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An Investigation of Bacterial Ribonucleases as an Antibiotic Target

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An Investigation of Bacterial Ribonucleases as an Antibiotic Target

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by

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ABSTRACT

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Antibiotics have been commonly used in medical practice for over 40 years. However, the misuse and overuse of current antibiotics is thought to be the primary cause for the increase in antibiotic resistance.

Many current antibiotics target the bacterial ribosome. Antibiotics such as aminoglycosides and macrolides specifically target the 30S or 50S subunits to inhibit bacterial growth. During the assembly of the bacterial ribosome, ribosomal RNA of the 30S and 50S ribosomal subunits is processed by bacterial ribonucleases (RNases). RNases are also involved in the degradation and turnover of this RNA during times of stress, such as the presence of an antibiotic. This makes ribonucleases a potential target for novel antibiotics.

It was shown that *Escherichia coli* mutants that were deficient for RNase III, RNase E, RNase R, RNase G, or RNase PH had an increase in ribosomal subunit assembly defects. These mutant bacterial cells also displayed an increased sensitivity to neomycin and paromomycin antibiotics. My research has also shown that an inhibitor of RNases, vanadyl ribonucleoside complex, potentiated the effects of an aminoglycoside and a macrolide antibiotic in wild type *Escherichia coli*, methicillin sensitive *Staphylococcus aureus*, and methicillin resistant *Staphylococcus aureus*.

RNases are essential enzymes in both rRNA maturation and degradation. Based on this and previous work, the inhibition of specific RNases leads to an increased sensitivity to antibiotics. This work demonstrates that the inhibition of RNases might be a new target to combat antibiotic resistance.

DEDICATION

This manuscript is dedicated to:

My mom and dad: Thank you for your support and confidence. I couldn't have done this without you. Both of you have always led by example and for that I am grateful. I wouldn't be the person that I am today without you. I love you both!

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Antibiotic Overview

Antibiotic resistance is a growing concern in the medical and research fields and is thought to occur due to excessive use of current antibiotics in treating humans, animals, and agriculture. In some cases, hospital-acquired bacterial infections have become almost untreatable due to increasing antibiotic resistance. A summary diagram of antibiotic use for humans, animals, and in the environment is seen in Figure 1.1 and depicts how the use of antibiotics in one group can migrate to affect other groups (Davies and Davies 2010; Giedraitienė and others 2011; Rosen 2011; Tenover 2001). In addition to drug resistance among the pathogenic bacteria, there is also a documented increase in antibiotic resistance of the normal flora. An example of this is *Escherichia coli*, a gram negative bacterial species that is found in the normal flora of the human bowels. This bacterial species has become a leading cause of urinary tract infections (Erb and others 2007). Previous studies have demonstrated the increase in antibiotic resistance is not due solely to hospital-acquired infections. Antibiotic resistant bacteria were originally thought to be nosocomial, but these data indicates that community-acquired infections are becoming more common, confirming that the spread of resistance is becoming more and more serious.

Before the 1940s, common treatments for bacterial infections were to insert a surgical drain into the site of the infection and use antiseptics, but this was not an adequate treatment and many bacterial infections were still fatal. A few years after antibiotics were first used to treat bacterial infections, penicillin resistance began to occur. Since then, resistance has grown to encompass various other antibiotics, including methicillin and vancomycin (Zinner 2005; 2007). By the

1990s, methicillin resistant *Staphylococcus aureus* was no longer limited to hospital-acquired infections, and community-acquired *S. aureus* began to appear more often (Hawkey and Jones 2009; Zinner 2007). Multiple bacteria species are now developing resistance to currently used antibiotics, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* (Giedraitienė and others 2011). In order to alleviate this growing problem, researchers are attempting to identify new antibiotic targets and to create novel antibiotics that contain unique structures to which resistance has not developed (Anderson 1999; Cars and others 2011; Högberg and others 2010). Antibiotic resistance is thought to occur by multiple different mechanisms. Some of these mechanisms include acquisition of resistance genes, up-regulation of genes encoding cellular efflux pumps, and spontaneous mutations (Zinner 2007). However, a better understanding of current antibiotic targets and how they function in cells will allow for increased efficiency in antibiotic research.

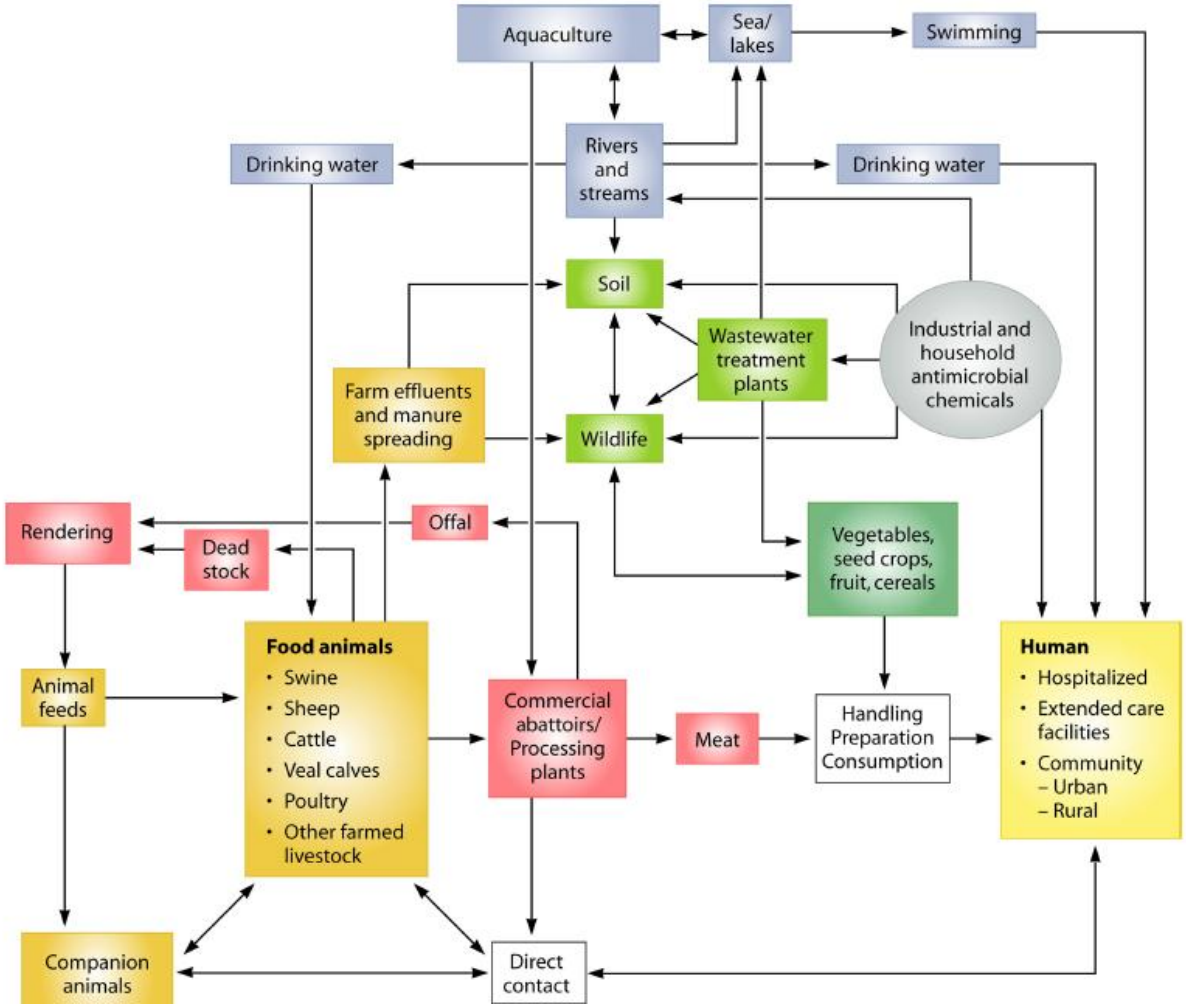

Figure 1.1: Overview of antibiotic use (Davies and Davies 2010). This figure indicates the use of antibiotic in the ecosystem is not restricted to that group. The use of antibiotics in the environment affects animals, humans, and the use of antibiotics in each of these groups also affect the environment. (Used with permission)

Antibiotic Targets

Bacterial ribosomes are composed of a small 30S and a large 50S subunit (Champney 2006). The 30S ribosomal subunit is composed of 16S ribosomal RNA (rRNA) and 21 proteins, while the 50S ribosomal subunit is composed of 23S and 5S rRNA and 34 proteins. Both the large and small subunits must be present to create the bacterial ribosome. Each subunit contains a specific center essential to translation. The 30S subunit contains a decoding center, and the 50S subunit

contains a peptidyl-transferase center. The decoding center is essential to the A binding site, and the PTC center is essential to the P binding site (Wilson 2009). Research has shown that antibiotics targeting these essential centers of the 30S and 50S subunits inhibit both subunit assembly and bacterial protein synthesis (Champney 2006; McCoy and others 2011; Siibak and others 2011). This indicates that inhibition of ribosomal subunit assembly and translational inhibition might be a synergistic process (Champney 2006).

Many antibiotics act as translational inhibitors (Champney 2006; McCoy and others 2011; Wilson 2009). During translation, mRNA brings a genetic code to the ribosome, and tRNA carries the amino acids to the ribosome. For processing to continue, the mRNA and the tRNA must move through the ribosome, i.e. progression must occur from the A site to the P site and finally through the E site of the ribosome. The ribosome serves as a platform for the tRNA to read the mRNA and create a nascent polypeptide chain. This is known as an elongation cycle that must involve the decoding of the mRNA (at the small ribosomal subunit by tRNA), the formation of a peptide bond, and release of the tRNA molecule at the peptidyl-transferase center (PTC) of the large ribosomal subunit (Kaczanowska and Rydén-Aulin 2007). Decoding of the mRNA by tRNA occurs within the A site. Next the tRNA carrying the nascent polypeptide chain is moved to the P site for peptide bond formation. Finally, the tRNA that is ready to exit the ribosome is moved to the E site. This tRNA is ready to exit the ribosome because it has transferred the amino acid to the nascent chain and is now uncharged (Wilson 2009). Once the mRNA has been completely read, the recycling of ribosomal subunits of the 70S ribosome is the final step in the protein synthesis cycle and must be completed in order to repeat the cycle (Borovinskaya and others 2007; Kaczanowska and Rydén-Aulin 2007; McCoy and others 2011; Poehlsgaard and Douthwaite 2005; Ramakrishnan 2002; Yonath 2005). A diagram of the

mechanism of translation is seen in Figure 1.2 (Ramakrishnan 2002). Many antibiotics act by targeting the decoding and PTC centers to inhibit protein synthesis in a bacterial cell (Wilson 2009). In addition to translation, another target of many antibiotics is the formation of the bacterial ribosome (Champney 2001; 2006; Wallis and Schroeder 1997).

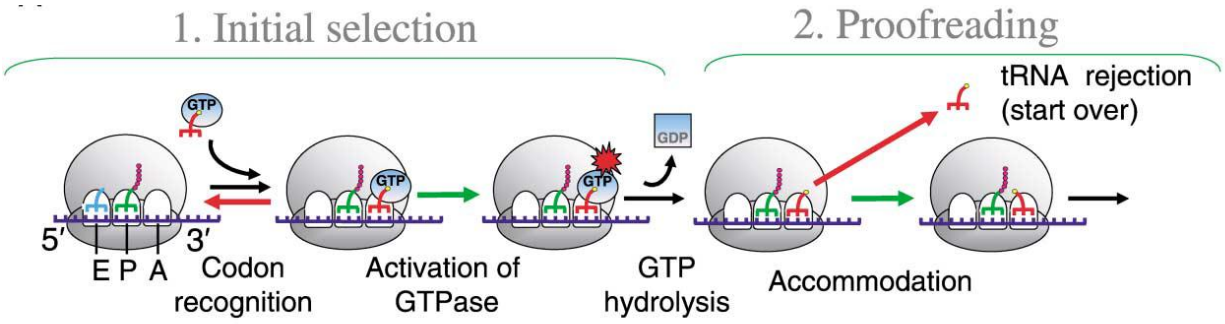

Figure 1.2: A diagram of translation (Ramakrishnan 2002). The following figure indicates the progression of tRNA through the A, P, and E sites of the bacterial ribosome as it reads mRNA. (Used with permission)

When assembly of either of the ribosomal subunits is inhibited, ribonucleases (RNases) have been found to degrade the ribosomal subunit assembly intermediates as seen in Figure 1.6 (Champney 2006). RNases are involved in rRNA processing to generate the 30S and 50S ribosomal subunit and RNA degradation. When an inhibitor, such as an antibiotic, is introduced specific RNases function in the recycling of the ribosomal precursors via rRNA turnover. This makes bacterial ribosome formation and RNases two important targets for the development of novel antibiotics to help fight antibiotic resistance. In order to understand antibiotic resistance, it is important to first have a basic knowledge of antibiotic functions.

Two families of antibiotics that specifically target both translation and ribosomal subunit assembly are the aminoglycoside and macrolide antibiotics. Aminoglycosides specifically target the bacterial ribosome and not eukaryotic ribosomes due to differences in amino acids at the target site on the ribosome and in cell membrane permeability (McCoy and others 2011).

Neomycin and paromomycin are two aminoglycosides that have been demonstrated to inhibit 30S ribosomal subunit assembly in wild type *E. coli* (Foster and Champney 2008; Mehta and Champney 2002) and in *S. aureus* (Mehta and Champney 2003). Aminoglycosides have also been shown to bind to the 50S ribosomal subunit (Borovinskaya and others 2007; Campuzano and others 1979; Scheunemann and others 2010). The aminoglycoside antibiotics have a positive charge that allows for the strong attraction to the negatively charged RNA (Shakil and others 2008). These antibiotics work by targeting both 30S and 50S ribosomal subunit assembly. Treatment with neomycin (Figure 1.3), a member of the aminoglycoside family, has been found to degrade the bacterial ribosome subunits by 60% in glucose starved cells (Zundel and others 2009). One interpretation of the decrease is that ribosomal subunits that are not assembled into active ribosomes are subject to degradation by bacterial ribonuclease.

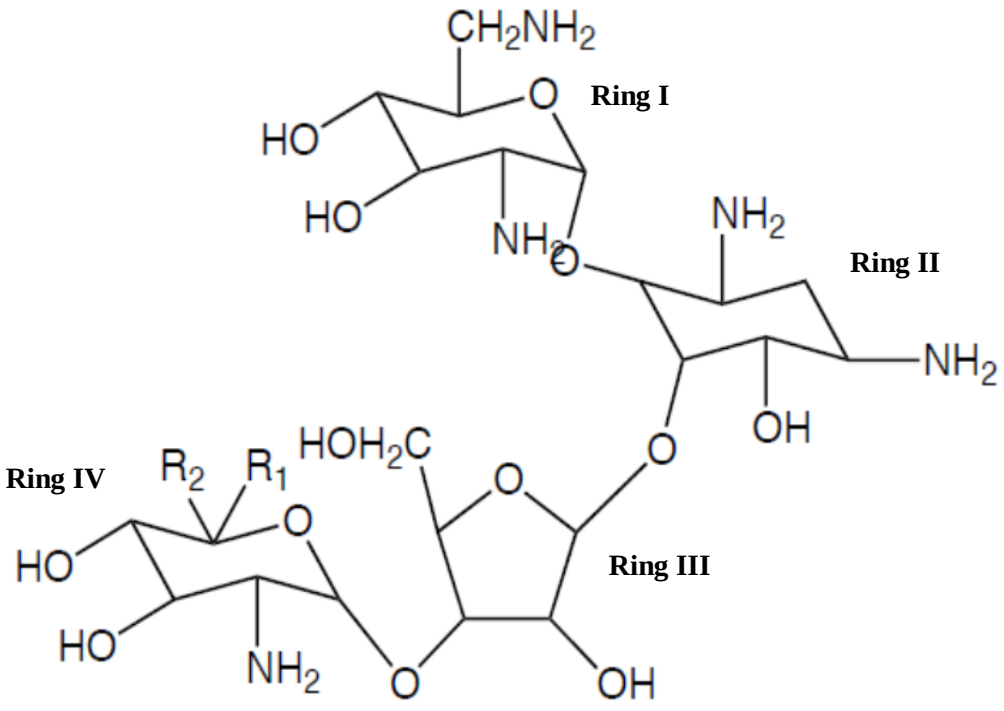

Figure 1.3: Structure of neomycin

Research indicates that the two main binding sites for aminoglycosides are 16S rRNA helix 44 and 23S rRNA helix 69 (Feldman and others 2010; Scheunemann and others 2010). The binding reduces, or in some cases completely inhibits, the ability of the ribosome recycling factor to recycle ribosomes by blocking this area of 23S rRNA. Helix 69 is also responsible for forming a bridge with the smaller 30S ribosomal subunit and the A and P sites of the ribosome. When the A site is blocked, the transfer from the A site to P site malfunctions and mistranslation occurs (Borovinskaya and others 2007; Hirokawa and others 2005; Scheunemann and others 2010; Schroeder and others 2000; Yonath 2005).

When binding to the 30S subunit, aminoglycosides bind to helix 44 of 16S rRNA. This binding displaces two adenine residues and leads to a stabilized confirmation of one or more of the four inter-subunit bridges. These two adenine residues are located in the A site of the ribosome and when displaced, the mRNA and tRNA binding specificities to this site are decreased leading to mistranslation and eventual cell death (Sutcliffe 2005). Paromomycin (Figure 1.4) is thought to function by binding to the 16S rRNA. In this antibiotic, ring IV makes contact with both sides of the helix 44 while ring I inserts itself into the RNA helix and is directly responsible for the displacement of adenine 1492 and adenine 1493 (Carter and others 2000). This displacement inhibits the movement of the 70S ribosome, thereby inhibiting the translocation of transfer RNA to a new position on the ribosome. The increased stabilization also prevents dissociation of the 30 and 50S ribosomal subunits and subsequent ribosome recycling (Carter and others 2000; Długosz and Trylska 2009; Sutcliffe 2005; Tenson and Mankin 2006).

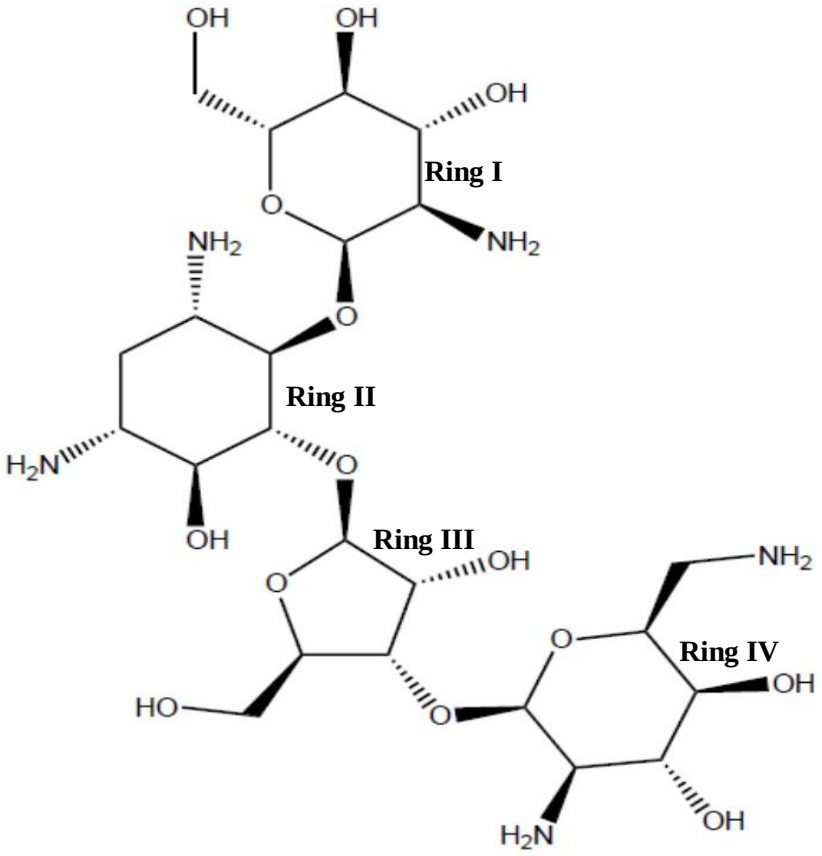

Figure 1.4: Structure of paromomycin

Macrolide antibiotics are a family of antibiotics that have a 12-16 membered macrolactone ring and include the antibiotics erythromycin and azithromycin (Figure 1.5). Macrolides have not been shown to affect 30S subunit formation even at high antibiotic concentrations (Silvers and Champney 2005). These antibiotics function by binding to the upper portion of the peptide exit tunnel, below the peptidyl-transferase center of the 50S ribosome. The binding occurs at nucleotides A2058 and A2059 via hydrogen bonds. This binding places the lactone ring of macrolides against the exit tunnel and blocks it so that the newly made peptide chain cannot elongate. The growth of the peptide chain is hindered depending on the bulk of the macrolide antibiotic, so that the bulkier antibiotics will result in a shorter peptide chain

(Bogdanov and others 2010; Kannan and Mankin 2011; Sutcliffe 2005; Tenson and Mankin 2006). Macrolides, such as azithromycin, are derivatives of the original macrolide, erythromycin, and are more flexible due to having a 15 membered macrolactone ring rather than the original 14 membered ring. The altered ring also has an increased size and leads to the antibiotic occupying more space in the exit tunnel. With more space being occupied in the exit tunnel, the antibiotic efficiency for inhibiting the elongation of the protein in increased (Yonath 2005). When binding to the ribosome, only one molecule of azithromycin can bind to a single ribosome (Petropoulos and others 2009). In addition to blocking polypeptide elongation, macrolide antibiotics can also bind to an intermediate of the 50S subunit, the 32S precursor. When wild type *S. aureus* cells or RNase E deficient *E. coli* cells were incubated with erythromycin, there was an accumulation of 23S rRNA and 32S precursor. These data are indicative of stalling of the assembly of the 50S ribosomal subunit (Pokkunuri and Champney 2007; Usary and Champney 2001). It has also been determined that macrolide selectivity for bacteria is based on the adenine at position 2058 of 23S rRNA. This adenine is conserved in bacteria but in eukaryotes it is a guanine residue (Mankin 2008; McCoy and others 2011; Starosta and others 2010).

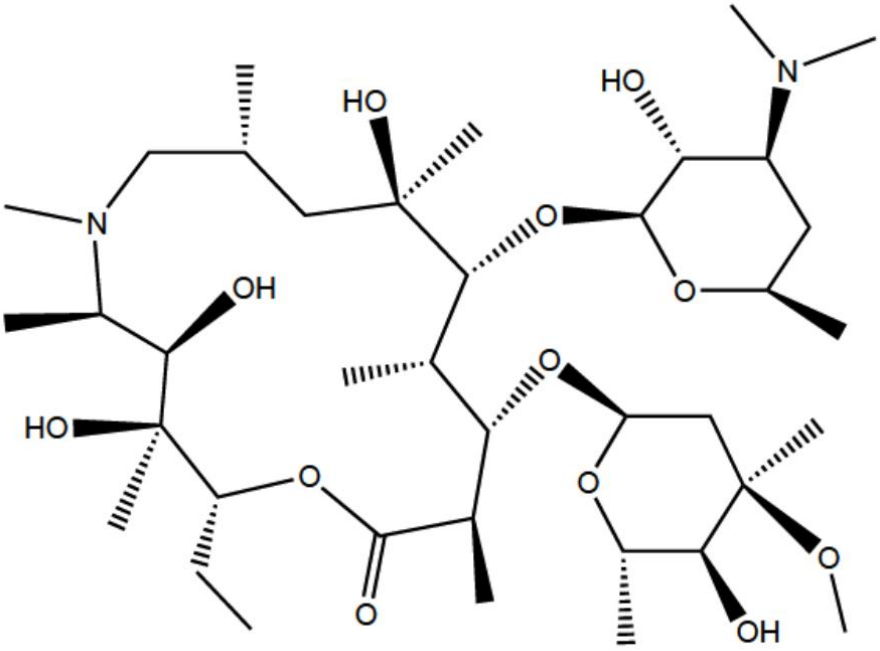

Figure 1.5: Structure of azithromycin

These data, taken together, describe the mechanisms of action for two classes of antibiotics. By better understanding the mechanisms of actions of current antibiotics, new targets for novel antibiotics can be explored to fight against antibiotic resistance.

