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Characterization of a 30S Ribsomal Subunit Intermediate Found in *Escherichia coli* Cells Growing with Neomycin and Paromomycin.

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Characterization of a 30S Ribosomal Subunit Assembly Intermediate Found in *Escherichia coli* Cells Growing with Neomycin or Paromomycin

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by

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ABSTRACT

Characterization of a 30S Ribosomal Subunit Assembly Intermediate Found in *Escherichia coli* Cells Growing with Neomycin or Paromomycin

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The bacterial ribosome is a target for inhibition by numerous antibiotics. Neomycin and paromomycin are aminoglycoside antibiotics that specifically stimulate the misreading of mRNA by binding to the decoding site of 16S rRNA in the 30S ribosomal subunit. Recent work has shown that both antibiotics also inhibit 30S subunit assembly in *Escherichia coli* and *Staphylococcus aureus* cells. This work describes the characteristics of an assembly intermediate produced in *E.coli* cells grown with neomycin or paromomycin. Antibiotic treatment stimulated the accumulation of a 30S assembly precursor with a sedimentation coefficient of 21S. The particle was able to bind radio labeled antibiotics both *in vivo* and *in vitro*. Hybridization experiments showed that the 21S precursor particle contained 16S and 17S rRNA. Ten 30S ribosomal proteins were found in the precursor after inhibition by each drug *in vivo*. In addition, cell free reconstitution assays generated a 21S particle during incubation with either aminoglycoside. Precursor formation was inhibited with increasing drug concentration. This work examines features of a novel antibiotic target for aminoglycoside and will provide information that is needed for the design of more effective antimicrobial agents.

DEDICATION

I first give praise and honor to my Lord and Savior Jesus Christ for His grace has truly guided through this journey. I thank my parents and family for their unconditional love and support of the years. They encouraged me to excel above and beyond what my eyes could see, even when things did not seem possible. My most heartfelt and deep gratitude goes to my grandmother, who has always been my number one fan and cheerleader. I will forever be grateful for my godparents Charlena Turner and Phillip Young for accepting me as their own and always believing in me. They truly have been a gift from God. I also thank Salima Smith, Monique Scott, and Kimberly McDaniels for their listening ears. I am also grateful for the congregation at Friendship Baptist Church as well as Jim and Louise Kelly for their prayers and support.

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ABBREVIATIONS

30S …… Small subunit of ribosome

p30S …… *In vivo* 30S ribosomal intermediate

$p30S_{neo}$ …… *In vivo* precursor from neomycin treated cells

$p30S_{paromo}$ …… *In vivo* precursor from paromomycin treated cells

RI …… *In vitro* reconstitution intermediate

RI_{neo} …… *In vitro* neomycin treated intermediate

RI_{paromo} …… *In vitro* paromomycin treated intermediate

50S …… Large Subunit of the ribosome

DNA …… Deoxyribonucleic acid

RNA …… Ribonucleic acid

rRNA …… Ribosomal RNA

S-buffer, R-buffer …… Subunit buffer, Ribosome buffer

TSB …… Tryptic soy broth

RNase …… Ribonuclease

SDS …… Sodium dodecyl sulfate

EDTA …… Disodium ethylenediamine-tetraacetate

MOPS …… 3-(N-Morpholino)-propanesulfonic acid

2-D …… Two-dimensional

Bis-Tris …… bis-tris(hydroxymethyl) aminomethane

CPM …… counts per minute

$_{d}$H2O …… deionized water

e.u. …… equivalence units of ribosomal proteins

ND …… no drug (antibiotic) present

PCR …… polymerase chain reaction

SSC …… standard saline citrate

TEMED …… N,N,N',N'-tetramethylethylenediamine

TP30 …… total proteins from 30S subunits

Tris …… tris(hydroxymethyl) aminomethane

GTP …… guanosine tri phosphate

tRNA …….. transfer RNA

BSA …… bovine serum albumin

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CHAPTER 1

INTRODUCTION

Antimicrobial Resistance

The increasing emergence of bacterial strains resistant to antimicrobial agents has been a persistent medical challenge for decades and is recognized as a grave public health problem. For many microorganisms second and third generation antibiotics are becoming less effective in treating infection and for others a single antibiotic is the only treatment available. Thus, the discovery of novel compounds to counter resistance remains one of the most important goals for medical research (Anderson 1999).

The design of more effective antimicrobial agents has focused on modifying existing drugs that rely on the same molecular targets (Knowles 1997). It has been the goal of pharmaceutical companies over the past decades to design compounds that combat resistance mechanisms. Since the discovery of penicillin in the 1940s, companies have been continuously improving antibiotic effectiveness by manufacturing second, third, and fourth generation antimicrobials. This effort to modify existing compounds began 20 years after the discovery of penicillin. Yet, by the 1980s almost 80% of *Staphylococcus aureus* isolates had become resistant to penicillin. By 2002, more than 57% of *S. aureus* isolates were resistant to the new classes of antibiotics, leaving the cell wall inhibitor vancomycin, as the last drug of choice (Mills 2006). Eventually, vancomycin resistant strains emerged and the lipopeptide antibiotic daptomycin

was the next compound of choice. Not surprisingly, within a year of FDA approval daptomycin resistant strains emerged (Mangili and others 2005).

A resolution to this problem now focuses on identifying new targets that inhibit essential bacterial functions (Chu and others 1996). However, this has not been a straightforward task. In the past 20 years there has been a decrease in the number of FDA approved drugs and of the 10 approved since 1998, only daptomycin has a novel drug site. And this was only 43 years following its discovery (Spellberg and others 2004; Norrby and others 2005).

It is very clear that identifying new drug targets should be of foremost priority. The targets with the greatest potential have been defined by their biological importance. Therefore, examining universal and essential cellular functions would be of utmost significance (Lerner and Beutel 2002). Protein biosynthesis is a fundamental cellular process and is a well-characterized antibacterial target. A number of antibiotic classes function by interacting with the fully formed 50S or 30S ribosomal subunits. However, resistance mechanisms that modify ribosomal RNA sequences in the fully formed subunits have compromised the utility of these compounds (Shaw and others 1998; Beauclerk and Cundliffe 1987). On the other hand, little attention has been given to the formation of these subunits, which also happens to be an important cellular process (Champney 2006). Accurate assembly of ribosomes is undeniably essential to the bacterial cell. For this reason, drugs preventing bacterial ribosome assembly may be a more fundamental target.

30S Subunit Structure and Function

The bacterial ribosome is a large macromolecular complex whose primary task is deciphering the genetic code and stimulating peptide bond formation. This is accomplished by both the 30S and 50S subunits. The small subunit sediments at 30S and binds mRNA, initiation factors, and the large subunit. It also participates in tRNA selection, thus playing a critical role in translational fidelity. The large subunit sediments at 50S and is responsible for peptide bond formation and movement of the ribosome along the mRNA sequence (Noller 1991).

Resolution of the crystal structure has provided insight into the 30S subunit architecture. About two-thirds of the subunit is RNA and the remaining one-third is ribosomal proteins. The secondary structure (Figure 1A) consists of four domains that organize into the structural regions shown in Figure 1B denoted as the head, body, shoulder, and the platform. The 5' rRNA domain forms the body and the central domain establishes the platform. The 3' minor and major domains assemble into the head and shoulder regions respectively (Wimberly and others 2000). Although non-functional, each of the 16S RNA domains can assemble separately (Nierhaus 1991; Powers and others 1993; Weitzman and others 1993). These four domains are connected by a region known as the neck, which is critical for the final 30S conformation and function (Wimberly and others 2001).

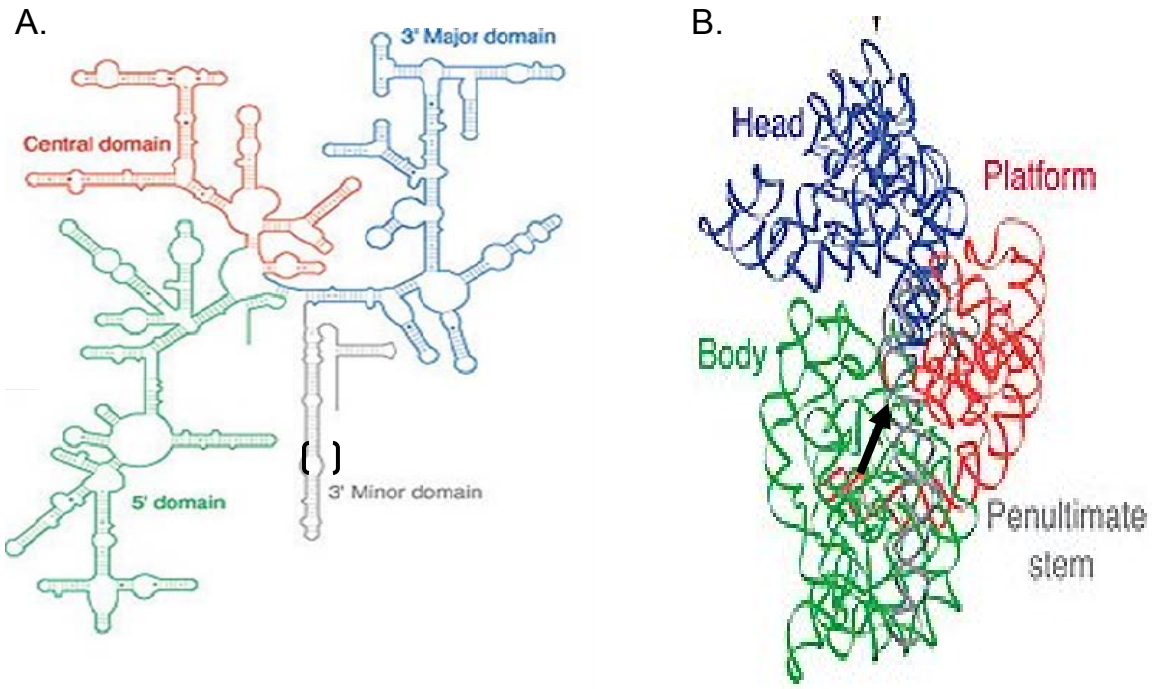


Figure 1 Secondary and Three Dimensional Structures of *E.Coli* 16S rRNA and 30S Subunit. A.) Secondary structure of 16S rRNA domains. The central, 3' major and minor, and 5' domains are colored; red, blue, purple, and green respectively. The 16S rRNA region enclosed by brackets represents the decoding region. B.) 3D structure of the 30S subunit. The head, platform, penultimate stem, and body are formed by the RNA domains shown in A and the decoding center is shown by the arrow (adapted from Holmes and Culver 2004).

Ribosomal Protein Structure

While mainly composed of RNA, the significance of ribosomal proteins to 30S function must not be underestimated. Both by direct and indirect interactions, ribosomal proteins are paramount to the organization of the subunit and RNA catalytic activity of the subunit depends on protein association. For example, direct binding of ribosomal proteins assist in domain assembly by initiating the tertiary fold between RNA helical junctions (Brodersen and Ditlev 2005). In addition, direct contacts with long protein extensions help stabilize the final structure. These extensions are highly basic thereby neutralizing the negatively charged RNA backbone. These narrow extensions are able to make direct and specific contacts with the RNA, anchoring the protein deep within the RNA folds. On the other hand, hydrophobic protein-protein interactions stabilize the structure indirectly by docking between RNA domains (Brodersen and Ditlev 2005). Overall, ribosomal proteins assist in domain organization to create functional binding centers.

The decoding center on the 30S subunit is the binding site for aminoglycoside antibiotics. Located between the head and the body, it is primarily composed of RNA. It is joined by helix 44 in the 3' minor domain with loop 530, and projections of 4 ribosomal proteins (Moore and Steitz 2002). Some of the current work on 30S structure and function involves characterizing the interactions of 16S rRNA with aminoglycoside antibiotics within this site.

Structure of Aminoglycosides

Isolated from *Streptomyces fradiae* in 1949, neomycin and paromomycin are two of several aminoglycosides that bind to the decoding center. They are effective against a number of aerobic and facultative anaerobic microorganisms (Kotra and others 2000). This makes them useful in treating abdominal and urinary tract infections and preventing endocarditis and bactermia. In most instances, these antibiotics are fast acting bactericidal agents and are synergistically effective against nosocomial infections (Jana and Deb 2006).

The aminoglycosides are four member-ring compounds composed of a 2-deoxystreptamine linked to several amino sugars. The positions of the linkages distinguish the different aminoglycoside classes. The 4,5 disubstituted class consists of neomycin and paromomycin. As shown in Figure 2, paromomycin and neomycin differ in chemical structure by the functional group attached to the C'6 of ring 1. Paromomycin has a hydroxyl group at this position, while neomycin possesses an amino group (Benveniste and Davies 1973, Schroder and Wallis 2001). These RNA binding antibiotics have several features that contribute to their antimicrobial activity. Their high positive charge enhances their attraction to the negatively charged RNA backbone. In addition, these features also permit promiscuous interactions with several other types of RNA motifs (reviewed in Tor 2006).

