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The Growth And Metabolic Effects Of Pseudouridine Synthase Gene Deletion On Escherichia Coli Strain Bl21

Lisa Marie Burnette

THE GROWTH AND METABOLIC EFFECTS OF PSEUDOURIDINE SYNTHASE GENE DELETION ON *ESCHERICHIA COLI* STRAIN BL21

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Lisa Marie Burnette
Bachelor of Science, University of North Dakota, 2007

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

(Chairperson)

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

Wayne Swisher
Dean of the School of Graduate Studies

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Lisa M. Burnette

December 17th, 2015

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You knew I'd be here long before I did.

ABSTRACT

During the process of ribosome assembly and maturation, the rRNA is heavily modified. The process of modification is ubiquitous throughout all kingdoms of life and includes cleavage by RNases, methylation and pseudouridylation of RNA species. Interestingly, while rRNA modification is ubiquitous and modification locations are frequently conserved, in many species the modification enzymes are dispensable for normal growth. This non-essentiality has made understanding the functions of RNA modification difficult.

Pseudouridines are one of the most common rRNA modifications and, in *Escherichia coli*, comprise nearly one third of the modifications found within the ribosome. Pseudouridylation is the post-transcriptional base isomerization of uridine to pseudouridine and is completed by a group of enzymes called pseudouridine synthases. In *E. coli*, the 6 synthases responsible for modifying the 23S rRNA are RluA, RluB, RluC, RluD, RluE and RluF, and the single synthase responsible for modifying the 16S rRNA is RsuA. Currently the few hypotheses as to the function of these modifications involve the effects of the unique chemical properties of a pseudouridine base on the RNA structure, but few studies have shown any effect on ribosome profiles *in vivo*.

Through construction of single pseudouridine synthase deletion strains we were able to identify several new phenotypes associated with deletion of either *rluC* or *rluE*. All the identified phenotypes were associated with the ability of the bacteria to tolerate low-oxygen conditions including altered glucose fermentation products, increased ability to utilize nitrate

in oxidative metabolism, and increased growth in low oxygen environments. Additionally, a series of multiple deletion mutants were constructed in order to investigate cumulative effects that might occur from loss of multiple pseudouridines from a single ribosomal RNA. Interestingly, no new growth alterations were noted, even in strains lacking all 7 of the rRNA pseudouridine synthase genes, indicating that under the tested conditions, there are not likely to be ribosomal assembly or function defects. While we were not able to determine a specific function for pseudouridines from this work, the additional phenotypes and the construction of a strain lacking all known synthase genes will provide new tools for furthering our understanding.

CHAPTER I

INTRODUCTION

Protein synthesis utilizes a significant portion of cellular energy: one study found that as much as 90% of bacterial energy is committed to the process (1). Production of the protein-manufacturing apparatus, ribosomes, accounts for a significant portion of this energy expenditure (2); in rapidly growing bacterial cultures, ribosomes can make up as much as 40% of the dry mass of each cell (3). The production of these ribonucleoprotein complexes is tightly regulated and can respond to changes in demand through a variety of cellular signals (4–7). Once assembled, ribosomes are also capable of affecting cellular processes or through the effects of transcription-translation coupling and the sensing of nutrient availability (8–10). This role as both a production factory and a regulatory factor explains why ribosomal structures are generally conserved throughout the kingdoms of life (11).

Ribosomal Structure and Assembly

The ribosome complex consists of two subunits, each constructed from ribosomal RNA (rRNA) and many ribosomal proteins (r-proteins). In *Escherichia coli*, the large (50S) subunit consists of two rRNAs, the 23S and 5S, along with 34 different r-proteins, while the small (30S) subunit contains only one rRNA species, the 16S, and 21 r-proteins (6). The two subunits associate during the process of translation initiation to form a functional 70S ribosome. *E. coli* has seven rRNA operons that each contain a single copy of the three rRNAs (6). Each operon has two promoters that are both considered very efficient (6), as their transcripts are needed for the "housekeeping" purpose of protein synthesis, but are also

sensitive to a wide variety of transcriptional regulation and signaling (4, 5, 12, 13). The rRNA transcript contains the three rRNA species along with a combination of several different tRNA species, all of which must be separated via RNase cleavage.

The rRNA processing begins while transcription is still underway. The 23S, 16S and 5S rRNAs are separated from each other and from the multiple tRNAs located within each of the rRNA operons by the endonuclease RNase III. The further modification of rRNA is completed as the ribosome assembles (14). While chaperones and helicases help guide the formation of rRNA secondary structure, the rRNA modifying enzymes use these topological additions to the rRNA to guide further modifications (11, 15–17).

The addition of ribosomal proteins to the growing ribosome complex occurs in several stages. As ribosomal modifications and rRNA secondary structure begin to form, the primary r-proteins for each of the two subunits are able to bind to the rRNA. With both subunits, the addition of the first proteins helps guide the binding of the secondary proteins and guide further modification (3, 11, 17). The tertiary proteins are added in a similar fashion. Compared to the 30S subunit, the 50S subunit is much larger and contains numerous additional proteins as well as a second rRNA species. Therefore, in the larger subunit this process is more complex, but both follow the same general steps in assembly, with the secondary and tertiary proteins being unable to bind until certain primary proteins are bound correctly. Identifying the specific steps at which rRNA modifications are introduced has proven more difficult. For example, RluD is known to modify a partially assembled ribosomal particle more efficiently than bare 23S rRNA, indicating that it is likely not involved in the very earliest steps of ribosome assembly (17). Also, as several of the prokaryotic pseudouridine synthases modify multiple sites that share limited sequence

similarity, it is believed that bound proteins and secondary structures help guide the enzymes to the correct sites (3, 18). However, as ribosomes can be assembled *in vitro* without any of the rRNA modifications, it is clear that none of them are essential for this process (19, 20).

Ribosome Assembly Factors

There are numerous ribosome assembly factors that, while not essential to the process, serve to reduce energy requirements, chaperone unfolded proteins and RNAs, or guide the folding of secondary structures (3). Because these proteins all facilitate the assembly of such an important cellular machine, deletion of the coding genes often results in a similar collection of associated phenotypes. For example, the DEAD box helicase family, members of which all recognize the common D-E-A-D amino acid motif, are associated with the ability to dissociate short RNA duplexes and several are involved in the assembly of the large ribosomal subunit (21). Deletion of genes from this family usually results in a cold-sensitive phenotype and, although these proteins are closely related, over-expression of one cannot complement for the cold sensitivity of another, indicating that they are all needed for correct ribosome assembly at cold temperatures (22). Ribosome assembly can be affected by other environmental stresses like osmotic pressure. This was made evident by studies which have shown that another ribosome assembly factor, RsgA, has been associated with resistance to high salt concentrations (23).

However, to confirm the role of a specific protein as a ribosome assembly factor there must be a ribosome assembly defect including an abnormal ribosome assembly profile and/or rRNA processing defects. The deletion of *deaD* or *bipA*, both ribosome-associated GTPases, results in a decrease in 50S ribosomal subunits at 20°C showing that they are both required for efficient ribosome assembly at cold temperatures (22, 24). Interestingly, the phenotypes

associated with deletion of *bipA*, including the cold sensitivity and ribosome assembly defects, can be alleviated by the deletion of the gene coding for RluC, one of the rRNA modifying enzymes (24, 25). This suggests that at least one of the known ribosome assembly factors interacts with rRNA modifications to facilitate ribosome assembly.

rRNA Modifications

Beyond the cleavages necessary to produce the three properly sized rRNA species, all organisms expend significant energy imparting a wide variety of post-transcriptional modifications to their rRNA (6). As much as 0.8% of the entire coding capacity of *E. coli* is devoted to the process of rRNA modification (26). Despite these significant genetic and metabolic expenditures, deletion of the rRNA-modifying enzymes from various laboratory organisms, including both prokaryotes and eukaryotes, rarely results in growth defects or other phenotypes and therefore most are considered non-essential. This lack of phenotypes has made understanding the role of rRNA modifications difficult, and to date, there are few hypotheses as to their role in ribosomal function.

Both the base and sugar moieties can be modified by several mechanisms. Modifications to the sugar backbone can include the addition of carbonyl, amino, or thio groups and bases have been shown to be modified by methylation to both purine and pyrimidine species as well as by the isomerization of uridine to pseudouridine (6). *E. coli* ribosomes have at least 36 rRNA base modifications, including 11 to the 16S rRNA species and 25 to the 23S rRNA species. The 16S rRNA contains 10 methylations and a single pseudouridine while the 23S rRNA contains at least 14 methylations, 9 pseudouridylations, one methylated pseudouridylation and one location containing a modification that has yet to be classified (6).

Complete ribosomes can be assembled *in vitro*; however, if the rRNA transcripts come from an *in vitro* rather than a "native" source, the catalytic activity of the assembled ribosomal particles is significantly impaired (20). Early studies linked this phenomenon to a lack of post-transcriptional modifications in an 80 nucleotide region spanning bases 2445-2523 of the 23S rRNA. This region contains 7 different modifications and the functional defect would suggest that one or more of these modifications may be necessary for proper ribosome assembly and function. Subsequent studies, however, have shown that several of the modifications, notably the pseudouridines at locations 2457 and 2504, are dispensable for growth *in vivo*, thus implicating one or more of the other modifications in the functional defects observed in the original study (18, 24, 25, 27). Interestingly, none of the 11 base modifications found on the 16S rRNA are necessary to form a fully functional 30S subunit in vitro (19). The lack of essentiality for modification in the ribosome assembly process has contributed significantly to the poor understanding of the spatial and temporal mechanisms behind rRNA modifications (14).

Pseudouridines

One of the most commonly found rRNA modifications is the isomerization of uridine to pseudouridine (28, 29). These modifications were one of the first identified ribonucleic acid modifications and were deemed "the fifth nucleoside," as they make up a majority of the RNA nitrogenous base modifications found, with as much as 4% of the cellular RNA being pseudouridines (28, 29). Sites of modification in the rRNA are typically found within conserved and/or catalytically important regions of the rRNA including the peptidyl-transferase center of the ribosome (29–34). While pseudouridylation in prokaryotes is believed to be limited to the stable RNAs, pseudouridylation of non-stable RNAs in

eukaryotes does occur and is thought to play a role in mRNA regulation and general RNA stability (35–41).

Pseudouridylation does not alter Watson-Crick base pairing, as pseudouridines present the same molecular face to the base-pairing face of the RNA molecule (Figure 1). However, the isomerization does change both the atoms exposed to the major groove and the glycosyl bond (Figure 1). The nitrogen atoms exposed to the major groove bring with them additional electrons that are not present with uridine bases and thus serve as extra electron donors. While the exact role or roles for these electrons have not been confirmed, it is thought that they may serve to facilitate interactions with ribosomal proteins through hydrogen bonding and/or strengthen rRNA structures during RNA folding (14, 16, 31, 32, 37, 42, 43). During isomerization from uridine to pseudouridine, the glycosyl bond is

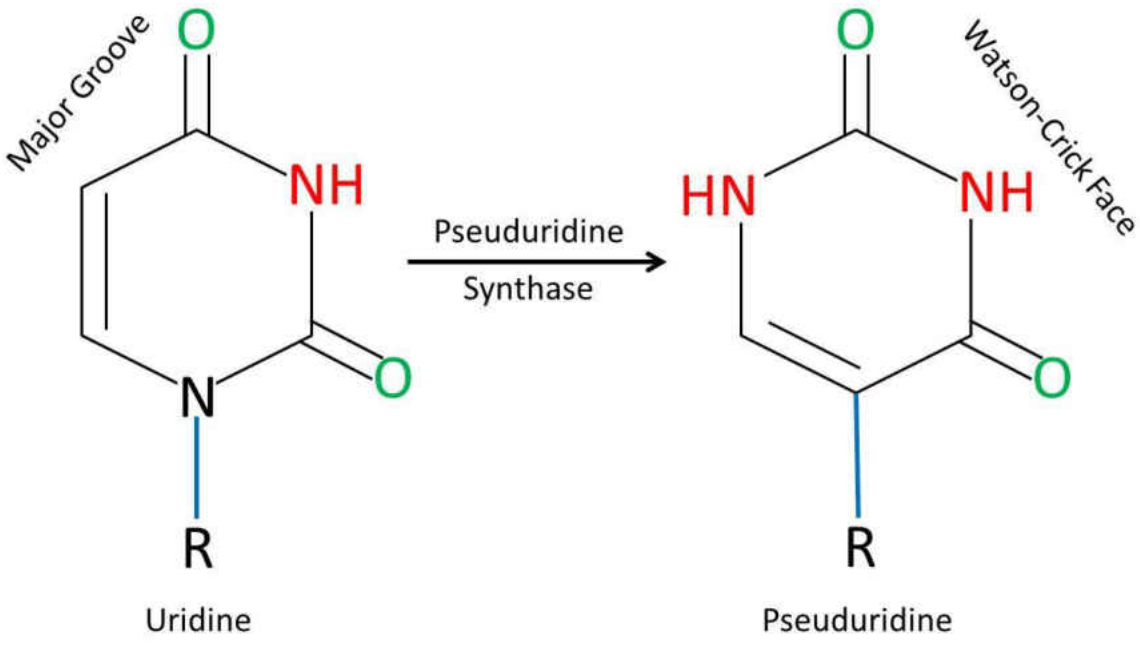

Figure 1: Comparison of uridine and pseudouridine bases: The pseudouridine synthase enzyme isomerizes uridine to pseudouridine and thus alters the chemical characteristics of the base. Blue represents the glycosyl bond. Red represents electron donors. Green represents electron acceptors. Also indicated: The Major groove face and Watson-Crick pairing face.

reformed as a C-C bond rather than the C-N bond found in all other nucleotides (42). This bond increases the potential conformational flexibility of the pseudouridine base and, when found in the *anti* configuration, has been shown to position the bases for successful alignment of water molecules with the 5' phosphates (16, 42). The stability of the RNA structures is optimized through these water molecule interactions and the increase in cooperative base stacking that the pseudouridine residue provides (16, 42). Although crystal structures have shown that RNA modifications do alter RNA structures, ribosomes can efficiently assemble when lacking specific RNA modifying enzymes and these mutant ribosomes do not necessarily show rRNA processing defects (24). Because ribosomes lacking pseudouridines can assemble and function without rRNA-processing defects, it implies that the evolutionary role for pseudouridines may be in fine-tuning ribosome assembly or function, rather in an essential assembly or functional role.