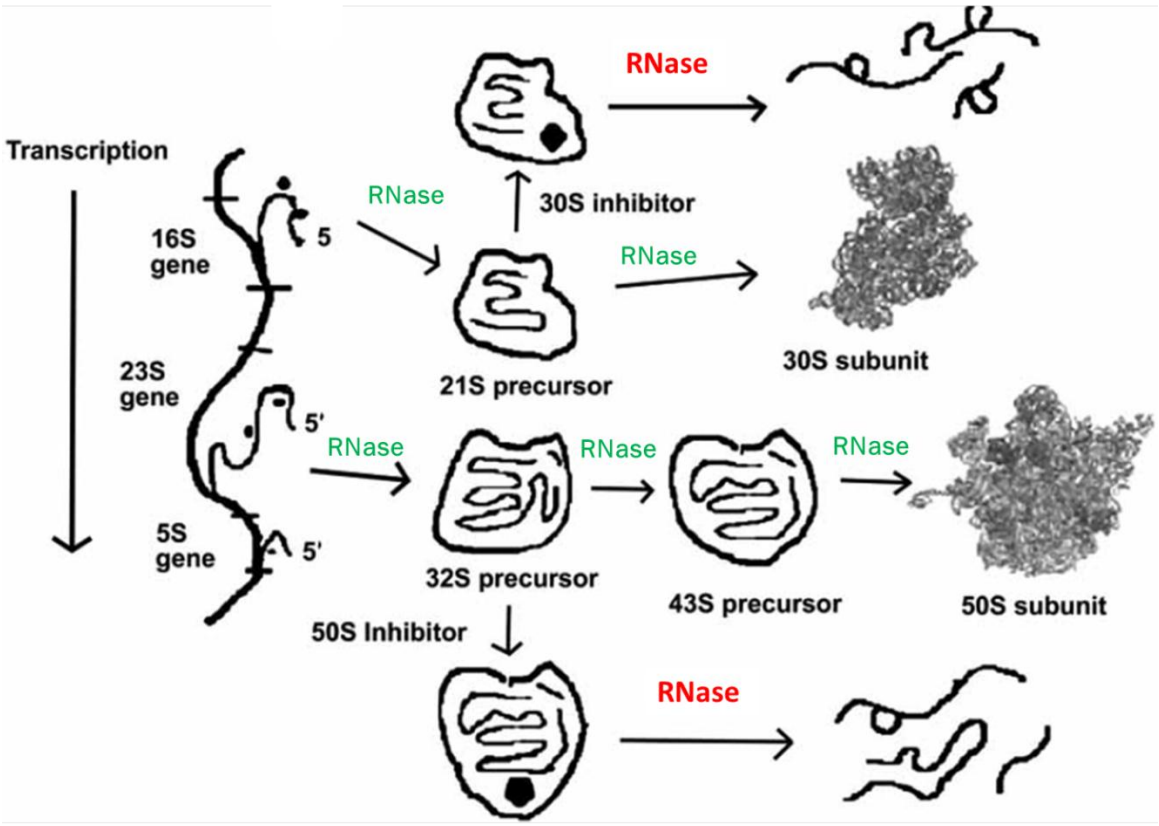

Figure 1.6: Diagram of bacterial ribosome assembly. This diagram shows that RNases are involved in ribosomal precursor processing and rRNA turnover (Champney 2006)

Bacterial Ribonucleases

Extensive research has been conducted to determine the functions of various ribonucleases in rRNA maturation, rRNA degradation, and ribosomal subunit assembly. In the bacterial cell, there are three main categories of RNA: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Between these categories, rRNA is the most abundant, and mRNA has the largest turnover rate (Li and others 2002). RNases play key roles in the degradation and maturation of each of these types of RNA. For example, RNase PH has been found to be involved in the processing and degradation of tRNA (Kelly and others 1992; Li and Deutscher 1994). mRNA degradation begins with the endonucleolytic cleavage by RNase E. RNase II then functions in degradation of the poly(A) tail and subsequent degradation of mRNA

(Mohanty and Kushner 2000). Once bound to the ribosome for translation, a region of 15 nucleotides in mRNA is protected from degradation by RNases (Rauhut and Klug 1999). These are just a few of the functions of RNases involving mRNA and tRNA. RNases also have significant roles in maturation and in degradation of the most abundant RNA in a bacterial cell, ribosomal RNA.

During assembly of the 30S and 50S ribosomal subunits, ribonucleases have been shown to play a key role in rRNA turnover when an inhibitor is present (Champney 2006). It was previously concluded by Silvers and Champney that *E. coli* strains deficient for certain RNases were enhanced in their sensitivity to the macrolide, azithromycin (Silvers and Champney 2005). Strains deficient in RNase E or polynucleotide phosphorylase (PNPase) were hypersensitive to azithromycin. In addition to an increased sensitivity to azithromycin, *E. coli* with these RNase deficiencies showed an accumulation of 23S rRNA and a reduced recovery rate of subunit formation after the removal of the antibiotic. It was ascertained that azithromycin decreased the assembly of the 50S ribosomal subunit and increased the accumulation of the 32S precursor to the 50S ribosomal subunit (Silvers and Champney 2005). Figure 1.6 depicts the pathway for 50S synthesis (Champney 2006). After azithromycin removal, the recovery rate of the 50S ribosomal subunit was slowest in RNase E deficient, PNPase deficient, or RNase II deficient *E. coli* strains. These data suggest that RNase E, RNase II, and PNPase play an important role in 23S rRNA turnover and identify a possible mechanism for antibiotic resistance (Silvers and Champney 2005). It is possible that an increase in RNases leads to a decrease in antibiotic sensitivity and an inhibition of specific RNases will lead to an increase in antibiotic sensitivity. A summary of these data can be seen in Table 1.1.

Table 1.1: Effect on ribosome deficiency on azithromycin sensitivity and 50S subunit assembly in *E. coli*. Adapted from Silvers and Champney (Silvers and Champney 2005)

		Function		Azithromycin	Effect	
RNase	**Type**	**Synthesis**	**Degradation**	**Sensitivity**	**Inhibit 50S Assembly**	**Increased rRNA Fragmentation**
I	Endo		+			
II	Exo		+	+	+	+
III	Endo	+	+			
E	Endo	+	+	+	+	+
PNPase	Exo		+	+	+	+

Silvers and Champney's work illustrates the importance of RNases in the turnover of 23S rRNA in the presence of a macrolide antibiotic (Silvers and Champney 2005). A better understanding of the function of some of the currently recognized RNases can provide a more focused use for antibiotics. A list of some of the known ribonucleases is shown in Table 1.2. Based on the functions of RNases and previous work showing that *E. coli* deficient for specific RNases displayed a reduction in ribosomal subunit formation (Silvers and Champney 2005; Usary and Champney 2001), it is possible that RNases could be used as a novel antibiotic target. Compounds which subsequently block RNases that function in ribosome assembly or degradation would be predicted to affect the sensitivity of current antibiotics.

Table 1.2: Summary of ribonuclease functions

		Function	
RNase	**Type of Ribonuclease**	**Maturation**	**Degradation**
I	Endo		Yes
II	Exo	Yes	Yes
III	Endo	Yes	Yes
E	Endo	Yes	Yes
G	Endo	Yes	
R	Exo		Yes
PNPase	Exo		Yes
PH	Exo	Yes	Yes

RNase I

RNase I is an endoribonuclease located in the periplasmic region of the bacterial cell. It functions in cleavage of RNA and is used as a defense mechanism against invasion by bacteriophage (Arraiano and others 2010; Raziuddin and others 1979). Degradation of RNA can occur by this enzyme due to cell damage. When the cell is damaged, it is hypothesized that RNase I is able to leave the periplasmic region, enter into the cell, and degrade ribosomal RNA. It is theorized that RNase I functions to forage nucleotides from the extracellular environment (Arraiano and others 2010). However, the enzyme is normally kept inactive by unknown mechanisms (Deutscher 2006; 2009).

RNase III

RNase III is an endoribonuclease encoded by the *rnc* gene and is responsible for cleavage of the primary rRNA transcript (Allas and others 2003; MacRae and Doudna 2007). This cleavage separates rRNA precursors into 17S, 25S, and 9S precursors, as seen in Figure 1.7, and initiates ribosomal subunit RNA processing (Kaczanowska and Rydén-Aulin 2007). Figure 1.7 is a diagram of how various RNases process ribosomal RNA (Davies and others 2010). RNase III is

involved in the cleavage of dsRNA (Xiao and others 2009). 16S and 23S rRNA sequences form double-stranded regions at the 3' and 5' ends that must be removed in order to further process the rRNA into the mature forms. King et al. determined that in an RNase III deficient *E. coli* strain, the 16S rRNA had a mature 5' end and 3' end while the 23S rRNA did not have a mature 5' or 3' end (King and others 1984). These results further demonstrated that RNase III was mechanistically the first RNase to cleave the primary RNA transcript (King and others 1984). Further research has found that in α-proteobacteria, RNase III cleaves 23S rRNA at helix 9 of the 3' terminus. This cleavage is important to the maturation of the 3' terminus (Evguenieva-Hackenberg and Klug 2000).

Final maturation of both 23S and 16S occurs via additional ribonucleases. Without RNase III and the initial cleavage, rRNA maturation will not occur. RNase III has been shown to interact with the 70S ribosome and the ribosomal subunits (Allas and others 2003). This interaction is thought to play a role in rRNA precursor maturation. RNase III cleaves the 23S rRNA precursor 3 nucleotides downstream of the mature 5' terminus in the ribosome. RNase III has also been implicated in final maturation of ribosomal RNA, which occurs when RNase III binds to the 70S ribosome to facilitate final 23S rRNA maturation within the ribosome (Allas and others 2003).

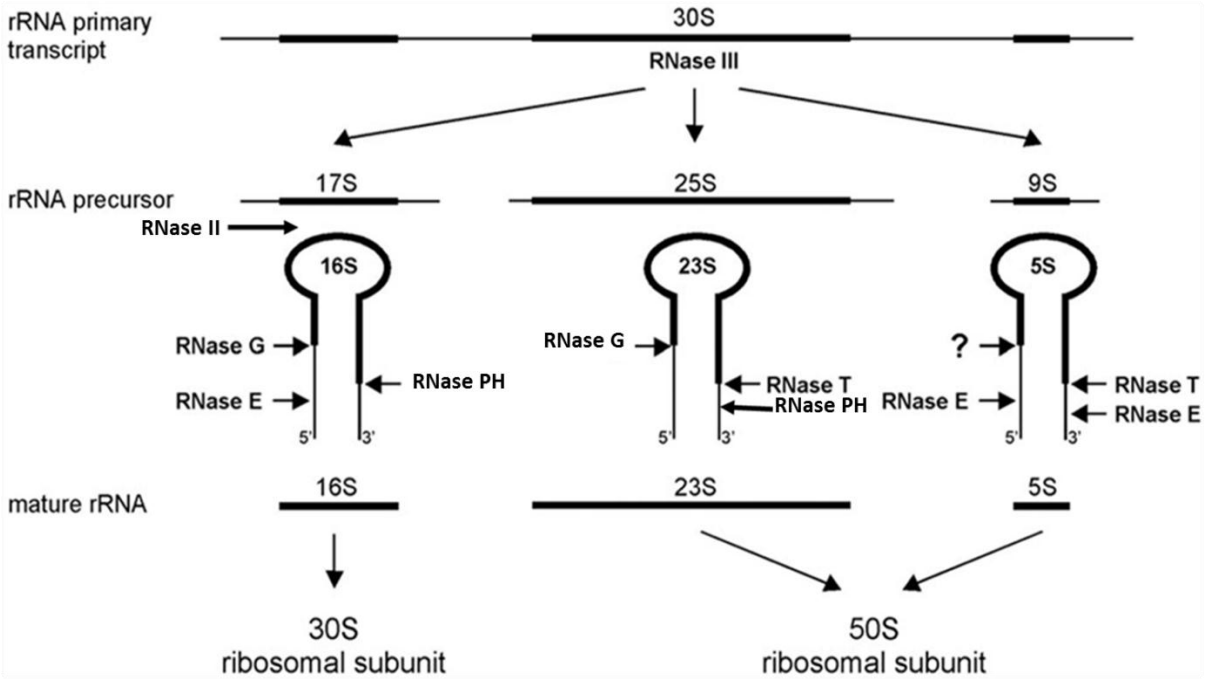

Figure 1.7: A diagram indicating significant functions of some RNase in RNA maturation. Modified from *Davies et al. 2010* (Davies and others 2010). The diagram shows that RNase III is responsible for the initial cleavage of the rRNA transcript. In order to produce functional 16S, 23S, and 5S rRNA, additional RNases are necessary for maturation. (Used with permission)

RNase E

RNase E is an endoribonuclease, encoded by the *rne* gene, that forms part of the degradosome particle with the enzyme polynucleotide phosphorylase (PNPase), an exoribonuclease involved in RNA decay (Arraiano and others 2010). These two ribonucleases, along with enolase and the DEAD-box helicase RhlB interact to form the degradosome (Vanzo and others 1998). This complex is responsible for degradation of rRNA in addition to mRNA. RNase E is an important enzyme in this complex because the other three components of the degradosome each bind to the carboxyl-terminal end of RNase E. This further demonstrates the significance of RNase E in RNA degradation. Along with the ribonucleases, RhlB is responsible for unwinding the RNA to allow for degradation. Enolase has an undefined function within the

degradosome (Vanzo and others 1998; Worrall and Luisi 2007). Studies have revealed that mutations eliminating the C-terminus of RNase E result in a disruption of the degradosome particle. However, the loss of the C-terminus also led to a reduction in cell growth, an increase in stabilized mRNA, and 5S rRNA maturation remained unaffected. This suggested that the C-terminus of RNase E is involved in degradation and not rRNA processing (Lopez and others 1999). Once assembled, the degradosome functions in degradation of 16S, 23S, and 5S rRNA. Evidence has shown that the cleavage associated with the degradosome is due to RNase E cleavage and not the ribonucleolytic cleavage activity of PNPase (Bessarab and others 1998). *E. coli* cells deficient for RNase E have been found to be hypersensitive to erythromycin. These cells also have a 70% reduction in the 50S ribosomal subunit and an increase in 50S precursor in the presence of the antibiotic. These data indicate that RNase E plays an important role in degradation of antibiotic impaired ribosomal subunits (Usary and Champney 2001).

Studies have shown that in addition to degradation, RNase E is involved in maturation of rRNA (Figure 1.7). RNase E, along with RNase G, has been implemented in the cleavage of *E. coli* 16S rRNA. The enzyme removed 115 nucleotides from the 5' terminus of the rRNA precursor to produce a mature 16S rRNA 5' end (Li and others 1999; Wachi and others 1999). RNase E is involved in the maturation of 5S rRNA by cleaving both the 3' and 5' termini (Arraiano and others 2010; Gutgsell and Jain 2012). In α-proteobacteria, RNase E has been implicated in the processing of 23S rRNA at the 5' end. This processing occurs at helix 9 following the initial cleavage by RNase III (Klein and Evguenieva-Hackenberg 2002). It stands to reason that RNase E might be involved in 23S rRNA processing in other gram negative bacteria similar to that of α-proteobacteria. However, no evidence has been published to-date. Taken together, the data demonstrate that during rRNA maturation, RNase E is an essential

ribonuclease that processes 16S and 5S rRNA. Some research also indicates an involvement in 23S rRNA maturation, depending on the bacteria. Furthermore, research has revealed an importance of RNase E in rRNA degradation (Bessarab and others 1998).

RNase G

RNase G is an endoribonuclease homolog of RNase E and has been shown to act similarly to RNase E (Tock and others 2000). Like RNase E, RNase G is involved in the maturation of the 5' end of 16S rRNA. Strains deficient for RNase G lack a mature 5' end of 16S rRNA but retain a mature 3' end. Without the mature 5' end of 16S rRNA, translational function is decreased (Li and others 1999; Roy-Chaudhuri and others 2010). In strains that are deficient for RNase G, there is an accumulation of a 16S rRNA precursor. These data further indicate that without RNase G, 16S rRNA cannot be processed correctly. While RNase E and RNase G are similar ribonucleases, they have different recognition sites on the rRNA as indicated in Figure 1.7 (Wachi and others 1999). Studies have shown that the cleavage of the 5' terminus of the rRNA is accelerated by a monophosphate residue that serves to stimulate RNase G (Jiang and Belasco 2004). RNase G is also involved in the processing of 23S rRNA, where it functions by processing the 5' end of the ribosomal RNA (Song and others 2011). Taken together, these studies show that RNase G functions to process 16S and 23S rRNA at the 5' end but, unlike RNase E, has not been shown to function in RNA degradation.

RNase R

RNase R is a 3'-5' exoribonuclease encoded by the *rnr* gene. RNase R is responsible for the degradation of 16S and 23S rRNA; however, it does not degrade 5S rRNA very well (Cheng and Deutscher 2002). This enzyme has been shown to increase during conditions of stress and is involved in pathogenesis by various microorganisms (Chen and Deutscher 2010; Matos and others 2009). When *E. coli* cells lack both PNPase and RNase R, the cells are no longer viable. Due to the fact that cells deficient for RNases R and PNPase are not viable, it is possible that these two ribonucleases are a common function that is essential for cell survival. This common function might be the degradation and turnover of rRNA. Cells lacking PNPase and RNase R showed an increase in 16S and 23S rRNA fragmentation. It has been suggested that this accumulation of RNA fragments is lethal to the bacterial cell (Cheng and Deutscher 2003).

RNase R degrades RNA fragments by binding to a 3' terminal overhang of at least seven nucleotides (Vincent and Deutscher 2006). It is the only ribonuclease that has been found to degrade double stranded RNA without the help of a helicase, provided that the 3' end overhang is single-stranded. Research has also demonstrated that RNA must thread through the RNase R protein before degradation can occur, indicating that the enzyme does not merely attach to RNA to begin degradation. RNase R does not degrade RNA via the 5' end. It has been determined that the RNB domain or C-terminal of the enzyme is essential for RNA degradation. This domain is crucial because of Asp^{280}, which must be present for degradation to occur. This amino acid contributes to the ribonuclease's ability to degrade RNA but not to the binding of RNA (Matos and others 2009; Vincent and Deutscher 2006).

As previously stated, many RNases are regulated by the presence or absence of other RNases. The cleavage of the *rnr* gene is regulated by RNase E. When RNase E is absent or

down-regulated in cells, the *rnr* operon stability is increased and RNase R protein is increased. This increased stability and protein production is possibly due to the lack of the degradosome (Cairrão and Arraiano 2006). These data further demonstrate the close connection and regulation of various bacterial ribonucleases. In addition to its regulation by RNase E, RNase R bears a resemblance to RNase II. The two enzymes share a 60% homology with each other. PNPase, RNase R and RNase II are the three major exoribonucleases in a bacterial cell (Awano and others 2010; Matos and others 2011).

RNase R is up-regulated in a cell during stress, such as starvation or dramatic temperature changes. Under conditions of stress, the enzyme is stabilized; however, during the normal exponential phase of cellular growth RNase R is very unstable. This raises the possibility that the cell increases RNase R during times of stress to degrade damaged ribosomal RNA but is not used as much for quality control purposes (Chen and Deutscher 2005; 2010). This RNase R-mediated degradation of rRNA during stress may be important since an accumulation of fragmented RNA can be toxic to the bacterial cell.

RNase II

Like RNase R, RNase II is a 3'-5' exoribonuclease. It is encoded by the *rnb* gene (Matos and others 2011). RNase II is thought to be involved in the removal of the poly (A) tail to protect mRNA from degradation and its presence has been shown to be regulated by PNPase. RNase III and RNase E indirectly affect RNase II by modulating PNPase and degrading RNase II mRNA respectively (Arraiano and others 2010; Zilhão and others 1995). RNase II is able to bind both DNA and RNA but distinguishes between the two based on the Tyr^{313} and Glu^{390} of the enzyme. Tyr^{313} of RNase II is responsible for the recognition of RNA, and Glu^{390} of RNase II is

responsible for recognition of DNA. For RNA degradation to occur, Asp^{209} of the enzyme must be present. This exoribonuclease has been shown to become a "super enzyme" when a site-directed mutation occurs that converts Glu^{542} to Ala^{542}. Once converted to a super enzyme, exoribonucleolytic cleavage is increased approximately 100-fold and RNA binding is increased by about 20-fold (Arraiano and others 2010; Matos and others 2011; Zuo and Deutscher 2001).

Under normal circumstances, RNase II is involved in both rRNA maturation and degradation. It has been documented that when RNase II is absent from *E. coli* cells, there is an accumulation of the 30S and 50S ribosomal precursors (Corte and others 1971). The data also showed an increase in the 16S rRNA precursor, 17S rRNA, when RNase II was inactive. These results indicated a role of RNase II in ribosomal precursor maturation and in the maturation of 17S to 16S rRNA (Corte and others 1971). Studies have shown that *E. coli* cells deficient for RNase II display a large reduction in the formation of the 50S subunit and an increase in 23S rRNA fragmentation (Silvers and Champney 2005). Taken together, these data indicate a role in maturation of 16S rRNA and the degradation and turnover of 23S rRNA.

Silvers and Champney found that *E. coli* cells deficient for RNase II demonstrated a decrease in the 50S ribosomal subunit amounts and an increase in 23S rRNA when the macrolide antibiotic, azithromycin, was present. Further research revealed that when azithromycin was removed, there was a significantly reduced rate of recovery in these cells. These data indicated that RNase II is important to the degradation and turnover of antibiotic-stalled ribosomes (Silvers and Champney 2005).

RNase II and RNase R are homologs of each other and share approximately 60% similarities. These enzymes do, however, have a few key differences. One difference between these two members of the RNase II super family is the type of final end product that is released.

For its final product, RNase II releases a fragment of four nucleotides in length. RNase R releases a two nucleotide fragment. This difference is due to the aromatic amino acid locations in the RNase. For RNase II, Tyr^{253} and Phe^{358} lock onto the RNA. Tyr^{253} is responsible for determining the end fragment size. For RNase R, Tyr^{324} and Phe^{429} lock onto the RNA and Tyr^{324} is responsible for the end fragment size. While Tyr^{324} is responsible for determining fragment size, studies have shown that the position of the phenylalanine determines how tightly the enzyme fastens onto the RNA. The final end product is an important distinction because studies have shown that the four nucleotide product must be further degraded to a two nucleotide product in order to be recycled (Matos and others 2009; Matos and others 2011). Another important difference between the two enzymes is the C-terminal tail. RNase R does not need the assistance of a helicase in order to degrade RNA while RNase II does. This is due to the presence of a lysine-rich C-terminal tail in RNase R that is not found in RNase II (Matos and others 2011). These differences are important distinctions between two degradative ribonucleases and further exemplify their functions.

PNPase

Polynucleotide phosphorylase (PNPase) is a 3'-5' exoribonuclease and, along with RNase PH, is a member of the PDX family (Arraiano and others 2010). PNPase is encoded by the *pnp* gene and functions solely in RNA degradation. This enzyme is part of a larger complex in bacterial cells, known as the degradosome (Mohanty and Kushner 2003). When a bacterial cell is deficient for RNase II or RNase III, PNPase levels are increased. PNPase functions to regulate RNase II by degrading the RNase II mRNA (Arraiano and others 2010). Studies have found that RNase E has a direct interaction with PNPase, enolase, and RhlB. While PNPase appears to bind

with RhlB, there does not appear to be any direct binding of PNPase with enolase and the interaction must occur through RNase E (Burger and others 2011).

PNPase and RNase PH are the only RNases that require a phosphate group at the end of the RNA group in order to break the phosphodiester bond and cleave the RNA (Zhou and Deutscher 1997). In order to begin the process of degradation, a single stranded RNA must have an RNA overhang that ranges from 7-10 nucleotides in length at the 3' end (Arraiano and others 2010). *E. coli* cells deficient for PNPase have been shown to have an accumulation of 23S rRNA in the presence of azithromycin and a reduced recovery rate when the antibiotic was removed. This information indicates an importance of PNPase in rRNA degradation when an antibiotic is present (Silvers and Champney 2005).

In *E. coli* cells deficient for RNase R and temperature sensitive for PNPase, there is a large accumulation of 16S and 23S rRNA, suggesting that these two enzymes play a key role in the degradation and turnover of this RNA (Zhou and Deutscher 1997). *E. coli* cells deficient for both PNPase and RNase PH grow much slower than wild type *E. coli* cells. These cells also show a decrease in the 50S ribosomal subunit and an increase in RNA degradation products sedimentating in sucrose gradients between 4S and 16S. These results suggest that the subunit is unable to assemble correctly which leaves the rRNA susceptible to degradation (Zhou and Deutscher 1997).

RNase PH

RNase PH is a 3'-5' exoribonuclease belonging to the PDX family (Arraiano and others 2010; Worrall and Luisi 2007). This enzyme binds to the last 4 nucleotides of single stranded RNA to begin degradation (Lorentzen and Conti 2005). RNase PH has been shown to be

involved in tRNA maturation; however studies are revealing an increasingly important role of the enzyme in rRNA degradation and maturation. RNase PH has been implicated in rRNA degradation during starvation, and it has been shown that the degradation of 16S rRNA at the 3' terminus is facilitated by RNase PH (Basturea and others 2011). Studies have found that the deletion of RNase II and RNase PH or the deletion of RNase PH and PNPase results in a large increase in the 23S rRNA precursor. Analysis of the RNA in these cells revealed unprocessed nucleotides of the 23S rRNA that are approximately 5-6 nucleotides in length (Gutgsell and Jain 2012). RNase PH was concluded to be the ribonuclease preferred to cleave 23S rRNA due to the following factors. First is that in the precursor form, 23S rRNA 3' end has a short overhang of approximately three nucleotides followed by base pairing with the 5' end. RNase II and PNPase are not as effective as RNase PH at cleaving this region. Second, the presence of two cytosines at the 3' end of 23S rRNA, which inhibit final rRNA maturation by RNase T. Together, these data show that RNase PH can initiate maturation by cleavage of the duplex region and removal of the CC sequence. This then allows RNase T to cleave and mature the 3' end of 23S rRNA (Gutgsell and Jain 2012). These data point to RNase II and PNPase playing a role in 23S rRNA degradation and also indicate that RNase PH plays a pivotal role in this process.

In summary, the eight RNases shown in Table 1.2 have been found to function in rRNA maturation and degradation. Various studies have indicated increasingly important roles of RNases when antibiotics are present (Silvers and Champney 2005; Usary and Champney 2001). However, ribonuclease deficient mutants are not naturally occurring. Treatment with an RNase inhibitor might act to mimic the results seen with various RNase deficient mutant bacteria, and potentially provide a novel mechanism for increasing the effectiveness of antibiotics which act by blocking ribosome assembly.

Vanadyl Ribonucleoside Complex

Vanadyl ribonucleoside complex (VRC) is a RNase inhibitor. This complex is commonly used to reduce RNA degradation during the isolation of RNA (Berger 1987; Berger and others 1980). While it is unknown which RNases are inhibited, it is theorized that VRC functions to inhibit endoribonucleases (Berger 1987). Lee et al. found that 10mM VRC inhibited recombinant RNase H activity by 90% (Lee and others 1997). To-date, there has been no published research indicating the use of RNase inhibitors to inhibit ribonuclease function in a cellular system. Previous data has shown that *E. coli* cells deficient for RNases are more susceptible to antibiotics (Silvers and Champney 2005; Usary and Champney 2001). It is possible that the use of an RNase inhibitor to inhibit bacterial RNases in the cell would serve as a novel antibiotic therapy.

