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***yciL, yfgB, ygdP and ybcJ* are novel genes that affect the process of protein synthesis in *Escherichia coli***

Submitted by

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in partial fulfilment of the requirements

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# Abstract

Translation is one of the most complex and essential cellular processes. During translation ribosomes, along with initiator tRNA, bind to mRNAs and synthesize peptide chain based on the genetic information in mRNAs.

Proteins that functionally related to each other, often physically interact with one another. Accordingly, one way to investigate novel gene function is to study the interactions that target proteins make with other characterized proteins.

Here, using tandem affinity purification (TAP) tagging we investigate that YciL, YfgB, YbcJ, and YgdP make interactions with other proteins. Based on the protein-protein interaction evidence, we hypothesize that these proteins are involved in protein synthesis and apply them to further investigations. We observed that once deleted, they all cause a decrease in translation fidelity. Their deletions also cause alterations in cell sensitivity to different translation drugs. Deletion of *yfgB* further affected the ribosome profile of the mutant strain. All together we show that *yfgB, yciL, ygdP,* and *ybcJ* have novel roles in translation.

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# List of Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| °C | Temperature in degree Celsius |
| CBP | Calmodulin-binding Peptide |
| g | gram[s] |
| g/l | gram/litre |
| h | hour |
| GTP | Guanosine Triphosphate |
| LB | Luria-Bertani |
| L | Litre[s] |
| MALDI-TOF | Matrix Assisted Laser Desorption/Ionization-Time of Flight |
| mRNA | messenger RNA |
| mg | milligram[s] |
| ml | millilitre[s] |
| µg | microgram[s] |
| µl | microlitre[s] |
| mM | micromole |
| MS | Mass Spectrometry |
| nm | nanometre |
| nt | nucleotide |
| OD | Optical Density |

| | |
|---|---|
| ONPG | Ortho-Nitrophenyl-β-D-Galactopyranoside |
| ORF | Open Reading Frame |
| PCR | Polymerase Chain Reactions |
| PPIS | Protein-Protein Interactions |
| Ram | Ribosomal ambiguity mutants |
| RBS | Ribosomal Binding Site |
| RF | Release Factor |
| rpm | revolutions per minute |
| RRF | Ribosomal Recycling Factors |
| s | second |
| S | (Svedberg) units for velocity of sedimentation |
| SDS-PAGE | sulphate polyacrylamide gel electrophoresis |
| SD | Shine-Dalgarno |
| TAP | Tandem Affinity Purification |
| TEV | Tobacco Etch Virus |
| tRNA | transfer RNA |
| WT | Wild Type |
| Y2H | Yeast two Hybrid |

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# Chapter 1:

## Introduction

## 1.1 Translation in prokaryotes:

Translation is one of the most complex and essential cellular processes. During translation, the ribosome along with initiator tRNA, bind to mRNAs and start synthesizing proteins based on the genetic information on the mRNAs. In addition to the ribosome, mRNA, and tRNA, there are also other factors that influence protein synthesis. These factors include: translation factors (IF1, IF2, IF3, etc), released factors (RF1, RF2, and RF3), guanosine triphosphate (GTP), etc. In the classical view, translation is divided into three phases: initiation, elongation, and termination.

In bacteria, translation initiation begins after the dissociation of 70S ribosome into small (30S) and large (50S) subunits. Binding of IF3 to the ribosome helps this dissociation (Petrelli *et al.*, 2001). The bacterial ribosome has three well-characterized tRNA binding sites: the aminoacyl (A), peptidy (P), and exit (E) binding sites. Initiation factor IF1 binds to the A-site of the ribosomal 30S subunit and directs the initiator tRNA to this site. tRNA has two recognition sites that help it to provide the correct amino acids, based on the mRNA sequence. One site binds to a specific amino acid and the other contains an anticodon that base-pairs with a codon in mRNA. Unlike eukaryotes, prokaryotic mRNAs contain Shine-Dalgarno (SD) sequences which are used to attract the 30S ribosome to the start codon (usually AUG). Translation initiation completed when the ribosomal large subunit joins the 30S subunit and tRNA on a mRNA (Weaver, 2005).

During elongation, ribosomes add amino acids one at a time to the initiating amino acid forming polypeptide chains. Elongation begins with the binding of the second aminoacyl-tRNA

to the P-site of ribosome. The first tRNA gets shifted to the E-site of ribosome and the first amino acid joins the second one. This process needs energy provided by GTP. Elongation continues until ribosome encounters a stop codon (UAG, UAA or UGA).

Release factors RF1, RF2 and RF3 recognize the stop codons and cause translation to stop. At this time, the ribosome dissociates into the large and small subunits ready to enter another cycle of translation. mRNA, tRNA, and the newly synthesized polypeptide chain are also released from the translation complex (Laursen *et al.*, 2005).

### 1.1.1 Initiation of translation in prokaryotes:

The process of translation initiation in prokaryotes is significantly different from that in the eukaryotic cells. In bacteria, initiation always follows two important pre-initiation events. First is the dissociation of ribosomes into two subunits and the other is charging of tRNA with a proper amino acid.

The acceptor stem in tRNA is where tRNAs bind to an amino acid. All tRNAs have the same three bases (CCA) at their 3'end. Terminal adenosine of the tRNA is the target for amino acid binding. Aminoacyl-tRNA synthetase is the enzyme which catalyzes the binding of tRNA to amino acids. Charging of tRNA occurs in two steps. In the first reaction, the amino acid is activated by adenosine triphosphate (ATP). The product of this reaction is aminoacyl-AMP which is called activated amino acid. In the second reaction, the energy in the aminoacyl-AMP is used to transfer the amino acid to tRNA. Aminoacyl-tRNA synthetase plays a crucial role in placing the correct amino acid in the tRNA.

Early evidence for dissociation of ribosomes was provided by Melselson and colleagues (1968) (Boelens *et al.*, 2002). They demonstrated that the *E.coli* 70S ribosome dissociate into 30S and 50S subunits using sucrose density gradient centrifugation. It is known that the three initiation factors (IF1, IF2, and IF3) play an important role in ribosome dissociation. IF1 binds to 30S subunit and promotes ribosome dissociation, whereas IF3 binds to the free 30S ribosomal particle and prevents its reassociation with a 50S particle. In the mean time, IF2 supports binding of IF3 and IF1 to the 30S subunit. All three factors are not able to do their tasks without the assistance of one to another.

The pre-initiation complex is formed by the binding of 30S ribosomal subunit to initiator tRNA and mRNA. The ribosomes read mRNA in the 5'→3' direction. The initiation codon in prokaryotes is usually AUG. Occasionally GUG and UUG are also utilized. In bacteria, it is shown that the initiation codons are not the binding site of the 30S ribosomal particle to the mRNA. The region upstream of the initiation codon is the ribosomal binding site, and is called the Shine-Dalgarno sequence (SD sequence). The consensus SD sequence is 5'- AGGAGGU-3'and is complementary to the 3'end of the 16S rRNA. All three initiation factors (IF1, IF2 and IF3) are also thought to affect the binding of ribosome to the mRNA. It seems that IF3 has an essential role in this process and the other two factors assist this task. IF2 is the primary factor involved in binding initiator tRNA to the mRNA. GTP is also required for tRNA binding, but it is not hydrolyzed until the 50S ribosomal subunit joins the complex (Søresen *et al.,* 2002).

The 70S initiation complex forms when the 50S ribosomal subunit joins the 30S subunit. At this time, IF3 and IF1 discharge the complex. GTP is then hydrolyzed to GDP and IF2 leaves

the complex. GTP hydrolysis facilitates the removal of IF2 and stabilizes the 70S initiation complexes for the elongation process (Kozak, 1999; Sonenberg *et al.*, 2000; Allen and Frank, 2007).

### 1.1.2 Elongation processes:

In contrast to the initiation process, elongation in prokaryotes and eukaryotes are very similar. Elongation occurs in three steps which are repeated over and over: binding aminoacyl-tRNA to the A site of ribosome, peptide bond formation, and translocation.

Elongation factors (EF-Tu, EF-Ts, and EF-G) are the three essential proteins for the elongation process. EF-T requires GTP for binding aminoacyl-tRNA to the ribosome. Unlike initiation, GTP does not need to be hydrolyzed to promote aminoacyl-tRNA binding to the ribosome.

There is also evidence suggesting that EF-Tus and EF-Gs are as necessary as EF-Ts for tRNA binding to the ribosome. To transfer tRNA to the ribosome, EF-Tu and GTP form a complex with an affinity for tRNAs. After binding to the tRNA, the tRNA is delivered to the ribosomal A-site. Then, GTP is hydrolyzed and the EF-Tu –GDP complex dissociates from the ribosome. In this process, the role of EF-TS is to convert EF-Tu –GDP to EF-Tu-GTP by nucleotide exchange.

During the second step of elongation, ribosomes form the peptide bonds between amino acids, thus forming polypeptide chains. The peptide bond forming reaction is performed by ribosomal 50S particles. Elongation factors are not involved in this part of the process. X ray

studies have revealed (Steitz and Moore, 2003) that it is the 23S rRNA that has the peptidyl transferase catalytic activity.

Translocation is the final step of elongation. During translocation, the ribosome is moved one codon length in the 3' direction, 3nt, on the mRNA. Simultaneously, peptidyl-tRNA is moved to the P-site and the deacylated tRNA is released from the ribosome. The translocation process requires the elongation factors EF-G and GTP (which is hydrolyzed after translocation is completed). After each round of the elongation, EF-G is released from the ribosome via GTP hydrolysis (Watson *et al.*, 2008).

### 1.1.3 Termination:

The final step of translation is termination. Termination occurs when the ribosome encounters one of the stop codons UAG, UGA, or UAA. Three releasing factors, RF1, RF2, and RF3 are known to mediate prokaryotic translation termination. RF1 recognizes the termination codons UAA and UAG, whereas RF2 recognizes UAA and UGA. RF3 is a GTP-binding protein that enables RF1 and RF2 to bind to the ribosome.

Nonstandard termination can happen in prokaryote cells in two ways: due to the presence of a nonsense mutation (pre-mature stop codon) and as a consequence of stalled ribosomes. Premature stop codons refer to termination codons that stop the translation process prematurely, in the middle of an mRNA, and produce incomplete proteins. Authentic stop codons are located at the end of the open reading frame (ORF) on all mRNAs. Nonesense mutations can generate a stop codon anywhere in the mRNA. Stalled ribosomes form when the

translating mRNAs contain no termination codons. In this case, the ribosome translates through the mRNA and then halts. Stalled ribosomes lead to non-canonical termination.

Termination is not the final step of translation. Ribosomes still need to be released from the mRNA and dissociate into subunits. In bacterial cells, ribosomal recycling factors (RRF) help the release of the ribosomes. They bind to the A site of ribosome, like aminoacyl-tRNA, and use a still unknown mechanism to promote ribosome discharge from the mRNA. This is an essential process for cell survival and deficiency in this process is not tolerated by the cell (Watson *et al.*, 2008).

## 1.2 Ribosome:

Ribosomes are large enzymatic macromolecular complexes that catalyze peptide bond formation and translate a nucleic acid based code into an amino acid code. The ribosome is composed of large and small subunits. A bacteria ribosome has a relative sedimentation rate of 70S and a mass of 2.4 MDa. The large subunit has a relative sedimentation rate of 50S and a mass of 1.5 MDa. The small subunit has a relative sedimentation rate of 30S and a mass of 0.8 MDa. Approximately, two-thirds of the ribosome consists of RNA and one-third consists of proteins (Steitz and Moore, 2003). The structure of ribosome is stabilized by three types of interaction: RNA-RNA interactions, RNA-protein interaction, and the Mg bridge. Mg bridges are formed by magnesium ions that mediate interactions between two or more phosphate groups on the ribosomes. The RNA-RNA interactions occur by the interaction between the adenine and the backbone hydroxyl groups of the RNA or simply by nucleotide base pairing of the RNA

molecules. The RNA-protein interactions are generally formed between sugar phosphate backbone of the RNA and various proteins (Traub and Normura, 1968; Cundliffe *et al.*, 1990).

Ribosome biogenesis refers to the overall assembly of mature ribosomes or reconstitution of ribosomal subunits. This process consists of four stages: synthesis of ribosomal RNA, processing, modification, and assembly of ribosomal RNA and proteins. Like eukaryotes, in prokaryotes the reconstitution of ribosome is a very complex event and requires numerous assembly factors to properly unite three ribosomal RNAs (5S, 16S, and 23S) with 55 ribosomal proteins (Nierhaus, 1991; Berk and Cate, 2007).

Synthesis of rRNAs occurs during the transcription of rRNA genes. rDNA is a genomic region that encodes rRNA. To transcribe the rRNA genes, RNA polymerase binds to the transcription initiation site (promoter), and catalyzes synthesis of rRNA. The product of rRNA transcription is the precursor RNA, which is longer than the final mature product (Wireman and Sypherd 1974; Karp, 2005).

The next step is the processing of the rRNA precursor or rRNA maturation. This process consists of two stages: the trimming of the rRNA precursor to the mature molecule and modification of the rRNA (for example methylation and pseudouridine synthesis) (Srivastava *et al.*, 1990). The maturation process starts before transcription of an rrn operon is finished (Apirion *et al.*, 1984). In the trimming stage, Rnase III cleaves the spacer sequence between the 16S, 23S and 5S rRNAs. Studies showed that the processing of 5S rRNA requires an extra element; Rnase E. Rnase E mediates the maturation of pre- 5S by adding three nucleotides to both the 5' and 3'ends of the RNA molecule (Feunteun *et al.*, 1972). The last stage of the

maturation is modification. In bacteria, most of the modifications of pre-rRNA are the conversion of uridines to pseudouridines and methylation. Methylation is the process by which methyl groups are attached to 2'ribose position of the rRNAs. Methylation of rRNA is thought to protect rRNA molecules against Rnase attack (Stiege *et al.*, 1988).

