ANALYSIS OF A NOVEL 30S RIBOSOMAL RNA PROCESSING PATHWAY INDEPENDENT OF RNASE III CLEAVAGE IN ESCHERICHIA COLI

by

GARGI CHAUDHURI

(Under the Direction of Sidney R. Kushner)

ABSTRACT

The coordinated synthesis and assembly of ribosomal subunits and ribosomal RNA (rRNA) in Escherichia coli is a highly complex and well-regulated process. One aspect of this control that is often overlooked is the post-transcriptional regulation of rRNA. The work described in this dissertation analyzes the processing of rRNAs in E. coli.

In E. coli all the three rRNAs are co-transcribed as 30S primary transcript from seven rRNA operons which is initially cleaved by RNase III to generate pre-rRNA species and are subsequently processed by various endo- and exoribonucleases to generate the mature ends. Here we show that the 30S primary transcript is efficiently processed by RNase E, RNase G and YbeY in absence of RNase III. This alternative processing pathway which works in absence of RNase III is a backup mechanism for cells to process 30S rRNAs. Moreover, the alternative processing pathway generates multiple cleavage sites which are unique from the RNase III-dependent pathway.

We also found that after the initial cleavages of 30S rRNA, the pre-16S and pre-23S rRNA species are separated from each other and are subsequently processed by the
secondary processing enzymes-RNase P, RNase Z and exoribonucleases to generate the mature rRNA species. We have also identified three new genes (YhgF, YraN and YhbQ) with possible enzymatic activity which play a role in the processing of pre-23S rRNA in the alternative processing pathway.

Here we also analyzed the alternative processing pathway in the individual rRNA operons and showed that the heterogeneities seen in the gene organization among the seven rRNA operons influence the 30S rRNA processing pattern. The primary processing enzymes of the alternative processing pathway has different efficiencies in *rrnD* and *rrnG* rRNA operon. In fact, we also showed that in the alternative processing pathway, the 5′ ends of some of the cleavage products in *rrnD* rRNA operon are not the same as in *rrnG* operon. Additionally, we also showed that the presence of the spacer tRNA in between the 16S and 23S rRNA is essential for 30S rRNA processing in absence of RNase III. While this work is not complete yet, we have constructed the tools required for the analysis of significance of spacer tRNA in rRNA processing in *E. coli.*

INDEX WORDS: 30S primary transcript, rRNA, RNase III, RNase E, RNase G, YbeY, spacer tRNA.
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DEDICATION

To my parents, I would not be the person I am today without your love, guidance, and support. I hope that this achievement will complete the dream that you had for me many years ago. To my niece, Ruhani, for giving me endless joy and happiness which kept me going in these six years.
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

All living cells require high levels of protein synthesis. The ribosome is the cellular machine that is responsible for the active protein synthesis in cells from every life form. The key catalytic activity of ribosomes is to create a chemical bond (peptide bond) between two amino acids during the process of protein translation. Surprisingly, the peptide bond formation is catalyzed by RNA components of the ribosome - ribosomal RNAs (rRNAs). Therefore, studying the processes by which the ribosomal subunits are assembled along with a clear understanding of rRNA transcription, processing, maturation and modification in *Escherichia coli* provides an excellent model system for understanding the basics of cellular metabolism and regulation. Evidence suggesting the possibility that rRNA processing steps in bacteria can regulate ribosome biosynthesis has opened new avenues of research in this field. Here we examine in detail the extensive work done over the last forty years with regard to ribosome assembly and the various ribonucleases that are involved in rRNA processing and maturation.
Ribosome assembly and ribosomal RNA

Ribosomes are complex ribonucleoprotein (RNP) particles whose primary function is to serve as a site and catalyst for protein biosynthesis\(^1\). In prokaryotes, ribosomes sediment as 70S particles and are formed from two subunits, namely the 30S and 50S subunits, which during translation assemble together to form the 70S functional ribosome. The 70S ribosome in \textit{E. coli} is constituted of roughly two-thirds RNA and one-third protein\(^2\). The smaller 30S subunit contains a single 16S ribosomal RNA (rRNA) molecule and 22 ribosomal proteins, while the larger 50S subunit contains one molecule of 23S rRNA and one of 5S rRNA along with 35 ribosomal proteins. The assembly of this conglomerate of proteins, rRNAs and the ribosomal subunits into a functional complex is of paramount importance for translation and therefore cellular viability.

Rapidly dividing cells produce hundreds of new ribosomes every minute\(^3\) to meet the demands of protein synthesis. Therefore, the process of ribosome assembly must be accurate and stringently controlled, so that the level of protein synthesis matches the growth rate of cells\(^4\). Additionally, in \textit{E. coli} the ribosomes form around 30\% of the dry mass and around 40\% of the energy produced by the cell is used for ribosome biogenesis. This requires a high level of coordination between the synthesis and ordered binding of ribosomal proteins and metal ions concomitant with rRNA processing, modifications and sequential conformational changes. The coordinated assembly is not only a prerequisite but also a necessity for the energy consumption of the cells.
In the last few decades, extensive research has been done to elucidate the process of ribosome assembly in detail, yet a lot remains to be investigated. A major breakthrough occurred in the late 1960s, when it was demonstrated that *in vitro* an active 30S subunit can be assembled from free rRNA and ribosomal proteins without any additional components\(^5\). These findings suggested that all the information necessary for ribosome assembly was encoded within the rRNA and proteins themselves. *In vitro* reconstitution experiments showed that the 30S ribosome assembly occurred in three steps\(^6\). First, the primary binding proteins bound to the mature 16S rRNA at low temperatures, followed by the binding of secondary ribosomal proteins. At this point the assembly is stalled to form the reconstitution intermediate (RI) which sediments at 21S. At higher temperatures the RI changes its conformation to RI* resulting in a 26S particle which then binds to the tertiary proteins to complete the 30S ribosome assembly\(^6\). Additionally, it was shown that the assembly process has a polar nature which progresses from the 5′ to 3′ direction of 16S rRNA.

During the first stage of assembly, 40% of the changes were localized at the 5′ domain, 31% at the central domain, and 19% and 14% at the 3′ major and minor domains of 16S rRNA, respectively. As the assembly transitions from RI to RI*, the changes observed are more concentrated toward the 3′ end of 16S rRNA with 19% localized in the 5′ domain, 14% located in the central domain, and 62% and 5% located in the 3′ major and minor domains of 16S rRNA, respectively. Lastly, with the final transition from RI* to 30 S subunit changes observed were, 12% localized in the 5′ domain, 8% located in the central domain, and 76% and 4% located in the 3′ major and minor domains of 16S.
rRNA, respectively. These results suggest that ribosome assembly is a co-transcriptional process *in vivo*\(^7\)–\(^9\) which re-emphasizes the importance of rRNA synthesis and the initial processing of the 30S rRNA primary transcript to separate individual rRNAs.

Similarly, *in vitro* experiments for 50S subunit assembly showed that it occur in four steps at different reaction conditions, producing three reconstitution intermediates\(^10,11\). The first intermediate which sediments at 33S, is formed by the interaction between 23S rRNA, 5S rRNA and 20 proteins. Subsequently, it changes conformation at a higher temperature and sediments at 41-43S, at which time the rest of the ribosomal proteins bind to it to form an inactive intermediate which sediments at 48S. The final step of assembly takes place at higher temperature and magnesium ion concentrations to promote the transition to active 50S subunits\(^12\). Based on the reconstitution experiments, 23S rRNA is essential for early assembly whereas 5S rRNA is necessary for late assembly but, dispensable for early assembly steps\(^13\).

However, these *in vitro* experiments were done using mature rRNA whereas *in vivo* they are all co-transcribed as a 30S rRNA primary transcript which needs to be cleaved to separate the individual rRNA species. Also they differ in their secondary structures which can affect their binding sites at different locations. Moreover, it did not include conditions where the ribosomal proteins could bind to the rRNAs before their transcription was completed, suggesting that the experiments did not provide a complete picture of the ribosome assembly\(^14\)–\(^16\). As a result, the ribosome assembly steps from *in vitro* reconstitution experiments did not provide an accurate picture of what happens *in vivo*.
Subsequently, *in vivo* studies revealed that there are two 30S intermediates which sediment at 21S and 30S unlike the three intermediates observed *in vitro* \(^{15,17,18}\). Similarly, 50S assembly had three intermediates *in vivo* instead of four intermediates as seen *in vitro*. Furthermore, the precursor intermediates had different protein components\(^{15,18}\). The most abundant precursor seen *in vivo* co-sedimented at 30S and 50S and they contained all the associated proteins of the mature subunits but had incompletely processed rRNAs (precursor rRNA)\(^{18}\). In fact, the final maturation of precursor rRNA was suggested to take place in polysomes\(^{18-21}\), further emphasizing that ribosome assembly and rRNA maturation are coupled.

Ribosome assembly is a fast and efficient process which takes approximately two minutes at 37°C *in vivo*. Concomitant with ribosome assembly, the rRNAs, which are initially transcribed as precursor molecules, must undergo nucleolytic processing to generate the mature molecules found in the functioning 70S ribosomes. Owing to the complexity of the ribosome assembly process, errors in assembly can occur due to misprocessed or misfolded rRNAs, misordered addition of ribosomal proteins or improper conformational rearrangements.

Such altered ribosomes are not competent for translation and are subjected to a quality control process that leads to their elimination *in vivo*. In addition, large rRNAs form metastable structures which can lead to errors in assembly. Therefore, coordination between the ribosomal proteins, proper rRNA processing and subsequent folding along with other external assembly factors is crucial to the fidelity of ribosome assembly.
Disrupting this coordination leads to defective translation as it has been previously shown that accumulation of rRNA precursors result in diverse ribosome assembly defects\textsuperscript{4,22}.

Further studies have also shown that rRNA processing occurs more efficiently when they adopt specific conformations that occur during the assembly process. When these conformations are not attained due to assembly defects, processing of rRNAs become defective. Another aspect of ribosome assembly that has been studied extensively in the past few years, relates to understanding whether the rRNA processing defects can themselves influence ribosome assembly. In order to answer this question, it is important to study and characterize the rRNA processing mechanisms in detail.

**Initial Understanding of rRNA Processing**

In most prokaryotes, the rRNA genes are organized into ribosomal RNA operons (\textit{rrn}). In \textit{Escherichia coli}, there are seven rRNA operons with the same basic organization of the individual rRNAs (Figure 1). Each operon has the 16S rRNA at the 5′ end of the 30S rRNA transcript after the promoter region, followed by a spacer tRNA, 23S rRNA and 5S rRNA with one or two tRNAs (depending on the type of the \textit{rrn} operon) at the distal end prior to the Rho-independent transcription terminator\textsuperscript{23–26}. Similar organization is seen in other prokaryotes such as \textit{Bacillus subtilis}\textsuperscript{27} and \textit{Clostridium perfringens} which has ten \textit{rrn} operons\textsuperscript{28}, \textit{Lactococcus lactis} which has six\textsuperscript{29}, \textit{Mycoplasma capricolum} which has two\textsuperscript{30} and the archaebacterium \textit{Halobacterium cutirubrum} which possesses only one\textsuperscript{31}. 
In *E. coli* based on sequence analysis it was shown, that the 16S and 23S rRNAs are each flanked by inverted repeat sequences which form two sets of stem structures with the mature 16S and 23S rRNAs looped out between the near perfectly paired stems of 36 and 28 base pairs (bp), respectively\(^{32,33}\) (Figure 2). In the late 1960s, a double-stranded RNA specific endoribonuclease RNase III was discovered which cuts the 36-bp and 28-bp stems on either sides to release 17S and pre-23S precursors (Figure 3), respectively\(^{34}\). In RNase III deficient cells, the full-length ribosomal operon transcript is produced, which is never seen when RNase III is present\(^{34}\). Moreover, when tested *in vitro* with purified RNase III, the 30S precursor, which accumulated in absence of RNase III was rapidly cleaved within the flanking double-stranded regions to generate 17S and pre-23S species, containing the sequences of mature 16S and 23S rRNAs as well as additional sequences at their 5′ and 3′ ends\(^{35}\). Subsequently, it was shown that in wild type cells, the 30S rRNA transcript was never seen.

These studies suggested that the initial 30S rRNA cleavage by RNase III is co-transcriptional. Additionally, it was shown that the first RNase III cleavage on 16S rRNA occurs even before the entire 30S rRNA is transcribed\(^{36}\). Therefore, the timing of rRNA transcription and processing are closely coupled and the coordination of transcription and RNase III processing are important for rapid folding of rRNAs and assembly of ribosome subunits.

However, RNase III cleavage does not generate mature ends of the 16S and 23S rRNAs indicating that other nucleases were involved in the final maturation steps. Subsequent work led to the discovery of an endoribonuclease identified as RNase M16,
which helped in the maturation of 16S rRNA. RNase M16 defective cells (named as BUMMER strain) accumulated an RNA molecule of 16.3S, which was smaller than the original 17S precursor generated by RNase III activity. This precursor had ~60 extra nucleotides at the 5’ end\(^{37}\). However, this 16.3S processing intermediate contained a mature 3’ terminus indicating that RNase M16 was not responsible for the maturation of the 3’ end of 16S rRNA\(^{38}\).

Additional work provided insights into 5S rRNA maturation. It was shown that following RNase III cleavage, RNase E processes the 9S rRNA precursor to generate a pre-5S rRNA with 3 extra nucleotides at both the 5’ and 3’ ends\(^{39,40}\) (Figure 3). This processing step is fast as the wild-type cell does not accumulate 9S rRNA at normal conditions\(^{41}\). However, temperature sensitive mutants of the essential endoribonuclease RNase E accumulated the 9S rRNA precursor confirming its role in 9S processing after RNase III cleavage\(^{39-41}\). The extra 3 nucleotides at the 3’ end after RNase E cleavage were suggested to be processed by the 3’→5’ exoribonucleases to generate mature 5S rRNA.

Not much was known about the 23S rRNA maturation, but one of the major conclusions made from these early studies was that rRNA maturation events occur in the context of an RNP particle, namely the assembling ribosome. It was suggested that the protein synthesis from the newly-assembled functional ribosome was necessary for the final maturation steps of rRNA.
Early studies provided strong evidence about the close connection between rRNA processing and ribosome assembly based on the numerous ribosome assembly mutants isolated, which showed severe rRNAs processing defects. However, analysis of the rRNA processing mechanisms in vitro from the ribosome assembly intermediates has been extremely challenging. What made it more difficult was to identify and isolate the pre-ribosomal substrates which might be potential substrates for a particular rRNA processing reaction. Therefore, further understanding of rRNA processing focused mainly on analysis of mutant strains lacking several known RNases, and not from in vitro biochemistry with rRNA or RNP substrates.

Another aspect of RNA metabolism that became the subject of extensive research was RNA degradation. It was observed that under certain stress conditions like starvation, nutritional downshift, stationary phase, or medium-imposed slow growth, rRNAs were extensively degraded. Such degradation can affect the preexisting ribosomes and newly synthesized rRNAs, if they were not already bound by the ribosomal proteins to assemble into functional active ribosomes. For instance, during very slow growth in E. coli, around 70% of the newly transcribed rRNAs fail to make it into ribosomes and are therefore degraded since free rRNA does not accumulate. These observations signified the importance of balanced synthesis of rRNA and ribosomal proteins, further emphasizing the importance of a clear understanding rRNA processing in detail.
Current understanding of rRNA processing

A. 16S rRNA maturation

In the last few decades, discoveries of new ribonucleases and analysis of their mutant strains have helped scientists to develop a better understanding of the rRNA processing pathway in *E. coli*. However, considering the importance of this pathway and the amount of time elapsed since the early studies described above, progress in this field has been rather slow and a lot still remains to be investigated.

As noted above, in *E. coli*, the rRNAs are co-transcribed as 30S rRNA primary transcripts, which undergo several nucleolytic processing steps to generate individual the mature rRNAs. The substrates for these initial rRNA processing steps are not naked RNAs but are incomplete, under-modified pre-rRNA ribosomal protein complexes. Both the pre-16S and pre-23S rRNAs are excised first from their primary 30S rRNA transcript by the action of RNase III, which has been shown to play two major roles: (a) it provides quality control by requiring the formation of processing stems and (b) it functionally replaces RppH and activates the rRNA substrate for the subsequent processing steps in rRNA maturation by generating 5’ monophosphorylated cleavage products.

Moreover, in *E. coli*, RNase III cleavage is co-transcriptional and thus, the 16S rRNA is separated from the rest of the transcript even before the RNA polymerase finishes transcribing the entire 30S rRNA transcript. This initial RNase III cleavage product (17S rRNA) contains extra 115 nucleotides (nt) at its 5’ end and 33 nt at the 3’ end of mature 16S rRNA, which must be removed to generate the mature species.
As stated above, early studies\textsuperscript{37} had identified a ribonuclease RNase M16 which when inactivated generated the mutant strain BUMMER, and accumulated a 16.3S intermediate containing \( \sim 60 \) extra nts at 5’ and a mature 3’ terminus suggesting that conversion of the 17S precursor to mature 16S rRNA required at least a two-step process. Nevertheless, the RNase activity from this ribonuclease in the context of rRNA processing in \textit{E. coli} remained unclear. However, a major breakthrough occurred in the understanding of 16S rRNA maturation with the discovery of RNase E and RNase G.

In the past few years, RNase E, has been identified as the most active endoribonuclease in \textit{E. coli}. It was discovered independently by two groups, as an essential enzyme involved with RNA turnover and processing, first as the \textit{ams} gene (\textit{alteration of mRNA stability}), named because of its role in the stabilization of a huge number of mRNAs caused by inactivation of the protein using a temperature-sensitive allele\textsuperscript{43,44}, and subsequently as RNase E via the biochemical methods to identify genes responsible for the processing of 5S rRNA\textsuperscript{40,41}. It was soon discovered that the \textit{ams} and \textit{rne} genes were actually the same gene, both encoding RNase E\textsuperscript{45}. Subsequently it was shown that after the initial RNase III cleavage, the 17S rRNA is further processed at the 5’ end by RNase E to generate a precursor molecule having extra 66 nts at 5’ end of the mature 16S rRNA which is subsequently cleaved by RNase G (Figure 3) to generate the mature 5’ end of 16S rRNA\textsuperscript{46}. The absence of the activity from either gene resulted in a dramatic 16S rRNA processing defect and inactivation of both the enzymes blocked the 5’ processing of 16S rRNA completely.
Further study of these enzymes revealed that in the absence of RNase E, a mature 5’ terminus of 16S rRNA can still be generated by RNase G, but it is a very slow process. Additionally in the absence of RNase G, a small amount of 16S rRNA with a mature 5’ end was produced with four or five extra residues at the 5’ end. Surprisingly the precursor that accumulated in the BUMMER strain\textsuperscript{37} was found to be exactly the same as the precursor that accumulated when RNase G (encoded by \textit{rng}) was inactivated\textsuperscript{46}. Moreover, complementation analysis showed that the BUMMER strain lacked active RNase G, and sequence analysis confirmed that the RNase G gene in the BUMMER strain had an 11 bp deletion at nucleotide residues 729–739 leading to a truncated RNase G protein\textsuperscript{47}.

\textit{In vitro} analysis further confirmed the role of RNase E and RNase G in 5’ maturation of 16S rRNA when it was shown that in the \textit{rne rng} or \textit{rng} mutant strains rRNA substrates were partially processed and accumulated extra 115 and/or 66 extra 5’ residues, respectively. These residues were removed when they were treated with RNase G overexpressing strains or with purified RNase E. Based on these observations, it became obvious that earlier identified endoribonuclease RNase M16 is RNase G. Additionally, it was seen that inactivation of RNase E and RNase G together caused a delayed maturation at the 3’ end of 16S rRNA, suggesting the possibility of some degree of coupling between their maturation processes\textsuperscript{46}. The requirement for 5’ processing is not absolute as it has been seen that blocking 5’ maturation still yielded 3’ mature terminus of 16S rRNA, but at reduced rates.
However, the exact mechanism of 3' end maturation of 16S rRNA remained a mystery for a long time until the discovery of the YbeY protein in the late 2000. YbeY, which is a heat shock protein in *E. coli* is highly conserved among bacteria\textsuperscript{48}. Based on the sequence similarity seen with known metal-dependent hydrolases, this gene was suggested to possess potential hydrolytic activity. It was subsequently shown that YbeY is a metal-dependent single-strand RNA specific endoribonuclease which acts at the 3’ end of precursor 16S rRNA after RNase III cleavage and generates 3’ phosphate\textsuperscript{49}. Furthermore, it was suggested that it processes the extra 33nts at the 3’ end of 16S rRNA (Figure 3) along with the exoribonucleases RNase R or polynucleotide phosphorylase (PNPase) to generate the mature end\textsuperscript{49,50}.

These conclusions were based on observations made in strains where YbeY was inactivated along with either RNase R or PNPase. It was seen that inactivation of these enzymes resulted in very little 16S rRNA with mature 3’ termini\textsuperscript{50}, suggesting the possibility of RNase R and PNPase to be potential candidates to carry out the final 3’ end processing. RNase R has been previously observed to cleave RNA substrates having a 3’ phosphate\textsuperscript{51} and has been implicated in the processing of the 3’ terminus of 16S rRNA in other organisms\textsuperscript{52,53}. Similarly, PNPase has been seen to play a crucial role in the 3’ processing of the 23S rRNA in *Arabidopsis thaliana* chloroplasts\textsuperscript{54}.

Another potential candidate that has been predicted to help YbeY in the final 3’ processing of 16S rRNA is YbeZ, a *phoH* paralog (usually found in the same operon as *ybeY*). YbeZ has been predicted to function as a phosphohydrolase\textsuperscript{55} to remove the 3’ phosphate generated by YbeY, thereby its possible role in the 3’ processing of 16S rRNA.
cannot be completely ruled out. Additionally, a potential role of GTPases in the 16S rRNA processing has been observed. Era, an essential and highly conserved cellular GTPase which has been implicated in ribosome maturation\textsuperscript{56}, is also an attractive candidate. Inactivation of Era has been seen to cause a loss of polysomes, accumulation of precursor 16S rRNA, and an increase in the relative amount of free 30S and 50S subunits compared to 70S ribosomes\textsuperscript{57}. Interestingly, Era is co-transcribed with RNase III and they are translationally coupled\textsuperscript{58}.

It is also found in the same operon as \textit{ybeY} in many bacteria, while in some cases like \textit{Clostridiales} BVAB3 str. UP119-5, \textit{ybeY} and \textit{era} are fused into a single gene. Previous work has also shown that Era interacts with the 3’ terminus of the 16S rRNA and generates a product which has three nucleotides of the mature 3’ terminus of 16S rRNA protruding out of the Era/16S rRNA complex\textsuperscript{59}. Therefore, a possible role of Era in 16S rRNA maturation cannot be completely ruled out and requires detailed study to elucidate its exact function.

However, there has been a lack of clarity regarding the actual mechanism of 3’ maturation of 16S rRNA. Another laboratory has shown that four of the known exoribonucleases in \textit{E. coli} namely, RNase II, RNase R, RNase PH and PNPase can process the extra 33 nts at the 3’ end. It was suggested that the absence of these enzymes slow down the 3’ 16S rRNA processing dramatically, but with different efficiencies\textsuperscript{60}. The most severe processing defect was seen in the absence of RNase PH. Mutant strains lacking all the four exoribonucleases were essentially seen to be unable to generate mature 16S rRNA. Additionally it was shown that the absence of 3’ maturation of 16S
rRNA affected the maturation process at the 5’ end too by reducing the processing efficiency of RNase E and RNase G. Moreover, mutant 30S particles accumulated immature 16S rRNA and formed 70S ribosomes very poorly. Therefore, the exact mechanism of 3’ maturation of 16S rRNA still remains unresolved. It could be that there are multiple pathways for this 3’ maturation process.