Pseudouridine Synthases

Chemical analysis of the isomerization process has revealed much about the function of the pseudouridine synthases. All identified synthases can be classified into five groups, named for five *E. coli* enzymes that represent these groups (42). In *E. coli*, the TruA, TruB, and TruD families all modify tRNAs exclusively (42). The RsuA family contains the RsuA, RluB, RluE and RluF proteins and this family is responsible for modifying both the 16S rRNA and three of the ten pseudouridine locations in the 23S rRNA. The final family is the RluA family and in *E. coli*, and consists of RluA, RluC, and RluD. This family is responsible for the final seven of the pseudouridines in the 23S rRNA (29, 42).

While there is little amino acid sequence similarity between members of the different families, all share a common central functional core fold and a conserved cleft region (29, 32,

42, 44). There is also a single conserved aspartate residue that is essential for catalytic activity in this region (42). This conserved central region has led some to hypothesize that these enzymes may be the result of gene duplications of an ancestral pseudouridine synthase gene (42). There is also very little sequence identity between the targeted modification sites, therefore, the remaining regions of the proteins have been implicated in target site sequence and structure recognition (29, 32, 42, 44).

While all organisms possess stand-alone synthases that have internal site recognition capabilities, some eukaryotic and archaeic pseudouridine synthases utilize an alternative mechanism that provides more sequence flexibility (29, 39, 41, 42). Because these organisms have a larger number of pseudouridine modifications, they utilize a synthase that can recognize and associate with multiple small nucleolar RNAs (snoRNAs) and utilize these to identify and modify target sequences (29, 39, 41, 42). This system allows for the modification of nearly 100 sites in yeast and over 200 in humans (41).

In *E. coli*, there are a total of 11 pseudouridine synthases. The TruA, TruB, TruC, and TruD proteins modify tRNAs exclusively (29, 37, 42). The remaining seven synthases, RluA, RluB, RluC, RluD, RluE, RluF and RsuA, are responsible for all of the modifications to the rRNAs (29, 31, 42). Although these are all considered stand-alone synthases, TruA, RluA, RluC and RluD can all modify multiple sites and the RluA modification targets are located on both the 23S rRNA and tRNA species (29). Despite this apparent flexibility in *E. coli* pseudouridine synthases, they are very specific in their modification sites. Deletion of a specific synthase results in the loss of modification at the associated site(s) and other synthases have never been shown to complement for one another (27, 29, 32, 42, 45, 46).

Pseudouridine Synthase Deletion Phenotypes

The deletion of any of the rRNA pseudouridine synthases is possible in *E. coli*, and rarely results in observable growth defects or other phenotypes. This is surprising given the ubiquitous and conserved nature of both the modifications and the synthase enzymes. The few phenotypes that have been identified in *E. coli* involve either a defect in competition against their parental strain, as was the case for deletion of *rluA* in MG1655 or BL21 (47) or were evident only in specific genetic backgrounds and were not visible in other contexts as was noted for *rluC* and *rluD* deletions (24, 25, 30, 45, 48, 49).

The deletion of *rluA* in either the MG1655 or BL21 background did not result in exponential phase growth defects when these strains were grown alone in culture (47). This was true in either rich or minimal media at a wide range of temperatures (47). However, when grown in competition with the parental strain, there was a clear disadvantage for the *ΔrluA* strain, not seen when either a *ΔrluC* or *ΔrsuA* strain was grown under similar competitive conditions (47). While interesting, this competitive disadvantage is ambiguous in nature, and has not led to a deeper understanding of the role for the *rluA* modifications.

The phenotypes for deletion of *rluD* have been noted in the MG1655 background (30, 33, 45, 48). These phenotypes include significant defects in ribosome assembly and function (30, 33, 45, 48), including defects in translation termination resulting in poor growth (33, 48). This poor growth was linked to significant read-through at all three stop codons, resulting in high levels of peptide turn-over (33, 45, 48). In particular, ribosomal protein S7 exhibited high levels or read-through that resulted in SsrA tagging which targets incomplete proteins for rapid degradation (48). The loss of the r-protein, S7, which is essential for the proper

function of the translation apparatus further hinders the ability of cells to produce new proteins, leading to a feed-back mechanism that causes significant growth inhibition.

Because of the growth impairment in these strains, suppressor mutations have been readily identified and frequently occur in the *prfB* gene (49). This gene codes for Release Factor 2 (RF2) in *E. coli*, one of the two class 1, codon specific, release factors in this organism (50). Interestingly, MG1655 possesses an unusual allele of *prfB* that is found rarely in other *E. coli* strains and the suppressor mutations identified in these studies resembled *prfB* genes from other *E. coli* strains, notably BL21 and other B strains (49). Replacement of the MG1655 *prfB* allele with the BL21 gene resulted in significantly increased growth in the MG1655*ΔrluD* strain (49). This implies that the three pseudouridines placed by RluD interact with, or facilitate RF2 interactions with the ribosome, at least in the context of the unusual *prfB* allele in MG1655. However, the role for the RluD-associated pseudouridines outside of the context of the MG1655 allele remains unclear.

The phenotypes associated with deletion of *rluD* are not the only strain-dependent phenotypes associated with the deletion of an *E. coli* pseudouridine synthase. Deletion of the *bipA* gene in MG1655 results in a collection of phenotypes including cold-sensitive growth, ribosomal assembly defects, and altered expression of stress response genes (24, 25, 51). However, deletion of *rluC* from an MG1655*ΔbipA* strain suppresses the *ΔbipA* defects in cold sensitivity, gene expression, rRNA processing, and ribosome assembly while not exhibiting any phenotypic alterations alone (24, 25). The phenotypes of a *bipA* deletion point towards a role for *bipA* in ribosome assembly, in particular at cold temperatures. Deletion of *rluC* in MG1655 results in ribosomes that are *bipA*-independent. The *rluC* deletion does not

display phenotypes without the *bipA* deletion in these strains, thus demonstrating another instance of strain-dependence for a pseudouridine synthase deletion.

Even fewer phenotypes are available in other organisms. The *Francisella tularensis* live vaccine strain (LVS), is attenuated *in vivo* and results in macrophage inflammasome activation (52). However, when *FTL-0699,* a gene which is annotated to code for an RluD-like pseudouridine synthase, is mutated in this strain it loses the *in vivo* attenuation and can inhibit activation of macrophage inflammasomes. This implicates pseudouridine synthases in *F. tularensis* infection, however, the mechanism of this process has not been studied further in this organism. Also, deletion of specific individual modification sites in yeast ribosomal rRNA results in minimal growth or assembly defects, but there appears to be a cumulative effect on both growth and ribosomal assembly when several sites of modification are lost in these cells (53).

Several studies have suggested a role for mRNA pseudouridylation in gene regulation. In *Saccharomyces cerevisiae* and other fungal species, several sites of conserved pseudouridine modifications have been identified, but there were no obvious alterations in mRNA stability or translation rate so their role in this context also remains unclear (35, 40). A similar connection has been made in human mRNAs with at least 89 human mRNAs modified to different degrees during different cellular growth states (40). Defects in one of the human pseudouridine synthases, dyskerin, have been implicated in the X-linked congenital disorder, dyskeratosis congenita (X-DC), and cancer because of its involvement in essential cellular processes like telomere stabilization (54). Ribosomes from cells depleted in dyskerin show a decrease in rRNA uridine modification levels and altered translation fidelity (54). The use of pseudouridylated mRNA as a therapeutic gene-replacement tool has been

investigated in human cell lines (55). Pseudouridines have been shown to reduce the immunogenicity of these transfected mRNAs by reducing the response of the RNA-sensing Toll-like receptors, TLR3, TLR7, and TLR8 (55, 56). These connections between pseudouridine modifications and their potential roles in human disease and disease therapies makes understanding their general functions even more imperative (54, 57, 58).

Strain Differences Between MG1655 and BL21(λDE3)

As noted above, several of the phenotypes associated with pseudouridine synthase deletions in *E. coli* are strain-dependent. The *prfB* allele in MG1655 is different than the allele found in almost all other *E. coli* strains and it was this difference that made the *rluD* deletion phenotype apparent (49). While MG1655 and similar K-12 strain derivatives have been a common choice for genetic and cell biology work for many decades (59, 60), BL21 and other B strains have been the more popular choice for work understanding phage genetics and more recently the BL21(λDE3) strain has become a common choice for laboratories doing protein expression work (59–61).

With the advent of whole genome sequencing, several studies have been performed analyzing the genetic differences that separate these two equally important, yet obviously distinct strains (60–63). Phage integration, UV mutagenesis treatments, transductions, and countless generations of segregated growth have produced significant genomic differences (60). One study in particular identified 426 single nucleotide polymorphisms (SNPs), 11 single base pair deletions, 25 multi-base pair insertion/deletion mutations as well as several insertion element differences and defective prophage (60). The λDE3 prophage integrated into the BL21 genome is one of the primary reasons this strain is favored for biotechnology and other protein production work, as it encodes the T7 RNA polymerase (60, 61). Other

notable differences between the strains include the loss of the *lon* gene, which codes for the Lon protease, in BL21 due to the incorporation of an insertion element and a SNP at codon 141 of *fnr*, which is altered in the BL21 strains to code for an amber stop codon, rather than one of several other functional amino acids (61, 64).

FNR and the Low Oxygen Response

There are two major regulatory networks that control growth under varying oxygenation conditions, the ArcAB two-component regulatory system and FNR. While both systems can serve a duel-regulatory role and their regulons do overlap, the ArcAB system is known to suppress the stress-response sigma factor, σ^s, and is not typically associated with regulation of genes under anaerobic conditions(65, 66).

However, FNR is a global transcription regulator that controls the transition to low oxygen and anaerobic growth environments (67–71). Because the ability to respond to decreases in oxygen availability are crucial to maintaining cellular ATP levels, transcription and translation rates of *fnr* are consistent, regardless of the environmental oxygenation conditions (68, 71). This consistent FNR availability allows cells to respond immediately to changing conditions without having to wait for FNR production in cells that may be lacking the energy necessary to complete transcription and translation. This indicates that FNR activity must be regulated through the sensing of oxygen, as the transcription of some FNR-regulated genes do not reach maximum levels unless under anaerobic conditions (68, 72).

When purified under anaerobic conditions, FNR has been shown to contain a complex iron-sulfur cluster that promotes dimerization of FNR molecules (71). It is in this dimeric conformation that FNR can bind DNA to regulate gene transcription (68, 71). The dimerization domain and DNA binding regions are in the C-terminal half of the FNR protein

with the oxygen-sensing region being at the N-terminal region (69, 71). However, when oxygen is present, this iron-sulfur cluster is disrupted and the homodimer falls apart, resulting in inactive FNR molecules (68, 71). The concentration of oxygen that has been found to be necessary to inactivate at least 50% of FNR molecules is 1μM, which is also the estimate for the cellular concentration of oxygen necessary to support cellular metabolism (71). Thus, as any system that plays a vital role in cellular metabolism should be, this system for gene regulation is finely tuned to cellular needs.

FNR is considered the primary regulator of the response to low oxygen conditions (68, 71). The FNR regulon is broad, with over 70 genes in 31 different operons being part of the traditional FNR regulatory network (68). This is not surprising, however, as making the conversion from an environment of high oxygen to low oxygen requires both the inhibition of genes requiring oxygen for their function and the activation of genes necessary for growth and metabolism under low oxygen conditions. This traditional network includes the gene networks one commonly associates with low oxygen tolerance like carbon metabolism, macromolecule synthesis, regulatory functions, and cell division (68, 72). However, FNR has been implicated in the regulation of many other processes like cell chemotaxis and drug sensitivity, as well as in the regulation of many genes of unknown function (68, 72).

Notably, FNR mutants have been shown to be defective in their ability to utilize nitrate or fumarate as a final electron receptor under anaerobic conditions (61, 71). Strains lacking FNR have also been shown to be defective in the production of hydrolases responsible for gas production during glucose fermentation (61). Regulation by FNR is pervasive and by also serving as a regulator of the transcription factors, the reach of FNR regulation is extended even further into the bacterial genome (72).

Summary and Hypotheses

Ribosomal RNA modification has been highly conserved throughout evolution yet a majority of rRNA modifications do not appear to be essential to ribosomal assembly or function. In particular, pseudouridines can alter the chemical capabilities of RNA without disrupting base pairing, however, there is no clear evidence to show what value pseudouridylation has *in vivo*. The few identified phenotypes are either associated with a vague, competitive disadvantage or are strain-specific and are not universal within even a single species, like *E. coli*. As deletion of pseudouridine synthase genes from model organisms like *E. coli* and *S. cerevisiae* has yielded few phenotypes from which to draw conclusions regarding potential functions, any new insights will be useful in the process of understanding these ubiquitous modifications.

The loss of a single pseudouridylation site rarely results in defective growth and in several instances, multiple sites have been lost with no negative effects. Deletion of up to three pseudouridine synthases, with an accumulated loss of up to five modification sites is possible, in *E. coli* strain MG1655, without obvious growth defects in laboratory conditions; however, in some model yeasts, the loss of several pseudouridine modification sites appears to result in an accumulated defect.

Since little information is known regarding the biological function of pseudouridines, we set out to construct a series of pseudouridine synthase deletions, all in a single background strain. Because of the *rluD* phenotypes seen in the MG1655 background, we chose the BL21(λDE3) strain background because we believed we would be able to delete all of the *E. coli* pseudouridine synthase genes without any growth defects or other aberrations. Surprisingly, we identified a growth-related phenotype for deletion of two of the

pseudouridine synthase genes in this background. We chose to investigate these phenotypes further in hopes of providing new information about the function of ribosomal pseudouridines.