Ribosomal assembly requires rRNAs, ribosomal proteins, and several other factors. rRNAs do not carry genetic information. They form a backbone to which ribosomal proteins and other translation elements can be attached. The 50S large subunits are formed by two rRNA (23S and 5S) and 33 proteins (L1 to L36), whereas the small 30S consists of one rRNA (16S) and 21 proteins (S1 to S21) (Srivastava, 1990). In bacterial cells, ribosomes represent up to 30% of the dry mass. This amount consumes more than 40% of the total energy production in a cell. Therefore, coordinated protein synthesis is essential for the economic consumption of cellular energy especially in the ribosome biogenesis process (Nomura *et al.*, 1984).

The reconstitution of the ribosome can be performed either *in vivo* or *in vitro*. *In vitro*, assembly of ribosome requires much longer incubation times, higher temperature, and nonphysiological ionic conditions, whereas in vivo assembly takes only a few minutes at 37 ℃. This difference raises the possibility of the existence of ribosomal factors that assist in assembly *in vivo* that are not available in the vitro assay (Maki *et al.,* 2002). In *E. coli*, new certain ribosome –associated proteins have been discovered which have crucial roles in the assembly of 50S subunits. Some of these proteins possess GTPase, methyltransferase, pseudouridine synthase, RNA helicase, and chaperones activities. Others have unknown functions (Bharat *et al.*, 2006).

The small subunit contains a messenger decoding site where the codon of a mRNA interacts with the anticodon site of a tRNA. This mechanism refers to the codon –anticodon recognition. The ribosome has an important role in controlling translational fidelity by monitoring the complementary between the anticodon and the codon. The small subunit also contains the anti-SD site to which SD sequence on the mRNA interacts and attracts the mRNA to the ribosome. The large subunit of ribosome contains the polypeptide transferase site where polypeptide chains are formed. Thus, the catalytic activity of the ribosome is contained within the large subunit (Garret *et al.*, 2000; Ramakrishnan, 2002; Green and Noller, 1997).

### 1.2.1 Effect of antibiotics on ribosome:

Due to its crucial role in the survival of the cell, the ribosome is a major target for natural and synthetic antibiotics. Most antibiotics block protein synthesis by binding either the large or the small ribosomal subunits. Due to its large size, ribosome contains many different sites to which small inhibitory molecules can bind. Most often, inhibitory compounds target the rRNA rather than ribosomal proteins (Gale *et al.*, 1981; Cundliffe *et al.*, 1981; Yonath, 2005; Poehlsgaard and Douthwaite, 2005).

Although the process of protein synthesis is highly conserved between prokaryotes and eukaryotes, there exist many antibiotics that work specifically on prokaryotic translation machinery and hence have clinical applications. Some of these antibiotics such as spectinomycin, tetracycline, aminoglycosides, paromomycin, and streptomycin bind to the 30S ribosomal subunits. Generally they exert their activity by interfering with decoding and proofreading process of ribosomes. Others such as, macrolides, lincosamides,

chloramphenicol, streptogramins, and thiostrepton bind to the large subunits (Cundliffe et *al.*, 1981; Champney and Burdine, 1995). Generally, these drugs interfere with the translocation step of translation (Mazzei *et al.*, 1993). Recent studies show that these compounds may also prevent the formation of 50S particles in bacteria cells without interfering with 30 subunit synthesis (Champney, 1999).

## 1.3 Translational fidelity:

Translational fidelity refers to the accuracy of protein synthesis. Error in translation can occur in any phase of protein synthesis. In addition to antibiotics, mutations in different translation elements can also affect translational fidelity. As mentioned before, ribosomes are the macromolecules responsible for transferring the genetic code to polypeptide sequences. Mutations in ribosomal protein are shown to increase or decrease the accuracy of translation. Unlike DNA replication, due to the absence of repair pathway, translation in bacteria is not considered to be very accurate (Rosenberger and Hilton, 1983). On other hand, mistakes made by ribosomes in the initial recognition steps, may be recovered by the rejection of the wrong aminoacyl-tRNA before GTP hydrolysis. This activity of the ribosome is known as proofreading. It is generally accepted that the accuracy of translation is related to the rate of the translation. The faster the rate of translation, the less accurate it is in decoding the genetic code. This is thought to be the consequence of lack of sufficient time to properly position the codon with its corresponding anticodon. In contrast, slow translation reduces the growth rate of the cell.

Consequently, in a living cell, there is a balance between speed and accuracy of the translation. Interestingly, mutations in genes encoding S12, S17 and L6 are shown to increase

fidelity (Bollen *et al.*, 1975; Kühberger *et al.*, 1979; Topisirovic *et al.*, 1977), whereas certain mutations in proteins S4, S5, L7, andL12 decrease the accuracy of the translation (Piepersberg *et al.*, 1975; Kirsebom and Isaksson, 1985; Kirith *et al.*, 2006).

In general, an error in protein synthesis occurs when one amino acid is substituted for another one. This substitution is called a missense error. Missense errors are the result of error in charged transfer RNA (tRNA) or an anticodon-codon mismatch on the ribosome. The former is known as mischarging and the latter is called misreading. Another factor which can cause an error in translation is nonsense mutation. This mutation causes termination in a non-codon specific manner.

Natural suppression is when a native tRNA reads over a stop codon which is generated as a result of a nonsense mutation. If this happens at any natural stop codon, it is called read-through. In *E.coli*, it has been shown that most read-throughs are the result of translational leakiness at a particular stop codon (Rosenberger and Hilton, 1983). The UGA codon has often been characterized as a very leaky stop signal (Roth, 1970). Another mechanism that causes alternation in protein synthesis is frame shifting. The frame shift errors are classified as 2-base or 4-base translocations. A 2-base translocation occurs when ribosomes move backwards or 5'slips on the mRNA and is called -1 frame shift. A 4-base frame shift refers to a +1 frame shift. It is known that mutations in ribosomal proteins play crucial roles in increasing ribosomal frame shifting. In addition, it has been shown that amino acid starvation can also have an effect on the rate of frame shifting by ribosomes (Weiss and Gallant, 1983; 1986).

Last but not the least, the accuracy of the protein synthesis may also be affected by the structure of the members of the translational apparatus as well as their relative concentrations within the cell. Altering the ratio of enzymes, proteins factors, and release factors can also lead to mischarge, misread, and read-through of stop codons (Parker, 1989).

## 1.4 mRNA and translation:

During translation, mRNA interacts with tRNA and the 30S ribosomal subunit. The region where the ribosome covers the mRNA in the translation initiation step is called the ribosomal binding site (RBS). Most mRNAs contain at least one RBS. In contrast to eukaryote mRNA, a bacterial mRNA may contain more than one RBS resulting in polycistronic mRNAs. The polycistronic mRNA posses multiple signals for initiation and termination of protein synthesis. RBS on mRNAs are not only characterized by the presence of a recognizable initiation, but they also contain additional elements that are necessary to promote correct initiation. Upstream from the initiation codon is the 5'untranslated region (5'-UTR). This region contains the SD sequence, which base pairs to the 3'of the 16S rRNA of the 30S ribosomal subunit and locates the mRNA at proper site of the ribosome. The distance between the SD sequence and the initiation codon is known to affect the efficiency of translation. Similarly the extent of SD involvement in secondary structure is shown to also affect the level of mRNA synthesis (Shine and Dalgarno, 1974; Gualerzi and Pon, 1990; Yusupova *et al.*, 2001).

Bacterial mRNAs can be divided into two categories: canonical and leaderless. Canonical mRNAs contain well-defined 5'- UTR elements, whereas leaderless mRNAs have no or very few nucleotides upstream of the initiation codon. Binding of leaderless mRNAs to the ribosome

involves a mechanism that is somewhat different from the binding of canonical mRNAs. The leaderless mRNA binding is dependent on the presence of the initiator tRNA, whereas canonical mRNAs bind independently of the initiator tRNA (Benelli *et al.*, 2003). Recently, a cell-free translation system was used to show that leaderless mRNAs preferentially interact with 70S ribosomes and are able to proceed from the initiation to the elongation phase even in the absence of initiation factors (Laursen *et al.*, 2005).

## 1.5 Protein-protein interactions:

Proteins control and perform the majority of biological activities in a living cell. Most proteins carry out their function in association with partner proteins by forming, protein-protein interactions (PPIS) and stable or transient complexes. PPIS are involved in various cellular activities such as signal transduction, protein synthesis, and protein degradation. In biological level, it is not common to find a single protein which operates alone in a cell. Studying protein interactions not only contributes to advancement in understanding of the function of genes, but also adds to our knowledge of the biology of a cell as a system. Studying of PPIS can also lead to our better understanding of certain human conditions such as cancer and early embryonic development.

PPIS investigations are generally divided into two groups: the top-down proteomic approach and the bottom-up genomic approach. The top-down approach may involve mass spectrometric (MS) analysis of native protein complexes purified by affinity processes. In the bottom-up approach, each protein encoded in the genome of interest is expressed for the examination of mutual interactions (Blackstock and Weir, 1999).

Since proteins that interact with each other generally are functionally related, numerous recent investigations have used PPIS as a method to study novel gene function. For example, if the protein of interest interacts with a number of transcription factors, then it is reasoned that the target protein also plays a role in transcription.

PPIS can be identified using various systematic approaches such as yeast two hybrid (Y2H) analysis and tandem affinity purification (Tap-tag). In YH2, the activation of a reporter gene is analyzed by the binding of a transcription factor to an upstream activating sequence. In this technique, the transcription factor is separated in two parts, the activating domain and the binding domain. The split protein is not able to work unless the two parts are contacted physically. To make the two parts come in contact, two protein fusions are created: the protein of interest (bait) which is constructed to have a DNA binding domain and its potential binding partner (prey) which is fused to an activation domain. If bait proteins interact with prey proteins, the binding of these two proteins will result in activation of the transcriptional factor which causes transcription of the reporter gene. The amount of the reporter products can be used as a measure for the strength of the interaction between the protein of interest and its associated protein (Chien *et al.*, 1991; Stagljar and Fields, 2002).

Each technique for measuring PPIS is useful but it has its own drawbacks. For example, Y2H is subject to high rates of false negatives and false positives, whereas affinity purification suffers from co-purifications of contaminants. In general, the best data will probably come from a combination of different techniques.

### 1.5.1 Tandem affinity purification (TAP) tag:

Tandem affinity purification (TAP) tag method allows for rapid purification of complexes without prior knowledge of the complex composition, activity, or function (Drakas *et al.*, 2005). The concept of the TAP is the fusion of a double protein (TAP cassette) tag to the target protein under natural level of protein expression. The tagged-protein, along with its associated proteins, is then extracted via a two-step affinity purification using selection columns (Zeghouf *et al.*, 2004; Gregan *et al.*, 2007).

The TAP cassette consists of two IgG binding domains of protein A of *Strephlococcus aureus* (portA), and a calmodulin-binding peptide (CBP), separated by a Tobacco etch virus (TEV) protease cleavage site. CBP is designed for efficient selection and specific release from the affinity column under normal conditions. PortA binds tightly to the IgG matrix, and can only be released under denaturing conditions at a low pH. TEV protease cleaves a specific recognition sequence, which allows the release of bound materials under natural conditions, and minimizes the risk of cleaving the bait protein or associated proteins eliminating the need for denaturing condition (Krogan *et al.*, 2002).

There are two types of TAP cassette: N-terminal and C -terminal tags. The difference between the two is the location of the tag, which is either at the extreme N- or C-terminus of the fusion protein. Both affinity tags have been selected for efficient recovery of proteins present at low concentration (Rigaut *et al.*, 1999). However, some proteins are thought to prefer one type of tag to the other. For example, proteins with specific signals at C-terminus are thought to retain their activity only if tagged at the N-terminus. In general, however, it is

thought that the C-terminal tagging is more efficient for maintaining the wild type activity of the protein.

TAP tag methodology may be broken down into four steps: tagging, purification, separation, and identification.

Tagging refers to the fusion of the TAP tag to the target protein within the host and is dependent on the methods available to introduce the TAP cassette into the host cell. The TAP cassette can either be inserted into a plasmid or directly into linear chromosome of organisms. If inserted into a plasmid, then there is a risk of over-expressing the target protein. This is not desired for the identification of true interactions as protein over-expression can lead to the formation of non-natural interactions. Therefore tagging at chromosomal location is highly desired (Puig *et al.*, 2001; Gavin *et al.*, 2002).

Tagging the chromosome of the host cell can be performed by one step PCR product transformation, utilizing the homologous recombination machinery within the host. Homologous recombination is a process of physical rearrangement of two strands of DNA, which is dependent upon the complementarity between two DNA strands (Hamilton *et al.*, 1989; Link *et al.*, 1997). This approach works efficiently in some cells such as yeast, *Saccharomyces cerevisiae*, whereas in others, such as *E.coli,* cells it is not as efficient. In *E.coli*, rapid degradation of linear DNA by intercellular exonucleases reduces the chance of recombination of the PCR product with the chromosome (Yu *et al.*, 2000). Homologous recombination in *E.coli* can be enhanced by transforming in the presence of the bacteriophage

lambda recombination system [λ-Red] which suppresses linear DNA degradation (Thaler *et al.*, 1987).

Tap purification consists of two phases. The first step involves the use of affinity column containing IgG beads which binds to Port A of the tagged protein and its associated complexes. The unbound proteins are washed out. TEV protease is then added cleaving the tag. This leaves the first affinity tag on the column and releases the target protein (and complex). The second affinity step removes TEV protease and contaminants remaining after the first column purification. After a few washes, the bound material is released using EGTA buffer under physiological conditions (Gregan *et al.*, 2007).

The isolated complex is separated into individual proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Silver staining is commonly used to visualize the separated protein bands (Zilbetstein *et al.*, 2007).

To identify the separated proteins, MS is used. Individual bands are cut out and applied to in gel trypsin digestion. Each sample is then analyzed using Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) MS (Gingras *et al.*, 2005).

TAP tag is a powerful, rapid, reliable, and affordable technique for purification of protein complexes. This method can also be applied to large scale studies of different types of cells. Since the complexes are purified under natural conditions, the purified complexes can be directly used for follow-up biochemical analysis. Since there is no over-expression of the proteins, it is generally accepted that TAP tag has a low level of false positives.