B. 23S rRNA maturation

In *E. coli*, after the initial RNase III-mediated cleavage, a precursor-23S rRNA molecule is produced. This precursor has three or seven extra nts at the 5’ end and seven to nine extra nts at the 3’ end. Based on previous studies, it was suggested that the extra 5’ residues were removed by endonucleases whereas the extra 3’ nucleotides were processed by exoribonucleases to produce mature 23S rRNA.

However, very little is known about the exact mechanism of this maturation process despite decades of research in this field. Recent work has suggested the possibility of the removal of the extra 5’ nucleotides by an endoribonuclease as no partially shortened products were observed. However, since it could also be due to the processive 5’→3’ activity by an exoribonuclease, additional and detailed analysis is necessary to conclusively establish an endonucleolytic mechanism. However, it should be noted that there is currently no evidence that *E. coli* contains a 5’→3’ exonuclease.

Recently, it has been suggested that RNase G facilitates 5’ maturation of 23S rRNA. Their work showed that inactivation of RNase G led to the accumulation of 23S rRNA precursor intermediate containing extra 77 nucleotides at its 5’ end. Furthermore, *in vitro* cleavage assays showed that RNase G cleaves synthetic RNA containing
containing the extra 77 nucleotides upstream of 5’ terminus of mature 23S rRNA at two sites present in single-stranded regions. However, their data was not adequate to unequivocally prove the role of RNase G in 5’ maturation of 23S rRNA. It could be an activity of ribosomal proteins, considering that precursor 23S rRNA was still observed in assembled ribosomes. Also the possibility of a previously unidentified enzyme cannot be completely ruled out.

On the other hand, 3’ maturation of 23S rRNA has been shown to depend on the activity of exoribonuclease, RNase T. In cells where RNase T was inactivated, precursors with extra 3’ residues accumulated, with little or no 3’ mature 23S rRNA. In vitro studies further confirmed the role of RNase T in 3’ maturation of 23SrRNA when it was shown that on using purified RNase T, the 23S rRNA precursors from an RNase T mutant ribosomes could be rapidly and completely converted to the mature size. Surprisingly, it took much longer to convert the naked 23S precursor to its mature size and many products that were one nt shorter than the mature form were also generated. Based on these observations, two main conclusions were drawn. Firstly, it demonstrated a direct role of RNase T in 3’ maturation of 23S rRNA, and secondly it showed that the later stages of 23S rRNA maturation occur in the ribosome as it was seen to be more efficient and accurate when the rRNA precursor was derived from the ribosome.

The fact that maturation occurred so efficiently with fully assembled ribosomes also suggested that the final 3’ maturation is a very late event in ribosome biogenesis. Initially it was shown that the 5’ processing of 23S rRNA was unaffected by events at the 3’ end. Thus, even though both the ends of 23S rRNA were base paired to form a double-
stranded stem, maturation of the 5’ end was initially considered to be an independent process. Nevertheless, recent studies have seemed to contradict this theory. A recent study has shown that the 5’ and 3’ immature ends of 23S rRNA ends were processed with similar kinetics either in wild-type cells or under conditions with impaired rRNA processing\(^6^4\). These results suggested that the processing events at both ends are coupled and may be co-regulated. Furthermore, it was found that the processing defects which led to the formation of immature 3’ end also caused a reduced rate of 5’ end maturation with some precursor intermediate accumulation\(^6^4\). These observations further indicated that 3’ end processing probably facilitates 5’ end maturation of 23S rRNA.

Although RNase T is known to be required for the removal of the last few residues from the 23S rRNA precursor, a possible role of other exoribonucleases in processing the extra residues at the 3’ end could not be entirely ruled out. Recent work has further helped in elucidating their importance in 3’ maturation of 23S rRNA. It has been suggested that RNase PH and RNase II are the other major enzymes involved in the 3’ maturation process, as their absence resulted in an accumulation of precursor 23S rRNA\(^6^5\).

Moreover, when RNase PH and RNase II were inactivated, defects like delayed ribosome assembly were observed\(^6^6\). Interestingly, the absence of RNase T, which is responsible for the final step of 23S rRNA maturation, conferred a less dramatic defect in ribosome assembly, as compared to when RNase PH and RNase II were inactivated. Therefore, it was concluded that ribosomal assembly is influenced mainly by the processing defects that occur at an early stage of 23S rRNA processing\(^6^6\). Additionally, in
the absence of RNase T, 23S rRNAs with extra residues at 3’ terminus were still observed to be incorporated into ribosomes, with very little effect on the cell growth rate, indicating that final 3’ maturation is not essential for functioning ribosomes\textsuperscript{66}.

At low temperatures, defects in processing of 23S ribosomal RNA at both the 5’ and 3’ ends have also been observed in strains with inactivated DEAD-Box RNA helicases, namely DeaD and SrmB\textsuperscript{67,68}, but not at 37°C. The processing defects seen in the absence of these helicases included the accumulation of precursors containing three to seven unprocessed nucleotides at the 5’ end and seven to nine unprocessed nucleotides at the 3’ end, suggesting their possible role in 23S rRNA maturation. Although their exact role in 23S rRNA maturation has not been completely understood, there has been speculation that they could help in unwinding the double-stranded stem, generating the single-stranded RNA that is more accessible to the ribonucleases and thereby facilitating the processing of 23S rRNA.

C. 5S rRNA maturation

RNase III cleavage also separates a 9S rRNA\textsuperscript{40} from the 30S primary transcript which is then processed by RNase E to release precursor 5S rRNA with three nucleotides on each side of the mature sequence\textsuperscript{39,40,69}. At present, it is ambiguous as to how the three extra nucleotides at the 5’ terminus are removed. However, studies have revealed that the mode of maturation at the 5’ end of 5S rRNA is essentially identical to that observed with the 5’ maturation of 23S rRNA\textsuperscript{63}. Likewise in 23S rRNA, the 3’ maturation of 5S rRNA requires RNase T, as cells lacking the enzyme do not produce any mature 5S rRNA\textsuperscript{69}. 
Additionally, the absence of RNase T, produced a processing intermediate containing two extra 3’ residues and a mature 5’ end. When further investigated, it was seen that this precursor molecule was assembled into functioning ribosomes. Thus, it was concluded that the 5’ maturation of 5S rRNA is independent of events at the 3’ terminus, and the presence of extra 3’ residues has little effect on ribosome function.

This observation was further confirmed from in vitro analysis where it was shown that purified RNase T can accurately and efficiently remove the extra 3’ residues from ribosomes derived from RNase T-deficient cells to generate the mature 3’ end. Moreover, like the 23S rRNA study, when the same experiment was performed with naked RNA substrate, it took a longer time to process and the final product generated lacked the normal 3’ terminal residue of mature 5S rRNA. These data further emphasized the importance of the ribosome context for efficient and accurate rRNA maturation.

There was striking similarity seen in the processing pattern of the 3’ termini of 23S and 5S rRNA as in both the cases, only RNase T performed the final removal of the 3’ residues to generate the mature 3’ terminus. This similarity of RNase T can be explained based on previous studies where it has been shown that RNase T can process residues close to a double-stranded stem. In case of mature 5S rRNA, there is only a single unpaired 3’ residue following the stem, and in 23S rRNA, there are two unpaired residues. Therefore, in each case, removal of the last precursor specific residues would require action adjacent to a stem, which RNase T has been shown to efficiently process. For both 23S and 5S rRNA, initial shortening can be done by others in the absence of
RNase T, but for removal of the last few residues, RNase T is essential. Moreover, like 23S rRNA, 3’ maturation of 5S rRNA has also been suggested to be a very late event during ribosome biogenesis.

D. Maturation of tRNAs contained in the seven rRNA operons

As shown in Figure 1, there are 14 tRNAs that are contained within 7 ribosomal RNA operons. Extensive research over the last few decades has shown that the essential endoribonuclease RNase P is responsible for the 5’ maturation of the tRNAs after the initial cleavage of the 30S primary transcript by RNase III. It hydrolytically cleaves the 5’ leader sequences from tRNA precursors to produce the mature 5’-termini, which are essential for generating functional tRNAs. The 3’ maturation of these tRNAs is primarily carried out by RNase T, although other known exoribonucleases facilitate 3’ maturation of tRNA with different efficiencies.

Role of primary and secondary 30S rRNA processing enzymes in other aspects of RNA metabolism

RNase III – In E. coli, RNase III (encoded by rnc) exists as an active dimer of two identical 25.4- kDa polypeptides. Only one subunit is required for catalytic activity and substrate specificity. It is a Mg²⁺-dependent nuclease, which has been shown to cleave phosphodiester bonds, creating 5’ phosphate and 3’ hydroxyl termini with an overhang of 2 nts. RNase III is unique among all the other known endoribonucleases in E. coli due to its ability to cleave double-stranded RNA molecules. It requires a substrate with a double-stranded region of 15–20 base pairs.
RNase III is highly conserved and is found in bacteria, fungi, plants, insects and mammals. Based on their structural organization these enzymes have been broadly grouped into three classes. Class 1 includes *E. coli* RNase III and its counterparts from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, Rntp1 and PacI. These enzymes contain one RNase III (RIII) domain and one double-stranded RNA Binding Domain (dsRBD). *Drosophila melanogaster* Drosha, which possesses two RIII domains (RIIIa and RIIIb) and a dsRBD, belongs to Class 2. This group of enzymes is mostly restricted to animals. Lastly, the members of class III include human Dicer and homologs are found in *S. pombe*, plants and animals.

Studies have shown that other than its role in rRNA processing, bacterial RNase III can also alter gene expression either by cleaving dsRNA or by binding without cleaving and changing RNA structure. In *E. coli* RNase III has been shown to autoregulate its own expression by cleaving the stem-loop structure located upstream of the *rnc* coding sequence, resulting in destabilization of its own mRNA\textsuperscript{76,77}.

Moreover, RNase III regulates the expression of PNPase (encoded by *pnp*) by cleaving the stem loop located near the 5ʹ end of the *pnp* mRNA\textsuperscript{78} However, in the case of bacteriophage lambda *CIII* gene regulation, binding of RNase III to a dsRNA structure without subsequent cleavage is suggested to alter the structure of the mRNA and thereby stimulate its translation\textsuperscript{79}. Additionally, recent studies using tiling microarrays have shown that approximately 12% of genes in *E. coli* are affected in an RNase III deletion strain, and the half-lives of total pulse-labeled RNA were not significantly affected in an RNase III deletion, indicating that RNase III plays a minor role in the mRNA decay.
pathway\textsuperscript{80,81}. Besides its role in rRNA processing and mRNA regulation, RNase III also participates in maturation of some tRNAs. However, surprisingly RNase III is not essential in \textit{E. coli}\textsuperscript{82}.

\textbf{RNase E} – This essential enzyme is a homotetramer of a large, 1061-amino-acid multi-domain protein that can be roughly divided into two halves. The N-terminal half of RNase E contains the catalytic core containing five subdomains; an RNase H domain, a S1 RNA binding domain, a 5′ sensor region, a DNase 1 domain, and a Zn\textsuperscript{2+} link\textsuperscript{83}. The S1 RNA binding domain interacts with the 5′ monophosphorylated RNA and permits access to the catalytic site of single-stranded RNA.

As a result, mutations in this region lead to thermolabile RNase E activity as seen in \textit{rne-1} (a point mutation at residue 66, used in this study) allele\textsuperscript{84}. Moreover, a truncated RNase E protein produced from the deletion of Zn\textsuperscript{2+} link region, was still seen to be sufficient to maintain the cell viability\textsuperscript{85}. All these results indicated that tetrameric quaternary structure is not essential for the core enzymatic function of RNase E.

The central region of the RNase E protein contains an arginine-rich RNA-binding site (ARRBS), which when deleted yields strains with almost identical growth properties and only a slight defect on mRNA decay compared with the full length protein\textsuperscript{86,87}.

The C-terminal domain of RNase E, which is an exceptionally long stretch of natively unstructured protein with no known enzymatic activity but it has been shown to serve as a scaffold for the formation of a multi-component complex called the RNA
degradosome\textsuperscript{88–90}. The RNase E-based degradosome contains the 3' → 5' phosphorolytic exoribonuclease polynucleotide phosphorylase (PNPase), a DEAD-box ATP-dependent RNA helicase (RhlB), and the glycolytic pathway enzyme enolase\textsuperscript{88,91–95}.

RNase E is a multifunctional enzyme in \textit{E. coli}, which has been shown to play significant role in many aspects of RNA metabolism in \textit{E. coli}. It is involved in the maturation of tRNAs\textsuperscript{86,96,97}, the processing of 16S rRNA precursors\textsuperscript{46,47}, the maturation of the M1 subunit of RNase P\textsuperscript{86,98}, the regulation of DNA synthesis by ColE1-type plasmids\textsuperscript{99}, the tmRNA mediated control of mRNA quality\textsuperscript{100}, the decay of small noncoding regulatory RNAs\textsuperscript{101,102}, and in the degradation of most mRNAs\textsuperscript{44,87,103–105}. RNase E cleaves a single-stranded RNA with a preference for A/U-rich sequences, and generates products with a free 3'-hydroxyl group and a 5'-monophosphate\textsuperscript{106–108}. In addition, RNase E seems to be a 5' end-dependent endoribonuclease\textsuperscript{109,110}, although under certain circumstances, the enzyme can cleave at internal sites without any interaction with the 5'-terminus\textsuperscript{111}.

Biochemical characterization of RNase E has revealed that it preferentially cleaves substrates with a 5'-monophosphate over those with a 5'-OH or triphosphate group\textsuperscript{109,112,113} and degrades the substrates processively or quasi-processively in the 3'→5' scanning mode\textsuperscript{114}. RNase E autoregulates its own synthesis by controlling the degradation rate of its mRNA (rne), such that its half-life varies inversely with cellular RNase E activity\textsuperscript{115,116}.
The C-terminal region of RNase E has also been implicated in the autoregulation of protein levels\textsuperscript{87,117}. In addition, the synthesis of RNase E is also affected by the cellular poly(A) levels and the composition of growth medium\textsuperscript{118,119}. Overall, it has been seen that the cellular level and activity of RNase E in \textit{E. coli} is tightly controlled at many different levels.

\textbf{YbeY}- The recently identified endoribonuclease YbeY (encoded by \textit{ybeY}) has been shown to play a role in ribosome quality control\textsuperscript{49} as well as functioning in 3′ maturation of 16S rRNA. Based on recent data it has been observed that inactivation of the \textit{ybeY} gene causes severe 30S ribosome assembly defects thereby affecting translation fidelity\textsuperscript{48}. Subsequently, it was shown that YbeY acts as a sensor for defective 70S ribosomes that contain defective 30S subunits\textsuperscript{49}. The Walker lab, proposed a model which suggested that once the cell senses the defective ribosomes, it initiates degradation of 70S ribosomes by making one or more YbeY mediated endonucleolytic cleavages in the rRNA. Subsequently, RNase R unwinds the rRNA with its helicase activity and degrades the rRNA\textsuperscript{49}.

Moreover, recent work has suggested an additional role of YbeY in transcriptional antitermination, which is critical for the production of ribosomal subunits and therefore explains the defect observed in rRNA maturation. It has been shown that YbeY is essential for \textit{rrn} transcription of regions that contain the antitermination sequences. In absence of YbeY, transcription from the P1 promoter of \textit{rrn} was shown to be unaffected, but in \textit{rrn} operons containing P2 promoter along with \textit{nut}-like sequences which constitute the antitermination region, the transcription was almost abolished\textsuperscript{120}. These findings were
compatible with the previously identified role of YbeY in 3’ maturation of 16S rRNA\textsuperscript{50} as correct transcription antitermination process is essential for accurate maturation of rRNAs. Moreover, YbeY has also been shown to play a key role in small RNA (sRNA) regulation and modulate Hfq-dependent and independent sRNA-mRNA interactions\textsuperscript{121}, therefore emphasizing the importance of this enzyme in RNA metabolism in \textit{E. coli}.

\textbf{RNase G} –RNase G is a paralogue to RNase E and shares around 34\% sequence identity and 50\% sequence similarity to the 470 amino acids near the N-terminal end of the RNase E protein\textsuperscript{122,123}. However unlike RNase E, RNase G is nonessential\textsuperscript{47} and is expressed at much lower levels (~3\%) than RNase E\textsuperscript{124}. Nevertheless, it has been shown that if RNase G is significantly overproduced in an \textit{rne} mutant, it can partially complement the effect of \textit{rne} mutation\textsuperscript{125}. Moreover, recent work has further confirmed its role in complementing the RNase E activity by using the \textit{rng-219} which can substitute for RNase E at normal levels\textsuperscript{126}. Additional roles of RNase G include processing of 6S RNA\textsuperscript{127}, some tRNAs\textsuperscript{128,129}, and specific mRNA transcripts\textsuperscript{130}.

\textbf{RNase P} –RNase P is an essential endoribonuclease, which was initially discovered in attempts to elucidate the biosynthesis of tRNA precursors in \textit{E. coli}\textsuperscript{131}. It is highly conserved and is found in all the three domains of life\textsuperscript{71,132}. A unique property of RNase P in \textit{E. coli} is that it is made up of two components, a catalytic RNA subunit known as the M1 RNA, encoded by the \textit{rnpB} gene\textsuperscript{133–135}; and a protein component, known as C5, encoded by the \textit{rnpA} gene\textsuperscript{135,136}.
Previous research has shown that the M1 RNA can process tRNA precursors in the absence of the C5 protein, but the presence of the C5 protein greatly influences the efficiency of cleavage and is required for RNase P activity and cell viability *in vivo*\(^{137,138}\).

The enzyme has also been shown to be involved in maturation of a number of other RNAs including the 5′-end of 4.5S RNA\(^{139}\), tmRNA\(^{100}\), small RNAs, tRNAs\(^{128,129,140,141}\), as well as processing polycistronic mRNA transcripts\(^{142}\). A genome-wide survey of RNase P activity has identified cleavage sites within the intercistronic regions of numerous polycistronic operons, indicating that RNase P has a very limited role in mRNA turnover\(^{142}\).

**Summary**

In *E. coli*, the three rRNAs are co-transcribed as part of a 30S rRNA primary transcript. In wild type cells, RNase III initially cleaves the 30S rRNA to separate the individual rRNAs. The initial cleavages are suggested to be co-transcriptional as in wild-type cells and the 30S rRNA accumulation is not observed. Subsequent to the RNase III cleavages, other endo- and exoribonucleases further process the rRNAs to generate their mature species. The cleavage of the 30S rRNA precursor is one of the best known functions of RNase III *in vivo*. When steady-state RNA is examined from exponentially growing *E. coli* RNase III deficient strains, the 30S precursor can be visually identified on agarose gels\(^{82}\). However, based on preliminary studies, we observed that the amount of detectable 30S rRNA precursor in *rnc* mutants accounted for only a small fraction (<5%) of the total processed rRNAs in the cell. In the absence of RNase III, 23S rRNA
processing defects were seen, but 16S rRNA maturation appeared normal. These results indicated the presence of an effective RNase III-independent 30S rRNA processing pathway in which other ribonucleases were able to process the 30S rRNA transcript very efficiently.

The work described in Chapter 2 identifies some of the enzymes involved in the alternative RNase III-independent 30S rRNA processing pathway. Our data suggest that the primary processing enzymes for the RNase III-independent pathway, namely RNase E, RNase G and YbeY perform the initial cleavage of 30S rRNA in absence of RNase III. Inactivation of these three enzymes in absence of RNase III makes the cell extremely sick. Furthermore, there is a large accumulation of 30S rRNA and very little or no mature 16S rRNA is produced. We also show that the alternative RNase III-independent 30S rRNA processing pathway has unique processing sites. When these processing sites were mapped, some of them were found in the AU rich regions which is a preferred substrate for RNase E activity\(^{108}\). These results support our hypothesis that RNase E is a primary processing enzyme in the alternative processing pathway.

However, some of the processing sites were located in the double-stranded stem region of 16S and 23S rRNA in the alternative processing pathway, suggesting the possible activity of a double-strand specific endonuclease or some helicase activity. Moreover, 23S rRNA was still produced in the absence of RNase III, RNase E, RNase G and YbeY, further emphasizing on the possibility of other enzyme(s) being involved in the pathway.
In the search for additional enzymes involved in this pathway, we did bioinformatics analysis in which we identified genes with possible endonucleolytic activity. The work described in Chapter 3 explores the possibility of new enzymes being involved in this alternative 30S rRNA processing pathway. Here we show that the newly identified genes - YhgF, YraN and YhbQ act as secondary processing enzymes in the alternative processing pathway. Their function is not essential when RNase E, RNase G or YbeY are expressed at wild-type levels in the cell. But, in the absence of these processing enzymes, inactivating YhgF or YraN or YhbQ makes the cell very sick. Moreover, absence of these enzymes led to a large increase in the precursor-23S rRNA accumulation with very little mature 23S rRNA being produced. Using northern blot analysis, we have further identified the length of this precursor-23S rRNA intermediate, which is a product of the initial cleavage of 30S rRNA in the alternative processing pathway. We found that it extends from the spacer region downstream of mature 3’ terminus of 16S rRNA to the 3’ terminus of 5S rRNA. Our data suggests that YhgF, YraN and YhbQ help in the processing of this precursor intermediate to generate mature 23S rRNA. However, we have not established the exact nature of the enzymatic activity of these newly identified genes.

In *E. coli* the rRNAs are co-transcribed as 30S rRNA from seven different ribosomal operons (*rrn A, rrnB, rrnC, rrnD, rrnE, rrnG* and *rrnH*) as shown in Figure 1. Although the basic organization of the seven operons are same with a 16S rRNA near the promoter region followed by a spacer tRNA, a 23S rRNA and a 5S rRNA at the 3’ end, there are some differences among them with respect to the tRNA identity, tRNA copy number, 5S
rRNA copy number and the spacer sequences. These heterogeneities can affect the cleavage sites of the processing enzymes. The first section of Chapter 4 focuses on understanding the effect of these heterogeneities on the processing sites of the enzymes involved in the alternative processing pathway. In order to study the 30S rRNA processing from different rrrn operons, we first constructed strains where chromosomally encoded rrrn operons were deleted and the cells were kept viable using a single rrrn operon plasmid and a tRNA plasmid. Our study focused mainly on the rrrnD and rrrnG operons since they had the maximum differences among them with regard to the organization of RNAs (Figure 1). The rrrnD operon has two spacer tRNAs in between 16S and 23S rRNA as compared to one tRNA in rrrnG (Figure 1). Moreover, rrrnD has two 5S rRNAs, whereas rrrnG has just one 5S rRNA (Figure 1). Therefore, in light of the difference in organization they have, there is a possibility that the processing sites in the alternative pathway may differ between them. In order to study if this hypothesis is correct, RNase III, RNase E and YbeY were inactivated in the single rrrn genetic backgrounds. Subsequently, northern blot analysis showed that inactivating the primary processing enzymes from both the RNase III-dependent and -independent pathway had more impact on the 30S rRNA accumulation in rrrnD operon as compared to rrrnG. Moreover, using primer extension analysis we showed that the alternative processing pathway had different processing sites in different rrrn operons.