Research Hypothesis

Many current antibiotics operate by inhibiting the assembly of the bacterial ribosome (Champney 2003; Champney and Burdine 1998a; Champney and Miller 2002; Champney and Rodgers 2007; Champney and Tober 2000; Chittum and Champney 1995; Mehta and Champney 2002; 2003). It is known that ribonucleases play an important role in bacterial ribosomal subunit assembly (Awano and others 2010; Gutgsell and Jain 2012; King and others 1984; Klein and Evguenieva-Hackenberg 2002; Li and others 1999; Song and others 2011; Xiao and others 2009). RNases also play a key role in rRNA degradation. When an inhibitor such as an antibiotic is introduced to the bacterial cell, under normal circumstances, RNases function to degrade the ribosomal precursors and to allow the rRNA nucleotides to be recycled and reused (Figure 1.6). Previous research has shown that when *E. coli* cells are deficient for RNases II, E, or PNPase, these cells display a hypersensitivity to azithromycin (Silvers and Champney 2005).

It is hypothesized that the loss of RNases will increase the sensitivity of *E. coli* to aminoglycoside antibiotics, neomycin and paromomycin. Aminoglycoside antibiotics are chosen for this research study because, like macrolides, they function by blocking 50S subunit assembly. However, unlike macrolides, aminoglycosides also block 30S subunit assembly (Campuzano and others 1979; Foster and Champney 2008; Mehta and Champney 2002; Silvers and Champney 2005; Tenson and Mankin 2006). The *E. coli* mutants that are found in this study to have an increased sensitivity to aminoglycosides are not found in nature. Therefore, I want to determine if a RNase inhibitor can be used to mimic the enhanced antibiotic sensitivity of RNase mutant *E. coli* cells. It is hypothesized that an inhibitor of ribonucleases can inhibit bacterial RNases and potentiate the effects of both a macrolide antibiotic and an aminoglycoside antibiotic.

Specific Research Aims

Aim 1: To determine whether RNase deficient *Escherichia coli* cells will display increased sensitivity to aminoglycoside antibiotics. Results are found in Chapter 2

Frazier, A. D., and W. S. Champney. 2012. Inhibition of ribosomal subunit synthesis in aminoglycoside treated ribonuclease mutants of *Escherichia coli*. Archives of Microbiology**:**In Submission.

Aim 2: To determine whether the use of vanadyl ribonucleoside complex will potentiate the effects of an aminoglycoside and a macrolide in wild type *Escherichia coli* cells. Results are found in Chapter 3

"Inhibition of Ribosomal Subunit Synthesis in *Escherichia coli* by the Vanadyl Ribonucleoside Complex"

Aim 3: To determine whether the use of vanadyl ribonucleoside complex will potentiate the effects of an aminoglycoside and a macrolide in methicillin sensitive and methicillin resistant *Staphylococcus aureus* cells. Results are found in Chapter 4

Frazier, A. D., and W. S. Champney. 2012. The vanadyl ribonucleoside complex inhibits ribosomal subunit formation in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy**:**In Submission.

CHAPTER 2

Inhibition of ribosomal subunit synthesis in aminoglycoside treated ribonuclease mutants of *Escherichia coli*

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Abstract The bacterial ribosome is a major target for current antibiotic therapy. During ribosomal subunit biogenesis, ribonucleases (RNases) play an important role in rRNA processing. Aminoglycoside antibiotics bind to both 30S and 50S subunits and stall subunit assembly. *E. coli* cells deficient for specific RNases are predicted to have an increased sensitivity to neomycin and paromomycin. It is shown that *E. coli* strains deficient for the rRNA processing enzymes RNase III, RNase E, RNase R, RNase G, or RNase PH have enhanced subunit assembly defects. These mutants showed an increased sensitivity to both aminoglycoside antibiotics. An increase in 16S and 23S rRNA fragmentation was detected in *E. coli* cells deficient for these enzymes. This research identified ribonucleases involved in rRNA processing as important in the effectiveness of aminoglycoside inhibition.

Introduction

Resistance to the most commonly used antimicrobial agents is increasing. Well examined mechanisms include acquisition of resistance genes, up-regulation of genes encoding cellular efflux pumps and spontaneous mutations in target genes (Zinner 2007). Development of novel antibacterial agents and the identification of additional bacterial targets have become important research endeavors.

One important target of many antibiotics is the bacterial ribosome (Wallis and Schroeder 1997; Champney 2006). The prokaryotic ribosome consists of a large 50S and a small 30S subunit. The 50S subunit is composed of 23S and 5S rRNA and 34 proteins. 30S subunits contain 16S ribosomal RNA and 21 proteins. During subunit biogenesis, 16S, 23S, and 5S rRNA transcripts and ribosomal proteins combine to form intermediate precursors. The rRNA in the precursor particles is cleaved by endo- and exoribonucleases to produce the mature subunits (Kaczanowska and Rydén-Aulin 2007; Deutscher 2009). In addition to maturation, ribonucleases (RNases) are involved in degradation of rRNA. When an inhibitor, such as an antibiotic, is introduced into a bacterial cell during ribosomal formation, specific RNases are utilized to degrade the rRNA to eliminate the stalled precursor (Champney 2006).

Some antibiotics function by targeting both the 30S and 50S subunits. Neomycin and paromomycin are two aminoglycoside antibiotics that have been shown to inhibit 30S subunit assembly in various bacteria, including *E. coli* and *Staphylococcus aureus* (Mehta and Champney 2002; Mehta and Champney 2003). These antibiotics bind to helix 44 of 16S rRNA and stimulate mistranslation of mRNA. Aminoglycosides also bind to helix 69 of 23S rRNA. By binding to helix 69, the ability of the ribosome recycling factor to recycle the ribosome is inhibited. It can be postulated, based on previous research, that the binding of aminoglycosides

to Helix 69 inhibits the ribosome recycling factor from dissociating the 30S and 50S ribosomal subunits and recycling the ribosome (Hirokawa et al. 2005; Borovinskaya et al. 2007; Scheunemann et al. 2010).

This important role of RNases in rRNA processing and subunit assembly makes these enzymes potential targets for novel antibiotics. It has been previously shown that strains of *E. coli* deficient for RNase E, RNase II, or polynucleotide phosphorylase (PNPase) displayed an increased sensitivity to erythromycin or azithromycin (Usary and Champney 2001; Silvers and Champney 2005). These mutant *E. coli* strains also demonstrated an accumulation of 23S rRNA and a reduced rate of recovery when the antibiotic was removed. The importance of RNases in rRNA processing led us to propose that mutant *E. coli* strains would also be more sensitive to aminoglycosides. This work shows that *E. coli* strains deficient for RNase III, RNase E, RNase R, RNase G, or RNase PH have an increased sensitivity to neomycin and paromomycin, reflected in reduced subunit synthesis and enhanced rRNA turnover.

Materials and methods

Analysis of cellular growth and viability

Escherichia coli strains that were used are listed in Table 2.1. Cultures were grown at 37^{o}C (or 32^{o}C for *ts* mutants) in tryptic soy broth (TSB). Strains SK5665, SK5729, SK6639 and SK7622 were supplemented with 4μg/mL thymidine. Growth rates were measured as an increase in cellular density over time using a Klett-Summerson colorimeter. At a Klett reading of 20, neomycin or paromomycin at 5 or 10μg/mL were added to the appropriate cultures. At a Klett

reading of 80, cellular viability was determined by TSB agar plate colony counting after serial dilutions (Jett et al. 1997).

Analysis of ribosomal subunit assembly

Cell cultures were grown in TSB as described. At a Klett reading of 20, neomycin or paromomycin at 5µg/mL were added to the appropriate cultures. After 15 minutes of growth with the antibiotics, ^{3}H uridine (30 Ci/mmol, Am. Radiochemicals) at a concentration of 1µCi/mL and uridine at a concentration of 2µg/mL were added. The cells were allowed to grow for two cellular doublings. At that time, uridine was added to 50µg/mL and the cells were incubated for an additional 15 minutes. Cells were collected by centrifugation and stored frozen at -70^{o}C.

Cellular lysates were prepared with lysozyme and DNaseI as previously described (Silvers and Champney 2005). The samples were centrifuged through 5-20% sucrose gradients in S buffer in an SW41 rotor at 187813 *x g* for 3.5 hours. Following centrifugation, fractions were collected by pumping them through an ISCO Model UA-5 absorbance monitor set at 254nm. The fractions were collected into vials and mixed with 3mL Scintisafe gel before measuring the ^{3}H uridine radioactivity by liquid scintillation counting.

Analysis of total cellular RNA with the Agilent Bioanalyzer

Bacterial cells were grown as described above. At a Klett reading of 80 or an approximate density of 4x10^{8} cells/mL, the cells were collected by centrifugation and RNA was extracted

from the cell pellet. Total RNA from cell samples was isolated by phenol/chloroform extraction and ethanol precipitation (Rio et al. 2011). Typically 0.5 to 1µg of RNA was examined using an Agilent Bioanalyzer 2100 and the RNA 6000 nano chip.

Table 2.1 *E. coli* strains used in this study

Strain	Phenotype	Genotype	Reference or source
SK901	None	F- malA thi-	(Kushner et al. 1977)
D10-1	I	HfrH met- rna-1 relA	(Gesteland 1966)
SK7622	III	F-thyA715 rncD38::kanR	(Babitzke et al. 1993)
SK5665	E	F-thyA715 rne1	(Arraiano et al. 1988)
SK4803	II	F-gal thi ton sup hasdR4 endAsbcB15 rnb296	(Donovan and Kushner 1986)
N7060	I, II, PNPase	F- metB1 tryA451 rpsl478 rna919rnb464 pnp13	(Weatherford et al. 1972)
MG1655 I^-R^-	I, R	Δrna Δrnr::cam	(Chen and Deutscher 2010)
GW11	G	F- zce-726::Tn10, TetR, rng::cat CmR	(Li et al. 1999)
SK6639	PNPase	F-thyA715, CmR, pnp-200, rph-1, λ-	(Cheng and Deutscher 2003)
SK5729	I, II, PNPase, PH	F- thyA715, rna-19, rnb-500, pnp-7, λ-, rph-1	Sidney Kushner

Analysis of 16S rRNA and 23S rRNA by Northern blot hybridization

Biotinylated 16S and 23S specific probes were constructed as previously described (Silvers and Champney 2005). The 16S (241 bp) and 23S (146 bp) DNA probes were amplified from plasmid pKK3535 DNA (Brosius et al. 1980) using the polymerase chain reaction. The 23S primers used were 23S F: TAG GGG AGC GTT CTG TAA G and 23S R: CCC ATT AAC GTT GGA C (nt. nos. 1188-1334). The 16S primers used were 16S F: GGA GGA AGG TGG GGA TGA CG and 16S R: ATG GTG ACG GGC GGT GTG (nt. nos. 1173-1414). The primers were from Life Technologies. PCR products were purified by extraction with equal amounts of phenol and chloroform before precipitating with 2 volumes of ethanol. The pellets were resuspended in

30µL of sterile water. The purified DNA probes were labeled with biotin using the Label-IT biotin labeling kit (Mirus) per the manufacturer's instructions (Silvers and Champney 2005).

Six micrograms of total RNA was denatured by heating at 55^{o}C for 10 minutes and separated on a 5% TAE PAGE gel as previously described (Rio et al 2011). After destaining overnight in sterile water, RNA was transferred onto Nytran nylon membranes using a Turbo blot apparatus (Schleicher & Schuell). The membranes were pre-hybridized in 15mL of 1X pre-hybridization solution (MRC, Inc.) at 42^{o}C for 30 minutes. The membranes were then hybridized overnight at 42^{o}C with 6mL hybridization buffer (50% formamide, 5X SSC, 0.1% sarkosyl, 0.02% SDS and 200µg/mL BSA), 1X background quencher (MRC, Inc.), and 4pmol of the denatured 16S or 23S specific probe (Silvers and Champney 2005). After hybridization, the membranes were washed and the probe detected with horseradish peroxidase using the North2South chemiluminescent hybridization kit (Pierce Chemical Co.). Analysis of the rRNA fragmentation was conducted by image analysis in the G Box Imager (SynGene).

Statistical analysis

Statistical differences were determined using Student t-test. Each wild type or RNase deficient mutant sample incubated with an antibiotic was compared to the control cells without antibiotics for that RNase strain. In each table, an asterisk indicates a statistical significance of $P<0.05$.

Results

An initial test was performed to determine whether *E. coli* mutants deficient for any of eight RNases would show an enhanced sensitivity to the aminoglycosides, neomycin and paromomycin. Previous research has shown that strains missing RNase II, E or PNPase demonstrated an increased sensitivity to the macrolide antibiotic azithromycin (Silvers and Champney 2005). As Table 2.2 shows, strains missing the rRNA processing enzymes RNase III, E, R, G or PH revealed a large reduction in viability after growth with either aminoglycoside. All the percentages were determined by comparing the antibiotic treated samples with that strain's control. In particular, neomycin at 10µg/mL reduced the viability of RNase III, E, R, or PH mutants to approximately 10% of the untreated control strain (Table 2.2).

Table 2.2 Effect of aminoglycosides on viability of wild type and mutant *E. coli* cells

		% Control total viable cell count			
Strain	**RNase mutation**	**Neomycin**		**Paromomycin**	
		5µg/mL	**10µg/mL**	**5µg/mL**	**10µg/mL**
SK901	None	15.4±12.8	26.3±9.0	7.7±5.1	3.0±0.5 *
D10-1	I	18.2±5.0	14.2±4.1	20.7±19.8	17.4±17.4
SK7622	III	0.32±0.001 *	5.7±4.2 *	4.6±3.5 *	2.5±1.2 *
SK5665	E	51.9±33.4	3.6±0.4 *	12.5±8.4 *	0.89±0.001 *
SK4803	II	44.4±25.9	5.2±4.9	11.1±3.3	1.1±0.9
N7060	I, II, PNPase	5.2±5.0 *	6.1±2.4 *	33.5±15.8	3.3±0.2 *
MG1655 I^-R^-	I, R	13.4±7.5 *	11.5±7.3 *	5.1±1.5 *	4.2±0.4 *
GW11	G	10.6±7.1 *	23.2±8.8 *	10.7±6.3 *	2.4±0.9 *
SK6639	PNPase	75.4±21.1	5.2±1.8 *	51.4±4.9	3.2±0.7 *
SK5729	I, II, PNPase, PH	80.0±39.3	3.0±1.9 *	15.0±6.1 *	2.0±0.6 *

Results are the means ± standard error of 4 independent experiments. (*) statistically significant with a *P value <0.05*

Aminoglycoside antibiotics can bind to both 30S and 50S ribosomal subunits (Borovinskaya et al. 2007; Scheunemann et al. 2010). Therefore, the mutants deficient for RNase I, E, III, R, G, PH or a combination of mutations were examined for impaired 30S and 50S ribosomal subunit assembly. *E. coli* deficient for more than one mutation (N7060) were also included in determining impairment of 30S and 50S ribosomal subunit assembly. This is because we wanted to show that the decrease in 30S and 50S subunit assembly for MG1655 I^-R^- and SK5729 was due to RNase R and RNase PH deficiencies respectively. Cells were labeled with ^{3}H-uridine during growth without and with antibiotics. Ribosomal subunit amounts were measured after separation by sucrose gradient centrifugation. Figure 2.1 shows the gradient profiles for each selected strain. Both aminoglycosides promoted a reduction in 30S subunit amounts in most of the RNase mutant *E. coli* strains. 50S subunit levels were reduced in every case after drug treatment except in the wild type and RNase G deficient strains. Significantly, a major increase in labeled RNA was seen in the top gradient fractions (Table 2.3), indicative of rRNA degradation (Silvers and Champney 2005). For example the RNase E mutant strain showed a 14 and 24% increase in labeled RNA at the top of the gradient when neomycin or paromomycin where added at a concentration of 5µg/mL. *E. coli* deficient for RNase PH showed a 10% decrease in the 30S ribosomal subunit when either antibiotic was added. Additionally, the 50S ribosomal subunit was decreased by 5 and 13% when 5µ/mL neomycin or paromomycin were added. In every case, the labeled RNA lost from the subunits was accounted for by an approximate amount present in the top gradient region. Strain N7060 is deficient for RNases I, II, and PNPase. Due to the findings that this strain displayed a decrease in 30S and 50S ribosomal subunits (Table 2.3), it was determined that any statistical significance found in strain SK5729 is due to the loss of RNase PH and not to the other mutations.

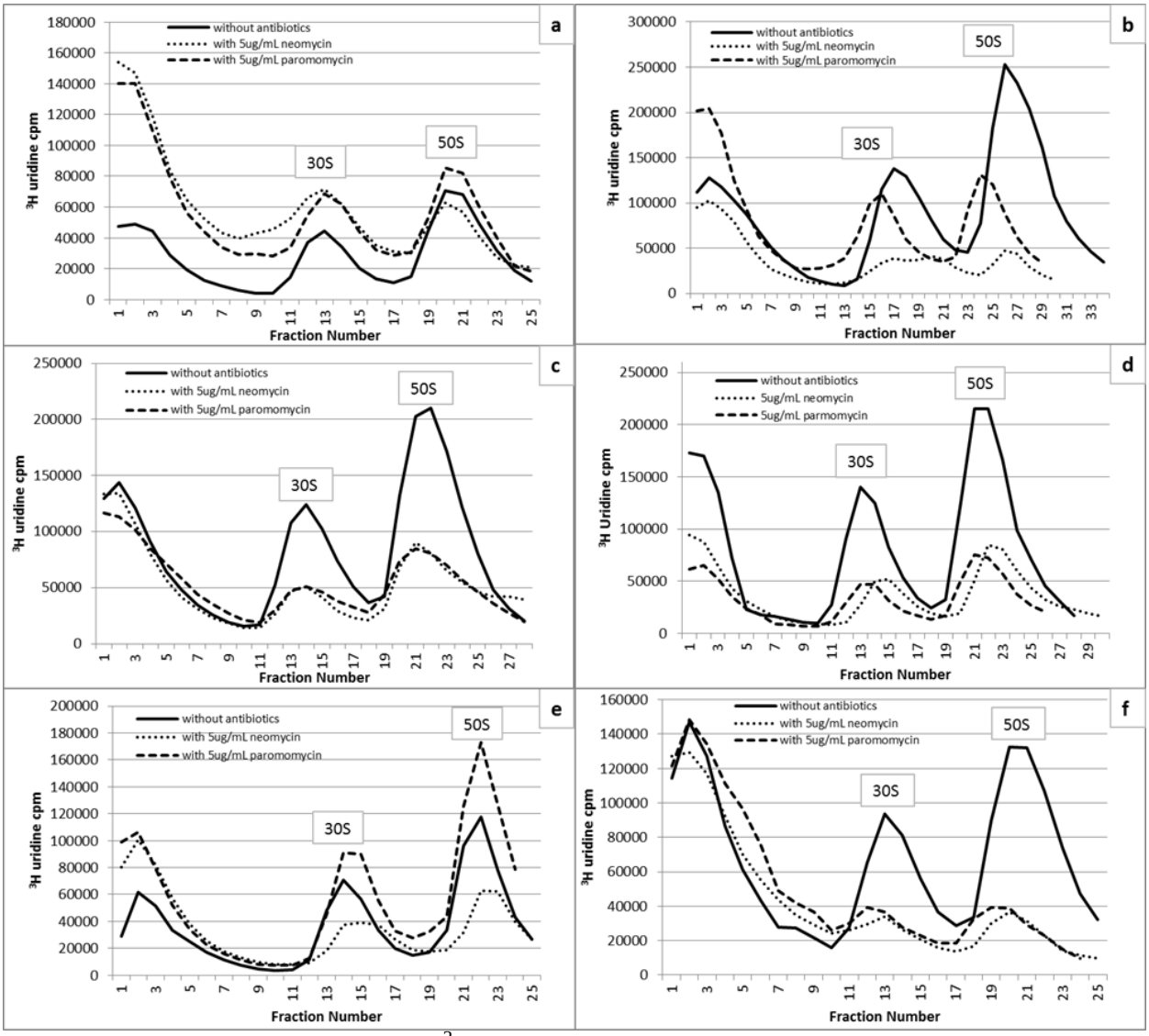

Fig. 2.1 Sucrose gradient profiles of ^{3}H uridine labeled ribosomal subunits isolated from *E. coli* cells grown with or without 5µg/mL antibiotics. Gradient profiles for wild type (A), RNase III deficient (B), RNase E deficient (C), RNase R deficient (D), RNase G deficient (E), and RNase PH deficient (F)

Table 2.3 Distribution of ^{3}H uridine labeled RNA in sucrose gradient regions

		% Total gradient radioactivity								
Strain	**RNase mutation**	**Control**			**Neomycin**			**Paromomycin**		
		Top	**30S**	**50S**	**Top**	**30S**	**50S**	**Top**	**30S**	**50S**
SK901	None	29.01±0.5	24.84±1.1	43.09±0.3	47.52±0.5 (166.8) *****	24.63±1.3 (105.9)	20.27±1.0 (47.0) *****	42.80±2.2 (149.2) *****	23.74±0.7 (95.6)	27.85±0.5 (66.9) *****
D10-1	I	27.92±3.1	22.94±0.8	45.57±1.8	45.52±5.0 (163.0)	22.49±2.0 (105.1)	27.55±8.4 (62.8)	40.32±8.6 (146.7)	19.32±3.2 (84.2)	35.49±6.1 (79.7)
SK7622	III	23.99±1.3	23.84±0.5	48.70±.05	44.06±6.3 (187.0)	23.73±3.2 (99.5)	24.93±3.9 (51.2) *****	41.95±2.7 (176.7) *****	22.57±1.1 (94.7)	30.37±3.2 (62.4) *****
SK5665	E	31.55±0.9	23.97±0.4	40.57±1.4	47.61±6.6 (150.9)	20.07±1.0 (83.7)*	27.83±5.5 (68.6)	37.64±3.8 (119.3)	27.12±4.5 (113.1)	33.78±2.6 (83.3)
N7060	I, II, PNPase	25.60±1.6	25.41±0.06	46.15±2.9	37.12±2.7 (145.0)	22.20±1.1 (87.4)	36.92±3.9 (80.0)	24.78±1.8 (96.8)	21.50±0.1 (84.6)*	45.77±0.06 (99.2)
MG1655I^{-}R^{-}	I, R	30.28±1.0	24.37±0.4	41.56±1.5	44.98±6.2 (148.5)	22.05±1.9 (90.5)	28.76±5.4 (69.2)	34.40±2.9 (113.6)	24.32±0.9 (99.8)	37.48±4.0 (90.2)
GW11	G	29.04±2.4	24.75±1.5	43.38±1.6	42.28±1.8 (145.6) *****	19.79±0.8 (80.0)	26.48±2.8 (61.0)	33.34±1.7 (114.8)	23.74±0.005 (95.9)	40.00±2.1 (92.2)
SK5729	I, II, PNPase, PH	38.31±1.9	26.42±3.8	31.78±2.6	62.42±4.4 (162.9) *****	16.85±0.7 (63.8)	16.47±3.6 (51.8) *****	62.03±3.0 (161.9) *****	17.31±1.0 (65.5)	15.46±2.3 (48.6) *****

Cells were grown with 5µg/mL aminoglycosides. Radioactivity in gradient fractions corresponding to the top, 30S, and 50S regions were summed and calculated as a percent of the total radioactivity in the gradients. Percentage of the 30S and 50S regions in comparison to the mutant or wild type strain controls is shown. Results are the means ± standard error of 2 independent experiments. (*) statistically significant with a *P value <0.05*

Total RNA was isolated from each selected strain after growth without and with the aminoglycosides, and the status of rRNA in all cells was examined by Agilent chip analysis. An enhanced rRNA degradation was observed in the mutants, particularly after growth with the antibiotics at 10µg/mL (Figure 2.2 and Table 2.4). In addition, RNase R and RNase PH mutant strains displayed an increase in small RNA oligonucleotides. As with the gradient rRNA distribution, the loss of 16S and 23S rRNA could be accounted for by an increase in small RNA species. For example, the RNase PH mutant strain's small RNA was increased by more than 130% when either antibiotic was added at 10µg/mL. In the RNase R mutant strain, 16S rRNA was decreased by 19-23% when neomycin or paromomycin were added at a concentration of 10µg/mL. The RNase PH deficient strain showed a 35% and 16% decrease in 23S rRNA when either neomycin or paromomycin were added at a 10µg/mL concentration (Table 2.4). Finally, the analysis revealed the accumulation of a band the approximate size of 16S precursor RNA in all mutant strains with the exception of the RNase G deficient strain. This 16S precursor band can be seen directly above the 16S band in the Agilent gel image (Figure 2.2).

