The transition state of the enzyme-catalyzed hydrolysis involves an intermediate which is either predominantly dissociative or associative (Maegley, Admiraal *et al.* 1996; Scheffzek, Ahmadian *et al.* 1997; Li and Zhang 2004; Grigorenko, Nemukhin *et al.* 2007). In a more dissociative transition state, negative charge would build up on the γ-β-phosphate-bridging oxygen and the other β-phosphate oxygens, and

the transition states involved would have very weak bonds between the γ-phosphate and the β-oxygen and attacking water (Maegley, Admiraal *et al.* 1996; Scheffzek, Ahmadian *et al.* 1997; Li and Zhang 2004; Grigorenko, Nemukhin *et al.* 2007). In a more associative transition state, the bonding between both β-oxygen and attacking nucleophile is strong, and negative charge builds up on the oxygens of the γ-phosphate (Maegley, Admiraal *et al.* 1996; Scheffzek, Ahmadian *et al.* 1997; Li and Zhang 2004; Grigorenko, Nemukhin *et al.* 2007). Most recent evidence has supported a predominantly dissociative mechanism for Ras, which is consistent with the solution hydrolysis of GTP (Maegley, Admiraal *et al.* 1996; Li and Zhang 2004; Grigorenko, Nemukhin *et al.* 2007). The catalytic residues for P-loop GTPases would be those that stabilize charges that build up during a transition state, or orient the attacking nucleophilic water (Li and Zhang 2004). In Ras, the water is oriented by Gln61 of the switch II region (**DxxGQ**), but this orientation only occurs in the activated conformation of the enzyme (Scheffzek, Ahmadian *et al.* 1997; Li and Zhang 2004). Other GTPases have alternative residues at this position, including YihA/YsxC which have a Tyr residue at the equivalent position (Joyce and Grindley 1982; Trach and Hoch 1989; Sprang 1997; Arigoni, Talabot *et al.* 1998; Dassain, Leroy *et al.* 1999; Leipe, Wolf *et al.* 2002). The other catalytic residues are those that stabilize charge on the bridging oxygen between the β and γ phosphates, especially Ras Gly13 (Asn36 in YihA) the backbone amide of which hydrogen bonds to the bridging oxygen, and multiple residues that stabilize charge buildup on the oxygens of the β-phosphate (Maegley, Admiraal *et al.* 1996; Scheffzek, Ahmadian *et al.* 1997; Li and Zhang 2004). GAP activation of hydrolysis in Ras involves an arginine residue from

the GAP (the "arginine finger", Arg789 of Ras-GAP) which helps orient the attacking water molecule and further stabilizes the bridging oxygen between β and γ phosphates (Scheffzek, Ahmadian *et al.* 1997; Li and Zhang 2004). Not all eukaryotic GTPases have GAPs with arginine fingers, notably Ran, ARF, Rap, EF-G, EF-Tu, and it appears therefore that most important function of GAPS is correct orientation of the active site catalytic residues through interactions with the flexible switch regions (Sprang 1997; Li and Zhang 2004).

1.4 GTPases of Unknown Function and The Bacterial Ribosome

Functional bacterial ribosomes (70S ribosomes) are large complexes of ribosomal ribonucleic acids (rRNAs) and proteins which first form two subunits, called the 30S and 50S, based on their apparent sedimentation coefficients (Svedberg units) (Hosokawa, Fujimura *et al.* 1966; Traub and Nomura 1968; Algranati, Gonzalez *et al.* 1969). Prokaryotic rRNAs, specifically called 16S, 23S and 5S, are co-transcribed from one of several operons and processed before ribosomal proteins are added (Wilson and Nierhaus 2005; Wilson and Nierhaus 2007). The small 30S ribosomal subunit includes the 16S rRNA, and 21 ribosomal proteins, whereas the large 50S subunit includes a 5S rRNA and a 23S rRNA onto which 33 ribosomal proteins are assembled (Wilson and Nierhaus 2005; Wilson and Nierhaus 2007). Preliminary studies of prokaryotic ribosome assembly using prokaryotic ribosomes involved purified ribosomes being chemically deconstructed to yield rRNA, then the addition of pools of proteins or single purified proteins to the rRNA to determine the possible sequences of incorporation (Hosokawa, Fujimura *et al.* 1966;

Nomura and Traub 1968; Traub and Nomura 1968; Nomura, Traub *et al.* 1969; Kischa, Moller *et al.* 1971; Nierhaus and Dohme 1974; Rohl and Nierhaus 1982; Nierhaus 1991). Ribosomal subunits can be reconstituted *in vitro*, but the efficiency of this process is far less than that of the *in vivo* process, and requires non-physiological temperatures and chemical conditions (Nierhaus 1991). The process is much better understood in eukaryotes, and multiple co-factors have been shown to have roles in assembly *in vivo*, including nearly 200 proteins and over 70 non-coding RNAs in yeast (Fromont-Racine, Senger *et al.* 2003; Hage and Tollervey 2004; Karbstein 2007). Collectively, these facts suggest the existence of unknown co-factors for prokaryotic ribosome assembly, and highlight a need for a more complete understanding of this complicated process in a cellular context (Nierhaus 1991; Hage and Tollervey 2004; Karbstein 2007). Although many modern antibiotics target bacterial ribosomes, ribosome assembly has yet to be intentionally exploited as a drug target, and therefore represents a potential source of novel antibiotics (Champney 2003; Hermann 2005; Poehlsgaard and Douthwaite 2005; Sutcliffe 2005; Comartin and Brown 2006).

In recent years, ongoing work in *Escherichia coli* and *Bacillus subtilis* has highlighted a role for an increasing number of cofactors in ribosome assembly or function (Karbstein 2007; Wilson and Nierhaus 2007). Currently, not less than 30 cofactors are known to modify 16S and 23S rRNA, some at multiple bases, and some 26 non-ribosomal proteins are thought to act in either regulation of assembly or ribosome regulation (Wilson and Nierhaus 2007). Interesting among these proteins are several representatives of the p-loop GTPases, namely EngA (Der/YphC), Era, Obg (CgtA/YhbZ), RbgA (YlqF),

RsgA (YjeQ/YloQ), EngB (YihA/YsxC), and most recently YqeH has been shown to be involved in ribosome assembly (Wilson and Nierhaus 2007; Loh, Morimoto *et al.* 2007; Uicker, Schaefer *et al.* 2007; Robinson, Hwang *et al.* 2002; Bharat, Jiang *et al.* 2006; Schaefer, Uicker *et al.* 2006; Matsuo, Morimoto *et al.* 2006; Uicker, Schaefer *et al.* 2006; Meier, Peery *et al.* 2000; Maki and Culver 2005).

The key phenotype of factors involved in ribosome assembly is an atypical ribosome profile. In the case of all of these factors, depletion leads to the accumulation of 30S and 50S or precursors thereof, and loss of 70S, typically demonstrated by analytical ultracentrifugation or centrifugation through a sucrose gradient followed by fractionation with absorbance monitoring at 254 nm or 260 nm wavelengths to detect RNA species (Bharat, Jiang *et al.* 2006; Schaefer, Uicker *et al.* 2006; Inoue, Alsina *et al.* 2003; Matsuo, Morimoto *et al.* 2006; Uicker, Schaefer *et al.* 2006). So called "ribosome profiles" are thus generated which have characteristic peaks for each subunit and 70S ribosomes, the ratios of which are consistent in wild-type *E. coli* under non-stressing physiological conditions. In addition, a majority of these genes were identified in preliminary studies as being involved in cell division because of the formation of elongated filamentous cells with multiple copies of the chromosome but apparent problems with failed septation (Gollop and March 1991; Dassain, Leroy *et al.* 1999; Morimoto, Loh *et al.* 2002; Datta, Skidmore *et al.* 2004; Campbell, Daigle *et al.* 2005).

1.5 The Study of Essential Genes of Unknown Function

The study of essential genes has traditionally been challenging because of the difficulties in determining phenotypes that would result from genetic perturbation of the gene. For essential genes, clean deletions can not be generated, so the phenotype of a deletion remains obscure. Multiple methodologies have been developed to address this problem, including: reverse chemical genetic methods which use small molecule inhibitors of the specific protein, generation of temperature sensitive mutations in the essential gene which result in a protein inactive at the non-permissive temperature; complementation *in trans* from a temperature-sensitive plasmid which is cured at a non-permissive temperature; and conditional complementation from a metabolite-inducible promoter such as P_{BAD}, taking advantage of physiological metabolic regulation (Stockwell 2000; Stockwell 2000; Hwang and Inouye 2001; Smukste and Stockwell 2005; Schaefer, Uicker *et al.* 2006; Terpe 2006; Kawasumi and Nghiem 2007). The simplest of these methods is the generation of strain where the gene of interest is under control of a tightly regulated inducible promoter. The P_{BAD} arabinose inducible promoter system has been extensively studied and well characterized in *E. coli* (Lee, Gielow *et al.* 1981; Johnson and Schleif 1995;Ogden, Haggerty *et al.* 1980; Martin, Huo *et al.* 1986; Huo, Martin *et al.* 1988; Schleif 2003; Terpe 2006).

Methods have been recently developed which allow for the efficient generation of strains of *E. coli* in which the expression of an essential gene is placed under control of an inducible promoter. These strains are generated using phage-based recombination-assistance plasmids and linear PCR products to facilitate chromosomal replacement of the

araBAD locus with the gene of interest and a resistance marker, and subsequent replacement of the wild-type gene with a second resistance marker (Datsenko and Wanner 2000; Campbell and Brown 2002; Bharat, Jiang *et al.* 2006). This system has been of great utility in multiple studies of essential genes involved in ribosome assembly (Bharat, Jiang *et al.* 2006). Using bacteria engineered in this way, phenotype is observable as a result of "depletion" of the gene product in liquid media by successive rounds of growth in the absence of the inducer, in this case L-arabinose. This provides an excellent system for controlling gene dosage and observing otherwise cryptic effects of the loss of an essential gene while the bacteria are in a state of stress prior to death (Bharat, Jiang *et al.* 2006). It is in this depleted state of stress that ribosome profiles are observed to be altered in the cases of EngA, YsxC, YlqF, and YqeH among others (Bharat, Jiang *et al.* 2006; Schaefer, Uicker *et al.* 2006; Uicker, Schaefer *et al.* 2007).

1.6 Chemical Enhancement Screening

In recent years there have been major advances in the use of chemicals as probes of physiological processes (Stockwell 2000; Stockwell 2000; Smukste and Stockwell 2005). Many antibiotics and bioactive molecules have specific targets identified *in vivo*, and as such are useful as probes for those aspects of physiology that they perturb. The concept of chemical synthetic lethality has been used successfully to probe the functions of genes of unknown function (Campbell, Daigle *et al.* 2005). When a genetic pathway is perturbed by the depletion or deletion of a given gene of unknown function, bioactive molecules which affect the same pathways or related cellular processes will show changes

in their minimum inhibitory concentrations whereas bioactive molecules that affect unrelated pathways will remain equally effective (Campbell, Daigle *et al.* 2005). This methodology is useful where collections of compounds are available for which targets are well characterized, and where gene functions have proved difficult to probe using traditional genetic techniques (Campbell, Daigle *et al.* 2005). A wide variety of antibiotics have been shown to bind the ribosome and to act against translation or its fidelity, and in many cases their binding sites on the ribosome have been mapped by x-ray crystallography (Hermann 2005; Sutcliffe 2005; Poehlsgaard and Douthwaite 2005). The use of targeted chemical collections has recently been used to investigate the function of YjeQ/YloQ on the ribosome, demonstrating the potential of this technique in probing activities on the bacterial ribosome (Campbell, Daigle *et al.* 2005). Using other antibiotics with known activities, it should be possible to probe a diverse set of cellular pathways for chemical genetic interactions with genes of unknown function.

1.7 Research Objectives

The efforts at the outset of this study were directed towards understanding the functions of *yihA* in *E. coli*, a gene of unknown function that based on limited circumstantial evidence, had a suspected role in ribosome assembly. To study this essential gene, construction of an *E. coli* strain where *yihA* expression was tightly regulated and dependent on l-arabinose was first necessary. Using this strain, the impact of YihA depletion on cellular morphology was confirmed, and the impact of its depletion on bacterial ribosomes was investigated. To gain greater insight into the areas of

ribosome physiology and function and other aspects of cellular physiology affected by YihA depletion, chemical sensitization of a strain depleted of YihA to a number of antibiotics with known targets involved in multiple cellular processes and various aspects of ribosome function were investigated. With the emergence of published data concerning the function of YsxC on the *B. subtilis* ribosome, the focus shifted towards the YihA protein and the relationship between specific residues and its function. To facilitate *in vitro* biochemistry i.e. antibody preparation and recombinant protein expression, plasmid constructs for over-expression of two variants of YihA were prepared, one with a cleavable hexa-histidine amino-terminal tag and the other with a carboxy terminal hexa-histidine tag. After verifying the ability of one of these constructs to complement the depletion of YihA, a subset of basic residues and active-site residues were selected for targeted mutagenesis within that construct in order to identify residues essential for YihA function *in vivo*. Using the techniques described, the uncharacterized physiological function and importance of *yihA*, a gene of unknown function in *Escherichia coli*, were investigated.

CHAPTER 2 – EXPERIMENTAL PROCEDURES

2.1 Bacterial Strains and Culturing Methods

Table 2.1 lists the strains constructed and used in the present study, and Table 2.2 lists plasmids constructed during this study. A complete list of primers utilized is shown in Table 2.3. Strains were cultured in Luria-Bertani (LB) broth with appropriate antibiotic selection (see Table 2.1, Table 2.2), at 37°C shaking at 250 rpm unless otherwise indicated. Solid media plates were made using LB-agar, with antibiotic selection (see Table 2.1, 2.2). Concentrations of antibiotics were 100 μg/ml ampicillin, 25μg/ml chloramphenicol, and 50μg/ml kanamycin. Where necessary, L-arabinose (Bioshop, Burlington, Ontario) was added to the media to a final concentration of 0.2% (w/v).

Electrocompetent cells were prepared using the protocol recommended by the Electroporator manufacturer's protocol (Bio-Rad, Hercules, California). This protocol was occasionally adapted for reduced culture sizes by changing the volumes of the glycerol rinses in proportion to the size of the culture being prepared. Ten percent glycerol was autoclaved for this procedure, and all handling of liquids was done under flame to prevent contamination.

Table 2.1 Bacterial Strains Used in This Study

Strain	Genotype	Reference/Source
MG1655	*F-labmda-*	George Church, Harvard Medical School
Novablue	*endA1 hsdR17(rk12-mk12+)supE44 thi-1 recA1 gyrA96 relA1 lac[F' pro A+ B+ lacIqZ delta M15::Tn10] cR*	Novagen.
BL21-AI	*F-ompT hsdSB (rB- mB-) gal dcm araB::T7RNAP-tetA*	Invitrogen.
EB1830	MG1655, *araBAD::P_{BAD}yihA-kan^R, pKD46*	This study.
EB1981	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA*	This study.
EB1982	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST14$_{yihA\text{-}c\text{-}HIS}$*	This study.
EB1983	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA}$*	This study.
EB1984	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}R119A\text{-}H120A}$*	This study.
EB1985	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K39A\text{-}S40A\text{-}R119D\text{-}H120D}$*	This study.
EB1986	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}G38A\text{-}K39A\text{-}S40A}$*	This study.
EB1987	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K156I\text{-}R163L}$*	This study.
EB1988	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K149I}$*	This study.
EB1989	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}T55A\text{-}E131G\text{-}R155L\text{-}K156I}$*	This study.
EB1990	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}Q127R\text{-}K188M\text{-}K192I}$*	This study.
EB1991	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K92M\text{-}R93S\text{-}K94E\text{-}R97L}$*	This study.
EB1992	MG1655, *araBAD::P_{BAD}yihA-kan^R,ΔyihA, pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}fragment}$*	This study.