The second section of Chapter 4 is focused on understanding the significance of the conservation of location of the spacer tRNA in between the 16S and 23S rRNA in all the seven operons. In order to study the role of spacer tRNA, we constructed a rrrnB operon
plasmid (pGCK-1) where the spacer tRNA in between the 16S and 23S rRNA was deleted and replaced with a unique restriction site. Subsequently, pGCK-1 was used to displace the *rrnB* operon plasmid in wild-type and RNase III deficient strains. We found that presence of the spacer tRNA in between 16S and 23S rRNA was not essential in presence of RNase III. However, in an *rnc* deletion strain, we were unable to displace the *rrnB* operon plasmid with pGCK-1, suggesting the possibility that the presence of a tRNA in between 16S and 23S rRNA is probably essential in the alternative processing pathway. Such a requirement indicates that in absence of RNase III, the spacer tRNA changes its secondary structure and probably folds itself into a conformation such that it facilitates the activity of the alternative 30S rRNA processing enzymes. Nevertheless, further analysis needs to be done in order to confirm the spacer tRNA’s role in the alternative processing pathway. Overall, the work presented here provides a detailed understanding of the 30S rRNA processing pathway in *E. coli* which in future, will help in a better understanding of RNA processing in higher organisms.

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Figure 1.1. Ribosomal RNA operons in *E. coli*.

Each of the seven operons are identified on the left side by their respective *rrn* designations. Mature rRNAs or tRNAs are indicated by the blue, yellow, pink and red bars. This model is not drawn to scale.
Figure 1.2. 30S Ribosomal RNA primary transcript in *E. coli*. The mature 16S (blue) and 23S rRNA (pink) is looped out in the stem-loop structure. The double-stranded stems for 16S and 23S rRNA have lengths of ~36 and ~28 base pairs respectively. Mature 5S rRNA and tRNA is indicated by the yellow and red bars. This model is not drawn to scale.
**Figure 1.3. Model of *E. coli* rRNA processing.** The 30S pre-rRNA transcript is first cleaved by RNase III at two physical locations, cleaving the double-stranded rRNA formed by the spacer sequences adjacent to mature 16S and 23S rRNAs, generating 17S, 25S, and 9 pre-rRNAs (labeled in green). 17S rRNA is cleaved initially by RNase E and then by RNase G to generate the mature 5’ end of 16S rRNA. YbeY along with RNase processes the extra 33nts at the 3’ end of 17S rRNA to generate the mature 3’ terminus of 16S rRNA. RNase E also cleaves 9S three nt on each side of the mature termini of 5S rRNA forming a p5S precursor. RNase P cleaves to produce the mature 5’ end of the tRNA. Exoribonucleases (primarily RNase T) are responsible for the 3’ end maturation of the tRNA, 23S rRNA, and 5S rRNA, but the endoribonuclease(s) responsible for the maturation of 5’ end of 23S, and 5’ end of 5S rRNAs remain unidentified (labeled in red). This model is not drawn to scale.
CHAPTER 2

ANALYSIS OF 30S rRNA PROCESSING IN \textit{ESCHERICHIA COLI} IN ABSENCE OF RNASE III

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ABSTRACT

For many years it has been assumed that RNase III is solely responsible for the initial processing of the 30S primary ribosomal RNA (rRNA) transcript in *Escherichia coli*. However, RNase III is not essential and very little 30S rRNA (<5% of total RNA) accumulates in its absence, suggesting the existence of an effective alternative processing pathway for the 30S primary transcript. Here we show that in the absence of RNase III a significant fraction of 30S rRNA is initially processed by a combination of RNase E, RNase G and YbeY to separate the pre-16S rRNA from the rest of the transcript. The inactivation of RNase E, RNase G and YbeY in a Δrnc genetic background led to a severe processing defect with ~14-16-fold increase in 30S rRNA levels and a concomitant decrease in the amount of mature 16S, 23S and tRNA compared to RNase III single mutant. Multiple cleavages within the 30S rRNA that are unique from the RNase III- dependent pathway have been identified by primer extension analysis. Our data suggest that beyond RNase E, RNase G, RNase P and Ybey there are additional enzymes involved in 30S rRNA processing pathway, particularly at 44°C.
INTRODUCTION

Ribosomal RNAs (rRNAs) - 16S, 23S and 5S rRNAs in Escherichia coli are matured from the larger 30S primary transcripts (Figure 1), which is initially processed by RNase III, a double-stranded RNA specific endoribonuclease encoded by rnc. RNase III which is conserved in all forms of life, has also been shown to be involved in the initiation of decay of a small, but significant proportion of mRNAs. In higher eukaryotes, Dicer, a member of the RNase III family, plays a crucial role in RNA interference (RNAi). In E. coli RNase III cleaves at four specific locations within the primary 30S rRNA transcripts to release 17S (pre-16S), 25S (pre-23S) and 9S (pre-5S) rRNA precursors (Figure 1). The pre-16S species are subsequently processed by RNase E and RNase G (encoded by rne and rng, respectively) to generate the mature 5′ end of 16S rRNA. Pre-5S rRNA is further processed by RNase E within 3 nt on each side of the mature sequence. The 3′ ends of 5S and 23S rRNA are matured by the 3′→5′ exoribonuclease, primarily RNase T. However, not much is known about the final maturation steps of 3′ end of 16S rRNA and the 5′ ends of the 23S and 5S rRNAs. A newly identified ribonuclease YbeY, encoded by ybeY has been suggested to process the extra 33 nt at the 3′ end of pre-16S rRNA to help generate the mature species.

In addition to RNase III, E. coli contains six additional well-characterized endonucleases, RNase E, RNase P, RNase G, RNase I, RNase LS and RNase Z but these are all specific for single-stranded RNA. RNase E, considered to be the most important among these is an essential enzyme with multiple functions including mRNA decay, rRNA maturation and tRNA processing. Its two well established roles in rRNA maturation are the removal of 66 nt from the 115 nt leader at the 5′ end of the pre-16S
rRNA\(^{19,20}\) and the processing of 9S rRNA (the precursor of 5S rRNA) after the initial RNase III cleavages of the 30S primary transcript\(^8\) (Figure 1).

RNase P, another essential enzyme in *E. coli*, is a ribonucleoprotein consisting of a protein (C5) and a catalytic RNA (M1) subunit\(^{21,22}\) responsible for the removal of the extra nucleotides from the 5′ end of tRNAs\(^{23}\). RNase P has also been shown to be involved in the maturation of 4.5S (a component of the essential signal recognition particle)\(^{24}\), other small RNAs\(^{25}\), the processing of a few polycistronic mRNAs\(^{26}\) and polycistronic tRNAs\(^{27–29}\).

RNase G has been shown to generate the mature 5′ terminus of the 16S rRNA after RNase E has shortened the 5′ leader of the pre-16S rRNA\(^{20}\). There is currently no evidence to suggest that RNase I, RNase LS or RNase Z play any role in the maturation of rRNAs.

Interestingly, it has previously been shown that functional ribosomes can still be made in an RNase III-deficient strain in which normal maturation of 23S rRNA does not occur, but mature 16S rRNA is still made\(^{30}\). Cells can use pre-23S rRNA that has not been matured completely in ribosome biosynthesis without a significant effect on growth and viability\(^6\). In contrast, pre-16S rRNA species cannot be incorporated into functional 30S ribosomes\(^{31}\). Overall, not much is known about how the 30S primary transcript is processed in absence of RNase III.

Here we show that the 30S primary transcript accounts for less than 5% of the total rRNA in a strain of *E. coli* carrying a complete deletion of the *rnc* gene. The Δ*rnc* mutant has a generation of 50 minutes compared to 30 minutes for the *rph-1* control. Analysis of multiple mutants in the Δ*rnc* genetic background indicates that RNase E, RNase G and
YbeY in combination play important roles in releasing the pre-16S species from the primary transcript and are required for the further maturation of the 23S and 5S species. RNase P is involved in the maturation of the 5′ termini of the tRNAs embedded in the rRNA operon transcripts. RNase Z may play a secondary role in rRNA maturation, but RNase I, RNase LS, two DEAD box RNA helicases (SrmB and Dead) as well as the three potential CRISPR/Cas endonucleases (CasE, Cas3 and Cas2) have no roles in rRNA maturation. Additionally, in the alternative processing pathway described here none of the 23S rRNA species have the 5′ terminus found in strains carrying a functional RNase III protein.

RESULTS

Elimination of various candidates for alternative processing pathway

The demonstration that RNase III was not essential in *E. coli* involved the use of either the *rnc*−14 allele (a Tn10 insertion)\(^{32}\) or the *Δrnc38* mutation that has 38 amino acids deleted from the coding sequence\(^{10}\). Initially both the *Δrnc38* and *rnc*−14 were used to construct a series of multiple mutants. In our first experiments we constructed strains carrying either the *Δrnc38* and *rnc*−14 alleles and deletions in the structural genes for RNase LS and RNase I. Since mutations in the DEAD-box RNA helicases SrmB and Dead have been shown to cause defects in ribosome assembly and rRNA processing\(^{33}\), we also examined strains carrying deletions of these two enzymes. Moreover, endonucleases which are normally thought to be inactive in *E. coli*, such as the members of the CRISPR/Cas genome defense system (Cas E, Cas 2 and Cas 3)\(^{34-38}\) were also candidates for testing since the CRISPR/Cas system is repressed by a small RNA which is dependent on RNase III for activity\(^{39}\). However, our analysis of various multiple
mutants indicated that RNase LS, RNase I, SrmB, DeaD, Cas E, Cas 2 and Cas 3 were not involved in the rRNA processing in the absence of RNase III (data not shown).

**Construction of a complete rnc deletion**

Both the Δrnc38 and rnc-14 alleles are marked with drug resistance markers that cannot be removed. Additionally, in our initial attempts to construct multiple mutants containing either the Δrnc38 and rnc-14 alleles and mutations in the genes for RNase E, RNase G and RNase P, we frequently encountered significant technical problems (explained in Materials and Methods). Some of these issues appeared to occur because of the synthesis of truncated RNase III proteins (data not shown). In fact, using a monoclonal antibody against RNase III, we detected a small amount of a truncated RNase III protein in an Δrnc38 strain (SK7622). Accordingly, we set out to construct a complete deletion of the rnc coding sequence which was marked with a drug resistance cassette and could be removed by employing the Flp recombinase. Since the rnc gene is the first open reading frame (ORF) in the rnc era recO operon and the era gene is essential for cell viability, we replaced the entire rnc coding sequence except for the translation start and stop codons with an apr cassette flanked with FRT sites. In addition, the bla promoter was added immediately upstream of the era coding sequence to transcribe the era and recO genes.

**30S rRNA is efficiently processed in the RNase III deficient strain**

Initial 30S rRNA processing to pre-rRNAs is absolutely required for generation of mature rRNAs. Since the timing of rRNA transcription and RNase III processing are closely coupled, the coordination of these two processes is important for rapid folding of rRNAs and assembly of ribosomal subunits. However, it has been shown previously that
functional ribosomes are made in the absence of RNase III. In order to assess the importance of RNase III in *E. coli* rRNA processing, we compared the growth rate and accumulation of 30S rRNA in an RNase III deletion (*Arnc::apr*) mutant compared to a wild type control. The *Arnc* mutant had a doubling time of 50 minutes compared to 30 minutes for the *rph-1* control strain (Figure 2). Most importantly, high resolution synergel/agarose gel analysis showed that in exponentially growing cells, where rRNA transcription rate is the highest, 30S rRNA constituted only 2-6% of total RNAs in *Arnc* mutant under steady-state conditions (Figure 3B, lane 2). No 30S primary transcript was observed in the *rph-1* strain (Figure 3B, lane 1). The levels of mature 23S (*M*23) and 16S rRNAs (*M*16) were only reduced by ~9-10% in the *Arnc::apr rph-1* strain compared to the *rph-1* control. A species (I) larger than the mature 23S rRNA was also present in the *Arnc::apr rph-1* mutant (Figure 3B, lane 2). Taken together, these data suggested that there was an efficient RNase III-independent 30S rRNA processing pathway in *E. coli*.

**RNase P and YbeY contribute in 30S rRNA processing in the absence of RNase III**

In order to determine which enzymes were responsible for 30S rRNA processing in the absence of RNase III, we initially tested if RNase P and YbeY played any role. RNase P was selected because of its role in the processing of the 5' termini of tRNAs and all seven rRNA operons have at least one tRNA embedded between the 16S and 23S species in the 30S transcripts43. YbeY was also an interesting candidate because of its function in pre-16S processing12. Accordingly, we examined the 30S rRNA processing under steady-state conditions in *Arnc::apr rnpA49 rph-1, Arnc::apr AybeY::cat rph-1* and *Arnc::apr Aybe::catY rnpA49 rph-1* mutant strains grown at either 30°C or 44°C by northern analysis using rRNA-specific oligonucleotide probes (Figure 4A).
We first probed the blot with an oligonucleotide complementary to the mature 5′end of 16S rRNA (Figure 4A, probe a). As expected, the *rph-1* strain only had mature 16S rRNA (Figure 4B, lanes 1-2) at both temperatures compared to the Δ*rnc::apr rph-1* double mutant where some 30S rRNA was observed in addition to the mature 16S rRNA species (Figure 4B, lanes 3-4). However, the level of the 30S rRNA increased ~4.5-fold in Δ*rnc::apr rnpA49 rph-1* mutant (Figure 4B, lanes 5-6) and ~5.5-fold in the Δ*rnc::apr ΔybeY::cat rph-1* mutant (Figure 4B, lanes 7-8) compared to the Δ*rnc::apr rph-1* double mutant, indicating a role for both RNase P and YbeY in the alternative processing pathway. Furthermore, 16S rRNA processing was significantly altered in the Δ*rnc::apr ΔybeY::cat rph-1* triple mutant. At the non-permissive temperature, the level of both mature 16S and 17S rRNAs was reduced significantly and with an increase in 16S* (a slightly smaller species) rRNA species (Figure 4B, lanes 7-8). The data was consistent with recently identified role for YbeY, suggesting that it helps in the 3′ maturation of 16S rRNA. It should be noted that in all the strains tested (Δ*rnc::apr rph-1*, Δ*rnc::apr rnpA49 rph-1*, Δ*rnc::apr ΔybeY::cat rph-1*) the levels of the primary 30S transcript decreased at 44°C.

Similar results were obtained when the blot was probed with an oligonucleotide that was complementary to the mature gltT tRNA (four of the seven *rrn* operons have a glt tRNA in between the 16S and 23S species and all the glt tRNAs have the same mature sequence) and 23S rRNA (probes b and c respectively, Figure 4A). In both cases, the steady-state levels of 30S species were significantly higher in Δ*rnc::apr rnpA49 rph-1* and Δ*rnc::apr ΔybeY::cat rph-1* triple mutants compared to the Δ*rnc::apr rph-1* double
mutant (Figure 4B). The 23S rRNA specific probe c detected a prominent species-the pre-23S precursor processing intermediate (labeled as I in Figure 4B), which might have been a product of a slow processing mechanism in absence of RNase III. Furthermore, the processed fraction (PF) of mature gltT tRNA was reduced to ~29% in the \( \Delta rnc::apr \Delta yeY::cat rph-1 \) strain and ~31% in the \( \Delta rnc::apr rnpA49 rph-1 \) triple mutant compared to ~50% in \( \Delta rnc::apr rph-1 \) double mutant, suggesting that the inactivation of either YbeY or RNase P affected the maturation of the tRNA in absence of RNase III (Figure 4B). Interestingly, there was no significant defect in the maturation of 23S rRNA, as there were comparable levels of mature 23S rRNA compared to the \( rph-1 \) control (Figure 4B). Surprisingly, we have not been able to make the \( \Delta rnc::apr rne-1 rph-1 \) strain after several attempts.

**RNase E and YbeY carry out the initial cleavages of the 30S rRNA transcript in the absence of RNase III**

Based on the data shown in Figure 4, we hypothesized that YbeY and probably RNase E played more significant roles in the alternative 30S rRNA processing pathway compared to RNase P. In order to test this idea, we constructed multiple mutants containing mutations in RNase III, RNase E and YbeY (\( \Delta rnc::apr rne-1 \Delta yeY::cat rph-1 \), \( \Delta rnc::apr rnpA49 \Delta yeY::cat rph-1 \) and \( \Delta rnc::apr rne-1 rnpA49 \Delta yeY::cat rph-1 \)). We examined 30S rRNA processing in these strains along with a \( \Delta rnc::apr rph-1 \) double mutant using northern analysis and RNA-specific oligonucleotide probes a, b and c (Figure 5A). With steady-state RNA isolated from cells grown at 30°C and a probe specific for 16S rRNA (probe a), the highest level of 30S rRNA was seen in \( \Delta rnc::apr \)
rne-1 ΔybeY::cat rph-1 quadruple mutant (∼7.7 fold, Figure 5B lanes 3-4). When the cultures were shifted to non-permissive temperature to inactivate RNase E, very little mature 16S rRNA (labeled as M₁₆) was produced (the processed fraction (PF) of mature 16S rRNA dropped from 39% to 19%) with a concomitant increase in 18S and 16S* rRNA intermediates. In addition, with the appearance of the smaller species (16S* rRNA), the precursor 17S rRNA also became barely visible. These results suggested that in absence of RNase III, when RNase E and YbeY, which are both instrumental in the 16S rRNA maturation process in the RNase III-dependent pathway, the cell could not effectively process the 17S rRNA intermediate to generate any mature 16S rRNA leading to the generation of a truncated processing product, 16S* rRNA.

The increase in the accumulation of the smaller 16S* rRNA species was indicative of defective 16S rRNA processing. However, the increase in 18S rRNA could be because of a very slow and inefficient processing of the existing 30S primary transcript by residual RNase E activity. Moreover, inactivating RNase P in addition to RNase E and YbeY did not lead to an increase in the amount of the 30S transcript, but it did alter the number and amount of the various 16SrRNA species (Figure 5B, lanes 5-8).

When the blot was probed with an oligonucleotide complementary to mature 5' glutT tRNA (Figure 5A, probe b), there was around ∼11-fold more 30S rRNA in the Δrnc::apr rne-1 ΔybeY::cat rph-1 quadruple mutant as compared to RNase III deficient strain at the permissive temperature. Interestingly, when the cells were shifted to the non-permissive temperature, the increase in the level of the 30S primary transcript decreased to 9-fold, but the amount of the pre-23S rRNA intermediate increased dramatically (Figure 5B, lane 4).
A similar effect was observed when the blot was probed for the mature 5' 23S rRNA (Figure 5A, probe c). This was because the ribosome assembly and downstream translation process was severely affected at higher temperature in the absence of processing enzymes. As a result, the feedback system senses the low level of translationally competent ribosomes and reduces transcription initiation at the rrn P1 promoters\textsuperscript{44,45}. Even though the transcription of new RNA would be stalled, the existing 30S rRNA is probably processed slowly by residual activity of RNase E activity, generating the precursor intermediate I (Figure 5B, lane 4).

Additionally, with the shift to the non-permissive temperature, there was a significant reduction in mature 23S rRNA (M\textsubscript{23}) that accompanied the increase in the precursor intermediate (I) in both the Δrnrc::apr rne-1 ΔybeY::cat rph-1 (~26% less) and Δrnrc::apr rne-1 rnpA49 ΔybeY::cat rph-1 (~15% less) strains. These results indicated that the residual RNase E activity was not sufficient to process the precursor I to further generate mature 23S rRNA and gltT tRNA species.

The composition of intermediate I was subsequently determined by probing the blot with oligonucleotides that were complementary to various regions of the operon. The probes complementary to different regions in the spacer region downstream of the 3' end 16S rRNA hybridized to this species. Species I also hybridized to probes complementary to 5' and 3' ends of spacer tRNA, 23S rRNA and 5S rRNA but not to probe a. These results indicated that the intermediate I extend from the spacer region downstream of 3' 16S rRNA end to the 3' end of 5S rRNA.

Since this precursor is generated only in absence of RNase III, it indicated that the initial cleavage of 30S rRNA in the alternative processing pathway occurs at the 5' end of
this intermediate. Moreover, the highest 30S rRNA and precursor I accumulation was seen in $\Delta rn:\text{::apr} \text{rne-1} \Delta ybe:\text{::cat} \text{rph-1}$ strains both at permissive and non-permissive temperatures (Figure 5B, lanes 3-4), with a concomitant reduction in the mature RNA species irrespective of the probes used, suggesting that RNase E and YbeY act as primary processing enzymes, aiding in the initial separation of 16S and 23S rRNA in the alternative processing pathway. Furthermore, it also appeared that RNase P only played a secondary role in the alternative 30S rRNA processing pathway.

**RNase G acts as a primary processing enzyme in the alternative processing pathway**

Based on our northern blot analysis using probes a, b and c (Figures 4 and 5), it appeared that RNase E and YbeY were primarily responsible for the initial cleavages of 30S rRNA in the alternative processing pathway. Furthermore, the inactivation of RNase P produced less 30S rRNA as compared to inactivation of RNase E and YbeY in absence of RNase III.

Thus, we concluded that in the alternative processing pathway RNase P acted as secondary processing enzymes, which helps in the processing and maturation of rRNAs but not the initial 30S rRNA cleavage. However, since RNase G has been shown to be responsible for the 5′ maturation of 16S rRNA$^{19,20}$, we constructed an $\Delta rn:\text{rne-1} \text{rng::cat} \Delta ybe:\text{rph-1}$ pentuple mutant for northern blot analysis.

When probed with an oligonucleotide complementary to the 5′ of 16S rRNA (Figure 6A, probe a), it was seen that the $\Delta rn:\text{rne-1} \text{rng::cat} \Delta ybe:\text{rph-1}$ mutant (Figure 6B, lanes 5-6) produced considerably more 30S rRNA as compared to the $\Delta rn:\text{::apr} \text{rne-1} \Delta ybe:\text{::cat} \text{rph-1}$ strain (Figure 6B, lanes 3-4) and the $\Delta rn:\text{::apr} \text{rph-1}$ strain (Figure 6B, lanes 1-2). Moreover, the mature 16S rRNA was not produced in the $\Delta rn:\text{rne-1} \text{rng::cat}$
ΔybeY rph-1 strain at either permissive or non-permissive temperatures. Additionally, at the non-permissive temperature multiple 16S precursor intermediates were produced, but none of them had the same size as mature 16S rRNA.

Subsequently, the same blot was probed with 5' mature terminus of gltT tRNA (Figure 6A, probe b) and 5' mature terminus of 23S rRNA (Figure 6A, probe c). Similarly, the 30S rRNA seen in the Arnc rne-1 rng::cat ΔybeY rph-1 mutant was significantly more as compared to Arnc::apr rne-1 ΔybeY::cat rph-1 quadruple mutant. When quantitated it was seen that there was ~14-16 fold more 30S rRNA in the Arnc rne-1 rng::cat ΔybeY rph-1 strain (Figure 6B, lanes 5-6) as compared to the Arnc::apr rne-1 strain (Figure 6B, lanes 1-2). In addition, inactivation of RNase G in Arnc::apr rne-1 ΔybeY::cat rph-1 background (Figure 6B, lanes 5-6), produced ~5-6 fold more 30S rRNA as compared to the Arnc::apr rne-1 ΔybeY::cat rph-1 strain.