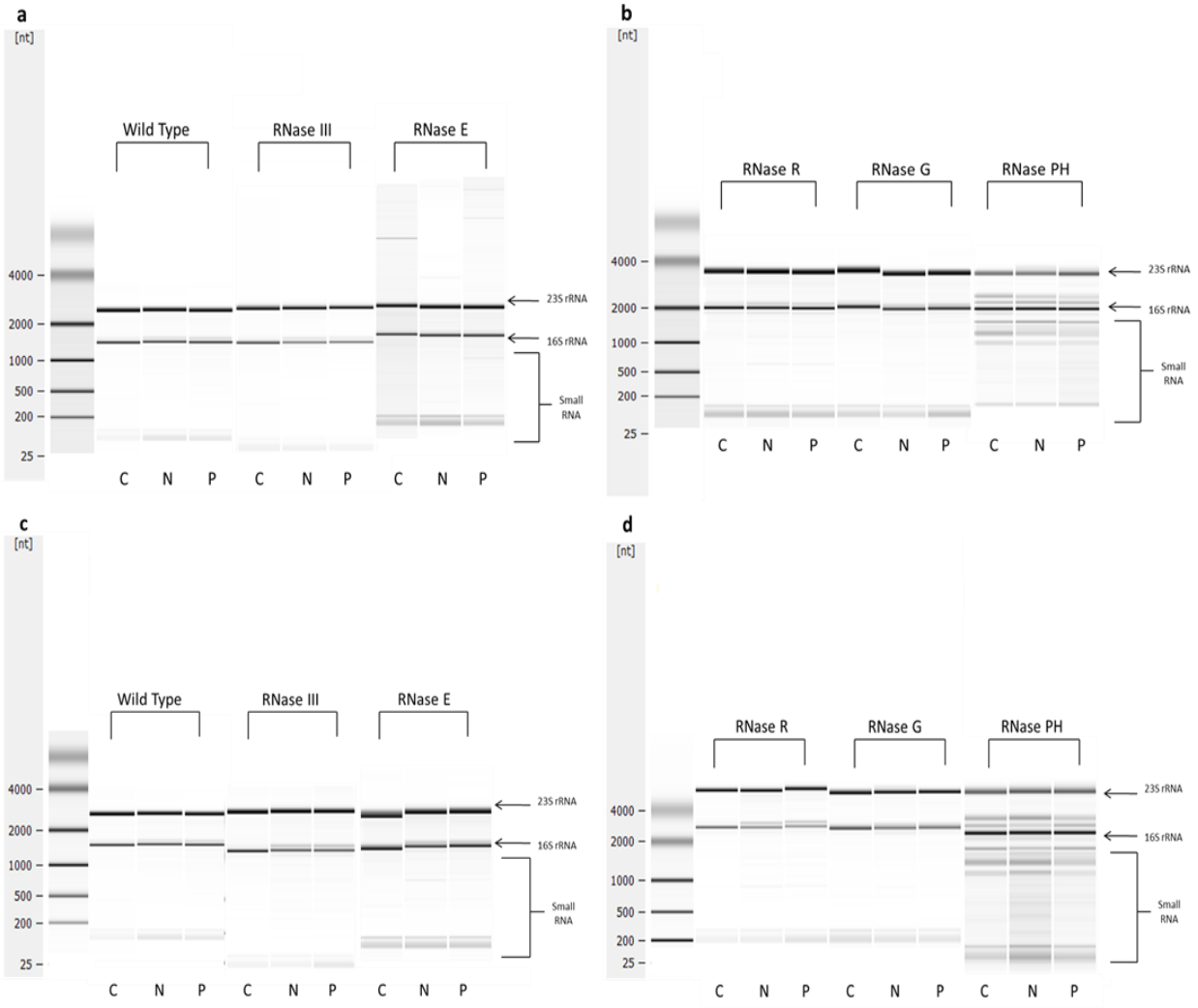

Fig. 2.2 Agilent gel analysis of total RNA. RNA samples were analyzed by the Agilent Bioanalyzer. Agilent chip analysis of RNA from wild type and mutant cells grown with 5µg/mL antibiotics (a) and (b). Agilent chip analysis of RNA from wild type and mutant cells grown with 10µg/mL antibiotics (c) and (d). Control (C), Neomycin (N), Paromomycin (P)

Table 2.4 Percentage distribution of small, 16S, and 23S rRNA by Agilent gel analysis

% Total area													
Strain	**RNase mutation**	**Control**				**Neomycin 5µg/mL**				**Paromomycin 5µg/mL**			
		RIN	**Small**	**16S**	**23S**	**RIN**	**Small**	**16S**	**23S**	**RIN**	**Small**	**16S**	**23S**
SK901	None	9.8± 0.1	11.53± 4.4	26.80±1.3	40.80±3.2	9.5±2.3 (96.9)	17.37±4.7 (150.7)	23.03±1.3 (85.9)	38.87±2.7 (95.3)	8.7±0.8 (88.5)	18.83±3.2 (163.3)	23.78±1.4 (88.7)	34.70±1.9 (85.0)
SK7622	III	8.8± 1.1	17.87±6.6	29.73±3.3	44.27±5.3	8.8±1.0 (100.0)	12.80±5.3 (71.6)	30.93±5.2 (104.0)	46.73±4.1 (105.6)	9.2±0.7 (104.5)	14.90±3.1 (83.4)	37.37±11.2 (125.7)	33.97±8.9 (76.7)
SK5665	E	8.8± 0.8	17.50±6.9	22.85±3.6	36.60±6.7	9.3±0.3 (105.7)	15.10±5.3 (86.3)	24.00±2.8 (105.0)	37.38±4.1 (102.1)	9.3±0.3 (105.7)	19.18±7.0 (109.6)	25.55±2.0 (111.8)	39.70±2.8 (108.5)
MG1655 I-R-	I, R	9.1± 0.5	13.80±3.4	19.80±2.4	32.57±6.7	8.2±0.8 (90.1)	15.83±3.5 (114.7)	15.50±4.7 (78.3)	26.07±8.3 (80.0)	9.0±0.1 (98.9)	17.57±1.2 (127.3)	19.77±1.3 (99.8)	33.23±2.8 (102.0)
GW11	G	9.7± 0.1	18.00±2.3	23.50±1.0	34.47±1.8	9.4±0.1 (96.9)	17.03±3.7 (94.6)	21.33±0.7 (90.8)	33.37±1.6 (96.8)	8.5±0.9 (87.6)	20.73±3.8 (115.2)	17.97±4.3 (76.5)	26.70±6.9 (77.5)
SK5729	I, II, PNPase, PH	6.6± 0.005	24.20±0.2	14.40±0.1	19.35±0.7	7.0±0.1 (106.1) *****	29.70±0.2 (122.7) *****	15.65±0.1 (108.7) *****	20.10±0.3 (103.9)	6.8± 0.005 (103.0) *****	34.90±2.0 (144.2) *****	12.70±0.1 (88.2) *****	15.25±0.3 (78.8) *****

% Total area													
Strain	**RNase mutation**	**Control**				**Neomycin 10µg/mL**				**Paromomycin 10µg/mL**			
		RIN	**Small**	**16S**	**23S**	**RIN**	**Small**	**16S**	**23S**	**RIN**	**Small**	**16S**	**23S**
SK901	None	9.5± 0.2	12.05±0.2	24.40±1.0	42.10±3.7	9.8±0.2 (103.4)	13.70±0.7 (113.7)	26.85±4.55 (110.0)	44.25±4.5 (105.1)	8.8±1.3 (92.6)	20.10±3.7 (166.8)	28.00±3.2 (114.8)	44.00±3.6 (104.5)
SK7622	III	9.0± 0.4	16.25±1.5	23.35±0.9	32.50±1.0	7.7±1.4 (85.6)	20.55±1.1 (126.5)	23.50±1.5 (100.6)	38.90±9.8 (119.7)	9.4±0.2 (104.4)	17.10±0.1 (105.2)	23.25±0.6 (99.6)	34.00±4.5 (104.6)
SK5665	E	10.0± 0.1	6.60±0.4	27.70±1.0	48.10±5.6	9.2±0.005 (92.0)	5.10±0.9 (77.3)	23.8±0.8 (85.9)	40.85±2.8 (84.9)	9.3±0.1 (93.0)	9.45±1.1 (143.2)	22.65±0.9 (81.8)	37.80±2.2 (78.6)
MG1655 I-R-	I, R	9.9± 0.1	13.8±1.4	25.4±0.5	43.65±2.65	8.9±0.1 (89.9)	13.05±0.3 (94.6)	20.55±0.1 (80.9) *	40.80±1.3 (93.5)	9.0±0.1 (90.9)	16.95±0.1 (122.8)	19.65±0.4 (77.4) *	40.50±1.9 (92.8)
GW11	G	9.8± 0.1	18.45±2.8	23.65±1.2	34.80±0.6	9.6±0.3 (98.0)	15.65±3.3 (84.8)	21.80±1.1 (92.2)	34.80±2.9 (100.0)	9.8±0.3 (100.0)	15.20±1.0 (82.4)	25.4±2.0 (107.4)	40.15±2.3 (115.4)
SK5729	I, II, PNPase, PH	6.7± 0.1	23.50±0.7	13.50±0.7	19.05±0.5	6.1± 0.005 (91.0) *****	33.60±0.5 (143.0) *****	9.45±0.3 (70.0) *****	12.70±0.4 (65.1) *****	6.7±0.1 (100.0)	31.30±0.2 (133.2) *****	13.35±0.3 (98.9)	15.95±0.2 (83.7) *****

Total RNA in gel bands from Figure 2.2 was computed by analysis of the electropherograms using Agilent software. Percentage of the total RNA in comparison to the wild type or mutant strain controls in each gel region is shown. The RNA integrity number (RIN) is indicated. Results are the means ± standard error of 2 independent experiments. (*) statistically significant in comparison to no drug controls for each strain with a *P value <0.05*

Degradation of 16S and 23S rRNA stimulated by antibiotic treatment was further examined by Northern hybridization analysis. Figure 2.3 shows the result of Northern blots hybridized with a 16S rRNA probe, and Figure 2.4 shows the same *E. coli* samples examined for rRNA fragmentation with a 23S rRNA probe. With the exception of the RNase E mutant, enhanced 16S rRNA fragmentation was observed (Figure 2.3). Enhanced 23S rRNA fragmentation was also detected by Northern hybridization analysis for the RNase III, R and PH deficient strains after antibiotic treatment (Figure 2.4). A measurement of the fragment sizes was determined for both 16S and 23S rRNA by G box Analysis (Figure 2.3 and Figure 2.4). These results substantiate the observations made by sucrose gradient and gel analysis of rRNA degradation.

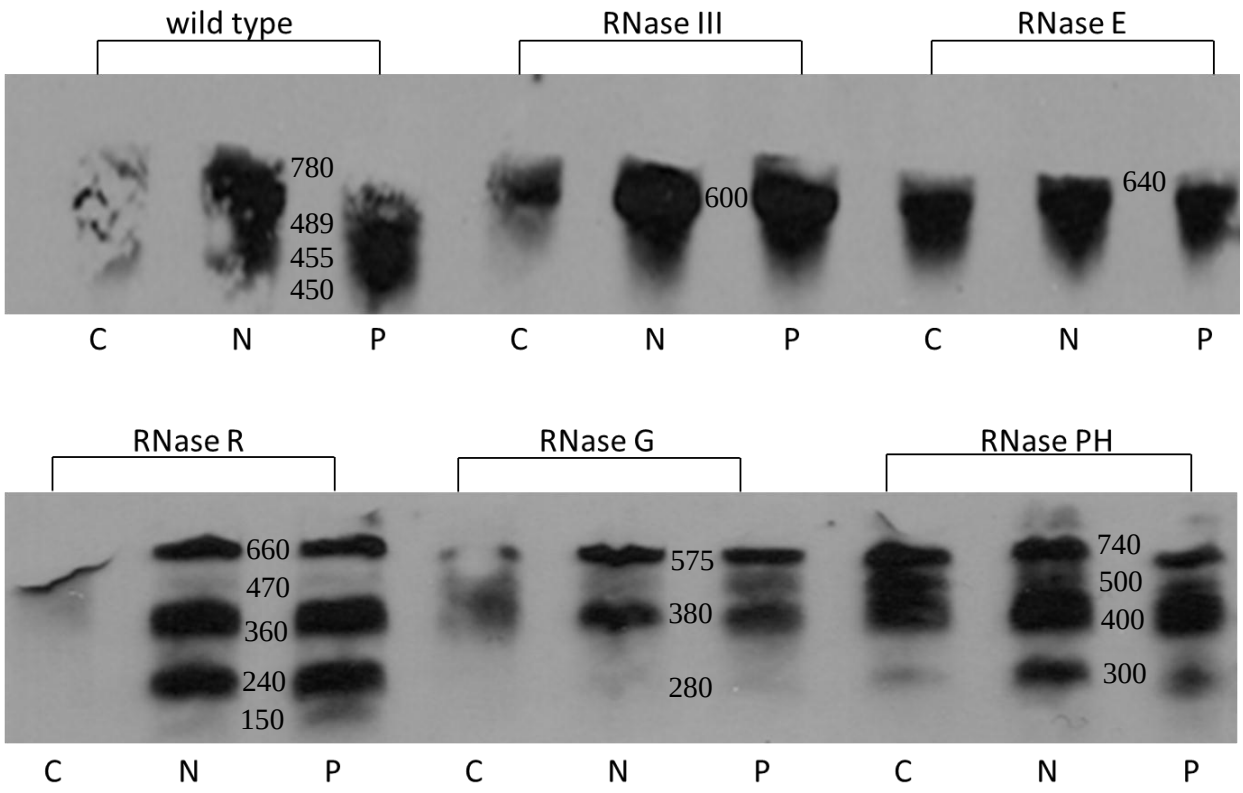

Fig. 2.3 Northern hybridization analysis of 16S rRNA fragmentation for wild type and RNase mutant *E. coli* cells. RNA was isolated from cells grown as indicated with 10µg/mL aminoglycosides. RNA sequences were identified by hybridization with a 16S DNA probe and nucleotide sizes are indicated. Control (C), Neomycin (N), Paromomycin (P)

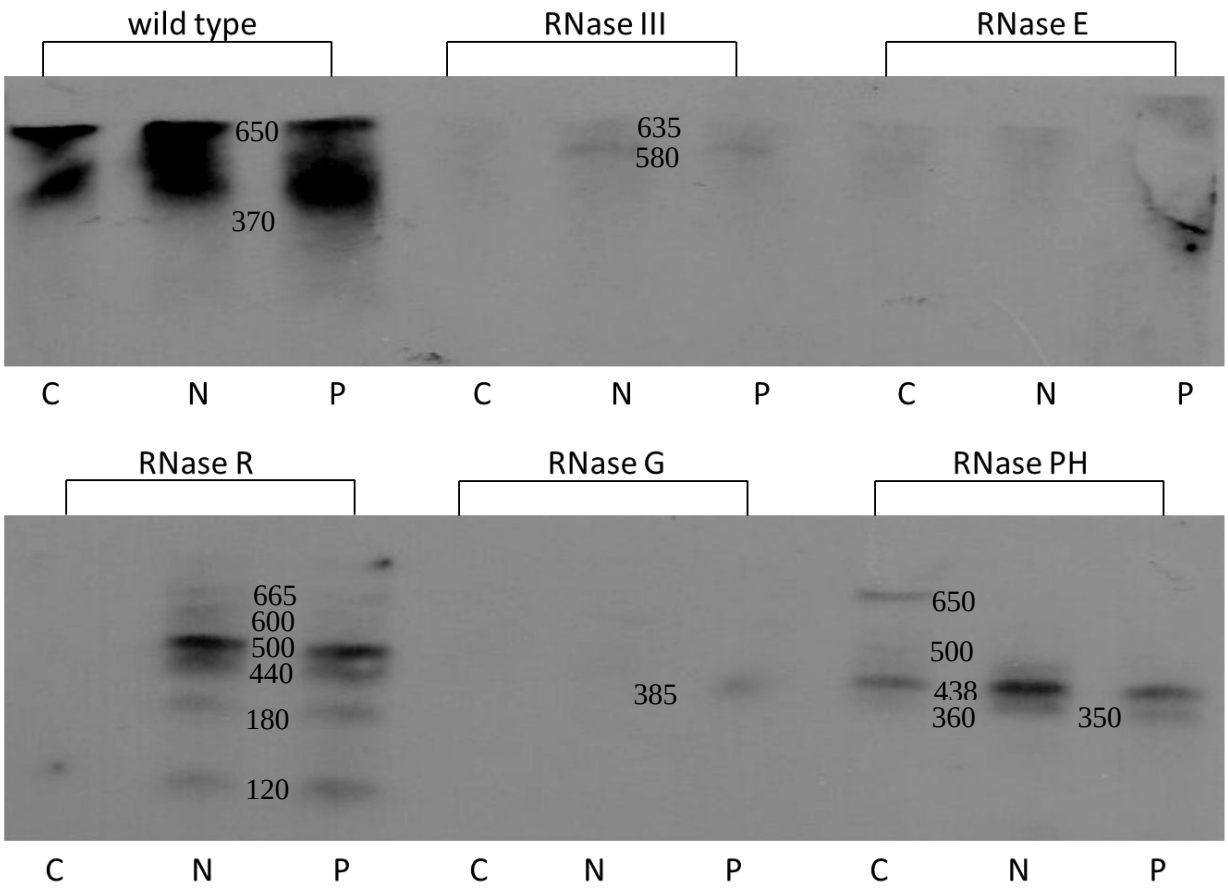

Fig. 2.4 Northern hybridization analysis of 23S rRNA fragmentation for wild type and RNase mutant *E. coli* cells. RNA was isolated from cells grown as indicated. RNA was isolated from cells grown as indicated with 10µg/mL aminoglycosides. RNA sequences were identified by hybridization with a 23S DNA probe and nucleotide sizes are indicated. Control (C), Neomycin (N), Paromomycin (P)

Discussion

Aminoglycosides are a widely used family of antibiotics that target the bacterial ribosome (Sutcliffe 2005). They bind to both subunits of the ribosome and affect both translational accuracy and ribosomal subunit recycling. Neomycin and paromomycin are structurally similar compounds and interact with the subunit rRNAs in similar ways. Both antibiotics stall the assembly of the 30S subunit in addition to impairing translation (Champney 2006).

Ribonucleases function in rRNA processing and degradation (Deutscher 2009). RNases III, E, G, and PH are involved in the processing of precursor rRNA during subunit synthesis. RNase III is responsible for the initial cleavage of the precursor rRNA (Deutscher 2009). RNase G and E function in processing of the 5' end of 16S rRNA (Li et al. 1999). Additionally, RNase G and PH are involved in processing of 23S rRNA (Song et al. 2011; Gutgsell and Jain 2012). RNase III, RNase E, and RNase PH are also involved in rRNA degradation in addition to RNase II, R, and PNPase (Deutscher 2009; Arraiano et al. 2010). RNase E, PNPase, enolase, and the DEAD-box helicase Rh1B form a complex known as the degradosome. The degradosome is responsible for degradation of rRNA under some circumstances (Vanzo et al. 1998). RNase R initiates rRNA degradation by binding to a 3' terminus overhang (Vincent and Deutscher 2006).

Elimination of specific RNases by mutation is predicted to enhance the effectiveness of antibiotics which stall ribosomal subunit formation by reducing the ability to recycle ribosomal subunits. *E. coli* cells with a mutation in RNase E showed enhanced sensitivity to erythromycin and the accumulation of a precursor to the 50S subunit (Usary and Champney 2001). *E. coli* cells deficient for RNase II or PNPase showed an enhanced sensitivity to azithromycin and an impairment of 23S rRNA function. Turnover of the RNA was stimulated and 50S ribosomal subunit formation was impaired by azithromycin treatment. In addition, recovery of subunit formation after antibiotic removal was impaired (Silvers and Champney 2005). Based on these findings, it was hypothesized that *E. coli* cells deficient for specific RNases would display an increased sensitivity to aminoglycosides.

The enzymes identified in this study to affect sensitivity to aminoglycosides are, for the most part, different from the enzymes shown to affect erythromycin and azithromycin sensitivity (RNase E and RNase II and PNPase). The difference in RNases in the two cases may result from

the differences in the effects of macrolides and aminoglycosides on the subunit assembly sequence. In this study, *E. coli* deficient for RNases III, E, R, G, or PH displayed an increased sensitivity to neomycin or paromomycin. Aminoglycosides affect both 30S and 50S ribosomal subunit synthesis while macrolides only affect 50S ribosomal subunit assembly (Champney and Tober 2000; Usary and Champney 2001; Mehta and Champney 2002). During transcription, 16S rRNA is transcribed first and initiates 30S precursor assembly prior to 23S and 5S rRNA synthesis for 50S precursor formation. Aminoglycoside antibiotic stalling of 30S formation leads to the accumulation of a 21S precursor, whose rRNA will not be further processed in specific RNase deficient mutants. Macrolide antibiotic stalling of 50S assembly generates a 32S precursor to the 50S particle which would need to be removed by the rRNA degradative enzymes RNase II and PNPase as observed (Silvers and Champney 2005).

The somewhat larger effects of the aminoglycosides on subunit synthesis and rRNA degradation in strain SK5729 may be due to additional RNase mutations in this strain. The strain lacks RNases I, II, PH and PNPase. However, examination of a mutant strain (N7060) deficient for RNase I, II, and PNPase showed no significant effects on subunit synthesis or rRNA degradation in the presence of either aminoglycoside (A. F. unpublished). We would conclude that the aminoglycoside effects in SK5729 are therefore likely due to the loss of RNase PH and not to the lack of the other ribonucleases.

In summary, this research has identified rRNA processing RNases as important in the effectiveness of aminoglycoside antibiotic inhibitory activity. Inhibition of processing RNases by small molecule inhibitors or RNA interference methods could represent an attractive new antimicrobial target.

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References

Arraiano CM et al. (2010) The critical role of RNA processing and degradation in the control of gene expression. FEMS Microbiol Rev 34:883-923

Arraiano CM, Yancey SD, Kushner SR (1988) Stabilization of discrete mRNA breakdown products in ams pnp rnb multiple mutants of *Escherichia coli* K-12. J Bacteriol 170:4625-4633

Babitzke P, Granger L, Olszewski J, Kushner SR (1993) Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. J Bacteriol 175:229-239

Borovinskaya MA et al. (2007) Structural basis for aminoglycoside inhibition of bacterial ribosome recycling. Nat Struct Mol Biol 14:727-732

Brosius J, Dull TJ, Noller HF (1980) Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli*. Proc Natl Acad Sci U S A 77:201-204

Champney WS (2006) The other target for ribosomal antibiotics: inhibition of bacterial ribosomal subunit formation. Infect Disord Drug Targets 6:377-390

Champney WS, Tober CL (2000) Specific inhibition of 50S ribosomal subunit formation in *Staphylococcus aureus* cells by 16-membered macrolide, lincosamide, and streptogramin B antibiotics. Curr Microbiol 41:126-135

Chen C, Deutscher MP (2010) RNase R is a highly unstable protein regulated by growth phase and stress. RNA 16:667-672

Cheng ZF, Deutscher MP (2003) Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. Proc Natl Acad Sci U S A 100:6388-6393

Deutscher MP (2009) Maturation and degradation of ribosomal RNA in bacteria. Prog Mol Biol Transl Sci 85:369-391

Donovan WP, Kushner SR (1986) Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. Proc Natl Acad Sci U S A 83:120-124

Gesteland RF (1966) Isolation and characterization of ribonuclease I mutants of *Escherichia coli*. J Mol Biol 16:67-84

Gutgsell NS, Jain C (2012) Role of precursor sequences in the ordered maturation of *E. coli* 23S ribosomal RNA. RNA 18:345-353

Hirokawa G, Nijman RM, Raj VS, Kaji H, Igarashi K, Kaji A (2005) The role of ribosome recycling factor in dissociation of 70S ribosomes into subunits. RNA 11:1317-1328

Jett BD, Hatter KL, Huycke MM, Gilmore MS (1997) Simplified agar plate method for quantifying viable bacteria. Biotechniques 23:648-650

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

Kushner SR, Maples VF, Champney WS (1977) Conditionally lethal ribosomal protein mutants: characterization of a locus required for modification of 50S subunit proteins. Proc Natl Acad Sci U S A 74:467-471

Li Z, Pandit S, Deutscher MP (1999) RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. EMBO J 18:2878-2885

Mehta R, Champney WS (2002) 30S ribosomal subunit assembly is a target for inhibition by aminoglycosides in *Escherichia coli*. Antimicrob Agents Chemother 46:1546-1549

Mehta R, Champney WS (2003) Neomycin and paromomycin inhibit 30S ribosomal subunit assembly in *Staphylococcus aureus*. Curr Microbiol 47:237-243

Rio DC, Ares M, Jr., Hannon GJ, Nilsen TW (2011) RNA: A Laboratory Manual. Cold Spring Harbor Protocols
Scheunemann AE, Graham WD, Vendeix FA, Agris PF (2010) Binding of aminoglycoside antibiotics to helix 69 of 23S rRNA. Nucleic Acids Res 38:3094-3105
Silvers JA, Champney WS (2005) Accumulation and turnover of 23S ribosomal RNA in azithromycin-inhibited ribonuclease mutant strains of *Escherichia coli*. Arch Microbiol 184:66-77
Song WS, Lee M, Lee K (2011) RNase G participates in processing of the 5'-end of 23S ribosomal RNA. J Microbiol 49:508-511
Sutcliffe JA (2005) Improving on nature: antibiotics that target the ribosome. Curr Opin Microbiol 8:534-542
Usary J, Champney WS (2001) Erythromycin inhibition of 50S ribosomal subunit formation in *Escherichia coli* cells. Mol Microbiol 40:951-962
Vanzo NF et al. (1998) Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. Genes Dev 12:2770-2781
Vincent HA, Deutscher MP (2006) Substrate recognition and catalysis by the exoribonuclease RNase R. J Biol Chem 281:29769-29775
Wallis MG, Schroeder R (1997) The binding of antibiotics to RNA. Prog Biophys Mol Biol 67:141-154
Weatherford SC, Rosen L, Gorelic L, Apirion D (1972) *Escherichia coli* strains with thermolabile ribonuclease II activity. J Biol Chem 247:5404-5408
Zinner SH (2007) Antibiotic use: present and future. New Microbiol 30:321-325

CHAPTER 3

Inhibition of Ribosomal Subunit Synthesis in *Escherichia coli* by the Vanadyl Ribonucleoside Complex

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ABSTRACT

The increase in antibiotic resistant microorganisms has driven a search for new antibiotic targets and novel antimicrobial agents. A large number of different antibiotics target bacterial ribosomal subunit formation. Several specific ribonucleases are important in the processing of rRNA during subunit biogenesis. This work demonstrates that the ribonuclease inhibitor, vanadyl ribonucleoside complex (VRC), can inhibit RNases involved in ribosomal subunit formation. The ribosomal subunit synthesis rate was significantly decreased and ribosomal RNA from the subunit precursors was degraded. VRC had no inhibitory effect on translation. VRC also enhanced the inhibitory effects of an aminoglycoside and a macrolide antibiotic.