Table 2.2. Plasmids Used in This Study

Plasmid	Description	Reference
pDONR	Gateway cloning vector	Invitrogen ™
pDEST17	Gateway Expression vector	Invitrogen ™
pDEST14	Gateway Expression Vector	Invitrogen ™
$pDEST14_{yihA\text{-}c\text{-}HIS}$	pDEST14 encoding a carboxy-terminally hexa-histidine tagged YihA	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$	pDEST17 encoding YihA with an amino-terminal hexa-histidine tag linked by a tobacco etch virus protease cleavable sequence	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}R119A\text{-}H120A}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K39A\text{-}S40A\text{-}R119D\text{-}H120D}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}G38A\text{-}K39A\text{-}S40A}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K156I\text{-}R163L}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K149I}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}T55A\text{-}E131G\text{-}R155L\text{-}K156I}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}Q127R\text{-}K188M\text{-}K192I}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}K92M\text{-}R93S\text{-}K94E\text{-}R97L}$	Derivative of $pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ with mutations.	This study
$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA\text{-}fragment}$	$pDEST17_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ expressing only 12 of the amino-terminal YihA residues.	This study
pkd46	Helper plasmid expressing *β,γ exo*	(Murphy 1998; Datsenko and Wanner 2000)
pkd3	Template plasmid for knockout PCR	(Datsenko and Wanner 2000)
pcp20	Temperature sensitive expression of FLP protein for excision of knockout resistance marker	(Cherepanov and Wackernagel 1995; Datsenko and Wanner 2000)
pCA24N-*yihA*	ASKA plasmid expressing amino-terminally histidine tagged YihA	(Kitagawa, 2005)
pBS-*araBAD-Flank-Kan*	Plasmid for knock-in PCR Product	Campbell and Brown 2002.

Table 2.3 Primers Used in This Study

Primer Name	Sequence (5' to 3')	Purpose
pBAD-seq_check-5'	gaacccgtattggcaaatattgacggccag	sequencing
pBAD-seq_check-3'	gcgtttcactccatccaaaaaaacggg	sequencing
CAT-KO-conf.-rev	caccatgggcaaatattatacgcaaggcga	knockout confirmation
yiha-KO-n-short	tcgccacaaagctttgactgacggagac gaccgctgtgtaggcttggagctgcttc	generating knockout
yiha-KO-n-med	ccgcctttattatggttcaggtataatcg ccacaaagctttgactgacggagacgaccg ctgtgtaggcttggagctgcttc	generating knockout
yiha-KO-n-long	cgtatggcttttacttacaacgctcacgccg aaaaatccgcctttattatggttcaggtat aatcgccacaaagctttgactgacggagacg accgctgtgtaggcttggagctgcttc	generating knockout
YihA-KO-C-short-new	gggccggatacgccacatccggcacaagcat taaggcaagaaaacatatgaatatcctcctta	generating knockout
YihA-kO-C-med-new	tgtaatctattgaatttacgggccggatacgct tcacatccggcacaagcataaggcaagaaaaca tatgaatatcctcctta	generating knockout
YihA-KO-C-long-new	gcctgatgcgctatgtttatcaggccaacggta gaattgtaatctattgaatttacgggccggata cgccacatccggcacaaggcattaaggcaagaa aacatatgaatatcctcctta	generating knockout
YihA-KI-MTLNL-15UP-EcoRV-F	gatatcacggagacgaccgctttgactaatttg aattatcaacagacg	generating knock-in
YihA-EcoRV-Reverse	gatatccccggggcggccgcttattcgccgtcc tctgcgttt	generating knock-in
YihA-R119DH120D-F	gtgctaatggatattgacgatccgctgaaagat ttg	Mutagenesis
YihA-R119DH120D-R	caaatctttcagcggatcgtcaatatccattag cac	Mutagenesis
YihA-R119AH120A-F	gtgctaatggatattgccgctccgctgaaagat ttg	Mutagenesis
YihA-R119AH120A-R	caaatctttcagcggagcggcaatatccattag cac	Mutagenesis
YihA-G38V-Forward	ggccgttccaacgcagttaaatccagcgcgctg	Mutagenesis
YihA-G38V-Reverse	cagcgcgctggatttaactgcgttggaacggcc	Mutagenesis
YihA-K39AS40A-Forward	cgttccaacgcaggtgcagccagcgcgctgaac acg	Mutagenesis
YihA-K39AS40A-Reverse	cgtgttcagcgcgctggctgcacctgcgttgga acg	Mutagenesis
YihA-S40A-Forward	tccaacgcaggtaaagccagcgcgctgaacacg	Mutagenesis
YihA-S40A-Reverse	cgtgttcagcgcgctggctttacctgcgttgga	Mutagenesis
YihA-K39A-Forward	cgttccaacgcaggtgcatccagcgcgctgaac	Mutagenesis
YihA-K39A-Reverse	gttcagcgcgctggatgcacctgcgttggaacg	Mutagenesis
YihA-NTD-Internal-Forward	agtgcgcctgatattcgccacctaccttcc	Sequencing/Gene detection
YihA-CTD-Internal-Reverse	Aaaccaggtatccagtttctgccg	Sequencing/Gene detection

Table 2.3 Continued

Primer Name	Sequence (5' to 3')	Purpose
YihA-SWII-92-94 and 97-Forward	gcggaagtcccggaagagatgatgagcgaatgg cagcttgcgctcggcgaatacctc	Mutagenesis
YihA-SWII-92-94 and 97-Reverse	gaggtattcgccgagcgcaagctgccattcgct catcatctcttccgggacttccgc	Mutagenesis
YihA-K149I-Forward	ctggtgctgctgaccaaagcggacatactggca agcggcgcacgtaaagcgcaattgaat	Mutagenesis
YihA-K149I-Reverse	attcaattgcgctttacgtgcgccgcttgccag tatgtccgctttggtcagcagcaccag	Mutagenesis
YihA-R155S-K156I-Forward	ccaaagcggacaaactggcaagcggcgcactta tagcgcaattgaatatggtgcgtgaagctgtac tggcg	Mutagenesis
YihA-R155S-K156I-Reverse	cgccagtacagcttcacgcaccatattcaattg cgctataagtgcgccgcttgccagtttgtccgc tttgg	Mutagenesis
YihA-K156I-R163I-Forward	gcggacaaactggcaagcggcgcacgtatagcg caattgaatatggtgcttgaagctgtactggcg	Mutagenesis
YihA-K156I-R163I-Reverse	cgccagtacagcttcaagcaccatattcaattg cgctatacgtgcgccgcttgccagtttgtccgc	Mutagenesis
YihA-K182M-K183I-Forward	ggttgaaacgttttcttcgttgatgatacaagg cgtggacaagctgcgg	Mutagenesis
YihA-K182M-K183I-Reverse	ccgcagcttgtccacgccttgtatcatcaacga agaaaacgtttcaacc	Mutagenesis
YihA-K182M-K192I-Forward	cgttgaagaaacaaggcgtggacatgctgcggc agatactggatacctggtttagcgagatgcagcc	Mutagenesis
YihA-K182M-K192I-Reverse	ggctgcatctcgctaaaccaggtatccagtatc tgccgcagcatgtccacgccttgtttcttcaacg	Mutagenesis
YihA-pD17-F-New	ggggacaagtttgtacaaaaaagcaggcttagat tacgatatcccaacgaccgaaaccctgtattttc agggcactattttgaattatc	Gateway cloning, mutagenesis
YihA-pD17-Rev	gggccactttgtacaagaaagctgggtattattc gccgtcctgcgtttcttc	Gateway cloning
YihA-pD14-F-New	gggacaagtttgtacaaaaaagcaggcttcgaag gagatagaaccatggtcgatgagtgcgcctgata ttcgccaccta	Gateway cloning
YihA-pD14-Rev	ggggccactttgtacaagaaagctgggtattagt ggtggtggtggtggttcgccgtcctgcgttt cttctacagg	Gateway cloning
BAD-a	aaggaaaaaagcggccgcaccgcgaatggtgaga ttgagaatata	Knock-In generation (Campbell and Brown 2002)
BAD-d	cgcacgcatgtcgacttcagacgggcattaacga tagtg	Knock-in generation (Campbell and Brown 2002)
BAD-e	atgcaggatttttgcccaga	Control for Knock-in (Campbell and Brown 2002)

2.2 General Cloning Methods and Protocols

Throughout this work, purification of plasmids from *E. coli* was performed using QiaPrep Miniprep kits (Qiagen Inc., Mississauga, Ontario) as per manufacturer's protocols, with all recommended rinse steps and elution in either 30 or 50 μL sterile distilled water. Agarose gels were prepared by addition to a final concentration of 1% agarose (w/v) to buffer tris-acetate-ethylene-diamine-tetraacetic acid (EDTA) (TAE), subsequent heating in a microwave until completely dissolved, and cooling in a gel-box until solidified (Sambrook, Fritsch *et al.* 1989). For purification, DNA samples were loaded with the addition of DNA-loading buffer, and gels stained by immersion in ethidium bromide in TAE for 10 to 30 minutes (Sambrook, Fritsch *et al.* 1989). Gels were visualized using a UV transilluminator, and pictures taken using a Canon digital camera were subsequently processed using Adobe Photoshop software such that images were made monochromatic (grey-scaled) and light intensity maximized (auto-adjust levels). DNA was isolated from agarose gels by excision using a razor blade after staining on the UV transilluminator, and subsequently purified by Qiagen kit as per manufacturer's instructions with all recommended rinses: Mini-elute gel extraction kits were used for low-abundance or small molecular weight fragments (<500 bp), whereas for abundant fragments or plasmids QiaQuick gel extraction kits were used (Qiagen Inc., Mississauga, Ontario). Where indicated, PCR reactions were cleaned using Qiagen QiaQuick gel extraction kits and manufacturer's recommended protocols for PCR purification.

E. coli chromosomal DNA was prepared from overnight 5 ml or 10 ml cultures grown in LB broth, which were pelleted in Eppendorf tubes by micro-centrifugation, re-suspended in 250 µL Tris-EDTA buffer, and treated with 25µL of lysozyme for 45 minutes at 25°C (Sambrook, Fritsch *et al.* 1989). Fifty microliters of freshly prepared STEP buffer was then added, and tubes were incubated at 50°C for 1 hour. Three hundred microliters phenol was then added to the tube, which was shaken by hand for 3 minutes, then micro-centrifuged for 15 minutes at 13,200 rpm. The top layer of the extraction was then pipetted off and added to twenty-five µL ammonium acetate (3M, pH 5.2). Two hundred microliters of ice-cold absolute ethanol was then added to each tube, and tubes were incubated at -20°C for 30 minutes or longer. Precipitated DNA was then spooled onto a glass rod, and solubilized in 100 µL water with RNAse-A (2µL of 10mg/ml) (Roche, Mississauga, Ontario). Concentration of all DNA samples in this study was performed using absorbance at 260nm in water in a quartz micro-cuvette, and the assumption that 1.0 absorbance unit at 260 nm is equivalent to 50µg/ml of DNA (Sambrook, Fritsch *et al.* 1989).

Polymerase chain reactions (PCRs) to generate DNA for the purposes of cloning used VENT high-fidelity polymerase and manufacturer's provided buffers (New England Biolabs, Pickering, Ontario) , whereas colony-PCR and other diagnostic PCRs were performed using HotStar Taq Polymerase and manufacturer's provided buffers (Qiagen Inc., Mississauga, Ontario). Reaction cycle times and temperatures, as well as magnesium, primer, and template DNA concentrations were varied for each application as needed to achieve successful amplifications. PCR reactions were verified by agarose

gel electrophoresis as described, and purified for cloning using either PCR-purification or gel-extraction as described, and quantified upon purification as described.

Restriction digests were performed using New England Biolabs restriction enzymes and manufacturer's recommended buffers and protocols (New England Biolabs, Pickering, Ontario). DNA was ligated using T4 DNA-ligase and buffers from New England Biolabs. Verification of digest products was performed by agarose gel electrophoresis.

Transformations of electrocompetent *E. coli* cells were performed with ice-chilled electroporation cuvettes (BioRad, Hercules, California), using a BioRad MicroPulser electroporation system on setting EC2. Two microliters of DNA were transformed per reaction into 45 µl of cells, and recovery was in 1 ml LB shaking at 30°C or 37°C as indicated, for between 1 hour and 24 hours. After recovery, 25 µl and 75µl of culture was plated, and the remainder was pelleted by centrifugation, then resuspended in 100µl LB and plated on a third plate of LB-agar with drug as indicated in each case.

2.3 General Methods: Protein

Sodium dodecyl sulfate (SDS) poly-acrylamide gel electrophoresis (PAGE) was performed using 15% SDS gels. Four 15% gels were made from the following solutions: Separating gel was cast from 3.4 ml water, 5 ml separation buffer, 200 µl 10% (w/v) SDS solution in water, 10 ml 30% acrylamide:bis 29:1 solution (BioRad, Hercules, California), 140 µl 10% (w/v) ammonium-per-sulfate (APS), 10 µl tetramethylethylenediamine (TEMED) (BioRad) and 200 µl 10% glycerol in water (Sambrook, Fritsch *et al.* 1989).

Stacking gel was cast on top of solidified separating gel using a solution of 6.1 ml water, 2.5 ml stacking buffer, 100µl 10% SDS (w/v) in water, 1 ml 30% acrylamide:bis 29:1 solution (BioRad, Hercules, California), 140 µl 10% APS, 10 µl TEMED. Gels were cast and electrophoresis performed using BioRad Protean gel systems.

Protein concentrations were determined using either the BioRad Bradford assay as per manufacturer's recommended protocol, or by denaturation and absorbance spectroscopy at 280 nm based on extinction co-efficients calculated using ProtParam software (ExPASy, http://ca.expasy.org/, Swiss Inst. Of Bioinformatics, Geneva, Switzerland).

For analysis of whole-cell protein contents, boiled whole cell preparations were performed as follows: 5 or 10 ml of cell culture were prepared and cells pelleted by centrifugation in Eppendorf 1.5 ml tubes. Cultures were then resuspended in 1 part buffer TE, and 1 part 2X SDS loading buffer was then introduced (Sambrook, Fritsch *et al.* 1989). Eppendorf tubes were then heated in a heating block to over 100°C for at least 20 minutes to denature proteins, and subsequently samples were analyzed by SDS PAGE.