Moreover, the amount of the precursor 23S intermediate in the Arnc rne-1 rng::cat ΔybeY rph-1 mutant (Figure 6B, lanes 5-6) was around ~1.5-2.2 fold more than in the Arnc::apr rne-1 ΔybeY::cat rph-1 strain (Figure 6B, lanes 3-4) suggesting a possible role of RNase G in the alternative processing pathway. With the increase in the 30S rRNA and precursor 23S intermediate levels, there was a concomitant reduction in the mature 23S rRNA levels. Furthermore, inactivation of RNase G further reduced the mature 23S rRNA levels by 25-35% in Arnc::apr rne-1 ΔybeY::cat rph-1 background. Overall, inactivation of RNase E, RNase G and YbeY in absence of RNase III had the maximum effect on 30S rRNA processing. This suggests that these enzymes are the primary processing enzymes in the alternative 30S rRNA processing pathway.
RNase Z functions as a secondary processing enzyme in the alternative processing pathway

Based on the data illustrated in Figures 4-6, it appeared that RNase E, RNase G and YbeY carry out the initial cleavages of the 30S primary transcript in the alternative processing pathway. In order to investigate if any other endoribonuclease(s) were involved in this initial cleavage, we included RNase Z in our study. When RNase Z was inactivated, the 30S rRNA accumulation was not significantly higher than Δrncl::apr rph-1, whereas inactivation of both RNase P and RNase Z (Figure 7B, lanes 7-10) showed significantly more 30S rRNA accumulation than in Δrncl::apr rph-1 (Figure 7B, lanes 3-4). When probed with b and c, although it was seen that the 30S rRNA accumulation in the Δrne-1 rnpA48 Δrnz::apr ΔybeY rph-1 strain (Figure 7B, lanes 9-10) was less than in the Δrne-1 ΔybeY::cat rph-1 mutant (Figure 7B, lanes 5-6) and the Δrne-1 rnpA48 ΔybeY::cat rph-1 strain (Figure 7B, lanes 7-8), the precursor I accumulation was significantly more than in the Δrne-1 ΔybeY::cat rph-1 strain at both the temperatures. The drop in PF for mature glutT tRNA was comparable to that of Δrne-1 ΔybeY::cat rph-1 (20% to 7%) but more severe than Δrne-1 rnpA48 ΔybeY::cat rph-1 (18% to 16%). This result suggested that even though inactivation of RNase Z did not have a large impact on the 30S rRNA levels, there was a considerable effect on the precursor I accumulation indicating its possible role in the processing of this precursor species.

When RNase P was inactivated in the Δrne-1 ΔybeY::cat rph-1 genetic background, a decrease in the precursor accumulation was observed, but when both
RNase P and RNase Z were inactivated in the same genetic background, the precursor accumulation increased. This result suggested that in absence of RNase P, it was RNase Z that might have aided in the processing of the precursor I after the initial 30S rRNA cleavage, but when both the enzymes were inactivated, the precursor I was no longer processed to generate mature RNAs, hence its levels increased. However, we predicted that if RNase Z was involved in the initial separation of 16S and 23S rRNA, even inefficiently, inactivating it in Δrnc::apr rne-1 ΔybeY::cat rph-1 background will lead to a further increase in the 30S rRNA levels with a decrease in the mature rRNAs, but no such effect was seen. Moreover, when probed with a, it had the intact mature 16S rRNA with less 18S, 17S 16S* rRNA as compared to Δrnc::apr rne-1 Δrnc::apr rph-1, suggesting that RNase Z does not play a significant role in 16S rRNA processing in the alternative pathway.

Inactivation of RNase E and YbeY along with RNase III causes a severe growth defect in the cells

In E. coli, the number of ribosomes per cell varies in proportion to the growth rate to meet the cell’s changing demand for protein synthesis. At any time, the number of active ribosomes in the cell is proportional to the rate of synthesis of total rRNA. This control and regulation of ribosome content is exerted at the level of transcription from all the seven rRNA operons (rrn)\textsuperscript{46–48}. It has also been suggested that the redundancy of rRNA operons exists in E. coli, in order to support the high levels of ribosome production necessary for rapid growth rates\textsuperscript{49}.
Based on the above data presented in Figures 3-6, we predicted that if RNase E, and YbeY drive the initial cleavage of the 30S rRNA primary transcript in absence of RNase III, there would be a significant growth defect in the strain where all three enzymes were inactivated. Accordingly, we determined the growth rates of various multiple mutants. All the strains were initially grown at 30°C into early exponential phase and then the cultures transferred to 44°C to inactivate the essential enzymes RNase E and RNase P. The Δrnc::apr rph-1 double mutant had a doubling time of 50 min compared to the rph-1 single mutant (Figure 2).

The growth defect became more severe in the Δrnc::apr rne-1 ΔybeY::cat rph-1 genetic background. At 30°C, even though RNase III and YbeY were completely inactivated and RNase E was partially active, it took around 4 hours to reach the exponential phase. Additionally, as the cells were shifted to 44°C, where RNase E gets completely inactivated, the growth defect became worse and the cells stopped growing within 45-60 minutes of shift. The severe growth defect is in agreement with the 30S rRNA processing defects observed in these strains.

The alternative 30S rRNA processing pathway has unique cleavage sites from the RNase III-dependent pathway

Although the data in Figures 4-7 clearly identified the composition of rRNA processing intermediates, the northern analyses did not provide detailed insights into the precise cleavage sites of the alternative 30S rRNA processing pathway. In order to study this, primer extension analysis was done at 5′ termini of both 16S rRNA and 23S rRNA using radiolabeled primers (a and d, respectively) specific for their 5′ mature ends, as
shown in Figure 8A. When a oligonucleotide complementary to the mature 5′ of 16S rRNA was used, the rph-1 control strain had two major bands (A4 and A5) other than the mature 5′ end labeled as M_{16} in Figure 8B, lanes 1-2. A4 is the known RNase III cleavage site and A5 is the RNase G cleavage site in the RNase III dependent pathway. In contrast, the \( \Delta rnc::apr \) rph-1 strain (Figure 8B, lanes 3-4) had three more bands- A1, A2 and A3 in addition to A5. Also, it had the mature 5′ of 16S rRNA (M_{16}) but the band intensity was lower than rph-1 control strain, suggesting some 16S rRNA maturation defect.

Moreover, inactivating RNase E, RNase P and YbeY in addition to RNase III (Figure 8B, lanes 5-8), led to a large decrease in M_{16} but the mature 5′ end was still made. This result corroborated with the fact that in absence of RNase E, the 5′ maturation of 16S rRNA slows down dramatically, but the maturation still takes place by RNase G\(^{19}\). With the reduction in the mature end, there was an increase in the bands A1, A2 and A3. When mapped, it was seen that bands A1 and A2 corresponded to the promoter P1 and P2 sites in the \( rrn \) operons and A3 was in the spacer region just upstream of the double stranded 16S rRNA stem structure (shown in Figure 9). The increase in band intensity of A1 and A2 probably represented increased transcription as a part of the feedback regulation seen in \( E. \ coli \). However, the \( \Delta rnc \) rne-1 rnpA49 \( \Delta rnz::apr \) AybeY rph-1 mutant (Figure 8B, lanes 9-10) did not have much difference as compared \( \Delta rnc::apr \) rph-1. It still had comparable levels of mature 5′ of 16S rRNA with a minor increase in bands A1 and A2, corroborating with the northern data suggesting the secondary role of RNase Z in the alternative pathway.
Appearance of a new cleavage site, A3 in the single-stranded spacer region upstream of 5' 16S rRNA further confirmed that the alternative processing pathway has cleavage sites unique from the RNase III dependent processing pathway.

Similarly, in case of the 5' end of 23S rRNA as shown in Figure 8C, the mature end labeled as M23 was seen in rph-1 (Figure 8C, lanes 1-2) but was absent in the Δrnc::apr rph-1 strain (Figure 8C, lanes 3-4). In addition, a series of cleavage sites (labeled as B1-B10) appeared both upstream and downstream of the mature 5' end, further supporting our hypothesis that the RNase III-independent alternative processing pathway has multiple cleavage sites that are different from the known RNase III-dependent processing pathway. The presence of multiple cleavage sites could be due to a very non-specific binding of the enzymes involved in the pathway. Although all the seven rrn operons have the same basic organization in E. coli, the region in between 16S and 23S rRNA varies significantly among the seven rRNA operons. Four of the seven operons contain tRNA\textsuperscript{Glu} in their spacer regions, and three contain tRNA\textsuperscript{Ala} plus tRNA\textsuperscript{Ile}. In addition to differences in types and numbers of tRNAs encoded in between 16S and 23S rRNA, the operons also contain sequence heterogeneities\textsuperscript{50}. Therefore, presence of multiple bands at the same region could be because of single cleavage event occurring at the spacer region which differs among the seven different rrn operons, resulting in multiple bands of different sizes.

Moreover, some of the cleavage sites seen in the RNase III deficient strain disappeared when RNase E and YbeY were inactivated (indicated as B4-10 in Figure 8C, lanes 5-6). In case of the RNase III deficient strain, RNase E and YbeY were still expressed and were carrying out the cleavages, thereby generating products of sizes
corresponding to bands B4-10. But when they were inactivated, the processing at that location were hindered and therefore the bands, B4-10 almost disappeared, emphasizing their key role in the alternative pathway. When mapped, the bands B4-B7 were located at the spacer region between the tRNA and 23S rRNA (Figure 9), at around 110, 104, 46 and 38 nucleotides upstream of mature 5’ of 23S rRNA, respectively. Bands B4 and B6 corresponded to an AU rich region in the spacer sequences. This re-emphasized the crucial role RNase E plays in separating 16S and 23S rRNA based on the sequence preference, as RNase E has been previously shown to cleave AU rich sequences\textsuperscript{51,52}.

Moreover, band B8 corresponded to 13 nucleotides region extending from the spacer to the double stranded 23S rRNA stem structure, whereas both the bands B9 and B10 were located on the stem of 23S rRNA. Owing to the heterogeneity seen in this spacer region among the seven different \textit{rrn} operons, multiple bands with a few nucleotide differences are produced, possibly due to the same cleavage event. In addition, the cleavage product (band B10) located internally in mature 23S rRNA, also reduced in band intensity when RNase E and YbeY were inactivated in absence of RNase III, confirming their role in the alternative pathway.

Surprisingly, the cleavage sites indicated as B1-3 increased in \textit{Anrc::apr rne-1 AybeY::cat rph-1} (Figure 8C, lanes 5-6) as compared to the \textit{Anrc::apr rph-1} (Figure 8C, lanes 3-4) at both temperatures. This maybe because of other enzyme(s) involved in this alternative 30S rRNA processing pathway, which was still actively expressed to carry out the cleavages at that specific location. One possible candidate can be RNase P and in order to confirm it, we also studied \textit{Anrc::apr rne-1 rnpA49 AybeY::cat rph-1} (Figure 8C, lanes 7-8).
The two bands collectively labeled as B3 decreased significantly and it was barely visible at higher temperature where RNase P was completely inactivated. When mapped, we found that B3 is located at the 5′ end of the spacer tRNAs *ala* and *glt* which is in agreement with the role of RNase P in tRNA maturation. The top band of B3, corresponded to the mature 5′ end of *glt* tRNA from operons *rrnB*, *rrnG*, *rrnC* and *rrnE*, whereas the bottom band of B3 corresponded to the mature 5′ end of *ala* tRNA from the rest of the seven operons - *rrnA*, *rrnD* and *rrnH*. The difference in intensities between the two bands for B3 could be due to differences in rate of transcription from these individual operons as it has been previously observed that the seven *rrn* operons have different copy numbers per cell and the gene dosage varies with the medium conditions53,54. Similarly, there was a reduction in the band B2 as well, which corresponded to an AU rich region (for operons *rrnBGCE*) 121 nucleotides downstream of the mature 3′ of 16S rRNA, but it had a less impact on inactivating RNase E, RNase P and YbeY in absence of RNase III. Likewise, inactivation of these four enzymes had a minor effect on band B1, which corresponded to the known RNase III processing site at the 3′ end of 16S rRNA as shown in Figure 9.

Inactivating RNase Z in addition to the above mentioned four enzymes did not have any major impact on bands B1-2 (Figure 8C, lanes 9-10) as it had comparable band intensities with Δrnc::apr rne-I rnpA49 ΔybeY::cat rph-I (Figure 8C, lanes 7-8), confirming our hypothesis that RNase Z acts as a secondary processing enzyme in the alternative pathway. It does not play a major role in the initial separation of 16S rRNA from the rest of the transcript. However, cleavage sites corresponding to ds-RNA region for bands B1, B8-10 suggested the possibility of another ds-RNA specific enzyme
existing other than RNase III in *E. coli*, unlike the accepted belief. It also emphasized on the possibility of enzymes in addition to RNase E, RNase P and YbeY being involved in the separation of 16S and 23S rRNA.

Therefore, in order to confirm the role of RNase G in the alternative pathway, primer extension analysis was done using oligonucleotides complementary to the 5′ end of 16S (probe a) and 23S rRNA (probe d) as shown in Figure 8A. In case of 16S rRNA, the 5′ mature terminus was barely produced in Δ*rnc rne-1 rng::catΔybeY rph-1* (Figure 8D, lane 7) at the permissive temperature. Furthermore, at higher non-permissive temperature the 5′ mature 16S rRNA almost disappeared (Figure 8D, lane 8). When compared with Δ*rnc::apr rne-1 ΔybeY::cat rph-1*, it was seen that 16S rRNA maturation was also significantly affected in absence of RNase E (Figure 8C, Lanes 5-6) but the effect was more pronounced when both RNase E and RNase G were inactivated (Figure 8D, lanes 7-8). However, in strains where RNase E and RNase G was expressed in wild type levels (Figure 8D, lanes 1-4), no such processing defect was seen at the 5′ mature terminus of 16S rRNA.

Additionally, bands A2 and A3 were reduced significantly in Δ*rnc rne-1 rng::catΔybeY rph-1* (Figure 8D, lane 7). Surprisingly, band A3 almost disappeared at higher temperature when RNase E was completely inactivated (Figure 8D, lane 8), suggesting that both RNase E and RNase G are required for processing at that location.

Similar processing defects were seen at the 5′ mature terminus of 23S rRNA in absence of RNase III, RNase E, RNase G and YbeY. The 5′ mature terminus of 23S rRNA labeled as M23 was present in *rph-1* (Figure 8E, lanes 1-2) but was absent in Δ*rnc::apr rph-1* (Figure 8E, lanes 3-4) corroborating with previous work which has
shown that RNase III inactivation affects 23S rRNA maturation\textsuperscript{30}. Inactivation of RNase E and YbeY in absence of RNase III (Figure 8E, lanes 5-6) reduced the intensities of bands B4-10, but the effect was more distinct when RNase G was inactivated (Figure 8E, lanes 7-8). Band B10 located internally in the 5’ mature 23S rRNA disappeared completely when RNase G was inactivated in $\Delta rnc::apr\ rne-1\ \Delta ybeY::cat\ rph-1$ genetic background, indicating that RNase G was responsible for the cleavage at that location. This result corroborated with previous work where it has been shown that RNase G plays a role in the maturation of 5’ 23S rRNA\textsuperscript{55}. Furthermore, there was a large decrease in bands B1 and B2, (located on the double-stranded stem at the spacer sequence downstream of mature 3’ of 16S rRNA) when RNase G was inactivated suggesting possible RNase G processing activity at this location. Therefore, based on our northern data and primer extension analysis it was evident that RNase G is one of the primary processing enzymes in the alternative 30S rRNA processing pathway. Together, RNase E, RNase G and YbeY are responsible for the initial cleavages on 30S rRNA to release the pre-16S rRNA from the rest of the transcript in absence of RNase III. However, it is still not clear if these enzymes act sequentially or as a complex in the alternative processing pathway.

**DISCUSSION**

The data presented here demonstrated that in the alternative 30S rRNA processing pathway, RNase E, RNase G and YbeY does the initial cleavages on 30S rRNA to release the pre-16S rRNA from the rest of the transcript in absence of RNase III (Figure 10), as shown in our proposed model in Figure 9. This is evident from the $\sim$14-16-fold increase
in 30S rRNA levels when RNase E, RNase G and YbeY were inactivated in the absence of RNase III (Figures 3-5). Absence of RNase E, RNase G and YbeY also led to severe 16S and 23S rRNA processing defects with no mature 16S rRNA produced. Moreover, with the decrease in the mature rRNA levels, the pre-23S rRNA increased dramatically. All these indicate the key role RNase E, RNase G and YbeY play, in the initial cleavage of 30S rRNA in the alternative processing pathway. Once the pre-16S rRNA is separated from the rest of the transcript which contains the spacer tRNA, 23S rRNA and the 5S rRNA, they are acted upon by the secondary processing enzymes RNase P and RNase Z along with the exoribonucleases to generate their mature species (Figure 10). The pre-16S rRNA is further processed by RNase E, RNase G at the 5′ end and YbeY, RNase R at the 3′ end to generate mature 16S rRNA.

Interestingly, in the alternative 30S rRNA processing pathway a unique 23S precursor intermediate (I) was generated as compared to the RNase III-dependent processing pathway. Based on our northern data, this intermediate extends from the known RNase III processing site at the 3′ of 16S rRNA to the 3′ end of 5S rRNA (Figures 3-6). Moreover, this precursor has never been reported to be produced in presence of RNase III suggesting it to be a unique cleavage product from the alternative processing pathway. Understanding the cleavage sites in between the 16S and 23S rRNA is crucial as it elaborated on where the 16S and 23S rRNAs are separated from each other. Using primer extension analysis, the cleavage sites were mapped (shown in Figure 8F) which were unique from the RNase III-dependent pathway further supporting our hypothesis. The cleavage bands which appeared in absence of RNase III at the 5′ of 23S rRNA, decreased significantly in absence of RNase E, RNase G and YbeY in Arnc::apr rph-1
genetic background, confirming their role in processing at those locations. Interestingly, when RNase E, RNase G and YbeY were inactivated in presence of wild type RNase III, 30S rRNA was still processed. No such significant processing defects were seen except for 16S rRNA. Therefore, it suggests that the RNase III-dependent pathway is a primary processing pathway in *E. coli* and the alternative processing pathway is a backup pathway to it.

However, based on our data the possibility of other enzyme(s) being involved in the separation of 16S and 23S rRNA could not be entirely ruled out. Inactivation of RNase E, RNase G and YbeY in absence of RNase III still produced some mature 23S rRNA, albeit significantly less than RNase III deficient strain. Moreover, based on primer extension analysis at the 5′ of 23S, cleavage sites were mapped which corresponded to the double stranded region on the 16S and 23S rRNA stem structure. Band B1 was located at the known RNase III processing site at the 3′ end of 16S rRNA ds-stranded stem. Cleavage at this double stranded region in absence of RNase III could be because of two possibilities. It could either involve the unwinding activity from the helicases in *E. coli*, which make the processing site at that location more accessible to the single-stranded RNA specific ribonucleases, RNase E, RNase G and YbeY. Alternatively, it could be from the activity of a previously unidentified enzyme which can cleave ds-RNA structures. Analyzing the 30S rRNA processing by inactivating the DEAD-box RNA helicases in Δrncrne-1 rng::cat AybeY rph-1 genetic background will give a vivid idea on the role of helicases in the alternative 30S rRNA processing pathway. Likewise, putative ds-RNA specific endonucleases can be identified from genome-wide searches for protein domains similar to RNase III and then further analyzed to study their role in the alternative processing
pathway. Lastly, the differences in the organization among the seven rRNA operons (rrn) with different tRNA copy numbers, tRNA identity, 5S rRNA copy numbers and the heterogeneity of spacer sequences can yield precursors of slightly different sizes. Therefore, the possibility of some differences in cleavage sites in the alternative processing pathway among the seven operons, could not be ruled out. Analyzing the effect of inactivating RNase E, RNase G and YbeY in absence of RNase III in the individual rrn operons on 30S rRNA processing, will in future help in the understanding of the alternative 30S rRNA processing pathway in more detail.

MATERIALS AND METHODS

Bacterial Strains and Growth Curves

All the strains used in this study are listed in Table 1. They are all isogenic derivatives of MG1693 (rph-1). The rph-1 is a naturally occurring mutation in the exoribonuclease RNase PH found in one of the first identified E. coli strain derivative of MG1655. The Δrnc38 and rnc-14 alleles were used initially for various strain constructions. However, we were unable to make multiple mutants in these genetic backgrounds without the presence of a rnc covering plasmid. Moreover, when Δrnc38 was tested using a monoclonal antibody for RNase III obtained from Donald Court laboratory, it was found to produce a truncated RNase III protein product. Therefore, a new rnc deletion strain Δrnc::apr was used for the construction of multiple mutants used for this study. The rne-1 and rnpA49 alleles used for this study, encode temperature-sensitive mutations which functionally inactivates RNase E and RNase P, respectively at 44°C. The strains were generated by the addition of chromosomal mutant alleles using P1
transduction and subsequently were selected for the antibiotic resistance marker associated with the mutant allele. The genotypes of transductants were confirmed by PCR, or by DNA sequencing in the case of \textit{rne-1} and \textit{rnpA49}.

Since most of the strains used for this study had temperature-sensitive mutations (\textit{rne-1} and \textit{rnpA49}), all strains were grown under similar conditions at 30°C with shaking at 225 rpm in Luria broth supplemented with thymine (50 µg/ml) and apramycin (100 mg/mL), chloramphenicol (20 mg/mL) or kanamycin (25 mg/mL), where appropriate, until they reached a cell density of approximately $1 \times 10^7$/ml (40 Klett units above background, no. 42 green filter). Subsequently, the cultures were shifted to the non-permissive temperature of 44°C. Cultures were diluted with pre-warmed growth medium as needed to maintain their exponential growth (Klett 40-80).

**RNA Isolation and Northern Analysis**

Total RNA was isolated from various exponentially growing strains under the growth conditions described above. The quality of RNA samples was checked by agarose gel electrophoresis and then quantified using a Nanodrop® ND-2000c spectrophotometer.

Glyoxal agarose northern analysis was conducted using samples prepared with NorthernMax®-Gly sample loading dye (Ambion) and electrophoresis in a 1% agarose gel in BPTE buffer run at 100 volts for approximately 3 hours. Gel transfer and subsequent steps were conducted as previously described\textsuperscript{27}. To analyze the processing pattern of the 30S rRNA transcript, northern blots were probed with γ-$P^{32}$-end-labeled
oligonucleotides labeled using T4 PNK (NEB) that were specific for different regions of the 30S rRNA transcript. The various oligonucleotide probes used are listed in Table 2. Quantification of band intensities were calculated using ImageQuant TL 7.0 software.

**Primer Extension Analysis**

Primer extension analysis for the 5’ of 16S and 23S rRNA were performed as described previously. The oligonucleotides used for the analysis are listed in Table 2.

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rRNA processing in Escherichia coli multiple mutants carrying a deletion in

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9S RNA molecule isolated from an rne mutant of E. coli. Cell. 1978;15(3):1055-


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Table 2.1: List of some of the bacterial strains used in this study

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Table 2.2: Oligonucleotide probes used in this study.