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Keywords: vanadyl ribonucleoside complex; *Escherichia coli;* ribosome assembly; protein synthesis

INTRODUCTION

The increase in antibiotic resistance is a global threat to both physicians and scientists (30). An important area of investigation is to discover novel antimicrobial agents to which resistance has not yet been developed. In addition, a significant effort is being made to identify new cellular targets for drug discovery (1, 4, 19).

The bacterial ribosome is a target for a diverse collection of different antibiotics (9, 36). Drug binding sites and molecular mechanisms of action have been described recently for many of these compounds based on refined crystal structures (7, 9, 14, 15, 25, 34). Most of these antibiotics bind to the 30S or 50S ribosomal subunit and inhibit translation of mRNA. Importantly, biogenesis of the subunits is an additional cellular target for many of these agents (8). When synthesis of either subunit is inhibited, specific ribonucleases (RNases) proceed to degrade the rRNA in the ribosomal subunit assembly intermediates (9). In particular, RNase E is important in the turnover of a 50S subunit precursor particle present in erythromycin-inhibited cells (34) and the enzymes RNase II and PNPase are also involved in rRNA degradation in cells inhibited by azithromycin (31).

In *Escherichia coli* cells, at least five different RNases are needed to generate and process the 5' and 3' ends of 16S, 23S and 5S rRNA (18, 23, 32, 35). Subunit assembly intermediates are the substrates for these activities. Mutant organisms missing any of these proteins show an enhanced sensitivity to both paromomycin and neomycin (16). These observations suggest that RNases in *E. coli* cells could be novel targets for antimicrobial agents. Inhibition of certain RNases is predicted to enhance the inhibitory effects of antibiotics targeting subunit formation (31, 34).

The vanadyl ribonucleoside complex (VRC) is a low molecular weight inhibitor of RNases that is commonly used during the isolation of RNA from cells (2, 28, 29). Although its target specificity is unknown, the compound is thought to target endoribonucleases (2). VRC has been shown to reduce the synthesis of both ribosomal subunits in *Staphylococcus aureus* (17). Cell viability was reduced in this organism and rRNA degradation was stimulated. The compound also enhanced the inhibitory effects of both paromomycin and azithromycin.

We hypothesized that VRC could therefore function as a novel antimicrobial agent in a gram negative organism, like *E. coli*. This work shows that VRC caused an inhibitory effect on ribosomal subunit formation in *E. coli* cells without affecting bacterial protein synthesis rates. VRC also enhanced the growth inhibitory effects of paromomycin and azithromycin in these cells. This work indicates that inhibition of RNases is a new drug target.

MATERIALS AND METHODS

Cellular growth and viability. *Escherichia coli* strain SK901 (22) cells were grown in a 37^{o}C water bath in tryptic soy broth (TSB). After 10 minutes of initial bacterial growth, 5mM vanadyl ribonucleoside complex (VRC, New England Biolabs) was added to the cells. Paromomycin or azithromycin were added to some cultures at concentrations of 5µg/mL or 10µg/mL. The cells were grown for two cellular doublings to approximately 4x10^{8} colony forming units (CFU)/mL. VRC caused a color change to the TSB media used here which made an estimate of the growth rate by turbidity changes difficult. Growth rates were measured as an increase in cell number with time after serial dilution and plating on TSB agar plates as previously described (20).

Rate of protein synthesis. Bacterial cultures were grown as above in the presence or absence of 0.5, 1, or 5mM VRC. After two cellular doublings, 1µCi/mL of ^{35}S-methionine (1175 Ci/mmol, MP Biomedicals) was added. Following the addition of the radioisotope, three 0.2mL samples were removed at 5 min intervals. Samples were precipitated in 10% TCA with 100µg of BSA, collected and washed on Whatman GF/A glass fiber filters. The filters were placed into vials containing 3mL Scintisafe gel. Radioactivity was measured by liquid scintillation counting.

Ribosomal subunit assembly. Cells were grown as described above. Following the addition of 5mM VRC at a Klett of 20, paromomycin, or azithromycin were added to the appropriate cultures. After 15 minutes of growth with the antibiotics, ^{3}H-uridine at 1µCi/mL (30 Ci/mmol, Am. Radiochemicals) and uridine at 2µg/mL were added. After the cells had grown through two doublings, 50µg/mL of uridine was added and the cells were incubated an additional 15 min. Cells were collected by centrifugation and were stored frozen at -70°C.

Cellular lysates were prepared by a lysozyme-freeze thaw method as previously described (14). The lysates were centrifuged through 5-20% sucrose gradients in S buffer in an SW 41 rotor at 187813 x *g* for 3.5 hours (31). Following centrifugation fractions were collected after pumping through an ISCO Model UA-5 absorbance monitor set at 254nm. The fractions were collected into vials and mixed with 3mL Scintisafe gel before measuring the radioactivity by liquid scintillation counting.

Uridine pulse and chase labeling. Cell cultures of 12ml were grown to a Klett of 20 and then 5mM VRC was added. Following one cellular doubling, the cells were pulse labeled with ^{3}H-uridine (1µCi/ml) for 90 sec and then chased with uridine at a concentration of 25µg/ml. At six

time intervals, 2ml samples were removed, collected by centrifugation, and stored frozen before analysis by sucrose gradient centrifugation as previously described (12, 31).

Agilent Bioanalysis of RNA. Bacterial cells were grown as described above. At a density of $4x10^8$ cells/mL, the cells were collected by centrifugation and RNA was extracted from the cell pellet using a chloroform/phenol extraction procedure as detailed by a published method (29). Total RNA was examined using an Agilent Bioanalyzer 2100 and the RNA 6000 chip. The sample preparation, loading procedure and run was carried out according to manufacturer's instructions for total RNA analysis. Generally 0.5 to 1µg of RNA was examined.

Northern blot hybridization. Biotinylated 16S and 23S specific probes were constructed by PCR as previously described (31). The 16S (241 bp) and 23S (146 bp) DNA probes were amplified from plasmid pKK3535 DNA (3) using the polymerase chain reaction with primers from Life Technologies. The 23S primers used were (23S F) TAG GGG AGC GTT CTG TAA G and (23S R) CCC ATT AAC GTT GGA C (nt. nos. 1188-1334). The 16S primers used were (16S F) GGA GGA AGG TGG GGA TGA CG and (16S R) ATG GTG ACG GGC GGT GTG (nt. nos. 1173-1414). PCR products were purified by extraction with phenol and chloroform and precipitation with 2 volumes of ethanol. The pellets were resuspended in 30µL of sterile water. The purified DNA probes were labeled with biotin using the Label-IT biotin labeling kit (Mirus) (31).

Six micrograms of total RNA was denatured by heating at 55^{o}C for 10 minutes and separated on a 5% TAE PAGE gel as previously described (29). RNA was transferred from the gel onto Nytran nylon membranes using a Turbo blot apparatus (S&S). The membranes were pre-hybridized in 15mL of 1X pre-hybridization solution at 42^{o}C for 30 minutes. The membranes

were then hybridized overnight at 42^{o}C with 6mL hybridization buffer, 1X background quencher, and 4pmol of the denatured 16S or 23S specific probe as previously described (31).

After hybridization, the membranes were washed and the probe detected via the North2South chemiluminescent hybridization kit (Pierce Chemical Co.) with strepavidin-conjugated horseradish peroxidase. Quantitative analysis of the rRNA fragmentation was determined by the G Box image analysis system (SynGene).

Statistical analysis. Statistics differences were determined by Student t-test. Each antibiotic or VRC sample was compared to the control cells without VRC or antibiotics. In each table, an asterisk indicates a statistical significance on $P<0.05$.

RESULTS

We have shown previously that 5mM VRC reduced the viability of *S. aureus* cells by over 90% and the compound also enhanced the effectiveness of two antibiotics in this microorganism (17). The effects of VRC and the same antibiotics on growth of *E. coli* cells were determined by colony counting of cell samples. *E. coli* cells grown with 5mM VRC alone showed a decrease in the viable cell counts or CFU (Table 3.1). Azithromycin alone led to an approximate 60% reduction in CFU; however, the inhibitory activities of azithromycin were enhanced in the presence of 5mM VRC. This inhibition was seen as an additive effect of the VRC. Paromomycin alone showed a significant decrease in the viable cell numbers, while the addition of VRC and paromomycin led to an increase in cell numbers comparable to the control cells without antibiotics or VRC (Table 3.1).

TABLE 3.1 VRC effects on cellular antibiotic sensitivity

	CFU ($x10^7$/mL)
Control	101±11.2
Paromomycin	8±1.0 (7.9) *****
Azithromycin	40±5.2 (39.6) *****
Control + VRC	87±7.9 (86.1)
Paromomycin + VRC	126±10.2 (124.8)
Azithromycin + VRC	18±2.4 (17.8) *****

The percentage reduction in viability compared with the control cells for each strain is listed in parenthesis. Antibiotics were used at 5µg/mL. Results are the means ± standard error of 3 independent experiments. (*) statistically significant with a *P value <0.05*

Based on the prior work in *S. aureus* (17), we tested whether the addition of VRC would lead to a decrease in ribosomal subunit amounts in *E. coli* cells. The distribution of ^{3}H-uridine labeled rRNA after sucrose gradient centrifugation was used to examine 30S and 50S subunit levels (Fig. 3.1a). Substantial reductions in the amounts of both particles were seen (Table 3.2). The addition of 5mM VRC led to a 26% reduction in 30S and a 45% reduction in 50S subunit levels (Table 3.2). In addition, a 26% increase in radio-labeled RNA was seen in the top gradient fractions, indicative of rRNA degradation (31). The increase was proportional to the loss of rRNA from both subunits (24%).

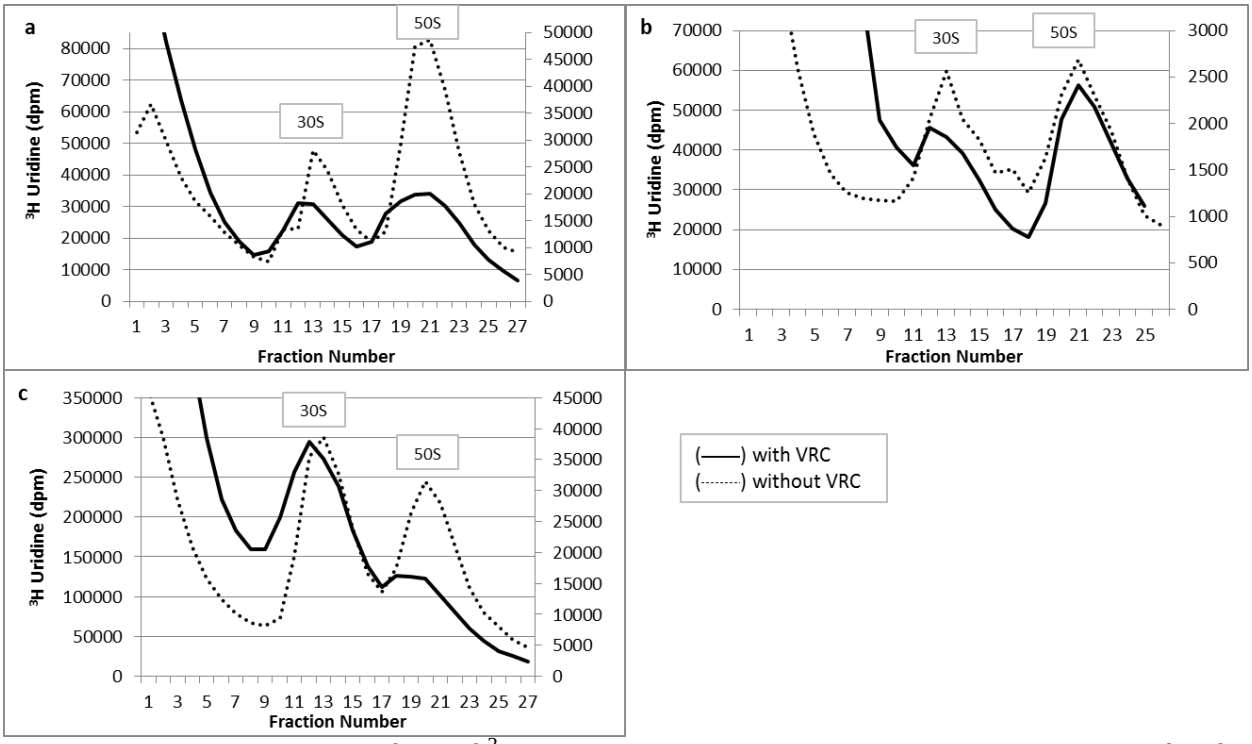

FIG 3.1 Sucrose gradient profiles of ^{3}H uridine labeled ribosomal subunits. Gradient profile for subunits from cells grown without and with VRC (a). Gradient profile for subunits from cells grown with VRC and 5µg/mL paromomycin (b). Gradient profile for subunits from cells grown with VRC and 5µg/mL azithromycin (c). Note the differences in the left (without VRC) and right (with VRC) axis.

TABLE 3.2 Distribution of ribosomal subunits in sucrose gradient regions

	% Total gradient radioactivity		
Treatment	**Top gradient region**	**30S region**	**50S region**
Control	29.09±2.6	23.83±0.7	41.19±2.5
Paromomycin	38.34±4.3 (131.8)	32.12±7.1 (134.8) *	24.92±3.7 (60.5) *
Azithromycin	42.45±7.6 (145.9)	30.74±4.1 (129.0) *	22.27±3.5 (54.1) *
Control + VRC	55.99±2.2 (192.5) *	17.84±1.5 (74.9) *	22.66±1.6 (55.0) *
Paromomycin + VRC	71.33±2.6 (245.2) *	12.29±2.6 (51.6) *	13.89±2.4 (33.7) *
Azithromycin + VRC	61.90±1.8 (212.8) *	23.4±1.40 (98.2)	10.05±0.3 (24.4) *

Cells were grown in the presence of 5µg/mL paromomycin or azithromycin and 5mM VRC. Radioactivity in gradient fractions corresponding to the top, 30S, and 50S regions was summed and calculated as a percent of the total radioactivity in the gradient. Percentage of the differences in the 30S and 50S regions in comparison to the control is shown. Results are the means ± standard error of 2 independent experiments. (*) statistically significant with a *P value <0.05*

When 5µg/mL of paromomycin or azithromycin was added to the bacterial cells, 30S and 50S subunit assembly was impaired (Fig. 3.1; Table 3.2). Degradation of rRNA was stimulated by both compounds (Fig. 3.1b, 3.1c). The addition of VRC in combination with the antibiotics led to a further reduction in 30S and 50S subunit amounts and an enhancement in rRNA degradation (Table 3.2). For example, when *E. coli* cells were incubated with 5µg/mL azithromycin there was a 129% increase in the 30S subunit assembly, a 46% reduction in the 50S subunit assembly and a 146% increase in the top of the gradient radio-labeled RNA. The increase in 30S subunit assembly could be attributed to an increase in the 50S precursor, 32S. When the *E. coli* cells were also incubated with 5mM VRC, there was a 2% reduction in 30S subunit assembly, a 76% reduction in 50S subunit assembly, and a 213% increase in the top of the gradient radio-labeled RNA compared to that of the untreated control cells. The proportional increase in RNA degradation was seen in each instance (Table 3.2). Table 3.3 shows the net reduction in the amounts of both subunits resulting from VRC inhibition alone and in combination with each antibiotic.

TABLE 3.3 Inhibition of subunit synthesis by VRC and antibiotics

	% Control amount	
Treatment	**30S**	**50S**
Control	100.0±13.3	100.0±14.6
Control + VRC	43.1± 8.1 *	31.4±5.5 *
Paromomycin + VRC	18.6±14.3 *	6.0±2.7 *
Azithromycin + VRC	47.3±15.5 *	11.1±3.5 *

Cells were grown in the presence of 5µg/mL paromomycin or azithromycin and 5mM VRC. Total radioactivity in the 30S or 50S subunit regions of the VRC treated samples was compared with radioactivity in the same gradient region of control cells. Percentage of the total 30S and 50S subunits in comparison to the control is shown. Results are the means ± standard error of 3 independent experiments. (*) statistically significant with a *P value <0.05*

The effect of VRC on subunit synthesis rates was measured by a ^{3}H-uridine pulse and chase labeling procedure. Figure 3.2 shows that the rates of 30S (Fig. 3.2a) and 50S (Fig. 3.2b) subunit formation in the absence of VRC. Assembly was complete in 60 min at 27°C (Figure 3.2). Growth with 5mM VRC lengthened the time for the rate of subunit assembly by about 24-fold, with 50% of net subunit formation completed in 120 min.

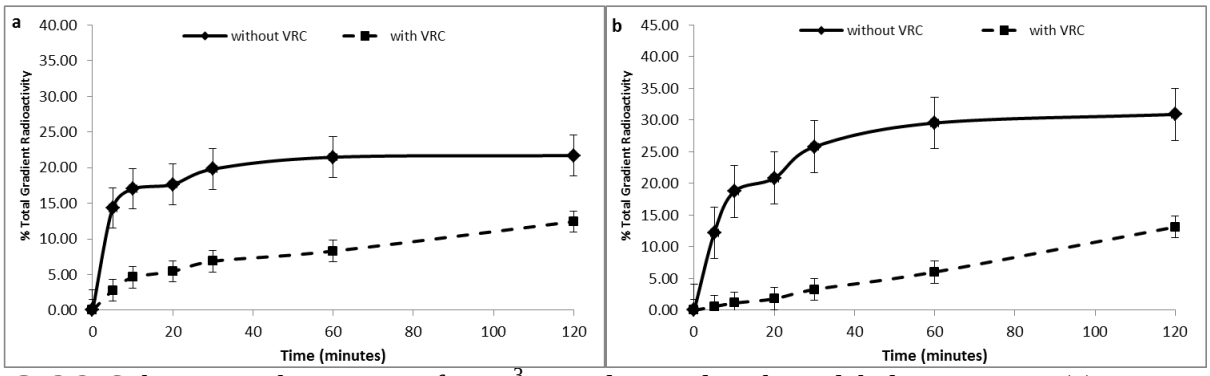

FIG 3.2 Subunit synthesis rates from ^{3}H uridine pulse-chase labeling assays. (a) Rates of formation of 30S subunits in cells growing with or without 5mM VRC. (b) Rates of formation of 50S subunits in cells growing with or without 5mM VRC. Results are the means ± standard error of 2 independent experiments.

Antibiotics which inhibit ribosomal subunit formation also affect translation (33). Protein synthesis rates in bacterial cells were measured in the presence of several concentrations of VRC to examine its effect on translation. The data in Figure 3.3 demonstrate that the rate of protein synthesis was increased slightly with increasing VRC concentrations but no inhibitory effects were seen. Combined with the decrease in 30S and 50S subunits, the protein synthesis results indicate that VRC affects the assembly of ribosomal subunits without affecting either subunit's function and further indicates that VRC inhibits rRNA processing ribonucleases.

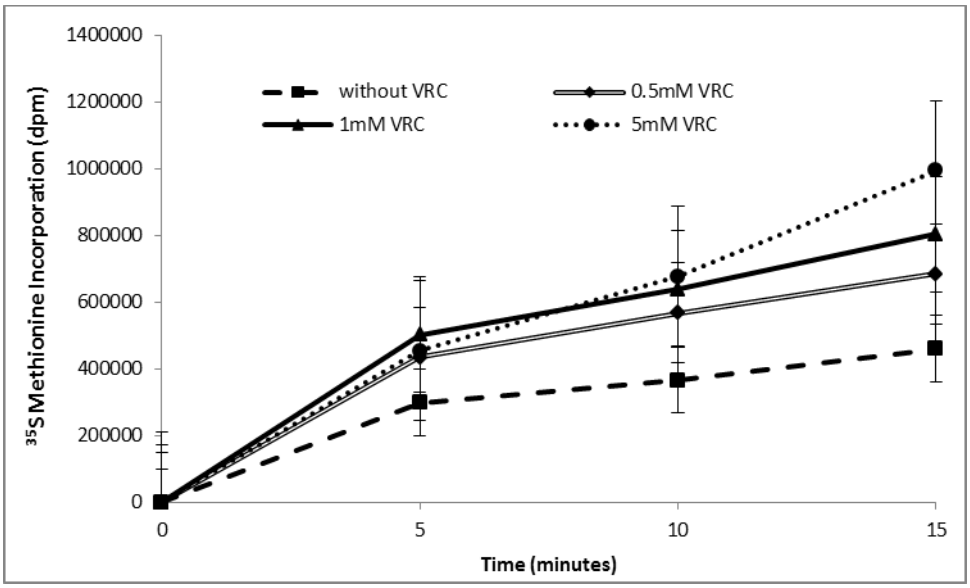

FIG 3.3 Protein synthesis rates for cells growing without and with VRC. Results are the means ± standard error of 2 independent experiments.

The effect of VRC and the two antibiotics on rRNA turnover was examined in more detail. The Agilent chip methodology was used to examine rRNA status in all cells. Figure 3.4 shows the gel pattern and Table 3.4 gives a quantitative analysis of the rRNA distribution. The amounts of both 16S and 23S rRNAs in *E. coli* were reduced by each experimental treatment compared to those in untreated control samples (Table 3.4). The treatment of antibiotics alone increased rRNA while decreasing 16S and 23S rRNA. VRC alone reduced the 16S and 23S rRNA amounts, and increased the RNA degradation observed when either antibiotic was added. Bands the approximate size of the precursor forms of 16S rRNA were apparent in each case where antibiotics were added and appeared to show more accumulation when VRC and antibiotics were present. The band representing the 16S precursor can be seen directly above the 16S rRNA band in the Agilent gel (Table 3.4).

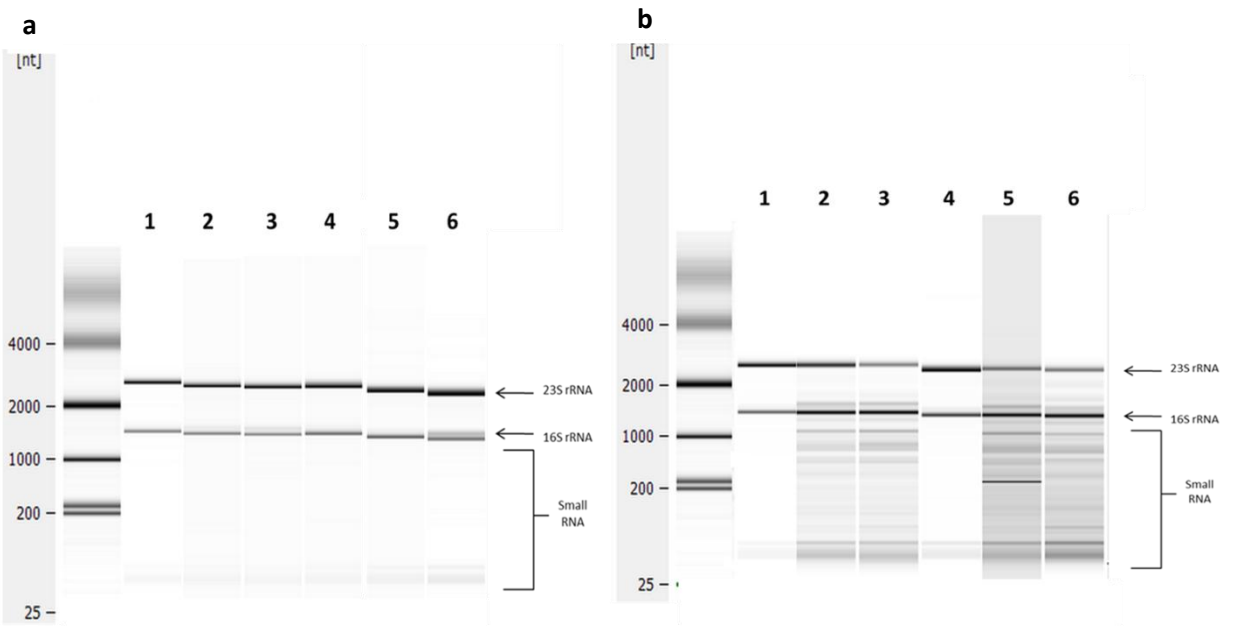

FIG 3.4 Agilent gel analysis of total RNA. RNA samples were analyzed by the Agilent Bioanalyzer. (a) RNA isolated from cells grown in the presence of 5µg/mL antibiotics. Lane 1, control cells; lane 2, cells grown with paromomycin; lane 3, cells grown with azithromycin; lane 4, cells grown with 5mM VRC; lane 5, cells grown with paromomycin and 5mM VRC; lane 6, cells grown with azithromycin and 5mM VRC. (b) RNA isolated from cells grown in the presence of 10µg/mL antibiotics. Lanes are as in (a)

TABLE 3.4 The amount and distribution of 23S, 16S, and small RNA species as determined by Agilent gel electrophoresis analysis

		% Total area		
Treatment	**RIN**	**Small RNA**	**16S rRNA**	**23S rRNA**
Control	9.2±0.7	22.63±13.1	31.47±4.5	43.73±5.7
Paromomycin	6.5±0.1 (71.2)	23.30±2.3 (103.0)	15.35±0.2 (48.8)	13.05±0.2 (29.8) *
Azithromycin	6.1±0.1 (65.8) *	27.25±3.7 (120.4)	15.10±0.1 (48.0)	7.10±0.1 (16.2) *
Control + VRC	9.6 ±0.5 (104.3)	13.53±3.0 (59.8)	24.57±2.6 (78.1)	35.93±10.1 (82.2)
Paromomycin + VRC	5.7±0.1 (62.0) *	38.55±0.2 (170.3)	12.00±0.1 (38.1) *	6.35±0.1 (14.5) *
Azithromycin + VRC	5.6±0.1 (60.9) *	38.90±2.3 (171.9)	8.65±0.2 (27.5) *	4.50±0.1 (10.3) *

Cells were grown in the presence of 10µg/mL paromomycin or azithromycin and 5mM VRC. RNA in each gel region from Figure 3.4 was computed by analysis of the electropherograms using Agilent software. Percentage of the total RNA in comparison to the control in each gel region is shown. The RNA integrity number (RIN) is indicated. Results are the means ± standard error of 2 independent experiments. (*) statistically significant with a *P value <0.05*

Northern hybridization analysis was used to examine the rRNA fragmentation with more specificity. Probes specific for 16S and 23S rRNA sequences were used to test for the degradation of each type of rRNA. The gel pattern is shown in Figure 3.5, and shows that equal amounts of RNA were loaded onto the gel. The probe hybridization results are shown in Figure 3.6, and indicate that VRC alone produced some fragmentation of 16S (Figure 3.6a). Each antibiotic stimulated 16S rRNA fragmentation as shown previously (15). However, when VRC and the antibiotics were used together, the fragmentation of both rRNA species was enhanced and there was an increase in the smaller RNA fragments (Fig. 3.6). The addition of VRC in combination with VRC led to an increase in 23S rRNA fragmentation (Figure 3.6b). These results confirm the degradation of rRNAs indicated by the gradient centrifugation and Agilent gel results.