2.4 Construction of *E. coli* with yihA under P_{BAD} Control

Construction of a strain where *yihA* expression was dependent on arabinose was performed in two steps following methods described previously (Datsenko and Wanner 2000; Campbell and Brown 2002). First, a copy of the wild-type *yihA* gene including 15 basepairs of upstream sequence was amplified by PCR from MG1655 *E. coli* chromosomal DNA using primers which included EcoRV sites on the 5' and 3' ends as

well as NotI and SmaI sites in the 3' primer (YihA-KI-MTLNL-15UP-EcoRV-F and YihA-EcoRV-Reverse), the PCR product was gel-purified and digested with EcoRV, and gel-extracted again. The *yihA* gene was then ligated into pBS derived plasmid provided by Tracey Campbell (Campbell and Brown 2002). The pBSaraBAD-flank-kan plasmid was pre-digested with PmeI, and ligation performed in the presence of PmeI to reduce the instances of re-ligation of the plasmid. The reactions were then transformed into electrocompetent Novablue cells, and transformants selected on LB-agar with kanamycin. Colonies were purified by re-streaking on LB-agar with kanamycin, and multiple colonies were used to make 10 ml liquid cultures from which plasmid DNA was purified as described, and from which stock strains were made in 15% glycerol LB and saved at -80°C. Orientation of the ligation was confirmed by SmaI digestion using restriction endonuclease specific cleavable sites 3' of the coding sequence of *yihA* and within the kanamycin resistance gene, yielding a 500bp product which was detected by gel electrophoresis. The DNA sequence of the plasmid was verified as correct by Mobix (McMaster University, Hamilton, Ontario) and it was used as template to amplify a 2.5 kbp PCR product with primers BAD-a and BAD-d (Campbell and Brown 2002). The knock-in PCR product was DpnI treated to ensure complete removal of plasmid that served as template, and gel purified at 2.5 kbp. The product was then transformed into MG1655 already containing pKD46 in the presence of 0.2% arabinose, and the resulting culture was incubated for 24 hours at 30°C, before plating on LB-KAN at 37°C for 16 hours. Colonies were likewise isolated and colony PCR was performed to identify strains with chromosomal integrations of *yihA* at the *araBAD* locus using primers yihA-NTD-

Internal-Reverse and BAD-d, and primers BAD-e and BAD-a as negative controls (Campbell and Brown 2002). A strain positive for *yihA* at *araBAD* was isolated, made electrocompetent, and transformed with pKD46 to make EB1830. The second step involved transformation of EB1830 with a linear PCR product including the chloramphenicol resistance gene from pKD3 and flanking sequence homologous to the regions flanking the wild-type locus of *yihA*. The linear PCR product was generated by first amplifying the chloramphenicol resistance gene from the plasmid with primers having approximately 60 bases of homology to the region surrounding *yihA*, subsequently purified, treating with 10 units DpnI for 21 hours under manufacturer's recommended conditions (New England Biolabs, Pickering, Ontario), and re-amplification using primers which generated longer flanking regions of 120 bases. The knock-out PCR product was then gel purified, and 2 µl was transformed into electrocompetent EB1830, with recovery at 30°C in LB-kanamycin-ampicillin with 0.2% L-arabinose present for approximately 20 hours. Seventy five microliters of the transformed cells were plated on LB-agar with kanamycin, chloramphenicol and 0.2% L-arabinose, and grown for 20 hours at 37°C. A single colony appeared after 24 hours and was re-streaked onto identical media. At 24 hours of growth, 4 colonies from the first re-streaking were streaked onto another plate ensuring colony purification had been achieved. From the second re-streaking, colonies were picked using a sterile loop and streaked onto LB-agar with kanamycin and chloramphenicol but without arabinose. At 24 hours, none of the cultures on media without arabinose showed single colonies, though trace smears of bacterial growth were observed on the plate. Differential interference contrast (DIC) light microscopy of this

bacterial growth showed cells that were remarkably elongated and suggested that the bacteria were dead or dying, indicating arabinose dependence. Single colonies on LB-kanamycin-chloramphenicol with 0.2% arabinose were picked and used to inoculate liquid cultures from which stock strains were made, and chromosomal DNA was isolated. The correct replacement of *yihA* at its native locus and presence of a complementing copy at *araBAD* was confirmed in one strain by PCR using multiple primer-sets, and the arabinose-dependence of this strain was confirmed by again streaking onto LB-agar-kanamycin-chloramphenicol with and without 0.2% arabinose. This strain was presumed to be an arabinose dependent strain with *yihA* under P_{BAD} control, and was made electrocompetent. The strain was transformed with the pCP20 plasmid, (Datsenko and Wanner 2000), recovered in LB-kanamycin-ampicillin with 0.2% arabinose at 30°C, then plated on LB-agar with the same antibiotics and 0.2% arabinose and grown 24 hours at 30°C. Single colonies from these plates were isolated, re-streaked on identical media, and grown at 30°C for 24 hours. Single colonies were then used to inoculate 5 ml liquid cultures in LB-kanamycin with 0.2% arabinose at 30°C, which were grown for 4 hours before being shifted to 43°C for 1 hour of growth, then shifted to 37°C for 12 hours to grow to saturation. Cultures were plated (50µl) on LB-agar with kanamycin and 0.2% arabinose, and colonies purified by re-streaking. A strain was isolated that showed chloramphenicol sensitivity, kanamycin resistance, and arabinose dependence as expected for a strain having lost the chloramphenicol resistance cassette. This strain was subsequently indexed as EB1981.

2.5 Growth Curves of *E. coli* with *yihA* under PBAD Control

Two sets of overnight cultures (one with 0.2% arabinose, one without) of EB1981 in LB-kanamycin and the same strain with pDEST17-N-His-TEV-*yihA*, or pDEST14-C-His-*yihA* in LB-kanamycin-ampicillin, or harboring an ASKA clone expressing amino-terminally hexa-histidine tagged YihA, and MG1655 in LB were grown to saturation at 37°C shaking at 250 rpm (see Table 2.2) (Kitagawa, Ara *et al.* 2005). Two microliters of each culture was used to inoculate 10 ml of the same mediuma (1/5000 dilution), and this was done in triplicate. The $O.D._{600}$ for each strain was then read at each time by sterile pipette extraction under flame of 200 µl of culture which was then placed in a clean 96 well CoStar flat-bottom plastic plate (Corning Inc., Corning, New York) read in a Spectramax Plus spectrophotometer. At approximately 4 hours ($O.D._{600}$ ~0.4), the strains were again subcultured by 1/5000. The data were analyzed using Microsoft Excel™; average $O.D._{600}$ for the triplicate cultures at each time point was calculated and standard deviation of the averaged values was determined and used to assign error bars in a line graph.

2.6 Ribosome Profiling by Sucrose Gradient Sedimentation

Ribosome purification and sucrose gradient fractionations were performed using protocols adapted from (Spedding 1990; Campbell, Daigle *et al.* 2005). For depletion, 10 ml cultures of EB1981 in LB-kanamycin with or without 0.2% arabinose were grown to saturation (optical density at 600 nm ($O.D._{600}$) was >1.0), and 100 µl of these cultures were used to inoculate 5 ml (1/50) cultures in LB-kanamycin which were then allowed to

grow to an $O.D._{600}$ of approximately 0.4 to 0.6. Once cultures had reached the desired $O.D._{600}$, 4 ml of each were used to inoculate 1L of the same media (1/250 dilution). The 1 L cultures were allowed to grow till $O.D._{600}$ of approximately 0.4 to 0.6, and chilled at 4°C for at least 20 minutes. MG1655 cultures were inoculated from saturated 5ml cultures in LB, used to inoculate (1/4000 dilution) 1L cultures of LB which were likewise grown to $O.D._{600}$ of approximately 0.2. Cells were harvested by centrifugation for 20 min at 7,000 *x g* in a Beckman-Coulter JLA 9.1000 rotor and Avanti J-25 floor-model centrifuge (Beckman-Coulter, Mississauga, Ontario), resuspended in 5 ml RNAse-free gradient buffer with 5 µl RNase-free DNAse (Roche Applied Sciences, Laval, Quebec) added, and lysed by sonication at 4°C, 3 passes of 1 minute each. Lysates were then clarified by centrifugation at 24,000 rpm in a Beckman Coulter MLA 80 rotor and Optima Max tabletop centrifugefor 45 minutes at 4°C. Where purification of ribosomes for *in vitro* biochemistry was desirable, clarified lysate was then pipetted onto equal volumes of 35% sucrose gradient buffer, and centrifuged at 150,000 *x g* for 15 hours in MLA 80 rotor at 4°C, after which pellets were resuspended in gradient buffer and frozen for later use. Where ribosome profiles were to be analyzed, the absorbance (260 nm) of lysate was measured, and between 100 and 200 absorbance units were loaded onto 32 ml sucrose gradients from 10% to 35% and centrifuged in an SW32 Ti rotor in a Beckman-Coulter Optima L100X for 16 hours at 18, 700 rpm. Gradients were displaced by addition of 60% glycerol in water to the bottom of the tubes using a Brandel Gradient Fractionator (Brandel, Gaithersburg, Maryland) set up in-line with an FPLC machine (Pharmacia Akta Prime) which monitored absorbance at 254 nm and collected fractions.

Profiles were constructed from absorbance at 254 nm (mili-absorbance units (mAu) vs. the volume of glycerol at which the absorbance was observed (ml) using raw data from the ACTA Prime imported as a text file into Microsoft Excel™.

2.7 Antibiotic Sensitization Screening

Saturated cultures of EB1981 lacking chloramphenicol resistance genes were grown in LB-kanamycin with 0.2% arabinose at 37°C with shaking, from which 100 µl was used to inoculate 10 ml fresh LB-kanamycin without arabinose under identical growth conditions. Once the 10 ml culture had reached an $O.D._{600}$ of 0.4 +/- 0.05, 50 µl of this culture was added to 100 ml of LB-kanamycin with or without 0.2% arabinose. From the culture, 200 µl was added to each well of a series of Corning CoStar 96 well clear-plastic plates (Corning Inc., Corning, New York). To such plates were added antibiotics which were two-fold serial diluted from left to right across the plate. Plates were prepared in triplicate and incubated at 37°C in a humidity controlled incubator without shaking for 20 hours. The $O.D._{600}$ of each well was read using a Spectramax plate-reading spectrophotometer, and plates were visually inspected for precipitated antibiotics which were omitted from analysis. The minimum inhibitory concentration (MIC) for each drug was defined as that which prevented growth above $O.D._{600}$ of LB alone in the 96 well plate for at least 2 of 3 replicates. The MIC for each antibiotic with arabinose present was compared to its MIC under depleted conditions, and the fold-sensitization was defined as the former divided by the latter. Where MICs were not found because of insufficient drug concentrations or irreproducible results to reproduce in

replicates, the experiment was repeated as necessary or the antibiotic in question was removed from the study. A total of 38 antibiotics were successfully tested and reported in this study.

2.8 Construction of Plasmids for Over-Expression of YihA

The *yihA* gene was amplified from the chromosomal DNA of MG1655 using either YihA-pD17-F-New/YihA-pD17-Rev or YihA-pD14-F-New/YihA-pD14-Rev primer pairs and cloned using the Gateway (Invitrogen, Burlington, Ontario) as per the manufacturer's instructions to create two over-expression constructs. One construct in pDEST17 plasmid had an amino-terminal tobacco etch virus (TEV) linker between the native protein and a hexa-histidine tag, while the other construct was made in pDEST14 and included a carboxy-terminal hexa-histidine tag on the otherwise native sequence. Upon completion of cloning, these constructs were transformed into electrocompetent BL21-arabinose inducible (BL21-AI) *E. coli* cells, and colony purified to yield clones which demonstrated inducible over-expression as determined by SDS PAGE of whole cell lysate. Gene sequences were also confirmed for the final products (Mobix Labs, McMaster University).

2.9 Generation of Plasmids Expressing YihA with Point-Mutations

Mutations were introduced to the *yihA* gene using crossover PCR, and the Gateway system (Invitrogen, Burlington, Ontario) was used to introduce these mutant genes into pDEST17 along with an amino-terminal hexa-histidine tag linked by a TEV

protease recognition sequence. Parallel PCR reactions were performed, the first using the 5' primer for Gateway™ cloning primer YihA-pD17-F-New and one of mutagenic primers, the second using the YihA-pD17-Rev and the reverse complement version of the primer used with YihA-pD17-F-New. The products of each were gel extracted at the expected molecular weights, and a second PCR performed including each pair of products and the two Gateway™ primers, and the products of these reactions were gel extracted at approximately 750 bp (the expected product size). At least 10 entry clone plasmids were isolated using Qiagen's Qiaprep Spin Miniprep kits and tested for insert by restriction digestion of plasmids using PstI which recognized a restriction endonuclease site within the pDONR vector and EcoRV which recognized an endonuclease site within *yihA* in a part of the gene sequence unaltered by mutations. Clones from the BP step of the Gateway (Invitrogen) protocol yielding digestion products were then sequenced before the LR reaction, and the sequences of the *yihA* variants in pDEST17 were confirmed after the final transformation into EB1981 (Mobix Lab, McMaster University). Strains EB1984 through EB1992 resulted from these plasmids being transformed into electrocompetent EB1981 cells, and subsequent purification of colonies.

2.10 Temperature Sensitization Screening of Mutants

Five millilitre cultures of EB1830, and EB1981-1992 were grown to saturation in LB-kanamycin-ampicillin with 0.2% arabinose. From each culture 100 µl was added to 100 µl of LB-kanamycin and the O.D. $_{600}$ of each was determined. The culture with the lowest O.D.$_{600}$ (0.28) was used as the basis for normalizing the O.D.$_{600}$ of the other

cultures after dilution into 100µl such that cultures with $O.D._{600}$ of 0.14 were achieved for each. The 12 cultures were each placed in one well of the 'A' row of wells in a 96 well CoStar plate (Corning Inc., Corning, New York), and 10 fold serial diluted down the plate yielding 8 concentrations of inoculum for each culture. One microliter of each inoculum for 2 sets of 6 strains was then plated using sterile pipette tips onto LB-agar-kanamycin-ampicillin, 3 plates with 0.2% arabinose 3 plates without, and one set of each plate type was incubated at 37°C, 42°C, and 16°C. Plates grown at 37°C were scanned after 19 hours of growth, whereas the plates grown at 16°C showed little growth at that time, and were accordingly scanned at 42 hours of growth.

2.11 Differential Interference Contrast Light Microscopy

VWR glass slides were prepared by treatment with 15 µl poly-l-lysine (Sigma, Oakville, Ontario) which was spread evenly over the surface using a sterile pipette tip, before being air-dried under flame. For imaging of cells from solid media, 10 µl PBS was then applied to the glass slide, and a sterile loop was used to pick a colony from the media, or in the case of a depleted YihA null, the sterile loop was used to collect cell lawns from the media. Cells were applied by gentle spreading in the PBS using the loop, and subsequently a square VWR Micro Cover Glass No. 2 cover slip was applied to the slide. For imaging of cells from liquid media, a volume of culture was pelleted and subsequently re-suspended in an equal volume of PBS. Ten microliters of cell suspension was then applied to a poly-l-lysine treated glass slide prepared as described, and a cover-slip added. Samples were imaged using oil-immersion light microscopy with an Olympus

Cx41 Microscope, and images captured by the attached Olympus Qcolour3 using Qcapture software. Pictures were re-sized after the addition of scale bars using Volocity Software, and modified by Adobe Photoshop to produce .JPEG files from the original .TIFF files for the sake of file size reduction.

CHAPTER 3 – RESULTS

3.1 The Generation of *E. coli* with *yihA* under P_{BAD} Control.

A strain of *E. coli* (EB 1981) was generated in which the only copy of the *yihA* gene was expressed under control of an arabinose inducible promoter using the method of Datsenko and Wanner (Datsenko and Wanner 2000). The *yihA* gene has been identified as essential in *E. coli* (Dassain, Leroy *et al.* 1999), and attempts to delete the gene without complementation were unsuccessful (data not shown). A complementing copy of *yihA* was integrated into the *araBAD* locus under the control of P_{BAD}, the native copy of the gene was replaced by a chloramphenicol resistance marker, and the genotype verified by multiple PCR reactions. The chloramphenicol resistance marker was later excised using a helper plasmid to yield a strain that was kanamycin resistant but chloramphenicol sensitive, EB1981. On LB-agar-kanamycin, the resulting strain was unable to form single colonies without arabinose present when streaked from frozen stocks. In liquid LB-kanamycin media however, the strain was capable of growth to saturation from frozen stocks, but upon successive subculturing became depleted and showed a reduction in growth rate. EB1981 was cultured in the presence and absence of arabinose to saturation, then sub cultured and grown to mid-log before being again subcultured into fresh LB-kanamycin for a final outgrowth. Figure 3.1 shows the growth of the EB1981 with and without arabinose during the first and second subcultures described. The strain showed nominal growth after 6 hours in the absence of arabinose, whereas in the presence of 0.2% arabinose growth was rapid and approached saturation. Thus EB1981 was shown to be arabinose dependent for growth under both solid and liquid growth conditions.