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<td>Before 5’ gltT</td>
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**Figure 2.1. Model of *E. coli* rRNA processing.**

The 30S pre-rRNA transcript is first cleaved by RNase III at two physical locations, cleaving the double-stranded rRNA formed by the spacer sequences adjacent to mature 16S and 23S rRNAs, generating 17S, 25S, and 9S pre-rRNAs (labeled in green). 17S rRNA is cleaved initially by RNase E and then by RNase G to generate the mature 5′ end of 16S rRNA. YbeY along with RNase R processes the extra 33nts at the 3′ end of 17S rRNA to generate the mature 3′ terminus of 16S rRNA. RNase E also cleaves 9S three nt on each side of the mature termini of 5S rRNA forming a p5S precursor. RNase P cleaves to produce the mature 5′ end of the tRNA. Exoribonucleases (primarily RNase T) are responsible for the 3′ end maturation of the tRNA, 23S rRNA, and 5S rRNA, but the endoribonuclease(s) responsible for the maturation of 5′ end of 23S, and 5′ end of 5S rRNAs remain unidentified (labeled in red). This model is not drawn to scale.
Figure 2.2. Growth curve for various mutants. Since most of the mutants were constructed in \textit{rne-1} and \textit{rnpA49} genetic backgrounds (temperature sensitive mutants), the cells were initially grown at 30°C until they reached the early exponential phase (40 Klett units, denoted by the black line). Subsequently, the cultures were shifted to 44°C. Cultures were diluted with pre-warmed growth medium as needed to maintain them in exponential growth. Measurements were taken at intervals of 30 minutes.
Figure 2.3. Agarose gel to study the effect of inactivation of RNase III on 30S rRNA processing. (A) The 30S primary transcript is shown with the 16S rRNA at the 5′ end followed by the spacer tRNA, 23S and 5S rRNA. The model is not drawn to scale. (B) RNA isolated from the strains were run in a 1% agarose gel. The mature 16S, 23S and gltT tRNA are denoted as M_{16}, M_{23} and M_{t}. I designate an intermediate that contains the tRNA, 23S rRNA and 5S rRNA.
Figure 2.4. Agarose Northern Blot analysis to study the effect of inactivation of RNase P and YbeY in absence of RNase III, RNase E and YbeY. (A) The location of the oligonucleotides used for the northern blot analysis are shown. (B) Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a 1% agarose gel. The genetic composition of each band is shown in the corresponding cartoon drawn in the right. The mature 16S, 23S and gltT tRNA are denoted as M<sub>16</sub>, M<sub>23</sub> and M<sub>i</sub>. I designate an intermediate that contains the tRNA, 23S rRNA and 5S rRNA.
Figure 2.5. Agarose Northern Blot analysis to study the effect of inactivation of RNase P in absence of RNase III, RNase E and YbeY. (A) The location of the oligonucleotides used for the northern blot analysis are shown. (B) Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a 1% agarose gel. The genetic composition of each band is shown in the corresponding cartoon drawn in the right. The mature 16S, 23S and gltT tRNA are denoted as $M_{16}$, $M_{23}$ and $M_I$. I designate an intermediate that contains the tRNA, 23S rRNA and 5S rRNA.
Figure 2.6. Agarose Northern Blot analysis to study the effect of inactivation of RNase G in absence of RNase III, RNase E and YbeY. (A) The location of the oligonucleotides used for the northern blot analysis are shown. (B) Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a 1% agarose gel. The genetic composition of each band is shown in the corresponding cartoon drawn in the right. The mature 16S, 23S and gltT tRNA are denoted as M₁₆, M₂₃ and Mᵢ. I designate an intermediate that contains the tRNA, 23S rRNA and 5S rRNA.
Figure 2.7. Agarose Northern Blot analysis to study the effect of inactivation of RNase Z in absence of RNase III, RNase E and YbeY. (A) The location of the oligonucleotides used for the northern blot analysis are shown. (B) Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a 1% agarose gel. The genetic composition of each band is shown in the corresponding cartoon drawn in the right. The mature 16S, 23S and gltT tRNA are denoted as $M_{16}$, $M_{23}$ and $M_I$. I designate an intermediate that contains the tRNA, 23S rRNA and 5S rRNA.
Figure 2.8. Identification of cleavage sites of the alternative processing pathway. (A) The location of the oligonucleotides used for the prime extension analysis are shown. (B-E) Primer extension analysis of the 5′ termini of 16S and 23S rRNA. Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a sequencing gel along with the sequencing ladder. The mature 5′ termini of 16S and 23S rRNA are denoted as $M_{16}$ and $M_{23}$ respectively. Bands A1-A4 and B1-B10 denote the multiple cleavage sites of the alternative processing pathway which are unique from the RNase III-dependent pathway.
Figure 2.9. Cleavage sites in the alternative processing pathway are unique from the RNase III-dependent pathway. The alternative processing pathway has multiple processing sites and they are denoted by the red arrows. Based on the primer extension analyses, bands A1-A4 and B1-B10 are mapped on the 30S primary transcript. The known processing sites of the RNase III-dependent pathway are denoted by the black arrows. The model is not drawn to scale.
P1,2- Promoter sites
III- RNase III
E- RNase E
G- RNase G
P- RNase P
Y- YbcY
X- Exoribonucleases
? - Unknown endoribonucleases
Figure 2.10. Primary vs Alternative 30S rRNA processing pathway in *E. coli*.

The primary RNase III-dependent pathway is indicated at the left side of the figure, whereas the RNase III-independent alternative processing pathway is drawn towards the right side of the figure. The 30S pre-rRNA transcript is first cleaved by RNase III or by RNase E, RNase G and YbeY to generate precursor intermediates. These precursors are further processed by secondary processing enzymes and exoribonucleases to generate the mature RNAs. This model is not drawn to scale.
Supplemental Figures. 2.S. Cleavage sites of alternative 30S rRNA processing pathway in *E. coli.* (S1) The bands A1-3 seen in primer extension at the 5′ end of 16S rRNA were mapped and their corresponding nucleotide locations are shown (black lined boxes). Bands A1 and A2 correspond to the transcription start sites. The blue colored box denotes the mature 5′ terminus of 16S rRNA. The green colored region denotes the region of mismatch seen among the selected three rRNA operons-rrnB, rrnG and rrnD which are most divergent among all the seven transcribed rRNA operons in *E. coli.* (S2) The bands B1-10 seen in primer extension at the 5′ end of 23S rRNA were mapped and their corresponding nucleotide locations are shown (black lined boxes). The blue and pink colored box denote the mature 3′ terminus of 16S rRNA and mature 5′ terminus of 23S rRNA respectively. The yellow boxes denote the mature 5′ and 3′ ends of the spacer tRNAs.
CHAPTER 3

STUDY OF THE NEWLY IDENTIFIED GENES THAT MAY PARTICIPATE IN
THE PROCESSING OF THE 30S rRNA PRIMARY TRANSCRIPT IN
ESCHERICHIA COLI

Gargi Chaudhuri, Joydeep Mitra, Bijoy K. Mohanty and Sidney R. Kushner, to be submitted to *Molecular Microbiology.*
INTRODUCTION

In *E. coli*, all three ribosomal RNAs (rRNAs) 16S, 23S and 5S rRNAs are co-transcribed along with various tRNAs as part of the seven distinct 30S primary transcripts (Figure 1). It has been shown that RNase III, a double-stranded RNA specific endonuclease encoded by *rnc*\(^1\), cleaves the 30S rRNA primary transcripts into respective pre-rRNA species. However, RNase III is not essential in *E. coli*\(^2\) and when the RNA from RNase III deficient strains was analyzed, the 30S rRNA accumulation constituted <5% of the total RNA. In absence of RNase III, normal maturation of 23S rRNA does not occur, but mature 16S rRNA can still be made\(^3\). Thus, it is apparent that the 30S rRNA primary transcript undergoes some alternative processing in the absence of RNase III.

As shown in Chapter 2, we have identified some of the primary processing enzymes involved in the alternative 30S rRNA processing pathway as RNase E, RNase G and YbeY. They drive the initial separation of 16S rRNA from the rest of the 30S rRNA transcript in absence of RNase III. This conclusion is supported by the 14-fold increase in 30S rRNA levels when RNase E, RNase G and YbeY were inactivated in absence of RNase III. The absence of RNase E, RNase G and YbeY also had a significant impact on the mature rRNA levels, especially in case of mature 16S rRNA.

In the alternative processing pathway after initial cleavages, there are two precursor intermediates formed, namely the precursor 16S rRNA and the precursor 23S rRNA (Figure 2). The precursor 23S rRNA intermediate, which contains the spacer tRNA, 23S rRNA and the 5S rRNA, is subsequently processed by RNase P and RNase Z, along with the exoribonucleases to generate the mature RNAs. Although, mature 23S rRNA was still
produced even in the absence of RNase III, RNase E, RNase G and YbeY, it was not clear how the pre-16S rRNA species was processed under these conditions. These observations suggested the possibility that other enzymes were involved in the alternative 30S rRNA processing pathway. Accordingly, we hypothesized that *E. coli* contained at least one additional previously unidentified endonuclease that was involved in the alternative processing pathway.

In order to identify putative endonucleases, a genome-wide search was performed utilizing protein domains from known bacterial ribonucleases. For example, the catalytic domain of RNase E, the primary endonuclease in *E. coli*, is accompanied by regions that facilitate physical interactions with other proteins in the degradosome, and the RNA chaperone Hfq. The N-terminal of RNase E contains the RNA binding S1 domain, which is found in a large number of RNA-associated proteins.

In contrast RNase Y, the functional counterpart of RNase E found in *B. subtilis*, contains a KH domain, which imparts its RNA recognition and binding function, and an HD domain, which is found in a wide variety of proteins with phosphohydrolase activity. Although this newly identified endoribonuclease in *B. subtilis* does not share any sequence similarity with RNase E, the functional similarity between the two enzymes is intriguing- both enzymes are essential endoribonucleases preferring 5′ monophosphorylated substrates, and they are the only RNases shown, thus far, to have a major impact on bulk mRNA degradation in their host organisms. Additionally, both of them are central members of their respective RNA degradosomes, and both are integral membrane proteins. The other major ribonuclease known to process ribosomal RNA in *B. subtilis* is RNase J1/J2, which shows both endonuclease and 5′→3′ exonuclease activity.
activity, and is composed almost exclusively of a metallo-beta-lactamase domain. Recent work has suggested that it is responsible for 5’ maturation of 16S rRNA in *B. subtilis*.

A search for the domains found in the above proteins were conducted in *E. coli* using the Interpro protein classification system. This search revealed multiple occurrences of these domains across the proteome. We selected a set of proteins which contained one or more ribonuclease related domains according to the Interpro database, that had no assigned function. Our top candidate was the hypothetical protein YhgF which contained an RNA binding S1 domain along with a RNase H-like domain. The N-terminal of YhgF also contains a tex-like domain, which is involved in transcriptional processes, and has been previously shown to preferentially bind with single-stranded RNAs.

Further study on Tex/YhgF from *P. aeruginosa* has revealed that although, it does not have any nuclease activity, it binds ssDNA ten-fold tighter than it binds ssRNA, dsDNA or dsRNA. However, it does show some binding activity on DNA/RNA hybrids in *P. aeruginosa*. Additionally, the *E. coli* yhgF gene is orthologous to the essential *B. pertussis* tex (toxin expression) transcription factor gene that negatively regulates toxin production. It is also homologous to the essential yeast transcription elongation factor Spt6, which is highly conserved from yeast to humans. All these features of YhgF made it a highly possible candidate for a putative endoribonuclease which could be involved in 30S rRNA processing in *E. coli* (Table 1).

The KH domain (responsible for RNA binding) of RNase Y is not found in *E. coli*. Although the HD domain is found in some RNA binding proteins, we were unable to
identify any potential endonucleases that contained these domains in our search. *E. coli* does not have an RNase J protein ortholog, but the closely related *S. enterica* has an RNase J1 homolog with identical functionality to the *B. subtilis* protein. However, we did identify two hypothetical proteins, YcaI and YcbL, that contained a metal binding beta-lactamase-like domain characteristic of RNase J1 as our other candidates (Table 1). Finally, we extended our list of candidate ribonucleases through keyword and gene ontology term searches across the Ecocyc database\textsuperscript{20,21}. This helped us identify YraN, a hypothetical protein with a domain resembling the archaeal tRNA endonuclease domain, and two predicted endonucleases YihG and YhbQ (Table 1).

Since inactivating RNase E and YbeY, in the absence of RNase III led to a large accumulation of 30S rRNA with very little mature 16S rRNA produced, we inactivated the above mentioned new genes in this genetic background using P1 transduction. We show here that when the RNA isolated from these multiple mutants was analyzed, we found that inactivation of YhgF had the maximum effect on 30S rRNA processing, with moderate effects seen in YraN and YhbQ deficient strains. Additionally, it appeared that YhgF, YraN and YhbQ play a key role in the processing and maturation of the precursor 23S rRNA intermediate. Nevertheless, our data suggests that their function becomes crucial only when RNase E and YbeY were inactivated, indicating their role as secondary processing enzymes in the alternative pathway. Similarly, when the role of YcaI and YcbL were investigated in the alternative pathway, it appeared that they do not have such significant processing defects as compared to YhgF, YraN and YhbQ. Lastly, YihG had no role in 30S rRNA processing as the amount of 30S rRNA was comparable to RNase III deficient strain.
RESULTS

Inactivation of YhgF, YraN or YhbQ has the maximum impact on 30S rRNA accumulation among the newly identified genes

Based on the results presented in Chapter 2, RNase E, RNase G and YbeY are responsible (Figure 2) for the initial cleavages of the 30S primary transcript in the alternative processing pathway. In order to investigate the role of the genes identified by bioinformatics analysis, we constructed an isogenic set of strains with deletions in the coding sequences (obtained from Keio collection) in our genetic background (MG1693) using P1 transduction. Deletion alleles of the six candidate genes were introduced into the Δ\text{rnc::apr} \ Δ\text{rne-1} \ Δ\text{ybeY::cat} \ Δ\text{rph-1} genetic background (Table 2). RNA isolated from these multiple mutants was used using northern blot analysis.

Since, RNase E is essential in \textit{E. coli}, for the purpose of this study the multiple mutants were constructed using the temperature sensitive RNase E mutant- \textit{rne-1}^{22} (a point mutation at residue 66). Therefore, the cells were grown first at permissive temperature (30°C) into early exponential phase and were subsequently shifted to the higher non-permissive temperature (44°C). Samples for RNA extraction were collected just before and 30 minutes after the temperature shift.

Since all the seven ribosomal RNA operons have the basic same organization of RNAs with a 16S rRNA at the 5’ end of the 30S rRNA transcript, we first probed with an oligonucleotide that was complementary to the mature 5’ end of 16S rRNA sequence (Figure 3A, probe a). In the \textit{rph-1} genetic background (Figure 3B, C lanes 1-2), no 30S rRNA accumulation was observed as the primary RNase III driven 30S rRNA processing pathway was active. The inactivation of RNase III, led to 30S rRNA accumulation which
constituted around 3% of the total RNA (Figure 3B, C lanes 3-4). Inactivation of the primary processing enzymes of the proposed alternative pathway along with RNase III (Δrnc::apr rne-1ΔybeY::cat rph-1), blocked both the processing pathways, thereby causing a significant increase (Figure 3B, C lanes 5-6, ~5 fold) in the 30S rRNA accumulation as compared to the RNase III deficient strain. Surprisingly, when the newly identified gene YhgF (which has RNase E-like S1 domain and RNase H domain) was inactivated in this genetic background, the amount of 30S rRNA accumulation further increased by another ~3 fold (Figure 3B, lanes 7-8). Inactivation of the genes with metal binding beta-lactamase-like domain of RNase J1 (YcaI and YcbL) had a moderate effect on 30S rRNA accumulation. Deletion mutation in ycaI led to ~1.8-2 fold more 30S rRNA accumulation (Figure 3B, lanes 9-10) as compared to Δrnc::apr rne-1ΔybeY::cat rph-1 whereas, inactivation of ycbL did not exhibit any such significant increase (Figure 3B, lanes 11-12). Similarly, when YraN, the hypothetical protein with a domain resembling the archaenal tRNA endonuclease was inactivated in the Δrnc::apr rne-1ΔybeY::cat rph-1 genetic background (Figure 3C, lanes 7-8), there was 3.3 fold increase in 30S rRNA as compared to Δrnc::apr rne-1ΔybeY::cat rph-1 strain. Moreover, inactivation of YhbQ (predicted endonuclease) had a similar effect on the 30S rRNA accumulation with 2.6 fold more 30S rRNA (Figure 3C, lanes 11-12) as compared to Δrnc::apr rne-1ΔybeY::cat rph-1. Surprisingly, inactivation of YihG (Figure 3C, lanes 9-10) led to a reduction in the 30S rRNA levels as compared to Δrnc::apr rne-1ΔybeY::cat rph-1. When quantitated, it had only 1 fold more 30S rRNA than Δrnc::apr rph-1, suggesting that it does not play a major role in the alternative 30S rRNA processing pathway.
Subsequently, the same blot was probed with 5’ mature gltT tRNA (Figure 3A, probe b) and 5’ mature 23S rRNA (Figure 3A, probe c). Similar results were observed with maximum 30S rRNA accumulation seen in strains with YhgF or YraN or YhbQ inactivated. A moderate effect on 30S rRNA accumulation was seen with inactivation of YcaI or YcbL and no effect at all with inactivating YihG. When quantitated, it was seen that $\Delta rnc::apr rne-1AybeY::cat AyhgF:::kan rph-1$ (Figure 3B, lanes 7-8), $\Delta rnc::apr rne-1AybeY::cat AyraN:::kan rph-1$ (Figure 3C, lanes 7-8) and $\Delta rnc::apr rne-1AybeY::cat AyhbQ:::kan rph-1$ (Figure 3C, lanes 11-12) had ~2 fold more 30S rRNA as compared to $\Delta rnc::apr rne-1AybeY::cat rph-1$, consistent with the data seen when the blot was probed with 5’ of mature 16S rRNA. Besides the increase in the 30S rRNA accumulation, all the three strains $\Delta rnc::apr rne-1AybeY::cat AyhgF:::kan rph-1$ (Figure 3B, lanes 7-8), $\Delta rnc::apr rne-1AybeY::cat AyraN:::kan rph-1$ (Figure 3C, lanes 7-8) and $\Delta rnc::apr rne-1AybeY::cat AyhbQ:::kan rph-1$ (Figure 3C, lanes 11-12) had significantly more precursor-23S rRNA intermediate (denoted as I in figure 3B and 3C) at the non-permissive temperature. This result suggested a possible role for these proteins in the processing of the precursor-23S rRNA intermediate which extends from the spacer region at 3’ terminus of mature 16S rRNA to the 3’ terminus of 5S rRNA.

Concomitant with the increase in this precursor accumulation, the amount of mature 23S rRNA produced in these strains were considerably less than $\Delta rnc::apr rne-1AybeY::cat rph-1$ parent. When quantitated it was seen that they had ~1.8-2.2 fold more precursor-23S rRNA accumulation with around 10-15% less mature 23S rRNA ($M_{23}$) produced. This result suggested that in the alternative 30S rRNA processing pathway, YhgF, YraN and YhbQ may play a crucial role in processing the precursor 23S rRNA
intermediate to further generate the mature RNA species. However, inactivating YcaI, YcbL or YihG did not have any significant effect on the precursor 23S rRNA accumulation (Figure 3).

**YhgF is involved in the processing of the precursor-23S rRNA intermediate in the alternative 30S rRNA processing pathway**

Based on the work presented in Figure 3, it appeared that YhgF, YraN and YhbQ play some role in processing of the precursor 23S rRNA intermediate (I) in the alternative 30S rRNA processing pathway. To confirm our hypothesis, we repeated the northern blot analysis using a biological replicate of the RNA isolated both at permissive and non-permissive temperatures from the multiple mutants with the newly identified genes inactivated in \( \Delta \text{rnc::apr rne-1} \Delta \text{ybeY::cat rph-1} \) genetic background. Since inactivation of the new genes (YhgF, YraN and YhbQ) had a major impact on the 23S rRNA maturation, an oligonucleotide complementary to the mature 5′ end of 23S rRNA (Figure 4A) was used. Consistent with our previous data, the maximum 30S rRNA accumulation was seen in \( \Delta \text{rnc::apr rne-1} \Delta \text{ybeY::cat yhgF:::kan rph-1} \) among all the multiple mutants (Figure 4B, lanes 1-2). Additionally, it had the most precursor-23S rRNA accumulation at both permissive and non-permissive temperature as compared to the other five multiple mutants. However, the effect was more pronounced at higher temperature when RNase E was inactivated (Figure 4B, lane 2).

To further support our hypothesis that YhgF acts as a secondary processing enzyme in the alternative 30S rRNA pathway, primer extension analysis was done using an oligonucleotide complementary to the 5′ end of 23S rRNA as shown in Figure 4A. The 5′ mature terminus of 23S rRNA labeled as \( M_{23} \) was absent in \( \Delta \text{rnc::apr rph-1} \) (Figure 4C, 120
lanes 1-2) corroborating with previous work which has shown that RNase III inactivation affects 23S rRNA maturation\(^3\). In addition, a series of cleavage sites (labeled as B1-B10) appeared both upstream and downstream of the mature 5′ terminus further supporting our hypothesis that the RNase III-independent alternative processing pathway has multiple cleavage sites unique from the known RNase III-dependent processing pathway. Since, the generation time of the \(\Delta rnc::apr\) \(rph-1\) strain is only incrementally slower than the wild-type control, these data corroborated with our theory that ribosomes containing the aberrantly processed 23S rRNA species was not significantly impaired in their ability to carry out protein synthesis. Additionally, the presence of multiple cleavage sites could either be due to a very non-specific binding of the enzymes involved in the pathway or because of the heterogeneity in RNA organization seen among the seven ribosomal RNA operons\(^23\).

In addition, some of the cleavage sites seen in the RNase III deficient strain disappeared when RNase E and YbeY were inactivated (indicated as B4-10 in Figure 4C, lanes 3-4). When mapped, the bands B4-B7 were located at the spacer region between the tRNA and 23S rRNA, at around 110, 104, 46 and 38 nucleotides upstream of mature 5′ of 23S rRNA respectively. Band B4 and B6 corresponded to an AU rich region in the spacer sequences. This result further confirmed the crucial role RNase E plays in separating 16S and 23S rRNA based on the sequence preference, as RNase E has been previously shown to cleave AU rich sequences\(^{24,25}\). Furthermore, band B8 corresponded to the 13 nucleotides region extending from the spacer to the double-stranded 23S rRNA stem structure, whereas, both the bands B9 and B10 were located in the stem of 23S rRNA.
Owing to the heterogeneity seen in this spacer region among the seven different \textit{rrn} operons, multiple bands with a few nucleotide differences were produced, possibly due to the same cleavage event. In addition, the cleavage product (band B10) was located internally in mature 23S rRNA, also reduced in band intensity when RNase E and YbeY were inactivated in absence of RNase III, confirming their role in the alternative pathway.

Surprisingly, the cleavage sites indicated as B1-3 increased in \textit{Δ}rrn::apr \textit{Δ}ybeY::cat \textit{Δ}yhgF::kan rph-1 (Figure 4C, lanes 5-6) as compared to the \textit{Δ}rrn::apr rne-1\textit{Δ}ybeY::cat rph-1 (Figure 4C, lanes 3-4) at both temperatures. This could be because in \textit{Δ}rrn::apr \textit{Δ}ybeY::cat vyhgF::kan rph-1, RNase E is still expressed at wild type levels and is thereby carrying out the cleavages at those locations. Similarly, there was an increase in the B10 band which also suggested that it was a product of RNase E activity. However, inactivating YhgF in the \textit{Δ}rrn::apr rne-1 \textit{Δ}ybeY::cat rph-1 background (Figure 4C, lanes 7-8), caused a significant reduction in the band intensities of B1-3.

When mapped it was seen that all these cleavage sites were located in the spacer region between 16S and 23S rRNA where the initial separation could take place. Band B1 overlapped with the known RNase III cleavage site at 33nts downstream of 3’ mature terminus of 16S rRNA. Band B2 corresponded to the spacer sequence between the 16S rRNA and the spacer tRNA while band B3 was at the 5’ end of the spacer tRNA.

These results indicated that YhgF helps in processing the precursor-23S rRNA intermediate at these specific locations. However, inactivation of YhgF did not have a significant impact on these bands in presence of RNase E (Figure 4C, lane 5). Thus it
appears that in the alternative processing pathway, YhgF activity only becomes important after the precursor 16S rRNA and precursor-23S rRNA are separated from each other by RNase E, RNase G and YbeY.

**Effect of inactivation of YhgF, YraN and YhbQ on the growth properties of cell**

In *E. coli*, rapidly dividing cells produce hundreds of new ribosomes each minute\(^\text{26}\) to meet with the increasing demands for protein synthesis. At any time, the number of active ribosomes in the cell is proportional to the rate of synthesis of total RNA. When the cell reaches a doubling time of around 20 min, there are as many as 70,000 ribosomes per *E. coli* cell, while at lower growth rates there may be only 20,000 ribosomes per cell\(^\text{27}\). Additionally, the bulk amounts of RNA transcribed and protein produced in the cell at any time are directly linked to the growth rate. Since the bulk of the bacterial RNA is ribosomal RNA (rRNA) constituting ~80% of the total RNA, processing and availability of mature rRNA is crucial to the cell’s survival.