## Objective:

The main focus of this study is to identify and characterize novel genes *yciL, ygdP, ybcJ,* and *yfgB* involved in the process of protein synthesis in *E.coli.* This is done by investigating the network of PPIS in *E.coli* to identify novel genes that interact with translation related proteins. Since functionally related protein often interact with each other, it is reasonable to assume that the interaction with translation proteins can be used as a starting point to identify new translation genes. Our initial starting point was the newly published network of *E.coli* PPIS (Butland *et al.*, 2005).

First, we confirmed some of the novel identified interactions between uncharacterized proteins and translation elements using TAP tagging in small-scale. We then characterized some of these interactions by deleting the target genes in *E.coli* cells and performing follow-up investigations to show their involvement in translation.

For this, we analyzed the effect of deletion of these genes in translational fidelity. We hypothesized that if these genes have roles in protein synthesis, their absence would increase or decrease the accuracy of translation. We also analyzed the rate of protein synthesis (translation efficiency). In addition, we performed ribosomal profiling experiments to identify the effect of these genes in the assembly of ribosomal subunits. To further confirm the involvement of these genes in protein synthesis, we analyzed the sensitivity of gene deletion strains to different drugs that target translation.

# Chapter 2:

# Materials & Methods

## 2.1 Bacteria strains& Plasmids:

The bacteria strains and plasmids used in this study are listed in Table 2. 1. Strain DY330 is described in (Yu *et al.*, 2000) and was constructed by transduction of ZH1141 with P1 phage grown on WJW23, followed by screening for the presence of a defective λ prophage, which causes temperature-sensitive cell growth at 42℃. *E.coli* DH5α was used as a plasmid host.

All *lacZ* plasmid mutants used in this study carried the *lacZ* gene on a tetracycline-resistant plasmids derived from pACYC184 (O'Conner *et al.*, 1992). The *lacZ* gene in the series of plasmids used here (the pSG *lac* series) was transcribed from the strong *tac* promoter (O'Conner *et al.*, 1993). Both mutant and wild type strains were retrieved from either the glycerol stock stored at -80℃ or from culture plates. *E.coli* cells were grown in liquid Luria-Bertani (LB) (10g/L NaCl, 10g/L bactotryptone, 5g/L yeast extract, pH: 7.5) or in LB agar plates (LB plus 1.5% agar). Growth medium for mutant strains was supplemented by 30μg/ml Kanamycin. One hundred μg/ml Ampicillin was used for plasmid selection. Cell cultures media was incubated at 30℃ in either bench-top incubator, or shaking bath (250 rpm) for 24 h. Culture growth was monitored by measuring the absorption at 600 nm.

| Strains | Genotype | *lacZ* plasmid(Ampr) | Relevant sequence in *lacZ* |
|---|---|---|---|
| DY330 | W3110 D*lac*U169 *gal*490 [*lcI*857 D(*cro–bio*A)] | pSG240 | GUG UA GGU(-1frameshift) |
| DH5α | | pSG400 | GUU UAG GCC(stop codon) |
| *yfgB*Δ | | pSG415 | GUG AAA UGA GCC(stop codon) |
| *ygdP*Δ | | 4pCCCU | UUA GAU CCCU GCU(+1frameshift) |
| *yciL*Δ | | | |
| *ybcJ*Δ | | | |

Table2.1: strains and plasmids used in this investigation

## 2.2 Extraction and preparation of plasmid:

Cultures (2 ml) of bacteria containing the desired plasmid were grown overnight at 37℃. Each culture was transferred to a 1.5 ml microfuge tube and centrifuge at 14,000 rpm for 2 min. The supernatant was discarded and the cell pellet was resuspended in a buffer as below. One of the following methods was used to extract plasmid DNA from bacteria.

## 2.2.1 Rapid preparation of plasmid:

Cells were resuspended in 100 µl TE (pH: 8.0) and left for 10 min at room temperature (RT). They were further treated with 200 µl of lysis solution (solution II) (0.2 M NaOH, 1% SDS), and placed on ice for 5 min. Then 150 µl of neutralization solution (solution III) (3Mpotassium acetate, pH: 4.8) was added, and the tubes were placed on ice for 5 min. The mixture was centrifuge 10min at top speed and the supernatant was treated with 900 µl (-20℃) absolute ethanol (EtOH) to precipitate DNA. After 10 min the DNA was pelleted by centrifugation at top speed for 2-5 min and resuspended in 2 µl TE buffer (pH: 8.0).

## 2.2.2 Plasmid mini-prep Kit (QIA prep)

QIAGENE mini-prep plasmid kit was used. The cell pellet was resuspended in 250µl of buffer P1 and transferred to a microfuge tube. Two hundred and fifty µl of buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times. Three hundred and fifty µl of buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4-6 times. The samples were centrifuged for 10 min at 13,000 rmp in a table-top microcentrifuge. The supernatant was transferred to the QIAprep spin column. The precipitate was pelleted by centrifugation at 14,000 rpm for 30-60 s and the flow through was discarded. The QIAprep spin columns were washed with 0 .5 ml of buffer PB and centrifuged for 30-60s. The flow-through was discarded.

The QIAprep spin column was washed by adding 0.75 ml buffer PE and centrifuged for 30-60 s. The flow-through was discarded and centrifuged for an additional 1min to remove

residual wash buffer. To elute DNA, the QIAprep spin column was placed in a clean 1.5 ml microfuge tube. Then 50µl of buffer EB or water were added to the center of each QIAprep spin column and centrifuged for 1min.

## 2.3 Transformation in Bacteria:

### 2.3.1 Preparation of competent cells:

The chemically prepared competent cells were obtained as described in Sambrook et al (2000). Basically, 1 ml of a fresh overnight culture of DH5α cells, derived from a single colony, was incubated with 100ml of LB medium on a shaker incubator (200 rpm) at 37℃ for 2 h. When, the culture reached an $OD_{600}$=0.5, it was transferred to a pre-cooled centrifuge bottle, left on ice for 10 min, and centrifuged at 4,000 rpm in a GSA rotor for 5min at 4℃. The supernatant was then poured off and the pellet was resuspended in 33 ml of RF1 buffer (100 mM $RbC_1$, 50 mM $MnCl_2$.4 $H_2O$, 50 mM potassium acetate, 10 mM $CaCl_2$.2$H_2O$, and 15% glycerol.pH:5.8) and placed on ice for 15 min. The cells were again recovered by centrifugation at 4,000rpm for 5min and then resuspended in 8µl of RF2 (10 mM MOPS, 10 mM $RbC_1$, 75 mM $CaCl_2$. 2$H_2O$ and 15% glycerol.pH:6.8). After the incubation on ice for 15 min, 200 µl aliquots were dispensed into pre-cooled microfuge tubes and transferred to -70℃ freezers.

## 2.3.2 Transformation of competent cells:

The chemical transformation protocol from Sambrook et al (2000) was followed. Typically, 100µl of the cell suspension was mixed gently with about 5µg DNA (10µl) and placed on ice for 30 min. The mixture was then transferred to 42℃ for 90 s and returned immediately to ice. Next, 800µl of SOC medium (2% bactotryptone, 0.5% yeast extract, 100 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the mixture and it was incubated at 37℃ on a shaker (200 rpm) for 1 h. Later, 20-100µl of the cell mixture was plated on LB medium containing ampicillin (100µg/ml) or chloramphenicol (170µg/ml) according to the type of the vector and its antibiotic resistance marker.

## 2.4 Preparation of cell extract:

Cells were grown in LB containing a proper antibiotic at 30℃ overnight. Then, 500 µl overnight cultures were added into 25 ml fresh LB media (no antibiotic). The mixtures were grown to OD600=0.4-0.6. The tubes were placed on ice for 5min. Then the samples were centrifuged for 10 min at 5,000 rpm. Seven ml phosphate buffer (0.1 M phosphate buffer) was added to the pellet. The mixture was sonicated (4 times-20s each time) at power 1.5 (Sonics &Material INC). The samples were placed on ice between each round of sonication. Next the samples were transferred to microfuge tubes, and centrifuged for 5-10 min at the top speed. The supernatant was collected as cell-extract.

## 2.5 Translational fidelity analysis:

The accuracy of translation in this study was analyzed by measuring β-galactosidase and β-lactamase activity in a construct containing *lac* Z gene mutated in N terminus with premature stop codons or ±1 frame shift.

### 2.5.1 β-galactosidase Assay:

The β-galactosidase activity was measured as described by Miller method (1972). The plasmids (pSG240, pSG400, pSG415, and 4pCCCU) were transformed to the wild type and mutant bacteria strains (DY330, *ybcJ*Δ, *yfgB*Δ, *yciL*Δ, and *ygdp*Δ) as described in the bacteria transformation protocol. The cell-extract was prepared as described above. Twelve µl of freshly made Mg buffer (10µl 1M $MgCl_2$, 35µl BME, and 550µl $H_2O$) was added to 120µl cell-extract. Then, 804µl of phosphate buffer was added to the samples. Next 264µl Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) was mixed rapidly with the mixture by inverting tubes. The time was recorded when the ONPG was added. The samples were incubated at 30℃ until a yellow color was apparent. At that point, 0.2µl of stop solution (1M $Na_2CO_3$) was added to the samples. The absorbance was read at 420nm.

### 2.5.2 β-lactamase Assay:

The activity of β-lactamase was measured as described in Jones et al (1982). The plasmids (pSG240, pSG400, pSG415, and 4pCCCU) were transformed into the competent cells of the wild type and mutant strains as describe in the bacteria transformation protocol. Cell containing

plasmids were grown at 30℃ overnight. One ml of overnight culture was mixed with 25ml of fresh media. The culture was grown to $OD_{600}$= 0.4-0.6. Cell -extract was prepared as described above. 2.4 mg/ml of CENTA (β-lactamase substrate) was mixed with 25mM phosphate buffer [$NaH_2PO_4$-$H_2O$ (M.W.120), $Na_2HPO_4$ (M.W.141.96) pH: 7.5]. Ten µl of this mixture (CENTA solution) was added to 990µl of (0.025M) phosphate buffer (pH=7.5). The spectrophotometer was calibrated by the phosphate buffer at 410nm. Then, the absorbance of the mixture of CENTA solution and phosphate buffer was read at 410nm (it should be around 0.077). Five ml of cell-extract was added in above solution. The absorbance was read at 420nm.

## 2.6 Protein synthesis efficiency analysis:

The protein synthesis efficiency was measured by incorporation of radioactively labeled amino acid ($^{35}$S-Met) into proteins as described by Meisenhelder and Hunter (1988). The wild type (DY330) and mutant strains cells (*yfgB*Δ, *ygdP*Δ, *yciL*Δ, and *ybcJ*Δ) were grown overnight at 30℃. Then, 800µl of each overnight culture was added to 25ml fresh LB media. The cultures were grown to $OD_{600}$=0.5. 1ml of each culture was mixed with 4µl ($^{35}$S-Met). The samples were incubated for 1h at 30℃, and centrifuged for 60min at the top speed. The cell pellets were washed four times by distilled water. Two µl of the samples were put on a filter paper. The filter paper containing samples was exposed to a phosphoimager screen, and kept overnight at dark. Next day, radiation exposure from each sample was quantified using a phosphoimager (Packard Bioscience Com).

## 2.7 Polysome profile analysis:

The ribosome profile experiments were analyzed as described in Charollais et al (2003). Cells were inoculated into 5ml of LB medium with antibiotics (Kanamycin for mutants and ampicillin for the wild type) at 30℃ overnight. The day after, 5ml of bacteria strains were inoculated into 100ml of LB (no antibiotic) and grown to $OD_{600}$=0.5.

To avoid polysome run off, chloramphenicol was added to a final concentration of 100μg/ml, 5min before harvesting. Cells were rapidly cooled, collected by centrifugation and washed with 30 volumes of buffer A (10 mM Tris-HCl, pH: 7.5, 60 mM KCl, 10 mM $MgCl_2$). Cells were resuspended in 2ml of buffer A and frozen on dry ice/ethanol bath for about 1h. After a first freeze-thaw cycle, cells were lysed by adding 660μl of lysozyme (0.4 mg/ml) freshly prepared in cold buffer A and frozen on dry ice/ethanol bath. Samples were stored in -80℃ freezer overnight. After a second freeze-thaw cycle, 0.25 ml of a solution containing detergents and DNase in buffer A (0.5%polyoxyethylene 20 cetyl ether, 0.5% deoxycholic acid, and 0.1 unit/ml promega RQ1 RNase-free DNase) was added. The mixture was incubated on ice for 20 min. The lysate was clarified by centrifugation in a microfuge at 16,000 g for 20min at 4℃. The extract concentration was estimated by measuring $OD_{260}$. To analyze polysome profiles, about seven $OD_{260}$units of lysate were layered onto 10-50%(w/v) sucrose gradient in buffer B (10 mM Tris-HCl, pH:7.5, 50 mM $NH_4Cl$, 10 mM $MgCl_2$, 1mM dithiothreitol

(DTT)) and centrifuged at 35,000 rpm for 3h at 4℃ in a Beckman SW40Ti rotor. The ribosome profiles were analyzed from the collected gradient solutions by monitoring $OD_{254}$.

## 2.8 Drug sensitivity analysis:

The sensitivity of mutant and wild type strains to antibiotics (kanamycin, streptomycin, tetracycline, and erythromycin) were measured using spot test. LB-agar medium was prepared as described above. The relevant antibiotics were added to the bottle of LB-Agar (~60°C). The media containing antibiotic were poured into sterile petri-dishes (approximately 20ml). After cooling down the agar plate, different dilution of overnight cultures ($10^{-4}$, $10^{-5}$, $2x10^{-5}$, and $10^{-6}$) were prepared (see below) and spotted on the agar plates.

Cells were grown overnight at 30℃. Ten µl of an overnight culture was mixed with 1ml of LB, and then 10µl of the mixture was added to 1ml of LB to make the dilution of $10^{-4}$. The mixture was spotted on the agar plate. To make dilution$10^{-5}$, 100µl of $10^{-4}$ dilution culture was mixed with 1ml LB. The mixture was spotted on the agar plate. Two hundred µl of the $10^{-5}$ dilution sample was mixed with 1ml LB and the mixture was spotted on the agar plate to make $2x10^{-5}$ dilution culture. The culture plates were kept in the incubator at 30℃ overnight. The following day, the growth rate of colonies was observed. The picture was taken from the culture plates.