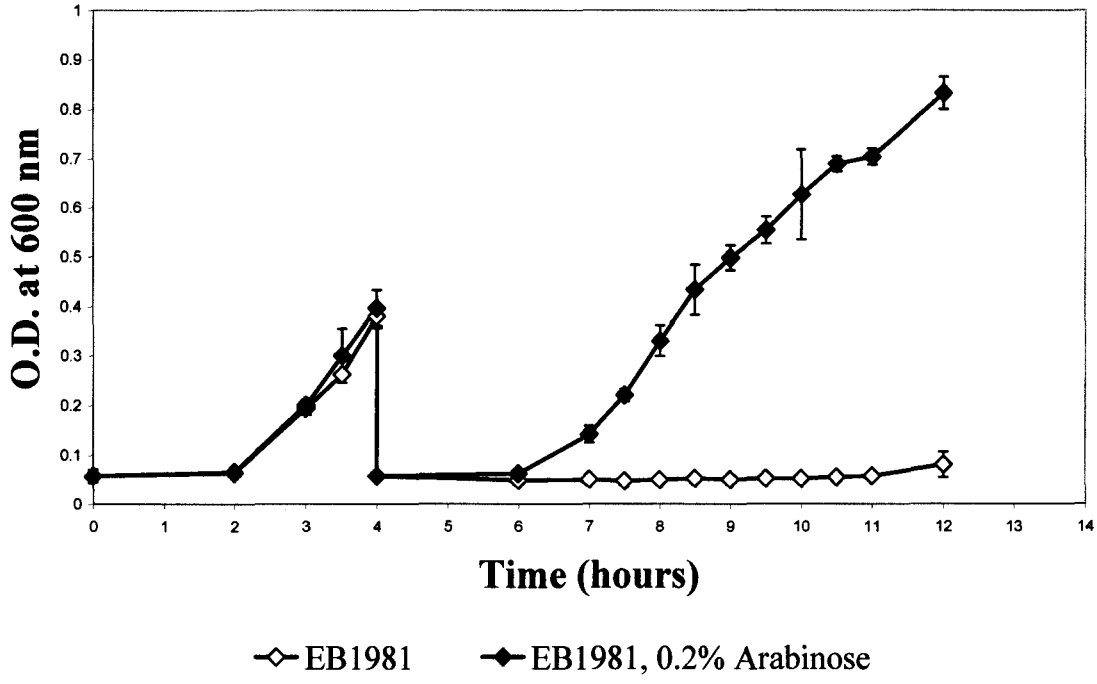


Figure 3.1. Arabinose Dependent Growth of EB1981 in Liquid Culture. The growth of the EB1981 was monitored by $O.D._{600}$ at 37°C with shaking 250 rpm in 10 ml LB-kanamycin with and without arabinose after subculturing from a saturated culture at 0 hours and subsequently again at $O.D._{600}$ of approximately 0.4 at 4 hours. Cultures were prepared in triplicate and the error bars represent one standard deviation from the mean of the $O.D._{600}$ of those triplicates.

3.2 Construction of Plasmids for Over-Expressing Tagged YihA Variants.

In order to study YihA *in vitro* it was useful to generate inducible over-expression vectors expressing histidine tagged variants of the protein suitable for use in batch purification. In order to express and rapidly purify a wild-type YihA protein, a tobacco etch virus (TEV) protease recognition sequence was used to link a hexa-histidine tag to the N-terminus of the YihA protein in a pDEST17 vector using the Gateway cloning system (Invitrogen, Burlington, Ontario). Once purified, this protein was proteolytically cleavable using recombinant TEV protease to yield a wild-type YihA protein with only the initiator methionine replaced with glycine (Kapust, Tozser *et al.* 2001). This method had been used previously to generate and easily purify TEV-cleavable histidine-tagged protein in the Brown lab (Daigle and Brown 2004). A second construct was generated in pDEST14 that expressed a carboxy-terminal hexa-histidine tagged variant of YihA. Expression from these two constructs (pDEST17-$_{\text{N-HIS-TEV-}yihA}$ and pDEST14-$_{\text{C-HIS-}yihA}$) was observed to be inducible in BL21-AI cells (data not shown) with the proteins migrating near their expected molecular weights (approximately 27,746 Da and 24,384 Da respectively vs. 23,561 Da for wild-type).

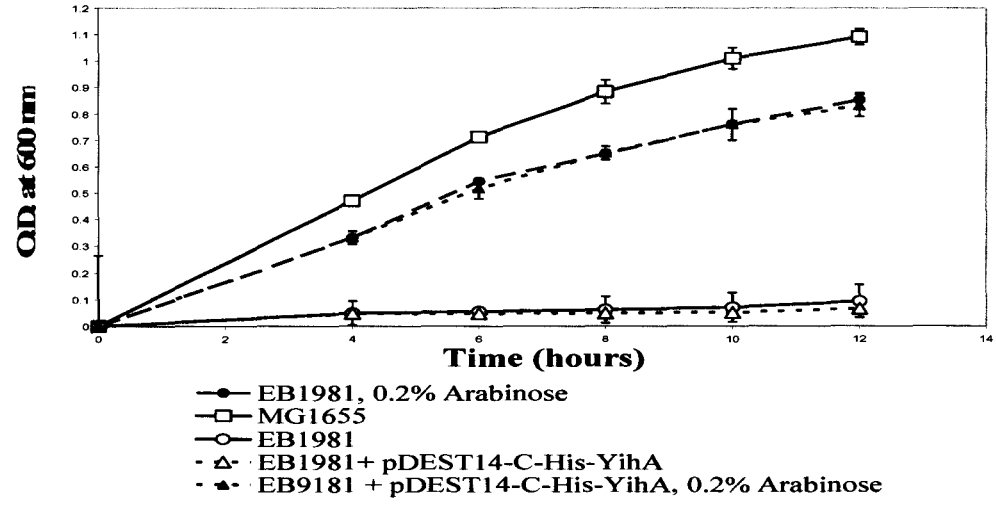
3.3 Complementation of EB1981 *in trans* From Plasmids Expressing YihA Variants.

The ability of wild-type YihA expressed in trans from a pDEST17 plasmid with a TEV-cleavable amino-terminal hexa-histidine tag to complement was tested. The presence of a copy of an essential gene under T7 promoter control on a pDEST vector has been shown previously within our lab to suppress the loss of the chromosomal copy for

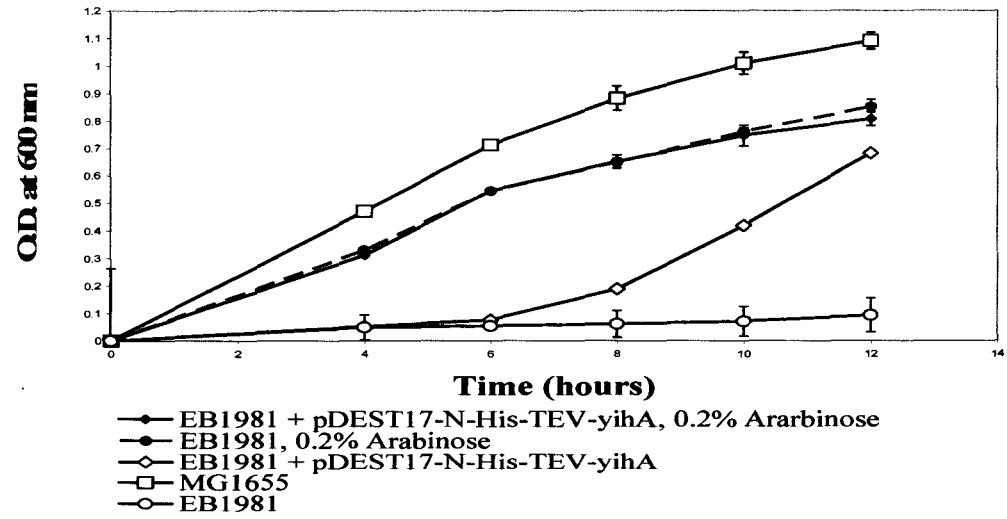
engA, a P-loop GTPase involved in ribosome biogenesis (A. Bharat, personal communication) (Bharat, Jiang *et al.* 2006; Schaefer, Uicker *et al.* 2006). The presence of pDEST17-$_{\text{N-HIS-TEV-}yihA}$ provided sufficient levels of expression to allow growth of the EB1981 without arabinose, though growth was slower than observed for EB1981in the presence of 0.2% arabinose (see Fig. 3.2). The ability of YihA expressed *in trans* from pDEST14-$_{\text{C-HIS-}yihA}$ plasmid to complement was also tested. In this case, the strain failed to grow in the absence of arabinose, indicating that the addition of 6 histidine residues to the carboxy-terminal end of YihA interfered with the activity of the protein, preventing complementation. The promoter (T7) and ribosome binding sites pDEST14 and pDEST17 are the same, and the inducible expression of a stable YihA migrating at the expected molecular weight in SDS PAGE experiments from the same construct was demonstrated in *E. coli* BL21-AI cells (data not shown), suggesting that levels of expression in the absence of inducer would be comparable from both plasmids. The growth rate of the EB1981 in the presence of arabinose appeared to be comparable to MG1655 wild-type *E. coli*, though cultures appear to reach saturation at a lower optical density.

The possibility of un-induced YihA expression from the pCA24N plasmid from the ASKA library (Kitagawa, Ara *et al.* 2005) complementing depletion ofYihA was investigated. The un-induced expression from PCA24N was also shown to be sufficient to complement (see Figure 3.2), which suggests the possibility of screening for multi-copy suppressors from this plasmid library.

A


OD at 600 nm
Time (hours)
EB1981, 0.2% Arabinose
MG1655
EB1981
EB1981+ pDEST14-C-His-YihA
EB9181 + pDEST14-C-His-YihA, 0.2% Arabinose

B


OD at 600 nm
Time (hours)
EB1981 + pDEST17-N-His-TEV-yihA, 0.2% Ararbinose
EB1981, 0.2% Arabinose
EB1981 + pDEST17-N-His-TEV-yihA
MG1655
EB1981

C

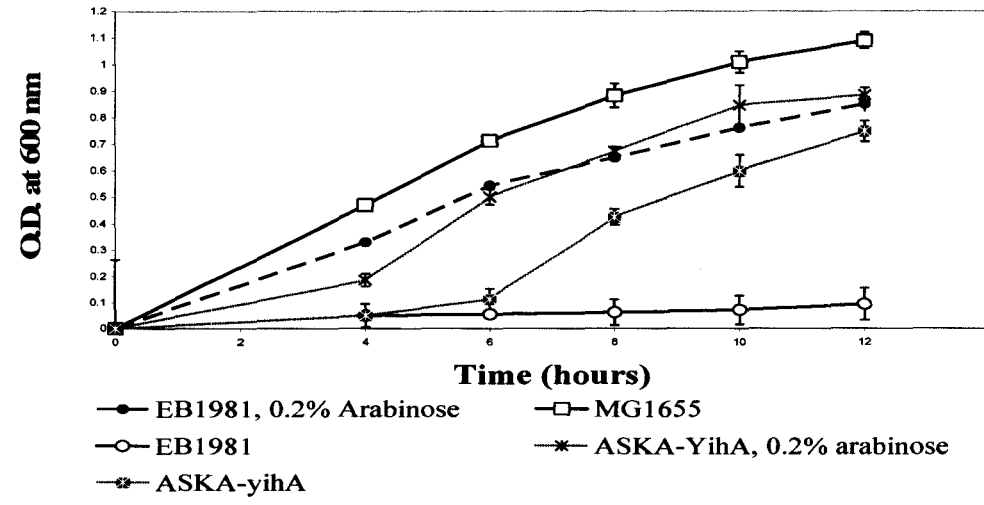

OD at 600 nm
Time (hours)
EB1981, 0.2% Arabinose
MG1655
EB1981
ASKA-YihA, 0.2% arabinose
ASKA-yihA

Figure 3.2 Growth of YihA-Depleted and Replete *E. coli* in Liquid Culture. The strains were grown in LB in triplicate as indicated with 0.2% arabinose or without arabinose and sub-cultured twice before optical density at 600 nm was monitored ($O.D._{600}$). Error bars represent one standard deviation from the mean of each set of three reads. **(A)** The growth of the EB1981 with or without a carboxy-terminal hexa-histidine tagged copy expressed from a pDEST plasmid (pDEST14-$_{C\text{-}His\text{-}yihA}$) in the presence or absence of arabinose is compared to wild type (MG1655) *E. coli*. **(B)** The growth of EB1981 with or without a copy of TEV-linked amino-terminal hexa-histidine tagged YihA from a pDEST plasmid (pDEST17-$_{N\text{-}His\text{-}TEV\text{-}yihA}$) in the presence or absence of arabinose is compared to wild type *E. coli*. **(C)** The growth of EB1981 with or without an amino-terminal hexa-histidine tagged YihA expressed from PCA24N from the ASKA clone set (Kitagawa, Ara *et al.* 2005) in the presence or absence of arabinose is compared to wild type *E. coli*. The growth profiles of wild-type *E. coli* and EB1981 without plasmid are the same data reproduced in three panels for comparison. Wild-type *E. coli* was cultured in LB through the same series of dilutions.

3.4 The Phenotype of *E. coli* Depleted of YihA

EB1981 was streaked out onto solid LB-agar with kanamycin and with or without 0.2% L-arabinose, and allowed to grow for 20 hours at 37°C. Single colonies were chosen from the arabinose-containing plate of the *yihA* null, and likewise single colonies of MG1655 on a plate of LB-agar were chosen and used to prepare slides for microscopy as described in Materials and Methods. The YihA-depleted strain did not form single colonies on solid media, so a sterile loop was used to collect cell debris from the lawn which formed where the initial inoculation from the stocks had occurred. D.I.C. light microscopy was performed at 100x magnification. The MG1655 cells and the YihA replete cells showed comparable sizes and morphologies (compare Fig. 3.3A and Fig. 3.3B), whereas YihA-depleted cells showed a drastically different phenotype (Fig 3.3C). The depleted cell shown in the left panel of Fig. 3.3C appeared to have formed a long filament before having lysed as indicated by the loss of density as indicated by several

white patches within the filament. The right panel of Fig. 3.3C shows cells from the same plate, picked from a denser area of growth. The cells shown in the right panel appear as a mixture of filaments and elongated cells of various lengths, none of which appear to have undergone lysis based on density.