We also investigated the ability of a non-pseudouridylated ribosome to support growth of *E. coli*. Several pseudouridine synthase deletions had been combined in a single *E. coli* strain without any obvious growth defects under laboratory conditions, and we hypothesized that this would be the case for a strain lacking the full complement of ribosomal pseudouridine synthase genes as well. However, the fact that yeast cells exhibited an additive effect that resulted in a noticeable defect from the loss of pseudouridylation at multiple sites indicated that there may be a limit to the number of pseudouridines. By constructing a series of single and multiple deletion strains we set out to determine the minimum number of pseudouridines necessary for *in vivo* ribosome function and, if any specific combinations of pseudouridine sites are necessary together. We would then undertake a comparison of these strains to strains lacking known ribosome assembly factors to determine if, as a whole, the modifications may be resulting in ribosome assembly defects.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and Media

All bacterial strains used are derivatives of either *Escherichia coli* (*E. coli*) K-12 or *E. coli* BL21 and are listed in the Appendix. The strains MG1655, a K-12 strain, and BL21(*λDE3*) were used as wild-type strains for most experiments and mutants that were generated were their isogenic counterparts. Strains were grown in Luria Bertani (LB) liquid media or on LB agar plates for most experiments (73). M63 minimal media was used for growth curves as indicated and to select against the donor strain, S-17 (*λpir*)/pJMSB8, after conjugation to remove the *res-npt-res* (kanamycin) cassette (74). When M63 media was used, carbon sources were added to a concentration of 0.4% for glucose or 0.8% all others. When necessary, antibiotics were used at the following concentrations: Ampicillin (100 mg/L), Kanamycin (50 mg/L), Tetracycline (20 mg/L).

Strain Construction Techniques

Transduction

Preparation of P1 phage lysate:

The donor strain was grown overnight in 5 ml LB broth at 37°C with continuous shaking. The following day, 50 µl of the overnight culture was added to 50 ml of LB containing 5mM $CaCl_2$ and 0.2% glucose. The culture was then incubated at 37°C with shaking until bacterial growth was barely visible, typically 30 min. At this point, 100 µl of $P1_{vir}$ phage lysate was added and growth at 37°C with shaking was resumed for another 2-3

hours until the culture cleared. 100 µl of chloroform was added and the culture was vortexed to lyse any remaining cells. The mixture was then centrifuged at 1200 x *g* for five minutes to pellet cellular debris. The supernatant was gently collected and transferred to sterile glass screw-capped tubes and an additional 100 µl of chloroform was added. Tubes were then vortexed to combine and stored at 4°C.

P1 transductions:

Recipient strains were grown overnight in LB broth at 37°C with continuous shaking. The following day, cultures were centrifuged at 1200 x *g* for five minutes to pellet cells and the supernatant was discarded. The pelleted cells were resuspended in 2.5 ml 10 mM $MgSO_4$, 5 mM $CaCl_2$. 100 µl of the resuspended cells were combined with 100 µl of phage lysate and incubated at 30°C for 30 minutes without shaking. Additional samples with only lysate or only resuspended cells were also incubated to be used as controls. After incubation 1 ml of LB containing 10mM sodium citrate was added to each of the samples and incubate continued at 37°C for an additional 45-60 minutes. The cultures were centrifuged at 1200 x *g* for five minutes and the supernatant was discarded. 100 µl of 1 M sodium citrate was used to resuspend the pelleted samples in addition to gentle vortexing. The samples were plated at on LB agar media containing the appropriate antibiotics and allowed to grow overnight at 37°C. Transductants were restreaked for purification prior to sample preparation for PCR verification (discussed later).

res-npt-res Resolution

Resolution of the *res-npt-res* cassette to remove the kanamycin resistance from a deletion strain was completed via conjugation of recipient strains with the donor strain , S17-1 *λpir*/pJMSB8 (74). The recipient and donor strains were grown overnight in 5 ml of LB

broth or LB broth containing ampicillin (for plasmid maintenance) respectively. The following day these strains were diluted 1:100 in LB broth containing ampicillin and grown at 37°C with shaking to mid-log phase. 100 µl of the recipient was combined with 400 µl of donor in an Eppendorf tube and centrifuged at 5000 x *g* for 5 min. The supernatant was discarded and the pelleted cells were resuspended in 50 µl LB broth. The mixture was seeded in the center of an LB agar plate and allowed to grow for approximately 8 hours without inverting at 37°C. After incubation, 1 ml of LB broth was added to the plate and used to resuspend the cultures for harvesting via gentle scraping of the agar surface. Harvested cells were serially diluted to 10^{-7}, and 50 µl of each of the final 3 dilutions, 10^{-5}, 10^{-6}, and 10^{-7}, were plated on M63 minimal media with glucose to select against the donor strain. After overnight incubation, colonies were patched onto LB plates and LB plates containing ampicillin or kanamycin to screen for loss of the antibiotic resistance from the original *res-npt-res* cassette as well as loss of pJMSB8, which codes for ampicillin resistance. Strains exhibiting the correct phenotypes were then streaked for purification prior to sample preparation for PCR verification (discussed later).

frt-kan-frt Resolution

The removal of the kanamycin resistance gene from strains containing the *frt-kan-frt* cassette was performed following a protocol first described by Datsenko and Wanner (75). The pCP20 plasmid was transformed into the recipient strain following the protocol described in the following section. After transformation, strains were plated on LB agar containing ampicillin and allowed to grow overnight at 30°C. The following day, several colonies from the agar plate were selected and combined into a test tube containing 5 ml of LB broth without antibiotics. This culture was allowed to grow at 37°C with shaking for 2-3

hours and then subcultured to 1:100. The growth and subculture procedure was repeated 2-3 times. This process allows for the loss of the temperature-sensitive pCP20 plasmid; while expression of the FLP recombinase facilitates the removal of the kanamycin resistance gene. After the final subculture period, the samples were diluted a final time at 1:100 and 50 µl were plated on LB agar plates without selection. After growing overnight at 37°C, individual colonies were patched onto LB agar plates without selection and LB agar plates containing either kanamycin or ampicillin to screen for both the loss of the pCP20 plasmid and the kanamycin resistance gene. The loss of the kanamycin gene was then confirmed using PCR of the relevant chromosomal region.

General Transformation

Preparation of competent cells:

Overnight cultures of recipient strains were grown in LB broth with shaking at 37°C. The following day, the overnight cultures were subcultured at a ratio of 1:100 into fresh LB media and grown to mid-log phase. Cultures were centrifuged at 3000 x *g* for 10 minutes to pellet cells. The pellet was then resuspended in a volume equal to the starting volume of sterile, pre-chilled 10 mM $CaCl_2$ and allowed to sit on ice for 20 min. The cells were then re-pelleted following the same procedure as above and resuspened in ½ volume of the calcium $CaCl_2$ and allowed to rest again for 20 minutes on ice. After this rest period, the cells were again pelleted and resuspended in 1/20 volume of 10mM $CaCl_2$. After an additional 20 minutes on ice the cells were divided into 50 µl aliquots. Aliquots were either frozen at -80°C until needed or preferably, used immediately.

Plasmid isolation:

Plasmid DNA was collected from 5 ml overnight bacterial cultures utilizing the Qiagen Miniprep plasmid isolation kit (Qiagen) following the manufacturer's instructions. The overnight cultures were pelleted by centrifugation at 18,000 x *g* for 5 minutes and resuspended in 250 µl of the supplied P1 buffer. After transfer to an Eppendorf tube, 250 µl of the provided buffer P2 was added and the samples were mixed by inverting the tube several times. Subsequently, 350 µl of the supplied N3 buffer were added and the samples were again mixed by inversion several times. Samples were then centrifuged at 18,000 x *g* for 10 minutes. After centrifugation, the supernatant was applied to the provided QIAprep column and centrifuged for 30-60 seconds at 18,000 x *g*. After discarding the flow-through, the column was washed using 750 µl of the supplied PE buffer and further centrifugation for an additional 30-60 seconds. The flow through was again discarded and the column was once more centrifuged for 60 seconds to remove any traces of ethanol from the wash buffer. The column was then transferred to an Eppendorf tube to collect the eluted DNA. Elution was completed using 50 µl of sterile, nuclease-free water and centrifuging the column for 60 seconds. Eluted DNA was stored at -20°C for future use.

Calcium chloride transformation:

A 50 µl aliquot of the prepared competent cells was combined with 1-5 µl of isolated plasmid DNA in an Eppendorf tube and incubated on ice for 20 min. After incubation, the samples were heat-shocked for 45 seconds in a 42°C heat block. Immediately following heat shock, 1 ml of LB broth was added to the samples and they were placed at 37°C for 60 minutes. After incubation, 50 µl of the culture was removed and plated on an LB agar plate containing appropriate antibiotics. The remaining culture was then centrifuge at 3000 x g to

pellet the cells. After discarding the supernatant, the pellet was resuspended in 50 µl of LB broth and transferred to another LB agar plate containing appropriate antibiotics. After incubation overnight, isolated colonies were tested for the presence of the plasmid by re-isolating plasmid as described above using the QIAprep mini kit and visualization of plasmid DNA on an agarose gel.

Polymerase Chain Reaction

DNA extraction for PCR:

200 µl of overnight culture of the bacterial strain of interest were combined with 800 µl of sterile, nuclease-free water in an Eppendorf tube. The mixture was boiled for 10 minutes and centrifuged at 18,000 x *g* for 5 minutes. The supernatant was collected in a separate Eppendorf tube and stored at -20°C for future use.

Polymerase chain reaction:

PCR reactions were performed using a 2400 Gene Amp Thermo Cycler (Perkin Elmer). Reactions to amplify chromosomal DNA containing suspected gene deletions were completed using Epicenter's Failsafe PCR kit. The contents of the kit included several premixes, each of which contained buffered salt solutions with dNTP's, various magnesium ion concentrations and a proprietary PCR enhancer. The kit also contained an enzyme mix with multiple DNA polymerases capable of amplifying fragments up to 20 kb in size. The premix that was determined empirically to provide the best results for a particular primer pair/template combination was used for further reactions. Reaction annealing temperatures were calculated based on primer melting temperatures ($T_{m)}$ that were calculated based on primer sequence. The PCR reactions were carried out for a minimum of 25 cycles.

Following PCR amplification, samples were electrophoresed on a 1% agarose gel and stained via ethidium bromide for visualization under UV light.

Bioscreen C Growth Curve Analyses

Growth curve analysis of various strains was completed using a Bioscreen C growth curve analyzer manufactured by Labsystems (Helsinki, Finland). Overnight cultures of strains were grown at 37°C with shaking. LB broth was utilized for any cultures that would be grown in an LB-based media and M63 minimal media with 0.4% glucose was used for strains to be grown in any of the minimal media studies. When appropriate, antibiotics were used for plasmid maintenance. Overnight cultures were subcultured into the desired media at a final OD_{600} of 0.01. Honeycomb plate wells were filled, in triplicate, with 300 µl of each subculture. All analyses were completed at 37°C with continuous shaking and measurements were taken at 15 minute intervals. Growth curves were generated using GraphPad Prism to plot OD_{600} vs. time. For clarity, some time points were omitted during graphing. OD_{600} values obtained from the Bioscreen C are not directly comparable to those obtained from a standard spectrophotometer due to the shorter path length of the wells in the honeycomb plates (25).

Amino Acid Growth Studies

Growth curves to determine the effects of growth in minimal media supplemented with amino acids were performed in the Bioscreen C. M63 minimal media with 0.8% glucose and 0.4% casamino acids was used for overnight growth of strains and amino acid groups were supplemented into M63 plus 0.8% glucose at a final concentration of 0.005% per amino acid with each media lacking a specific amino acid group. The groups were organized based on the biosynthesis pathways used to produce each amino acid in order to

identify any potential defects in amino acid synthesis and were as follows: Group1-tyrosine, tryptophan and phenylalanine. Group2-alanine, valine, leucine, and isoleucine. Group3-glutamate, glutamine, arginine and proline. Group4-aspartate, threonine, methionine, asparagine, and lysine. Group5-serine, glycine, cysteine, and histidine. As with the previous Bioscreen C growth curves, overnight cultures were subcultured to an OD_{600} of 0.01 and grown with the same settings. GraphPad Prism was utilized for plotting. For graphing clarity, certain data points were omitted as indicated in the figure legends.

Cold Sensitivity Assays

Growth Curves

Cold sensitivity assays were performed by manual growth curve analysis. Overnight cultures were grown in LB broth at 37°C with shaking. The following day, strains were subcultured into 125 ml baffled flasks to an OD_{600} of 0.01 into 25 ml of LB broth. Cultures were grown in a 20°C shaking water bath and growth was monitored by measuring the OD_{600} and the indicated time points. Growth curves were generated using GraphPad Prism to plot OD_{600} vs. time.

Colony-forming Ability

Colony-forming ability of mutant strains was assessed by observation of growth at 20°C. Both mutant and control strains were streaked for isolation on LB agar. Plates were placed inverted in a 20°C incubator for 72 hours. The ability of each strain to form isolated colonies was noted and plates were photographed. A minimum of 3 biological replicates were obtained.

Colony Forming Unit (CFU) assay

To confirm the ratio of OD_{600} to live cells for any given strain, a CFU assay was performed. Cultures were grown to stationary phase and OD_{600} values were measured. The samples were then serially diluted to 10^{-5} and 100 µl of each of the last 3 dilutions, 10^{-3}, 10^{-4}, 10^{-5}, were plated on LB media. After approximately 8 hours of growth, colonies were counted and an average CFU/ml calculated for each strain. These values were then divided by the original OD_{600} value of the strain to determine the CFU/OD_{600}. This experiment was completed with three biological replicates and the average CFU/OD_{600} was graphed using GraphPad Prism. P-values were calculated via a one-way ANOVA comparing each test strain to the parental BL21.

Metabolic Assays

Glucose Fermentation Assay

Carbohydrate fermentation assays were performed utilizing the method described in Microbiology: A Laboratory Manual (76). 5 ml of Difco Purple Broth Base (Becton, Dickinson and Company (BD), Franklin Lakes, New Jersey) with 0.5% glucose was added to 13 mm test tubes containing an inverted 6 x 50 Durham tube. A short (15 minute) autoclave session was used to sterilize the media and drive media into the inverted Durham tube. If appropriate, antibiotics were added for the purpose of plasmid maintenance. Media was stored at 4°C for future use. Before the media was inoculated, it was allowed to reach room temperature. 10 µl of overnight cultures grown in LB media were inoculated into the fermentation tubes and samples were allowed to incubate at 37°C without shaking for 48 hours. Samples were visually inspected for color change from purple to yellow, indicating the production of acidic products during fermentation. Qualitative inspection of the inverted

Durham tube allowed for determination of the production of gaseous products of fermentation through the visualization of a gas bubble trapped by the inverted tube.