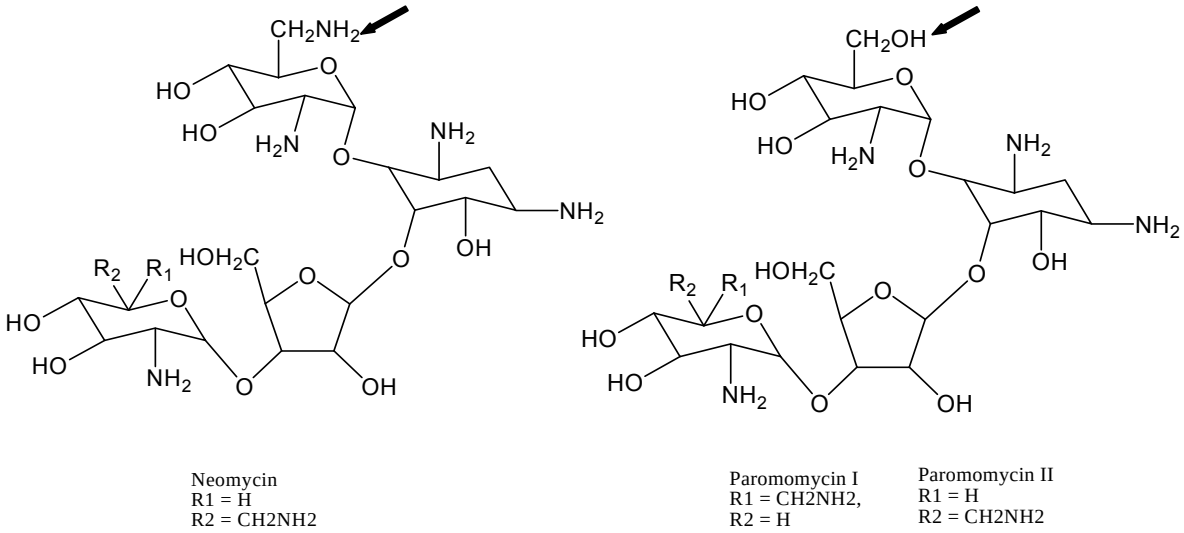


Figure 2 Structure of Neomycin and Paromomycin. Neomycin has an amino group and paromomycin has a hydroxyl group (indicated by the arrow).

Figure 3 shows paromomycin complexed to the ribosomal A site. Rings I and II mediate the direct and specific interactions of the antibiotic with the A site. The drug binds to the major groove within an RNA pocket created by bases A1408, A1492, and A1493. Ring I forms a pseudo base pair with A1408 and stacks above C1409:G1491. The amino groups at positions 1 and 3 shown in Figure 3 serve as hydrogen bond donors to U1495 and G1494. The amino groups at positions 1 and 3 of ring II are also essential for binding to the decoding site of 16S rRNA. This binding induces a conformational change, that facilitates additional binding of the antibiotic to the rRNA (Fourmy and others 1996,1998; Recht and others 1999). This same interaction is found with other distinct classes of aminoglycosides and explains the general specificity of the

neamine core for the A site (Blanchard and others 1998). Rings III and IV are accommodated into the major groove toward the lower stem of the A site. Although these rings do not make base specific contacts with the RNA, they contribute to the specificity by providing additional positive charges (Schroeder and Wallis 2001).

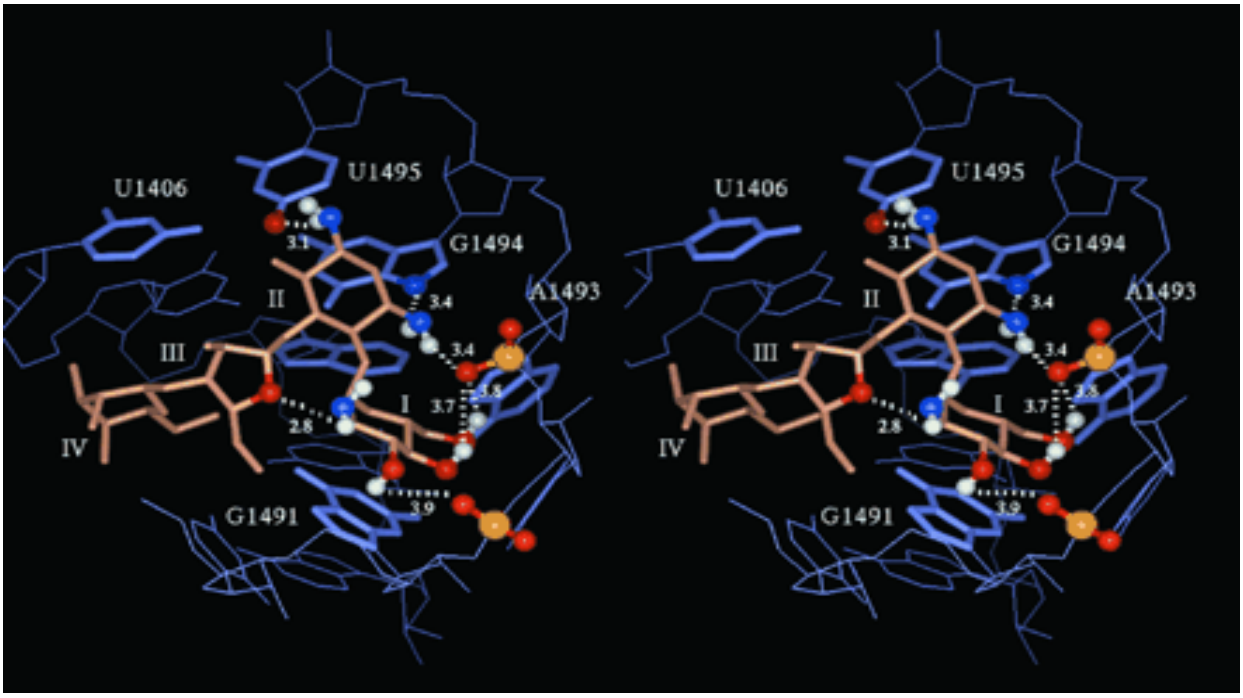


Figure 3 Structure of Paromomycin Complexed to the Ribosomal A Site. Paromomycin complexed to the A site is shown in stereo view. Rings I and II of paromomycin make base specific contacts with A1408, U1495, G1494, A1493, and G1491. Rings III and IV do not interact within the RNA pocket but contribute the antibiotics induced fit. The blue represents the RNA backbone and tan represents paromomycin. The dashed lines represent possible hydrogen bonds (adapted from Schroeder and Wallis 2001).

A detailed examination of aminoglycosides and A site interactions concluded that the 16S rRNA sequences within the decoding region were sufficient to elicit a conformational change in structure (Fourmy and others 1998). Footprinting experiments (Recht and others 1999) using an *E.coli* 16S rRNA 27-mer oligonucleotide identified the nucleotides that were critical for high affinity paromomycin binding. Subsequently, NMR studies revealed that drug binding to these nucleotides produced a change in the conformation of the decoding region of the 30S subunit (Lynch and Puglisi 2001) (Figure 4).

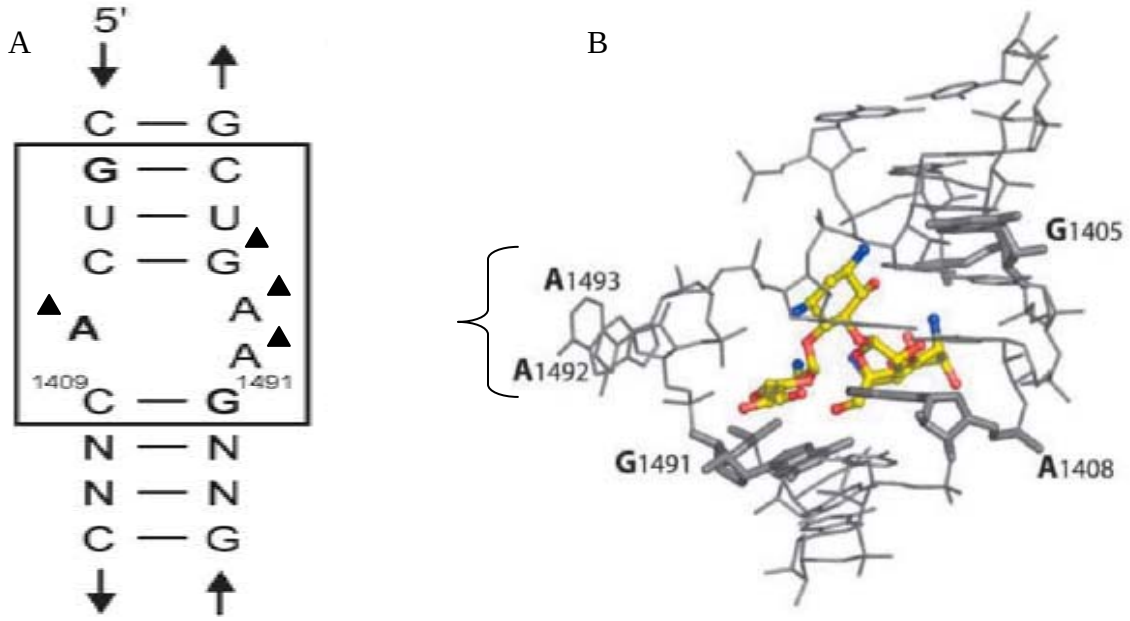


Figure 4 Paromomycin Complexed With the A Site Induces a Conformational Change. A.) Nucleotides of the ribosomal A site that are involved in paromomycin binding. The triangles represent bases protected from dimethylsulfate modification in the presence of antibiotic. B.) Binding of

paromomycin results in the “flipping out” of A1492 and A1493 (adapted from Pfister and others 2005).

Inhibition of Translation

Neomycin and paromomycin are specific for the 30S subunit and induce misreading of the genetic code and mistranslation of messenger RNA (Davies and others 1965,1968). In the absence of the antibiotics, translation begins with initial selection of an amino acyl-tRNA (cognate) through mRNA base paring. Recent structural data has shown that base pairing alone is not sufficient to ensure translational fidelity. Instead, the proofreading capacity depends on a highly energetic conformational change that is a prerequisite for cognate tRNA binding. Aminoglycosides bypass this discriminatory step facilitating a change in structure that allows non-cognate tRNA binding with high affinity (Figure 5). As a result, the error frequency is increased (Ogle and others 2001,2002,2003).

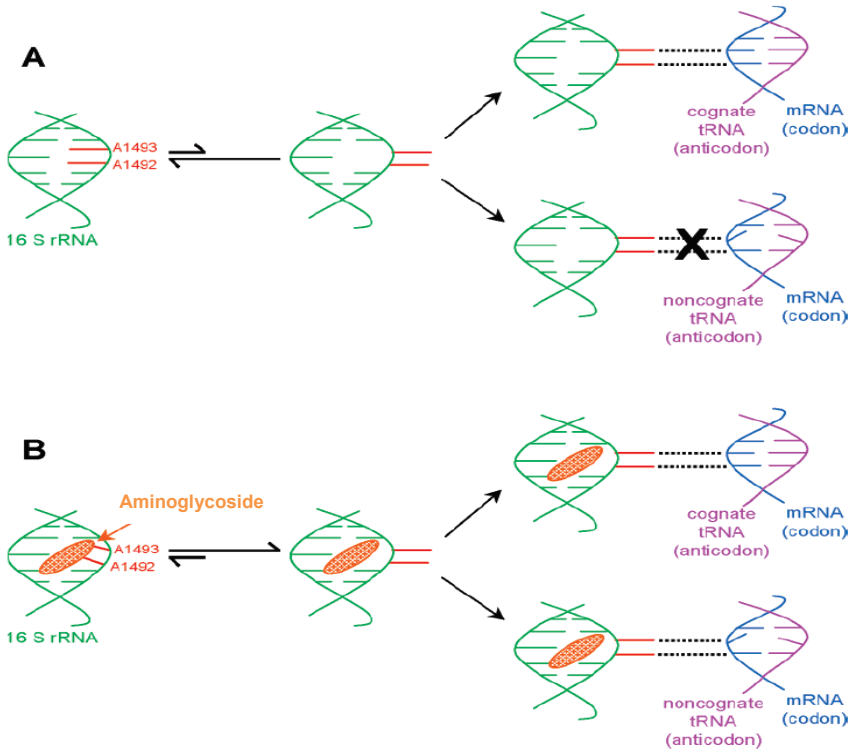


Figure 5 Conformational Dynamics of the Decoding Site During tRNA Selection and Antibiotic Binding. A.) A1492 and A1493 are in conformational equilibria between the "flipped in" or flipped out states, with the intrahelical states being favored in the absence of drug. In their extrahelical states, A1492 and A1493 are able to interact with the tRNA anticodon (magenta) and the mRNA codon (blue). This interaction being favored in the presence of the cognate tRNA anticodon and disfavored in the presence of a non-cognate tRNA anticodon. B.) The effects of aminoglycoside binding to the decoding region. The drug (shown in brown) binds to the 16S rRNA and shifts the conformational equilibria of A1492 and A1493 toward their "flipped out" states. As a result, A1492 and A1493 are now able to engage in favorable interactions with the codon-anticodon minihelix, even when the anticodon is non-cognate (adapted from Pilch and others 2005).