### 2.8.1 Preparation of antibiotics:

Antibiotics used in this study were listed in Table 2.2. To prepare water soluble antibiotics stock solution, (500 mg streptomycin, 500 mg erythromycin, and100 mg kanamycin) was added to 10ml distilled water and mixed well (stored at -20℃). For ethanol soluble antibiotics stock solution, (120 mg tetracycline) was mixed with 10ml of 95% ethanol (stored at -20℃ except erythromycin that kept at 2-8℃).

Table 2.2: Antibiotic used in this study

| Antibiotics | Stock conc (mg/ml) | Final conc (mg/ml) | Mode of action |
|---|---|---|---|
| Kanamycin | 10 | 0.5,0.9,1 | Inhibit protein synthesis, translocation and elicit miscoding |
| Streptomycin | 50 | 5,6,9 | Inhibit protein synthesis by binding to the S12 protein of the 30S ribosomal. |
| Tetracycline | 12 | 0.5,0.9,1 | Inhibit protein synthesis by preventing binding of aminoacy tRNA to the ribosome A site. |
| Erythromycin | 50 | 6,8,10,14,18 | Inhibit protein synthesis by interacting with 50S. |

## 2.9 Inactivation of the *E.coli yciL, yfgB, ybcJ*, and *ygdP* genes using *E.coli* homologose recombination method (in collaboration with Dr. Butland's lab, Berkely National Labs)

The genes of interest (*yciL, ybcJ, yfgB,* and *ygdP*) were knocked out by using homologose recombination (Zhang *et al.*, 1998; Lee *et al.*, 2001). A polypeptide chain reactions (PCR) product, which contains a kanamycin resistance cassette and primers, was amplified using standard PCR protocol. *E.coli* DY330 (containing red recombinase genes from phage λ) was grown at 30℃ to an OD600=0.4-0.6 in LB medium. Due to the presence of recombinase genes from phage λ, this *E.coli* strain has a reduced rate of linear DNA degradation. The cells were then shifted to 42℃ for 15 min to induce expression of λ recombination proteins followed by chilling in ice water for 10 min. Electrocompetent cells were prepared by washing the cells three times with 10% glycerol. 500 ngr of PCR products were boiled for 1 min per 500 bp and then chilled in ice-cold water. The denature DNA fragments were electroporated into 50μl of ice-cold competent cells. After electroporation, 1ml of LB medium was added to the cuvette, and the culture was incubated at 30℃ for 3h with shaking. The cells were then plated on LB in the presence of 50 μg/ml Kanamycin.

## 2.10 Protein purification (Tandem affinity purification/MS)

### 2.10.1 Primer design and TAP construct:

The PCR primers must be designed with a 5' region containing 40-50 nucleotides (nt) homologous to the genomic region being replaced by the tag and inner 3'conserved regions, which allows for amplification of the tag. TAP tag cassette consists of a calmodulin-binding peptide (CBP), a Tobacco etch virus (TEV) protease cleavage site, and an IgG binding domain of protein A *Staphylococcus aureus*. The tag is attached to the kanamycin (depending on the cassette) resistance marker. The forward primer must be immediately adjacent and in- frame with the stop codon of the gene being tagged.

### 2.10.2 PCR amplification:

Standard PCR reactions were performed using (T3 thermo cycler PCR machine from biometra) to amplify the tap construction and primers. Typical PCR reactions contained :2.5U of taq DNA polymerase, 5µl of 10x PCR buffer, 1.5µl $MgCl_2$ (50 mM), 1µl dNTP (10 mM), primers (25 pmoles of each), 0.5 µl template (highly variable), and $dH_2O$ (up to 50µl). After through mixing by pipette, 50µl of sterile mineral oil was added.

The standard reaction conditions were: denaturation at 95℃ for 5 min, followed by 30 cycles of 1 min denaturation at optimal temperature for primer, 1 min extension at 72℃. A final 10 min exention at 72℃ was usually included. Optimal primer annealing temperature was

calculated by adding 4℃ for each G/C and 2℃ for each A/T present in the primer; this value was then reduced by 0-4℃ depending on PCR results.

### 2.10.3 Purification of PCR product (QIA quick PCR purification Kit):

Five hundred µl of buffer PB was mixed with 100µl of PCR sample. The mixture was transferred to 2 ml pure filter column with a wash collector and centrifuged for 30-60s. The flow –through was discarded and the column was washed with 0.75 ml buffer PE and centrifuged for 30-60s. The flow –through was discarded and the QIA quick column was placed in a clean microfuge tube. DNA was eluted from column with 50µl of buffer EB or $H_2O$ for 1 min. The DNA concentration was assessed visually through agarose –gel electrophoresis (0.7-0.8%) in the presence of ethidium bromide against a standard of known concentration.

### 2.10.4 Tag Incorporation:

Five ml of the culture of *E.coli* strain DY330, containing the λ red prophage sequence under temperature-sensitive regulation, was inoculated in LB media and incubated at 32℃ overnight. The following day, 1 ml of overnight culture was transferred to a flask containing 50 ml of fresh LB media and incubated at 32℃ until the culture's OD600 reached between 0.4 and 0.6 (approximately 2-4 h). To induce cells for recombination activity, 10 ml of culture was transferred to a 50 ml sterile flask and incubated in water bath at 42℃ for 15 min. Then the tubes were incubated in an ice- water bath for 10 min.

Cells were transferred to a sterile 14 ml falcon tube and centrifuged at 7,000 rpm for 2 min. The supernatant was discarded and the pellet was resuspended in 1 ml ice-cold sterile water. Cells were transferred into a 1.5 ml centrifuged tube. The samples were centrifuged at maximum speed at 4℃ on a table-top microcentrifuge. The supernatant was discarded. Cells were washed for a second time, with 1 ml of ice-cold sterile water. Cells were then resuspended in 300 µl sterile ice-cold water and stored.

One hundred µl of competent cells (prepared as above) was added to 1 µl of linear PCR product (~5µg) in an electroportion cuvette. Cells were electroporated at setting of 1.8KV, 200Ω, and 25µFD in a Bio-Rad Gene Pulser or equivalent electroporation machine. Cells were transferred in a 1.5 ml microfuge tube and 1 ml of fresh LB medium was added. The samples were incubated at 30℃ for 1 h. Cells were centrifuged in a table-top microcentrifuge for 1 min at maximum speed. The supernatant was discarded and the pellet was resuspended in 100 µl of fresh LB. Cells were spread over a LB plate containing 50µl/ml kanamycin and incubated at 30℃ for 1-2 days. A selected transformant was inoculated in 4 ml of LB at 30℃. Cells were harvested at 7,000 rpm for 6 min and pellet was kept at -80℃ for further experiment.

## 2.10.5 TAP Purification:

TAP purification was conducted by using a protocol as described in Puig et al (2001). 100µl of IgG Sepharose beads, corresponding to 200µl of bead suspension, was transferred into the column (0.8x4-cm Poly-Prep columns). The beads were washed with 10 ml IPP150 (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Nonidet (NP-40)). The composition of the extract buffer was

adjusted to 10 mM Tris-Cl, pH8.0, 100 mM NaCl, and 0.1%NP-40. The extract was transferred into the column containing the washed beads and rotated for 2 h at 4℃.

Elution was done by gravity flow and the beads were washed three times with 10 ml of IPP150 and once with 10 ml of TEV cleavage buffer. Cleavage was done in the same column by adding 1 ml of TEV cleavage buffer and 100 units of TEV protease. The beads were rotated for 2 h at 16℃ and the elute was recovered by gravity flow.

One hundred µl of calmodulin beads, corresponding to 200µl of bead suspension, was transferred into a column and washed with 10 ml of IPP150 calmodulin binding buffer (10 mM Tris-Cl, pH8.0, 10 mM 2-mercaptoethanol, 150 mM NaCl, 1mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 % NP-40).

Three ml of IPP150 calmodulin binding buffer and 3µl of 1M $CaCl_2$ were added to the 1 ml of elute recovered after TEV cleavage. This solution was then transferred to the column containing washed calmodulin beads and rotated for 1 h at 4℃. After the beads were washed with 30 ml of IPP150 calmodulin binding buffer, the bound proteins were eluted with 1 ml of IPP150 calmodulin elution buffer (10 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 0.1% NP-40, 2 mM EGTA). Five elution fractions of 200µl each were collected.

## 2.10.6 Separation and characterization of interacting proteins:

The purified protein complexes were separated by SDS-PAGE as describe in Gavin et al (2002). To set up the apparatus, two glass plates were separated (one small front plate, one larger back plate) by a pair of spacers (1 mm) which were set to the outer left and right edges of the plates. The separated plates were screwed into clamp assembly to secure in place. The clamp assembly should be fit into a casting stand. The bottom of the plates was pressed firmly against the bottom of the casting stand and all sides were tightly sealed to prevent leakage.

To prepare separating gel, 2.14 ml of 30% acrylamide/bis acrylamide (97% acrylamide, 3% bisacrylamide, in sterile water) was added to 1.53 ml of distilled water, 1.25 ml of 1M Tris-HCl, 50µl 10% SDS, and 5µl TEMED. Then the mixture was transferred between the glass plates and the space between them to approximately 2 cm from the top. To prepare stacking gel, 0.233 ml 30% acryamide/bisacrylamide was mixed with 1.23ml sterile water, 0.5 ml 1M Tris-HCl pH: 6.8, 20 µl 10%SDS, and 1.3 µl TEMED. Then 13 µl ammonium persulfate was added and the mixture was used to fill the remaining space between the plates.

A comb was inserted and the gel was allowed to set for 30 min. The comb was removed and the gel was transferred from the casting stand to the electrophoresis tank. The tank was filled with electrode buffer (3 g Tris base, 14.4 g glycine and 1 g SDS, 1L water). One volume of protein sample was added to 1 volume of reducing buffer (1 ml 0.5M Tris-HCl pH: 6.8, 0.8ml glycerol, 1.6 ml 10% SDS, 0.4 ml mercaptoethanol, 4.2 ml distilled water, 10 µl bromophenol blue) and 10 µl of this sample was loaded in the wells. The gel was run at 180V for 45 min.

The gel from electrophoresis apparatus was separated and transferred to a container filled with 120 ml of sensitizing solution (200 mg sodium thiosulfate anhydrate, 1L distilled water) and was incubated with gentle rocking for 2 min. The sensitizing solution was discarded and the gel was rinsed twice with water. Silver staining solution (2 g silver nitrate, 50 ml water, 760µl formalin, and bring to 1L final volume with water) was added to the container and the mixture was incubated for 20 min. The staining solution was discarded and the gel was washed for 30s with developing solution (60 g sodium carbonate, 4 mg sodium thiosulfate anhydrate, 0.5 ml of formalin and bring to final volume of 1L). The solution was discarded and replaced with 300 ml of fresh developing solution. The mixture was incubated for 2-5 min. To developing solution, 50 ml of terminating solution was added and incubated with gentle rocking for 10 min. The gel was photographed and the size of protein bands was compared to the protein molecular weight marker. The gel was transferred to a clean glass plate. The visible bands were cut with a clean razor blade. The band was transferred to an eppendrof tube with acetonitrile for 15 min. The tube was then centrifuged and the acetonitrile removed. Next, the gel was covered with trypsin digestion buffer and incubated at 4℃ for 45 min. The supernatant was collected by centrifuge at max speed for 2 min. Twenty five mM ammonium bicarbonate was then added and the gel was incubated for 25 min. Ammonium bicarbonate was removed with a micropipette. The extracted peptides were incubated for 10 min at RT with 30µl of extraction solution and centrifuged. This was repeated three times. A speed–vac was used to evaporate extracted sample. The samples were then used for MS.

- Mass Spectrometry (MS):

To identify the TAP- purified proteins, individual protein bands treated with trypsin were subjected to MS. This was done in commercial laboratory (Affinium Biotech) using Matrix assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) MS analysis.

# Chapter 3:

# Results

## 3.1 Identification of proteins that interact with *E.coli* YciL, YfgB, YbcJ, and YgdP proteins:

To identify proteins that interact with our candidate proteins (thought to be involved in translation), the TAP cassette (which contains the calmodulin binding peptide (CBP), protein A, TEV protease cleavage site, and kanamycin resistance marker) was amplified by PCR, using primers sequences with homologies to the sequences upstream and downstream of the target genes . The PCR products were introduced into *E.coli* DY330 chromosome which contains λ-red under control of a temperature- sensitive repressor using homologous recombination. The protein complexes associated with the tagged-protein were affinity purified as described in Materials and Methods. Affinity-purified proteins were separated on 10% SDS- polyacrylamide gels. After silver staining, the protein bands were excised, and then subjected to trypsin hydrolysis to generate small polypeptides. The content of these peptides were analyzed by MALDI-TOF MS.

Figure 3.1 shows the proteins which were found to interact with the YciL, YfgB, YgdP, and YbcJ proteins. Generally, it is possible for YciL, YfgB, YgdP, and YbcJ tagged proteins to interact with other proteins in two ways: protein-protein interactions, or protein-RNA interactions. Therefore, in parallel experiments, RNase A (2μg/ml) was added to protein complexes prior to the affinity purification step to distinguish those interactions, which are independent of the RNA intermediate molecules (Fig 3.1, panels C, E, G, and I).

The proteins, which interact with YciL, YgdP, YbcJ and YfgB, can be divided into two groups: the ribosomal proteins and those independent of ribosomes. This is in agreement with

the inherent nature of translations proteins which are generally thought to function both in association with ribosomes and independent of ribosomes.

Shown in Figure 3.1 (B, C); Table 3.1, YbcJ tagged-protein interact with RpsB, and RpsC proteins which are essential 30S ribosomal subunits proteins. In addition, YbcJ protein interacts with other proteins such as SrmB and DeaD, which are involved in the assembly of 50S ribosomal subunits. The interaction with RpsB was eliminated by the addition of RNase indicating that it was mediated by a RNA molecule. In contrast presence of RNase enhanced an interaction with SrmB and YbcJ suggesting that the interactions of YbcJ with ribosomal proteins might be mediated by the ribosome itself (not true protein-protein interactions). This is a characteristic of transient translation proteins including ribosome assembly factors. All together, these data clearly indicate that YbcJ has a physical association with translation proteins and highlights a possible transient role for this protein in translation.