Nitrate Reduction Assay

Following the methods described in the text, Microbiology: A Laboratory Manual, strains were tested for their ability to reduce nitrates to nitrites (76). 5 ml of Difco Nitrate Broth (BD) was added to a 13 mm test tube and then sterilized by autoclaving. When appropriate, antibiotics were added to the media. Unused media was stored at 4°C until needed. 10 µl of overnight cultures grown in LB were added to the nitrate broth and tubes were allowed to incubate for 48 hours. After incubation 50 µl of Reagent A (6 g/L of *N*,*N*-Dimethyl-1-napthylamine in 5 M acetic acid) and 50 µl of Reagent B (8 g/L of sulfanilic acid in 5 M acetic acid) were added and cultures were observed for the rapid development of a red color. A strong reaction and red color development after 30 seconds was considered indicative of a positive response.

CHAPTER III

RESULTS

Growth and metabolic abberations associated with deletion of *rluC* or *rluE* in BL21(λDE3)

There are few documented phenotypes for strains lacking pseudouridine synthase genes. Of the few that are known, most have been shown to require specific genetic backgrounds to become apparent, and are thus strain-dependent (24, 25, 49). For example, deletion of the *rluD* gene in the MG1655 background results in significant defects in growth and translation termination (30, 33, 45). Suppressors of these defects have been found in the gene that codes for the protein, RF2, a translational release factor, of which MG1655 harbors an unusual allele (48, 49). Restoration of normal growth can be accomplished by substituting in an allele of the coding gene, *prfB*, from an alternative genetic background, specifically BL21 (49). A similar situation has been noted with deletion of *rluC*. Deletion of the gene *bipA* from MG1655 results in a series of defects in growth and ribosome assembly, particularly in cold temperatures (24, 51). Subsequent deletion of *rluC* from these strains results in restoration of these aberrant phenotypes, even though, in MG1655, *rluC* deletion alone results in no identified growth defects or other aberrant behaviors (24, 25). Although MG1655 and similar K-12 derivatives are commonly used background strains for research in this area, phenotypes from the deletion of the pseudouridine synthase genes have not been forthcoming in these strains. Therefore, we hypothesized that utilizing a different genetic

background for deletion of the pseudouridine synthase genes could yield new phenotypes and thus new information on the function of pseudouridines in the ribosome.

Deletion of rluC or rluE Affects Entry into Stationary Phase in BL21(λDE3)

A series of deletion strains were constructed in both MG1655 and BL21(λDE3), with each strain containing one *rlu* gene deletion. We chose not to include deletions of *rluD* in this study because previous research had already characterized the phenotypes resulting from *rluD* deletion in MG1655 and their relationship to the BL21 strain (49). Initial growth studies were performed in the Bioscreen C growth curve analyzer. All cultures were grown at 37°C with a setting of "continuous shaking" selected and cultures were grown until all strains reached stationary phase. Although all strains exhibited a similar growth rates we noted that MG1655 achieved a higher OD_{600} value, indicative of a higher cell density, before entry into stationary phase than BL21(Figure 2A). We also noted that, in agreement with previously published data (18, 27, 47), when grown at 37°C in LB broth, we did not see any growth abnormalities from the *rlu* deletions in MG1655, when compared to the parental strain (Figure 2B). Similarly, deletion of *rluA*, *rluB*, or *rluF* did not alter the growth of BL21 (Figure 2C). However, deletion of *rluC* or *rluE* in BL21 resulted in strains that attained significantly higher stationary phase OD_{600} value than their parent (Figure 2C). Interestingly, the growth of these two deletion strains was remarkably similar to the growth pattern of MG1655 (Figure 2A and Figure 2B).

OD_{600} values are only a proxy for actual bacterial numbers, as they are just a measure of the ability of the media and its contents to diffract light; therefore OD_{600} values increase as bacterial culture density increases. However, if cultures vary in cell size or morphology, the

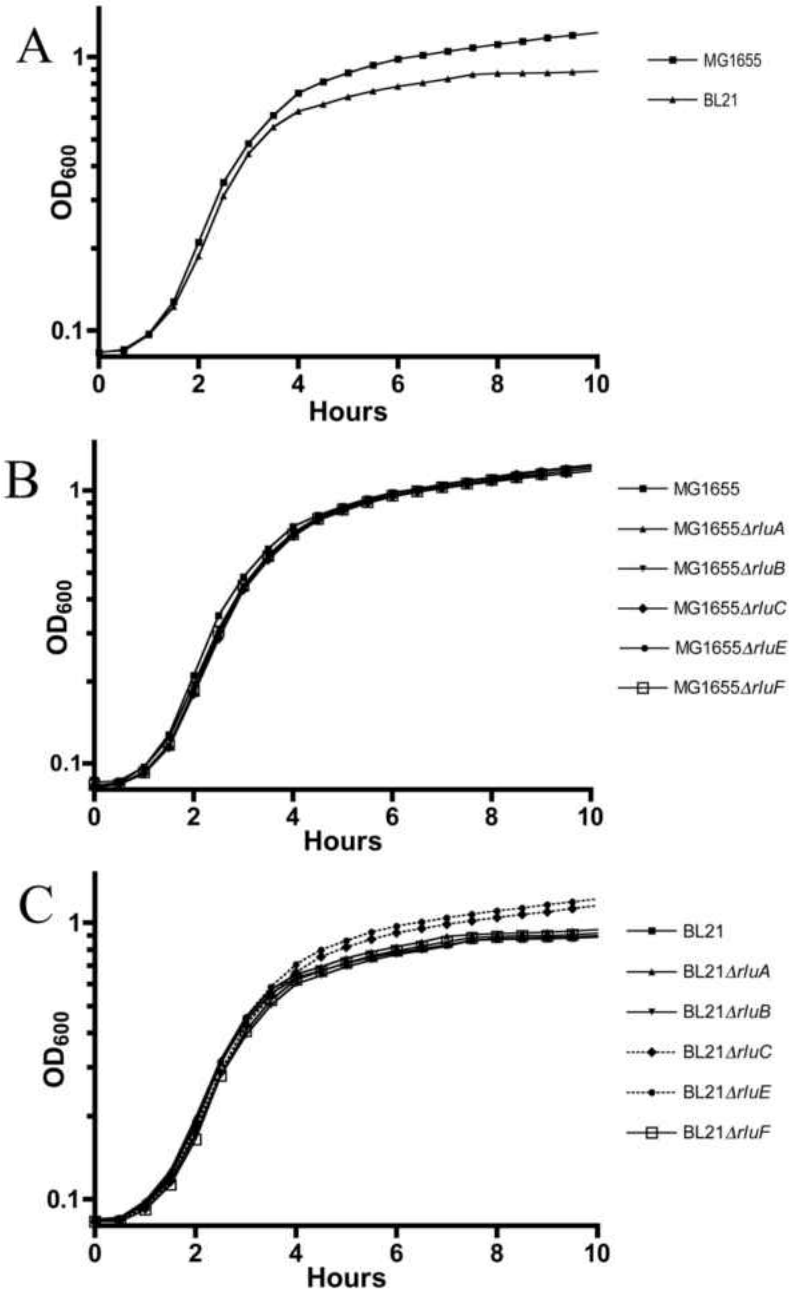

Figure 2: Deletion of *rluC* or *rluE* in BL21 results in increased stationary phase cell density: Strains were grown in a Bioscreen C growth curve analyzer in LB media as described in the materials and methods. Samples were run in triplicate and graphs are a representative of a minimum of three biological replicates. For graphing clarity only the 30 minute time points are shown. Error bars represent the standard deviation. (A) Comparison of the growth of the BL21 and MG1655 parental strains. (B) Comparison of the growth of the MG1655 and MG1655*Δrlu* strains. (C) Comparison of the growth of the BL21 and BL21*Δrlu* strains.

values may not be directly comparable. Therefore, to confirm that the increase in OD_{600} was due to increased cellular numbers and not to changes in other factors, colony-forming unit assays were performed. After the strains were allowed to grow to stationary phase, cultures were diluted and plated on LB agar plates. The following day, average colony counts from these plates were divided by the OD_{600} of the original stationary phase cultures to calculate the CFU/OD_{600}. No differences were seen in the CFU/OD_{600} for any of the strains, indicating that at a given OD_{600} all cultures would have a similar number of cells (Figure 3). This confirmed that the increased OD_{600} values observed were indeed from increased cell numbers and not likely from alterations in cellular morphology.

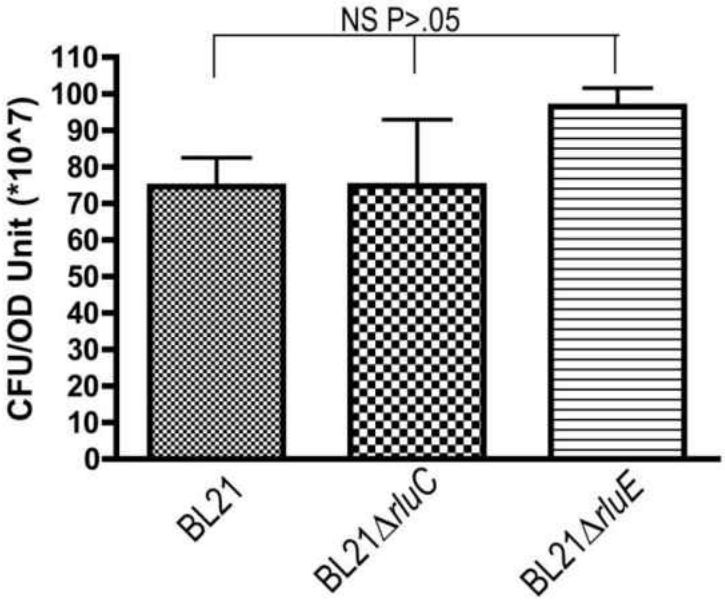

Figure 3: Colony-forming unit studies indicate that higher stationary phase OD_{600} values are indicative of an increase in viable cells: Cultures were grown to stationary phase and OD_{600} values measured. After sample dilution and plating as described in the materials and methods, colony counts were obtained and CFU values determined. Error bars represent standard deviation. No statistical differences were found at the 95% confidence interval.

Glucose as a Carbon Source Results in a Unique Growth Pattern

The increase in stationary phase cell density we described above may be a result of several factors including altered metabolism, sensitivity to metabolic waste products, or altered signaling for entry into stationary phase. We began further study into possible mechanisms by utilizing M63 minimal media containing a variety of carbon sources. This would allow us to look more closely at the effects of carbon metabolism on the previously observed phenotypes. Glucose, glycerol, acetate, and pyruvate were chosen because their diverse points of entry into the ATP synthesis pathways would provide insight into possible metabolic deficiencies. To increase the energy density of the media, when carbon sources other than glucose were used, the carbon source concentration of 0.8% was selected rather than the 0.4% used for glucose. This improved interpretation of the results by decreasing variability and increasing overall growth rates so that carbon sources could be compared to one another more easily.

For these experiments we utilized the Bioscreen C with identical settings to the previous experiments. While the overall growth rates were slower than in rich media, strains grown in media containing glycerol (Figure 4A) or pyruvate (Figure 4B) all exhibited a similar pattern to that seen with growth in LB broth (Figure 3C). We noted that with these carbon sources the BL21 parental strain reached a lower stationary phase OD_{600} than either the BL21*ΔrluC,* BL21*ΔrluE* strains or MG1655. As with rich media, the exponential growth rates were also similar between strains (Figure 4A and Figure 4B). When grown in acetate, even after 48 hours, the strains did not exhibit a leveling-off of their growth rate that would clearly indicate entry into stationary phase and so we were not able to draw clear conclusions about the stationary phase OD_{600} values (Figure 4C). However, it should be noted that in this

media, as with the glycerol and pyruvate media, all the strains had similar growth rates during exponential growth (Figure 4A-C).

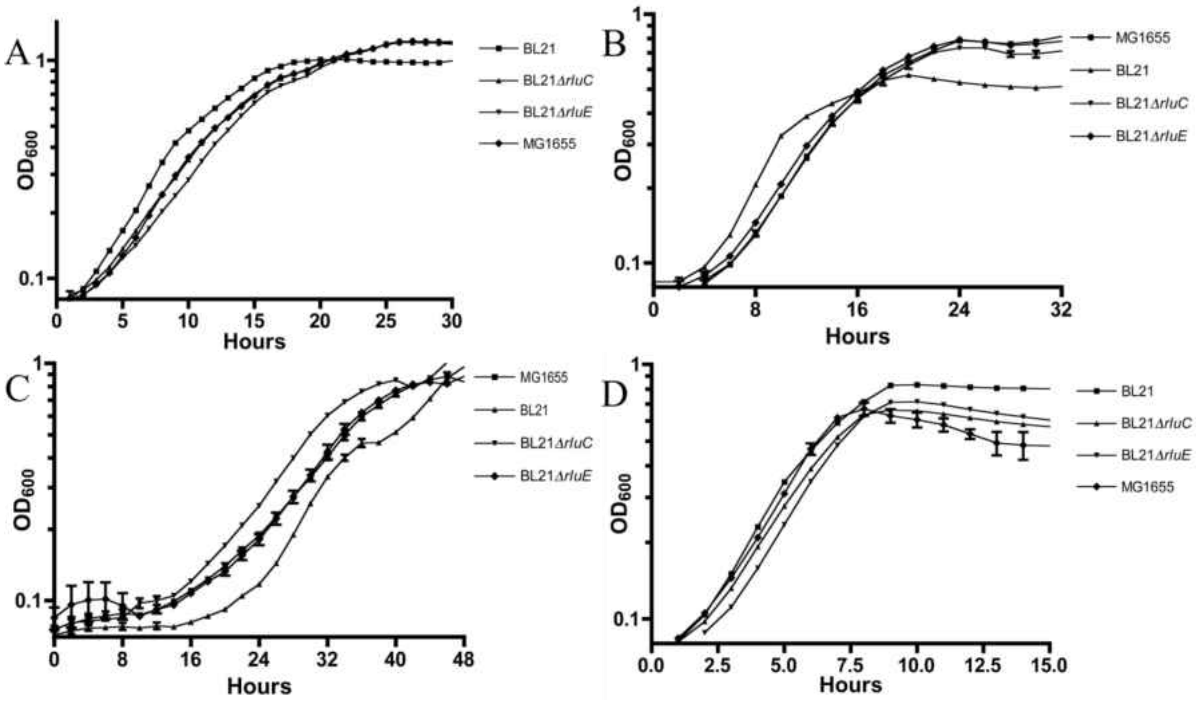

Figure 4: Glucose as a sole carbon source results in a unique growth phenotype for BL21*ΔrluC* and BL21*ΔrluE* strains: Strains were grown in a Bioscreen C in M63 media with various carbon sources as described in the materials and methods. Samples were run in triplicate and graphs are a representative of a minimum of three biological replicates. For graphing clarity only the 30 minute time points are shown. Error bars represent the standard deviation. (A) MG1655, BL21, and BL21*ΔrluC* (LMB274) or *ΔrluE* (LMB276) were grown in M63 media with glycerol as the sole carbon source. (B) The same strains as in "A" were grown in M63 media with pyruvate as the sole carbon source. (C) The same strains as in "A" were grown in M63 media with acetate as the sole carbon source. (D) The same strains as in "A" were grown in M63 media with glucose as the sole carbon source.