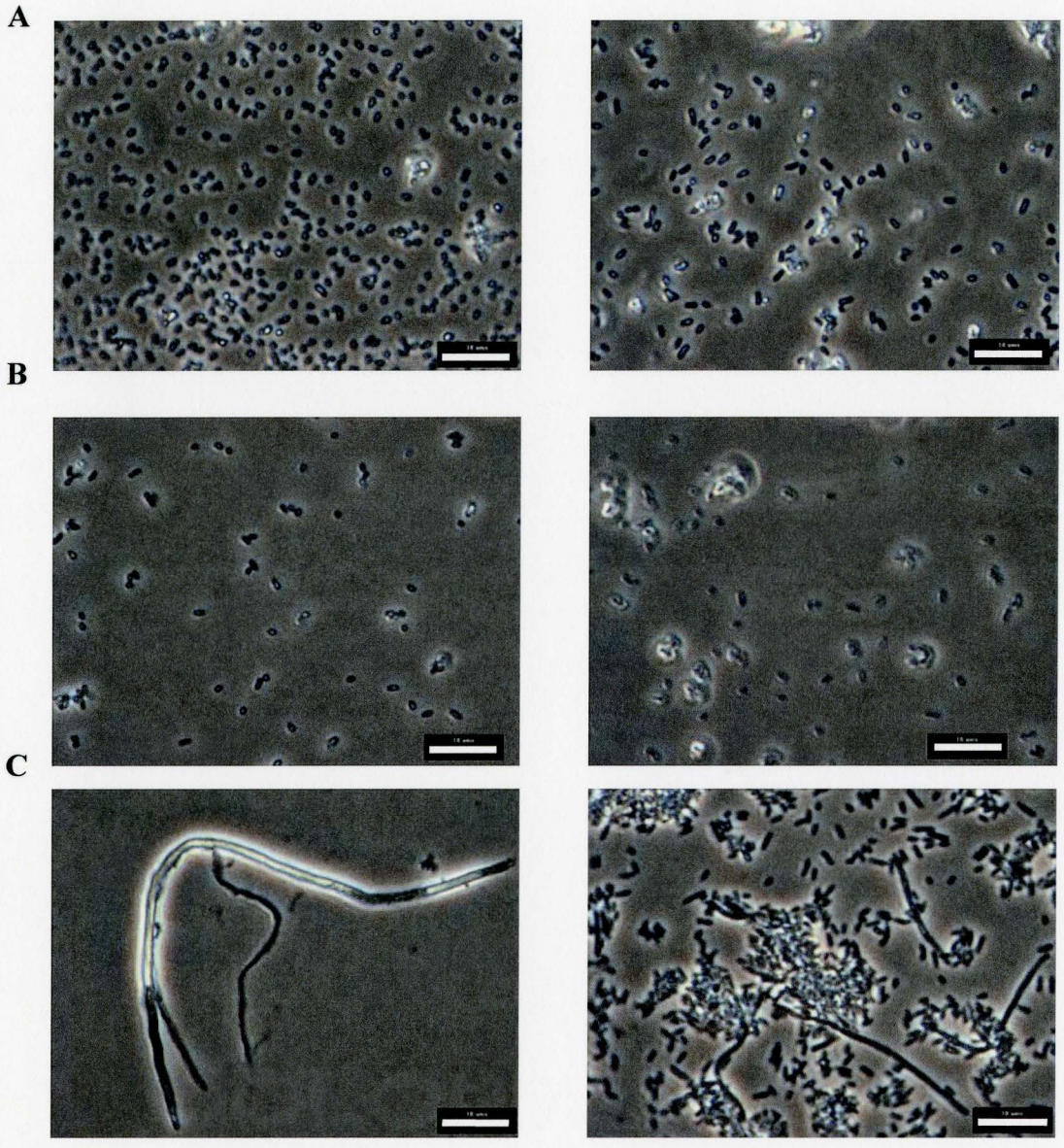


Figure 3.3. Phenotype of YihA-Depleted and Replete *E. coli* on Solid Media. Differential interference light microscopy was used to image *E. coli* grown on solid media. **A.** Two fields of view of MG1655 wild-type *E. coli*. **B.** EB1981 grown on LB-agar with kanamycin and 0.2% L-arabinose. **C.** Left Panel: A single cell from EB1981 grown on LB-agar with kanamycin under depleting conditions (no arabinose). Right Panel: A cluster of cells from a EB1981 grown on LB-agar with kanamycin under depleting conditions, picked from an area of higher density on the same plate as the single cell shown in the left panel. All pictures were taken at 100x magnification, scale bars are 10 μm.

3.5 Impact of YihA Depletion on Ribosomes in *E. coli*

The effect of reduced cellular levels of YihA on ribosomes *in vivo* was examined. During this study it was reported independently that the *Bacillus subtilis* ortholog of *yihA* (*ysxC*) was essential and depletion of that gene caused an increased level of 30S and 50S subunits and reduced the pool of 70S ribosomes in cells (Schaefer, Uicker *et al.* 2006; Wicker-Planquart, Foucher *et al.* 2008). Ribosome absorbance profiles at 254 nm after sedimentation through a sucrose gradient from 10% to 35% sucrose were collected for wild-type *E. coli* and a conditional *yihA* null under replete or depleted conditions. The *yihA* conditional strains were cultured then sub-cultured twice in LB-kanamycin with or without arabinose, and this experiment was repeated on more than three occasions. At mid-log in the third round of depletion, where growth was typically slowed in a depleted strain, ribosomes were extracted and examined. Figure 3.4 shows ribosome profiles collected using EB1981 sub-cultured multiple times in the absence of arabinose (Fig. 3.4C) and in the presence of arabinose (Fig. 3.4B) compared to wild-type (Fig. 3.4A). The wild type ribosome profiles show peaks for 30S, 50S and 70S particles as indicated. The conditional *yihA* null grown in the presence of arabinose likewise consists of three major absorbance peaks corresponding well to those identified in the wild-type. The depleted *yihA* null conversely shows a significant decrease in 70S levels; the 70S peak appears as the smallest of the three peaks compared to the wild-type profiles where the 70S form predominates. Coincident with the drastic loss of potentially functional 70S ribosomes is an accumulation of two major non-70S particles, likely corresponding to 30S and 50S or precursors thereof. In both examples of depleted profiles, 50S or its

precursor appears as the larger of the two peaks. It is important to note that in separate experiments the volume at which each peak was observed varied within approximately 2 ml (compare left vs. right panels for each of Fig. 3.4 A, B, C), which suggests analysis should be limited to relative levels of ribosomal species. The sucrose gradient centrifugation method used here does not provide for determination of actual sedimentation coefficients for macromolecules.

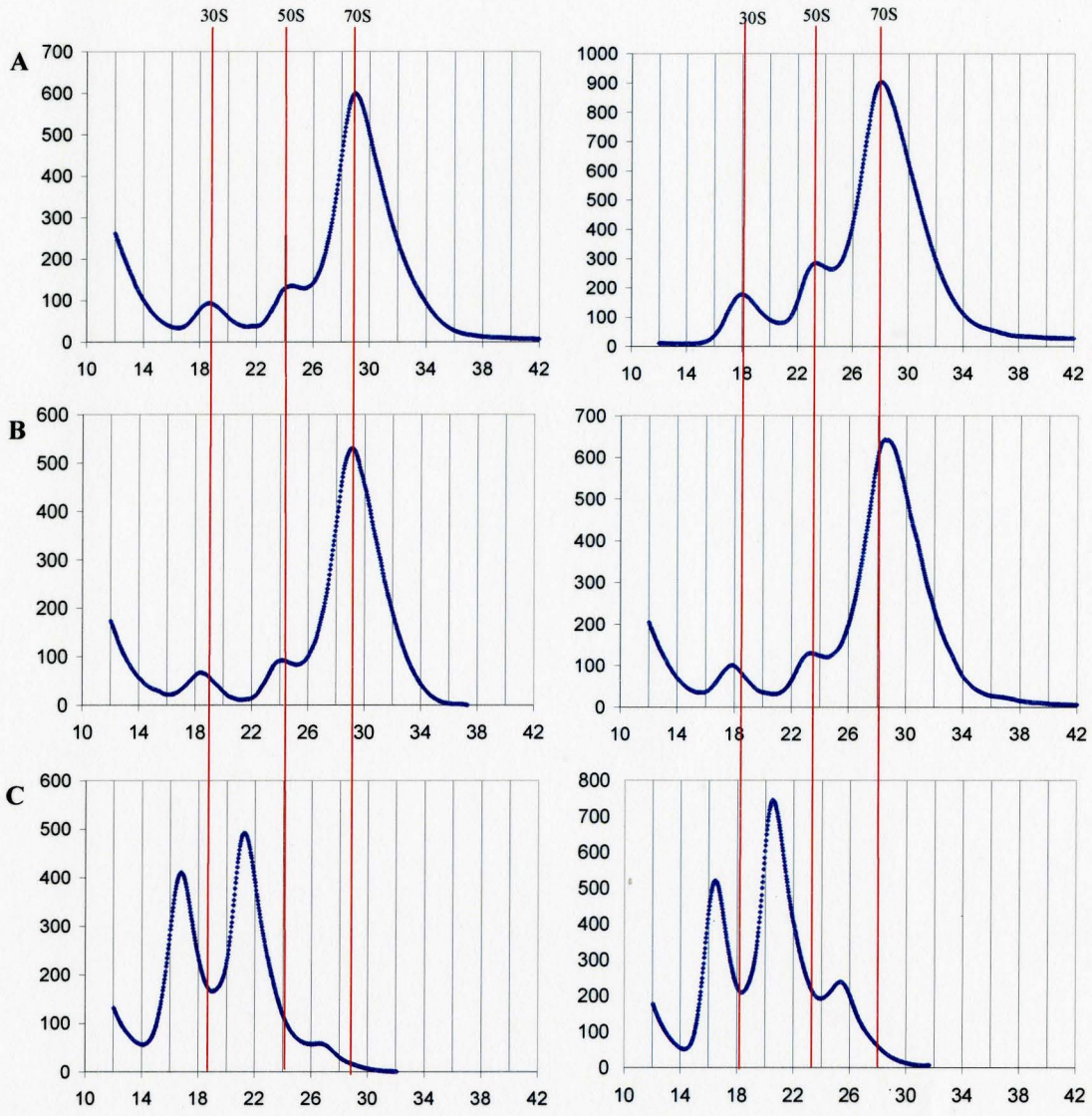


Figure 3.4. Impact of Decreased Levels of YihA on Ribosomal Subunit Distribution *in vivo*. The relative levels of 30S, 50S and 70S ribosomes were studied by monitoring the absorbance at 254 nm (mAu) (the y-axis) of sucrose gradients from 10% to 35% (w/v) as they were fractionated by upwards displacement with 60% glycerol in water (v/v). The x axis indicates volume of displacing glycerol used at each point, and the axes are aligned vertically. All cultures were grown to mid log ($OD_{600} \approx$ 0.4-0.6). Red bars correspond to 30S, 50S, and 70S peaks based on each panel in **(A). A.** Two ribosomes profiles from wild-type *E. coli* MG1655 grown in LB. **B.** Two ribosome profiles from YihA replete EB1981 cultured in LB kanamycin with 0.2% (left panel) and 0.002% (right panel) arabinose. **C.** Two ribosome profiles from YihA depleted EB1981 cultured in LB kanamycin without arabinose.

3.6 Antibiotic Sensitization Screening

Cultures of the EB1981 were grown to saturation, and sub-cultured into 96 well plates with or without 0.2% arabinose, where each row of cultures contained a serial dilution of antibiotic. The fold sensitization was determined by comparison of the apparent minimum inhibitory concentration (MIC) for each antibiotic in the 0.2% arabinose plates divided by the apparent MIC for each antibiotic in the absence of arabinose. Figure 3.5 shows the fold sensitization of a depleted strain to a series of 38 antibiotics clustered by their targets *in vivo*. Twelve antibiotics were found to have no change in their MICs regardless of depletion, while 14 antibiotics appeared to show an MIC against depleted cultures of ½ their MIC against cultures with arabinose present. The occurrence of 2 fold sensitization is expected within experimental variation, and so occurred regardless of antibiotic mechanism, however sensitization greater than 2 fold occurred only within antibiotics targeting either cell wall or the A-site of the ribosome. Six antibiotics; ampicillin, nafcillin, carbenicillin, vancomycin, bacitracin, and butirosin were observed to have 4 fold lower MICs against YihA depleted bacteria. Five antibiotics, namely; metampicillin, phosphomycin, amikacin, isepamicin, and chlorocycline were shown to have 6 fold lower MICs against depleted bacteria, while only gentamicin showed an 8 fold lower MIC. The antibiotics having 4 or greater fold decreases in MIC are all contained within either those acting upon cell envelope or those targeting the A-site of the ribosome (aminoglycosides or tetracyclines).

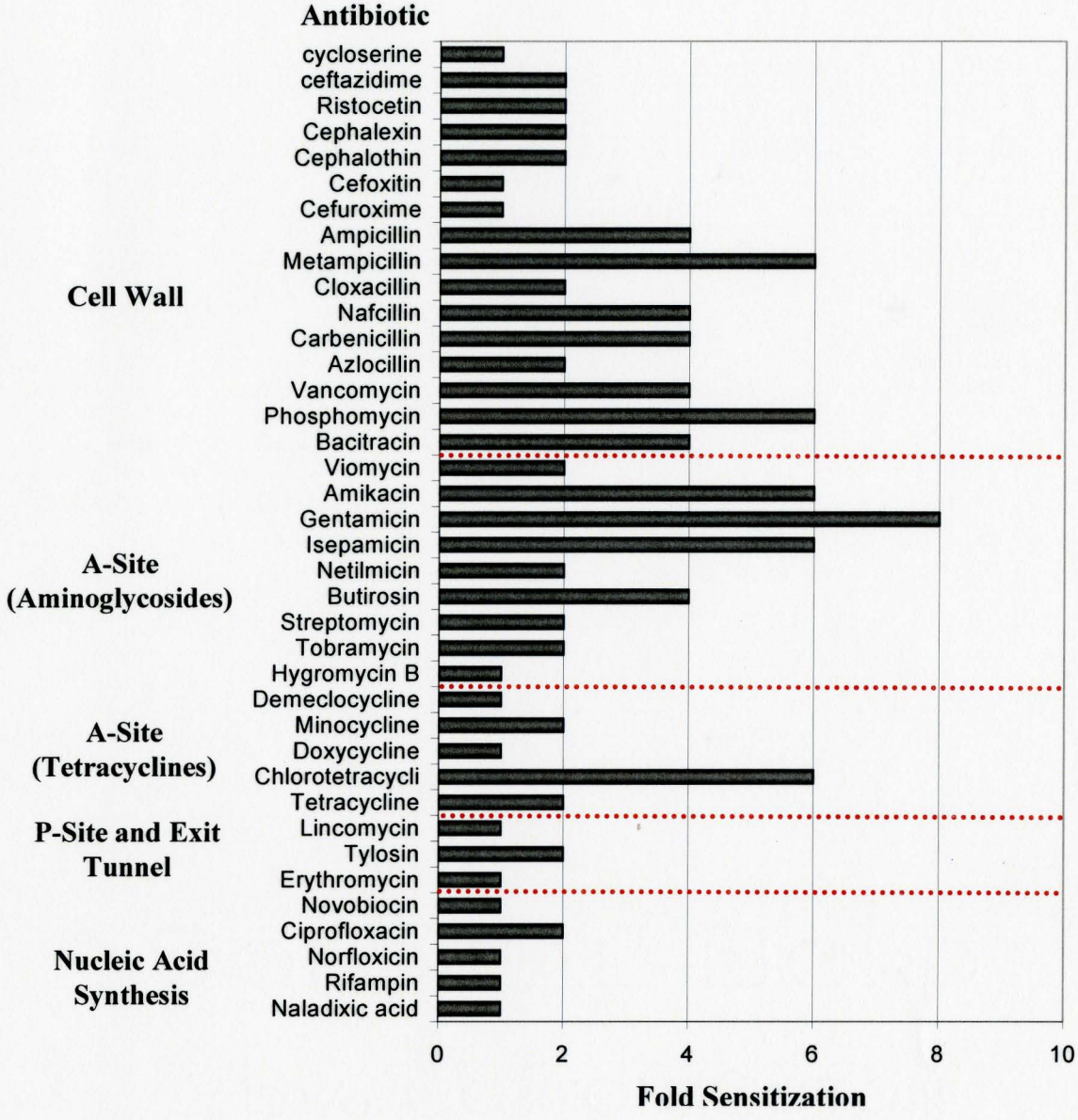


Figure 3.5. Antibiotic Sensitization of YihA-Depleted *E. coli.* The fold sensitization of EB1981 to a variety of antibiotics of known mechanism of action when grown in the absence of arabinose as compared to the same strain grown in the presence of 0.2% arabinose is shown. Antibiotics are sorted by general mechanism or target of action on the vertical axis and the ratio of minimum-inhibitory-concentrations (MICs) is shown on the horizontal axis (fold sensitization). Bacteria were cultured in 96 well plates in LB-kanamycin with or without 0.2% arabinose for 20 hours at 37°C with humidity control, and MICs were based on duplicate results of $O.D._{600}$ values for each. A 1 fold sensitization indicates equal sensitivity regardless of depletion.