In Figure 3.1 (D, E); Table 3.1, RpsA, RplF, SrmB, SpoT and RplE are example of proteins that are found to interact with the YgdP tagged-protein. RpsA and RplE are 30S and 50S ribosomal subunit proteins, respectively, and SpoT is an enzyme which coordinates variety of cellular activities in response to change in nutritional abundance. The interaction with RplE seemed to be RNA independent.

Some of the proteins, which interacted with YfgB tagged-protein (Figure 3.1 (F, G); Table 3.1), have functions as elongation factors or GTP binding in translation process. For example, FusA is a protein chain elongation factor EF-G, GTP binding and TufA is a protein chain

elongation factor EF-TU. These data may indicate that the YfgB protein may directly or indirectly affect the elongation step of translation.

Also as indicate in Figure 3.1 (H, I); Table 3.1, a number of proteins (RplA, RplB, and SrmB), which interact with YciL tagged-protein, are 50S ribosomal subunit proteins or have a function associated with the 50S subunits. These data may suggest that YciL protein may have a role which is closely associated with the function of 50S. Some of the observed interactions with 50S ribosomal proteins seemed independent of RNA molecules.

All together, the interaction data indicate that YfgB, YciL, YgdP, and YbcJ tagged-proteins interact with those proteins which play a role in translation. Therefore, we conclude that based on protein-protein interaction evidence *yfgB, yciL, ygdP* and *ybcJ* genes are involved in protein synthesis.

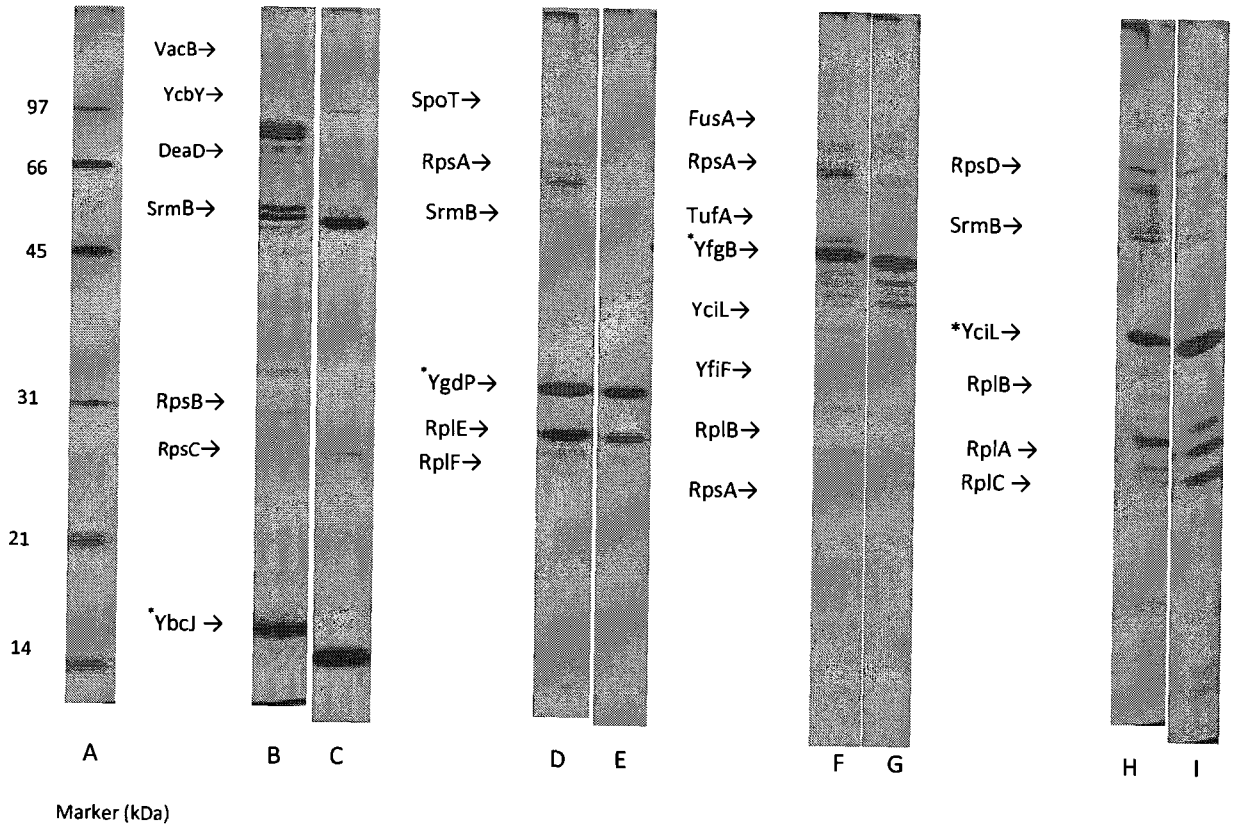

97
66
45
31
21
14
VacB→
YcbY→
DeaD→
SrmB→
RpsB→
RpsC→
*YbcJ →
SpoT→
RpsA→
SrmB→
*YgdP→
RplE→
RplF→
FusA→
RpsA→
TufA→
*YfgB→
YciL→
YfiF→
RplB→
RpsA→
RpsD→
SrmB→
*YciL→
RplB→
RplA →
RplC →
A
B
C
D
E
F
G
H
I
Marker (kDa)

Figure 3.1: Analysis of TAP purification of *E.coli* strain DY330 tagged for different target proteins. The protein complexes are affinity-purified and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by silver staining. Individual subunits of the purified complexes, recognized by distinct bands on the gel, were identified by in- gel trypsin digestion followed by MALDI-TOF MS analysis. Lane (A) represents molecular weight markers. (B), (D), (F) and (H) represent purifications for tagged YbcJ, YgdB, and YciL, respectively. (C), (E), (G) and (I) are identical to (B), (D), (F) and (H), respectively, expect that RNase A (2μg/ml) was added to these samples before the affinity purification step. Each band corresponds to a co-purified protein.* represents target tagged-proteins.

Table3.1: The target tagged-proteins and their interactors:

| Target proteins | Interacting protein | Property |
|---|---|---|
| YfgB | DeaD | ATP-dependent-RNA helicase |
| | DnaJ | Co-chaperone with Dnak |
| | FruR | DNA-binding transcriptional |
| | FusA | Elongation factor GTP binding |
| | RpsA | 30Sribosomal protein S1 |
| | RpsB | 30Sribosomal protein S2 |
| | RpsC | 30S ribosomal proteins S3 |
| | RpsD | 30S ribosomal proteinS4 |
| | RpsJ | 30 S ribosomal subunit |
| | RplB | 50S ribosomal subunit protein L2 |
| | RplC | 50S ribosomal protein L3 |
| | RplD | 50S ribosomal protein L4 |
| | SecA | Translocase protein |
| | SrmB | A Dead-box RNA helicase |
| | TufA | Protein chain elongation factor |
| YgdP | DeaD | ATP-dependent-RNA helicase |
| | RplA | 50S ribosomal protein |
| | RplB | 50S ribosomal subunit protein L2 |
| | RplE | 50S ribosomal subunit protein L2 |

| | | |
|---|---|---|
| | RpsA | 30Sribosomal protein S1 |
| | RpsB | 30Sribosomal protein S2 |
| | RpsD | 30S ribosomal proteinS4 |
| YbcJ | SrmB | A Dead-box RNA helicase |
| | DeaD | ATP-dependent-RNA helicase |
| | DnaJ | Co-chaperone with Dnak |
| | RplC | 50S ribosomal protein L3 |
| | RplL | 50S ribosomal subunit protein |
| | RpsC | 30S ribosomal protein S3 |
| YciL | VacB | Acts non-specifically on ribosomal RNA |
| | RplA | 50S ribosomal protein |
| | RplB | 50S ribosomal subunit protien L2 |
| | RplC | 50S ribosomal protein L3 |
| | RpsA | 30Sribosomal protein S1 |
| | RpsD | 30S ribosomal proteinS4 |
| | RpsJ | 30 S ribosomal subunit |
| | SrmB | A Dead-box RNA helicase |
| | PnP | Involved in mRNA degradation |

## 3.2 Effect of target gene deletions on translational fidelity:

If our target genes are involved in translation, then it might be possible that their deletions may alter translation fidelity. To examine this possibility, gene deletion mutant strains for yciL, yfgB, ygdP, and ybcJ were generated using a homologous recombination method as described in the Materials and Methods section. In this case, the target genes were replaced by a kanamycin or chloramphenicol resistance markers.

To study the effect of target genes in translation fidelity, the plasmid-encoded *lacZ* genes mutated in the N-terminal region with a premature stop codon (pSG400, pSG415) or a frame shift mutation (pSG240, 4pCCCU) were used. These plasmids were first introduced in the wild type (DY330) and gene deletion strains (*yfgB*Δ, *ygdP*Δ, *yciL*Δ and *ybcJ*Δ). The transformed strains were subjected to β-galactosidase analysis. The level of β-galactosidase in cell extract was measured using ONPG standard method (O'Conner *et al.*, 1992) as in the Materials and Methods sections.

The β-galactosidase was produced by translating ribosomes only if they read through an in frame stop codon or shifted reading frame. In this case, only those ribosomes that mistakenly do not recognize the stop codon, or randomly shift between reading frames would be able to produce β-galactosidase. The background level of stop codon read through and frame shifts was obtained from the wild type strain. For direct comparison between the wild type and gene deletion strains, the level of β-galactosidase activity in the wild type has been set at 1.00. Each experiment was repeated at least 4 times.

As indicated in Table 3.2, production of β-galactosidase was increased in the gene deletion strains containing the plasmid-encoded mutated *lac*Z gene. Levels of β-galactosidase activity in the *E.coli yfgB*Δ, *yciL*Δ, *ygdP*Δ, and *ybcJ*Δ deletion strains with UAG (pSG400) or UGA (pSG415) stop codons at the N-terminus of the *lac*Z gene, were 10-20 folds greater than the wild type transformant. As indicated, the UGA (pSG415) premature stop codon produced higher levels of β-galactosidase. It has been shown that the production of β-galactosidase in such expression systems that carries pre-mature stop codons or frame shifts, is a reflection of translational fidelity for the tested strains (O' Conner and Dahlberg, 1993). Therefore, our observations suggest that deletion of *E.coli yciL, yfgB, ygdP*, and *ybcJ* genes lead to decreased fidelity of the ribosome in translation. These results further confirm the involvement of the target genes in the process of protein synthesis.

**Table3.2:** Relative production of β-galactosidase in the wild type and gene deletion *E.coli* strains *yciL*Δ, *yfgB*Δ, *ygdP*Δ, and *ybcJ*Δ

| **Strains** | **pSG240 (-1 frame shift)** | **pSG400 UAG Stop Codon** | **pSG415 UGA Stop Codon** | **4pCCCU (+1 frame shift)** |
|---|---|---|---|---|
| DY330 (WT) | 1.3 | 1.5 | 1 | 1.8 |
| *yfgB*Δ | 2.5 | 22.7 | 86.3 | 6 |
| *yciL*Δ | 9.8 | 38.2 | 113.6 | 16.6 |
| *ygdP*Δ | 10.3 | 18.18 | 29.7 | 12.09 |
| *ybcJ*Δ | 6.18 | 12.09 | 36.18 | 8 |

**Table3.2:** Relative production of β-galactosidase in the wild type and gene deletion *E.coli* strains *yciL*Δ, *yfgB*Δ, *ygdP*Δ, and *ybcJ*Δ. The plasmids caring *lac*Z gene mutated in the N terminus of protein (pSG240, pSG400, pSG415, 4pCCCU) were introduced in the wild type and mutant *E.coli* strains. *E.coli* transformants were grown at 30℃ overnight. The cell-extract was prepared as described in Materials and Methods. The activity of β-galactosidase was measured by using the ONPG method. Each experiment was repeated more than 4 times with ± 25% or less variation. The amount of β-galactosidase in the wild type using pSG 415 is set at 1:00 and every other value is relative to this. WT is the wild type strain.

Differences in the plasmid numbers between strains might also explain the observed differences in β-galactosidase activities. To test for this possibility, the activity of β-lactamase in the above cell extracts was assayed using the CENTA method. The β-lactamase is encoded on the plasmid as an ampicillin-resistance marker. If plasmid copy number was being affected in different strains, then it is expected that β-lactamase activity would measured this (Thompson *et al.*, 2002). As shown in Table 3.3, the ratio activity of the β-galactosidase to β-lactamase enzyme was slightly increased in mutant transformants compare to the wild type. However these increases were not sufficient to justify the observed difference in the β-galactosidase activities, indicating that the increased levels of β-galactosidase were mainly due to decreased translation fidelities.

**Table3.3:** The ratio of β-lactamase and β-galactosidase enzyme in the wild type and mutant *E.coli* strains.

| Strains | pSG240 (-1frameshift) | | | pSG400 UAG Stop codon | | | pSG415 UGA stop codon | | | 4pCCCU (+1frameshift) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | β-Gal | β-lac | Normalized | β-Gal | β-Lac | Normalized | β-Gal | β-Lac | Normalized | β-Gal | β-lac | Normalized |
| DY330 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *yfgBΔ* | 1.8 | 1.15 | 1.5 | 14.7 | 1.2 | 12.25 | 86.3 | 1.7 | 50.76 | 3.3 | 3.1 | 1.06 |
| *yciLΔ* | 7.2 | 2.9 | 2.4 | 24.7 | 2.3 | 10.7 | 113.6 | 1.6 | 71 | 9.15 | 1.2 | 7.6 |
| *ygdPΔ* | 7.6 | 2.4 | 3.16 | 11.7 | 2.12 | 5.5 | 29.7 | 1.5 | 19.8 | 6.65 | 1.9 | 3.5 |
| *ybcIΔ* | 4.5 | 1.6 | 2.8 | 7.8 | 2.2 | 3.5 | 36.18 | 1.9 | 19.04 | 4.4 | 1.7 | 2.5 |

**Table3.3:** The ratio of β-lactamase and β-galactosidase enzyme in the wild type and mutant *E.coli* strains. The activity of β-lactamase was measured using the CENTA assay. The amount of β -lactamase and β-galactosidase in the wild type were set at 1:00 and other values are relative to them.