This control and regulation of ribosome content is exerted at the level of transcription from all the seven rRNA operons (*rrn*)\(^\text{28–30}\). It has also been suggested that the redundancy of rRNA operons exists in *E. coli*, in order to support the high levels of ribosome production necessary for rapid growth rates\(^\text{31}\). It further emphasizes the importance of 30S rRNA processing once it is transcribed, because intact 30S rRNA cannot bind to the ribosomal subunits which will affect downstream translation process.

Therefore, in light of the importance of 30S rRNA processing with regard to cell’s survival, it was possible that blocking both the primary and alternative 30S rRNA processing pathways would lead to inviability. In order to confirm this hypothesis,
bacterial growth curves were carried out on the various mutants. Since RNase E is essential in *E. coli*, we used the temperature sensitive mutant *rne*-1 allele. All the cells were initially grown at 30°C until they reached early exponential phase and were then shifted to 44°C to inactivate RNase E.

It was seen that *Δrnc::apr rph*-1 had a doubling time that was ~30 minutes more than *rph*-1 control (Figure 5A). In addition, when YhgF was inactivated along with RNase III (Figure 5A, *Δrnc::apr ΔyhgF::kan rph*-1), the cells grew almost at the same rate as *Δrnc::apr rph*-1 double mutant. This result suggested that inactivating YhgF alone was not sufficient to block the alternative processing pathway. However, inactivating one of the primary processing enzymes of the alternative pathway, YbeY along with RNase III, significantly increased the generation time and the cells grew much more slowly at 44°C. It took almost 2 hours for the cells to reach the exponential phase. The growth defect phenotype became more severe in *Δrnc::apr rne*-1 *ΔybeY::cat rph*-1 quadruple mutant. Even at 30°C, where RNase III and YbeY were completely inactivated, and RNase E was only partially active, it took the culture many hours to reach exponential phase. After this strain was shifted to 44°C, where RNase E was inactivated, the cells stopped growing within 60 minutes of the shift. The severe growth defect phenotype was in agreement with the 30S rRNA processing defects seen in the strain.

This result indicated that the absence of functional RNase III along with inactivated RNase E and YbeY effectively blocked both the primary and alternative 30S rRNA processing pathways. Therefore, no functional 16S, 23S and 5S rRNA were produced at
this point. Moreover, the inability to process 30S rRNA probably leads to a reduction in the number of mature tRNAs (which were a part of the polycistronic 30S rRNA transcripts) in the cell and hence may have affected the downstream protein synthesis. Additionally, the lack of mature rRNAs may have also affected the ribosome assembly, thus producing defective ribosomes which cannot take part in protein translation and affecting the equilibrium between the free, non-translating and translating ribosomes. As a result, the feedback regulation shifted the equilibrium towards the buildup of the free fraction resulting in the repression of rRNA and tRNA synthesis\(^{32}\).

The growth defect became even more pronounced when YhgF was inactivated in the *Δrnc::apr rne-1 ΔybeY::cat rph-1* genetic background. This observation corroborated our northern data, where *Δrnc::apr rne-1 ΔybeY::cat ΔyhgF::kan rph-1* multiple mutant had more 30S rRNA accumulation as compared to *Δrnc::apr rne-1 ΔybeY::cat rph-1*. However, when RNase E and YbeY were expressed at wild-type levels, inactivation of YhgF did not have any major impact on the growth properties, confirming our hypothesis that YhgF acts as a secondary processing enzyme. Its expression becomes essential only when RNase E, YbeY and RNase III are all inactivated.

Based on our initial data (Figure 3C), inactivation of YraN (*Δrnc::apr rne-1 ΔybeY::cat ΔyraN::kan rph-1*) and YhbQ (*Δrnc::apr rne-1 ΔybeY::cat ΔyhbQ::kan rph-1*) also had large amounts of 30S rRNA levels as compared to *Δrnc::apr rne-1 ΔybeY::cat rph-1* control. In order to investigate their role as primary or secondary processing enzymes in the alternative processing pathway, an isogenic set of strains were constructed where YhgF, YraN and YhbQ were inactivated in absence of RNase III.
Subsequently, their growth properties were studied both in liquid and on solid medium. In liquid cultures at 37°C, inactivation of YhgF in absence of RNase III did not have a major impact (Figure 5B) on the growth properties, as previously observed. However, inactivating YraN made the cells grow slightly more slowly than Δrnc::apr ΔyhgF::kan rph-1 strain. When YhbQ was inactivated, the cells grew significantly slower than Δrnc ΔyhgF ΔyraN::kan rph-1 multiple mutant. When grown on solid medium, similar results were observed where Δrnc::apr rph-1 and Δrnc::apr ΔyhgF::kan rph-1 grew almost at the same rate. But, Δrnc ΔyhgF ΔyraN ΔyhbQ::kan rph-1 was the sickest strain that took longest to grow single colonies. Nevertheless, none of the strains had the severe growth defect observed in Δrnc::apr rne-1 ΔybeY::cat rph-1, further confirming that YhgF, YraN and YhbQ are secondary processing enzymes whose function becomes essential only in absence of RNase E and YbeY in the alternative processing pathway.

**Inactivation of YhgF, YraN and YhbQ does not affect 30S rRNA processing in the presence of RNase E, RNase G and YbeY**

Since inactivation of YhgF or YraN or YhbQ in Δrnc::apr rne-1 ΔybeY::cat rph-1 genetic background had maximum significant effect on 30S rRNA processing in the alternative processing pathway, we investigated if they act as primary or secondary processing enzymes in this pathway. Based on our growth curve analysis of strains Δrnc::apr ΔyhgF::kan rph-1, Δrnc ΔyhgF ΔyraN::kan rph-1 and Δrnc ΔyhgF ΔyraN ΔyhbQ::kan rph-1, it appeared that YhgF, YraN and YhbQ played a secondary role in presence of RNase III, RNase E and YbeY, since their inactivation did not have any significant effect on growth. To confirm this hypothesis, RNA was isolated from these strains grown at 37°C and analyzed using northern blot analysis (Figure 6).
When probed with an oligonucleotide complementary to mature 5′ terminus of 16S rRNA (Figure 6A, probe a), the rph-1 strain only had mature 16S rRNA (M_{16}) and no 30S rRNA accumulation was observed as previously shown in Chapter 2 (Figure 6B, lane 1). In absence of RNase III, some 30S rRNA was not completely processed (Figure 6B, lane 2), since RNase E, RNase G and YbeY were still expressed at wild-type levels. Inactivating YhgF alone in absence of RNase III did not increase the steady-state levels of 30S rRNA (Figure 6B, lane 3). Surprisingly, subsequent inactivation of YraN (Figure 6B, lane 4) reduced the amount of 30S rRNA levels as compared to Δrnc::apr ΔyhgF::kan rph-1 strain and it was barely visible in Δrnc ΔyhgF ΔyraN ΔyhbQ::kan rph-1 (Figure 6B, lane 5). Similar results were observed when probed with mature 5′ terminus of gltT tRNA (Figure 6A, probe b) and 5′ terminus of 23S rRNA (Figure 6A, probe c). In case of probe b, Δrnc::apr ΔyhgF::kan rph-1 had ~0.6 fold more 30S rRNA as compared to Δrnc::apr rph-1, but this level was reduced with subsequent inactivation of YraN and YhbQ in presence of RNase E and YbeY. Moreover, a similar pattern was seen with regard to the precursor-23S rRNA intermediate accumulation. Comparable amounts of this precursor intermediate were produced in Δrnc::apr rph-1 and Δrnc::apr ΔyhgF::kan rph-1, but this level was reduced with the inactivation of YraN and YhbQ.

**DISCUSSION**

The data presented here demonstrated that the newly identified gene YhgF which contains RNA binding domains that are similar to those found in RNase E, plays a role in the alternative 30S rRNA processing pathway. Based on our data, inactivating YhgF, YraN or YhbQ in Δrnc::apr rne-1 ΔybeY::cat rph-1 background led to an increase in 30S rRNA accumulation along with a significant increase in the precursor 23S rRNA
intermediate at both the temperatures (Figure 3B and C). However, at higher temperature these mutants had a slight reduction in 30S rRNA levels with a significant increase in precursor I. This could be because of the inactivation of the alternative processing enzymes- RNase E, YbeY and YhgF along with RNase III which essentially blocks both the primary and alternative 30S rRNA processing pathway. As a result, the balance between the amount of 30S rRNA transcribed in the cell and the amount of mature rRNAs produced is disrupted. Such a shift in equilibrium triggers the feedback regulation system in the cell, causing it to increase the rate of transcription and actively use the residual RNase E produced from before the temperature shift. However, the residual RNase E can only facilitate the initial separation of 16S rRNA from the precursor-23S rRNA intermediate, but not in the processing of the precursor intermediate in absence of YhgF. As a result, at higher temperature the amount of precursor-23S rRNA further increases in Δrnc::apr rne::kan ybeY::cat yhgF:::kan rph-1, Δrnc::apr rne-1 ybeY::cat yraN:::kan rph-1 and Δrnc::apr rne-1 ybeY::cat yrbQ:::kan rph-1 genetic background.

Additionally, these strains took more than two days to grow using either solid or liquid medium. Furthermore, as shown in our growth curve analysis, the cells were extremely sick and stopped growing within 45-60 minutes of temperature shift with the RNase E completely inactivated (Figure 5A). All these data indicated that these three new genes play an important role in the alternative processing pathway.

Based on our northern data as shown in Figure 4B, inactivating YhgF produced the highest steady-state levels of 30S rRNA as compared to the other five genes tested. There was a large amount of a precursor-23S rRNA intermediate produced with a concomitant
reduction in the mature 23S rRNA. This was the first time that we observed a reduction in the mature 23S rRNA levels. The data also indicated that YhgF plays a crucial role in the processing of precursor-23S rRNA intermediate to generate mature 23S rRNA. However, our work requires further analysis in order to confirm their role as primary or secondary processing enzymes in the alternative processing pathway.

Using high resolution primer extension analysis at the 5′ of 23S rRNA, we were able to identify the cleavage sites of YhgF (Figure 4C) in the alternative processing pathway. However, it appeared that the absence of YhgF had no effect when RNase E is expressed, suggesting that YhgF acts like a secondary processing enzyme in the alternative processing pathway. Furthermore, this observation was confirmed when Δrnc::apr ΔyhgF::kan rph-1 strain was analyzed. It had the same growth phenotype as Δrnc::apr rph-1 with almost comparable levels of 30S rRNA produced (Figure 5B, 6B). This result confirmed our hypothesis that YhgF acts as a secondary processing enzyme in the alternative 30S rRNA processing pathway and that its function becomes essential only when RNase III, RNase E and YbeY are inactivated.

However, no such processing defects were seen when YcaI, YcbL and YihG were inactivated. Moreover, inactivating YihG in Δrnc::apr rne-1 ΔybeY::cat rph-1 further reduced the amount of 30S rRNA produced; thereby confirming that it does not participate in 30S rRNA processing.

To confirm the endonucleolytic activity of these newly identified genes, in vitro assays need to be done. Based on our data, we now know that YhgF plays a role in the processing of the precursor-23S rRNA intermediate. Therefore, substrates for in vitro assays could be designed accordingly which mimics the precursor-23S rRNA. There is a
possibility that YhgF is a double-stranded RNA specific endoribonuclease as inactivating YhgF in Δrnc::apr rne-1 ΔybeY::cat rph-1 reduced the band intensities of cleavage sites denoted as B1-B3 (Figure 4C, lanes 7-8). B1 corresponds to the known RNase III processing site in the double stranded-stem located at the 3’ end of 16S rRNA. Additionally, bands B8 and B9 which were reduced in Δrnc::apr ΔybeY::cat ΔyhgF::kan rph-1 (Figure 4C, lanes 5-6) as compared to Δrnc::apr rne-1 ΔybeY::cat rph-1 (Figure 4C, lanes 3-4) is also located on the double-stranded stem flanking the mature 23S rRNA, therefore indicating it’s possible role in cleaving double-stranded RNA. Once the enzymatic activity and substrate specificity of YhgF, YraN and YhbQ are established, it will be much easier to understand their exact role in RNA metabolism in E. coli.

Though this project is not finished yet, a significant amount of information has been obtained from the data presented here. It is now clear that presence of the primary processing enzymes – RNase E, RNase G and YbeY is essential for the alternative 30S rRNA processing pathway. After the initial 30S rRNA cleavage by these enzymes, secondary processing takes place which helps in the final maturation of the rRNAs. The newly identified genes (YhgF, YraN and YhbQ) with possible endonucleolytic activity act as some of the secondary processing enzymes. However, their function is not essential if the primary processing enzymes are expressed at wild-type levels, suggesting the possibility that there are multiple secondary processing enzymes present in the cell which can facilitate the final maturation of rRNAs once the initial cleavage is done. However, the role of RNase E, RNase G and YbeY, which drives the initial 30S rRNA cleavage in the alternative pathway, is indispensable.
MATERIALS AND METHODS

Bacterial Strains and Growth Curves

All the strains used in this study are listed in Table 2. They are all isogenic derivatives of MG1693. The *rne-1* allele used for this study, encodes a temperature-sensitive mutation\(^{22}\) which functionally inactivates RNase E at 44°C. The deletions in the newly identified genes were obtained from the *E. coli* Genetic Stock Center (Yale University) and were constructed as part of the Keio Collection\(^ {33,34}\). The strains were generated by the addition of chromosomal mutant alleles using P1 transduction and subsequently were selected for the antibiotic resistance marker associated with the mutant allele. The genotypes of transductants were confirmed by PCR, or by DNA sequencing in the case of *rne-1*.

Bacterial strains for growth curve analysis and RNA isolation purposes were grown under one of two conditions: For experiments with temperature-sensitive mutations (*rne*-1), all strains were grown under similar conditions at 30°C with shaking at 225 rpm in Luria broth supplemented with thymine (50 µg/ml) and apramycin (100 mg/mL), chloramphenicol (20 mg/mL) or kanamycin (25 mg/mL), where appropriate, until they reached a cell density of approximately 1x10^7/ml (40 Klett units above background, no. 42 green filter). Subsequently, the cultures were shifted to the non-permissive temperature of 44°C. Cultures were diluted with pre-warmed growth medium as needed to maintain them in exponential growth (Klett 40-80).
For experiments not involving strains with temperature-sensitive mutations, strains were grown at 37°C with shaking at 225 rpm in Luria broth supplemented with thymine (50 µg/ml) and antibiotics, where appropriate, until they reached a cell density of approximately 50 Klett units above background. In both the cases, measurements were taken every 30 minutes for growth curve analysis, whereas for RNA isolation samples were collected before and 30 minutes after temperature shift. Finally, the growth curves were replicated in triplicate and values averaged and then plotted.

RNA Isolation and Northern Analysis

Total RNA was isolated from various exponentially growing strains as described previously under the growth conditions described above. The quality of RNA samples was checked by agarose gel electrophoresis and then quantified using a Nanodrop® ND-2000c spectrophotometer.

Glyoxal agarose northern analysis was conducted using samples prepared with NorthernMax®-Gly sample loading dye (Ambion) and electrophoresis in a 1% agarose gel in BPTE buffer run at 100 volts for approximately 3 hours. Gel transfer and subsequent steps were conducted as previously described. To analyze the processing pattern of the 30S rRNA transcript, northern blots were probed with γ-P32-end-labeled oligonucleotides labeled using T4 PNK (NEB) that were specific for different regions of the 30S rRNA transcript. The various oligonucleotide probes used are listed in Table 3. Quantification of band intensities was done using ImageQuant TL 7.0 software.
Primer Extension Analysis

Primer extension analysis for the 5′ of 16S and 23S rRNA were performed as described previously\textsuperscript{36}. The oligonucleotides used for the analysis are listed in Table 3.

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24. McDowall KJ, Lin-Chao S, Cohen SN. A+U content rather than a particular


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Table 3.1: List of candidate endonucleases based on domain and keyword matches.

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<th>Gene</th>
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<th>Matches in protein</th>
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<td>yhgF</td>
<td>b3407</td>
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<td>b0927</td>
<td>&quot;Beta-lactamase-like domain&quot;, Metal ion binding</td>
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<td>&quot;tRNA-endonuclease-like domain&quot;/&quot;Restriction endonuclease type II-like&quot;</td>
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Table 3.2: List of bacterial strains used in this study

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Table 3.3: Oligonucleotide probes used in this study.

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<td>16S exc RNase III</td>
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<td>16S 1586 (probe a)</td>
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<td>16S N5</td>
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<td>Before 5’ gltT</td>
<td>TCGTGTAGGGTGAGCTTTTCATTAATA</td>
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<td>23S 3580 (probe c)</td>
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<td>23S 2873</td>
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<td>5S 3’</td>
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<td>23S inc RNase III</td>
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**Figure 3.1. Model of *E. coli* rRNA processing.** The 30S pre-rRNA transcript is first cleaved by RNase III at two physical locations, cleaving the ds rRNA formed by the spacer sequences adjacent to mature 16S and 23S rRNAs, generating 17S, 25S, and 9S pre-rRNAs (labeled in green). 17S rRNA is cleaved first by RNase E and then by RNase G at the mature 5’ end of 16S rRNA. YbeY along with RNase R processes the extra 33nts at the 3’ end of 17S rRNA to generate the mature 3’ terminus of 16S rRNA. RNase E also cleaves 9S three nt on each side of the mature termini of 5S rRNA forming p5S. RNase P cleaves at the mature 5’ end of the tRNA. Exoribonucleases (primarily RNase T) are responsible for the 3’ end maturation of the tRNA, 23S rRNA, and 5S rRNA, but the endoribonuclease(s) responsible for the maturation of 5’ end of 23S, and 5’ end of 5S rRNAs remain unidentified (labeled in red). This model is not drawn to scale.
Figure 3.2. Primary vs Alternative 30S rRNA processing pathway in *E. coli*.

The primary RNase III- dependent pathway is indicated at the left side of the figure, whereas the RNase III-independent alternative processing pathway is drawn towards the right side of the figure. The 30S pre-rRNA transcript is first cleaved by RNase III or by RNase E, RNase G and YbeY to generate precursor intermediates. These precursors are further processed by secondary processing enzymes and exoribonucleases to generate the mature RNAs. This model is not drawn to scale.
Figure 3.3. Agarose Northern Blot analysis to study the effect of inactivation of the new genes in absence of RNase III, RNase E and YbeY. (A) The location of the oligonucleotides used for the northern blot analysis are shown. (B, C) Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a 1% agarose gel. The genetic composition of each band is shown in the corresponding cartoon drawn in the right. The mature 16S, 23S and gltT tRNA are denoted as M$_{16}$, M$_{23}$ and M$_{t}$. I designate an intermediate that contains the tRNA, 23S rRNA and 5S rRNA.
Figure 3.4. YhgF is a secondary processing enzyme in the alternative processing pathway. (A) The locations of the oligonucleotides used for the northern Blot and primer extension analysis are shown. (B) Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a 1% agarose gel. The 30S rRNA levels were highest in \( \textit{Arnc::apr rne-1 \Delta ybeY::cat \Delta yhgF::kan rph-1} \) as compared to the other strains. There was a large increase in the precursor-23S rRNA accumulation (I) with a simultaneous reduction in mature 23S rRNA (\( \textit{M}_{23} \)) levels, indicating its role in processing this intermediate and thereby helping its separation from precursor-16S rRNA. (C) Primer extension analysis of the 5’ ends of 23S rRNA. The cleavage sites were marked as bands B1-10. The mature 5’ terminus of 23S is denoted as \( \textit{M}_{23} \) in the figure which is produced only in \( \textit{rph-1} \) control stain and is absent in all the mutant strains.
Figure 3.5. Growth curves for various mutants (A) Since most of the mutants were constructed in *rne*-I genetic background (temperature sensitive mutant), the cells were initially grown at 30°C until they reached the early exponential phase (40 Klett units, denoted by the black line). Subsequently, the cultures were shifted to 44°C. Cultures were diluted with pre-warmed growth medium as needed to maintain them in exponential growth. Measurements were taken at intervals of 30 minutes. (B) Similar to (A) except that the cells were grown at 37°C, since these strains were not temperature sensitive.
Figure 3.6. Agarose Northern Blot analysis to study the role of YhgF, YraN and YhbQ in presence of RNase E and YbeY. (A) The location of the oligonucleotides used for the northern blot analysis are shown. (B) Cells were initially grown at 30°C (P) into early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA isolated at both the temperatures and were run on a 1% agarose gel. The mature 16S, 23S and gltT tRNA are denoted as $M_{16}$, $M_{23}$ and $M_t$. 
CHAPTER 4

ANALYSIS OF THE PROCESSING OF INDIVIDUAL RIBOSOMAL OPERONS
IN *ESCHERICHIA COLI* AND THE SIGNIFICANCE OF CONSERVATION OF
SPACER tRNA IN BETWEEN 16S AND 23S rRNA

Gargi Chaudhuri and Sidney R. Kushner, to be submitted to *Molecular Microbiology.*

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INTRODUCTION

Bacteria possess a sophisticated genetic machinery to optimize their growth rate in response to various environmental conditions. Growth rate is directly related to the level of ribosome synthesis, the cellular machine responsible for the active protein synthesis in cells from every life form. The number of ribosomes produced per cell in a growing bacteria is directly proportional to the growth rate\(^1\). Moreover, the intracellular level of RNA polymerase, the key apparatus of transcription is directly correlated with ribosome levels\(^2,3\).

In *Escherichia coli*, rapidly dividing cells produce hundreds of new ribosomes each minute\(^4\) to meet the demands of protein synthesis. At any time, the number of active ribosomes produced in the cell is proportional to the rate of synthesis of total RNA. When the cell reaches doubling times of around 20 min, there are as many as 70,000 ribosomes per *E. coli* cell, while at lower growth rates there may be only 20,000 ribosomes per cell\(^5\). Additionally, the bulk amounts of RNA transcribed and protein produced in the cell at any time are directly linked to the growth rate. Since the bulk of the bacterial RNA is ribosomal RNA (rRNA), ~80% of the total RNA, processing and availability of mature rRNA is crucial to the cell’s survival.

To provide appropriate levels of rRNA, bacterial genomes typically include multiple copies of *rrn* operons in their genome and at high growth rates most of the RNA polymerase molecules are assigned to the transcription of these operons\(^6\). In *E. coli*, ribosomes are composed of three species of rRNA (16S, 23S and 5S rRNA) and a total of 22 species of ribosomal protein (r-proteins) for the 30S ribosomal subunit and 35 species
of r-proteins for the 50S ribosomal subunit. The synthesis of these rRNA species and r-proteins are highly coordinated for efficient assembly of ribosomes.

The *E. coli* K-12 genome contains seven rRNA operons, which are similar in the basic gene organization, with each consisting of tandem promoters (P1 and P2), 16S rRNA gene, spacer tRNA genes (tRNA$^{\text{ala}}$ and tRNA$^{\text{ile}}$ in the *rrnA*, *rrnD* and *rrnH* operons and tRNA$^{\text{glu}}$ gene in the *rrnB*, *rrnC*, *rrnE* and *rrnG* genes), 23S rRNA gene, 5S rRNA gene and distal tRNA genes at 3′ termini (tRNA$^{\text{thr}}$ gene in the *rrnD*, tRNA$^{\text{asp}}$ genes in the *rrnC* and *rrnH* operon and tRNA$^{\text{trp}}$ in *rrnC*), respectively (Figure 1).