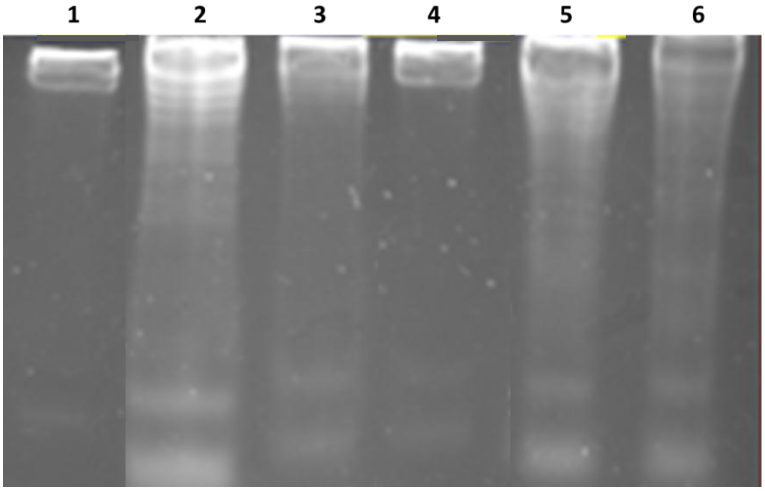

FIG 3.5 Analysis of rRNA fragmentation by gel electrophoresis. RNA samples are: lane 1, control cells; lane 2, cells grown with 10µg/mL paromomycin; lane 3, cells grown with 10µg/mL azithromycin; lane 4, cells grown with 5 mM VRC; lane 5, cells grown with 10µg/mL paromomycin and VRC; lane 6, cells grown with 10µg/mL azithromycin and VRC.

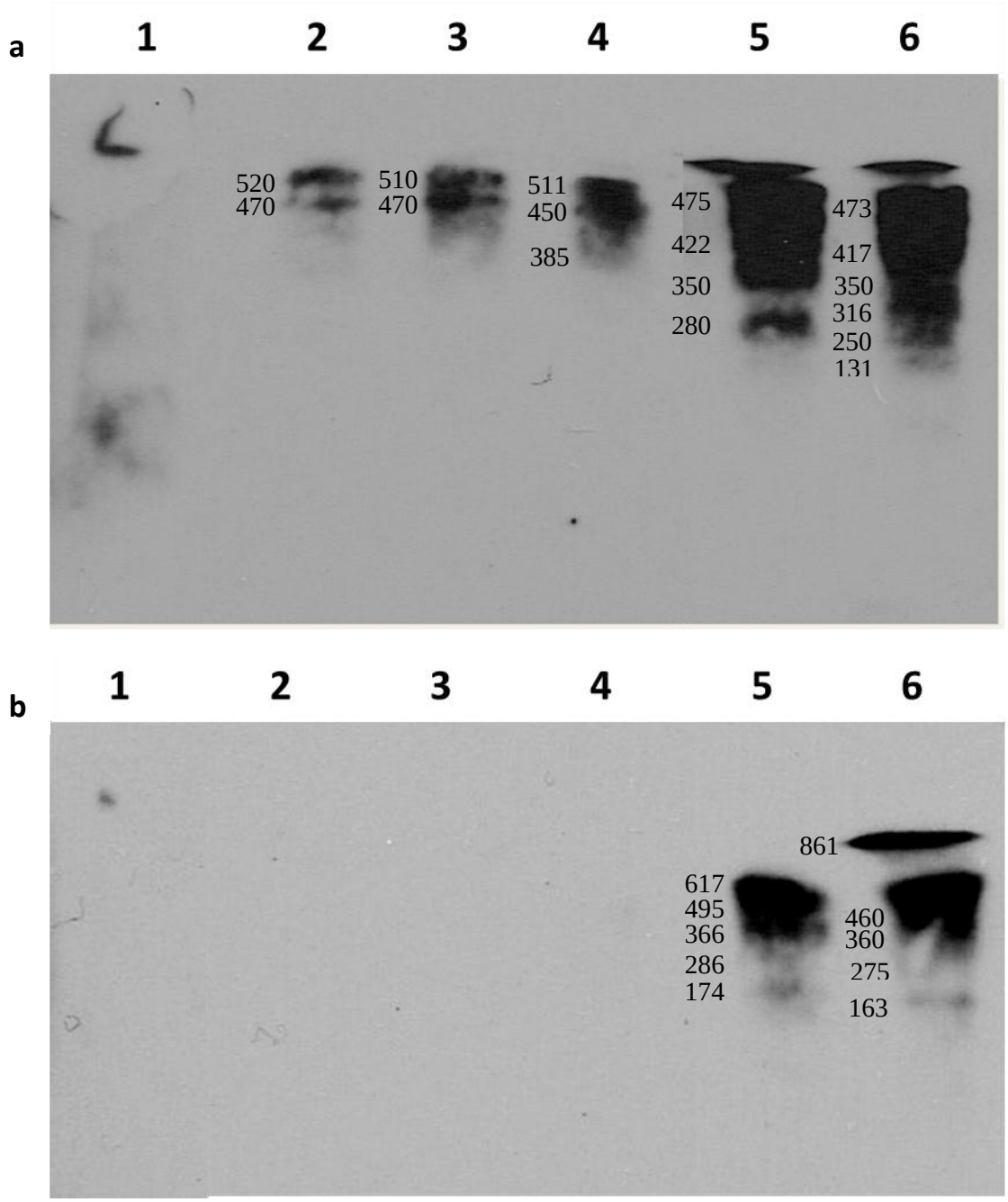

FIG 3.6 Northern hybridization analysis of rRNA fragmentation. RNA was isolated from cells grown in the presence of 10µg/mL antibiotics and/or 5mM VRC. (a) 16S rRNA hybridization. The samples are lane 1, control cells; lane 2, cells grown with paromomycin; lane 3, cells grown with azithromycin; lane 4, cells grown with 5 mM VRC; lane 5, cells grown with paromomycin and VRC; lane 6, cells grown with azithromycin and VRC. (b) 23S rRNA hybridization. Lanes as in (a). Position of estimated sizes of the rRNA fragments are indicated.

DISCUSSION

Bacterial ribosomes are essential structures in all prokaryotic cells. Expression of genetic information relies on accurate and rapid translation of mRNA sequences. A very large number of antimicrobial agents target the translation process causing either bacterial stasis or bacteriocidal effects (9, 24). Ribosomal subunit formation is an essential process for bacterial cell growth and viability and is also a target for many of these same translational inhibitors (9).

The present work builds on several of our previous findings regarding various aspects of antibiotic inhibition of bacterial ribosome formation (6, 8, 13, 15, 25, 26, 34). A large number of different translational inhibitors can stall ribosomal subunit formation in growing cells (10-12). Mutant strains of *E. coli* deficient in specific RNases showed an enhanced sensitivity to both macrolide (31, 34) and aminoglycoside (16) antibiotics, and subunit formation was impaired in each case while rRNA degradation was stimulated. In addition, treatment of *S. aureus* with an inhibitor of RNases, VRC, promoted similar effects, slowing subunit formation and enhancing rRNA turnover (17). The present work extends the evidence showing that impairment of subunit formation by mutation or small molecule inhibitors is detrimental to cell growth. It is hypothesized that the increase in viable cells when paromomycin and VRC are present is due to an effect on messenger RNA turnover (21, 27). This is because VRC is efficient at decreasing ribonucleases and ribonucleases have been shown to be important in mRNA turnover (2, 5). One surprising observation was increased viability of *E. coli* cells treated with paromomycin and VRC (Table 3.1). At this time, we have no explanation for the increase in cell viability in these cells since they have also been shown to have a significant decrease in subunit assembly and an increase in 16S and 23S rRNA fragmentation.

The specific ribonuclease(s) inhibited by VRC in these cells are unknown. RNase III is one likely target since the formation of both subunits was impaired to the same extent. This enzyme generates both precursor 16S and 23S rRNAs from the primary transcript (37) and is a endoribonuclease, the likely substrate for VRC (2). Other processing endo-RNases like RNase E, G, R or PH could also be affected by VRC (16). VRC may certainly have other effects on tRNA or mRNA metabolism in cells in addition to the inhibitory effect on subunit formation. These remain to be investigated.

A separation of the inhibition of subunit formation and subunit function in translation is a finding from this research. A large reduction in the rate of synthesis of both subunits was revealed without a concomitant reduction in translation rates. Preexisting ribosomes in VRC treated cells seem capable of normal rates of protein synthesis. This data further indicates that VRC is reducing the rate of ribosomal subunit formation without affecting the function of the bacterial ribosome. Due to the lack of inhibition of translation (Figure 3.3) the effects of VRC are more than likely due to assembly inhibition and not an effect on translation.

The results described here can be interpreted as follows. Either of the two antibiotics is capable of binding to a subunit precursor particle and stalling subunit maturation, as we have previously demonstrated (15, 25). VRC alone likely impairs the processing of rRNA precursor sequences, a process essential for subunit maturation as well (17). Combining VRC with an antibiotic will generate a stalled subunit precursor in a cell with a reduced rRNA processing capability, thus enhancing the effectiveness of the antimicrobial agent.

Inhibition of rRNA processing RNases indicates a new antimicrobial target in cells. A search for other small molecule inhibitors of RNase activity or the application of current RNA interference methods may promote the development of more effective current or novel antimicrobial therapies.

ACKNOWLEDGEMENTS

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REFERENCES

1. **Anderson, R. M.** 1999. The pandemic of antibiotic resistance. Nat Med **5:**147-149.
2. **Berger, S. L.** 1987. Isolation of cytoplasmic RNA: ribonucleoside-vanadyl complexes. Methods Enzymol **152:**227-234.
3. **Brosius, J., T. J. Dull, and H. F. Noller.** 1980. Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli*. Proc Natl Acad Sci U S A **77:**201-204.
4. **Cars, O., A. Hedin, and A. Heddini.** 2011. The global need for effective antibiotics-moving towards concerted action. Drug Resist Updat **14:**68-69.
5. **Carter, A. P., W. M. Clemons, D. E. Brodersen, R. J. Morgan-Warren, B. T. Wimberly, and V. Ramakrishnan.** 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature **407:**340-348.
6. **Champney, W. S.** 2003. Bacterial ribosomal subunit assembly is an antibiotic target. Curr Top Med Chem **3:**929-947.
7. **Champney, W. S.** 2001. Bacterial ribosomal subunit synthesis: a novel antibiotic target. Curr Drug Targets Infect Disord **1:**19-36.
8. **Champney, W. S. (ed.).** 2008. New Antibiotic Targets. Humana Press Inc., Totowa, New Jersey 65-67.
9. **Champney, W. S.** 2006. The other target for ribosomal antibiotics: inhibition of bacterial ribosomal subunit formation. Infect Disord Drug Targets **6:**377-390.
10. **Champney, W. S., and R. Burdine.** 1996. 50S ribosomal subunit synthesis and translation are equivalent targets for erythromycin inhibition in *Staphylococcus aureus*. Antimicrob Agents Chemother **40:**1301-1303.
11. **Champney, W. S., and R. Burdine.** 1998. Macrolide antibiotic inhibition of translation and 50S ribosomal subunit assembly in methicillin-resistant *Staphylococcus aureus* cells. Microb Drug Resist **4:**169-174.
12. **Champney, W. S., and W. K. Rodgers.** 2007. Retapamulin inhibition of translation and 50S ribosomal subunit formation in *Staphylococcus aureus* cells. Antimicrob Agents Chemother **51:**3385-3387.
13. **Champney, W. S., and C. L. Tober.** 2000. Specific inhibition of 50S ribosomal subunit formation in *Staphylococcus aureus* cells by 16-membered macrolide, lincosamide, and streptogramin B antibiotics. Curr Microbiol **41:**126-135.
14. **Chittum, H. S., and W. S. Champney.** 1995. Erythromycin inhibits the assembly of the large ribosomal subunit in growing *Escherichia coli* cells. Curr Microbiol **30:**273-279.
15. **Foster, C., and W. S. Champney.** 2008. Characterization of a 30S ribosomal subunit assembly intermediate found in *Escherichia coli* cells growing with neomycin or paromomycin. Arch Microbiol **189:**441-449.
16. **Frazier, A. D., and W. S. Champney.** 2012. Inhibition of ribosomal subunit synthesis in aminoglycoside treated ribonuclease mutants of *Escherichia coli*. Archives of Microbiology**:**In Submission.
17. **Frazier, A. D., and W. S. Champney.** 2012. The vanadyl ribonucleoside complex inhibits ribosomal subunit formation in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy**:**In Submission.
18. **Gutgsell, N. S., and C. Jain.** 2012. Role of precursor sequences in the ordered maturation of *E. coli* 23S ribosomal RNA. RNA **18:**345-353.

19. **Högberg, L. D., A. Heddini, and O. Cars.** 2010. The global need for effective antibiotics: challenges and recent advances. Trends Pharmacol Sci **31:**509-515.
20. **Jett, B. D., K. L. Hatter, M. M. Huycke, and M. S. Gilmore.** 1997. Simplified agar plate method for quantifying viable bacteria. Biotechniques **23:**648-650.
21. **Kennell, D.** 2002. Processing endoribonucleases and mRNA degradation in bacteria. J Bacteriol **184:**4645-4657; discussion 4665.
22. **Kushner, S. R., V. F. Maples, and W. S. Champney.** 1977. Conditionally lethal ribosomal protein mutants: characterization of a locus required for modification of 50S subunit proteins. Proc Natl Acad Sci U S A **74:**467-471.
23. **Li, Z., S. Pandit, and M. P. Deutscher.** 1999. RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. EMBO J **18:**2878-2885.
24. **McCoy, L. S., Y. Xie, and Y. Tor.** 2011. Antibiotics that target protein synthesis. Wiley Interdiscip Rev RNA **2:**209-232.
25. **Mehta, R., and W. S. Champney.** 2002. 30S ribosomal subunit assembly is a target for inhibition by aminoglycosides in *Escherichia coli*. Antimicrob Agents Chemother **46:**1546-1549.
26. **Mehta, R., and W. S. Champney.** 2003. Neomycin and paromomycin inhibit 30S ribosomal subunit assembly in *Staphylococcus aureus*. Curr Microbiol **47:**237-243.
27. **Mohanty, B. K., and S. R. Kushner.** 2003. Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. Mol Microbiol **50:**645-658.
28. **Reddy, K. J., and M. Gilman.** 2001. Preparation of bacterial RNA. Curr Protoc Mol Biol **Chapter 4:**Unit4.4.
29. **Rio, D. C., M. Ares, Jr., G. J. Hannon, and T. W. Nilsen.** 2011. RNA: A Laboratory Manual. Cold Spring Harbor Protocols.
30. **Rosen, T.** 2011. Antibiotic resistance: an editorial review with recommendations. J Drugs Dermatol **10:**724-733.
31. **Silvers, J. A., and W. S. Champney.** 2005. Accumulation and turnover of 23S ribosomal RNA in azithromycin-inhibited ribonuclease mutant strains of *Escherichia coli*. Arch Microbiol **184:**66-77.
32. **Song, W. S., M. Lee, and K. Lee.** 2011. RNase G participates in processing of the 5'-end of 23S ribosomal RNA. J Microbiol **49:**508-511.
33. **Tenson, T., and A. Mankin.** 2006. Antibiotics and the ribosome. Mol Microbiol **59:**1664-1677.
34. **Usary, J., and W. S. Champney.** 2001. Erythromycin inhibition of 50S ribosomal subunit formation in *Escherichia coli* cells. Mol Microbiol **40:**951-962.
35. **Wachi, M., G. Umitsuki, M. Shimizu, A. Takada, and K. Nagai.** 1999. *Escherichia coli* cafA gene encodes a novel RNase, designated as RNase G, involved in processing of the 5' end of 16S rRNA. Biochem Biophys Res Commun **259:**483-488.
36. **Wallis, M. G., and R. Schroeder.** 1997. The binding of antibiotics to RNA. Prog Biophys Mol Biol **67:**141-154.
37. **Xiao, J., C. E. Feehery, G. Tzertzinis, and C. V. Maina.** 2009. *E. coli* RNase III(E38A) generates discrete-sized products from long dsRNA. RNA **15:**984-991.

CHAPTER 4

The vanadyl ribonucleoside complex inhibits ribosomal subunit formation in *Staphylococcus aureus*

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Keywords: vanadyl ribonucleoside complex; *Staph. aureus;* ribosome formation; protein synthesis; macrolides; aminoglycosides

ABSTRACT

Objectives: The discovery of new antibiotic targets is important to stem the increase in antibiotic resistance against most currently used antimicrobials. The bacterial ribosome is a major target for a large number of antibiotics which inhibit different aspects of translation. Some of these antimicrobial agents also inhibit ribosomal subunit formation as a second cellular target. Precise subunit assembly requires the activity of several distinct RNases for proper rRNA processing. The present work shows that the vanadyl ribonucleoside complex (VRC) inhibited RNases involved in ribosomal subunit formation without an effect on translation.

Materials and methods: Methicillin sensitive and methicillin resistant strains of *Staphylococcus aureus* were examined for the inhibitory effects of VRC on cell viability by colony counting. Protein synthesis rates were measured by isotopic methionine incorporation. Ribosome synthesis was measured by isotopic uridine incorporation into ribosomal subunits as displayed on sucrose gradients. Pulse and chase radiolabeling was used to measure subunit synthesis rates. RNA turnover was determined by Agilent assay.

Results: The rates and amounts of subunit synthesis were significantly reduced in the presence of the compound. Ribosomal RNA was degraded and cell viability was reduced as a consequence. VRC also enhanced the inhibitory effects of macrolide and aminoglycoside antibiotics on ribosome formation.

Conclusions: Bacterial ribosomal subunit synthesis was specifically impaired in VRC treated cells with the rates and amounts of both subunits reduced. Cell viability was significantly reduced and rRNA turnover was stimulated.

Introduction

Antibiotics play an important role in global health. However, antibiotic resistance is becoming an increasing problem world-wide [1]. The discovery of novel drug targets and new types of antimicrobial agents is necessary to control infections caused by resistant microorganisms [2].

A major cellular target for various, currently used antimicrobial agents is the bacterial ribosome. Many steps in the complex process of translation can be inhibited by different compounds. Information on the specific inhibitory effects of many translational inhibitors has been reviewed recently [3]. The biogenesis of the large and small ribosomal subunit in bacteria is a second target for many of these same antibiotics [4].

Bacterial ribosomal subunit assembly begins with specific ribosomal proteins adding to the nascent 16S, 23S, and 5S ribosomal RNA transcripts. For both 30S and 50S synthesis, an intermediate precursor particle is formed initially. These particles contain a subset of the total ribosomal proteins found in the mature subunit and precursor forms of the 16S and 23S rRNA [5]. Endo- and exonucleolytic cleavage of the precursor rRNAs by ribonucleases is essential for generation of the mature subunits [6]. Mutant strains of *E. coli* deficient in specific RNases are hypersensitive to azithromycin [7] and to aminoglycoside antibiotics (A. F., unpublished). Maturation of the precursors into mature subunits is delayed in these mutants and an increase in the degradation of rRNA can be observed [7]. Antibiotics targeting subunit assembly inhibition has been investigated [8]; however, the use of RNase inhibition to potentiate the effects of currently used antibiotics has not been examined.

The vanadyl ribonucleoside complex (VRC) is a low molecular weight inhibitor of RNases that has been used during the isolation of RNA from cells [9-11]. It is an effective inhibitor of cellular RNases although its target specificity is unknown. We reasoned that VRC could target

and inhibit RNases involved in ribosomal subunit assembly and thus function as a novel antimicrobial agent. This work shows that VRC can specifically inhibit ribosomal subunit formation in both methicillin sensitive (MSSA) and methicillin resistant (MRSA) *S. aureus* cells without an inhibitory effect on protein synthesis. VRC was found to enhance the inhibitory effects of paromomycin and azithromycin in these organisms. The results indicate that RNases may be a novel antibiotic target.

Material and Methods

Cellular growth and viability

Methicillin sensitive *Staphylococcus aureus* RN1786 [12] and the methicillin resistant strain A1024 [13] were grown at 37^{o}C in tryptic soy broth (TSB). After one hour of initial bacterial growth, 5mM VRC (New England BioLabs) was added to the cells. For some experiments, paromomycin or azithromycin were added at a concentration of 1.5µg/mL or 5µg/mL respectively. Growth rates were measured as an increase in cellular density over time using a Klett-Summerson colorimeter as previously described [14]. Cellular viability was determined by colony counting on TSB agar plates after serial dilution as described [15].

Protein synthesis assay

Cells were grown as described above in the presence or absence of 5mM VRC. After two cellular doublings, 1µCi/mL of ^{35}S-methionine (1175 Ci/mmol, MP Biomedicals) was added. Following the addition of the isotope, three 0.2mL samples were removed at 5 minute intervals. Each sample was precipitated in 10% TCA with 100µg/ml of BSA, collected and washed on

Whatman GF/A glass fiber filters. The filters were placed into vials containing 3mL Scintisafe gel. ^{35}S- Methionine radioactivity was measured by liquid scintillation counting.

Uridine pulse and chase labeling

Two 12ml cultures of cells, one control and one with VRC at 5mM were grown to a Klett of 40. The cells were pulse labeled with 1µCi/ml ^{3}H-uridine (30 Ci/mmol, Am. Radiochemicals) for 90 sec and then chased with uridine at 25µg/ml. At intervals, 2ml samples were removed, collected by centrifugation, washed and stored frozen before lysis for sucrose gradient centrifugation as described [7, 16].

Ribosomal subunit assembly

Bacterial cell cultures were grown in TSB as described. Following the addition of VRC at a Klett of 20, paromomycin or azithromycin was added to the appropriate culture. After 15 minutes of growth with the antibiotics, ^{3}H uridine at a concentration of 1µCi/mL and uridine at a concentration of 2µg/mL were added. The cells were allowed to grow for two cellular doublings. At that time, uridine was added to 50µg/mL and the cells were incubated an additional 15 minutes. Cells were collected by centrifugation and stored frozen at -70^{o}C.

Cellular lysates were prepared with lysostaphin, DNase I and Trition X100 as previously described [16]. The samples were centrifuged through 5-20% sucrose gradients in S buffer in an SW41 rotor at 187813 x *g* for 3.5 hours [7]. Following centrifugation, sample fractions were collected by pumping them through an ISCO Model UA-5 absorbance monitor set at 254nm. The fractions were collected into vials and mixed with 3mL Scintisafe gel before measuring the ^{3}H uridine by liquid scintillation counting.

Agilent bioanalysis of RNA

Bacterial cells were grown as previously described above. At a density of $4x10^8$ cells/mL, the cells were collected by centrifugation and RNA was extracted from the cell pellet. Total RNA was isolated by a spin column procedure with the RNA isolation kit from Norgen (Norgen Biotek Corp). RNA was examined using an Agilent Bioanalyzer 2100 and the RNA 6000 chip. Typically 0.5 to 1µg of RNA was analyzed via chip analysis conducted according to the manufacturer's instructions for total RNA analysis.

Eukaryotic cell growth

Growth of eukaryotic cells in tissue culture was performed as follows: 20,000 macrophage (RAW 264.7 cells; [17]) or 10,000 fibroblast cells (BJ cells; ATCC) were seeded into a 96-well plates with 200µL supplemented RPMI 1640 or DMEM media respectively. Both macrophage and fibroblasts were grown at 37°C in a 5% CO_2/95% air humidified atmosphere. After two or twelve hours of initial growth respectively, 0.5, 1, or 5mM VRC were added to the appropriate wells. After an additional two hours of growth for each cell type, 10µg/mL of paromomycin or azithromycin was added to the wells. The cells were allowed to grow for approximately 32 additional hours before performing a Cell Titer 96® AQueous one solution cell proliferation assay as per manufacturer's instructions (Promega).

Statistical analysis

Statistical differences were determined by Student t-test. Each antibiotic or VRC sample was compared to the control cells without VRC or antibiotics. In each table, an asterisk indicates a statistical significance of $P<0.05$.

Results

The VRC was initially tested to see if it had an inhibitory effect on cell growth and viability. In both methicillin sensitive and methicillin resistant *S. aureus* cells, the addition of VRC decreased cell viability (Table 4.1). When 5mM VRC was used, cell numbers were decreased by over 90% in both strains. The addition of paromomycin alone led to a 92% and 18% reduction in cell viability for MSSA and MRSA cells respectively, while azithromycin led to a 92% and 95% decrease. VRC also enhanced the inhibitory effects of paromomycin and azithromycin on cell viability. The addition of VRC led to an increase in the efficiency of the antibiotics as seen by a statistically significant decrease in total viable cell counts compared with the effects of the antibiotics alone (Table 4.1).