Ribosome Assembly

Rearrangement of the 30S subunit by a molecule less than 1000 times its molecular weight has revolutionized current understanding of ribosome structure and function. More than 40 years of investigation on this massive structure have revealed details of interactions within the fully formed particle as well as its subunits. In contrast, details of how ribosomal subunits are assembled still remain elusive (Culver 2003).

Several in vitro techniques have been employed to characterize the assembly pathway of the 30S subunit (Nomura 1973, Stern and others 1989). Ribonuclease cleavage and proteolytic digestion has been used to identify elements of 16S rRNA that interact with proteins (Powers and Noller 1995). Ribosomal reconstitution and protection experiments have been performed to examine protein dependent RNA rearrangements that occur to form the 30S subunit (Traub and Nomura 1968; Nashimoto and others 1971; Powers and others 1993). Altogether, these data revealed that assembly is a multistep cooperative process coupled with RNA transcription in cells.

The precursor 16S RNA transcript results from cleavage of a larger precursor RNA by ribonuclease III. This results in an immature 16S sequence with a 115 nt and 33 nt extension at the 5' and 3' ends respectively. While RNA maturation is coupled with the addition of ribosomal proteins, the precursor sequences may help to promote a conformation required for subunit assembly

(Nicholson 1999; Deutscher 2003). During the initial stage, major RNA rearrangements occur forming many of the protein binding sites. This would include sites for primary binding proteins shown in Figure 6A. As assembly proceeds, major changes in protection occur in the 5' domain, then shifting to the 3' domain. This is consistent with data revealing a subset of protein dependent protections in the 5' and central domains in the 21S intermediate (Figure 6B). The addition of tertiary binding proteins results in structural rearrangements with protected nucleotides in the 3' minor and major domains, a pattern consistent with mature 30S subunits (Holmes and Culver 2004, 2005).

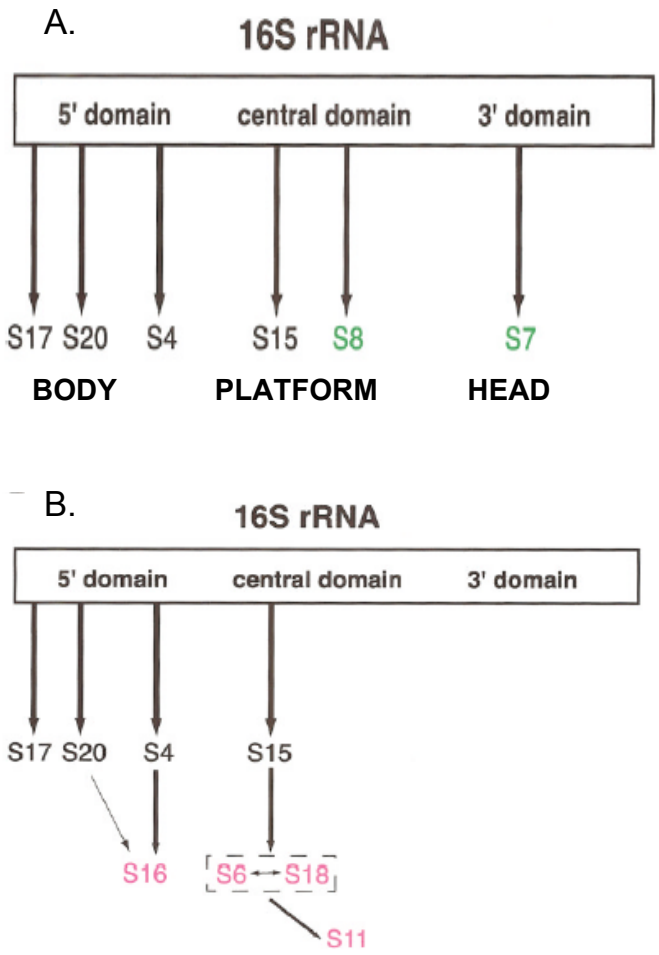


Figure 6 5' to 3' Polarity of Ribosomal Protein Binding to 16S RNA. A.) The primary binding proteins shown in black bind to the 5' and central domain during the early stages of assembly. Proteins shown in green are also primary binding proteins but associate midway during assembly. The tertiary domains that are formed by each protein is also shown. B.) Secondary proteins that bind to the 5' and central domain are shown in pink (adapted from Culver 2003).

These data revealed that three major transitions in RNA structure take place during subunit formation. First, initial binding of primary proteins nucleate assembly and the subsequent addition of secondary proteins produces a 21S intermediate (RI). Shown in Figure 7 is a possible structural arrangement of this particle and its proteins. *In vitro* reconstitution assays revealed that the second RNA conformational change is solely energy dependent and does not require additional protein binding. This particle sediments at 26S and is critical in forming the additional binding sites for the tertiary ribosomal proteins (Mizushima and others 1970; Held and others 1973,1974). Figure 8 is a 30S assembly map that shows the interdependencies of protein binding for 30S subunit formation.

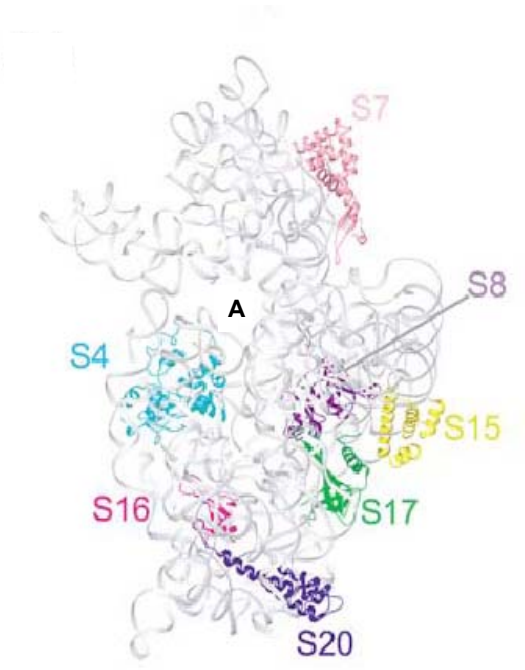


Figure 7 Schematic Representation of the 21S Ribosomal Intermediate. A representation of the structural arrangement of the in vitro 21S ribosomal intermediate (RI). This structure was prepared using the x-ray data of Wimberly and others (2000) for the Thermus thermophilus 30S ribosome to 3A resolution (PDB Id 1J5E). The neomycin and paromomycin binding site is indicated by A. No tertiary proteins are shown in this particle (adapted from Holmes and Culver 2005)

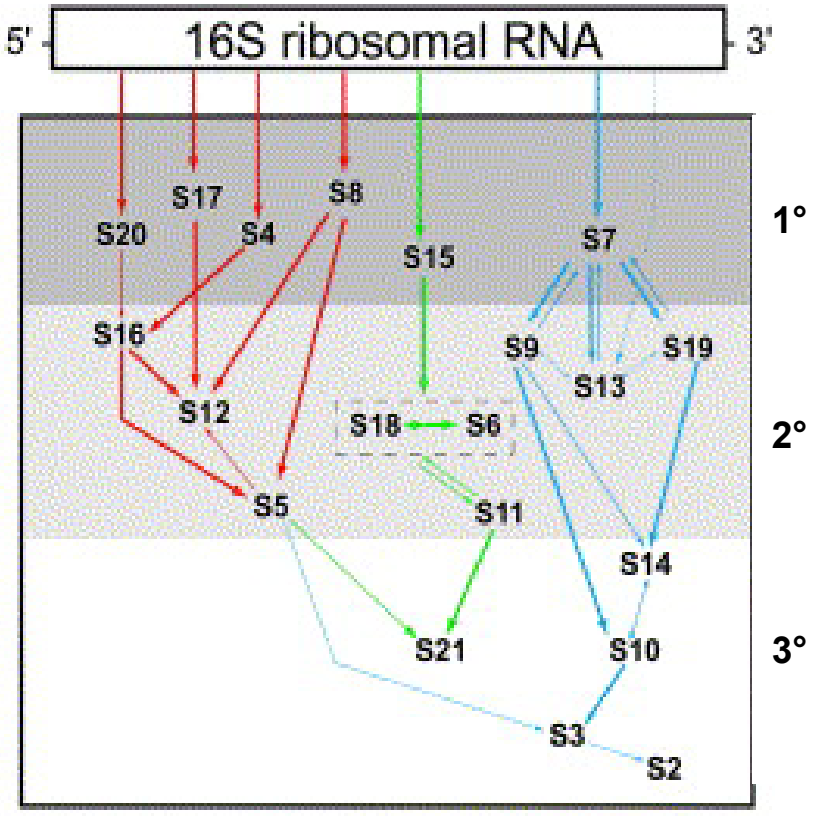


Figure 8 Representation of 30S Subunit Protein Assembly Map. Primary binding proteins are shown in the first row. Secondary and tertiary proteins are shown in second and third row respectively. The arrows represent the interdependence of each protein. Proteins in red, green, and blue bind to the 5', central, and 3' domains respectively (adapted from Culver 2003).

Previous Studies

Collectively these results suggest that a subset of proteins are critical for 30S subunit formation and their cooperative role is to facilitate the conformational changes in 16S rRNA to form a functional subunit. How this process is impaired by aminoglycosides and what 16S rRNA conformational change is needed to bind these antibiotics remains unclear (Culver 2003; Champney 2006).

Recently, the work of Mehta and Champney revealed that aminoglycosides have a second inhibitory target on the 30S subunit. These results were prompted by the work of previous investigations showing that a number of compounds that inhibit translation also prevent ribosome assembly in growing cells (Mehta and Champney 2002, 2003). The macrolides, ketolides, lincosamides, and streptogramin B compounds prevent assembly of the 50S subunit with little or no effect on the small subunit (Champney 2003). Inhibition by the macrolide antibiotic erythromycin produced an intermediate particle that sedimented in the 30S region of a sucrose gradient. Gel electrophoresis revealed that the assembly intermediate was composed of 23S and 5S ribosomal RNA from the large subunit, as well as 18 of the 34 ribosomal proteins (Usary and Champney 2001). It has been shown in both *E. coli* and *S.aureus*, that neomycin and paromomycin inhibit 30S ribosomal subunit assembly in growing cells in a similar manner to 50S inhibition, producing a 21S assembly intermediate. It was shown that these antibiotics are specific for 30S assembly, with little or no effect on 50S assembly (Mehta and Champney 2002, 2003). This

work was the first identification that aminoglycosides inhibit 30S subunit assembly *in vivo*. Unlike the 50S assembly intermediate, the 21S precursor (p30S) remains uncharacterized.

The model for assembly inhibition in Figure 9 proposes that in the absence of antibiotic, assembly proceeds normally forming a functional subunit with a binding site (A). In the presence of neomycin or paromomycin at the IC_{50}, half of the cells go on to form functional subunits that can be inhibited in translation (Figure 9B) and the other half are inhibited during assembly. It remains unclear whether these pathways are identical. Last, the intermediate would be degraded by cellular ribonucleases in a similar manner as the large subunit (Silvers and Champney, 2005).

This work is intended to explore the observation that many antibiotics that inhibit the translational functions of the bacterial ribosome also prevent its assembly in microbial cells. 30S assembly inhibition by neomycin and paromomycin has been examined *in vivo* by characterizing the rRNA and protein composition of the precursor particle.