Each of the seven rRNA operons is transcribed from two promoters, upstream P1 and downstream P2, which are separated by approximately 120 bp in length (Figure 1). It has been shown that P1 plays a major role in high-level synthesis of rRNA during exponential growth in nutrient-rich media, while P2 accounts for the basal-level synthesis of rRNA at low growth rates and in stationary phase. The cis-acting elements namely, AT-rich ‘UP element’ and GC-rich ‘discriminator’ located within the promoter region along with the trans-acting protein factors (Fis, H-NS, Lrp and DksA) are also involved in the regulation of rRNA synthesis. In addition, recent work has shown that the two nucleotide factors, pppGpp$^{11-13}$ and ‘initiating nucleotide’ iNTPs$^{14}$, also participate in this rRNA synthesis regulation.$^{6,9,15}$

Initially the seven rRNA copies were believed to be identical in their structure and function. However, recent analysis of the seven rRNA operons in *E. coli* has revealed significant differences in their expression pattern. It has been suggested that the seven rRNA operons has the growth rate- and growth phase-dependent differences in their
expression\textsuperscript{16,17}. Moreover, even though the sequences of seven \textit{rrn} operons are almost identical, further analysis revealed significant differences in their tRNA identities, tRNA copy number and 5S rRNA copy number (Figure 1).

All the seven \textit{rrn} operons in \textit{E. coli} are located in non-contiguous sites around the genome and are transcribed in the same direction as genome replication\textsuperscript{18}. The redundancy of rRNA operons in \textit{E. coli} has been considered to be an adaptive mechanism which has evolved in order to support the high levels of ribosome production necessary for rapid growth rates\textsuperscript{19,20}. All the seven rRNA operons are needed for the optimal adaptation to changing physiological conditions\textsuperscript{21}. Studies on \textit{E. coli} strains with the deletion of several rRNA operons\textsuperscript{22,23}, have shown that the level of growth reduction is directly related to the number of deleted rRNA operons. However, the presence of even a single rRNA operon on the genome can produce as much as 56\% of wild-type levels of rRNA\textsuperscript{23}. Subsequently, the correlation between the number of \textit{rrn} operons and the rate of cell growth was confirmed by studies using a set of engineered rRNA operon copy-number variants\textsuperscript{24}. Based on these findings, it has been suggested that \textit{E. coli} requires an excessive level of ribosome production in order to keep a certain level of ribosome storage. Moreover, the unused ribosomes have been seen to be stored in bacterial cells as inactive forms by forming ribosome dimers after interaction with dimerization factors such as ribosome modulation factor RMF\textsuperscript{25}. Therefore, these results indicate that the redundancy of \textit{rrn} operons in \textit{E. coli} is an adaptive mechanism by which it stores the necessary machinery required for survival in adverse conditions.
In *E. coli*, all three ribosomal RNAs (rRNAs) 16S, 23S and 5S rRNAs are co-transcribed along with various tRNAs as the 30S primary transcripts (Figure 2). It has been shown that RNase III, a double-stranded RNA specific endoribonuclease encoded by *rnc*\(^{26}\), cleaves the 30S rRNA primary transcript into their respective pre-rRNA species. However, RNase III is not essential in *E. coli*\(^{27}\) and when the RNA from RNase III deficient strains has been analyzed, the 30S rRNA accumulation constituted <5% of the total RNA. In addition, it has been previously shown that functional ribosomes can still be made in RNase III-deficient strain, therefore indicating the presence of an efficient alternative 30S rRNA processing pathway. In Chapter 2 and 3, we have identified some of the enzymes involved in this alternative 30S rRNA processing pathway. We have shown that RNase E, RNase G and YbeY carry out the initial cleavages of the 30S primary transcript to separate the 16S rRNA from the rest of the transcript in absence of RNase III (Figure 2). Once the pre-16S rRNA is separated from the rest of the transcript, which contains the spacer tRNA, 23S rRNA and the 5S rRNA, they are acted upon by the secondary processing enzymes RNase P, RNase Z, Yhgf, YraN and YhbQ along with the exoribonucleases to generate the functional species (Figure 2). We have also identified the cleavage sites of the alternative processing pathway which are unique from the RNase III-dependent processing pathway.

However, the strains used in Chapters 2 and 3 were constructed with all the seven chromosomally encoded *rrn* operons intact. Therefore, rRNA was actively produced from all seven of them. As an additional potential complication, since each of the seven rRNA operons in *E. coli* are different from each other in terms of nucleotide sequence of the exterior and interior transcribed spacer regions, the length of the 30S transcript, rRNA
and tRNA copy numbers, and tRNA identities (Figure 1), the alternative processing pathway produced a large number of products. The lack of homogeneity among the rRNA operons also raised the question of whether they were all processed by the same enzymes at the same locations with similar efficiencies, further complicating the analysis of processing by generating operon-dependent cleavage patterns.

In order to study the processing pattern from the individual rrn operons in the alternative 30S rRNA processing pathway, we have developed a system in which we could investigate rRNA processing from a single rRNA operon. Studying the processing from individual operons should potentially reduce the number of processing intermediates allowing us to study if all the rRNA operons were processed in the same manner by the alternative pathway.

Accordingly, we obtained a strain of E. coli from the Cathy Squires laboratory, which lacks all seven rRNA operons in the MG1655 genetic background. The mutant was kept viable through two plasmids, one a pSC101 derivative (6-8 copies per cell) that contains the rrnB operon, and one a p15A derivative (~15 copies per cell) that contains all of the tRNAs normally found within the deleted rRNA operons (Cathy Squires, unpublished results).

Although the generation time of this strain in rich medium was approximately 10 minutes longer versus a rph-1 control containing all seven rrn operons, this particular strain has been proven to be phenotypically more robust than previously published strains that lacked all chromosomal rRNA operons (Mark Stead, unpublished results). However, analysis of the the plasmid carrying the rrnB operon in the Squires strain (pK4-16 in strain SQ2158, see Table 3), revealed that the plasmid could not be used to do
plasmid displacement to study other \textit{rrn} operons. Therefore, a new series of pSC101 origin plasmids was developed using pWSK29 derivatives\textsuperscript{29} (pMSK2-4, Table 1, Mark Stead, unpublished results).

For the purpose of this study, three rRNA operons were chosen in order to address the effect of heterogeneity seen amongst the seven operons: \textit{rrnB}, \textit{rrnG}, and \textit{rrnD}. The \textit{E. coli} rRNA operons can be broadly classified into two groups with \textit{rrnB}, \textit{rrnC}, \textit{rrnE}, and \textit{rrnG} being very similar, and \textit{rrnA}, \textit{rrnD}, and \textit{rrnH} being very similar to each other (Figure 1). Among them, the two operons representing the greatest divergence from each other are \textit{rrnD} and \textit{rrnB}. Therefore, these two operons, along with \textit{rrnG} (as \textit{rrnG} is very similar to \textit{rrnB} and both operons should be processed similarly if nucleotide sequence is the primary determinant for cleavage) were cloned to generate pMSK2-4 (Table 1), respectively. The individual ribosomal operon plasmids were constructed such that each contains a different selectable drug marker from each other. Since \textit{E. coli} can only maintain a single pSC101 origin plasmid in the absence of selection, the introduction of a new \textit{rrn} operon-containing plasmid with appropriate drug selection will essentially displace the resident \textit{rrn} operon plasmid. Thus, using the plasmid displacement method\textsuperscript{30} and P1 transduction various multiple mutants were made in order to study the alternative 30S rRNA processing pathway.

Here, we show that there are some differences in the alternative 30S rRNA pathway between the \textit{rrnG} and \textit{rrnD} operons. Inactivating RNase III, RNase E and YbeY resulted in slightly higher 30S rRNA levels when rRNAs were produced from the \textit{rrnD} operon as compared to \textit{rrnG} operon. Moreover, more 23S rRNA precursor intermediates were produced with a simultaneous reduction in mature 23S rRNA levels suggesting a more
pronounced 23S rRNA processing defects for the *rrnD* operon. In contrast, the 16S rRNA processing defect was more severe for the *rrnG* operon as compared to *rrnD* operon. Additionally, the two strains had differences in their processing sites in the alternative pathway further confirming that the heterogeneity seen in the individual operons has an impact on the alternative 30S rRNA processing pattern.

Moreover, since the location of the spacer tRNA between the 16S and 23S rRNA is highly conserved among all the seven operons, it raised the question if it helps in the separation of the rRNAs in the alternative processing pathway. In order to study the role of the spacer tRNA in the alternative pathway, a ribosomal operon plasmid (*rrnB* operon plasmid, pGCK1, Table 1) was constructed in which the spacer tRNA gene was deleted and replaced with a unique restriction site. Using the plasmid displacement method, the *rrnG* operon plasmid was replaced with the *rrnB* plasmid in the presence and absence of RNase III. We show that in the presence of RNase III, the spacer tRNA does not play any role in the separation of 16S and 23S rRNA. However, in absence of RNase III, the presence of the spacer tRNA between 16S and 23S rRNA becomes essential, thereby suggesting its possible role in the alternative processing pathway.

**RESULTS**

**Analysis of the alternative 30S rRNA processing pathway in strains containing single rRNA operon**

Based on the results presented in Chapter 2, RNase E, RNase G and YbeY are responsible for the initial cleavages of the 30S primary transcript in the alternative processing pathway. In order to investigate the processing pattern of the alternative
pathway in individual rRNA operons, we constructed a set of isogenic strains with RNase III, RNase E and YbeY inactivated carrying either the \textit{rrnD} or \textit{rrnG} operon plasmids. These triple mutants were constructed such that all the chromosomally encoded \textit{rrn} operons were deleted and they were kept viable using either the \textit{rrnD} or \textit{rrnG} plasmids along with the tRNA plasmid (pTRNA67) containing all of the tRNAs normally found within the deleted rRNA operons. Subsequently, RNA was isolated from each strain and examined using northern blot analysis.

Since RNase E is essential in \textit{E. coli}, the multiple mutants were constructed using the temperature sensitive RNase E mutant \textit{rne-I} \textsuperscript{31} (a point mutation at residue 66). Therefore, the cells were grown first at permissive temperature (30°C) into early exponential phase and were subsequently shifted to the non-permissive temperature (44°C). Samples for RNA extraction was collected just before and 30 minutes after the temperature shift. We first probed with an oligonucleotide that was complementary to the mature 5' end of 16S rRNA coding sequence (Figure 3A, probe a). In the \textit{rph-1} control, both the \textit{rrnD} (Figure 3B, lanes 1-2) and \textit{rrnG} (Figure 3B, lanes 7-8) derivatives had mature 16S rRNA, without any 30S rRNA beng present as previously observed. However, inactivation of RNase III in both \textit{rrnD} (Figure 3B, C lanes 3-4) and \textit{rrnG} strains (Figure 3B, C lanes 9-10) inactivated the primary processing pathway, leading to the presence of a 30S rRNA species which constituted around 3-5% of the total RNA. When quantitated, inactivation of RNase III in the \textit{rrnD} strain produced \textasciitilde{}0.56 fold more 30S rRNA compared to the \textit{rrnG} strain. However, since the alternative 30S rRNA processing pathway was still functional, most of the 30S rRNA primary transcript was processed to generate mature 16S rRNA (M\textsubscript{16}). Furthermore, inactivation of some of the
primary processing enzymes of the alternative pathway along with RNase III (\textit{Arnc::apr rne-1 \textit{AybeY::cat rph-1}}) in both single operon strains, blocked the primary and alternative processing pathways, thereby causing a large increase in the levels of 30S rRNA as compared to the \textit{Arnc::apr rph-1} strain. In case of \textit{rrnD} operon (Figure 3B, lane 5), there was \~7.3 fold more 30S rRNA produced as compared to the \textit{Arnc::apr rph-1} double mutant (Figure 3B, lanes 3). For the \textit{rrnG} operon (Figure 3B, lane 11), there was \~8.6 fold more 30S rRNA as compared to \textit{Arnc::apr rph-1} double mutant (Figure 3B, lanes 7). It was evident that inactivating RNase E and YbeY in absence of RNase III had a large impact on 30S rRNA processing suggesting that both \textit{rrnD} and \textit{rrnG} operons require RNase E and YbeY activity for their processing.

However, there were some notable differences in 16S rRNA processing pattern between the two operons. The processed fraction of mature 16S rRNA in \textit{rrnG} was around 23-29\%, whereas in \textit{rrnD} it was comparatively more \~31-37\%. Additionally, at non-permissive temperature the larger 18S precursor increased with a concomitant reduction in the amount of 17S rRNA precursor with no significant effect on the mature 16S rRNA levels for the \textit{rrnD} operon. In case of the \textit{rrnG} operon, along with the increase in the 18S precursor intermediate and 16S* rRNA truncated processing product, there was a concomitant reduction in both 17S and mature 16S rRNA levels at higher temperature, but not in \textit{rrnD}. These differences in precursor intermediate levels could be due to differences in the efficiency of the processing enzymes involved in the alternative processing pathway for the individual operons. Subsequently, the same blot was probed with an oligonucleotide complementary to 5’ mature terminus of 23S rRNA (Figure 3A, probe c). Similar results for both the operons were observed with maximum 30S rRNA.
levels in strains where RNase E and YbeY were inactivated in absence of RNase III, confirming that they both require the activity of these enzymes for the initial cleavages of 30S rRNA. Furthermore, there was a larger accumulation of the 23S rRNA precursor intermediate (I) in the triple mutant for the *rrnD* operon as compared to the *rrnG* operon. When quantitated it was seen that at non-permissive temperature this precursor was around 8.9 fold more in *Arnc:*apr *rne-1 Aybey:*cat *rph-1* strain (Figure 3B, lane 6) as compared to *Arnc:*apr *rph-1*. In contrast the increase in *rrnG* operon (Figure 3B, lane 11) was only 2 fold more but it was not as pronounced as for the *rrnD* operon.

In addition, it appeared that the 23S precursor intermediate (I) was composed of two distinct bands of nearly comparable sizes for the *rrnD* operon. The presence of two bands for this precursor could be due to the presence of two spacer tRNAs in between 16S and 23S rRNA in the *rrnD* operon which are probably cleaved successively to be separated from the 23S rRNA. This result suggested that the processing of the precursor 23S rRNA intermediate I in *rrnD* operon takes place in multiple steps, which first requires the removal of the tRNA\textsubscript{ile} gene from the rest of the precursor intermediate followed by the removal of the next tRNA\textsubscript{ala} gene. But the *rrnG* operon has only one spacer tRNA and as a result we do not see two distinct bands for the precursor I. Thus, based on the northern data, it was clear that the heterogeneity seen among the different *rrn* operons had an impact on the processing pattern in the alternative 30S rRNA processing pathway.

To further confirm this hypothesis, primer extension analysis was done at both the 5′ terminus of 16S (Figure 3A, probe a) and 23S rRNA (Figure 3A, probe d). As previously observed, the mature 16S rRNA was produced from both *rrnD* and *rrnG* operons even in the absence of RNase III (denoted as M\textsubscript{16}, Figure 3C and D, lanes 1-4). However, in
absence of RNase III, four new bands (A1-4) appeared (Figure 3C, D, lanes 3-6) which were absent in *rph-1* control strain (Figure 3C, D, lanes 1-2), *rrnD* and *rph-1, rrnG* (Figure 3D, lanes 1-2). When mapped, bands A1 and A2 corresponded to the promoter sites of their respective operons. Band A3 corresponded to an AU rich region located 15 bp upstream of the known RNase III processing site. In case of *rrnD* operon, there were two bands for A3 which were located very close to each other. The second band for A3 was not seen in *rrnG* rRNA operon suggesting that the processing enzymes which cleave at this location make multiple cuts in *rrnD* operon but not in *rrnG*. Surprisingly, the mature 16S rRNA (M16) and the bands denoted as A4 were produced in significantly reduced amounts in *Arnc::apr rne-1 AybeY::cat rph-1, rrnG* strain as compared to *Arnc::apr rph-1, rrnG* and *Arnc::apr rne-1 AybeY::cat rph-1, rrnD* strains. This result corroborated our northern data where we have observed that the mature 16S rRNA was barely produced from the *rrnG* operon in absence of RNase III, RNase E and YbeY, indicating that inactivating these enzymes has a more severe 16S rRNA processing defect in *rrnG* operon as compared to *rrnD* operon.

Similarly, when the cleavage sites for the alternative pathway at the 5′ terminus of 23S rRNA was mapped, there were differences seen between *rrnD* and *rrnG* operons. In both *rph-1, rrnD* and *rph-1, rrnG* control strains the 5′ mature terminus of 23S rRNA was seen along with the known RNase III restriction sites (Figure 3E, F, lanes 1-2), whereas in absence of RNase III, the 5′ terminus was completely absent (Figure 3E, F, lanes 3-4). Instead, a series of new cleavage sites appeared which are denoted as B1-11 in Figure 3D and E (lanes 3-4). Inactivation of RNase E and YbeY in absence of RNase III (Figure 3E, F, lanes 5-6) significantly reduced the band intensities of B4-B9 for both the operons.
suggesting that RNase E and YbeY cleave at these locations for both the operons in the alternative processing pathway. When mapped, it was seen that they were located in the spacer region in between the spacer tRNA and 23S rRNA. Additionally, these bands were mostly concentrated in AU rich regions which is a preferred substrate for RNase E activity, therefore confirming that RNase Ecleaves at these locations in the alternative pathway. However, bands B2 and B4 appeared only when RNase E and YbeY were inactivated in absence of RNase III, indicating the activity of other enzymes in the alternative processing pathway.

Furthermore, the increase in band intensity of B1, B3 and B11 on inactivation of RNase E, RNase III and YbeY (Figure 3E, F, lanes 5-6) also suggested the activity of other enzymes involved in the pathway. When the band intensities of B4-10 observed in Δrne::apr rne-1 Δybey::cat rph-1, rrnD and Δrne::apr rne-1 Δybey::cat rph-1, rrnG strains were compared, inactivation of RNase III, RNase E and YbeY had a more significant effect at the 5′ terminus of 23S derived from the rrnD operon as compared to rrnG operon. The bands were significantly reduced in rrnD operon as compared to rrnG. This result was in agreement with our northern data where we saw that with the rrnD rRNA operon the 30S and 23S rRNA precursor levels produced were higher as compared to rrnG operon. This result indicated that in the alternative processing pathway, RNase E and YbeY has different efficiencies to process individual rRNAs depending on the operon.
Construction of rRNA operon plasmid without the spacer tRNA

The presence of a spacer tRNA in between the 16S and 23S rRNA is highly conserved among all the seven *rrn* operons (Figure 1). The conservation of the tRNA in that location raised the question on whether it helps in the proper folding of the rRNAs such that it increases the processing efficiencies of RNase III or the enzymes in the alternative processing pathway. It is also possible that it plays an important role in the separation of the 16S rRNA and 23S rRNA in the 30S rRNA alternative processing pathway. In order to investigate its role, we constructed a ribosomal operon plasmid (pGCK-1, *rrnB*) where the spacer tRNA<sup>glu</sup> gene was deleted using overlap PCR (Figure 4) and replaced with a unique restriction site. The primers used for this overlap PCR is shown in Table 2. This construction was done using the *rrnB* plasmid (pMSK3) which is a pSC101 origin plasmid and a pVMK94 derivative.

Role of spacer tRNA in presence of RNase III

In order to study the significance of the spacer tRNA, we first tried to displace an *rrnG* operon plasmid (containing the spacer tRNA gene) with the *rrnB* plasmid in presence of RNase III as described in Ow et. al<sup>30</sup> with some modifications. After three dilutions in presence of apramycin (antibiotic resistance marker for *rrnB*), the cells were spread on plates with the proper antibiotic. Subsequently the purified single colonies were tested to determine if they had become susceptible to ampicillin (antibiotic resistance marker for *rrnG*) which would indicate that it lost the resident plasmid *rrnG* (Figure 5A).
To confirm the loss of the resident plasmid, restriction digestion of the plasmids was
done and it was seen that apramycin resistant strains (Figure 5B, lanes 6-7) did not
produce fragments of the same size as \textit{rrnG} (Figure 5B, lane 2 and 5) indicating that it
had lost the resident plasmid. Instead, it now has a fragment produced by the unique
restriction site (\textit{NsiI}) from the \textit{rrnB*} (Figure 5B, lanes 6-7) that was absent in the \textit{rrnG}
plasmid (Figure 5B, lane 2 and 5), therefore confirming successful plasmid displacement.
It also suggests that in presence of RNase III, the presence of the spacer tRNA in between
the 16S and 23S rRNA is not essential.

Subsequently, RNA isolated from the \textit{rrnB*} displaced operon plasmid strain was
analyzed using a 1\% agarose gel (Figure 5C). The \textit{rph-1} strain containing all seven
operons intact produced mature 23S and 16S rRNA with no 30S rRNA accumulation
(Figure 5C, lane 1).

As previously observed, in the \textit{Arnc::apr rph-1} strain (containing all seven
chromosomal operons) 30S rRNA was observed which constituted \(~3\% of total RNA
along with mature 23S and 16S rRNA (Figure 5C, lane 2). There was a small amount of
precursor 23S rRNA intermediate observed suggesting a 30S rRNA processing defect.
RNA isolated from the cells with all the seven chromosomal \textit{rrn} operons deleted and kept
viable with the \textit{rrnG} operon plasmid (Figure 5C, lane 3), did not produce any 30S rRNA.
Additionally, it did not exhibit any defect in 23S or 16S rRNA processing.

A similar result was seen when the \textit{rrnG} operon was displaced with the \textit{rrnB*}
(Figure 5C, lane 4). The absence of the spacer tRNA did not lead to any 30S rRNA
accumulation and had mature 16S and 23S rRNA as seen in the \textit{rph-1} control. A similar
effect was seen with an independent biological isolate (Figure 5C, lane 5). These results confirmed our hypothesis that in presence of RNase III, the spacer tRNA had no significant role in 30S rRNA processing.

**Role of spacer tRNA in absence of RNase III**

Based on our plasmid displacement analysis, we showed that in presence of RNase III, the spacer tRNA had no significant role in 30S rRNA processing. However, the possibility that its presence become important in absence of RNase III could not be entirely ruled out. Therefore, in order to study the role of the spacer tRNA in the alternative 30S rRNA processing pathway, the *rrnB* plasmid was used to displace *rrnG* in the Δ*rnc38::kan rph-1* background. The Δ*rnc38::kan rph-1* strain was constructed by deleting a 40bp region in the coding sequence of RNase III and was replaced by a kanamycin cassette\(^{27}\). The resultant mutant retained the coding capacity for the first 71 of the 227 amino acid residues found in RNase III\(^{27}\). In this RNase III deficient strain, we were able to displace the *rrnG* operon plasmid with the *rrnB* (Figure 6A). The colonies were susceptible to Ampicillin (marked on *rrnG*) whereas they were resistant to apramycin (marked on *rrnB*).

Similarly, to confirm the plasmid displacement, restriction digestion of the plasmids were done and it was seen that both the isolates which had lost their resistance to ampicillin antibiotic marker (Figure 6B, lanes 4-5) had the same fragment sizes as *rrnB* (Figure 6B, lane 3) which was missing in *rrnG* (Figure 6B, lane 2). Thus, in Δ*rnc38::kan rph-1* background the presence of the spacer tRNA was not essential. Moreover, RNA isolated from these isolates containing the *rrnB* operon did not exhibit any significant
30S rRNA processing defects (data not shown). They had comparable levels of 30S rRNA compared to the Δrnc38::kan rph-1, rrnG, thus confirming the non-essential role of the spacer tRNA in this background.