Interestingly, when glucose was the sole carbon source a very different result was seen (Figure 4D). In this case, BL21 exceeded the stationary phase OD_{600} values seen with MG1655 and the BL21*ΔrluC* and BL21*ΔrluE* strains (Figure 4D). As before, growth rates during exponential phase remained similar between strains. Published literature regarding

the growth of BL21 strains and K-12 strains like MG1655 has identified metabolic differences between these strains in the areas of glucose metabolism and oxygen sensitivity (61, 62, 77) and our data support this. This indicates that there may be some metabolic process or other growth related factor unique to glucose metabolism and/or low-oxygen tolerance that is altered in the *rluC* or *rluE* deletion strains.

Supplementation of Amino Acids to Glucose Media Does Not Correct to a Rich Media Phenotype

Altering the protein manufacturing machinery of the cell by removing rRNA modifications may leave cells more sensitive to decreases in amino acid concentrations or perturbations in amino acid synthesis. We chose to investigate any role this phenomenon may be playing in our system by performing growth analyses in our M63 minimal media supplemented with amino acids. The groupings chosen for the amino acid supplementation were based on the various biosynthetic pathways in order to provide insight into potential defects in amino acid biosynthesis. The aromatic family, dubbed group 1, contains tyrosine, tryptophan and phenylalanine. Group 2, also called the pyruvate family, contains alanine, valine, leusine and isoleucine. We called the glutamate family group 3. It contains glutamate, glutamine, arginine and proline. Group 4 contains asparagine, threonine, methionine, aspartate, lysine and isoleucine, however, we chose to include isoleucine in group 2 and so it was left out of this group. Group 5 is comprised of serine, glycine, and cysteine, all members of the serine family as well as histidine. In each media type, all but one of the groups were supplemented.

In media lacking any one of the groups, apart from group 4, the *rluC* and *rluE* deletion strains reached a lower stationary phase OD_{600} value than either the parental BL21 strain or the BL21*ΔrluA* strain (Figure 5A-C and E). This strain was included as a control

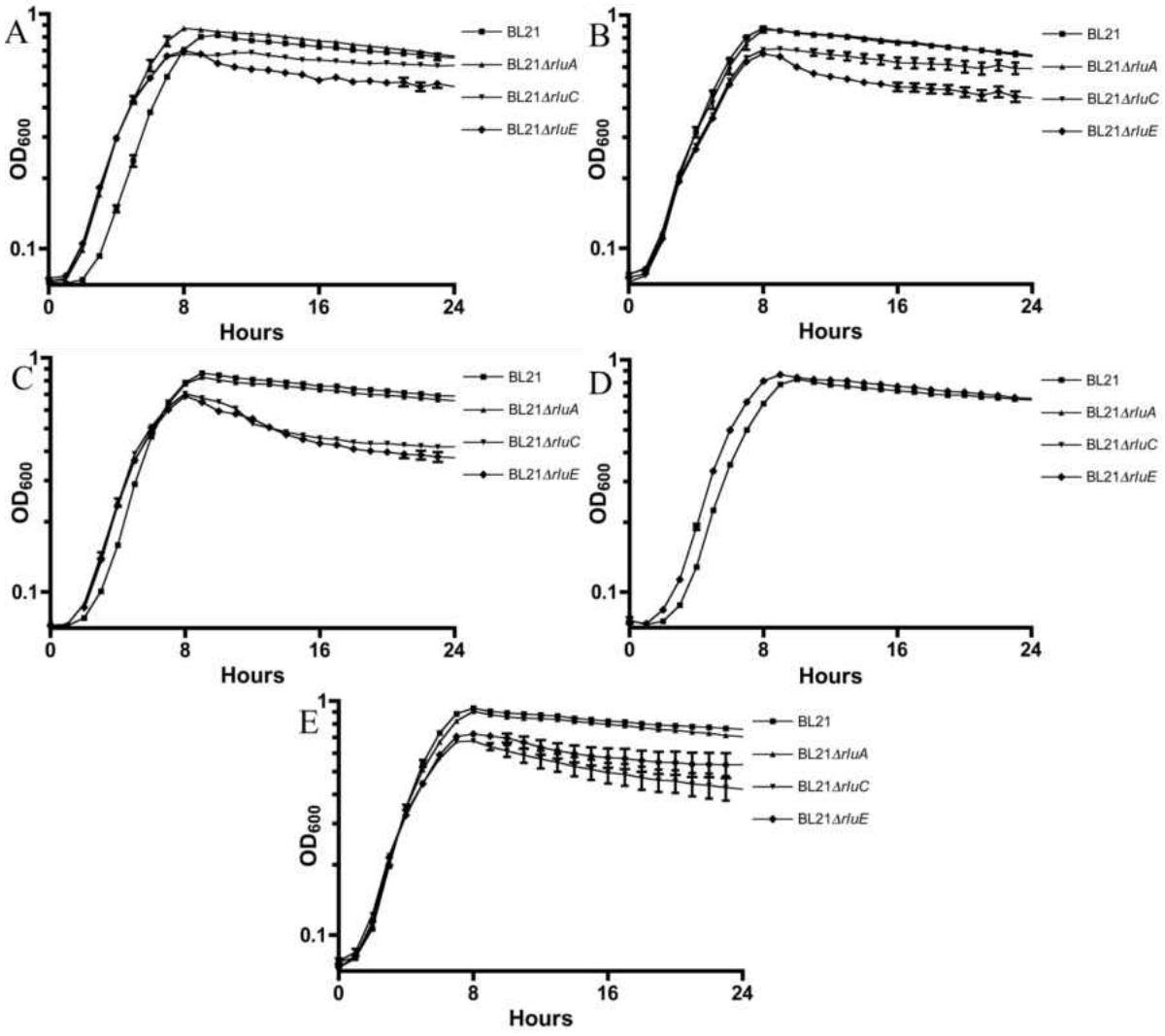

Figure 5: Growth alterations seen in the *rluC* and *rluE* deletion strains cannot be alleviated by amino acid supplementation: Strains were grown in a Bioscreen C in M63 media glucose and amino acids as described in the materials and methods. Samples were run in triplicate and graphs are a representative of a minimum of three biological replicates. For graphing clarity only the 30 minute time points are shown. Error bars represent the standard deviation. (A), BL21, and BL21*ΔrluC* (LMB274), *ΔrluE* (LMB276) or *ΔrluA* (LMB272) were grown in M63 media with glucose and all amino acid groups except group 1. (B) The same strains as in "A" were grown in M63 media with glucose and all amino acid groups except group 2. (C) The same strains as in "A" were grown in M63 media with glucose and all amino acid groups except group 3. (D) The same strains as in "A" were grown in M63 media with glucose and all amino acid groups except group 4. (E) The same strains as in "A" were grown in M63 media with glucose and all amino acid groups except group 5.

because it does not exhibit any of the other growth phenotypes we have yet observed (Figure 2C). This is the same growth pattern we noted for the M63 media with no supplementation (Figure 4D) and indicates that, under these conditions, amino acid production is not likely the reason for the altered growth we noted in the *rluC* and *rluE* deletion strains.

In media lacking the group 4 amino acids we see a slightly different pattern of growth, where the *rluC* and *rluE* deletion strains have an extended lag phase before beginning logarithmic growth with the logarithmic growth rates and stationary phase OD_{600} values being similar to the parental and *ΔrluA* strains (Figure 5D). While interesting at first glance, this aberration appears to be a result of the unique combination of amino acids in this group, as several of the group 4 amino acids have a high ATP manufacturing cost. Media lacking these amino acids will be significantly less favorable for growth and thus may be resulting in a unique metabolic state for these strains. This seems plausible as the slightly lengthened lag phase may be indicative of the *ΔrluC* and *ΔrluE* cultures needing to make more robust changes to their transcriptome than the other two strains. It is interesting to note that in media lacking either group 1 or group 2 amino acids, the BL21*ΔrluC* strain demonstrated more tolerance to the changes in nutrient availability than the BL21*ΔrluE* strain (Figure 5A and Figure 5B) while in the case of the group 5 amino acids the BL21*ΔrluE* strain was able to reach a higher OD_{600} than BL21*ΔrluC* (Figure 5E). The mechanism behind these differences is unclear and may prove a fruitful avenue for exploring any potential mechanistic differences between the phenotypes seen with these two strains.

Growth Phenotype Appears to be Oxygen-Dependent

The results obtained thus far indicate that the *rluC* and *rluE* deletion strains may be exhibiting altered glucose metabolism, particularly when cultures reach higher densities.

There are several possible explanations for variability in the entry into stationary phase including an alteration in stationary phase signaling or an altered metabolism during the environmental conditions present in the high cell density conditions. Because we identified a unique phenotype when glucose is the sole carbon source, general aberrations in stationary phase entry are not likely, as we would not necessarily expect to see differences with changes in metabolites. However, these data provide strong evidence to support the hypothesis that there is some environmental condition present when cell density increases that affects glucose metabolism.

When considering the environmental conditions present at high cell density in our original experiments, we noted several unique features of the Bioscreen C growth curve analyzer. In particular, the 100 well plate used has flat bottomed wells that measure just 7 mm in diameter (Figure 6). These small wells only allow for loading of a small sample volume and even when used on a setting of "continuous shaking" will provide minimal aeration due to the lack of baffling. Whereas minimal aeration may be sufficient when culture densities are low, as in the early stages of our growth studies, we hypothesize that, at higher cell densities, the minimal aeration provided becomes insufficient. Under these conditions of reduced oxygenation, the deletion of *rluC* or *rluE* alters the ability of BL21 to metabolize carbohydrates, in particular glucose.

To test our "poor oxygenation" hypothesis, we employed a custom-designed apparatus that would allow us to extract culture samples as frequently as we chose without introducing external atmosphere. This apparatus consisted of a 125 ml Erlenmeyer flask with a rubber stopper that would tightly seal the flask once inserted. Through this stopper, we inserted an 18-gauge syringe needle and to the tip of the needle we affixed a length of

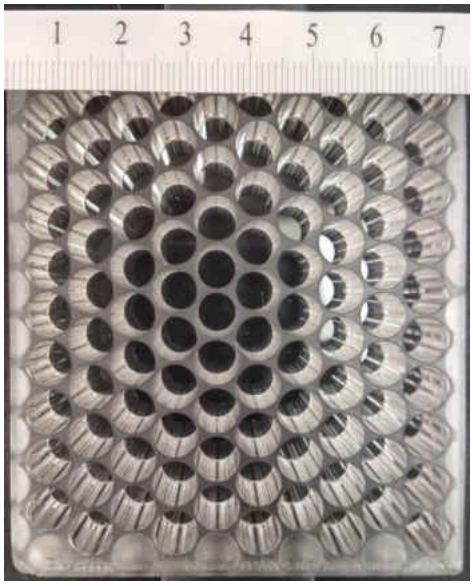

Figure 6: Bioscreen C automated growth curve analyzer honeycomb plate: The honeycomb plates utilized in the Bioscreen C have 100, flat-bottomed wells with a diameter of approximately 7mm.

capillary tubing which was long enough to extend to the bottom of the flask. After subculturing into LB media, we stoppered the flask and capped the exposed end of the needle with a 1 cc syringe. These cultures were grown without shaking at 37°C. Growth of these cultures was then compared against cognate samples grown in similar flasks with loose-fitting caps and vigorous shaking.

Our hypothesis would predict that under the highly oxygenating conditions we would see little to no differences between the BL21 parent strain and the *rluC* and *rluE* mutants and this is confirmed by our well-oxygenated condition growth curves (Figure 7A). Unlike the cultures grown in the Bioscreen C, the growth curves from all the strains, generated from these optimal aeration conditions appear nearly identical. Also in agreement with the above hypothesis, when aeration was limited during growth in our custom apparatus we observed an exacerbation of the growth changes we noted in the Bioscreen C curves (Figure 7B).

BL21 exhibited extremely poor growth and reached a very low final OD_{600} value while the BL21*ΔrluC* and BL21*ΔrluE* strains were far less affected by the lower oxygen levels (Figure 7B). While the growth of the BL21 parental strain plateaued at the 5 hour time point, the two deletion strains continued to increase cell density for an extended period of time and by the end of the analysis period had reached a far higher OD_{600} (Figure 7B). Interestingly, unlike many of the Bioscreen C growth curves, where the BL21*ΔrluC* and *ΔrluE* and the alternate parental strain, MG1655, grew to similar OD_{600} values, this extreme condition results in an

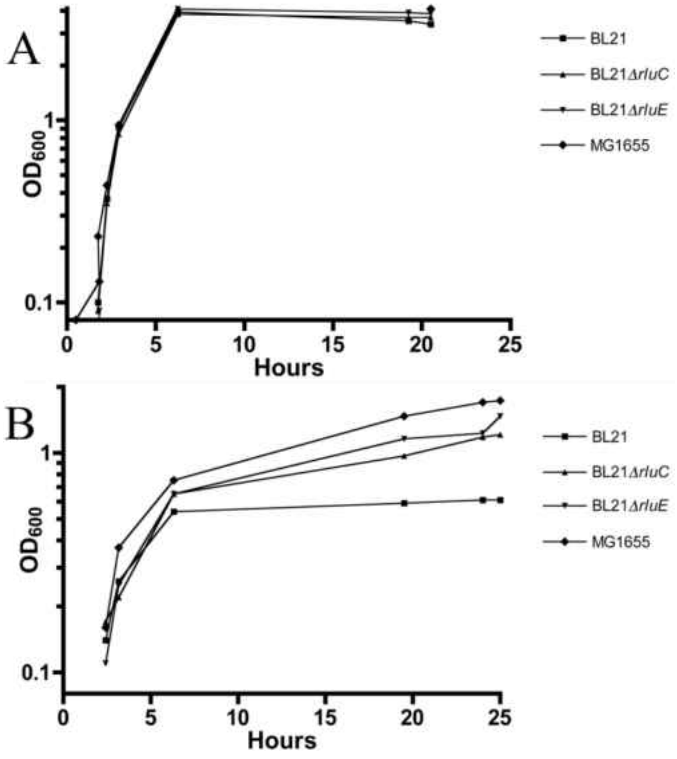

Figure 7: Culture aeration exacerbates the growth differences between the BL21*ΔrluC* and BL21*ΔrluE* strains and their parent: Strains were grown in either full aerated flasks or in the custom-designed flasks as described in the materials and methods. Growth was monitored at the indicated time points. Graphs are a representative of a minimum of three biological replicates. (A) MG1655, BL21, and BL21*ΔrluC* (LMB274) or *ΔrluE* (LMB276) were grown with vigorous shaking in baffled flasks with loose-fitting caps to maximize aeration. (B) The same strains as in "A" were grown in were grown in a custom-designed apparatus without shaking.

intermediate phenotype for the *rlu* deletion strains (Figure 7B). This indicates that, while in the less extreme environment provided in the Bioscreen C the *rluC* and *rluE* deletions result in an MG1655-like growth pattern, the deletions do not result in a robust complementation of the deficient growth of the BL21 strain under more extreme conditions. This evidence strongly supports our hypothesis that oxygenation plays a key role in the phenotypes we have observed.