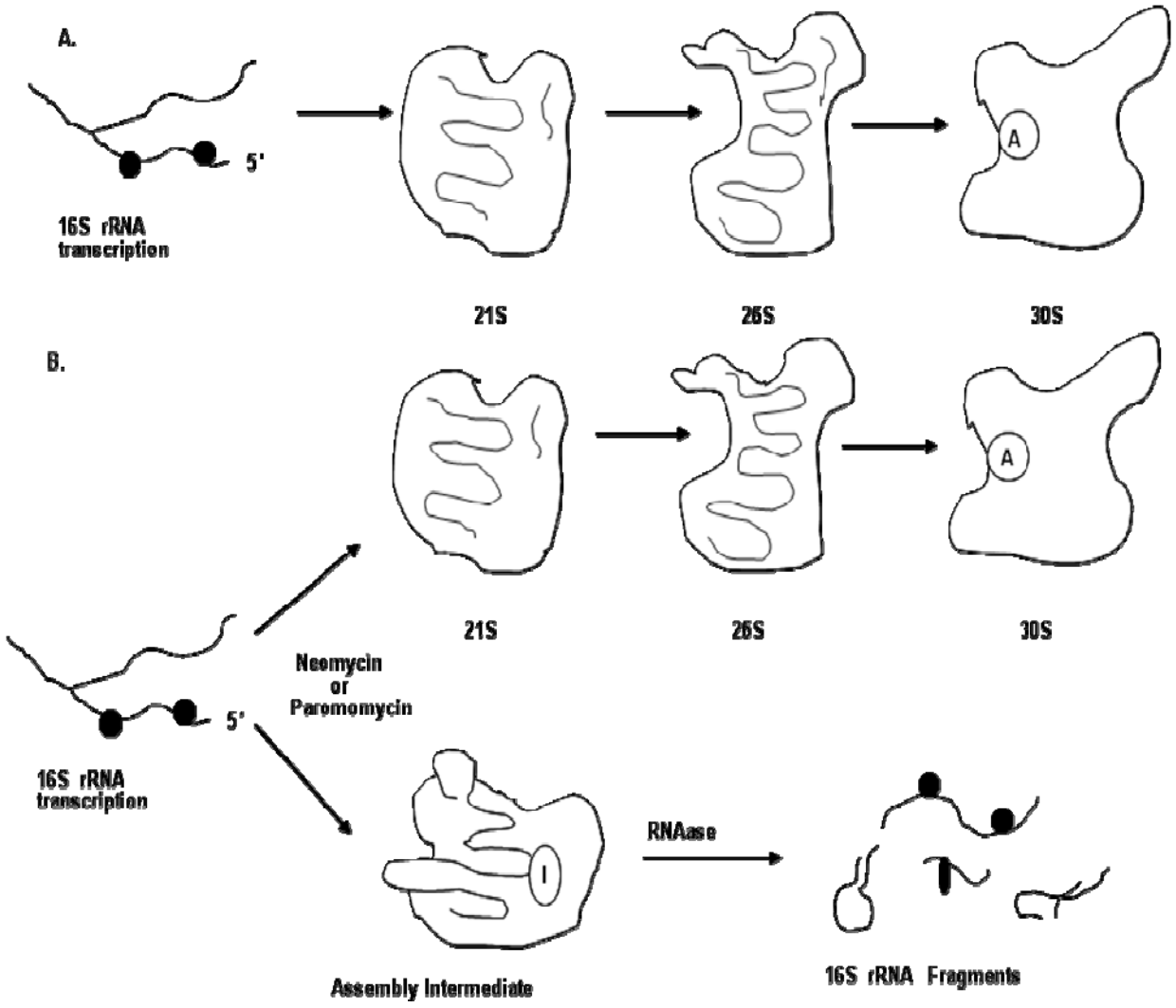


Figure 9 Model of 30S Subunit Assembly in *E.coli* Cells. A.) The pathway of normal 30S assembly. B.) In the presence of the aminoglycoside, subunit assembly stalls and an assembly intermediate accumulates with the drug bound in a site (I) similar to the site found on the mature 30S subunit (A). Cellular ribonucleases degrade the intermediate into rRNA oligonucleotides and free ribosomal proteins (adapted from Usary and Champney 2001).

CHAPTER 2

MATERIALS AND METHODS

Media

Tryptic Soy Broth: 30 g tryptic soy broth in 1 L $_dH_2O$.

Minimal Media: 100 ml 1X A-salts, 1 ml 20% glucose, 0.1 ml 20% $MgSO_4$, and 0.05 ml thiamin [1mg/ml].

5X A-salts: 52.5 g K_2HPO_4, 22.5 g KH_2PO_4, 5 g $(NH_4)\ 2SO_4$, and 2.5 g Na citrate×($2H_2O$) to 1 L with $_dH_2O$.

Buffers

S-Buffer: 10 mM Tris-HCl (pH 8.0), 50 mM NH_4Cl and 0.5 mM Mg Acetate.

R-Buffer: 10 mM Tris-HCl (pH 8.0), 50 mM NH_4Cl, 10 mM Mg Acetate, and 0.2 mM β-mercaptoethanol.

Ribosome Wash: 10% sucrose, 2M NH_4Cl, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0)

Binding Buffer: 50 mM Tris-HCl (pH 7.8), 80 mM KCl, 16 mM Mg Acetate

Wash Buffer: 25 mM Tris-HCl (pH 7.8), 60 mM KCl, 20 mM Mg Acetate

10X MOPS Buffer: 0.2 M MOPS (pH 7.0), 20 mM sodium acetate, 10 mM EDTA (pH 8.0).

RNA Resuspension Buffer: 150 µl formamide, 36 µl formaldehyde, 30 µl 10X MOPS buffer.

RNA Running Buffer: 900 ml sterile $_dH_2O$, 100 ml 10X MOPS buffer.

Alkaline Transfer Buffer: 3 M NaCl, 8 mM NaOH, and 2 mM Sarkosyl.

5X Neutralizing Buffer: 0.5M Na_2HPO_4, 0.5M NaH_2PO_4 in 1 L $_dH_2O$

Formamide Hybridization Buffer: 50% formamide, 5X SSC, 0.1 % sarkosyl, 0.02% SDS, 200 µg/ml BSA, with 1X background quencher.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

20X SSC: 3.0 M NaCl, 0.3 M Na citrate and HCl to adjust pH to 7.0.

Protein Electrophoresis Buffers

1st dimension sample buffer: 0.01 M Bis-Tris (pH 4.2), 7.6 M urea, 6mM acetic acid, 0.14 M β-mercaptoethanol in 10 ml $_dH_2O$.

1st dimension upper tank buffer: 0.01M Bis-Tris (pH 3.8) and 3.6 mM acetic acid in 1 L $_dH_2O$.

1st dimension lower tank buffer: 0.01 M Bis-Tris (pH 6.0) and 0.4 mM acetic acid in 1 L $_dH_2O$.

1st dimension separating gel solution: 0.4 M Bis-Tris (pH 5.5), 0.45 mM acetic acid, 4% acrylamide, 0.66% bis-acrylamide and 8M urea.

2nd dimension separating gel solution: 0.44M glacial acetic acid (pH 4.5), 0.44M KOH, 18% acrylamide, 0.5% bis-acrylamide and 6M urea.

2nd dimension running buffer: 0.93M glacial acetic acid (pH 4.0) and 0.93M glycine.

Reconstitution Buffers

Buffer #4: 20 mM Tris-HCl (pH 6.0), 4 mM Mg acetate, 400 mM NH_4Cl, 0.2 mM EDTA and 5 mM β-mercaptoethanol.

Buffer #5: 20 mM Tris-HCl (pH 6.0), 4 mM Mg acetate, 400 mM NH_4Cl, 0.2 mM EDTA, 5 mM β-mercaptoethanol, and 6M urea

Buffer #7: 10 mM Tris-HCl (pH 7.6) and 4 mM Mg acetate.

Buffer # 9: 110 mM Tris-HCl (pH 8.0), 4 mM Mg acetate, 4 M NH_4Cl, 0.2 mM EDTA and 20 mM β-mercaptoethanol.

Bacterial Cell Growth

Escherichia coli ribonuclease deficient strain D10-1, an RNase I mutant (Gesteland 1966), and SK901 (Mehta and Champney 2002) were used in this study. Cells were grown in either tryptic soy broth or minimal media (A salts and 0.2% glucose) (Miller 1972). Fresh media was inoculated with an overnight bacterial culture. The growth rate was measured by following the increase in cell density in a Klett Summerson calorimeter at 27°C. Neomycin, paromomycin, and streptomycin were added at a cell density of 15 Klett units. The cells were harvested after two cell doublings (60 Klett units).

Analysis of Ribosomal RNA

Primer Design

A 101 nt 16S DNA probe was generated by amplifying a region of 16S DNA from plasmid pKK3535 using the polymerase chain reaction. PCR reaction mixtures contained 45µl of PCR Supermix High Fidelity reagent (Gibco BRL), 1µl of plasmid DNA (6.5ng), 1µl (10 pmol) of 16S forward and reverse primers, and 2µl of sterile water. The forward primer was: GGAGGAAGGTGGGGATGACG and the reverse primer was ATGGTGACGGGCGGTGTG (nt. no. 1173-1414) from Life Technologies. Samples were amplified for 35 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec. The PCR product was purified by extraction with an equal volume of phenol and chloroform and precipitated with 2 volumes of ethanol. Single stranded DNA probes for 16S rRNA precursor sequences were purchased from One Trick Pony Corporation. The sequence for the 5' precursor is: 5'-CGC TCA AAG AAT TAA ACT TCG-3' and the 3' precursor is: 5'- ACG CTT CTT TAA GGT AAG G-3' (Figure 10). The PCR product and precursor probes were resuspended in sterile water and labeled with biotin using the Label-IT biotin labeling kit (Mirus).

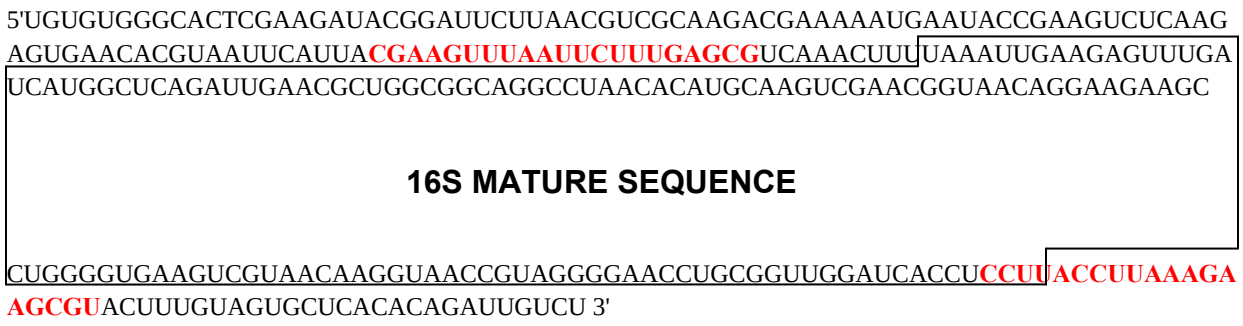


Figure 10 Upstream and Downstream Regions of 16S RNA Sequence. Nucleotides in red represent the probes for the precursor region. The 5' probe begins +10 nt upstream of the mature sequence. The 3' probe overlaps with the last four nucleotides of the mature sequence.

Slot Blot Hybridization

E. coli cells were harvested after two cell doublings by centrifugation in a Beckman centrifuge (J2-21) at 6,000 rpm for 12 minutes in a JA21 rotor. Cell pellets were washed with sterile S-buffer. Washed pellets were centrifuged again at 6,000 rpm for 12 minutes. The cell pellets were stored at -70°C before cell lysis.

Cells were lysed with lysozyme by the freeze thaw method. Washed cell pellets were thawed at room temperature and resuspended in 200 µl of S- buffer and 15 µl of a 5 mg/ml solution of lysozyme was added. Cells were allowed to incubate at room temperature for 15 to 20 minutes and were then subject to a freeze-thaw process. Cells were frozen for 5 minutes at -70°C and then thawed at room temperature. This procedure was repeated twice. DNA was digested in

cell lysates by adding 2 units of DNAase I to each sample. The samples were centrifuged at 6,000 rpm for 12 minutes.

Cells lysates were added to 5-20% sucrose gradients and spun at 39,000K for 5.5 hrs in a SW40 rotor. RNA for slot blot hybridization was prepared from sucrose gradient fractions. Any DNA that would present contamination for the slot blot analysis would have been separated on the gradient and not present in 30S and 50S subunit fractions. Each fraction from the sucrose gradient was digested with 10 µg of proteinase K (Fluka) in 0.1 % SDS for 2 hours at 44°C followed by ethanol precipitation (2 volumes) and resuspended in 0.2 mL of $_{d}H_2O$. RNA from each fraction was applied to a nylon membrane (Nytran) using a slot blot apparatus. The membranes were washed with 0.5 mL of TE buffer and RNA was cross linked using a UV oven. The membranes were placed in 50 ml plastic corex tubes with 15 ml of 1X pre-hybridization solution (MRC, Inc.) and allowed to incubate at 42°C for 30 minutes in a hybridization incubator (Fisher-Biotech). The pre-hybridization buffer was discarded and the membranes were hybridized with the biotinylated 16S DNA probe overnight at 42°C with 4 pmol of denatured 16S probe in 7 ml of hybridization buffer and 1X background quencher (MRC Inc.). The probe was denatured by mixing with 0.1 volume of Mirus Denaturation Buffer D1 and incubating at room temperature for 5 minutes. The solution was chilled on ice and mixed with 0.1 volume of Mirus Neutralization Buffer N1 and incubated at room temperature for 5 minutes.