However, the Δrnc38::kan rph-1, rrnG strain produced a truncated protein, which could alter the effects of spacer tRNA in the alternative processing pathway. In order to eliminate this possibility the rrnG plasmid in the complete rnc deletion (Δrnc::apr rph-1, rrnG) strain was used for further analysis. Since the Δrnc deletion was marked with apramycin flanked with FRT sites, it was first treated to remove the antibiotic cassette using the FLP recombinase from pCP20 plasmid. Subsequently, the rrnB* plasmid was used to displace rrnG plasmid in Δrnc rph-1 genetic background. Based on replica plating and antibiotic selection, it was observed that after three dilutions, some of the isolates lost the resident rrnG operon plasmid and retained only the rrnB* operon plasmid. However, when they were tested for plasmid restriction digestion, none of them had the fragment sizes of rrnG (Figure 6C, lane 2) or rrnB* (Figure 6C, lane 3). All of them had new bands of different sizes (Figure 6C, lane 5) suggesting that the new incoming rrnB* plasmid has recombined with the resident rrnG operon plasmid in Δrnc rph-1 genetic background. Moreover, the fragment produced from the restriction enzyme Nsil present only in the rrnB* was not observed in these recombined isolates indicating that the cells with rrnB* may have recombined with rrnG such that it retained the spacer tRNA and thereby lost the Nsil restriction site. Such a recombination event was not observed in Δrnc38::kan rph-1 genetic background (Figure 6C, lanes 4), where after the rrnB* operon plasmid displacement, it produced the fragments of the same size as rrnB* (Figure 6C, lane 3).
This was the first time when we had observed such a recombination event indicating that the cells probably cannot survive without the spacer tRNA in absence of functional RNase III produced. In order to confirm this hypothesis, the recA gene (responsible for homologous recombination in E. coli) was inactivated in the Δrnc rph-1, rrnG genetic background. Subsequently, the rrnB* plasmid was used to displace rrnG in the Δrnc ΔrecA::kan rph-1, rrnG background. However, after several attempts we have not been able to displace the rrnG plasmid in this genetic background, thereby suggesting that the spacer tRNA is probably essential for 30S rRNA processing in absence of RNase III in E. coli. To further confirm it, the plasmid displacement needs to be done in Δrnc rph-1, rrnG background with a rnc covering plasmid (pMSK9). If rrnB* can displace rrnG in this genetic background with RNase III produced from the covering plasmid, it would confirm the role of the spacer tRNA in separation of 16S and 23S rRNA in the alternative 30S rRNA processing pathway.

**DISCUSSION**

In E. coli the rRNAs are transcribed from seven different operons, which have the basic same general organization. Each of these seven operons consist of tandem promoters (P1 and P2) at the 5’ end followed by a 16S rRNA gene, spacer tRNA genes (tRNA{ala} and tRNA{ile} in the rrnA, rrnD and rrnH operons and tRNA{glu} gene in the rrnB, rrnC, rrnE and rrnG genes), a 23S rRNA gene, 5S rRNA gene and distal tRNA genes at 3’ termini (tRNA{thr} gene in the rrnD, tRNA{asp} genes in the rrnC and rrnH operon and tRNA{asp} in rrnC), respectively (Figure 1).
The data presented here has demonstrated that these heterogeneities seen in the individual rRNA operons affect the processing patterns in the alternative 30S rRNA processing pathway. For the purpose of this study, we focused mainly on *rrnB*, *rrnD* and *rrnG* operons since they have the most significant differences in their gene organization.

Based on our northern data analysis on the multiple mutants (Figure 3B, C), we showed that inactivation of RNase E and YbeY in absence of RNase III, had a more significant effect on the 16S rRNA processing in the *rrnG* operon as compared to the *rrnD* operon. In contrast, in the case of 23S rRNA processing, inactivation of these enzymes had a more significant effect on the *rrnD* operon as compared to the *rrnG* operon.

The processing defects observed in northern blots were confirmed by the primer extension analysis (Figure 3C-F). It was seen that in absence of RNase III, RNase E and YbeY hardly any mature 16S rRNA was produced from the *rrnG* operon. The band for mature 5′ terminus of 16S rRNA decreased drastically in absence of these enzymes for the *rrnG* operon (Figure 3D, lanes 5-6), although no such reduction was seen for the *rrnD* operon (Figure 3C, lanes 5-6). Similarly, some of the multiple cleavage sites that appeared at the 5′ terminus of 23S rRNA in absence of RNase III were not seen for the *rrnD* operon strain when RNase E and YbeY were inactivated (Figure 3E, lanes 5-6). The same impact was seen in *rrnG* operon but in much reduced levels (Figure 3F, lanes 5-6), suggesting that RNase E and YbeY are more efficient in processing 23S rRNA in *rrnD* operon as compared to *rrnG* operon.
However, we did not observe any significantly different processing intermediates or cleavage sites produced by the enzymes involved in the alternative processing pathway between the two individual operons. Overall, our data indicated that the heterogeneities seen in the individual operons affect the processing efficiencies of the enzymes involved in the alternative 30S rRNA pathway.

Despite the several heterogeneities seen among seven rRNA operons, one organizational aspect that has been highly conserved among all of them is the location of a spacer tRNA gene in between the 16S and 23S rRNA. In order to study the significance of the conservation of the spacer tRNA in that location, we have constructed a rRNA operon plasmid (rrnB*) where the spacer tRNA gene was deleted and replaced with a unique restriction site. This plasmid was used to displace rrnG operon plasmid both in presence and absence of RNase III. It was seen that in presence of RNase III, the spacer tRNA gene is not essential for the 30S rRNA processing and therefore does not affect the cell’s viability.

However, we have not been able to displace the rrnG operon plasmid with the spacer tRNA-less rrnB* operon plasmid in absence of RNase III, suggesting that the conservation of location of the spacer tRNA may have a role in the separation of 16S and 23S rRNA in the alternative pathway. To further support our theory, detailed analysis is required. In order to prove the essential role of spacer tRNA in the alternative pathway, some of the experiments that needs to be done in future are, 1) displace rrnG by the rrnB* operon plasmid, in presence of rnc covering plasmid in the Δrnc rph-1 genetic background and, 2) subsequently, displace the rnc covering plasmid with an empty vector in the same genetic background. Failure to displace the rnc covering plasmid by an empty
vector in Δrnc rph-1, rrnB* genetic background, will confirm the essential role of spacer tRNA in the alternative pathway. Lastly, it will also be interesting to see if rrnB* operon can displace rrnG in Δrnc::apr rne-1 ΔybeY::cat rph-1 background.

MATERIALS AND METHODS

Bacterial Strains

All the strains used in this study are listed in Table 3. They were all isogenic derivatives of MG1693. The rne-1 allele used for this study, encodes a temperature-sensitive mutation which functionally inactivates RNase E at 44°C. The deletion in the rnc gene was constructed by using overlap PCR (unpublished results). The ΔybeY::cat strain was obtained from Graham Walker laboratory. The deletion in the recA gene was obtained from the E. coli Genetic Stock Center (Yale University) and were constructed as part of the Keio Collection. The multiple mutants were generated using P1 transduction with subsequent selection for the antibiotic resistance marker associated with the mutant allele. The genotypes of transductants were confirmed by PCR or by DNA sequencing in the case of rne-1.

Plasmids

The plasmids used for this study are listed in Table 1. Plasmids carrying rRNA operons (pMSK2-4) were used for the construction of multiple mutants. The single operon plasmids were previously constructed by PCR amplification of the rRNA operon using primers specific to each particular operon (rrnD, rrnG, and rrnB, respectively). The amplified fragments also contained restriction sites needed for digestion and ligation into
pSC101 origin plasmids (pWSK29 for pMSK2 and pMSK4, and pVMK94 for pMSK3). These restriction sites were used later to confirm the identity of the \textit{rrn} operons in the multiple mutants.

The pGCK1 plasmid was constructed in a pSC101 origin plasmid. It is a derivative of pMSK3 (\textit{rrnB} operon plasmid). The spacer tRNA gene was deleted using primers listed in Table 2 and were replaced by the unique restriction site \textit{NsiI}. Using restriction enzymes \textit{PstI} and \textit{NsiI}, the absence of the spacer tRNA gene was confirmed. Furthermore, the deletion of spacer in \textit{rrnB*} operon was also confirmed by DNA sequencing.

**RNA Isolation and Northern Analysis**

Since \textit{rne-1} is a temperature sensitive mutant, all the bacterial cells were grown at permissive temperature till early exponential phase and were then shifted to non-permissive temperature. Samples were collected before and 30 minutes after temperature shift. Total RNA was isolated from various exponentially growing strains as described previously\textsuperscript{35}. The quality of RNA samples was checked by agarose gel electrophoresis and then quantified using a Nanodrop\textsuperscript{®} ND-2000c spectrophotometer.

Glyoxyl agarose northern analysis was conducted using samples prepared with NorthernMax\textsuperscript{®}-Gly sample loading dye (Ambion) and electrophoresis in a 1\% agarose gel in BPTE buffer run at 100 volts for approximately 3 hours. Gel transfer and subsequent steps were conducted as previously described\textsuperscript{36}. To analyze the processing pattern of the 30S rRNA transcript, the northern blots were probed with $\gamma$-$\text{P}^{32}$-end-labeled oligonucleotides labeled using T4 PNK (NEB) that were specific for different
regions of interest of the 30S rRNA transcript. The various oligonucleotide probes used are listed in Table 2. Quantification of band intensities was calculated using ImageQuant TL 7.0 software (GE Healthcare).

**Primer Extension Analysis**

Primer extension analysis for the 5’ of 16S and 23S rRNA were performed as described previously. The oligonucleotides used for the analysis are listed in Table 2.

**REFERENCES**


5. Bremer H, Dennis PP. Modulation of Chemical Composition and Other Parameters of the Cell at Different Exponential Growth Rates.

6. Jin DJ, Cagliero C, Zhou YN. Growth rate regulation in *Escherichia coli*. *FEMS*


31. McDowall KJ, Hernandez RG, Lin-Chao S, Cohen SN. The *ams*-1 and *rne*-3071 temperature-sensitive mutations in the *ams* gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. *J Bacteriol*. 1993;175(13):4245-4249.


Table 4.1: Plasmids used in this study

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<td>ptRNA672</td>
<td><em>Asp-1, Trp, Ile-1, Ala-1B and Thr-1</em> tRNAs, StrR/SpcR</td>
<td>Unpublished data, M. Stead</td>
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<td>pMSK2</td>
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<td>pMSK3</td>
<td><em>rrnB</em> rRNA operon, Apr&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMSK4</td>
<td><em>rrnG</em> rRNA operon, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pMSK9</td>
<td>*rnc+, Cm&lt;sup&gt;R&lt;/sup&gt; (P&lt;sub&gt;BAD&lt;/sub&gt; RNase III expression vector)</td>
<td>Unpublished data, M. Stead and G. Chaudhuri</td>
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<tr>
<td>pGCK1</td>
<td><em>(rrn)B</em> rRNA operon, spacer tRNA&lt;sup&gt;glu&lt;/sup&gt; deleted, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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Table 4.2: Primers used in this study

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<td>16S 1586 (probe a)</td>
<td>CGTTCGACTTGCATGTGTTA</td>
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<tr>
<td>23S 3580 (probe c)</td>
<td>CGACGCTTATCGCAGATTA</td>
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<tr>
<td>Reverse primer (probe d)</td>
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<td>Fwd16S_tRNA del</td>
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<tr>
<td>Rev23S_tRNA del</td>
<td>TAACGTTGGACAGGAAACCCTTGGTC</td>
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### Table 4.3: List of some of the bacterial strains used in this study

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<td>MG1693</td>
<td>$rph^{-1}$</td>
<td>$E. coli$ genetic stock center</td>
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<td>SK5072</td>
<td>$\Delta rnc::apr rph^{-1}$</td>
<td>Unpublished data, Valerie F. Maples</td>
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<tr>
<td>SQ2158</td>
<td>pK4-16 ($rrnB$ operon)</td>
<td>Unpublished data, Cathy Squires</td>
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<td>SK5191</td>
<td>$rph^{-1}, rrnD$</td>
<td>Unpublished data, Mark Stead</td>
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<td>SK5192</td>
<td>$rph^{-1}, rrnB$</td>
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<td>SK 5193</td>
<td>$rph^{-1}, rrnG$</td>
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<td>This study</td>
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<td>$\Delta rnc38::kan rph^{-1}, rrnG$</td>
<td>This study</td>
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<td>$\Delta rnc::apr rne^{-1} \Delta ybeY::cat rph^{-1}, rrnD$</td>
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<td>This study</td>
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<td>Cell Line</td>
<td>Description</td>
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<td>SK6218</td>
<td>pGCK1 (rrnB* operon)</td>
<td>This study</td>
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<td>SK6238</td>
<td>rph-1, rrnB*</td>
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<td>SK6246</td>
<td>Δrnc38::kan rph-1, rrnB*</td>
<td>This study</td>
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Figure 4.1. Ribosomal RNA operons in *E. coli*.

Each of the seven operons are identified on the left side by their respective *rrn* designations. Mature rRNAs or tRNAs are indicated by the blue, yellow, pink and red bars. This model is not drawn to scale.
Figure 4.2. Primary vs Alternative 30S rRNA processing pathway in *E. coli*.

The primary RNase III-dependent pathway is indicated at the left side of the figure, whereas the RNase III-independent alternative processing pathway is drawn towards the right side of the figure. The 30S pre-rRNA transcript is first cleaved by RNase III or by RNase E, RNase G and YbeY to generate precursor intermediates. These precursors are further processed by secondary processing enzymes and exoribonucleases to generate the mature RNAs. This model is not drawn to scale.
Figure 4.3. Alternative 30S rRNA processing pathway in *rrnD* and *rrnG* rRNA operons. (A) The locations of the oligonucleotides used for the northern blot analysis are shown. (B) Cells were initially grown at 30°C (P) till early exponential phase prior to shifting to 44°C (NP) for 30 minutes. RNA was isolated at both the temperatures and was run on a 1% agarose gel. The genetic composition of each band is shown in the corresponding cartoon drawn in the right. The mature 16S and 23S are denoted as M<sub>16</sub> and M<sub>23</sub>. (C, D) The cleavage sites of the alternative 30S rRNA processing pathway at the 5′ of 16S were mapped and were marked as bands A1-A5. The mature 5′ terminus of 16S is denoted as M<sub>16</sub> in the figure. (E, F) The cleavage sites of the alternative 30S rRNA processing pathway at the 5′ of 23S were mapped using primer Extension analysis. The cleavage sites were marked as bands B1-10. The mature 5′ terminus of 23S is denoted as M<sub>23</sub> in the figure which is produced only in *rph-1* control strain and is absent in all the mutant strains.
Figure 4.4. Construction of *rrnB* operon plasmid. The spacer tRNA<sup>glu</sup> gene from *rrnB* rRNA operon was deleted using overlap PCR and replaced by a unique restriction site, *NsiI*. The primers used for the PCR are enlisted in Table 2. The construction of this plasmid was done using a pSC101 origin plasmid which is marked with an apramycin antibiotic marker. The model is not drawn to scale.
Figure 4.5. Significance of the spacer tRNA gene in presence of RNase III. (A) The \textit{rrnB}* rRNA operon plasmid had the spacer tRNA$^{\text{glu}}$ gene deleted. This plasmid was used to displace \textit{rrnG} rRNA operon which had the intact spacer tRNA$^{\text{glu}}$ gene. After three dilutions, the bacterial cell cultures were streak purified and individual colony was tested for antibiotic resistance by replica plating. All the \textit{rrnB*}, Apr$^r$ transformants were susceptible to Ampicillin indicating successful plasmid displacement. (B) The plasmid displacement was confirmed with restriction digestion experiments. The plasmids isolated from the transformants were digested with \textit{PstI} and \textit{NsiI}. Lane 1 is a ladder used as a standard to determine the fragment sizes. Only the transformant which has the incoming \textit{rrnB*} plasmid will have the \textit{NsiI} corresponding fragment size as seen \textit{rrnB*} (lane3), whereas the ones which will have successful plasmid displacement will retain the resident plasmid \textit{rrnG} and will have the same fragment sizes as in Lane 2. The two independent isolates used for this study shows successful plasmid displacement (lanes 6-7). (C) The effect of absence of spacer tRNA in presence of RNase III was analyzed by running the RNA isolated from them in a 1\% agarose gel. No 30S rRNA processing defect was observed in absence of the spacer tRNA when wild-type levels of RNase III were produced in the cells (lanes 4-5).
Figure 4.6. Significance of the spacer tRNA gene in absence of RNase III. (A) The rrnB* rRNA operon plasmid which had the spacer tRNA$^{\text{glu}}$ gene deleted was used to displace rrnG rRNA operon in the Δrnc38::kan rph-1 genetic background. After three dilutions, the bacterial cell cultures were streak purified and individual colonies were tested for antibiotic resistance by replica plating. All the rrnB*, Apr$^\text{r}$ transformants were sensitive to ampicillin indicating successful plasmid displacement. (B) The plasmid displacement was confirmed with restriction digestion experiments. The plasmids isolated from the transformants were digested with PstI and NsiI. Lane 1 is a ladder used as a standard to determine the fragment sizes. Only the transformant which had the incoming rrnB* plasmid will have the NsiI corresponding fragment size as seen rrnB* (lane 3), whereas the ones which will not have successful plasmid displacement will retain the resident plasmid rrnG only and will have the same fragment sizes as in Lane 2. The two independent isolates used for this study showed successful plasmid displacement (lanes 4-5). (C) In order to eliminate the effect of the truncated RNase III protein produced from Δrnc38::kan rph-1, the plasmid displacement was done in a complete rnc deletion strain, Δrnc rph-1. The isolate which lost resistance to Ampicillin produced multiple bands (lane 5) of sizes different from rrnG (lane 2) or rrnB* (lane 3), indicating that a recombination event has occurred between rrnB* and rrnG plasmids.
CHAPTER 5

CONCLUSIONS

In *E. coli*, all three ribosomal RNAs (rRNAs) 16S, 23S and 5S rRNAs are co-transcribed as part of the seven distinct 30S primary transcripts which are cleaved and processed by various enzymes to generate their mature species. It has been shown that RNase III, a double-stranded RNA specific endoribonuclease encoded by *rnc*\(^1\), carry out the initial cleavages of the 30S rRNA primary transcripts. However, RNase III is not essential in *E. coli*\(^2\) and when the RNA from RNase III deficient strains was analyzed, the 30S rRNA accumulation constituted <5% of the total RNA. Moreover, in absence of RNase III, normal maturation of 23S rRNA does not occur, but mature 16S rRNA can still be made\(^3\). These results indicated that there is an alternative processing pathway in which 30S rRNA processing can take place in the absence of RNase III.

My dissertation research was mainly focused on analyzing the alternative 30S rRNA processing pathway in *E. coli*. In Chapter 2, the alternative processing pathway was studied in strains with all the seven chromosomal rRNA operons intact. The work demonstrated here showed that in absence of RNase III; RNase E, RNase G and YbeY carry out the initial cleavages of the 30S primary transcript. They separate the pre-16S rRNA from the rest of the transcript after the initial cleavages in the alternative pathway.
The pre-23S rRNA intermediate which extends from the spacer tRNA to the 5S rRNA is subsequently processed by RNase P, RNase Z and other exoribonucleases to generate the mature species. Surprisingly, the precursor-23S rRNA intermediate is unique to the alternative processing pathway. It is not produced in presence of RNase III, suggesting that the two 30S rRNA processing pathways act independent of each other and have different cleavage sites.

This was also confirmed from our primer extension analyses data, where we have shown that the alternative processing pathway has multiple cleavage sites in the region between 16S and 2S rRNA which are unique from the RNase III-dependent pathway. However, the work presented here was done in rne-1 mutant allele and it has been shown previously shown that this allele has residual RNase E activity. It could be the reason why we have observed 23S rRNA maturation still taking place. It will be interesting to study the 30S rRNA processing in a complete rne deletion strain.

Moreover, based on the mature 23S rRNA levels seen in absence of RNase III, RNase E, RNase G and YbeY, the possibility of other enzyme(s) involved in the alternative processing pathway could not be entirely ruled out. Additionally, we did not see any significant 30S rRNA processing defects in absence of the other known endoribonucleases in E. coli.

Therefore, using bioinformatics analyses we identified six putative endonucleases which may have a role in this alternative pathway. In Chapter 3 we show that among the newly identified genes, YhgF, YraN and YhbQ act as secondary processing enzymes in the alternative processing pathway. They help in the processing of the pre-23S rRNA intermediate. However, they do not play any significant role in the initial cleavages of the
30S primary transcript. Additionally, not much is known about the enzymatic activity and substrate specificity of YhgF, YraN and YhbQ. Therefore, it will be interesting to study and characterize these genes which help in pre-23S rRNA processing in the alternative pathway.

The strains used in Chapters 2 and 3 were constructed with all the seven chromosomally encoded rRNA operons (rrn) intact. Therefore, rRNA was actively produced from all seven of them. As an additional potential complication, since each of the seven rRNA operons in *E. coli* are different from each other in terms of nucleotide sequence of the exterior and interior transcribed spacer regions, the length of the 30S transcript, rRNA and tRNA copy numbers, and tRNA identities\textsuperscript{4}, the alternative processing pathway produced a large number of products.

The lack of homogeneity among the rRNA operons also raised the question of whether they were all processed by the same enzymes at the same locations with similar efficiencies, further complicating the analysis of processing by generating operon-dependent cleavage patterns. Therefore, in order to study the processing pattern from the individual *rrn* operons in the alternative 30S rRNA processing pathway, we developed a system in which we could investigate rRNA processing from a single rRNA operon.

In Chapter 4, we show that for the *rrnD* and *rrnG* operons which had the maximum differences with regard to RNA gene organization, RNase E and YbeY are some of the primary processing enzymes which carry out the initial 30S rRNA cleavages, but with different efficiencies. Inactivating RNase III, RNase E and YbeY resulted in slightly larger 30S rRNA levels from the *rrnD* operon as compared to *rrnG* operon.
Moreover, our primer extension analysis showed that they have different cleavage sites indicating that the heterogeneities observed in the organization of the seven rRNA operons can alter the alternative processing pattern among the individual rRNA operons.

We also studied the significance of conservation of the location of the spacer tRNA between the 16S and 23S rRNA among all the seven operons. In order to study the role of the spacer tRNA, we constructed a ribosomal operon plasmid in which the spacer tRNA gene was deleted and replaced with a unique restriction site. Using this modified rRNA operon plasmid, we showed that in the presence of RNase III, the spacer tRNA does not play any role in the separation of 16S and 23S rRNA. However, in absence of RNase III, the presence of the spacer tRNA between 16S and 23S rRNA becomes essential thus, suggesting its possible role in the alternative processing pathway. This is the first time where we have shown that the spacer tRNA becomes essential for 30S rRNA processing in the alternative pathway. It will be interesting to see the effect of the absence of spacer tRNA when RNase E, RNase G and YbeY are inactivated along with RNase III.

Overall, our work expanded the knowledge concerning the 30S rRNA processing in *E. coli*. The data contained in this dissertation has demonstrated the existence of a novel 30S rRNA processing pathway which acts efficiently in absence of RNase III. Interestingly, the secondary processing enzymes of the RNase III-dependent pathway, namely RNase E, RNase G and YbeY play the role of primary processing enzymes in absence of RNase III, indicating the highly flexible and sophisticated machinery bacterial cells have to maintain homoeostasis. Understanding the alternative pathways which bacteria use to survive and adapt in different conditions will in future help, in a better understanding of how more complicated species adapt to different conditions. It will also
help in solving the recent problem of bacteria becoming antibiotic resistant by designing drugs with multiple targets such that it can affect both the primary and alternative pathways efficiently. Therefore, the findings presented here will aid future work in this field, and help demonstrate how important post-transcriptional regulation truly is in the maintenance of biological systems.

REFERENCES