Table 4.1. Effect of VRC and antibiotics on cell viability of *S. aureus* cells

Strain	Inhibitor	Cell viability ($x10^7$/mL)
Methicillin sensitive *S. aureus* (MSSA)	none	209±66.7
MSSA	paromomycin	17±16.5 (8.1) *
MSSA	azithromycin	16±5.4 (7.7) *
MSSA	VRC	11±5.4 (5.3) *
MSSA	paromomycin + VRC	8±7.8 (3.8) *
MSSA	azithromycin + VRC	2±0.4 (0.96) *
Methicillin resistant *S. aureus* (MRSA)	none	306±85.9
MRSA	paromomycin	252±15.5 (82.4)
MRSA	azithromycin	17±6.2 (5.5) *
MRSA	VRC	5±1.3 (1.6) *
MRSA	paromomycin + VRC	11±5.9 (3.6) *
MRSA	azithromycin + VRC	1±0.6 (0.32) *

The percentage reduction in viability compared with the control cells for each strain is listed in parenthesis. Results are the means ± standard error of 5 independent experiments. (*) statistically significant with $P<0.05$

Ribonucleases are essential for both ribosomal subunit assembly and for turnover of mRNA after translation. The inhibitory effect of VRC on translation and subunit formation was examined separately. The incorporation of ^{35}S-methionine into total cellular proteins was examined in both strains in the absence and presence of VRC. As Figure 4.1 shows, the addition of VRC led to a slight increase in protein synthesis rates in both methicillin sensitive (MSSA) and methicillin resistant (MRSA) *S. aureus* cells; however, no significant inhibitory effect on the rate of translation was observed.

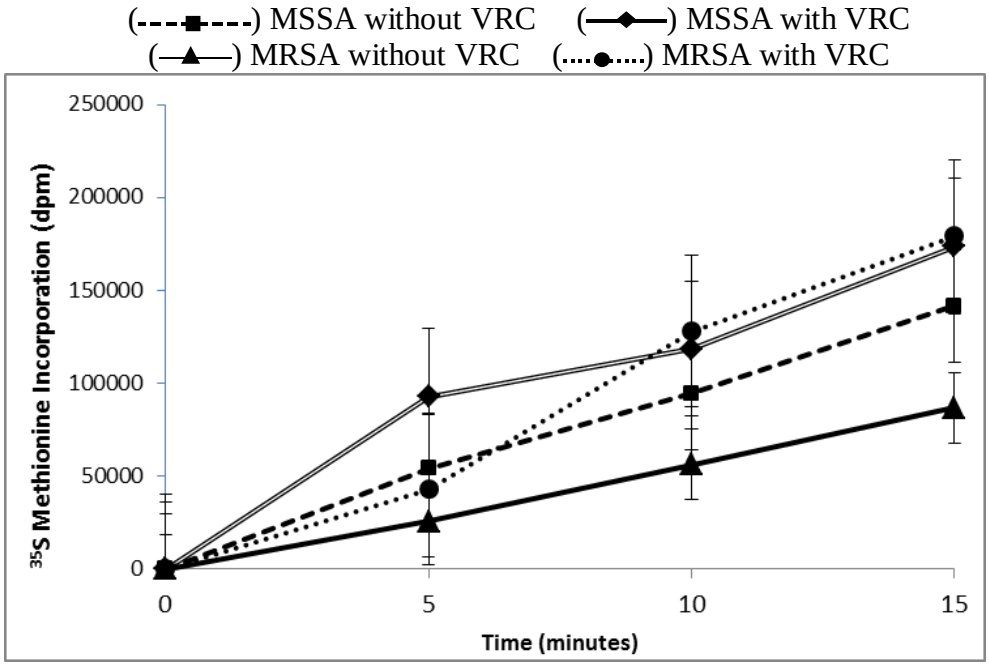

Figure 4.1. Protein synthesis rates for MSSA cells and MRSA cells grown without or with 5mM VRC. Results are the means ± standard error of 3 independent experiments.

Ribosomal subunit assembly was examined in both strains by ^{3}H uridine labeling during growth in the absence and presence of 5mM VRC. The subunits were separated by sucrose density gradient centrifugation to show the distribution of ^{3}H uridine into the ribosomal subunits.

Figure 4.2a and 4.2b show the sucrose gradient profiles for the labeled subunits. In both microorganisms there was an overall 90% reduction in the formation of both subunits in the VRC treated samples when compared with the control. The data showed a 15 to 25% increase in ^{3}H uridine labeled RNA in the slowly sedimenting top gradient fractions (Figure 4.2; Table 4.2). This result is indicative of rRNA degradation [7].

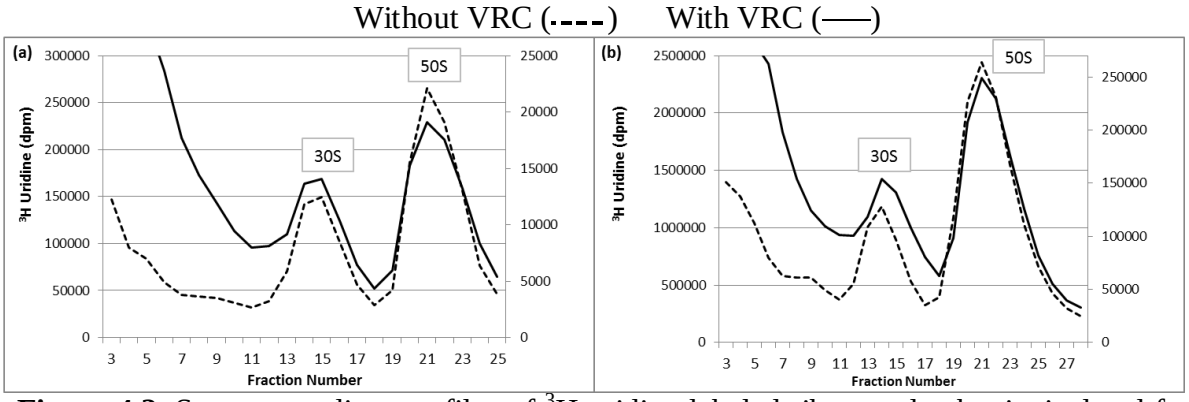

Figure 4.2. Sucrose gradient profiles of ^{3}H uridine labeled ribosomal subunits isolated from cells grown without or with 5mM VRC. (a) Gradient profile for MSSA cells. (b) Gradient profile for MRSA cells. Note the differences in the left (without VRC) and right (with VRC) axis.

Table 4.2. Distribution of ^{3}H uridine labeled RNA in sucrose gradient regions

Strain	**% Total gradient radioactivity[a]**		
	Top	**30S**	**50S**
Methicillin sensitive *S. aureus* Control	33.17±1.0	21.68±0.1	41.58±0.9
Methicillin sensitive *S. aureus* + VRC	57.03±1.8 (171.9) *	14.65±0.8 (67.6) *	24.57±0.8 (59.1) *
Methicillin resistant *S. aureus* Control	30.72±1.5	17.47±2.2	47.88±0.7
Methicillin resistant *S. aureus* + VRC	44.67±1.6 (145.4) *	17.68±0.3 (101.2)	32.98±2.0 (68.9) *

[a] Radioactivity in gradient fractions corresponding to the top, 30S and 50S regions was calculated as a percent of the total radioactivity in the gradient. Results are the means ± standard error of 3 independent experiments. (*) statistically significant with a *P value <0.05*

The influence of VRC on the rate of ribosomal subunit formation was examined by a ^{3}H uridine pulse and chase labeling procedure. The rates of subunit formation in the absence of the inhibitor were equivalent to those observed previously in *S. aureus* [16, 18] (Figure 4.3). Synthesis of both subunits was completed by 60 minutes at 27°C. Compared with control cultures, VRC reduced the rate of formation of both subunits in these organisms. The 50S subunit assembly rate was lengthened 4-fold under these conditions (Figure 4.3).

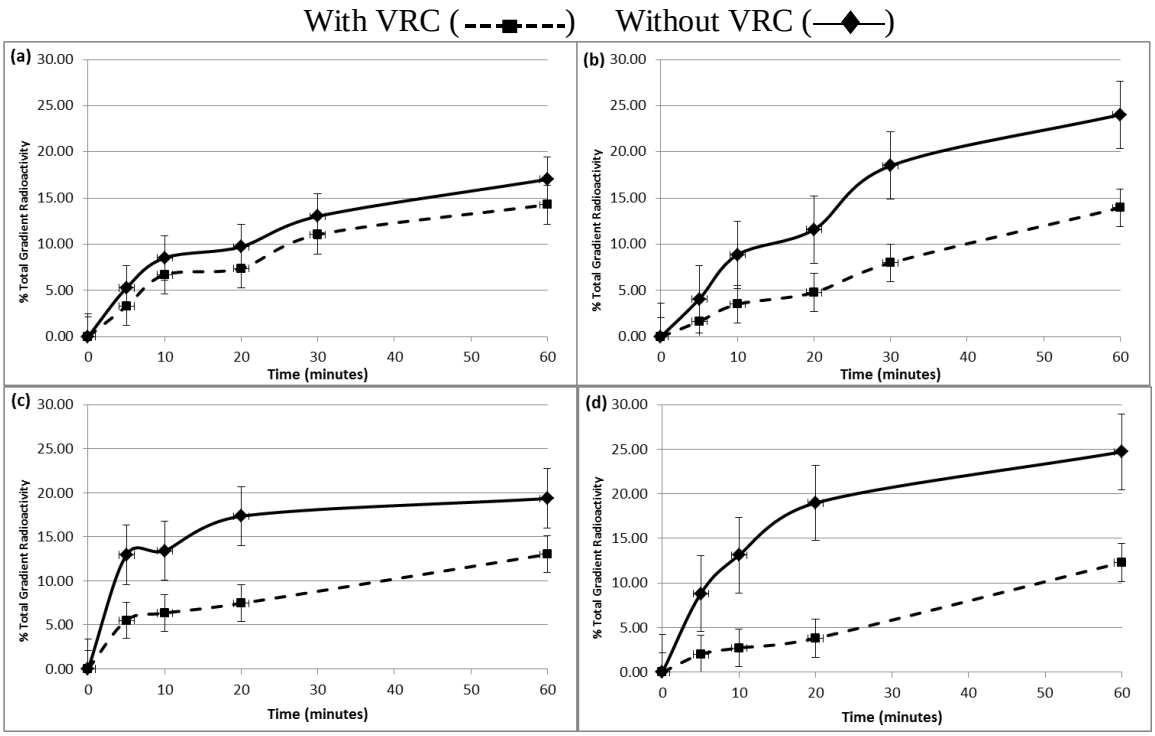

Figure 4.3. Kinetics of ribosomal subunit formation in cells growing with and without 5mM VRC. (a) Rates of formation of 30S subunits in MSSA cells. (b) Formation of 50S subunits in MSSA cells. (c) Rates of formation of 30S subunits in MRSA cells. (d) Formation of 50S subunits in MRSA cells. Results are the means ± standard error of 2 independent experiments.

The status of rRNA in control and VRC treated cells was examined by the Agilent Bioanalyzer analysis. Significant decreases in the relative amounts of both16S and 23S rRNA were observed in VRC treated cells of both strains (Figure 4.4). Bands representing the

approximate size of the precursor forms of both rRNA species could be seen directly above the 16S and 23S rRNA bands. Growth with both VRC and antibiotics led to an increase in fragmented rRNA in these cells (Table 4.3). In both strains, the addition of VRC alone led to a significant 2 to 3- fold increase in smaller RNA molecules. The addition of VRC and paromomycin or azithromycin also led to a significant increase in small RNA molecules in MRSA cells (Table 4.3).

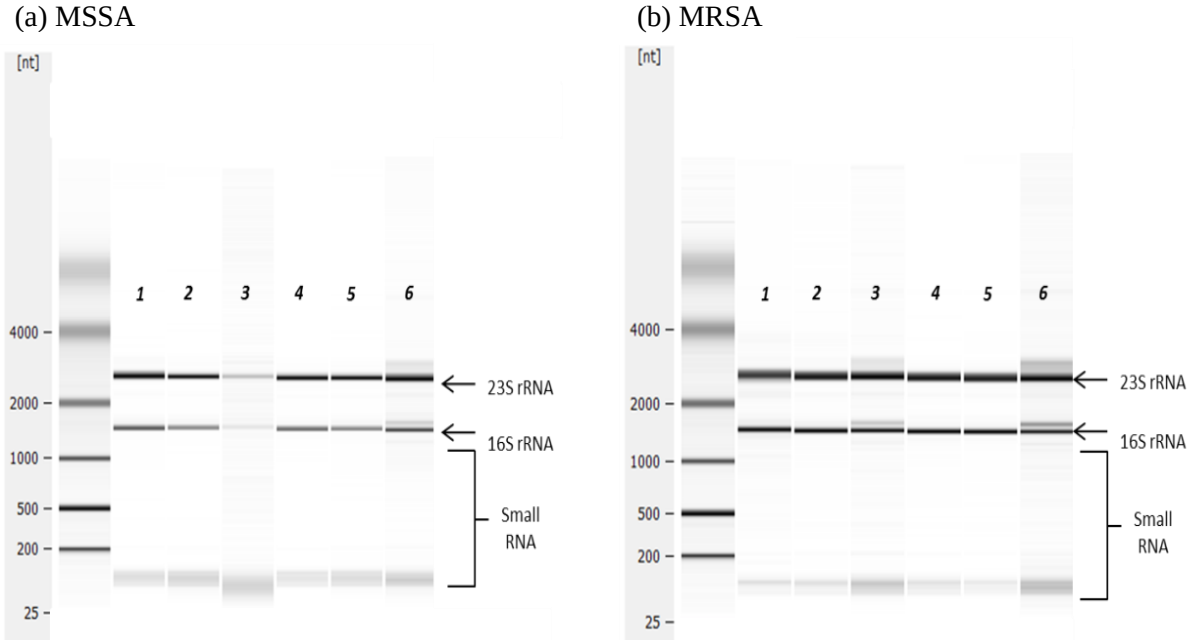

Figure 4.4. RNA samples analyzed by the Agilent Bioanalyzer. Total RNA was isolated from MSSA and MRSA cells grown without and with VRC and antibiotics. (a) Agilent chip analysis of RNA from MSSA cells. The RNA samples are: lane 1, control cells; lane 2, cells grown with paromomycin; lane 3, cells grown with azithromycin; lane 4, cells grown with 5mM VRC; lane 5, cells grown with paromomycin and 5mM VRC; lane 6, cells grown with azithromycin and 5mM VRC. (b) Agilent chip analysis of RNA from MRSA cells. Sample lanes are the same as in (a).

Table 4.3. Distribution of small RNA species after Agilent gel separation

Strain	Inhibitor	% Total area[a]
Methicillin sensitive *S. aureus* (MSSA)	none	15.60±0.6
MSSA	paromomycin	28.90±0.005 (185.3) *
MSSA	azithromycin	41.10±3.2 (263.5) *
MSSA	VRC	19.20±1.0 (123.1)
MSSA	paromomycin + VRC	19.70±0.2 (126.3) *
MSSA	azithromycin + VRC	26.70±0.8 (171.2) *
Methicillin resistant *S. aureus* (MRSA)	None	8.80±0.4
MRSA	paromomycin	14.35±0.5 (163.1)
MRSA	azithromycin	25.05±2.0 (284.7) *
MRSA	VRC	14.80±0.4 (168.2) *
MRSA	paromomycin + VRC	11.80±1.0 (134.1)
MRSA	azithromycin + VRC	31.10±0.1 (353.4) *

[a] Total RNA in gel bands smaller than 16S rRNA was computed by analysis of the electropherograms using Agilent software. Results are the means ± standard error of 2 independent experiments. (*) statistically significant with a *P value <0.05*

In order to determine if VRC could be used as an antimicrobial agent in eukaryotic cells, the compound and antibiotics were applied to fibroblasts and to macrophage cells in culture. When antibiotics were added to the eukaryotic cells, there was no significant change in overall cellular viability. However, when 1mM or 5mM VRC was added to the eukaryotic cell cultures, there was a 90% and 98% reduction in cell viability. This led to the conclusion that VRC could not be used to enhance antibiotic effectiveness in eukaryotic cells.

Discussion

The bacterial ribosome is an important target for current antibiotic treatments [3]. These antimicrobial agents target both translation and ribosomal subunit formation [4]. For ribosomal subunit biogenesis, a number of different endo-and exoribonucleases are required. These include RNases III, E, G, T and PH [6]. This processed RNA is used to form the subunit precursors and

without an inhibitor present, the precursors mature to generate the 30S and 50S ribosomal subunits respectively [5].

As previously stated, other endo- and exoribonucleases are required to complete the processing of 16S, 23S, and 5S rRNA. This processed RNA is used to form the 21S and 32S subunit precursors and without an inhibitor present, the precursors mature to form the 30S and 50S ribosomal subunits respectively [5]. When an inhibitor, such as an antibiotic, is present the precursor RNA is broken down. Ribonucleases are used by the cell to degrade the subunit precursors and their rRNA into nucleotides to be recycled [4, 5, 19]. RNase activity is therefore an important mechanism for both mRNA turnover and rRNA processing.

Our previous research has shown that *E. coli* strains deficient in RNase E, RNase II or polynucleotide phosphorylase (PNPase) were hypersensitive to azithromycin [7]. These mutants showed an enhanced accumulation of 23S rRNA, a stimulation of rRNA breakdown and a reduced recovery rate of 50S ribosomal synthesis after azithromycin removal. These results suggested that the use of an RNase inhibitor in bacterial cells could enhance the efficiency of current antibiotics. These mutant strains are increased in sensitivity to the aminoglycoside antibiotics, neomycin and paromomycin, while 16S rRNA turnover is stimulated in some of these mutant strains as well (A. F., unpublished).

The present results can be interpreted to suggest that the antibiotics alone stall subunit formation with precursor particle accumulation [20, 21]. VRC alone inhibits the activity of critical rRNA processing RNases. Either antibiotic with VRC causes the accumulation of a subunit precursor and the inhibition of rRNA processing, leading to an enhanced inhibitory effect on subunit formation and a further reduction in cell viability. The relatively high concentration of VRC used here (5mM) and its inhibitory effects on eukaryotic cells would preclude its use as a

human antimicrobial agent. Its effects on ribosome synthesis indicate its use as a model compound and suggest that inhibition of bacterial RNases can be a novel target for drug development.

It is important to note that VRC specifically inhibited subunit formation without an effect on translation. This reinforces the established idea that translation and subunit assembly are separate and equivalent targets for ribosomal antibiotics [4, 22-24]. Either process can be a targeted by specific antimicrobials. The cellular RNase target for VRC is unknown but RNase III is a likely target since the formation of both subunits was affected to the same extent. Inhibition of subunit assembly-specific RNases by VRC suggests that these RNases may be a target by other small molecule inhibitors or by RNA interference approaches. This research further indicates the importance of RNases as a novel target in antibiotic research.

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Transparency Declarations

None to report

References

1 Rosen T. Antibiotic resistance: an editorial review with recommendations. *J Drugs Dermatol* 2011; **10**: 724-733.
2 Champney WS. *New Antibiotic Targets*. Totowa, New Jersey: Humana Press Inc., 2008; 65-67.
3 Wilson DN. The A-Z of bacterial translation inhibitors. *Crit Rev Biochem Mol Biol* 2009; **44**: 393-433.
4 Champney WS. The other target for ribosomal antibiotics: inhibition of bacterial ribosomal subunit formation. *Infect Disord Drug Targets* 2006; **6**: 377-390.
5 Kaczanowska M, Rydén-Aulin M. Ribosome biogenesis and the translation process in *Escherichia coli*. *Microbiol Mol Biol Rev* 2007; **71**: 477-494.
6 Deutscher MP. Maturation and degradation of ribosomal RNA in bacteria. *Prog Mol Biol Transl Sci* 2009; **85**: 369-391.
7 Silvers JA, Champney WS. Accumulation and turnover of 23S ribosomal RNA in azithromycin-inhibited ribonuclease mutant strains of *Escherichia coli*. *Arch Microbiol* 2005; **184**: 66-77.
8 Champney WS. Bacterial ribosomal subunit assembly is an antibiotic target. *Curr Top Med Chem* 2003; **3**: 929-947.
9 Reddy KJ, Gilman M. Preparation of bacterial RNA. *Curr Protoc Mol Biol* 2001; **Chapter 4**: Unit4
10 Berger SL. Isolation of cytoplasmic RNA: ribonucleoside-vanadyl complexes. *Methods in enzymology* 1987; **152**: 227-234.
11 Lee YI, Hong YB, Kim Y et al. RNase H activity of human hepatitis B virus polymerase expressed in *Escherichia coli*. *Biochem Biophys Res Commun* 1997; **233**: 401-407.
12 Koepsel RR, Murray RW, Rosenblum WD et al. Purification of pT181-encoded repC protein required for the initiation of plasmid replication. *J Biol Chem* 1985; **260**: 8571-8577.
13 Champney WS, Burdine R. Macrolide antibiotic inhibition of translation and 50S ribosomal subunit assembly in methicillin-resistant *Staphylococcus aureus* cells. *Microb Drug Resist* 1998; **4**: 169-174.
14 Chittum HS, Champney WS. Erythromycin inhibits the assembly of the large ribosomal subunit in growing *Escherichia coli* cells. *Curr Microbiol* 1995; **30**: 273-279.
15 Jett BD, Hatter KL, Huycke MM et al. Simplified agar plate method for quantifying viable bacteria. *Biotechniques* 1997; **23**: 648-650.
16 Champney WS, Rodgers WK. Retapamulin inhibition of translation and 50S ribosomal subunit formation in *Staphylococcus aureus* cells. *Antimicrob Agents Chemother* 2007; **51**: 3385-3387.
17 Raschke WC, Baird S, Ralph P et al. Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* 1978; **15**: 261-267.
18 Mehta R, Champney WS. Neomycin and paromomycin inhibit 30S ribosomal subunit assembly in *Staphylococcus aureus*. *Curr Microbiol* 2003; **47**: 237-243.
19 Evguenieva-Hackenberg E, Klug G. New aspects of RNA processing in prokaryotes. *Curr Opin Microbiol* 2011; **14**: 587-592.
20 Foster C, Champney WS. Characterization of a 30S ribosomal subunit assembly intermediate found in *Escherichia coli* cells growing with neomycin or paromomycin. *Arch Microbiol* 2008; **189**: 441-449.

21 Usary J, Champney WS. Erythromycin inhibition of 50S ribosomal subunit formation in *Escherichia coli* cells. *Mol Microbiol* 2001; **40**: 951-962.
22 Borovinskaya MA, Pai RD, Zhang W et al. Structural basis for aminoglycoside inhibition of bacterial ribosome recycling. *Nat Struct Mol Biol* 2007; **14**: 727-732.
23 Champney WS, Burdine R. 50S ribosomal subunit synthesis and translation are equivalent targets for erythromycin inhibition in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1996; **40**: 1301-1303.
24 Champney WS, Tober CL, Burdine R. A comparison of the inhibition of translation and 50S ribosomal subunit formation in *Staphylococcus aureus* cells by nine different macrolide antibiotics. *Curr Microbiol* 1998; **37**: 412-417.

CHAPTER 5

SUMMARY

Antibiotics play an important role in society and global health. As the use and misuse of antibiotics increases, so does the prevalence of bacterial antibiotic resistance (Hawkey and Jones 2009; Rosen 2011; Tenover 2001). Due to this increasing problem, the development of novel antibiotic targets is a constant research endeavor.

Many commonly used antibiotics function by targeting the bacterial ribosome. Aminoglycosides have been shown to bind to the 30S ribosomal subunit to inhibit the recycling of the ribosomal subunit and affect the translational accuracy (Foster and Champney 2008; Mehta and Champney 2002; Sutcliffe 2005). Additionally, aminoglycoside research has demonstrated that these antibiotics also target the 50S ribosomal subunit (Borovinskaya and others 2007; Campuzano and others 1979; Scheunemann and others 2010). Macrolides specifically target the 50S ribosomal subunit but not the 30S subunit in the bacterial cell (Champney 2008; Champney and Burdine 1998b; Champney and Rodgers 2007; Chittum and Champney 1995; Silvers and Champney 2005; Usary and Champney 2001).

The bacterial ribosome is composed of a small (30S) and large (50S) subunit. Each of these subunits are made up of ribosomal RNA and various proteins. The 30S subunit is composed of 16S rRNA while the 50S subunit is composed of 23S and 5S rRNA (Champney 2003). In order for the proper formation of the subunits to occur, the ribosomal RNA must be processed from a primary RNA transcript into mature RNA. RNases are the enzymes involved in this process. Ribonucleases such as RNase III, E, G, and PH all play pivotal roles in rRNA processing as seen

in Figure 1.7 and Table 1.2 (Davies and others 2010; Gutgsell and Jain 2012; Kaczanowska and Rydén-Aulin 2007; Song and others 2011).

Along with rRNA processing, RNases also play important roles in rRNA degradation and turnover. Figure 1.6 illustrates the importance of RNases in rRNA degradation. When an inhibitor (such as an antibiotic) is introduced into a bacterial cell during ribosome formation, RNases play key roles in the breakdown of the 30S and 50S subunits. Once degraded, the rRNA can then be recycled back into the bacterial ribosome assembly process without the attached translational inhibitors (Champney 2006). RNases II, R, E, and PNPase are all involved in the degradation of rRNA as shown in Table 1.2 (Arraiano and others 2010; Cheng and Deutscher 2002; 2003; Vanzo and others 1998).

Prior RNase research by Silvers and Champney demonstrated that when *E. coli* cells were deficient for RNase E, RNase II, or PNPase, these cells displayed an increased sensitivity to the macrolide antibiotic, azithromycin (Silvers and Champney 2005). Additionally, these cells had an accumulation of the 32S precursor for the 50S ribosomal subunit and a reduced recovery rate of the 50S subunit once the antibiotics were removed. This particular work illustrated the importance of ribonucleases in 23S rRNA turnover and antibiotic sensitivity to azithromycin (Silvers and Champney 2005). This work also indicated that RNases might serve as a potential antibiotic target.