The probe was detected using the North to South Chemiluminescent Detection Kit (Pierce) using a horseradish peroxidase strepavidin conjugate for biotin detection. The membrane was covered with plastic wrap on a glass plate and exposed to Fuji Medical X-ray film. X-ray film was developed by soaking the film in Kodak GBX developer for 1-5 minutes, rinsing in H_2O, soaking in fixer and replenisher for 1-5 minutes, followed with a final rinsing in H_2O. The average spot intensity for each fraction was quantified by scanning the film using an Alpha Innotech image analysis system.

Northern Blot Analysis

Total RNA was isolated from *E. coli* cells using the Aqua Pure (Bio-Rad) RNA isolation kit. Total RNA (5 µg) from cells treated with or without antibiotic was denatured by heating at 55°C for 15 min and electrophoresed on a 1.8% agarose gel for 2.5 hours (Farrell 1993). RNA was detected by Northern blot analysis. The RNA was blotted onto nylon membranes using a Turboblot apparatus. RNA transfer was performed using an alkaline transfer buffer for 2 hours. After the transfer, gels were checked for RNA by ethidium bromide staining. The membrane was neutralized in 1X neutralizing buffer and the RNA was cross-linked to the membranes using a UV oven (Fisher-Biotech). Hybridization was performed as described for the slot blot experiment except the membranes were hybridized separately for 20 hrs with the 5', 3', and 16S probes at 32°C with hybridization buffer. The membranes were then washed in 6X SSC for 15 min. The second stringency wash contained 0.1X SSC and 0.5% SDS for

15 min at 50°C. Hybridization and stringency wash temperatures were performed at 42°C for the 16S probe. The North to South Chemiluminesent Kit was used for probe detection as previously described.

Two-Dimensional Polyacrylamide Gel Electrophoresis

Cells were grown in A salts minimal media (100 ml) with ^{35}S-methionine (5µCi/ml) and supplemented with 0.4 µg/ml of 19 amino acids excluding methionine. Radio labeled cell lysates were spun through 5-20% R buffer sucrose gradients as previously described for RNA analysis. Fractions containing the precursor region and 30S subunits were collected and pooled. To detect any contamination, the pooled region was respun on a sucrose gradient and the precursor region was collected. Non-radio labeled 30S subunits (100µg) were added to the pooled fractions as a carrier. Total ribosomal proteins were isolated by treating subunits with 0.1 volume of 1M Mg Acetate and 2.2 volumes of glacial acetic acid for 45 min at -20°C to extract the RNA. Next samples were spun at 10,000 rpm for 30 min. The RNA pellet was discarded and the ribosomal proteins were precipitated from the supernatant with 5 volumes of acetone for 3 hrs at 4°C. Following centrifugation at 10,000 rpm for 30 min, the pellet was dried and resuspended in sample buffer. The labeled proteins separated by two-dimensional gel electrophoresis as previously described (Geyl and others 1981; Usary and Champney 2001) the proteins were detected by Comassie Blue staining. The protein spots were excised from the gel, digested with 0.5 ml 30%

hydrogen peroxide at 85°C for 3 hrs and the amount of radioactivity was determined by liquid scintillation counting. Data were normalized by dividing by the number of methionine residues for each protein (Giri and others 1984).

Aminoglycoside Radio Labeling

Neomycin and paromomycin were radio labeled by reductive methylation with ^{3}H-formalydehyde as previously described (Champney 1989). During the reductive methylation process protons on the amino groups of the aminoglycoside were replaced with tritiated methyl groups from the $^{3}HCOH$. Antibiotics were incubated at 37°C for 30 minutes under the following conditions: neomycin and paromomycin (100µg), ^{3}H formaldehyde (20nmol), $NaCNBH_3$ (20µg), and 10mM $NaPO_4$ buffer pH 6.6. The labeled antibiotic was separated from free formaldehyde by chromatography on a Bio-Gel P2 column in 0.1M NH_4 acetate buffer. Fractions containing the labeled drug were pooled, lyophilized and resuspended in 200 µl of water. The specific activity for ^{3}H-neomycin and ^{3}H-paromomycin was 3.3mCi/mol, which was determined by measuring 2µl of the sample by liquid scintillation counting.

Thin layer chromatography was performed to determine the number of methylated amino groups (Roets and others 1995). Paromomycin and neomycin (labeled and unlabeled) were dissolved in water at a concentration of 4.0 mg/ml and 1.0 µl of each sample was applied to a cellulose TLC plate. The mobile phase solution consisted of methanol-20% sodium chloride (vol/volume) (15:85).

The mobile phase solution was equilibrated for at least 1 hour before use and the mobile phase ascended over 12 cm in 2 hours. The plate was dried and fluorography was performed by adding scintillator solution consisting of 7% (weight/volume) solution of 2,5-diphenyloxalazole (scintillation grade) in ethanol that was poured over the chromatograph as described (Toutchstone and Dobbins 1983). The chromatograph was used to expose x-ray film at -70°C for 2 days. Figure 11 shows the autoradiograph of the labeled neomycin and paromomycin. In addition to radio labeling, ^{3}H-paromomycin was also purchased from Moravek Biochemicals (0.6 Ci/mmol)

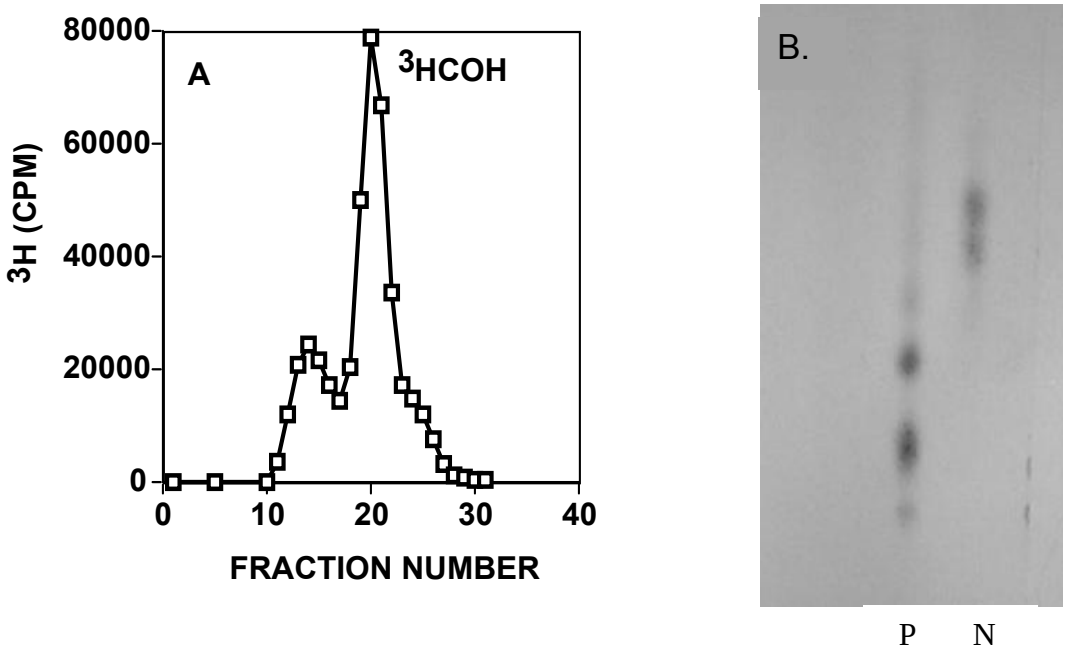


Figure 11 Identification of Radiolabeled NH_3 Groups of ^{3}H-Paromomycin and ^{3}H-Neomycin. A.) Radioactive profile of fractions collected from a Bio Gel P2 column containing ^{3}H-paromomycin. The smaller peak represents radio labeled

antibiotic and the larger free ^{3}H-formaldehyde. B.) Thin layer chromatography of aminoglycosides prepared by reductive methylation. P represents ^{3}H-paromomycin and N, ^{3}H-neomycin.

In Vivo Binding Assay

E.coli cells were grown in minimal media in the presence of ^{3}H-neomycin or ^{3}H-paromomycin (0.5µCi/ml) 6µg/ml, and streptomycin at 2µg/ml. The antibiotics were added to cells at a Klett of 20, corresponding to 1.6 x 10^{8} cells/ml. The cells were grown for two cell doublings and immediately harvested and lysed by the freeze thaw method as described above. Cell lysates were spun through 5-20% S buffer sucrose gradients for 5.5 hrs at 39,000 rpm in a SW40 rotor. Fractions were collected using an ISCO UV absorbance monitor and the amount of ^{3}H-antibiotic binding was measured by liquid scintillation counting.

In Vitro Binding Assay

30S, 50S, and p30S particles were isolated from sucrose gradients of cell lysates as described for protein analysis. Subunits and precursors were pelleted in a Ti50 rotor at 45,000 rpm for 16 hrs. The 21S precursor particle was dialyzed against 100 ml of R buffer to remove bound antibiotic. Increasing amounts of ^{3}H-paromomycin (Moravek Biochemicals) were incubated with 5 pmoles of p30S, 30S, or 50S in binding buffer at 37°C for 15 minutes. Particles with bound antibiotic were captured on a 0.45 µm Millipore filter and washed with 10mL of

wash buffer. Bound ^{3}H-paromomycin was detected by liquid scintillation counting.

Ribosomal Subunit Reconstitution

Isolation of RNA and Proteins

Ribosomal RNA was isolated from 30S subunits. These subunits were from sucrose gradient lysates that were pelleted at 45,000 rpm for 18 hrs in a Ti50 rotor. The pellets were resuspended in 1 ml of R buffer. The RNA was extracted using 0.1 volumes of 10% SDS and 1.2 volumes of phenol. The mixture was vortexed for 8 min and spun at 10,000 rpm for 10 min. Two volumes of ethanol was added to the aqueous phase to precipitate RNA. After 2 hrs at - 20°C the RNA was pelleted by centrifugation at 9,000 RPM for 45 min and resuspended in buffer #7.

Total ribosomal proteins were isolated from 30S subunits by adding 0.1 volume of 1M Mg Acetate and 2.2 volumes of glacial acetic acid for 45 min at - 20 ° C, followed by centrifugation at 10,000 rpm for 30 min. The RNA pellet was discarded and the ribosomal proteins were precipitated from the supernatant by adding 5 volumes of acetone for 3 hrs at 4°C. Following centrifugation at 10,000 rpm for 30 min, the pellet was dried and resuspended in 1 ml of buffer #5. The proteins were dialyzed for 2 hrs against 100 ml of buffer #4.

30S Subunit Reconstitution

Reconstitution was performed as previously described (Spedding 1990). ^{3}H-16S rRNA was isolated from cells grown with 1µg/ml ^{3}H-uridine. In order to halt further isotope incorporation after two cell doublings, uridine (50 µg/ml) was added in excess to each culture. After a 15 min chase period, ^{3}H-30S subunits were isolated from sucrose gradients and the ^{3}H-16S rRNA was extracted as described above. Then 22 µg of ^{3}H-16S rRNA and 200µg of total 30S ribosomal proteins were incubated at 40°C in buffer # 9 for 20 min and immediately added to ice. To examine assembly inhibition *in vitro*, neomycin and paromomycin were added to the reconstitution mixture at final concentrations of 0.04, 0.2, and 0.4µM. Mixtures were then layered on 5 to 20% reconstitution buffer sucrose gradients and centrifuged at 39,000 rpm for 5.5 hr in a SW40 rotor. Gradient fractions were collected and counted by liquid scintillation.

CHAPTER 3

RESULTS

Inhibition of Bacterial Cell Growth

E.coli cells were grown at 10μg/ml of neomycin or paromomycin, which was determined in previous studies (Mehta and Champney 2002), for experiments examining the assembly inhibition. In the absence of the antibiotics cell growth continued exponentially (Fig 12A). Cell growth with 10μg/ml of neomycin or paromomycin decreased the growth rate by more than 50%, with neomycin having a greater inhibitory effect. In certain experiments, streptomycin was used to enhance the binding of neomycin and paromomycin to ribosomal subunits (Lando and others 1978). Streptomycin is an inhibitor of protein synthesis, so at 2 μg/ml there was a small lag in cell growth compared to cells grown without antibiotic (Figure 12B). Cells grown with streptomycin in combination with neomycin or paromomycin, were still inhibited by 50%.