Metabolic Indicators of Low Oxygen Tolerance are Altered in Strains Lacking rluC or rluE

Our data up to this point indicated that deletion of *rluC* or *rluE* in BL21 resulted in improved tolerance to low-oxygen conditions and that these strains exhibited altered growth, particularly in glucose, when compared to the BL21 parent. These two phenomena led us to question if there were any differences in low-oxygen metabolism of glucose, in particular, in glucose fermentation. In order to look directly at the process of glucose fermentation, we employed a carbohydrate fermentation assay with glucose as the sole carbon source. By inspecting the media for color change from purple to yellow after incubation, we were able to determine if glucose was fermented to produce acidic products. This test also allowed for the detection of gaseous products of fermentation by trapping them in the inverted Durham tube. In agreement with published data (62), while the BL21 strain is able to reduce the pH of the fermentation media sufficiently to change the indicator color, the inverted Durham tube trapped no gaseous products (Figure 8 and Table 1). MG1655 also performs like previous data suggest (62) and both reduces the pH of the media and produces a robust gas bubble in the Durham tube (Figure 8 and Table 1). Unlike the parental BL21 strain, the BL21*ΔrluC*

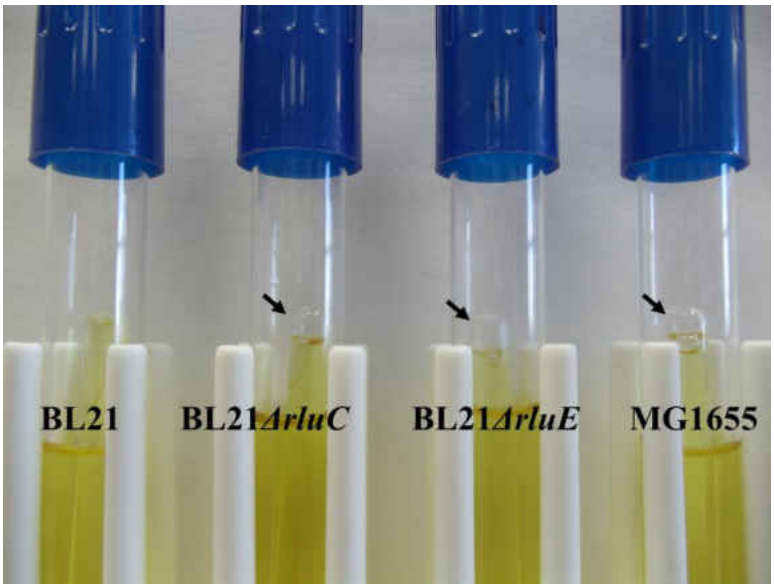

Figure 8: Deletion of *rluC* or *rluE* in BL21 results in increased gas production during glucose fermentation. Fermentation tubes were inoculated and allowed to incubate for 48 hours at 37°C without shaking. Acid production was identified by observation of color change from purple to yellow and gas production was noted by identification of a gas bubble trapped by the inverted Durham tube. Studies were performed with a minimum of three biological replicates. Arrows indicate gaseous products trapped by the inverted Durham tube.

Table 1: Deletion of *rluC* or *rluE* in BL21 results in increased gas production during glucose fermentation.

Strain	Acid Production	Gas Production
BL21	+	-
BL21*ΔrluC*	+	+
BL21*ΔrluE*	+	+
MG1655	+	+
BL21*Δfnr*	+	-
BL21*ΔrluCΔfnr*	+	+
BL21*ΔrluEΔfnr*	+	+
MG1655*Δfnr*	+	+

Fermentation tubes were inoculated and allowed to incubate for 48 hours at 37°C without shaking. Acid production was identified by observation of color change from purple to yellow and gas production was noted by identification of a gas bubble trapped by the inverted Durham tube. Studies were performed with a minimum of three biological replicates.

and BL21*ΔrluE* deletion strains both decrease pH and produce gas bubbles of a similar size to MG1655, indicating these strains have gained the ability to produce gaseous products during fermentation (Figure 8 and Table 1). These data strongly support our hypothesis that the phenotypes seen are a result of altered low-oxygen metabolism.

If the differences seen in the BL21*ΔrluC* and BL21*ΔrluE* strains are a result of an altered regulatory factor we would anticipate that these strains would behave differently from their parent in other low-oxygen-related metabolic assays. To confirm this, we conducted nitrate reduction tests, as the ability to utilize nitrate as an alternative electron acceptor is regulated in a similar fashion to other low oxygen processes. These regulatory networks include the production of the hydrolases responsible for gas production in glucose fermentation (68). As with the carbohydrate fermentation assays, both BL21 and MG1655 behaved as published data would suggest (61). BL21 was unable to reduce nitrates to nitrites, indicating this strain is unable to utilize this alternative electron acceptor (Table 2). MG1655, however, was readily able to reduce nitrates to nitrites and therefore had use of

Table 2: Deletion of *rluC* or *rluE* in BL21 results in increased nitrate to nitrite reduction.

Strain	Reduction to Nitrites
BL21	-
BL21*ΔrluC*	+
BL21*ΔrluE*	+
MG1655	+
BL21*Δfnr*	-
BL21*ΔrluCΔfnr*	+
BL21*ΔrluEΔfnr*	+
MG1655*Δfnr*	+

Nitrate reduction media was inoculated and allowed to incubate for 48 hours at 37°C without shaking. After addition of reagents "A" and "B" (described in Materials and Methods) media was observed for development of a bright red color. Color development within 30 seconds was considered a positive result. Studies were performed with a minimum of three biological replicates.

these alternative electron acceptors (Table 2). In agreement with our hypothesis, both the BL21*ΔrluC* and BL21*ΔrluE* strains showed robust reduction of nitrates to nitrites, demonstrating another gain-of-function in a low oxygen metabolic pathway (Table 2). When taken in context with the Bioscreen C and low oxygen growth curves, this metabolic information indicates that BL21 and MG1655 have different metabolic profiles under low oxygen conditions and that deletion of *rluC* or *rluE* alters the profile of the BL21 strain to more closely resemble MG1655.

Changes in the Cellular Levels of FNR Cannot Adequately Explain the Identified Phenotypes

As we have noted above, in many instances, the BL21*ΔrluC* and *ΔrluE* strains appear to behave more like MG1655 than their parent, BL21. We, therefore, investigated whether some known difference between BL21 and MG1655 could account for the phenotypic differences we see in the *ΔrluC* and *ΔrluE* strains. Interestingly, published work has demonstrated that BL21 is defective in FNR production, as the *fnr* gene in this strain contains a point mutation at codon 141 which results in a premature amber (UAG) codon and an unstable, rapidly degraded protein product (61). FNR is a global transcription factor that is the primary regulator of the transition from high to low oxygen growth (68). The regulon of FNR includes, among many others, the hydrolases responsible for gas production during glucose fermentation and the nitrate reductases that allow for the utilization of nitrogen as an alternative electron acceptor (68, 72). Thus, we hypothesized that alterations in the cellular expression of FNR could account for the collection of phenotypes associated with deletion of *rluC* or *rluE*.

Deletion of another of the pseudouridine synthase genes, *rluD*, has been associated with read through of stop codons in the presence of an unusual allele of the translation

release factor gene, *prfB* (49). It was because of this previous association with read-through of stop codons and deficient translation termination that we then hypothesized that deletion of *rluC* or *rluE* in the BL21 background may also result in read through, particularly at the premature stop codon of the BL21 *fnr* gene. This read-through could result in the production of stable, functional FNR and a resumption of proper FNR regulation. To determine if increased cellular levels of FNR could alter the growth phenotype seen with BL21, we complemented the defective *fnr* gene *in trans* with the pGS27 plasmid. This plasmid is constructed with the K-12 region of the chromosome that contains *fnr* and its promoter and has been shown to produce functional FNR *in vivo* (67). In support of our hypothesis, growth of the pGS27-containing strain grew to a higher stationary phase OD_{600} than the BL21 parental strain (Figure 9). Although the plasmid- containing BL21 strain was not able

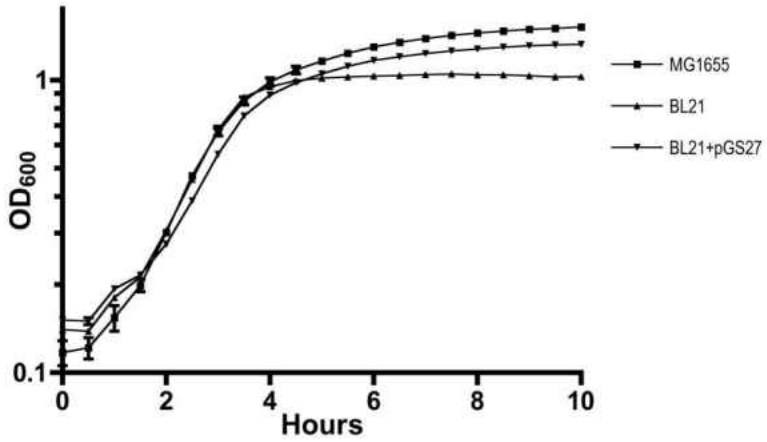

Figure 9: Complementation of *fnr in trans* partially alleviated the growth defect of BL21: Growth of BL21 containing the plasmid pGS27 was compared to both MG1655 and the non-complemented BL21 strain. Strains were grown in a Bioscreen C as described in the materials and methods. Samples were run in triplicate and graphs are a representative of a minimum of three biological replicates. For graphing clarity only the 30 minute time points are shown. Error bars represent the standard deviation.

to reach the same stationary phase OD_{600}, this indicates that at least some of the observed effects of the *rluC* or *rluE* deletions could potentially be attributed to increased regulation by functional FNR.

If the deletion of *rluC* or *rluE* in BL21 is resulting in read-through of the premature stop codon found in *fnr*, then we would predict that a complete deletion of *fnr* would result in similar phenotypes to the BL21 strain and that it would not be suppressible by the *rluC* or *rluE* deletions. That is, the metabolic defects of the BL21 strains would not be suppressible because read-through of the stop codon would require the presence of the defective *fnr* allele and by replacing that gene completely with an antibiotic cassette we would remove any chance of translation of any *fnr* product. If our hypothesis is correct, deleting *fnr* from either BL21*ΔrluC* or *BL21ΔrluE* would result in those strains losing their gain-of-function ability to produce gas during glucose fermentation or utilize nitrates as an electron acceptor. Disappointingly, in strains lacking both *fnr* and *rluC* or *rluE*, these metabolic functions were not lost (Table 1 and Table 2). Thus, these results indicate that read through of the premature stop codon in the *fnr* gene cannot be the functional explanation for these phenotypes. Further studies involving other regulators of the low oxygen response system may elucidate the mechanism behind this collection of phenotypes.

Construction and Characterization of an *Escherichia coli* Strain Lacking All Ribosomal Pseudouridine Synthases

While it has been shown that ribosomes lacking all pseudouridine modifications will function *in vitro*, their function *in vivo* has not been investigated (19, 20). Previous research has demonstrated that single deletions of the *rsuA*, the *rlu* genes *A*, *B*, *C*, *E,* and *F* as well as up to three of these synthases in various combinations can be deleted from MG1655 without adverse effects on growth (25, 27, 47, 78). However, in this strain background, deletion of *rluD* results in significant growth aberrations (30). Because of this, MG1655 would not be a suitable background for constructing a strain lacking all of the ribosomal synthase genes, as any variation in growth caused by the combining of multiple deletions would be masked by the strong phenotype of the *rluD* deletion. Interestingly, the *rluD* phenotypes seen in MG1655 are not observed in the BL21 background, as the MG1655 allele of the *prfB* gene, a translation release factor, is very unusual and not found in the *E. coli* B strains (49). Other than the phenotypes noted earlier in this work, there are no documented phenotypes for deletion of any of the ribosomal pseudouridine synthases in the BL21(λDE3) strain and therefore it was selected as a suitable background to attempt construction of a series of multiple deletion strains. Utilizing this strain collection we would then be able to look at growth and other phenotypes associated with ribosome assembly and function.

Construction of an E. coli Strain Lacking All Ribosomal Pseudouridine Synthases is Possible

Because pseudouridines may play a role in rRNA stability, rRNA structure and various RNA-RNA and RNA-protein interactions, it was possible that the ribosomes produced from strains lacking all or a majority of the ribosomal pseudouridine modifications may be too unstable to support cellular functions. Thus, we were unsure if we would be able to delete the entire repertoire of the seven ribosomal pseudouridine synthase genes, *rluA*, *B*,

C, *D, E*, *F* and *rsuA*. We chose to attempt construction of a series of strains lacking the synthase genes in various combinations, from single mutations up to all seven in a single strain if possible.

Deletions of *rluA*, *rluC*, and *rluF* were completed by phage transduction of a kanamycin resistance cassette that contained a resolvable antibiotic element flanked by "*res*" sites. The resolution of the kanamycin was completed via conjugation with the S17-1 *λpir*/pJMSB8 strain. The remaining deletions, *rluB*, *rluD*, *rluE* and *rsuA* were also kanamycin resistance cassettes, but were resolvable via flanking "*frt*" sites that would recombine via the FLP recombinase. A detailed explanation of these techniques can be found in Chapter II. Through these systems we were able to sequentially construct strains containing multiple deletions without needing multiple antibiotic markers and with minimal risk of polar effects.