3.7 Rescue of YihA depleted Strains By Variants of YihA At Multiple Temperatures.

The ability of a series of YihA variants to rescue a strain depleted of wild type YihA was tested. A pair-wise comparison of the growth of each strain with or without arabinose at 16°C, 37°C, or 42°C is shown in Figure 3.6. As shown previously in liquid media (see Fig. 3.2), EB1982 bearing a pDEST14-$_{\text{C-HIS-}yihA}$ plasmid was unable to compensate for the lack of wild-type YihA protein *in vivo*, and so residual growth of that strain at all three temperatures was taken to be representative of failure to complement. Conversely, pDEST17-$_{\text{N-HIS-TEV-}yihA}$ has been shown to complement for depletion of YihA (see Fig. 3.2), so the growth of EB1983 on solid media at all three temperatures was taken to be indicative of successful *in vivo* complementation. Interestingly, at 16°C, even in the presence of 0.2% arabinose, growth of EB1981 was greatly diminished relative to growth at 37°C and 42°C. No difference was observed between growth with or without arabinose for EB1983 at 16°C, and EB1983 only grew at one further dilution than EB1982. EB1992 expresses a hexa-histidine tag linked via TEV protease consensus sequence to the first 12 amino acids of YihA, acting as a plasmid-only control, and showed growth on solid media that was comparable to EB1982. Aside from negative controls, at 37°C and 42°C only EB1985 and EB1986 failed to complement. EB1985 and EB1986 are similar in that the proteins they encode have mutations to the consensus sequences of G1 which would be expected to abrogate nucleotide binding based on research done on other small GTPases (Sprang 1997; Bharat, Jiang *et al.* 2006). EB1986 has G38A, K39A, and S40A point mutations to G1, while EB1985 contains a combination of G1 mutations K39A and S40A, with R119D, H120D mutations which lie adjacent to the switch-II region of the

protein between G3 and G4 and are conserved throughout multiple species (Ruzheinikov, Das *et al.* 2004). A *yihA* gene encoding mutations of both the conserved residues R119 and H120 to alanine residues was expressed in EB1984, and complemented as well as the wild type gene at all temperatures. The point mutations in EB1987 through EB1991 appeared to be insignificant for growth at 37°C and 42°C under the tested conditions. However, the YihA mutants expressed in EB1984, and EB1987 through EB1991 appeared to grow better at 16°C than EB1983 in both complemented and depleted conditions. These mutant YihA proteins each encode one or more point mutations to non-active-site basic residues which convert them to non-basic residues (see Tables 2.1 and 2.2), none of which appear essential for viability at 37°C or 42°C according to the results of this experiment. Expression of the first 12 amino-acids from YihA linked to the amino-terminal tag in EB1992 did not positively affect growth at 16°C, nor did EB1985 or EB1986 indicating that this was not an effect of the tag or plasmid, but dependent on the presence of the mutated YihA proteins.

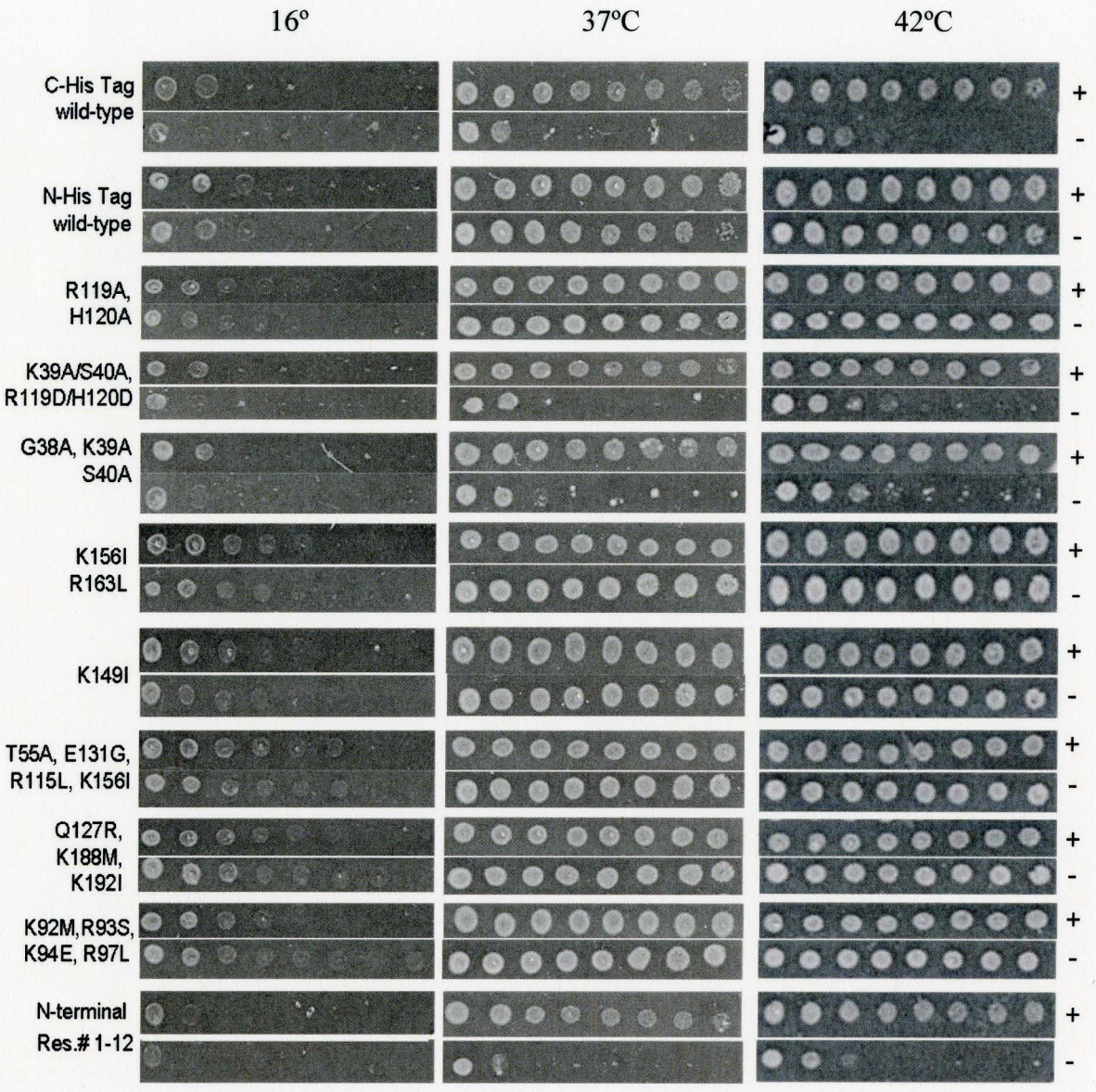


Fig 3.6 Complementation of YihA-Depleted *E. coli* by YihA Variants Expressed *trans* From a pDEST17 Plasmid. Cultures of nine conditional *yihA* null strains carrying plasmids expressing mutant versions of the gene (from top: EB1982-1992, see Table 2.1) were normalized to an OD_{600} of 0.1 in LB, and 10 fold diluted in series across a 96 well plate. From these cultures, pairs of LB-agar-kanamycin-ampicillin plates with (+) or without (-) 0.2% arabinose were seeded with 1µl of each culture, and grown at either 16°C, 37°C, or 42°C. Plates at 37°C and 42°C were grown for 20 hours, whereas 16°C plates were grown for 42 hours. EB1982 contains a plasmid known not to complement at 37°C, while EB1983 contains a plasmid shown to complement at 37°C.

4 Discussion

The study of GTPases of unknown function in bacteria has been an intensely active research area in recent years. The growing evidence of the involvement of a number of genes in ribosome assembly has given birth to a field that continues to shed light on the essential process of ribosome assembly and offer insights into this incredibly complicated process *in vivo*. The essential and conserved nature of many bacterial GTPases, as well as bacterial factor-facilitated ribosome assembly *in vivo*, suggest that these proteins and the process they are involved in may one day come to represent a valuable target for the development of much needed novel antibiotics (Comartin and Brown 2006). Research into GTPases of unknown function has been challenging, and in many cases their roles in ribosome assembly remain speculative and obscure. The investigation of these cryptic proteins has mandated the creative use of diverse genetic and chemical genetic techniques, and in many cases, studies raise more questions than they answer going forward. While we are far from a complete functional understanding of any of these GTPases, let alone the overall ribosome assembly process *in vivo*, progress continues towards these important goals and invaluable insights into essential bacterial physiology will ultimately result.

4.1 Construction of Genetic Tools for Studying YihA

The construction of a strain of *Escherichia coli* with the only allele of *yihA* under P_{BAD} regulation is reported in this study. In agreement with previous findings by Dassain *et al.* (1999), the strain showed a phenotype of elongation and failed cell division upon

depletion, forming long filaments which were not viable upon sub-culturing (Dassain, Leroy *et al.* 1999). Further, two plasmids were constructed, pDEST17-$_{\text{N-HIS-TEV-}yihA}$ and pDEST14-$_{\text{C-HIS-}yihA}$ for over-expression of the protein, and demonstrated that uninduced expression from the former but not the latter was sufficient to rescue a strain depleted for wild-type YihA. The larger tag is found on the pDEST17-N-HIS-TEV-*yihA* plasmid, however it is apparent that the placement of 6 histidine residues on the carboxy-terminus of YihA prevents either folding or the essential function of the protein *in vivo*, likely through interfering with interactions. Both constructs express in the soluble portion of cell lysate and migrate on SDS at their expected molecular weights (data not shown), suggesting that aggregation and or degradation of the protein is not an issue. The C-terminal residues of YihA are distal from the active site, and are predicted to comprise a helix based on existing structures for YsxC (Ruzheinikov, Das *et al.* 2004; Das, Sedelnikova *et al.* 2004). That the addition of positive charges to a protein expected to have significant interactions with the ribosomes interferes with binding suggests that there might be a steric clash preventing binding to an interacting protein or the ribosome. The results suggest the C-terminal region of the protein might be key for interacting with another protein or the ribosome, which is consistent with the essentiality of the C-terminal 23 amino-acids as shown previously in the *B. subtilis* ortholog YsxC (Pragai and Harwood 2000). The uninduced expression from another plasmid, amino-terminally tagged construct from the ASKA collection (Kitagawa, Ara *et al.* 2005), was shown to be able to complement for loss of the wild-type gene in the present study. This phenotype opens the possibility of screening for multi-copy suppressors among the ASKA clone-set,

which would allow for a genome-wide search for genetic interactions in *E. coli*. Previously, a genetic screen with the ASKA clone set has been used by T. Campbell to identify multi-copy suppressors of *yjeQ* deletion slow-growth phenotype (Campbell and Brown 2008). Indeed, a remarkable number of studies have taken advantage of multi-copy suppression and identified genetic interactions of genes of unknown function, especially those genes now thought to be involved in ribosome assembly (Yamanaka, Ogura *et al.* 1992; Dammel and Noller 1995; Lu and Inouye 1998; Tan, Jakob *et al.* 2002; Inoue, Alsina *et al.* 2003; Campbell and Brown 2008; Hwang and Inouye 2008). The application of this technique to the study of *yihA* would no doubt yield further avenues of research and offer insight into the function of this gene.

4.2 Impact of YihA Depletion on Ribosome Profiles

The conditional *yihA* null allowed for observation of phenotypes of cells prior to cell death under deplete conditions. During this study, results using a similar system in *B. subtilis* showed that depletion of YsxC led to accumulation of a precursor to the 50S subunit, termed 44.5S, at the expense of 70S translation competent ribosomes (Schaefer, Uicker *et al.* 2006; Wicker-Planquart, Foucher *et al.* 2008). The ribosome profiles of the *yihA* conditional-null strain under depleting conditions was examined, and accumulation of 30S and a species that sediments near where 50S subunits from wild-type *E. coli* sediments was observed, at the apparent cost of 70S subunits. The results described herein agreed qualitatively with those in *B. subtilis*, and similarly support a role for YihA/YsxC in ribosome assembly, but from the data it is impossible to determine if the

subunits accumulating were precursors to 30S and 50S, or assembled 30S and 50S (Wicker-Planquart, Foucher *et al.* 2008; Schaefer, Uicker *et al.* 2006). The sedimentation through sucrose is affected in part by the concentrations of the different species sedimenting, such that both 50S and 30S appear to migrate as smaller species when there is reduced 70S present in comparison to both the complemented null and wild-type profiles. The accumulation of subunits is the defining phenotype of several genes thought to be involved in ribosome biogenesis (Bharat, Jiang *et al.* 2006; Schaefer, Uicker *et al.* 2006; Uicker, Schaefer *et al.* 2007; Wicker-Planquart, Foucher *et al.* 2008). This study represents the first demonstration of altered ribosome profiles in *E. coli* depleted of YihA, which provides the strongest evidence yet for a role in ribosome assembly.

4.3 Rescue of YihA Depleted *E. coli* By Variants of YihA At Multiple Temperatures.

The literature on YihA/YsxC supported a role in ribosome assembly at the outset of this work, however a unique and troubling aspect of these proteins is their lack of recognizable domains other than the GTPase domain. The simplicity of these Ras-like GTPases kept their functions cryptic for a long time, and the failure of previous studies to identify interacting partners among soluble cellular proteins left little clue as to their specific mechanism of action (Butland, Peregrin-Alvarez *et al.* 2005). Indeed, aside from the wealth of knowledge available for GTPases of the Ras superfamily and the inference of similar function, very little is understood of YihA/YsxC function *in vivo*. To pursue a greater understanding of the protein's function and to define important residues in these proteins, selected basic residues and clusters of residues in YihA, as well as active-site

residues, were mutated to identify residues important for interaction with the ribosome (see Appendix 5.1, 5.2). Based on structural studies of YsxC and YihA, the following non-active-site residues were chosen for mutagenesis: R119 and H120, K156 and R163, K149, R155 and K156, K188M, K92 through K94 with R97, K156 and R163, K188 and K192. In addition, residues G38, K39 and S40 of the active site were targeted, where K39 and S40 have been shown previously in other GTPases to be necessary for binding of nucleotide, and G38 has been shown to be essential for nucleotide binding in YihA (Sprang 1997; Bharat, Jiang *et al.* 2006) .