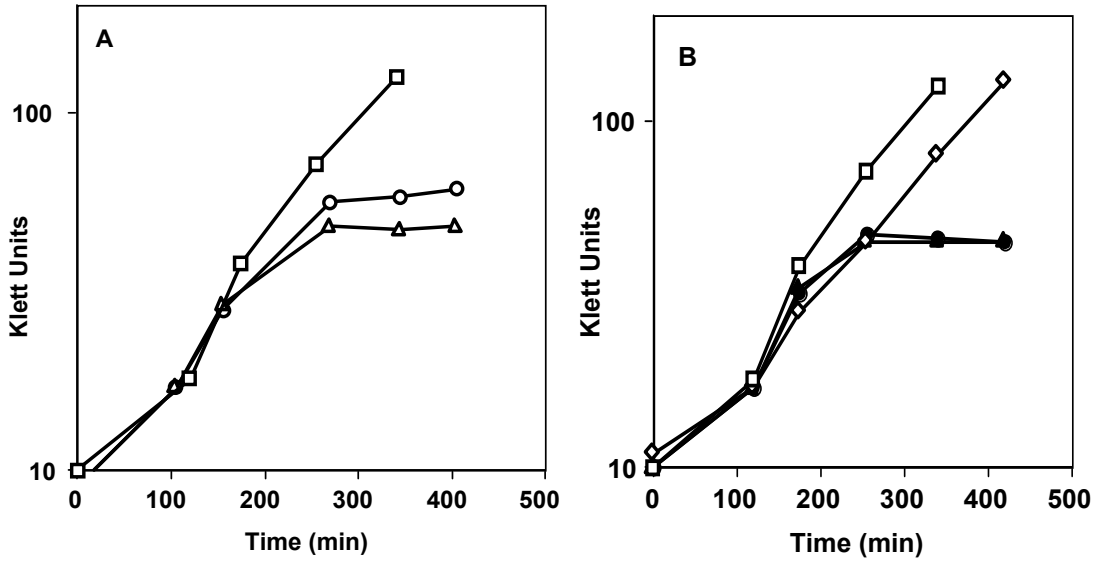


Figure 12 Cell Growth of *E.coli* SK901 Growing in the Absence and Presence of Neomycin, Paromomycin, and Streptomycin. A.) Cells were grown in the absence of the antibiotic (□), or with neomycin (10µg/ml) (∆), or paromomycin (10µg/ml) (○). B.) Cells grown with streptomycin alone at 2µg/ml (◊), or with streptomycin and neomycin (▲), or with streptomycin and paromomycin (●), or no antibiotics (□).

Identification of the 30S Ribosomal Assembly Intermediate

Mehta and Champney showed cells treated with neomycin and paromomycin produced a precursor particle with a sedimentation coefficient of 21S (2002). The intent of this experiment was to determine if this precursor particle could be successfully isolated from cells for further analysis. Sucrose gradient profiles show 30S assembly inhibition (Figure 13) and the accumulation of a p30S particle in cells grown with paromomycin or neomycin. Cells were

lysed and separated under high magnesium conditions (10mM) to ensure complete separation of the precursor from mature subunits. In the presence of the antibiotics, immature ribosomes are unable to associate with 50S particles and prevent 70S particle formation. When treated with antibiotics there was a greater accumulation of RNA in the precursor region of the gradient. The gradient profile in Figure 13B represents pooled fractions from sucrose gradient lysates from neomycin treated cells shown in Figure 13A. Mehta and Champney have already shown that the 30S assembly intermediate sediments in this region of the gradient (2002,2003) and Figure 13B confirms that in the presence of neomycin a 21S precursor particle does accumulate in this region and can be successfully isolated. Similar results were obtained using paromomycin (data not shown).

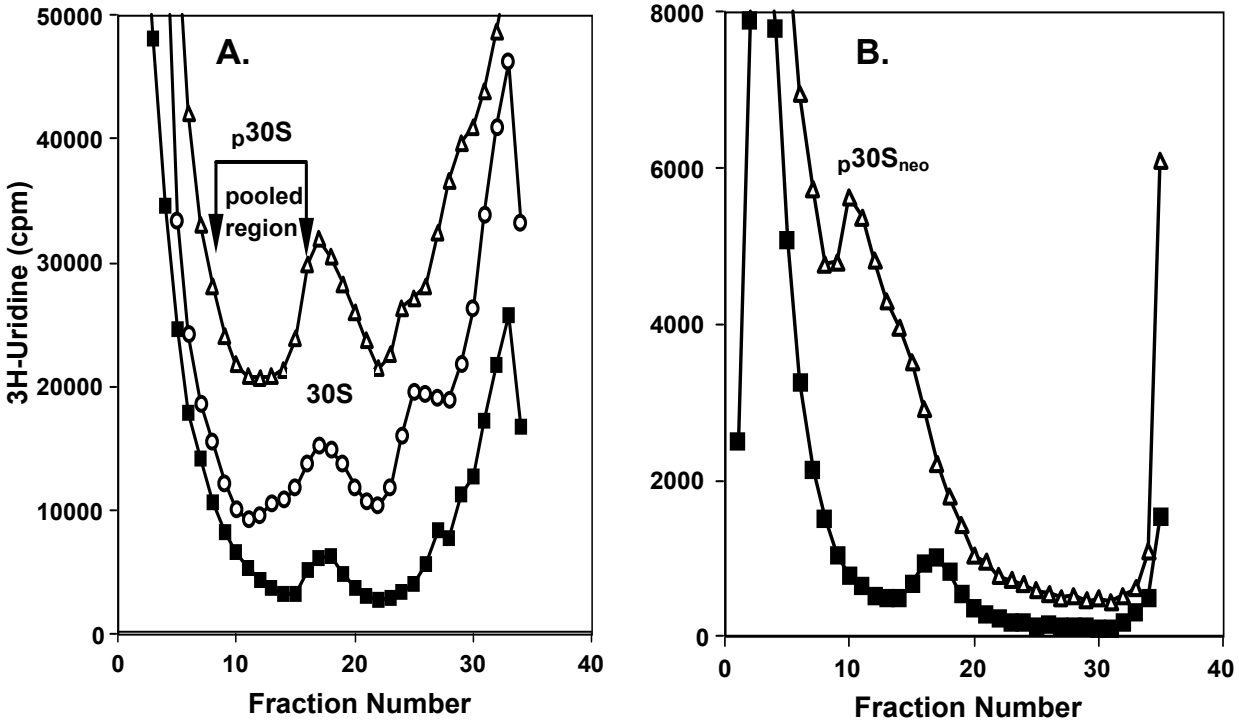
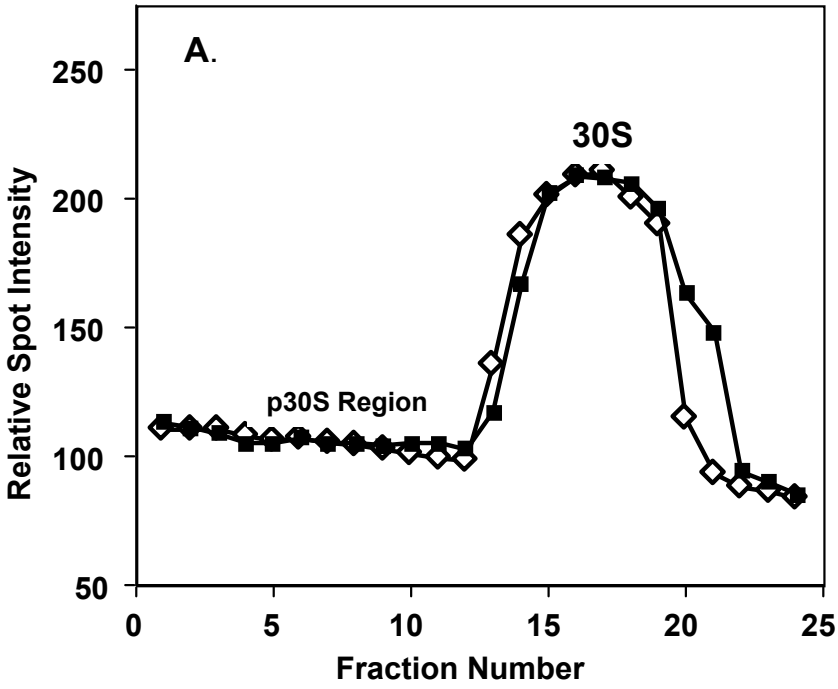
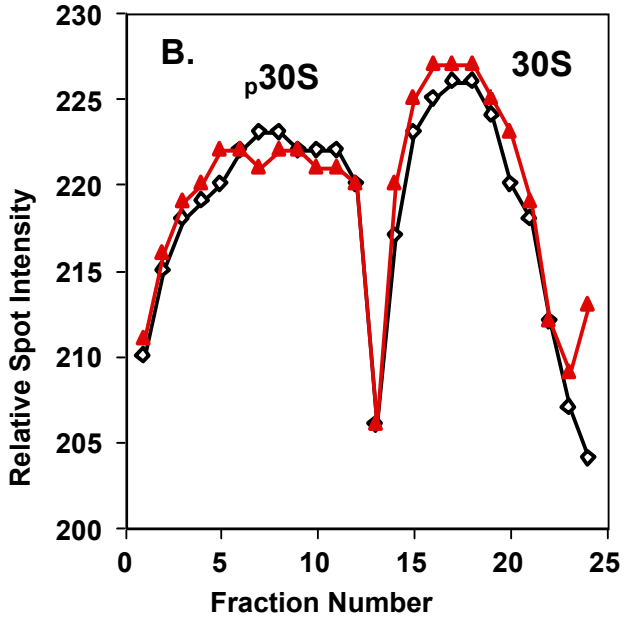


Figure 13 Sucrose Gradient Profiles of *E.coli* SK901 Treated With Neomycin or Paromomycin. A.) Sucrose gradient profiles of cell lysates. Cells were grown in the absence of the antibiotic (■), or with neomycin (10µg/ml) (Δ), or paromomycin (10µg/ml) (○).The cells were lysed and layered on R buffer sucrose gradients and ^{3}H-uridine incorporation determined by liquid scintillation. B.) The p30S$_{neo}$ assembly intermediate (Δ) collected from fractions 9-15 of the gradient from neomycin treated cells (Figure 13A). The same fractions were collected for the p30S particle from untreated cells (■).

Identification of 16S RNA in the 21S Intermediate

To substantiate that the p30S assembly intermediate was indeed a precursor to the 30S subunit, the rRNA content was examined in cells grown with neomycin and paromomycin. RNA isolated from sucrose gradient fractions of cell lysates treated with or without antibiotic was hybridized with a 16S DNA probe, and the signal was negative in the precursor region for control cells and cells treated with streptomycin (2μg/ml). While streptomycin is an inhibitor of protein synthesis, it has no effect on assembly inhibition (Figure 14A). Relative spot intensities of 16S rRNA hybridization of fractions from cells treated with neomycin (Figure 14B) shows the presence 16S rRNA in the p30S and 30S subunit regions of the gradient. Similar results were also found in cells treated with paromomycin, but with lesser amounts of 16S hybridization to smaller RNA fragments that sediment in fractions 1-5 of the gradient (Figure 14C).


A.
30S
p30S Region
Relative Spot Intensity
Fraction Number
250
200
150
100
50
0
5
10
15
20
25


B.
p30S
30S
Relative Spot Intensity
Fraction Number
230
225
220
215
210
205
200
0
5
10
15
20
25

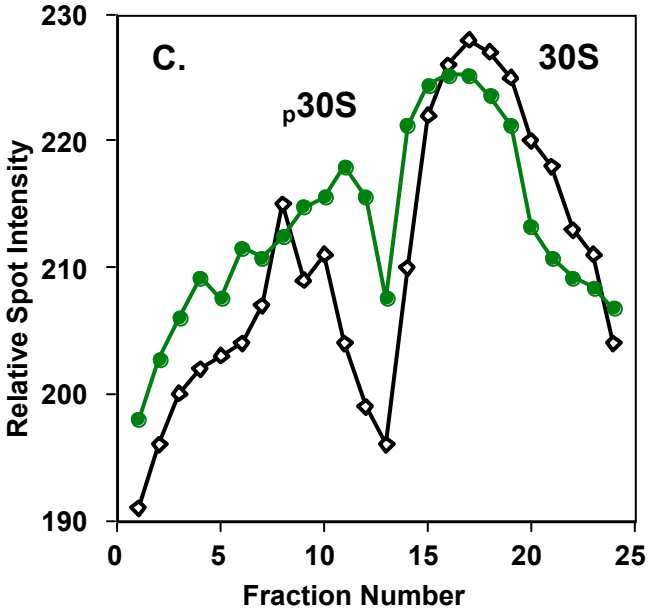

C.
30S
p30S
Relative Spot Intensity
Fraction Number
230
220
210
200
190
0
5
10
15
20
25

Figure 14 Hybridization Analysis of Sucrose Gradient Fractions From Antibiotic Treated and Control Cells. Slot blots of 16S rRNA hybridization of RNA isolated from sucrose gradient fractions of cells grown with or without antibiotic. A.) Cells grown without antibiotic (■) or with streptomycin (2μg/ml) (◊). B.) Cells grown with neomycin alone (10μg/ml) (▲), or with neomycin and streptomycin (2μg/ml) (◊). C) Cells grown with paromomycin alone (10μg/ml) (●), or with paromomycin and streptomycin (2μg/ml) (◊).