A study was conducted to first determine if the absence of RNases in *E. coli* cells would show an increased sensitivity to aminoglycosides (Chapter 2) as was seen for Silvers and Champney to a macrolide (Silvers and Champney 2005). The results showed that *E. coli* cells deficient for RNase III, E, R, G, or PH displayed a significant decrease in cell viability in the presence of neomycin or paromomycin. Additionally, RNase PH deficient cells demonstrated a

significant reduction in 30S and 50S ribosomal subunits and a significant reduction in 16S and 23S rRNA. RNase III is an essential enzyme in the cleavage of the primary RNA transcript (Deutscher 2009). RNases E and G are found to function in the processing of 16S rRNA (Li and others 1999). RNase G, along with RNase PH, is involved in maturation of 23S rRNA (Gutgsell and Jain 2012; Song and others 2011). In addition to rRNA maturation, RNase is involved in rRNA degradation by forming part of the bacterial degradosome (Deutscher 2009; Vanzo and others 1998).

The data from Chapter 2 and Silvers and Champney (Silvers and Champney 2005) indicated that the loss of certain RNases led to an increased susceptibility to currently used antibiotics. This finding is an important point because antibiotic resistance to aminoglycosides and macrolides is documented (Anderson 1999; Cars and others 2011; Högberg and others 2010; Zinner 2005). Novel antibiotic targets, such as ribonucleases, could hold an important key to the development of new drugs and the improvement of current ones.

Due to the fact that RNase mutants are not found in nature, the second aim of this study was to determine if the use of an RNase inhibitor would act to inhibit RNases important to RNA maturation and thereby potentiate the effects of an aminoglycoside and a macrolide (Chapter 3). Vanadyl Ribonucleoside Complex (VRC) is a low weight molecular inhibitor of RNases that is mainly used during RNA isolations (Berger 1987; Berger and others 1980). While it is unknown which specific RNases are inhibited by VRC, it has been proposed that VRC specifically targets endoribonucleases (Berger 1987). The work showed that VRC led in a slight decrease in viable cell counts and had an additive effect on *E. coli* when azithromycin was also added. Paromomycin and VRC led to an increase in viable cells that, while not completely understood, could possibly be attributed to a decrease in mRNA turnover. Additionally, the presence of VRC

was shown to decrease ribosomal subunit assembly and increase the fragmentation of 16S and 23S rRNA, especially when azithromycin was also present. This work showed that an inhibitor of RNases could be used in gram negative *E. coli* cells to potentiate the effects of the macrolide, VRC. VRC did not appear to increase the overall effectiveness of the aminoglycoside, paromomycin. The exact mechanism for why paromomycin and VRC led to an increase in *E. coli* cell viability is not known. The addition of VRC and azithromycin led to a decrease, and the addition of VRC and paromomycin or azithromycin led to a significant decrease in cell viability for both MSSA and MRSA cells. It is postulated that the reason for the increase in *E. coli* cells incubated paromomycin and VRC is due to the differences in bacterial cell walls in gram negative and gram positive bacteria and the different targets of aminoglycoside verses macrolide antibiotics.

In addition to use of VRC in *E. coli*, it was hypothesized that VRC would act similarly in different bacteria. Methicillin resistant strains of *Staphylococcus aureus* began as hospital acquired infections but as antibiotic resistance spread, the introduction of community acquired *S. aureus* began to appear (Hawkey and Jones 2009; Zinner 2007). The final aim of this research was to determine if VRC would potentiate the effects of an aminoglycoside and a macrolide in a gram positive bacterial species (Chapter 4).

Methicillin sensitive and methicillin resistant *Staphylococcus aureus* were incubated with VRC and paromomycin (an aminoglycoside) or azithromycin (a macrolide). The study demonstrated that VRC alone reduced the viable cell counts in both types of *S. aureus*. The addition of VRC also led to a reduction in the assembly of the 30S and 50S assembly in *S. aureus* cells. The results also showed that when either of the antibiotics was added, the effects of the antibiotics were potentiated by the VRC. This study further demonstrated that an inhibitor of

bacterial RNases is effective in not only gram negative bacteria, as seen in Chapter 3, but also potentially dangerous gram positive bacteria (i.e. methicillin resistant *S. aureus*). A summary of all three aims is seen in Table 5.1. The table shows that *E. coli* lacking specific RNases demonstrated an increased sensitivity to aminoglycosides and that the use of VRC led to an increase in the inhibitory effects of an aminoglycoside and a macrolide.

Together, this study demonstrates a novel antibiotic drug target in the bacterial ribonucleases. It also shows a new use for an RNase inhibitor to potentiate the effects of current antibiotics in both a gram negative, an antibiotic sensitive gram positive, and an antibiotic resistant gram positive bacterial species. While VRC was shown to have cytotoxic effects on eukaryotic cells (Chapter 4), future studies to identify different prokaryotic-selective RNase inhibitors could be useful to antibiotic research. It would be interesting to determine if antibiotic resistant bacterial strains up-regulate specific RNases to increase the rRNA turnover in addition to other methods of resistance. Other future areas of research could involve different RNase inhibitors to determine if different combinations will increase or decrease antibiotic susceptibility. With bacterial resistance to current antibiotics on the rise, it is essential that we research innovative antibiotic targets in order to improve global health and safety.

Table 5.1: Final dissertation summary

Strain	RNase Mutation	Aminoglycoside Sensitivity	Macrolide Sensitivity	Inhibit 30S Assembly	Inhibit 50S Assembly	Increased 16S rRNA Fragmentation	Increased 23S rRNA Fragmentation
SK901	None						
D10-1	I						
SK7622	III	+		+	+		+
SK5665	E	+		+			
SK4803	II						
N7060	I, II, PNPase						
MG1655 I R	I, R	+		+	+	+	+
GW11	G	+				+	
SK6639	PNPase						
SK5729	I, II, PNPase, PH	+		+	+	+	+
***Escherichia coli* + VRC**		+	+	+	+	+	+
Methicillin Sensitive *Staphylococcus aureus* + VRC		+	+	+	+		
Methicillin Resistant *Staphylococcus aureus* + VRC		+	+	+	+		

REFERENCES

Allas U, Liiv A, Remme J. 2003. Functional interaction between RNase III and the *Escherichia coli* ribosome. BMC Mol Biol 4:8.

Anderson RM. 1999. The pandemic of antibiotic resistance. Nat Med 5(2):147-149.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M et al. . 2010. The critical role of RNA processing and degradation in the control of gene expression. FEMS Microbiol Rev 34(5):883-923.

Arraiano CM, Yancey SD, Kushner SR. 1988. Stabilization of discrete mRNA breakdown products in ams pnp rnb multiple mutants of *Escherichia coli* K-12. J Bacteriol 170(10):4625-4633.

Awano N, Rajagopal V, Arbing M, Patel S, Hunt J, Inouye M, Phadtare S. 2010. *Escherichia coli* RNase R has dual activities, helicase and RNase. J Bacteriol 192(5):1344-1352.

Babitzke P, Granger L, Olszewski J, Kushner SR. 1993. Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. J Bacteriol 175(1):229-239.

Basturea GN, Zundel MA, Deutscher MP. 2011. Degradation of ribosomal RNA during starvation: comparison to quality control during steady-state growth and a role for RNase PH. RNA 17(2):338-345.

Berger SL. 1987. Isolation of cytoplasmic RNA: ribonucleoside-vanadyl complexes. Methods Enzymol 152:227-234.

Berger SL, Hitchcock MJ, Zoon KC, Birkenmeier CS, Friedman RM, Chang EH. 1980. Characterization of interferon messenger RNA synthesis in Namalva cells. J Biol Chem 255(7):2955-2961.

Bessarab DA, Kaberdin VR, Wei CL, Liou GG, Lin-Chao S. 1998. RNA components of *Escherichia coli* degradosome: evidence for rRNA decay. Proc Natl Acad Sci U S A 95(6):3157-3161.

Bogdanov AA, Sumbatyan NV, Shishkina AV, Karpenko VV, Korshunova GA. 2010. Ribosomal tunnel and translation regulation. Biochemistry (Mosc) 75(13):1501-1516.

Borovinskaya MA, Pai RD, Zhang W, Schuwirth BS, Holton JM, Hirokawa G, Kaji H, Kaji A, Cate JH. 2007. Structural basis for aminoglycoside inhibition of bacterial ribosome recycling. Nat Struct Mol Biol 14(8):727-732.

Brosius J, Dull TJ, Noller HF. 1980. Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli*. Proc Natl Acad Sci U S A 77(1):201-204.

Burger A, Whiteley C, Boshoff A. 2011. Current perspectives of the *Escherichia coli* RNA degradosome. Biotechnol Lett 33(12):2337-2350.

Cairrão F, Arraiano CM. 2006. The role of endoribonucleases in the regulation of RNase R. Biochem Biophys Res Commun 343(3):731-737.

Campuzano S, Vázquez D, Modolell J. 1979. Functional interaction of neomycin B and related antibiotics with 30S and 50S ribosomal subunits. Biochem Biophys Res Commun 87(3):960-966.

Cars O, Hedin A, Heddini A. 2011. The global need for effective antibiotics-moving towards concerted action. Drug Resist Updat 14(2):68-69.

Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature 407(6802):340-348.
Champney WS. 2001. Bacterial ribosomal subunit synthesis: a novel antibiotic target. Curr Drug Targets Infect Disord 1(1):19-36.
Champney WS. 2003. Bacterial ribosomal subunit assembly is an antibiotic target. Curr Top Med Chem 3(9):929-947.
Champney WS. 2006. The other target for ribosomal antibiotics: inhibition of bacterial ribosomal subunit formation. Infect Disord Drug Targets 6(4):377-390.
Champney WS. 2008. New Antibiotic Targets. In: Walker JM, editor. Methods in Molecular Medicine. Totowa, New Jersey: Humana Press Inc. p. 274.
Champney WS, Burdine R. 1996. 50S ribosomal subunit synthesis and translation are equivalent targets for erythromycin inhibition in *Staphylococcus aureus*. Antimicrob Agents Chemother 40(5):1301-1303.
Champney WS, Burdine R. 1998a. Azithromycin and clarithromycin inhibition of 50S ribosomal subunit formation in *Staphylococcus aureus* cells. Curr Microbiol 36(2):119-123.
Champney WS, Burdine R. 1998b. Macrolide antibiotic inhibition of translation and 50S ribosomal subunit assembly in methicillin-resistant *Staphylococcus aureus* cells. Microb Drug Resist 4(3):169-174.
Champney WS, Miller M. 2002. Linezolid is a specific inhibitor of 50S ribosomal subunit formation in *Staphylococcus aureus* cells. Curr Microbiol 44(5):350-356.
Champney WS, Rodgers WK. 2007. Retapamulin inhibition of translation and 50S ribosomal subunit formation in *Staphylococcus aureus* cells. Antimicrob Agents Chemother 51(9):3385-3387.
Champney WS, Tober CL, Burdine R. 1998. A comparison of the inhibition of translation and 50S ribosomal subunit formation in *Staphylococcus aureus* cells by nine different macrolide antibiotics. Curr Microbiol 37(6):412-417.
Champney WS, Tober CL. 2000. Specific inhibition of 50S ribosomal subunit formation in *Staphylococcus aureus* cells by 16-membered macrolide, lincosamide, and streptogramin B antibiotics. Curr Microbiol 41(2):126-135.
Chen C, Deutscher MP. 2005. Elevation of RNase R in response to multiple stress conditions. J Biol Chem 280(41):34393-34396.
Chen C, Deutscher MP. 2010. RNase R is a highly unstable protein regulated by growth phase and stress. RNA 16(4):667-672.
Cheng ZF, Deutscher MP. 2002. Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. J Biol Chem 277(24):21624-216249.
Cheng ZF, Deutscher MP. 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. Proc Natl Acad Sci U S A 100(11):6388-6393.
Chittum HS, Champney WS. 1995. Erythromycin inhibits the assembly of the large ribosomal subunit in growing *Escherichia coli* cells. Curr Microbiol 30(5):273-279.
Corte G, Schlessinger D, Longo D, Venkov P. 1971. Transformation of 17 s to 16 s ribosomal RNA using ribonuclease II of *Escherichia coli*. J Mol Biol 60(2):325-338.
Davies BW, Köhrer C, Jacob AI, Simmons LA, Zhu J, Aleman LM, Rajbhandary UL, Walker GC. 2010. Role of *Escherichia coli* YbeY, a highly conserved protein, in rRNA processing. Mol Microbiol 78(2):506-518.

Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74(3):417-433.
Deutscher MP. 2006. Degradation of RNA in bacteria: comparison of mRNA and stable RNA. Nucleic Acids Res 34(2):659-666.
Deutscher MP. 2009. Maturation and degradation of ribosomal RNA in bacteria. Prog Mol Biol Transl Sci 85:369-391.
Długosz M, Trylska J. 2009. Aminoglycoside association pathways with the 30S ribosomal subunit. J Phys Chem B 113(20):7322-7330.
Donovan WP, Kushner SR. 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. Proc Natl Acad Sci U S A 83(1):120-124.
Erb A, Stürmer T, Marre R, Brenner H. 2007. Prevalence of antibiotic resistance in *Escherichia coli*: overview of geographical, temporal, and methodological variations. Eur J Clin Microbiol Infect Dis 26(2):83-90.
Evguenieva-Hackenberg E, Klug G. 2000. RNase III processing of intervening sequences found in helix 9 of 23S rRNA in the alpha subclass of Proteobacteria. J Bacteriol 182(17):4719-4729.
Feldman MB, Terry DS, Altman RB, Blanchard SC. 2010. Aminoglycoside activity observed on single pre-translocation ribosome complexes. Nat Chem Biol 6(1):54-62.
Foster C, Champney WS. 2008. Characterization of a 30S ribosomal subunit assembly intermediate found in *Escherichia coli* cells growing with neomycin or paromomycin. Arch Microbiol 189(5):441-449.
Frazier AD, Champney WS. 2012a. Inhibition of ribosomal subunit synthesis in aminoglycoside treated ribonuclease mutants of *Escherichia coli*. Archives of Microbiology:In Submission.
Frazier AD, Champney WS. 2012b. The vanadyl ribonucleoside complex inhibits ribosomal subunit formation in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy:In Submission.
Gesteland RF. 1966. Isolation and characterization of ribonuclease I mutants of *Escherichia coli*. J Mol Biol 16(1):67-84.
Giedraitienė A, Vitkauskienė A, Naginienė R, Pavilonis A. 2011. Antibiotic resistance mechanisms of clinically important bacteria. Medicina (Kaunas) 47(3):137-146.
Gutgsell NS, Jain C. 2012. Role of precursor sequences in the ordered maturation of *E. coli* 23S ribosomal RNA. RNA 18(2):345-353.
Hawkey PM, Jones AM. 2009. The changing epidemiology of resistance. J Antimicrob Chemother 64 Suppl 1:i3-10.
Hirokawa G, Nijman RM, Raj VS, Kaji H, Igarashi K, Kaji A. 2005. The role of ribosome recycling factor in dissociation of 70S ribosomes into subunits. RNA 11(8):1317-1328.
Högberg LD, Heddini A, Cars O. 2010. The global need for effective antibiotics: challenges and recent advances. Trends Pharmacol Sci 31(11):509-515.
Jett BD, Hatter KL, Huycke MM, Gilmore MS. 1997. Simplified agar plate method for quantifying viable bacteria. Biotechniques 23(4):648-650.
Jiang X, Belasco JG. 2004. Catalytic activation of multimeric RNase E and RNase G by 5'-monophosphorylated RNA. Proc Natl Acad Sci U S A 101(25):9211-9216.
Kaczanowska M, Rydén-Aulin M. 2007. Ribosome biogenesis and the translation process in *Escherichia coli*. Microbiol Mol Biol Rev 71(3):477-494.

Kannan K, Mankin AS. 2011. Macrolide antibiotics in the ribosome exit tunnel: species-specific binding and action. Ann N Y Acad Sci 1241(1):33-47.
Kelly KO, Reuven NB, Li Z, Deutscher MP. 1992. RNase PH is essential for tRNA processing and viability in RNase-deficient *Escherichia coli* cells. J Biol Chem 267(23):16015-16018.
Kennell D. 2002. Processing endoribonucleases and mRNA degradation in bacteria. J Bacteriol 184(17):4645-4657; discussion 4665.
King TC, Sirdeshmukh R, Schlessinger D. 1984. RNase III cleavage is obligate for maturation but not for function of *Escherichia coli* pre-23S rRNA. Proc Natl Acad Sci U S A 81(1):185-188.
Klein F, Evguenieva-Hackenberg E. 2002. RNase E is involved in 5'-end 23S rRNA processing in alpha-Proteobacteria. Biochem Biophys Res Commun 299(5):780-786.
Koepsel RR, Murray RW, Rosenblum WD, Khan SA. 1985. Purification of pT181-encoded repC protein required for the initiation of plasmid replication. J Biol Chem 260(14):8571-8577.
Kushner SR, Maples VF, Champney WS. 1977. Conditionally lethal ribosomal protein mutants: characterization of a locus required for modification of 50S subunit proteins. Proc Natl Acad Sci U S A 74(2):467-471.
Lee YI, Hong YB, Kim Y, Rho HM, Jung G. 1997. RNase H activity of human hepatitis B virus polymerase expressed in *Escherichia coli*. Biochem Biophys Res Commun 233(2):401-407.
Li Z, Deutscher MP. 1994. The role of individual exoribonucleases in processing at the 3' end of *Escherichia coli* tRNA precursors. J Biol Chem 269(8):6064-6071.
Li Z, Pandit S, Deutscher MP. 1999. RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. EMBO J 18(10):2878-2885.
Li Z, Reimers S, Pandit S, Deutscher MP. 2002. RNA quality control: degradation of defective transfer RNA. EMBO J 21(5):1132-1138.
Lopez PJ, Marchand I, Joyce SA, Dreyfus M. 1999. The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing in vivo. Mol Microbiol 33(1):188-199.
Lorentzen E, Conti E. 2005. Structural basis of 3' end RNA recognition and exoribonucleolytic cleavage by an exosome RNase PH core. Mol Cell 20(3):473-481.
MacRae IJ, Doudna JA. 2007. Ribonuclease revisited: structural insights into ribonuclease III family enzymes. Curr Opin Struct Biol 17(1):138-145.
Mankin AS. 2008. Macrolide myths. Curr Opin Microbiol 11(5):414-421.
Matos RG, Barbas A, Arraiano CM. 2009. RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation. Biochem J 423(2):291-301.
Matos RG, Barbas A, Gómez-Puertas P, Arraiano CM. 2011. Swapping the domains of exoribonucleases RNase II and RNase R: conferring upon RNase II the ability to degrade ds RNA. Proteins 79(6):1853-1867.
McCoy LS, Xie Y, Tor Y. 2011. Antibiotics that target protein synthesis. Wiley Interdiscip Rev RNA 2(2):209-232.
Mehta R, Champney WS. 2002. 30S ribosomal subunit assembly is a target for inhibition by aminoglycosides in *Escherichia coli*. Antimicrob Agents Chemother 46(5):1546-1549.
Mehta R, Champney WS. 2003. Neomycin and paromomycin inhibit 30S ribosomal subunit assembly in *Staphylococcus aureus*. Curr Microbiol 47(3):237-243.

Mohanty BK, Kushner SR. 2000. Polynucleotide phosphorylase, RNase II and RNase E play different roles in the in vivo modulation of polyadenylation in *Escherichia coli*. Mol Microbiol 36(4):982-994.
Mohanty BK, Kushner SR. 2003. Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. Mol Microbiol 50(2):645-658.
Petropoulos AD, Kouvela EC, Starosta AL, Wilson DN, Dinos GP, Kalpaxis DL. 2009. Time-resolved binding of azithromycin to *Escherichia coli* ribosomes. J Mol Biol 385(4):1179-1192.
Poehlsgaard J, Douthwaite S. 2005. The bacterial ribosome as a target for antibiotics. Nat Rev Microbiol 3(11):870-881.
Pokkunuri I, Champney WS. 2007. Characteristics of a 50S ribosomal subunit precursor particle as a substrate for ermE methyltransferase activity and erythromycin binding in *Staphylococcus aureus*. RNA Biol 4(3):147-153.
Ramakrishnan V. 2002. Ribosome structure and the mechanism of translation. Cell 108(4):557-572.
Raschke WC, Baird S, Ralph P, Nakoinz I. 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 15(1):261-267.
Rauhut R, Klug G. 1999. mRNA degradation in bacteria. FEMS Microbiol Rev 23(3):353-370.
Raziuddin, Chatterji D, Ghosh S, Burma DP. 1979. Site of action of RNase I on the 50 S ribosome of *Escherichia coli* and the association of the enzyme with the partially degraded subunit. J Biol Chem 254(21):10575-10578.
Reddy KJ, Gilman M. 2001. Preparation of bacterial RNA. Curr Protoc Mol Biol Chapter 4:Unit4.4.
Rio DC, Ares M, Jr., Hannon GJ, Nilsen TW. 2011. RNA: A Laboratory Manual. Cold Spring Harbor Protocols.
Rosen T. 2011. Antibiotic resistance: an editorial review with recommendations. J Drugs Dermatol 10(7):724-733.
Roy-Chaudhuri B, Kirthi N, Culver GM. 2010. Appropriate maturation and folding of 16S rRNA during 30S subunit biogenesis are critical for translational fidelity. Proc Natl Acad Sci U S A 107(10):4567-4572.
Scheunemann AE, Graham WD, Vendeix FA, Agris PF. 2010. Binding of aminoglycoside antibiotics to helix 69 of 23S rRNA. Nucleic Acids Res 38(9):3094-3105.
Schroeder R, Waldsich C, Wank H. 2000. Modulation of RNA function by aminoglycoside antibiotics. EMBO J 19(1):1-9.
Shakil S, Khan R, Zarrilli R, Khan AU. 2008. Aminoglycosides versus bacteria--a description of the action, resistance mechanism, and nosocomial battleground. J Biomed Sci 15(1):5-14.
Siibak T, Peil L, Dönhöfer A, Tats A, Remm M, Wilson DN, Tenson T, Remme J. 2011. Antibiotic-induced ribosomal assembly defects result from changes in the synthesis of ribosomal proteins. Mol Microbiol 80(1):54-67.
Silvers JA, Champney WS. 2005. Accumulation and turnover of 23S ribosomal RNA in azithromycin-inhibited ribonuclease mutant strains of *Escherichia coli*. Arch Microbiol 184(1):66-77.
Song WS, Lee M, Lee K. 2011. RNase G participates in processing of the 5'-end of 23S ribosomal RNA. J Microbiol 49(3):508-511.

Starosta AL, Karpenko VV, Shishkina AV, Mikolajka A, Sumbatyan NV, Schluenzen F, Korshunova GA, Bogdanov AA, Wilson DN. 2010. Interplay between the ribosomal tunnel, nascent chain, and macrolides influences drug inhibition. Chem Biol 17(5):504-514.
Sutcliffe JA. 2005. Improving on nature: antibiotics that target the ribosome. Curr Opin Microbiol 8(5):534-542.
Tenover FC. 2001. Development and spread of bacterial resistance to antimicrobial agents: an overview. Clin Infect Dis 33 Suppl 3:S108-115.
Tenson T, Mankin A. 2006. Antibiotics and the ribosome. Mol Microbiol 59(6):1664-1677.
Tock MR, Walsh AP, Carroll G, McDowall KJ. 2000. The CafA protein required for the 5'-maturation of 16 S rRNA is a 5'-end-dependent ribonuclease that has context-dependent broad sequence specificity. J Biol Chem 275(12):8726-8732.
Usary J, Champney WS. 2001. Erythromycin inhibition of 50S ribosomal subunit formation in *Escherichia coli* cells. Mol Microbiol 40(4):951-962.
Vanzo NF, Li YS, Py B, Blum E, Higgins CF, Raynal LC, Krisch HM, Carpousis AJ. 1998. Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. Genes Dev 12(17):2770-2781.
Vincent HA, Deutscher MP. 2006. Substrate recognition and catalysis by the exoribonuclease RNase R. J Biol Chem 281(40):29769-29775.
Wachi M, Umitsuki G, Shimizu M, Takada A, Nagai K. 1999. *Escherichia coli* cafA gene encodes a novel RNase, designated as RNase G, involved in processing of the 5' end of 16S rRNA. Biochem Biophys Res Commun 259(2):483-488.
Wallis MG, Schroeder R. 1997. The binding of antibiotics to RNA. Prog Biophys Mol Biol 67(2-3):141-154.
Weatherford SC, Rosen L, Gorelic L, Apirion D. 1972. *Escherichia coli* strains with thermolabile ribonuclease II activity. J Biol Chem 247(17):5404-5408.
Wilson DN. 2009. The A-Z of bacterial translation inhibitors. Crit Rev Biochem Mol Biol 44(6):393-433.
Worrall JA, Luisi BF. 2007. Information available at cut rates: structure and mechanism of ribonucleases. Curr Opin Struct Biol 17(1):128-137.
Xiao J, Feehery CE, Tzertzinis G, Maina CV. 2009. *E. coli* RNase III(E38A) generates discrete-sized products from long dsRNA. RNA 15(5):984-991.
Yonath A. 2005. Antibiotics targeting ribosomes: resistance, selectivity, synergism and cellular regulation. Annu Rev Biochem 74:649-679.
Zhou Z, Deutscher MP. 1997. An essential function for the phosphate-dependent exoribonucleases RNase PH and polynucleotide phosphorylase. J Bacteriol 179(13):4391-4395.
Zilhão R, Régnier P, Arraiano CM. 1995. The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*. FEMS Microbiol Lett 130(2-3):237-244.
Zinner SH. 2005. Overview of antibiotic use and resistance: setting the stage for tigecycline. Clin Infect Dis 41 Suppl 5:S289-292.
Zinner SH. 2007. Antibiotic use: present and future. New Microbiol 30(3):321-325.
Zundel MA, Basturea GN, Deutscher MP. 2009. Initiation of ribosome degradation during starvation in *Escherichia coli*. RNA 15(5):977-983.
Zuo Y, Deutscher MP. 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. Nucleic Acids Res 29(5):1017-1026.

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