## 3.3 Drug Sensitivity:

If our target genes *yciL, yfgB, ybcJ*, and *ygdP* have a role in translation, then it might be expected that their deletion might increase or decrease the sensitivity of their gene deletion mutants to different drugs that target translation. It has been shown that tetracycline, kanamycin, and streptomycin bind to the 30S ribosomal subunit and interfere in protein synthesis. In contrast, erythromycin binds to 50S ribosomal subunits and block the way of polypeptide to release (Hansen *et al.*, 2002; Champney, 2003; Champney and Tober, 2000; Broderson *et al.*, 2000). We used these drugs to investigate the potential involvement of our target genes in translation.

To this end, the *E.coli* gene deletion and the wild type stains were spotted on LB agar media with different concentration of tetracycline (0.5 mg/ml, 0.9 mg/ml, and 1 mg/ml), kanamycin (0.6 mg/ml, 0.8 mg/ml, and 1 mg/ml), erythromycin (6 mg/ml, 8 mg/ml, 10 mg/ml, and 18 mg/ml), and streptomycin (5 mg/ml,6 mg/ml, and 9 mg/ml). The culture plates were incubated at 30℃ for one day. The control sample was grown on LB without antibiotic. The results indicate that the deletion of *E.coli yciL, yfgB, ygdP* and *ybcJ* genes cause alternations in the pattern for sensitivity of the strains to the tested drugs.

As shown in Figure 3.2, deletion mutation for all the target genes increased cell sensitivity to tetracycline. Of the gene deletion mutants, *ygdPΔ* seems to have the least

difference in sensitivity. Up to drug concentrations of 0.9 mg/ml, the pattern of its cell growth seems very similar to the wild type. On the other hand, *yfgBΔ* seems to be the most sensitive mutant. At a drug concentration of 0.9 mg/ml, there was significant reduction in growth. When the concentration of tetracycline was increased to 1 mg/ml, *yfgB*Δ cells showed almost no growth. *yciL*Δ and *ybcJ*Δ also showed increased sensitivity to tetracycline, but both showed some growth at drug concentration of 1mg/ml.

In Figure 3.3, deletion of some of the target genes showed increased sensitivity to kanamycin. Deletion strains seem to have no difference in sensitivity to drug concentration of 0.6 mg/ml. *yciL*Δ showed the highest level of sensitivity to kanamycin. It seems to have minimal growth at drug concentration of 1 mg/ml. In contrast, *ygdP*Δ showed very little sensitivity to kanamycin. The pattern of cell growth in *ybcJ*Δ seems to be similar to the wild type.

Figure 3.4 showed that deletion of all target genes increased cell resistance to streptomycin. *yfgB*Δ and ybcJ seems to be the most resistance mutants to the drug, even at drug concentrations of 9 mg/ml. It should be noted that the WT strain stopped growing at the drug concentration of 6 mg/ml. *ygdP*Δ and *yciL*Δ also increased resistance to streptomycin, but both showed some growth at concentrations of 9 mg/ml.

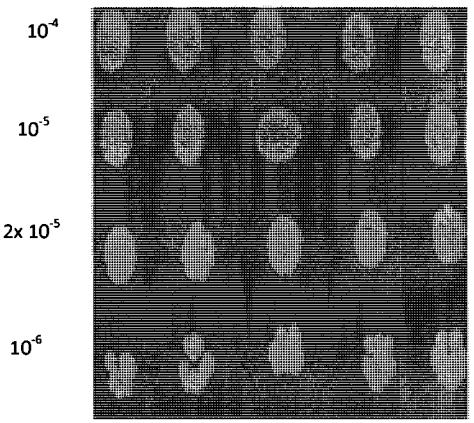
In Figure 3.5, deletion of all target genes showed alternation in the pattern of their growth on erythromycin. *yfgB*Δ showed increased cell sensitivity to erythromycin and showed almost no growth at the drug concentration of 6 mg/ml. In contrast, *ygdP*Δ showed increased level of cell resistance to erythromycin even at very high drug concentrations. Even at drug

concentrations of 18 mg/ml, this mutant seems to have some growth. The patterns of growth for *ybcJ*Δ and *yciL*Δ seem to be very similar to the wild type.

All together, deletions of *yciL, ybcJ, yfgB*, and *ygdP* genes resulted in alteration in the drug sensitivity patterns of the mutant cells for different translation drugs. This is in agreement with the involvement of these genes in translation and further supports the idea that *yciL, yfgB, ybcJ,* and *ygdP* are novel translation components.

**Figure 3.2:** Spot test drug sensitivity analysis for *E.coli* deletion strains *yciL*Δ, *yfgB*Δ, *ybcJ*Δ, *ygdP*Δ, and the wild type (DY330) to tetracycline. Drug concentration of 0.5 mg/ml, 0.9 mg/ml, and 1 mg/ml were used. Different dilutions of overnight culture of mutants and the wild type ($10^{-4}$, $10^{-5}$, $2x10^{-5}$, and $10^{-6}$, as indicated) were spotted on the plates. The plates were kept in the incubator at 30℃ overnight. The growth pattern of mutants strains were compared to that of the wild type. Panel (a) is the control plate which contains LB media without antibiotic.

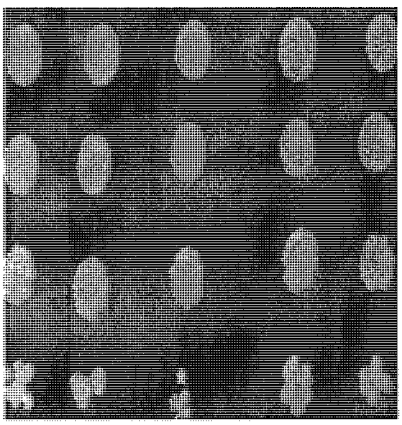
a




DY330 *ybcJ*Δ *ygdP*Δ *yciL*Δ *yfgB*Δ

(LB)

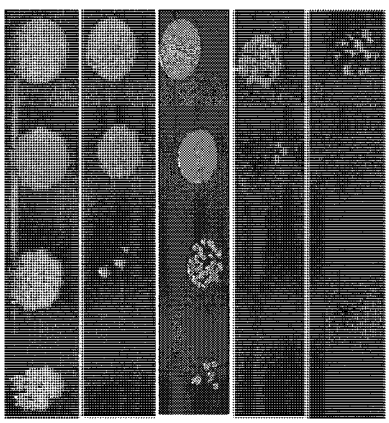
b



DY330 *ybcJ*Δ *ygdP*Δ *yciL*Δ *yfgB*Δ

(LB+ tetracycline 0.5 mg/ml)

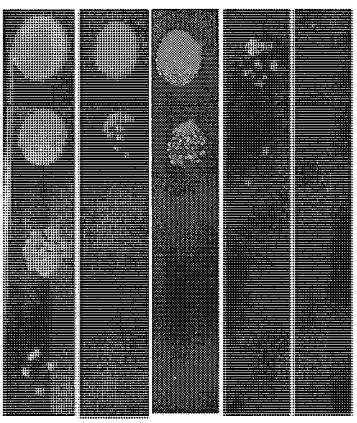
c



DY330 *ybcJ*Δ *ygdp*Δ *yciL*Δ *yfgB*Δ

(LB+ tetracycline 0.9mg/ml)

d

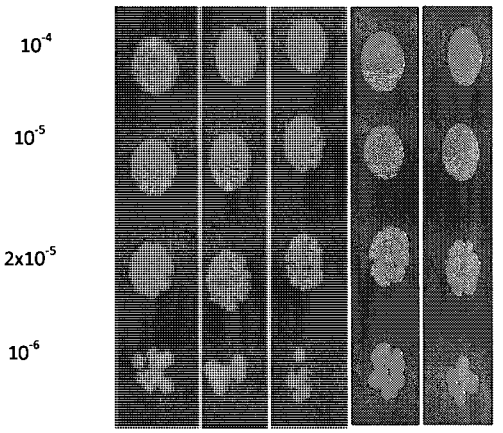


DY330 *ybcJ*Δ *ygdP*Δ *yciL*Δ *yfgB*Δ

(LB+ tetracycline 1mg/ml)

**Figure 3.3:** Spot test drug sensitivity for *E.coli* deletion strains *yciL*Δ, *yfgB*Δ, *ybcJ*Δ, *ygdP*Δ and the wild type (DY330) to kanamycin. Drug concentration 0.6 mg/ml, 0.8 mg/ml, 1 mg/ml were used. Different dilution of overnight culture of mutants and the wild type ($10^{-4}$, $10^{-5}$, $2x10^{-5}$, $10^{-6}$) were spotted on the plates. The plates were kept in the incubator at 30℃ overnight. The growth pattern of mutants strains were compared to that of the wild type. Panel (a) is the control plate which contains LB media without antibiotic.

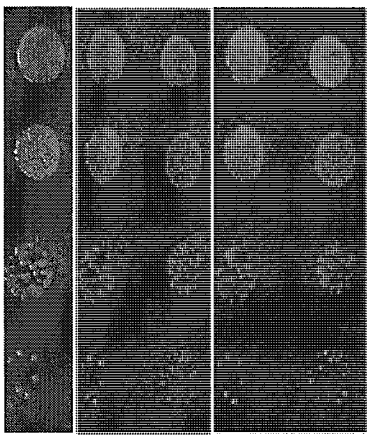
a




Dy330 ybcJΔ ygdPΔ yciLΔ yfgB Δ

(LB)

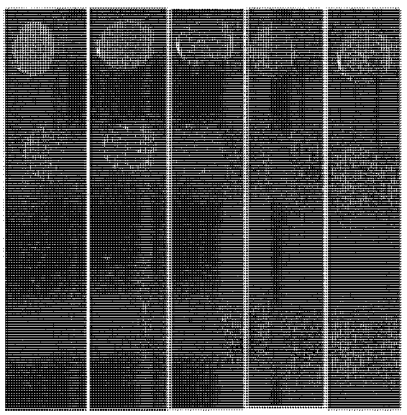
b



Dy330 ybcJΔ ygdPΔ yciIΔ yfgBΔ

(LB+ kanamycin 0.6 mg/ml)

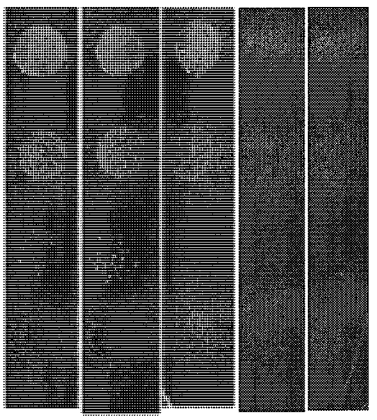
c



Dy330 *ybcJΔ* *ygdpΔ* *yciLΔ* *yfgBΔ*

(LB+ Kanamycin 0.8 mg/ml)

d

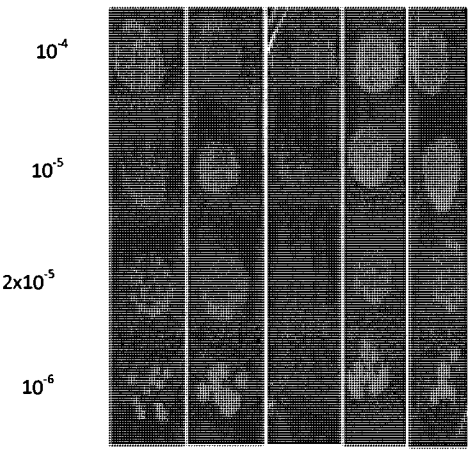


Dy330 *ybcJΔ* *ygdpΔ* *yciLΔ* *yfgBΔ*

(LB+ kanamycin 1mg/ml)

**Figure3.4:** Spot test drug sensitivity for *E.coli* deletion strains *yciL*Δ, *yfgB*Δ, *ybcJ*Δ, *ygdP*Δ and the wild type (DY330) to streptomycin. Drug concentration of 5 mg/ml, 6 mg/ml, and 9 mg/ml were used. Different concentrations of overnight culture of mutants ($10^{-4}$, $10^{-5}$, $2x10^{-5}$, $10^{-6}$) were spotted on the plates. The plates were kept in the incubator at 30℃ overnight. The growth pattern of mutants strains were compared to that of the wild type. Panel (a) is the control plate which contains LB media without antibiotic.