Northern Hybridization Analysis of 16S and Precursor RNA

Maturation of 16S rRNA is coupled with 30S subunit assembly and unprocessed RNA are not present in a mature functional 30S subunit. However, an accumulation of precursor rRNA occurs in cells defective in 30S or 50S subunit assembly (Himeno and others 2004; Kaczanowska and Aulin 2005). Therefore, gel electrophoresis was employed to examine the type of rRNA species in the p30S intermediates isolated from drug treated cells. Total RNA from drug treated cells was analyzed on a 1.8% agarose gel (Figure 15A). Lane 1 contains mature 16S RNA isolated from 30S subunits. Precursor RNA as well as mature 16S RNA was identified in control cells (Lane 2). The cells were grown at a lower than normal temperature (27°C) and because 16S maturation is coupled with assembly, small amounts of natural precursor (p30S) are available. A band slightly larger than 16S was identified in cells treated with neomycin and

paromomycin (Lanes 3 and 4) corresponding to the position of 17S RNA. The amount of 16S remained the same and treatment of cells with either antibiotic had no effect on 23S rRNA (Lanes 2-4).

Northern hybridization was performed using probes specific for the 3' and 5' precursor sequences of 17S rRNA to determine the effects on RNA processing. Hybridization with the 5' probe to RNA from the gel shown in Figure 15A identified immature RNA in both antibiotic treated and untreated cells (Figure 15B). However, greater amounts of 17S rRNA were observed with neomycin and paromomycin (lane 3 and 4) using the 5' probe. In contrast, 17S RNA detected using the 3' probe was seen only in antibiotic treated cells with increased levels in the presence of paromomycin (lane 4). The reduced amount of 16S RNA in drug treated samples was probably a result of RNA degradation.

Densitometry measurements of the relative amounts of RNA from Northern blots hybridized with mature and precursor 16S probes are shown in Figure 16. The amount of 16S RNA remained the same in absence and presence of antibiotic. In the absence of antibiotic, there was a greater amount of 5' precursor compared to 16S RNA and 3' immature RNA in drug treated cells. However compared to non-treated cells, treatment with neomycin caused a 2.5 fold increase in 3' precursor and a 1.5-fold increase in 5' RNA. In the presence of paromomycin, there was a 4.5 and 1.7 fold increase in 3' and 5' precursor RNA respectively.

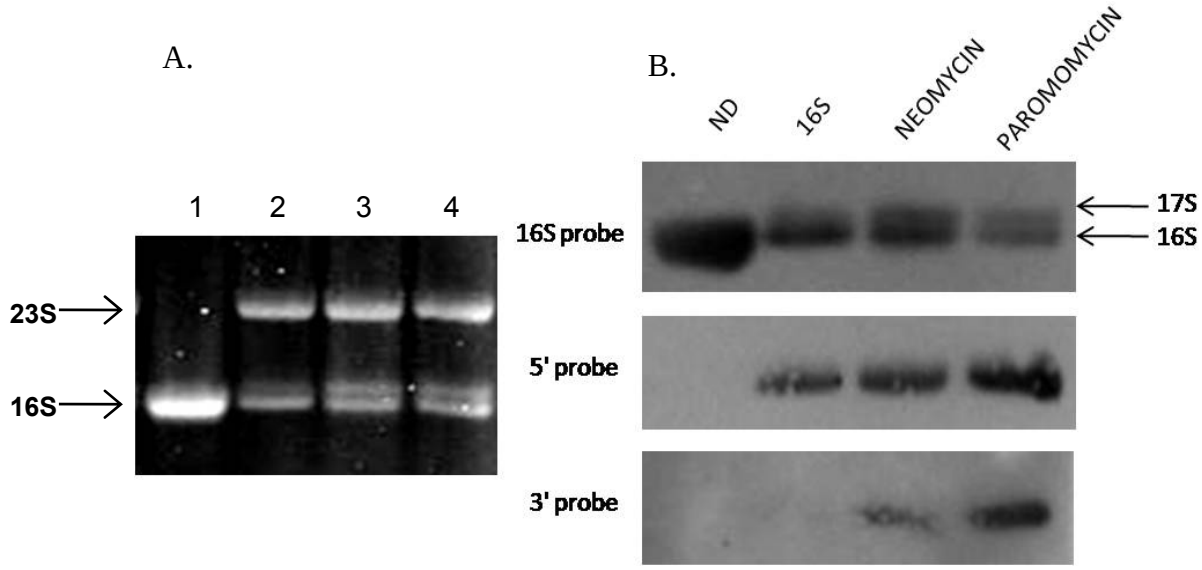


Figure 15 Identification of immature 17S rRNA of the 21S ribosomal assembly intermediate. A.) Agarose gel of rRNA from antibiotic treated cells showing the size of rRNA present in cells treated with or without antibiotic. Total RNA (5µg) was electrophoresed on a 1.8% MOPS-formaldehyde agarose gel for 2.5 hrs at 50 volts. 16S rRNA was identified in untreated and drug treated cells (lanes 2-4) as compared to mature 16S RNA isolated from 30S subunits (lane 1). The band above the 16S RNA represents the position of 17S RNA (lane 3 and 4). B.) A Northern blot of RNA from the agarose gel in 15A after hybridization with 5' and 3' probes specific for 17S RNA and an internal 16S probe.

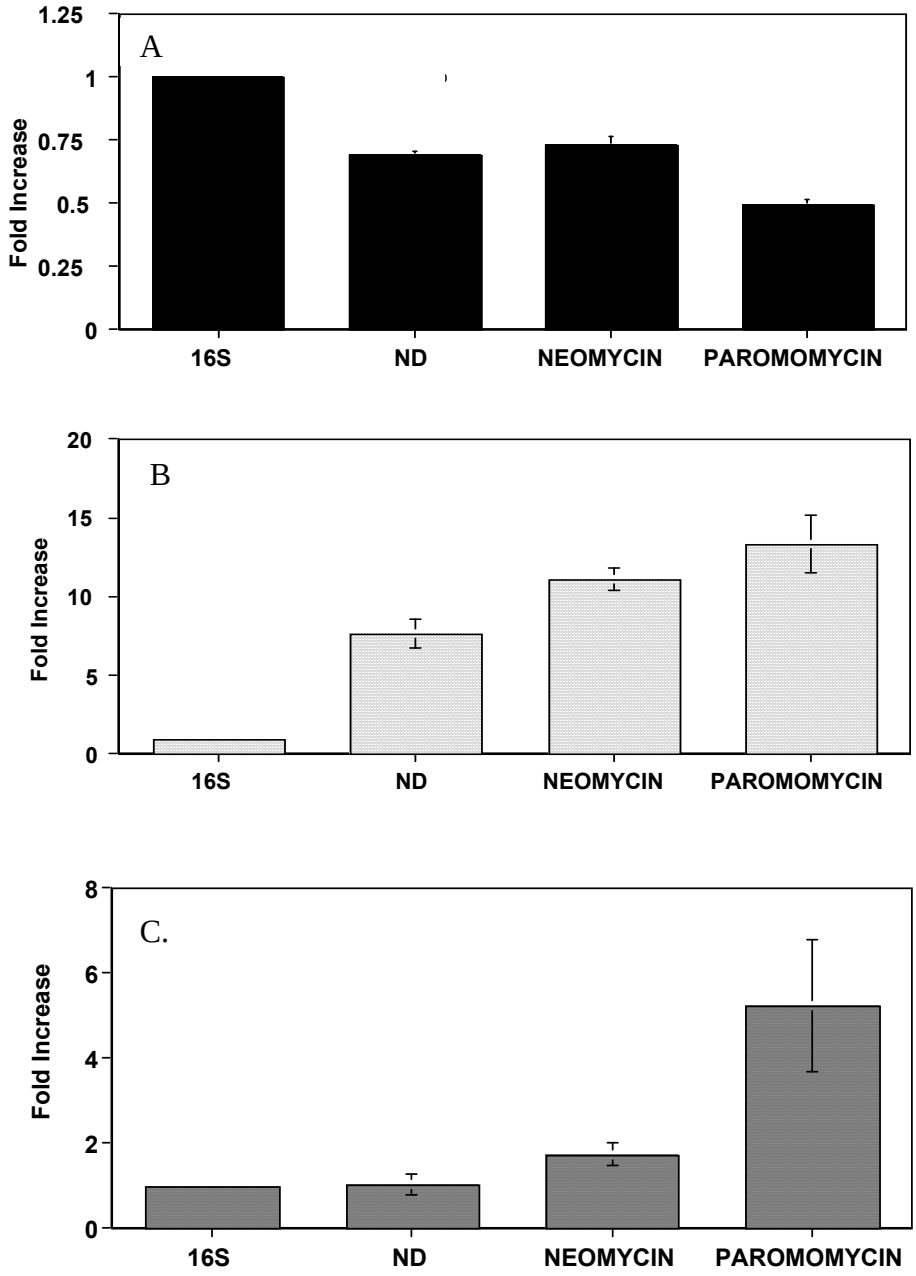


Figure 16 Relative Amounts of 17S rRNA from Cells Treated With Neomycin and Paromomycin. The relative intensity from integration of Northern blots hybridized with mature and precursor 16S probes are shown as a fold increase. The amount of 16S rRNA was set as one. A.) 16S probe B.) 5' precursor probe C.) 3' precursor probe. Error bars are SEM of three determinations.

Analysis of the *In Vivo* 21S Intermediate Ribosomal Protein Composition

Sequential addition of 30S ribosomal proteins *in vitro* revealed that a subset of proteins was critical to initiate folding of 16S rRNA. The organization and presence of these proteins alters the conformational state of the ribosomal complex. In addition, the alternate and transient conformations that are seen *in vitro* are likely to occur during assembly *in vivo* (Culver 2003). Characterizing the RNA structure and protein composition is critical for understanding the conformation of the p30S intermediate from drug treated cells. Two-dimensional gel electrophoresis was employed to identify which proteins were present in the p30S intermediate from drug treated cells. The p30S region was isolated from sucrose gradients of cells grown with ^{35}S-methionine treated with or without antibiotic as described in Figure 13. Proteins from p30S, $p30S_{neo}$, and $p30S_{paromo}$ were isolated and resolved on an 18% polyacrylamide two-dimension gel (Figure 17). Liquid scintillation counting of excised protein spots indicated an increase in radioactivity of fractions 9-16 pooled from the precursor region of sucrose gradients treated with antibiotic as compared to untreated samples. In the absence of antibiotic, cells are naturally producing some precursor; therefore, proteins present in these cells served as the background. For antibiotic treated cells, ^{35}S-methionine incorporation into proteins identified in this region equal to or above non-drug samples were considered present. Of the 21 30S ribosomal proteins, 11 were present in the precursor particle from cells treated with

neomycin ($p30S_{neo}$) as shown by the increase in ^{35}S-methione incorporation compared to cells without antibiotic (Figure 18A). Similar results were found in cells treated with paromomycin where 10 proteins were present. A comparison of the $p30S_{neo}$ and $p30S_{paromo}$ protein content with the p30S intermediate (Nomura 1973), and *in vitro* intermediate RI (Held 1974) is shown in Table 1.

The protein content of the $p30S_{neo}$ and $p30S_{paromo}$ was similar with the exception of S4 which was absent in the $p30S_{paromo}$ intermediate. There was a greater difference between the neomycin and paromomycin intermediate compared to the RI particle. These differences may reflect the use of mature RNA in reconstitution experiments versus precursor RNA in cells.