While published data has shown that construction of a strain lacking up to three of the ribosomal synthases was possible, it was unknown if four or more deletions in a single strain would result in functional ribosomes and viable cells. From a total of three different three-deletion strains, we constructed two different four-deletion strains, LMB387 and LMB388 (Table 3). A derivative from each of these was used to produce a five-deletion strain, LMB338 and LMB339 (Table 3). Transduction to remove *rluD* was completed with a derivative of each of these strains and resulted in LMB343, a strain lacking *rlus A-F* (Table 3). The final strain, lacking all six of the *rlu* genes and *rsuA* was dubbed LMB353 (Table 3). In no cases did construction of the various strains take more than two to three transduction attempts, confirming the non-essential nature of these genes, even when deletions were combined.

Table 3: Deletion of multiple pseudouridine synthase genes is possible in *Escherichia coli.*

Strain Name	Number of Deletions	Deleted Synthases Genes
LMB272	1	*rluA*
LMB273	1	*rluB*
LMB274	1	*rluC*
LMB276	1	*rluE*
LMB277	1	*rluF*
LMB281	2	*rluA, C*
LMB312	2	*rluC, D*
LMB283	3	*rluA, B, C*
LMB284	3	*rluA, C, E*
LMB285	3	*rluA, C, F*
LMB287	4	*rluA, B, C, F*
LMB288	4	*rluA, C, E, F*
LMB338	5	*rluA, B, C, E, F*
LMB339	5	*rluA, B, C, E, F*
LMB343	6	*rluA, B, C, D, E, F*
LMB353	7	*rluA, B, C, D, E, F, rsuA*

Utilizing a combination of techniques described in the materials and methods, deletions of various pseudouridine synthase genes were combined into a series of *E. coli* BL21 strains. Up to and including all seven ribosomal gene deletions were constructed in a single strain.

Growth of Synthase-lacking Strains Under Various Laboratory Conditions

As we demonstrated in the previous chapter, deletion of either *rluC* or *rluE* in the BL21 background results in increased stationary phase OD_{600} values when grown in LB media at 37°C in the Bioscreen C (Figure 1C). However, these differences were alleviated under conditions of high oxygenation (Figure 7A) and so, to eliminate any ambiguity resulting from the phenotypes of the *rluC* and *rluE* mutants, we chose to pursue studies of the combination mutants under conditions of high oxygenation. Growth of the combination mutants under these ideal conditions, in LB media at 37°C resembled their parental BL21 strain (Figure 10). We saw no differences in the length of lag phase, the growth rate during logarithmic phase or the stationary phase OD_{600} values attained as compared to the BL21 parental strain (Figure 10).

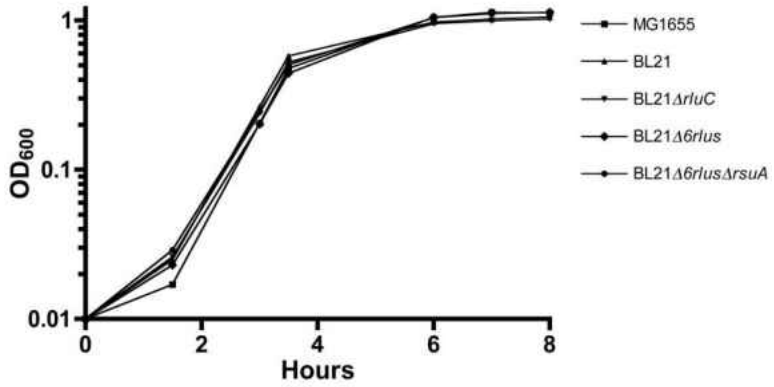

Figure 10: Under ideal conditions, deletion of all seven ribosomal pseudouridine synthase genes does not result in growth aberration. Cultures were grown in LB media at 37°C, in baffled flasks with vigorous shaking and growth was monitored via measurements of OD_{600} values at the indicated time points. Graph is a representative of three biological replicates.

As noted above, several suggestions for the function of rRNA pseudouridines involve their role in altering RNA structures and therefore the ability of ribosomes to properly assemble (79). One hallmark of deletion of ribosome assembly factors is poor growth in cold temperatures (22). Because ribosomal pseudouridines have been hypothesized to play a role in ribosome assembly (79), we chose to analyze the growth of the combination mutants under cold temperature conditions. When grown on solid LB media at 20°C, BL21 and both the 6 deletion and 7 deletion strains grew similarly (Figure 11). All the BL21 derivatives tested were able to form colonies in less than 72 hours (Figure 11). For comparison, strain LMB100 is an MG1655 mutant lacking *bipA*, and has been shown in several previous studies to be cold sensitive (24, 25, 51). This strain was unable to form isolated colonies on the LB agar at 20°C (Figure 11). This resistance to cold temperatures is also evident when strains are grown in liquid LB media. None of the single or combination mutants exhibited a growth defect when in well oxygenated liquid LB media grown at 20°C (Figure 12). These data agree with previous data from the single *rluC* deletion in MG1655, which has never demonstrated cold sensitivity (24, 25). While not definitive, the lack of cold sensitivity even

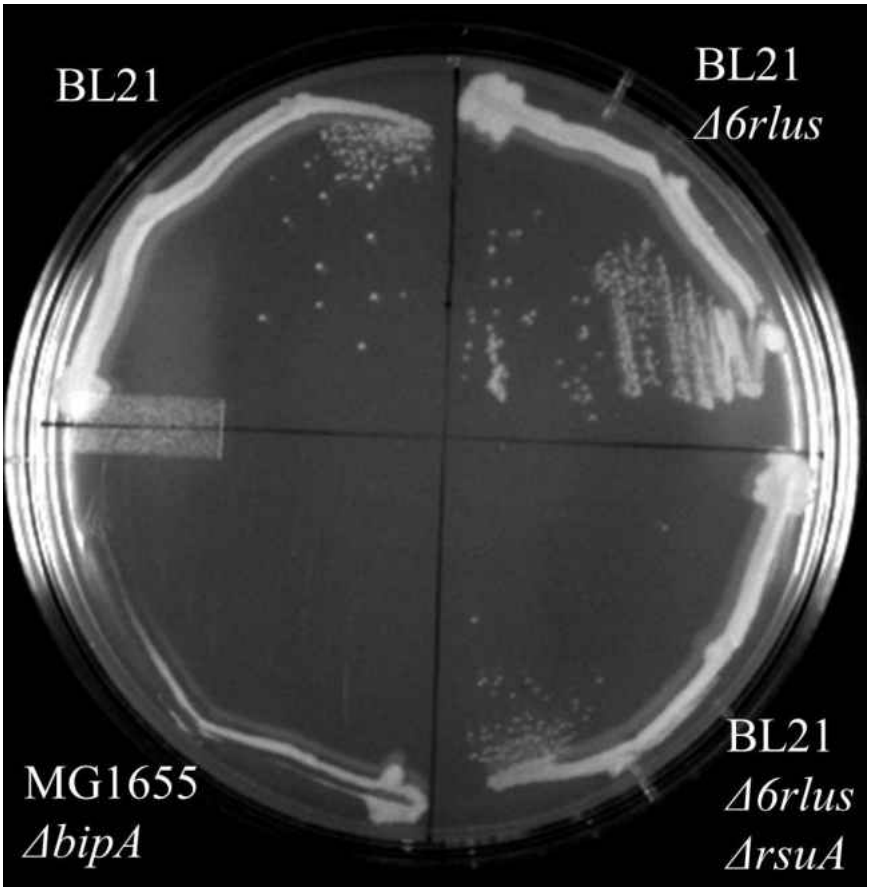

Figure 11: Deletion of all seven ribosomal pseudouridine synthase genes does not result in a loss of colony-forming ability in cold temperatures. Strains were streaked for isolation on LB agar and place at 20°C for 48 hours. After incubation, plates were photographed and inspected for the ability to form isolated colonies.

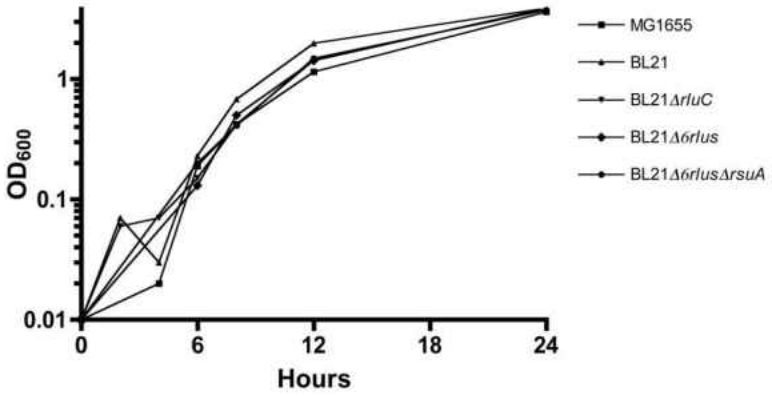

Figure 12: Deletion of all seven ribosomal pseudouridine synthase genes does not result in growth aberrations in cold temperatures. Cultures were grown in LB media at 20°C, in baffled flasks with vigorous shaking and growth was monitored via measurements of OD_{600} values at the indicated time points. Graph is a representative of three biological replicates.

in strains lacking all ribosomal pseudouridine synthases, serves to downplay the potential role for pseudouridines in ribosome assembly. Further studies involving other phenotypes associated with deletion of ribosome assembly factors as well as ribosome profiles and rRNA analyses will be needed to either confirm or deny the role for pseudouridines in ribosome assembly.

CHAPTER IV

DISCUSSION

Ribosomal pseudouridine modifications are highly conserved throughout evolution yet their biological functions remain largely unknown. However, none of the pseudouridine synthase genes are essential in *E. coli* and deletion rarely results in a phenotype under laboratory conditions. This has made these ribosomal modifications a conundrum, as evolutionarily conserved genes or processes typically serve a significant or essential role (80).

While the chemical properties of pseudouridines have led to the hypothesis that these modifications may influence RNA secondary structure formation or other process of ribosome assembly, deletions of many of the pseudouridine synthase genes have not been shown to exhibit any of the hallmarks of deficient ribosome assembly, such as cold sensitivity or changes in subunit ratios in ribosome profiles (24, 25). In certain genetic backgrounds deletion of *rluD* results in both rRNA defects and a significant growth defect; however, these phenotypes have been shown to be strain-dependent (30, 49). Deletion of this gene from an alternative laboratory strain, BL21(λDE3), has been shown to have minimal effects on growth (49). All other pseudouridine synthase gene deletions did not demonstrate an aberrant growth in the MG1655 strain and so, were already considered non-essential. It was because we would be able to delete all of the pseudouridine synthase genes in the same strain background that we chose to work in the BL21 background. This would allow us to

compare all of the single deletion strains and potentially combine multiple deletions all in the same background.

Identification and Characterization of New Phenotypes for Deletion of *rluC* or *rluE*

While performing preliminary growth analyses on the single *rlu* gene deletion strains, we identified a growth difference between the BL21 and MG1655 parental strains. However, when one considers the documented differences between these two common laboratory strains this difference is not unexpected. With several insertion elements and hundreds of SNPs different between these two strains, we were not necessarily surprised to see growth and metabolic aberrations. As we expected, deletion of *rluA*, *rluB*, *rluC*, *rluE*, and *rluF* in MG1655 did not alter growth. However, what we did not foresee was the difference in growth identified between the BL21 parental strain and the BL21Δ*rluC* and BL21Δ*rluE* strains. In several media types we noted that deletion of either *rluC* or *rluE* in the BL21 strain resulted in higher stationary phase OD_{600} values than the BL21 parent. To our knowledge, this is the first time a growth phenotype has been identified for deletion of either *rluC* or *rluE* and the first phenotype of any kind identified for the deletion of *rluE*.

Minimal media with glucose as the sole carbon source was the only tested media that altered the previously described phenotype. In this context, the OD_{600} values reached by the BL21Δ*rluC* and BL21Δ*rluE* mutants were less than that of the parental strain. This indicates that the observed phenotype was connected to the ability of these strains to utilize glucose. However, as the major differences in growth were only apparent during stationary phase, we hypothesized that there must also be some unique environmental factor or biological process associated with the entry into stationary phase that was altering the metabolism of glucose in the deletion strains.

When considering the environmental conditions present in our cultures during the transition from exponential growth to stationary phase, we noted that the honeycomb plates used in the Bioscreen C may not be providing ideal aeration especially when cultures become dense. The small wells have flat bottoms that are unlikely to provide adequate aeration, especially when the cell cultures become dense. Because of these culture conditions, we hypothesized that the *rluC* and *rluE* deletion strains were able to respond to low environmental oxygen levels differently than the BL21 parental strain, particularly with regard to the ability to metabolize glucose.

When we grew our cultures in an ideally oxygenated environment, meaning baffled flasks with vigorous shaking, all the tested strains grew with no discernible differences, including the two laboratory strains, BL21 and MG1655. However, our growth studies in our custom-designed, low-oxygen environment flasks demonstrated that the BL21 parental strain was impaired in its growth. The exponential phase growth rate was reduced and this strain reached a lower final OD_{600} than that of MG1655. Also, BL21Δ*rluC* and BL21Δ*rluE* did, in fact, show increases in both the exponential phase growth rate and the final OD_{600} value when compared to the parental BL21 strain. While deletion of *rluC* or *rluE* was not able to completely complement the growth defect of BL21, this result is in agreement with our hypothesis that low oxygenation may be a factor in the growth phenotype we noted in our earlier growth curves.

Bacterial cells must make significant changes to their metabolism in order to survive in low oxygen conditions and maintain a sufficient supply of ATP to power the necessary cellular processes. This is accomplished by utilizing alternative electron acceptors or shunting energy rich compounds through less efficient metabolic pathways. As we felt it was

highly unlikely that either pseudouridines or pseudouridine synthases play a role directly in glucose metabolism or growth in low oxygen conditions, we reasoned that deletion of *rluC* or *rluE* alters levels of either transcription, or more likely translation, of a metabolic regulator responsible for governing these processes. We examined two metabolic indicators of cellular ability to adapt to low oxygen environments, namely the ability to utilize nitrate as an alternative electron acceptor and the ability to ferment glucose to produce both acidic and gaseous end products. In both these cases we observed that deletion of *rluC* or *rluE* restored a defective metabolic process of the BL21 parental strain. The BL21 strain was not able to reduce nitrates to nitrites, and would therefore be at a significant disadvantage when growing under low oxygen conditions. Being able to utilize nitrates is pivotal to maintaining ATP production from the process of oxidative phosphorylation as without an alternative to oxygen as an electron acceptor the process will cease. Also, because the BL21 strain lacks the ability to produce gaseous products at the end of glucose fermentation, it would struggle to produce adequate amounts of ATP through the fermentative pathways. This pathway defect would result in the accumulation of potentially toxic products that would not diffuse out of the media as the gaseous products would. However, in both cases, deletion of *rluC* or *rluE* results in restoration of these abilities and improved growth under low oxygen conditions. This implicates some regulatory factor, one that controls metabolism under low oxygen conditions, as a mediator of this collection of phenotypes we have so far observed.