During cloning of these mutants, several of the key residues were mutated successfully, but sequencing revealed other non active site residues had also been changed, and in one case (EB1985) a combination of changes in active site residues K39 and S40 with changes in R119 and H120 occurred. In the case of EB1989, the intended R155L and K156I substitutions were made but accompanied by T55A and E131G mutations. T55 lies within the G2/Switch I region, but is not a catalytic residue (T62 is involved in Mg^{2+} co-ordination), while E131 lies between G3/Switch II and G4, and neither residue is well conserved in other GTPases. In the case of EB1990, which has K188M and K192I substitutions, an additional mutation of Q127R was present in the clones sequenced. Q127 lies between G3/Switch II and G4, and is not conserved even in *B. subtilis* (see Appendix 5.1). Therefore, in the case of both EB1989 and EB1990, no significant compensatory or deleterious impact from the unexpected mutations would be predicted, but the possibility cannot be eliminated. The fact that mutants were isolated that were unable to support viability, namely those with altered residues in G1, suggests

that sufficient care was taken during the cloning process to limit selective pressure, and supports the conclusion that these mutations were randomly occurring during PCR and were not compensatory for functions affected by targeted mutations.

Previous work by A. Bharat of the Brown lab on the essential GTPase EngA in *E. coli* suggested that single mutations of basic residues within that protein were insufficient to abrogate function *in vivo*, based on complementation assays similar to those employed in this study (A. Bharat, personal communication). Based on this finding, multiple residues were mutated in almost every case, hoping to target basic patches of residues in YihA which might collectively reduce affinity for ribosomes or other interacting partners as yet unknown. Based on these findings, none of the residues or groups of residues aside from the active-site residues (EB1985 and EB1986) significantly affected complementation. In the case of EB1991, which has 4 closely spaced basic residues (92-94, 97) mutated into 4 different residues; methionine, serine, aspartic acid and leucine respectively, it was surprising that no change in viability was observed. These four residues are found in switch II region, which is known to be highly dynamic in GTPases, having very different confirmations between GTP bound and apo forms of the enzyme. In the case of Ras, switch II has been implicated in effector binding based on mutagenesis (Marshall 1993; Sprang 1997). R97 is conserved in both *B. subtilis* and *E. coli* enzymes, and K92 in *E. coli* corresponds to an arginine residue in *B. subtilis*, so the possibility of this region interacting with an effector or rRNA was considered. The most surprising result was the finding that R119 and H120, which are part of a conserved patch of basic residues next to the nucleotide binding site, appear to tolerate mutations to alanine

residues (Ruzheinikov, Das *et al.* 2004). These residues are the YihA equivalent of R116 and H117 in *B. subtilis,* which along with R31 and K146 (YihA R34 and K149) were identified as part of the a conserved basic patch in a structural study of *B. subtilis* (Ruzheinikov, Das *et al.* 2004). The possibility remains that mutation of these two residues to alanine was insufficient to alter the overall characteristic charge of the region. Isolation of R119D/H120D double mutants was attempted unsuccessfully, in hopes of a more deleterious disruption of this basic patch of residues. The finding that R119A/H120A double mutants are viable may simply indicate that alteration of all 4 residues in this patch would be required, or that non-acidic residues are permissive. The ability of a K149I mutant in YihA to complement at 37°C and 42°C in this study is consistent with these interpretations.

K188 and K192, R155 and K156, K156 and R163 pair-wise mutations were selected based on their proximity on a surface rendered model of Apo-YihA (RCS-PDB 1PUI), and in every case showed no detectable effect on viability under tested conditions. K188 and K192 are the only two residues within the C-terminal 23 amino acids targeted in this study, and previous studies have shown it was not possible to isolate deletions of the last 23 residues in YihA (Pragai and Harwood 2000). The K149I mutation was selected as part of the conserved basic residue patch mentioned earlier in both the *B. subtilis* and *E. coli* enzymes, sitting as the first residue after the G4 consensus (N/T)KxD**K**) (Ruzheinikov, Das *et al.* 2004). This mutation also showed no effect on viability. Overall, random mutagenesis of positive residues failed to show any effect on viability, which could indicate failure to target the correct areas of the YihA protein, or

that mutagenesis to merely non-charged instead of acidic residues was insufficient to interfere with function. Based on this data, it seems reasonable to hypothesize that the interaction of YihA with the ribosome relies upon a variety of different contacts spread out over the protein. This model is appealing as there is a no identifiable RNA binding motif in this protein family, and given the successful mutagenesis of several conserved charged residues absent any growth phenotype. YihA may simultaneously recognize multiple ribosomal proteins and or ribosomal RNAs, so multiple residues and areas of the protein may facilitate binding and allow the enzyme to adapt a catalytic confirmation. Finally, the possibility cannot be dismissed that non-basic residues play key roles in YihA interactions.

The only mutations affecting viability at 37°C and 42°C were G38A, K39A and S40A. It is predicted based on homology and conservation among GTPases, as well as previous work on YihA and a related enzyme, EngA, that these residues are essential for nucleotide binding (Sprang 1997; Bharat, Jiang *et al.* 2006). Therefore, this finding is consistent with GTP-binding, and likely hydrolysis, being essential for YihA function. This result represents the first direct evidence that these activities of YihA are essential. It is not surprising in the absence of other known protein motifs or domains that this would be an essential function, based on the fact that YihA has all the conserved residues of a GTPase, but given that measurement of enzyme activity has been elusive to date in unpublished attempts (data not show) and previously published studies, this result is the first direct evidence that GTP hydrolysis is significant *in vivo* (Lehoux, Mazzulla *et al.* 2003).

The mutagenic studies discussed represent a preliminary investigation of the various residues of YihA, driven by the hypothesis of ribosome interaction mediated by basic charges. The apparent failure to identify and manipulate any basic patches which were essential for interactions may be due to the fact that the mutants described herein were not a comprehensive set across the protein, or involved alterations which did not introduce acidic residues. The finding that G39 and S40 are essential for viability demonstrates that the GTP binding function, and likely the catalyzing GTP hydrolytic function, of the protein is essential *in vivo*. Future studies may identify overlooked residues essential for YihA/YsxC interactions with ribosomal proteins and rRNA. Such work will be important for understanding the interactions between YihA and its binding partners. Alternatively, a cryo-electron microscopy or X-ray crystallography study of YihA bound to the ribosome or a precursor would provide excellent insight into the binding site of the protein, as these techniques have offered the greatest insights into the interactions of various molecules such as antibiotics or proteins such as Era with the ribosome (Yonath and Bashan 2004; Poehlsgaard and Douthwaite 2005; Sharma, Barat *et al.* 2005; Sutcliffe 2005).

It is worth noting that at 16°C wild-type YihA as expressed from P_{BAD} or as a tagged variant from pDEST17-$_{N\text{-}HIS\text{-}TEV\text{-}yihA}$ was unable to support growth after 42 hours incubation beyond the third dilution, whereas EB1984 and EB1987-through EB1991 appeared to show better growth regardless of arabinose concentration. In the case of EB1989 through EB1991, the constructs appeared to support growth more successfully in the absence of arabinose. This result was extremely surprising, as the lower temperature

was expected to further stress strains relying on mutated versions of YihA, rather than benefit their growth. It was expected that colder temperatures might impact ribosome assembly which is a complex process, shown *in vitro* to be aided by high temperatures when co-factors are not present (Traub and Nomura 1968; Nierhaus and Dohme 1974; Nierhaus 1991). Indeed, at 42°C growth was apparent at 10 fold lower concentrations of inoculum in the cases of several YihA depleted strains relative to the same strain depleted of YihA at 37°C. It can be proposed that better growth in the presence of the YihA mutants at colder temperatures was due to less efficient binding to the ribosome; if YihA were a checkpoint protein recognizing a specific confirmation along the 50S assembly landscape, then less efficient binding might result in a checkpoint that was more permissive of improperly assembled ribosomes, which might result in more 70S formation. The wild-type enzyme might stall more easily on the ribosomes at 16°C, locking them in an intermediate state which is inescapable at that temperature. This is necessarily speculative; however the observations herein warrant further investigation. It would be useful to examine the ribosome profiles of these cultures grown at 16°C, as well as growth curves to analyze the rates of growth and determine if the wild-type complemented nulls are not viable at 16°C or merely extremely slow-growing. It would also be useful to transform the plasmids bearing these mutated *yihA* genes into wild-type *E. coli*, and see if they offer a growth advantage at 16°C relative to over-expressed wild-type YihA, as the strains grown in the presence of arabinose displayed reduced growth at 16°C relative to those mutants growing without arabinose.

4.4 Antibiotic Sensitization Screening

In order to probe the impact of YihA depletion on various aspects of cellular function, a strain depleted of YihA was tested against 38 antibiotics having well established functions. A similar approach has been employed successfully in the investigation of the *B. subtilis yloQ* gene, which like *yihA* has been shown to impact on ribosome profiles and to have a potential role in ribosome biogenesis or function (Campbell, Daigle *et al.* 2005). The interaction of a genetic pathway with a small bioactive molecule was termed 'chemical synthetic lethality' in the *yloQ* study (Simons, Dafni *et al.* 2001; Campbell, Daigle *et al.* 2005). Demonstrating the utility of this technique, the researchers used 28 antibiotics with well defined targets *in vivo* and successfully identified multiple instances of chemical synthetic lethality (Campbell, Daigle *et al.* 2005). Interestingly, the chemical genetic interactions were limited to several antibiotics acting on the P-site of the ribosome as translation inhibitors, as well as phosphomycin and an RNA polymerase inhibitor (Campbell, Daigle *et al.* 2005). In this study, a comparable methodology was adapted employing a slightly different set of 38 antibiotics to probe the effects of YihA depletion on cellular physiology. These included many antibiotics which targeted different areas of ribosomes and ribosome function, as well as cell envelope and nucleic acid synthesis.

As expected, a significant number of antibiotics showed no sensitization or 2 fold sensitization, and so a cutoff of 4 fold sensitization was set. The depleted null showed sensitization of 4 fold or greater to a number of drugs targeting the cell envelope and the ribosomal A-site.

Multiple cell wall antibiotics showed chemical genetic interactions with *yihA*. Depletion caused an apparent 4-fold more sensitization to ampicillin, nafcillin, carbenicillin, vancomycin and bacitracin. Ampicillin, carbenicillin, metampicillin and nafcillin are all beta-lactam antibiotics derived from penicillin (Walsh 2003; Rolinson and Geddes 2007). Beta-lactam antibiotics inhibit penicillin-binding-proteins (PBPs), preventing them from cross-linking nascent peptidoglycan moieties in growing bacterial cell walls (Walsh 2003; Zapun, Contreras-Martel *et al.* 2008). Vancomycin is a glycopeptide antibiotic whose spectrum is normally limited to gram positive bacteria, and it acts to inhibit peptidoglycan biosynthesis by binding D-Ala-D-Ala repeats on newly synthesized N-acetyl-muramic acid linked pentapeptides and sterically hindering interactions with transglycosylase and transpeptidase enzymes (Reynolds 1989; Courvalin 2006). Bacitracin similarly is considered active primarily against gram positive bacteria, and targets a different aspect of peptidoglycan biosynthesis (Stone and Strominger 1971). The other cell wall antibiotic to which depleted cells were sensitized was phosphomycin, which is an inhibitor of the essential enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) which is involved in the first committed step of peptidoglycan biosynthesis (Marquardt, Brown *et al.* 1994; Brown, Vivas *et al.* 1995; El Zoeiby, Sanschagrin *et al.* 2003; Eschenburg, Priestman *et al.* 2005).

The activities of the cell wall-targeting antibiotics that were considered hits within the assay all centre on peptidoglycan biosynthesis. The apparent sensitization of YihA-depleted bacteria to drugs targeting peptidoglycan synthesis was unexpected, but has also been observed in EngA-depleted cells within the Brown lab (A. Bharat, personal

communication). As mentioned, *yloQ* showed chemical genetic interactions with phosphomycin, but not ampicillin, bacitracin, or vancomycin which were used in that study (Campbell, Daigle *et al.* 2005). The authors attributed the effect of phosphomycin to a potential "additional cellular target other than MurA" (Campbell, Daigle *et al.* 2005). In the present study, the interactions with multiple cell-wall antibiotics imply a specific effect of YihA depletion on some aspect of cell membrane or peptidoglycan structure. It is unclear why certain antibiotics targeting cell wall were not effective. The fact that YihA-depleted cells are affected by these antibiotics likely reflects a general destabilization of the cell wall due to some indirect effect of reducing the number of active ribosomes in a cell or some general stress response affecting membranes as a result of problems with ribosome assembly. There is no indication as to how ribosome assembly is linked to membrane structure and function aside from potential translational stresses. The reduction in active ribosomes might reduce the incorporation of membrane proteins important for maintenance of the cell wall. The ability of vancomycin and bacitracin to access the peptidoglycan layer of *E. coli* and presumably act to destabilize it suggests that the outer membrane is compromised to some extent, as these drugs normally are not active against gram negative bacteria. Absent any other evidence for a direct role of YihA in membrane structure or function, the simplest explanation of this result is that this is an indirect effect of stress resulting from ribosome depletion, consistent with similar results in EngA-depleted *E. coli* (A.Bharat, personal communication). The possibility of general translation stresses seems contradictory to the finding that lincomycin, tylosin, and erythromycin did not show interactions with *yihA*. Therefore,

the effects of YihA and EngA depletion respectively on cell wall stability may indicate an as-yet unidentified connection between ribosome assembly and cell wall structure. This finding remains speculative and suggests a need for further research into the structure of cell walls in gram negative bacteria where ribosome assembly has been perturbed.

The ribosome represents one of the most exploited antibiotic targets in bacteria. Extensive crystallographic studies have provided a significant understanding of the binding sites and mechanisms of action of drugs targeting the ribosome in recent years (Knowles, Foloppe *et al.* 2002; Auerbach, Bashan *et al.* 2004; Yonath and Bashan 2004; Hermann 2005; Poehlsgaard and Douthwaite 2005; Sutcliffe 2005; Borovinskaya, Pai *et al.* 2007). Therefore, ribosomal structure and function were investigated in a system where depletion of YihA had occurred, using antibiotics with well known binding sites and effects on ribosome function. Of 17 ribosome targeting antibiotics tested in this study, 5 showed significant chemical genetic interactions. The hits, namely amikacin, gentamicin, isepamicin, butrosin and chlorotetracyline all appear to target the A-site of the ribosome. Amikacin, gentamicin, isepamicin, and butrosin are aminoglycoside antibiotics (Walsh 2003; Hermann 2005; Poehlsgaard and Douthwaite 2005; Sutcliffe 2005). Aminoglycoside antibiotics affect the decoding site of the ribosome, which is a small rRNA loop (helix 44 of the 16S rRNA) found in the A-site (Walsh 2003; Hermann 2005; Poehlsgaard and Douthwaite 2005; Sutcliffe 2005). The interactions of these antibiotics with the decoding site cause a conformational change in the decoding site which mimics the conformation adopted when a correct amino-acylated tRNA is present in the A-site (Walsh 2003; Hermann 2005; Poehlsgaard and Douthwaite 2005; Sutcliffe

2005). The conformational change caused by aminoglycosides allows the incorporation of amino acids from non-cognate amino-acyl tRNA species, resulting in the mistranslation of mRNAs and generation of incorrect (and presumably inactive) proteins. Tetracyclines also primarily target the A-site, preventing tRNA from entering the site, but have multiple binding sites on the ribosome (Pioletti, Schlunzen *et al.* 2001; Brodersen and Nissen 2005; Ogle and Ramakrishnan 2005). Crystal structures were first solved of tetracycline bound to two sites on the 30S subunit; the primary site involves 16S helices H31 and H34 in the A-site, while the secondary site involves helices H27 and H11 found in a functionally important area of the 30S ribosome (Brodersen and Nissen 2005). The primary site was shown to be sterically incompatible with tRNA, while the secondary site seems to cause the ribosome to adopt an error-prone conformation which would cause mis-translation (Brodersen and Nissen 2005). Later, 5 additional sites for tetracycline binding to the ribosome were identified, however they had lower occupancies than the primary binding site and were distal to the decoding site (Pioletti, Schlunzen *et al.* 2001). The significance of the additional binding sites of tetracycline *in vivo*, if any, remains unclear (Pioletti, Schlunzen *et al.* 2001). The macrolide antibiotics lincomycin, tylosin and erythromycin were also included in the antibiotic screen. Macrolides are translation inhibitors that bind a site near the peptidyl-transferase centre (PTC) within the exit-tunnel (Hermann 2005; Poehlsgaard and Douthwaite 2005; Sutcliffe 2005). Macrolides bind the 23S rRNA of the 50S ribosomal subunit sterically preventing the progress of the nascent polypeptides through the exit tunnel, thereby inhibiting protein synthesis and causing dissociation of peptidyl-tRNA with only a few amino acids from the ribosome (Tenson,

Lovmar *et al.* 2003; Hermann 2005; Poehlsgaard and Douthwaite 2005; Sutcliffe 2005). All the ribosomal antibiotics studied here interact with 23S or 16S rRNA, with the exception of some of the non-primary tetracycline binding sites whose functional relevance remain unclear (Pioletti, Schlunzen *et al.* 2001).