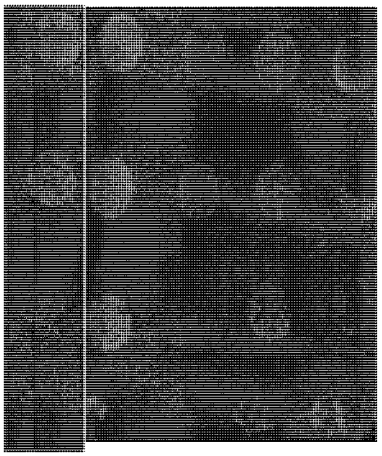
a




DY330 *ybcJ*Δ *ygdP*Δ *yfgB*Δ *yciL*Δ

(LB)

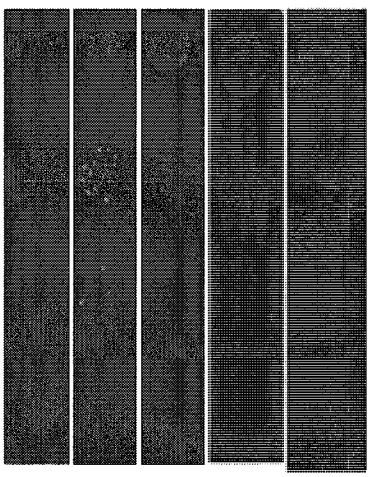
b



DY330 *ybcJ*Δ *ygdP*Δ *yciL*Δ *yfgB*Δ

(LB+ streptomycin 5mg /ml)

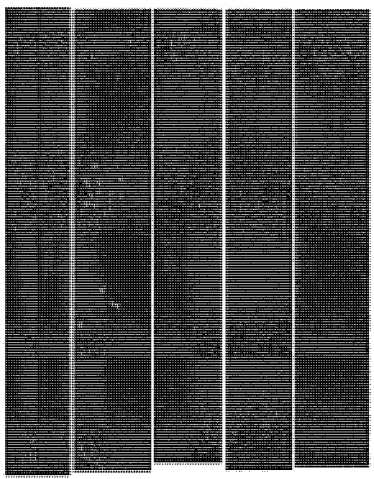
c



DY330 *ybcJ*Δ *ygdP*Δ *yciL*Δ *yfgB*Δ

(LB+ streptomycin 6mg /ml)

d

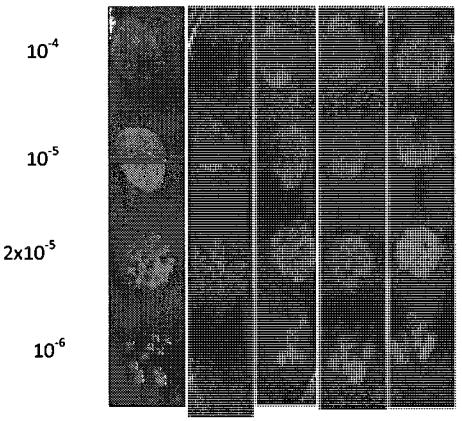


DY330 *ybcJ*Δ *ygdP*Δ *yciL*Δ *yfgB*Δ

(LB+ streptomycin 9mg /ml)

**Figure 3.5:** Spot test drug sensitivity analysis for *E.coli* deletion strains *yciLΔ*, *yfgBΔ*, *ybcJΔ*, *ygdPΔ* and the wild type (DY330) to erythromycin. Drug concentrations of 6 mg/ml, 8 mg/ml, 10 mg/ml, and 18mg/ml were used. Different dilutions of overnight culture overnight culture of mutants and the wild type ($10^{-4}$, $10^{-5}$, $2x10^{-5}$, $10^{-6}$) were spotted on the plates. The plates were kept in the incubator at 30℃ overnight. The growth pattern of mutants strains were compared to that of the wild type. Panel (a) is the control plate which contains LB media without antibiotic.

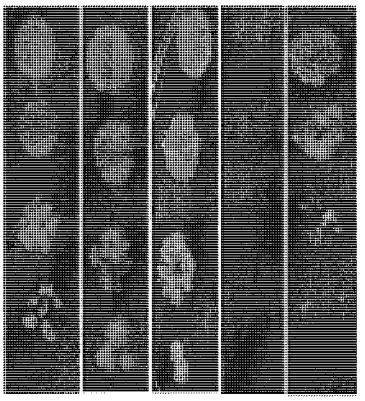
a




DY330 *ybcJΔ ygdPΔ yfgBΔ yciLΔ*

(LB)

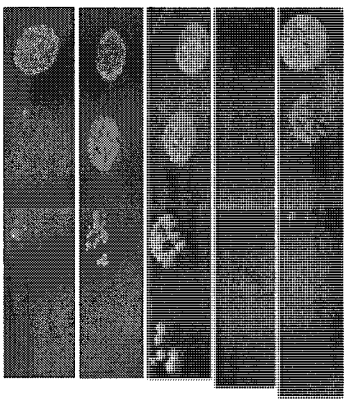
b



DY330 *ybcJΔ ygdPΔ yfgBΔ yciLΔ*

(LB+ erythromycin 6mg/ml)

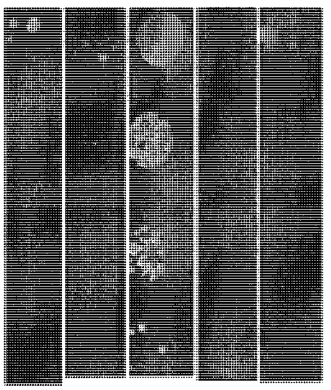
c



DY330 *ybcJΔ ygdPΔ yfgBΔ yciLΔ*

(LB+ erythromycin 8mg/ml)

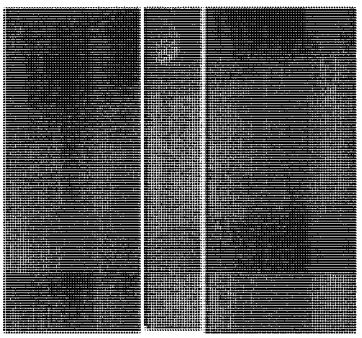
d



DY330 *ybcJΔ ygdPΔ yfgBΔ yciLΔ*

(LB+ erythromycin 10mg/ml)

E



DY330 *ybcJΔ ygdPΔ yfgBΔ yciLΔ*

(LB+ erythromycin 18mg/ml)

## 3.4 Protein labeling *in vivo*:

If the target genes are involved in translation then we may expect that deletion of them can affect the efficiency of translation. To investigate translational efficiency in the gene deletion and the wild type strains, [$^{35}$S]-methinione incorporation method was used. Two µl of [$^{35}$S]-methionine isotope was introduced into the growing cell at OD600 = 0.5 for one h. The cells were collected by centrifugation and washed three times. The third wash samples were screened by phosphoimager to measure total isotope incorporation. This experiment was repeated more than 4 times. If the candidate genes are, in fact, involved in protein synthesis, it might be expected that, based on their molecular activity, the deletion of some of them may cause the reduction (or perhaps an increase) in total protein synthesis within a cell. [$^{35}$S]-methionine incorporation measurements, shown in Table 3.4 indicate that the deletion of the target genes in *E.coli* can affect the rate of protein synthesis *in vivo*. The phosphate measurements for the wild type were set at 1:00 and represent a count of 93,000. As indicated (Table 3.4), $^{35}$S incorporation measurements suggest that the amount of protein synthesis increased in the *ygdP*Δ strain. This observation provides further support for the involvement of this gene in protein synthesis.

**Table 3.4:** Relative protein synthesis measurement *in vivo*.

| Strains | A600 nm | [ $^{35}$S]-methionine (1 μ Ci/ml ) | Phosphate measurement |
|---|---|---|---|
| DY330(WT) | 0.49 | 2 | 1.00 |
| YfgBΔ | 0.50 | 2 | 1.01 ± 0.05 |
| YciLΔ | 0.48 | 2 | 0 .93 ± 0.25 |
| YgdPΔ | 0.49 | 2 | 1.16 ± 0.05 |
| YbcJΔ | 0.50 | 2 | 0.94 ± 0.12 |

**Table 3.4:** Relative protein synthesis measurement *in vivo*. The rate of translation in *E.coli* mutants and the wild type was examined by measuring the incorporation of $^{35}$S-labeled amino acid Met into cellular protein. Two µl of $^{35}$S-methionine was added to 1ml of growing cultures at around OD=0.5. 1µl of each sample was used for $^{35}$S-labeled incorporation detection. The phosphate measurement for the wild type was set at 1:00 and represents a count of 93,000. Other measurements are relative to this value.

## 3.5 Ribosome profile analysis:

To further examine the involvement of the *E.coli yciL, ygdP, yfgB* and *ybcJ* genes in protein synthesis, we investigated the effect of their deletion on the distribution of the ribosome using ribosome profile analysis. In this experiment, the effect of the deletion of the target genes on ribosomal- subunits and polysome formations in the wild type and mutant strains were compared. After growth in LB medium at 30℃, cells were lysed, and ribosomal particles were separated by sucrose gradient sedimentation. As expected, as shown in Figure3.6, different distinct peaks were observed in the wild type strain corresponding to 30S, 50S, 70S and polysomes. Polysomes, as expected, are represented by a series of short peaks. This profile is altered when *yfgB* is deleted. The results show an increase in the proportion of free subunits, particularly 30S, with a reduction in polysomes. In contrast, when *yciL, ygdP, ybcJ* were deleted no difference was observed for their corresponding profiles (data not shown). The ratio of the 50S to 30S absorption peaks at 254nm is normally about 2 in the wild type, but in the *yfgB*Δ, this ratio was approximately 1.1 which may indicate a deficiency in 50S subunit formation. This is because the small and large subunits are thought to be in equilibrium with the monosomes and hence deficiency in the production of one subunit may cause an increase in the production of the other (Vlásek *et al.*, 2001). This activity of *yfgB* is supported by its interactions with those proteins which play a role in 50S subunit assembly and support a role for *yfgB* in ribosome biogenesis.

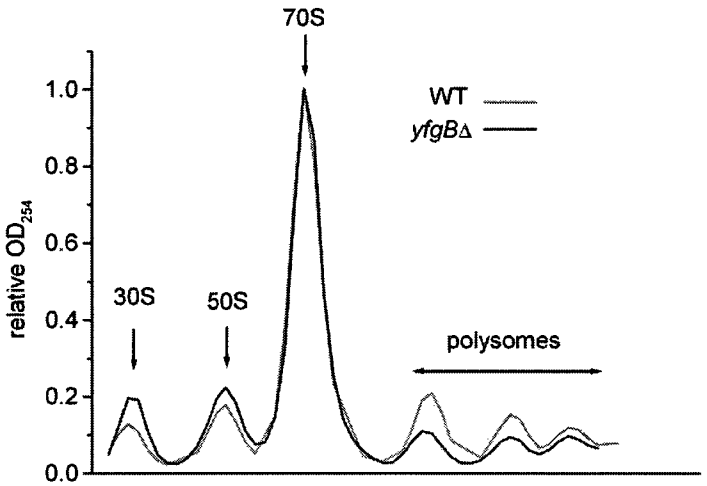

70S
WT
yfgBΔ
1.0
0.8
0.6
0.4
0.2
0.0
relative OD254
30S
50S
polysomes

**Figure3.6:** The ribosome profile of the wild type and *yfgB*Δ strains.

Deletion of *yfgB* leads to an increase in the proportion of 50S and 30S, and a reduction in polysomes. The wild type and *yfgB*Δ strains were grown in LB medium at 30℃. Ribosome profiles were analyzed as described in Material and Method. The peaks corresponding to the free 30S and 50S ribosomal subunits, 70S monosomes, and polysomes are indicated.

# Chapter 4:

# Discussion

Proteins that are functionally related often interact with one another and generate functional protein complexes. Accordingly, one way to investigate novel gene functions is to study the interactions that target proteins make with other characterized proteins. One of the best ways to analysis protein-protein interactions is the affinity method. In this study, we identified *E.coli yciL*, *ybcJ*, *ygdP*, and *yfgB* genes involved in protein synthesis based on the evidence that they interacted with different translation associated proteins.

In a previous study Butland et al. (2005) has shown that YciL, YfgB, YgdP, and YbcJ proteins were co-purified with several proteins that had reported roles in protein synthesis. These data were gathered by large scale study of protein- protein interactions using TAP tagging approach. Due to the high rates of false positives and false negatives associated with such large scale analysis, we repeated these experiments in a smaller setting with multiple repeats to confirm the observed associations.

To confirm the association of the above proteins with translation proteins, their corresponding genes were double tagged using homologous recombination approach in a background strain which supports linear DNA transformation (DY330 with λ phase under temperature sensitive CI857 repressor)(Zhang *et al.*,1998). This approach represents the reciprocal taggings of the co-purified proteins. The tagged YbcJ, YciL, YfgB, and YgdP proteins, along with their associated protein partners, were purified through affinity purification. The purified proteins were separated using SDS-PAGE electrophoresis and identified by MALDI-TOF MS. In this way, the proteins which physically interacted with the target proteins *in vivo* were

determined. Since the tagged proteins are not over-expressed, it is expected that the identified partners represent true interactions.

It is important to note however, that these interactions can be divided into two groups. Group one are those that are mediated via true protein-protein interactions and group two which are those that are mediated via a connecting RNA molecule. It should be noted that the ribosome is a huge RNA based molecule. Therefore, in theory, many of our observed interactions might be mediated by this molecule and hence they may not reflect true protein-protein interactions. Such interactions could complicate the interpretation of the results.

To eliminate RNA-protein interactions, RNase was added to the complex mixture before the affinity purification steps. The presence of RNase, in theory, will eliminate intermediate RNA molecules and hence can be used to differentiate between true protein-protein interactions from those mediated by RNA molecules. It should be noted that it is still possible that certain regions of the ribosome, that are more resistance to RNase treatment, mediate some of the observed interactions in the presence of the concentration of RNase that was used in this study (2 μg/ml). On the other hand, it has been shown that using higher concentrations of RNase interferes in the affinity purification process (Butland *et al.*, 2006). Therefore regardless of the presence of RNase, some of observed interactions may still be mediated via ribosomal or other types of RNA molecules.

Our TAP purification analysis indicated that the majority of interactors for YciL and YfgB proteins are independent of RNA molecules. For example, the interactions between YciL and RspA and RplB were independent of RNase treatment. In contrast, many of the YbcJ and YgdP

associated proteins seemed to be mediated via an RNA molecule. For example, the interactions between YbcJ and VacB and RpsB were eliminated by the addition of RNase.

Our PPIS analysis show that YfgB interacts with TufA, and FusA which have essential roles in elongation and GTP binding (an activity which is associated with elongation), in an RNA independent manner. It is also found that this protein interacts with ribosomal subunit proteins Rpsa, RpsB, RpsC, and RpsD. Interestingly, similar to the affect of *yfgB* on translation fidelity (see below) mutations in RpsD are reported to increase translational errors (Balashov and Humayun, 2003). DnaJ and GrpE (Maki *et al.*, 2000; Alix and Guerin, 1993) were also found to co-purify with tagged-YfgB. Maki et al (2002) has shown that DnaJ and GrpE, facilitate the assembly of the 30S ribosomal subunit *in vitro* in an ATP-dependent manner. YfgB was also found to interact with two RNA helicases SrmB and DeaD proteins. The SrmB protein has been shown to mediate the assembly of 50S subunit via remodeling pre-rRNA molecules (Charllais *et al.*, 2003). DeaD protein is a major cold shock protein that may fascinate destabilizing mRNA inhibitory structures during translation initiation (Butland *et al.*, 2006; Iost and Dreyfus, 2006) and is also involved in 50S ribosomal biogenesis (Pugh *et al.*, 1999; Charollais *et al.*, 2004). These data clearly indicate that YfgB interacts with a number of translation proteins and hence have a function in protein synthesis. Furthermore, this function seems to be targeted towards ribosome biogenesis.