Because the BL21Δ*rluC* and BL21Δ*rluE* strains behaved more like the alternative laboratory strain, MG1655, than their parent, BL21, we investigated whether there is some genetic difference between MG1655 and BL21 that may be linked to our observations. This difference would need to explain both the growth and the metabolic differences noted

between the two parent strains and be something that would be a likely candidate for alteration by deletion of either *rluC* or *rluE* deletion. As metabolism is such an essential cellular function, there are few master regulators that could be responsible for altering such intrinsic properties. In fact, in *E. coli*, the response to changing oxygen levels has only two major regulatory systems that control expression of this gene network, FNR and the ArcAB two-component system. Although the ArcAB system does regulate several genes responsible for metabolism, it has not been implicated in governing gene expression during anaerobic growth and therefore, we felt would not be the likely regulator of the responses we have noted.

As FNR has been described as the primary regulator of the low oxygen growth response, this made this regulon seem a more credible target for further investigation. Interestingly, the genetic studies comparing various laboratory strains of *E. coli* showed that BL21 is defective in FNR production (61). The *fnr* gene in this strain has a point mutation that results in a stop codon at amino acid 141. The resulting truncated protein cannot dimerize or bind to DNA and is therefore non-functional, unstable, and rapidly degraded (61). We also noted that the strain-dependent phenotype associated with deletion of *rluD* was a result of significant read-through of stop codons (48). From this we hypothesized that deletion of *rluC* or *rluE* in the BL21 allowed read-through to occur at the stop codon of the *fnr* gene, thus resulting in a small but significant quantity of functional FNR protein and the re-establishment of FNR regulation. While the phenotypes associated with deletion of *rluD* involve high levels of read through at all three of the stop codons, such unbridled termination failure would not be necessary in this case. As FNR is a transcriptional regulator, only a

small amount of full-length protein would be needed to allow for establishment of regulation at the high-affinity DNA binding sites.

In examining whether complementation of the defective FNR gene in the BL21 strain could indeed result in increased stationary phase OD_{600} values, we found that the pGS27 plasmid, which has a small region of the MG1655 chromosome containing that strain's functional *fnr* gene, did indeed increase growth in the less-than-ideal growth conditions of the Bioscreen C. This showed that by providing exogenous FNR we could replicate at least one of the observed phenotypes observed for the deletion of *rluC* or *rluE*. However, this did not confirm the importance of FNR itself in this process, as the exogenous FNR might alter the expression of another gene that is the actual functional reason for the phenotypes seen with deletion of *rluC* or *rluE*.

If, as our hypothesis states, read-through of FNR was the reason for the growth and metabolic differences, deletion of FNR from the BL21Δ*rluC* and BL21Δ*rluE* strains would abolish these phenotypes. Disappointingly this was not the case, as BL21 strains lacking both *rluC* and *fnr*, or *rluE* and *fnr* were still able to reduce nitrates to nitrites and produce gas during glucose fermentation. This indicates that FNR derived from the BL21 chromosome cannot be the source of the phenotypes we have identified. From this we concluded that alterations in gene expression must be occurring downstream of the regulation of FNR but within the FNR regulon, as the identified phenotypes all indicate that expression of genes from this regulatory network are altered in the BL21Δ*rluC* and BL21Δ*rluE* strains.

The RluE pseudouridine is placed at location 2457 of the 23S rRNA, and one of the three RluC modifications is place at location 2504, just 47 nucleotides away (18). These pseudouridines are located near one another in the peptidyl transferase region of the rRNA

and this proximity may be the reason that deletion of either one of these genes results in a similar collection of phenotypes (81). Interestingly, a strain lacking both the *rluC* and *rluE* genes did not exhibit any additive growth changes, therefore, it may be that the loss of a single modification to this conserved rRNA region is sufficient to alter ribosome function. However, there is currently no direct evidence for this hypothesis.

There are several unanswered questions regarding the above described phenotypes. First and foremost is: "What is the regulatory mechanism driving these metabolic changes?" Further work using quantitative RT-PCR may prove useful in determining which branches of the FNR gene network are dysregulated in the *rluC* and *rluE* deletion strains. These results will direct study to the transcription factors and other regulatory elements responsible for the more specific set of dysregulated genes and should clarify at which level of regulation these altered ribosomes mediate these effects. However, with currently available data any speculation as to what these targets may be would be very premature.

The other, and arguably more important, questions derived from this study are: "What role do ribosomal pseudouridines and pseudouridine synthases play in gene regulation?" and "What is the purpose of ribosomal pseudouridine modification in cell function?" With so little known about what roles rRNA modifications serve, these questions are truly at the heart of current research. While there is little out there to support any one hypothesis regarding specific roles, I propose the following:

1. Ribosomes exist in a heterogeneous mixture of populations, each population varying with regard to their modification status.

2. Each population of ribosomes provides preferential translation of a specific subset of mRNAs. This preference is guided by interactions either helped or hindered by the rRNA modification status.
3. The population ratios may change as different environmental pressures or cellular needs dictate, in a similar manner to how mRNA transcript levels are adjusted. This would provide another layer of cellular control over protein production.

In the context of this study, my hypothesis would argue that in ribosomes lacking the modifications placed by RluC or RluE, there would be preferential translation of mRNA from genes responsible for the identified phenotypes. Under conditions with high oxygen the naturally occurring population of ribosome may be small, as would quantity of the mRNA these ribosomes would preferentially translate and so you would see very little translation. In conditions of low oxygen, the hypothesis would predict that as there is an increase in the quantity of the mRNA for these genes, this ribosomal population would become more active and together these individual changes would lead to the significant increase in the expression of genes required for low-oxygen tolerance that we would expect. In the BL21 strain, which is lacking a functional regulator of the low-oxygen response and would have difficulty generating the necessary increase in mRNA, these cells would struggle to respond to the low oxygen environment. However, in the mutant strains, where the entire ribosome population is lacking the RluC or RluE modifications, the small amount of transcribed mRNA that is produced without the positive regulation from FNR, would be preferentially transcribed by the full complement of ribosomes and therefore may be expressed at much higher levels than in wild-type cells.

My hypothesis is not completely unfounded. mRNAs are known to be pseudouridylated differentially in several species of yeast and in human cells, based on growth phase of the cultures, indicating that pseudouridine synthases can be regulated based on cellular needs (40). Other rRNA modifications in *E. coli* have been shown to only exist in a subset of ribosomal RNAs, demonstrating that there are already known sub-populations of ribosomes (82). But, by far the most intriguing fact is that pseudouridylation status has been shown to alter expression of mRNAs and has been used to induce codon variation during translation (36, 38, 54, 83). Taken together, these support the above hypothesis that ribosomes exist as a collection of sup-populations, each one preferentially translates a subset of mRNAs, and that this preference is determined by the intrinsic chemical properties of the interacting RNA species, which can be altered by RNA modifications such as pseudouridines.

Construction and Characterization of an *Escherichia coli* Strain Lacking All Ribosomal Pseudouridine Synthases

While studies from *E. coli* indicated that functional ribosomes could be assembled lacking multiple pseudouridine modifications (20), others in eukaryotic organisms indicated that combining multiple deletions leads to additive growth defects (53). This ambiguity led us to question what minimum rRNA pseudouridylation state was necessary to support cellular function. We hypothesized that if we were able to determine the particular combination of pseudouridine modifications that would be necessary to support proper assembly and translation *in vivo*, we could determine if the modifications were contributing to specific necessary structural changes or binding of r-proteins or other accessory factors. We also believed that by constructing a series of pseudouridine synthase deletion strains, we would be able to test a majority of possible deletion combinations. Through these strains we hoped to determine if *E. coli* exhibited either an additive growth defect similar to that noted

in higher organisms, a growth defect when the modification from a certain region were missing, or possibly, no defect at all.

We found that deletion of all the pseudouridine synthase genes, the 6 large subunit synthase genes and the single synthase gene for the small subunit, was possible in BL21. A single strain lacking all 7 of these genes exhibited no obvious growth defects when grown under ideal laboratory conditions of 37° with rich media and vigorous shaking as well as in cold temperatures (20°C). As cold temperature growth defects are often associated with faulty ribosomal assembly, a lack of growth defect in these conditions suggests that these ribosomes assemble correctly even in this less-than-ideal environment.

The ability to delete all of the rRNA pseudouridine synthase genes from a single strain of *E. coli* both confirms the non-essential nature of the enzymes and the modifications under our laboratory conditions as well as demonstrates that, unlike some higher organisms, there are no cumulative growth defects associated with the loss of these modifications. Being able to study ribosomes lacking all of the various combinations of modifications may prove fruitful in determining what role these modifications play in the overall ribosomal structure.

Recent studies have shown that there is a market for bioengineered ribosomes in industrial settings and in designing these unique constructs, scientists have been able to refute some previously held assumptions regarding what is necessary for proper ribosomal function. In particular, the ability to engineer a ribosome with subunits that are tethered by a shared rRNA species has shown that subunits do not need to completely separate to allow for cycling on and off mRNAs (84). While many were skeptical that these tethered ribosomes could support growth, there was interest in developing a system that would ensure

specifically altered 16S and 23S rRNAs would combine into a homogeneous ribosome population in an organism with multiple rRNA operons. This unique construct was able to support growth of *E. coli* in the absence of wild type ribosomes and was used to create an orthogonal ribosome-messenger RNA system (84). Because targeted pseudouridylation of mRNA has been shown to alter translation efficiency and codon recognition (36, 38, 40, 85), it may be that the use of directed pseudouridylation in otherwise non-modified ribosomes may be another avenue to explore in the bioengineering of ribosomes.

APPENDIX

Appendix

List of Strains and Plasmids Used in this Study

Strain	Genotype	Reference
MG1655	*rph-1 ilvG rfb-50*	Laboratory collection
BL21(λDE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	Laboratory collection
S17-1 λpir(pJMSB8)	Tp^r Sm^r *recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn7 *λpir*	(74)
KM32	*argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-5 thi1-rpsL31 tsx-33 supE44 Δ(recC ptr recB recD)::Plac-bet exo cmr*	(86)
JW1328	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*fnr771*::*kan rph-1* (*rhaD-rhaB*)*568*	(87)
JW1261	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*rluB*::*kan rph-1* (*rhaD-rhaB*)*568*	(87)
JW1121	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*rluE*::*kan rph-1* (*rhaD-rhaB*)*568*	(87)
JW2171	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*rsuA*::*kan rph-1* (*rhaD-rhaB*)*568*	(87)
CH6960	BL21(λDE3) *ΔrluD::frt-kan-frt*	(48)
KK22	KM32 *ΔrluA::res-npt-res*	(25)
KK23	KM32 *ΔrluC::res-npt-res*	(25)
KK24	KM32 *ΔrluF::res-npt-res*	(25)
LMB101	MG1655 *ΔrluA::res-npt-res*	This study
LMB102	MG1655 *ΔrluB::frt-kan-frt*	This study
LMB103	MG1655 *ΔrluC::res-npt-res*	This study
LMB105	MG1655 *ΔrluE:: frt-kan-frt*	This study
LMB106	MG1655 *ΔrluF::res-npt-res*	This study
LMB272	BL21(*λDE3*) *ΔrluA::res-npt-res*	This study
LMB273	BL21(*λDE3*) *ΔrluB::frt-kan-frt*	This study
LMB274	BL21(*λDE3*) *ΔrluC::res-npt-res*	This study
LMB276	BL21(*λDE3*) *ΔrluE::frt-kan-frt*	This study
LMB277	BL21(*λDE3*) *ΔrluF::res-npt-res*	This study
LMB279	BL21(*λDE3*) *ΔrluC<res >*	This study
LMB317	BL21(*λDE3*) *ΔrluE< frt>*	This study

LMB297	BL21(λDE3)+pGS27	This study
LMB281	LMB279 *ΔrluA::res-npt-res*	This study
LMB282	LMB281 *ΔrluA<res >*	This study
LMB312	LMB279 *ΔrluD::frt-kan-frt*	This study
LMB340	LMB312 *ΔrluD< frt>*	This study
LMB314	MG1655 *Δfnr::frt-kan-frt*	This study
LMB315	LMB279 *Δfnr::frt-kan-frt*	This study
LMB316	BL21(*λDE3*) *Δfnr::frt-kan-frt*	This study
LMB318	LMB317 *Δfnr::frt-kan-frt*	This study
LMB283	LMB281 *ΔrluB::frt-kan-frt*	This study
LMB284	LMB281 *ΔrluE::frt-kan-frt*	This study
LMB285	LMB281 *ΔrluF::res-npt-res*	This study
LMB286	LMB285 *ΔrluF<res >*	This study
LMB287	LMB286 *ΔrluB::frt-kan-frt*	This study
LMB288	LMB286 *ΔrluE::frt-kan-frt*	This study
LMB336	LMB287 *ΔrluB< frt>*	This study
LMB337	LMB288 *ΔrluE< frt>*	This study
LMB338	LMB336 *ΔrluE::frt-kan-frt*	This study
LMB339	LMB337 *ΔrluB::frt-kan-frt*	This study
LMB341	LMB338 *ΔrluE< frt>*	This study
LMB342	LMB339 *ΔrluB< frt>*	This study
LMB343	LMB341 *ΔrluD::frt-kan-frt*	This study
LMB344	LMB343 *ΔrluD< frt>*	This study
LMB353	LMB344 *ΔrsuA::frt-kan-frt*	This study

Plasmid	Genotype	Reference
pCP20	*Eco*R1 digest of λG70 phage cloned into pBR325	(75)
pGS27	pCP20 Amp^R and Cm^R plasmid with Ts replication and thermal induction of FLP synthesis	(67)

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