The ribosome-targeting antibiotics to which YihA-depleted *E. coli* became hyper-sensitive in this study were only those of the aminoglycoside and tetracycline class. The aminoglycosides and tetracyclines share a common binding region of the ribosome, the A-site, and interact with 16S rRNA, whereas macrolides interact with 23S rRNA. The fact that all the binding and interaction data for YsxC to date involve the large subunit and proteins thereof suggests that the sensitization to 16S rRNA binding antibiotics does not relate to 30S structure, but rather some aspect of 50S or overall 70S structure. The other, and probably more significant commonality between aminoglycosides and tetracyclines versus macrolides is their impact on ribosome function. The drugs that register as hits in this screen affect translational fidelity by interfering in the active site, whereas the ones that did not register as hits block translation of proteins after decoding without affecting fidelity. The impact of translational infidelity in *E. coli* has been studied, and it is well documented that there are mutations to ribosomal proteins and rRNA that can decrease fidelity *in vivo* through their impact on overall ribosome structure (Janosi, Shimizu *et al.* 1994; Jerinic and Joseph 2000; Ogle and Ramakrishnan 2005; Fredriksson, Ballesteros *et al.* 2007). Results here indicate that ribosome profiles are affected by depletion of YihA, and work in YsxC has shown similar phenotypes, with the further implication that the 50S particles in depleted strains lack ribosomal proteins or

have altered structures (Schaefer, Uicker *et al.* 2006; Wicker-Planquart, Foucher *et al.* 2008). In a YihA-depleted cell, the reduced number of active 70S particles might include some imperfectly assembled but active 50S subunits. Ribosomes containing improperly assembled 50S subunits might display a reduction in translational fidelity, and in that case the sensitization to translation-fidelity antibiotics would be a logical result. The proteins apparently reduced in 50S subunits from YsxC depleted bacteria are L16, L27 and L36 (Schaefer, Uicker *et al.* 2006). Previous work has shown that L27 and L16 are individually non-essential for ribosome function, but deletion of L27 causes cold sensitivity, while ribosomes reconstituted *in vitro* without L16 require much longer incubations before assembly can be completed (Franceschi and Nierhaus 1990; Nierhaus 1991). L36, interestingly, is non-essential and has recently been found to be lacking from 70S ribosomes when crystallized, leading to the suggestion that it may not be a *de facto* structural protein of the 70S ribosome (Selmer, Dunham *et al.* 2006). L16 and L27 proteins interact with helices 38 and 89 of the 23S rRNA in the large subunit, close to the peptidyl-transferase centre (P-site) (Schaefer, Uicker *et al.* 2006; Selmer, Dunham *et al.* 2006; Wilson and Nierhaus 2007). L27 is thought to play a role in the placement of the acceptor stem of the P-site tRNA, has been shown to cross-link with both A- and P-site tRNA, and plays a separate role in the binding of the ribosome recycling factor (Wower, Wower *et al.* 1998; Wilson and Nierhaus 2005; Wilson and Nierhaus 2005). L16 has interactions with both the A- and P- sites of the 50S, and maybe involved with L27 in P-site tRNA acceptor-stem positioning and ribosome recycling factor binding, it may also help to position the A-site tRNA (Wilson and Nierhaus 2005). In addition, L16 has been

suggested to be important for initiation based on mutations to L16 conferring resistance to evernimicin, which is thought to inhibit 70S translation initiation complex formation (Belova, Tenson *et al.* 2001). The fact that L16 and L27 collectively have a role in positioning the tRNAs in the A-site and P-site suggests that if they were incorrectly oriented or absent from 70S in a YihA-depleted strain, there would be significant impacts on efficiency of translation especially around the tRNA interactions. Structural changes to L16/L27 positioning would therefore be consistent with the observed chemical genetic interactions with drugs that affect the recognition of tRNA in the A-site. In addition, it has been shown previously that ribosome recycling factor (*frr,* RRF) is important *in vivo* for translational fidelity (Janosi, Shimizu *et al.* 1994). If L16 and L27 structure are affected in a YihA-depleted background, RRF binding could be affected, contributing to translational infidelity (Wilson, Nierhaus, 2005).

The hypothesized explanation of the chemical synthetic lethality of A-site translation inhibitors does not explain why the depleted cells did not show a sensitization to general translation inhibition expected from macrolides. As was the case for cell wall antibiotics, it is unclear why only some of the aminoglycosides and tetracyclines showed chemical synthetic lethality in the YihA-depleted bacteria.

The antibiotic sensitizations described here are remarkably similar to those observed in a similar screen for EngA (A. Bharat, personal communication). The *B. subtilis* ortholog of EngA (YphC) has been elsewhere hypothesized to act at a step in 50S assembly close to that of YsxC, so the overlapping hits may reflect similar effects in the depletion of these two essential GTPases (Schaefer, Uicker *et al.* 2006). Conversely, a

study of the depletion of the non-essential GTPase YloQ in *B. subtilis* against a set of antibiotics, including many of the same ones used in this study, showed a sensitization exclusively to ribosome-targeted antibiotics acting at the P-site or exit-tunnel (Campbell, Daigle *et al.* 2005). The same study showed a sensitization to phosphomycin, but not to vancomycin or bacitracin, though being in a gram positive strain the cell wall would already be more sensitive to vancomycin and bacitracin, and such sensitization might be exclusive to gram negative bacteria. The function of YloQ/YjeQ *in vivo* remains obscure, though the cumulative evidence to date suggests an involvement in 30S ribosome assembly, and there is evidence of a cell-wall defect in depleted cells (Daigle and Brown 2004; Campbell, Daigle *et al.* 2005). It also seems that depletion or deletion of genes involved in different aspects of ribosome assembly show varied chemical synthetic lethality with ribosome targeting antibiotics, reflecting the varied nature of the structure and function of ribosomes in different genetic contexts. These findings imply that screening for ribosome assembly factors by looking for chemical synthetic lethality should be possible, though a large number of drugs would be necessary. It would be of interest to screen an ordered library of deletions to non-essential genes in *E. coli* against drugs targeting varied areas of the ribosome in the future, as it is predicted there are many as yet unidentified bacterial factors in ribosome assembly.

4.5 Concluding Remarks

In summary, the phenotypes of a YihA-depleted *Escherichia coli* are reported here to include the formation of long filamentous cells and the accumulation of 30S and 50S subunits or precursors at the expense of 70S ribosomes, consistent with previous reports (Dassain, Leroy *et al.* 1999; Schaefer, Uicker *et al.* 2006). The lethal phenotype of YihA depletion can be suppressed by expression *in trans* by leaky expression from a pDEST17 or PCA24N encoded amino-terminally tagged version of YihA, but not a pDEST14-encoded carboxy-terminally tagged YihA protein. The lethal phenotype of YihA depletion can likewise be suppressed by expression of a series of mutated versions of YihA with basic residues replaced by non-charged residues, but not by mutants for which nucleotide binding would be affected, suggesting that the essential function of YihA includes GTP binding and hydrolysis *in vivo*. Finally, chemical genetic interactions have been identified between *yihA* and antibiotics which affect translational fidelity at the A-site of the ribosome as well as antibiotics which impact cell wall structure. These findings are the first evidence suggesting YihA depletion affects translational fidelity and the structure of the bacterial cell wall *in vivo*. The work reported herein offers insight into the function of the cryptic *yihA* gene, and supports a role for this gene in ribosome assembly *in vivo*. In addition, these findings open the possibility of a number of interesting directions for further research addressing the role of this gene and the process of bacterial ribosome assembly which represents an increasingly attractive potential drug target.

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APPENDICES

5.1 Analysis of Mutated Residues in YihA Variants in This Study

A

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1
|         |         |         |         |         |         |
MTNLNYQQTHFVMSAPDIRHLPSDTGIEVAFAGRSNAGKSSALNTLTNQKSLARTSKTPG
---MKVTKSEIVISAVKPEQYPEGGLPEIALAGRSNVGKSSFINSLINRKNLARTSSKPG
   ::  ::.:*:** . .: *..   *:*:*****.**** :*:* *:*.*****..**

          |         |         |         |         |         |
RTQLINLFEVADGKRLVDLPGYGYAEVPEEMKRKWQRALGEYLEKRQSLQGLVVLMDIRH
KTQTLNFYIINDELHFVDVPGYGFAKVSKSEREAWGRMIETYITTREELKAVVQIVDLRH
:** :*:: : *  ::**:****:*:*.:. :. * * :  *: .*:.*:.:* ::*:**

          |         |         |         |         |         |
PLKDLDQQMIEWAVDSNIAVLVLLTKADKLASGARKAQLNMVREAVLAFNGDVQVETFSS
APSNDDVQMYEFLKYYGIPVIVIATKADKIPKGKWDKHAKVVRQT-LNIDPEDELILFSS
. .: * ** *:    .*.*:*: *****:..*  . : ::**:: * :: : ::  ***

                          210
          |         |         |
LKKQGVDKLRQKLDTWFSEMQPVEETQDGE
ETKKGKDEAWGAIKKMINR----------
 .*:* *:    :.. :..
```

B

Targetted Residue	G38	K39	S40	K92	R93	K94	R97	R119	H120	K149	K156	R163
% Conservation*	100	100	100	100	44	46	60	100	100	100	78	100

Figure 5.1 Analysis of Mutated Residues in YihA Variants in This Study. (A). ClustalW2 alignment of YihA (top) (NP_418301) and YsxC (NP_390697) (Ruzheinikov, Das *et al.* 2004) using default parameters. "*" indicates identical residues, ":" indicates conservative substitutions, and "." indicates semi-conservative substitutions (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Thompson, Higgins *et al.* 1994). Residues targeted for mutation in this study are bold and underlined, bars indicate 10 residue intervals. Residues mutated during the cloning process, but not specifically targeted, are bold but not underlined. **(B).** A Blast search of the RefSeq collection (BlastP) using YihA (NP_418301) was performed with default parameters , and the top 49 hits were aligned using ClustalW2 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). From the alignment, the percent conservation of each residue was calculated. *The aligned sequences in this case did not include YsxC.

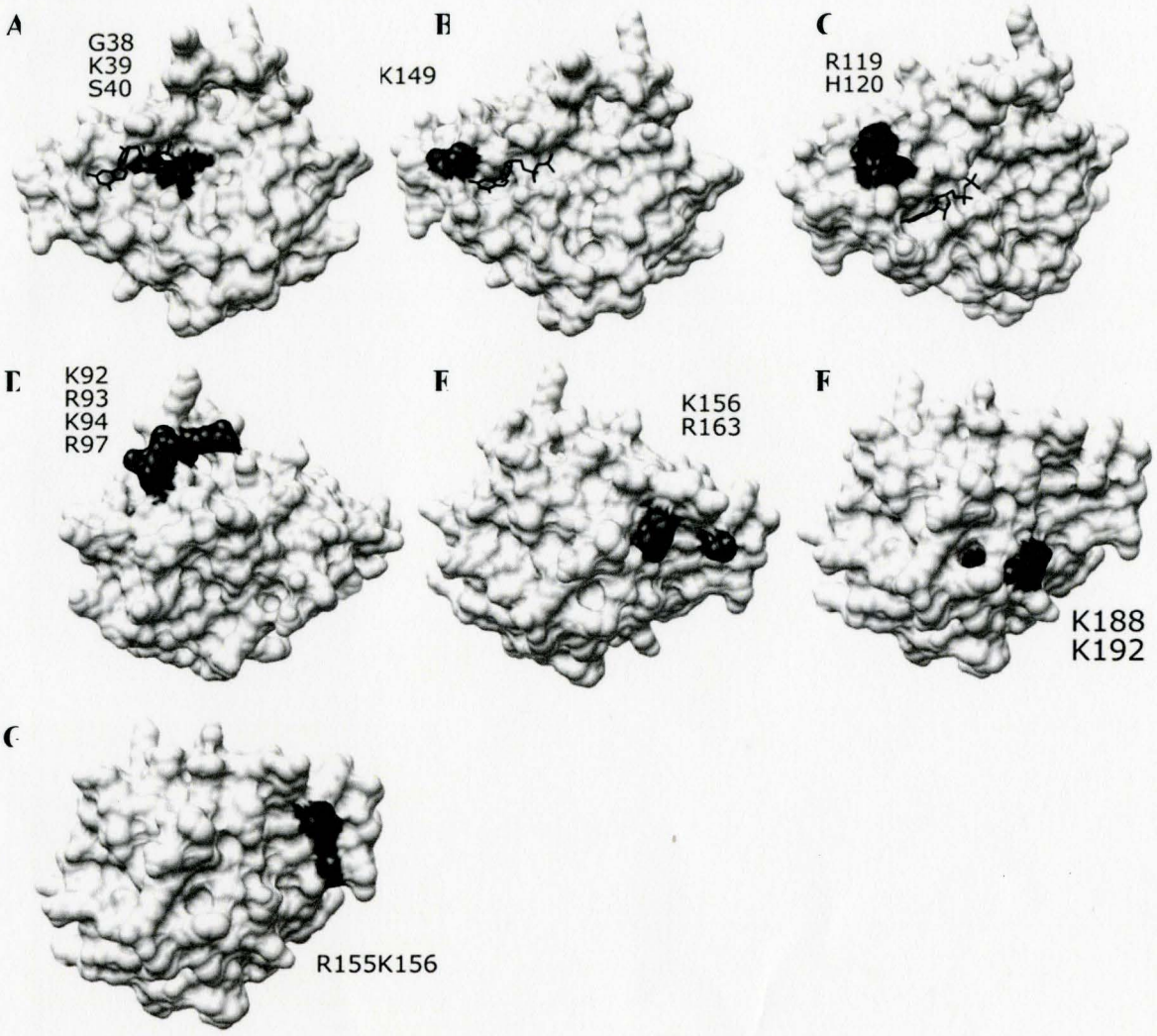

Figure 5.2. YihA Residues Targeted For Mutation in This Study. USCF Chimera rendered surface models of YsxC with bound GDP (RCSB PDB 1SVI) (Berman, Westbrook *et al.* 2000; Pettersen, Goddard *et al.* 2004; Ruzheinikov, Das *et al.* 2004) are shown with the residues corresponding to those mutated in YihA in the present study labeled and shown in black in each case (see Figure 5.1A). GDP is shown in black as a wire model. (**A-C**) View of the protein with active site nucleotide visible. (**D-G**) Models shown are approximately 180 degrees horizontally rotated relative to (**A-C**) to show mutations occurring on the back of the protein, relative to the nucleotide binding site.