The effect of *yfgB* on ribosome biogenesis was further supported by our ribosome profile analysis. The *E.coli* mutant strain for *yfgB* showed a decrease in translation polysomes along with a corresponding increase in free 30S and 50S subunits. There is a greater increase in

accumulation of the 30S subunit than 50S subunit. Since the free subunits are thought to be in equilibrium with the monosomes (70S), an increase in 30S subunit may suggest a defect in 50S biosynthesis (Vlásek *et al.*, 2001). Therefore, it is possible that *yfgB* affect ribosome biogenesis by affecting the assembly of 50S subunit. In *E.coli*, the 50S subunit is formed by two RNAs (23S and 5S) and 33 proteins, while the small 30S subunit consists of one RNA (16S) and 21 proteins (S1 to S21). The assembly of ribosomal subunits includes the maturation of rRNA precursor and assembly of ribosomal protein on the rRNA. At this point it is difficult to suggest which step of the 50S biogenesis might be the target of *yfgB*. Further analysis is required.

If a protein in involved in translation, based on its molecular function, its deletion may or may not affect the accuracy of translation. It has been shown that mutation in genes specifying the 30S ribosomal protein S4, S5, 16S RNA, 23S RNA, elongation factor (EF)-TU and release factors 1 and 2 decrease the accuracy of the translation (Parker, 1989; O'Conner *et al.*, 1997; Kirith *et al*, 2006) and confer a so-called ribosomal ambiguity mutation (Ram) phenotype characterized by increased translational errors (Roy-Chaudhuri *et al.*, 2008). In *E.coli*, mutations in the decoding center of the ribosome, or any of several region outside it, can affect the fidelity of translation (O'Conner *et al*,. 1992; Allen and Noller, 1991). These mutations may affect codon-anticodon interactions directly, or the interaction between the ribosome and EF-Tu or release factor during the elongation and termination phases of translation. The accuracy of translation is also enhanced by proofreading, which is associated with EF-TU-hydrolysis of GTP. Mutation in S4, S5 and EF- TU decrease proofreading functions in *E.coli*. It has been hypothesized that reduced proofreading by defective ribosome in Ram cells leads to an increase in translational errors (Anderson and Kurland, 1983).

Here, we measured translation fidelity using a plasmid-based β-galactosidase expression system which contains a premature stop codon or a frame shift. The data clearly indicate that, in the absence of *yfgB* translational fidelity is significantly decreased. At this point it is difficult to suggest whether this effect is exerted on the mechanism of translation or it proofreading activity.

To further investigate the involvement of the candidate genes in protein synthesis, total protein synthesis rate in gene deletion strains were measured *in vivo*. It has been shown that the translation initiation complex plays an important role in the rate of protein synthesis and functions as a rate limiting step. The translation initiation complex consists of a number proteins, RNAs, initiator factors, and tRNAs (Voges *et al.*, 2004; Gualerzi *et al.*, 2000). There are many factors that have an effect on protein synthesis efficiency such as Shine-Dalgarno (SD) sequence, the initiation codon, the distance between the Shine-Dalgarno sequence and the initiation codon, and the stability of binding between mRNA and 16S rRNA (Mori *et al.*, 2007 ). Ribosomal protein S1, which is involved in the ribosome binding site, has a positive effect on the affinity of 30S subunits and mRNA (De Smit and Van Duin, 1990). In contrast, codons with high G+C content directly after the start codon have a negative impact on the level of expression (Pedersen-Lane *et al.*, 1997). If our target proteins contribute to ribosome-mRNA binding, the activity of the S1 ribosomal protein, or the overall mechanism of translation then we may expect to see changes in the rate of proteins synthesis.

The rate of protein synthesis was measured by the incorporation of radioactively labeled methionine. To our surprise, deletion of *ygdP* was just only deletion strain that resulted

in an increase in the rate of protein synthesis. Other deletion strains (*yfgB, ybcJ*, and *yciL)* did not change the rate of protein synthesis. One way to interpret these results is deletion of *ygdP* might remove a blockage in the synthesis of new polypeptide. This blockage might be explained by the process of translational proofreading. If proofreading is eliminated then one may expect that the rate of protein synthesis will be increase along with a corresponding increase in translation error. In agreement with this, deletion of *ygdP* resulted in an increased rate of error in translation (see above).

Next we investigated if the absence of *yfgB* altered the sensitivity of the mutant strains to different translational block drugs. We observed that deletion of *yfgB* drastically increased cell sensitivity to erythromycin. Erythromycin is an effective inhibitor of translation (Chittum and Champney, 1995; 1994). The x-ray crystallography has shown that erythromycin binds to a site in the proximal part of polypeptide exit tunnel on the 50S and inhibits protein synthesis by blocking the passage of polypeptide down the exit tunnel. It is also thought to cause a reduction in the rate of 50S subunit formation (Steitz *et al.*, 2003; Zaman *et al.*, 2007). This observation is in line with the involvement of *yfgB* in 50S biogenesis.

Deletion of *yfgB* also resulted in a decrease in cell sensitivity to streptomycin and an increase in kanamycin sensitivity. Streptomycin disrupts translational accuracy by increasing the affinity for noncognate tRNA (Karimi and Ehrenberg, 1996; 1994). It causes extensive misincorpoation of incorrect amino acid by interfering in the recognition of the codon-anticodon interaction (Carter *et al.*, 2000; Springer *et al.*, 2001). It also has been shown that single mutation in different translation genes confer resistance to streptomycin (Bohman *et al.*,

1984 ; Frattali *et al.*, 1990 ; Montandon *et al.*,1986). Similarly, Recht and Puglisi (2001) have shown that kanamycin interferes with translational accuracy by increasing the affinity for noncognate tRNA selection. They have also shown that resistance in *E.coli* results from decreased binding affinity of kanamycin to helix44 upon mutation of site U1406 A or A 1408G in 16S rRNA. These changes in drug sensitivity for *yfgB*-D are in agreement with the observation that deletion of *yfgB* increased translation error (see above).

We also observed that the deletion of *yfgB* increased cell sensitivity to tetracycline. Tetracycline is one of the primary antibiotics that have been used against a wide range of gram-negative and gram-positive bacteria since 1940 (Chopra *et al.*, 1992). Tetracycline binds primarily to the 30S ribosomal subunit and inhibit protein synthesis by blocking the binding of aminoacylated tRNA to the A site (Ross *et al.*, 1998). Tetracycline also prevents binding of both release factors RF1 and 2 during termination (Brown *et al.*, 1993). The fact that deletion of *yfgB* increased cell sensitivity to tetracycline is in agreement with a function for *yfgB* in translation.

YbcJ was found to interact most likely with those proteins (RpsB, RpsC, RplA, RplL, and RplC) which play essential roles in 30S and 50 S ribosomal subunits assembly (Yamazaki *et al*,. 2008;http://www. shigen. nig. ac, jp/ecoli/pec/). In agreement with these observations, it has been previously shown that *ybcJ* has distant sequence similarity with the larger family of ribosomal protein S4 (Aravind and Koonin, 1999). Our PPIS analysis also shows that YbcJ protein interacted with DnaJ which is a heat shock protein with a chaperone activity. DeaD and SrmB were also found to co-purify with tagged YbcJ. These two proteins belong to a large family of putative RNA helicase which play important roles in RNA processing associated with translation

(Mohr *et al.*, 2002). Structural studies have reported the presence of αL motif in positive charged residue on *ybcJ* structure which may function to bind –vely charge molecules such as rRNA (Volpon *et al.*, 2003). In a recent study, Jiang et al (2007) also revealed a weak association for *ybcJ* with the 50S particle. Taken together, our PPI results confirm the involvement of *ybcJ* in processes of protein synthesis.

We observed that deletion of *ybcJ* gene lead to decreased cell sensitivity to streptomycin and increased cell sensitivity to tetracycline. Streptomycin has an inhibitory activity against the function of the 30S subunit of the ribosome and disrupts translational accuracy by increasing the affinity for noncognate tRNA (Karimi and Ehrenberg, 1996; Hansen *et al.*, 2002). Interestingly, our translational fidelity investigation show that deletion of the *ybcJ* resulted in an increase in translational errors. This is in agreement with the role of *ybcJ* in maintaining of the translational accuracy. The effect of deletion of *ybcJ* on tetracycline is another evidence for the involvement of *ybcJ* in protein synthesis. All together, our findings support a role of *ybcJ* in protein synthesis.

Our PPIS investigation shows that YgdP interacted with several essential ribosomal proteins (RplD, RplE, RplF, RpsA, RpsB, RpsD, and RpsE). Previous studies have reported that YgdP comigrate with a particle slightly bigger than the 30S particle. They also hypothesized that YgdP might be involved in hydrolyzing dianosine pentaphosphate, an alarmone signaling molecule related to the stress response (Bessman *et al.*, 2001). In our study, YgdP was found to interact with RpsD protein. Mutations of RpsD are known to compromise translational fidelity, a phenotype which we also observed for YgdP deletion mutant strain. In addition, SrmB , which is

involved in 50S biogenesis, is found to co-purify with tagged YgdP. Moreover, we found an interaction between YgdP and RplB, with a known function is 50S assembly along with other activities. Together these analyses indicate an involvement for YgdP in translation, with a possible role in ribosome assembly.

In addition, we also observed *ygdP* deletion strain is strongly resistance to erythromycin, kanamycin, and tetracycline. It is a possible that mutation of this gene may cause a conformation change which inhibits the binding of these drugs to the 50S subunit particles. It has been shown that many cases of antibiotic resistance in mutation strains can be related to alternation of a specific nucleotide of the 23S rRNA within the peptidyl transferase centre (Vester and Douthwaite, 2001). Further, we observed that deletion of *ygdP* resulted in a decreased translational fidelity which is in accordance with a role for *ygdP* in protein synthesis.

Our PPIS investigation show that the YciL protein interacted with several 50S ribosomal subunit proteins. RplB is one of those interactors which have an important role in 50S activity. These interactions might be in agreement with Del Campo et al (2001) who suggested a Pseudouridine 2605 synthase for *yciL in vivo*. Pseudouridine 2605 are found at a functionally site of 23S ribosomal subunits, and are known to have a crucial role in protein synthesis (Raychaudhuri *et al.*, 1999). We also observed an interaction for YciL protein with RpsD protein. As before RpsD has a role in translational fidelity which may explain a reduction in translational fidelity that we observed in the absence of *yciL*. We also found YciL protein interacted with SrmB protein which is known to be involved in ribosome biogenesis. In addition, deletion of *yciL* gene increased the level of sensitivity to streptomycin, tetracycline and kanamycin. Since these

antibiotics directly affect the fidelity of translation, our findings are in direct agreement with the observation that *yciL* affects translational fidelity.

Translation fidelity is generally associated with the 30S subunits, however, our PPIS analysis hints to a role for *yciL* which might be more associated with 50S subunit. These seemingly contradictory observations are not common. For example mutations of the 50S ribosomal subunit protein L7 and L12 , and certain mutations in 23S rRNA are shown to affect translation fidelity in a similar fashion to the mutation in 30S ribosomal protein S4 and S5 or 16S rRNA (Goringer *et al.*, 1991; Chaudhuri *et al.*, 2008). Such observations further interconnect the classical activities of 30S and 50S subunits.

We observed that deletion of *yciL, ygdP,* and *ybcJ* do not seem to have an effect on ribosome profile under our experimental conditions. This however does not exclude the possibility that these genes may, in fact, play a role in ribosome biogenesis under different physiological conditions. Charollais et al (2004) showed that the deletion of DeaD and SrmB genes have no effects on the ribosomal profile when cells were grown at 37˚C. However, when the growth temperature was reduced to 20°C, DeaD and SrmB proteins exerted a direct role in the assembly of ribosomes, suggesting a role for these proteins in ribosome biogenesis under cold shock. Interestingly, we observed that YbcJ, Ygdp, and YciL proteins interact with both SmrB and DeaD proteins. This may suggest a role for these proteins in ribosome assembly at low temperatures. This possibility however was not investigated in our analysis.

Similarly, our analysis cannot rule out a role for *yciL, ygdP, yfgB*, and *ybcJ* in ribosomal subunit formation/ function and/or stability. Of 55 ribosomal proteins, only eight ribosomal

proteins (S4, S5, S12, L4, L13, L20, L22, and L24) are needed for initiation of ribosome assembly process (Nishi and Schnier, 1986; Nierhaus, 1991). Other ribosomal proteins play a role in the late assembly process. Mutation in the late assembly proteins have no effect on ribosomal subunit formation, but change the assembly rate, accelerating the formation of ribosomal particle *in vivo* (Francesch and Neihaus, 1990). It is very much possible that *yciL, ydgP, yfgB,* and *ybcJ* may in fact affect ribosome biogenesis during in the late assembly steps.

We have also observed that YciL, YfgB, YbcJ, and YgdP proteins have interactions with a number of proteins that have no known function in bacterial translation. These interactions may fall into three general categories. Some of them might be false positives. Co-purification of contaminants during TAP affinity purification is well-established (Puig *et al.*, 2001). Alternatively, some interactors may represent cross-talk between translation and other cellular processes. Another possibility is that some proteins may have multiple cellular functions which are involved in two (or more) different cellular processes. In TAP analysis, these interactors cannot be separated from one another.

## Future work:

For future study, since many translation genes act in a temperature dependent manner, the effect of the above gene deletions on the growth rate in different temperatures can be monitored to study the involvement of the target proteins as cold/heat shock elements. The spot test asses drug sensitivity and ribosome profile experiment can also be conducted at different temperature. *In vivo* experiments can be design and used to verify the functions of the target genes. The effect of over-expression of the target genes can be analyzed to further study their molecular activities. High throughput genetic interaction analysis can also be applied to better understand the activity of the target genes.

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