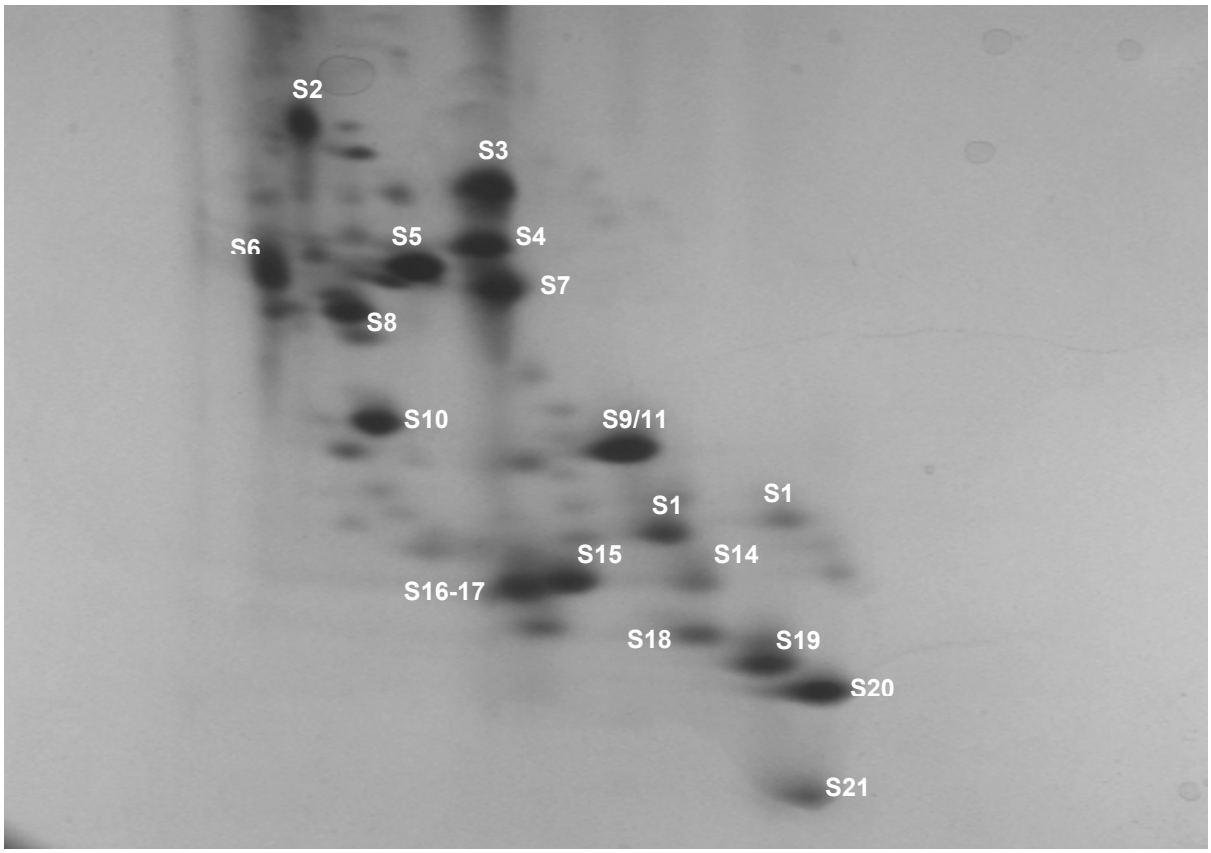
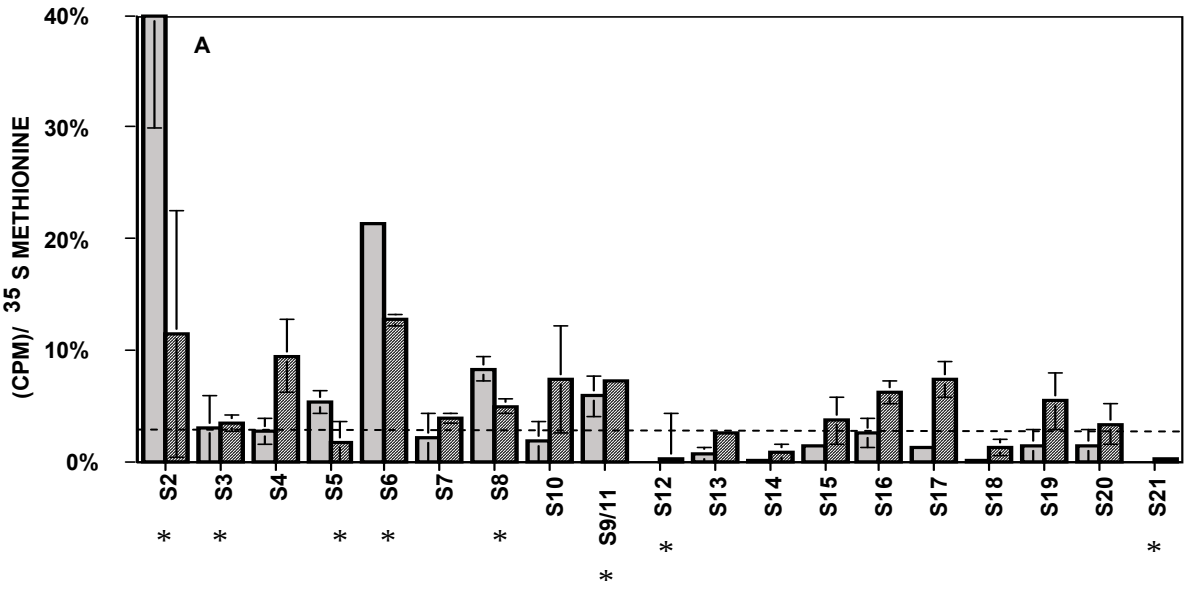


Figure 17 2D Gel Electrophoresis Pattern of 30S Ribosomal Proteins. An 18% polyacrylamide gel of ribosomal proteins from the p30S intermediate. The protein pattern was also the same for the $p30S_{neo}$ and $p30S_{paromo}$ intermediates. Prior to protein extraction, each ^{35}S-methionine labeled intermediate particle was mixed with 100 μg of non radio labeled 30S proteins for spot detection. Electrophoresis in the first dimension was 5 hrs at 100V and the second dimension 18hrs, followed by Comassie Blue staining. Each spot was excised from the gel, digested with 30% hydrogen peroxide and the radioactivity in the proteins were detected by liquid scintillation counting.



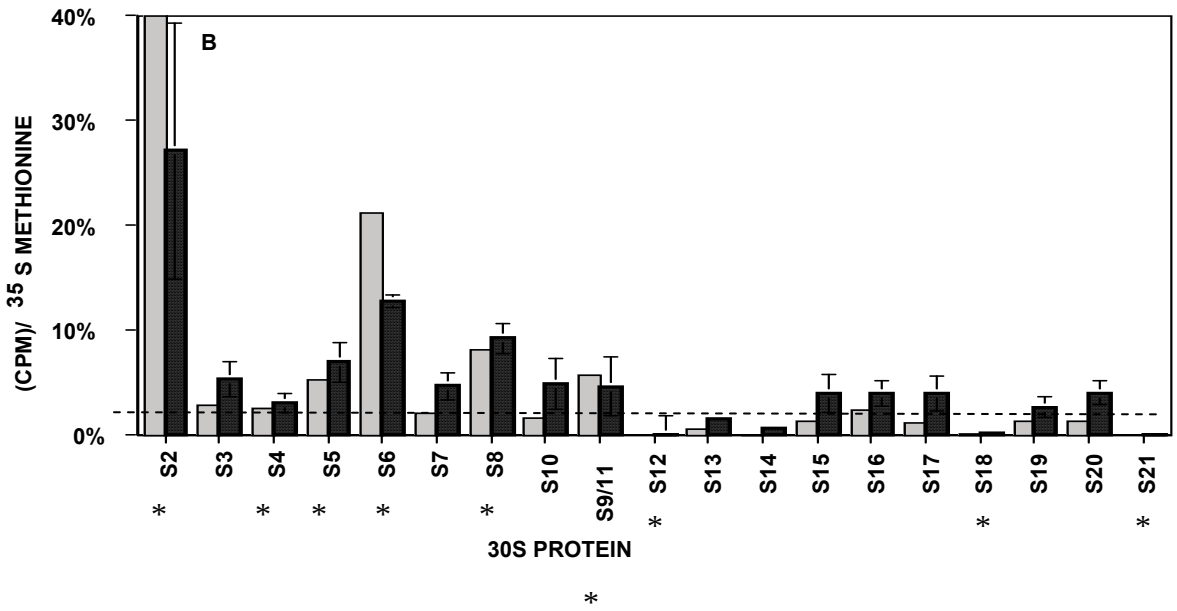


Figure 18 Radio labeled Ribosomal Proteins Present in the Assembly Intermediate from Antibiotic Treated Cells. A.) Ribosomal proteins present in the precursor particle from cells treated with neomycin (10μg/ml) or B.) paromomycin (10μg/ml). Solid grey bars represent the threshold level of radioactivity of proteins isolated from the precursor from cells without antibiotic treatment (same in A and B). A representative threshold level for each protein is represented by the dashed line. However, each ribosomal protein in the $p30S_{neo}$ and $p30S_{paromo}$ was compared to the corresponding protein from the p30S region of untreated cells. The amount of radioactivity is represented as the percent of the total radioactivity in each gel and mean of three experiments. The differences in methionine content in the ribosomal proteins were normalized by a procedure described by Giri and others by dividing the radioactivity present in each protein by the number of methionine residues for the protein (Giri and others 1984). The proteins with

an asterisk were considered to be absent in the antibiotic treated intermediates. Error bars represent the SEM of the mean of 3 gels.

Table 1 30S Ribosomal Proteins Present In The Precursor Region of Sucrose Gradients From Antibiotic Treated Cells.

Proteins that were judged to be present in the isolated 30S precursor labeled with methionine are indicated with a (+). The third column represents ribosomal proteins present in the natural 21S assembly intermediate (p30S) (Nomura 1973) and the fourth column the *in vitro* 21S reconstitution intermediate (RI) (Held 1974; Culver 2005).

Protein	Neomycin	Paromomycin	*In vivo* 21S intermediate '	*In vitro* 21S intermediate'
S1				
S2				
S3		+		
S4	+		+	+
S5				+
S6			+	+
S7	+	+	+	+
S8			+	+
S9				+
S10	+	+		
S11				+
S12				+
S13	+	+	+	+
S14	+	+		
S15	+	+	+	+
S16	+	+	+	+
S17	+	+	+	+
S18	+			+
S19	+	+	+	+
S20	+	+	+	+
S21				

Antibiotic Binding to the *In Vivo* Assembly Intermediate

Hybridization experiments (Figure 14B-C) identified 16S rRNA in the p30S ribosomal intermediates in cells grown with neomycin and paromomycin. Other analysis have shown that aminoglycosides can bind to 16S rRNA in the absence of ribosomal proteins. It is therefore likely that the p30S intermediate particle that contains a partial protein content is capable of binding antibiotic as well. *In vivo* and *in vitro* binding assays were conducted to determine if the precursor particle was capable of binding radio labeled antibiotics. Streptomycin was added to enhance neomycin and paromomycin binding (Lando and others 1976) and the data revealed that binding of paromomycin increased by 60 % (Figure 19). Figure 20 shows sucrose gradient profiles from cells grown with radio labeled antibiotics. ^{3}H-antibiotic binding to rRNA was seen in the p30S region of the gradient. As expected, the sucrose gradient profile also shows specific binding to the 30S subunit and this was comparable to the binding shown for neomycin to the 21S intermediate (Figure 20A). Paromomycin had a less inhibitory effect on cell growth and decreased binding to the 21S particle is evident in Figure 20B. Neomycin and paromomycin binding was observed to 50S subunits as well (Figure 20 A-B).

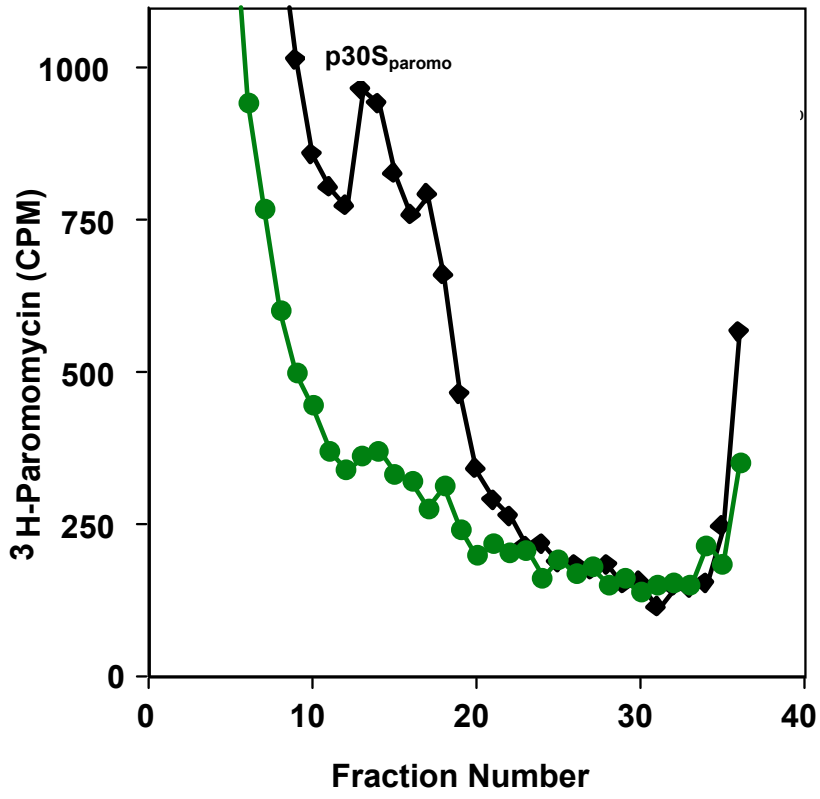


Figure 19 Streptomycin Enhances Paromomycin Binding to the *In Vivo* 30S Assembly Intermediate. Cells were grown in the presence of ^{3}H-paromomycin (4µg/ml) (●) or with streptomycin (♦) (2µg/ml) and ^{3}H-paromomycin (4µg/ml). Cell lysates were separated on 5-20% R buffer sucrose gradients. Without streptomycin maximum binding occurred at 300 cpm (●) (fraction 13) and with streptomycin at 1000 cpm (♦) (fraction 13).

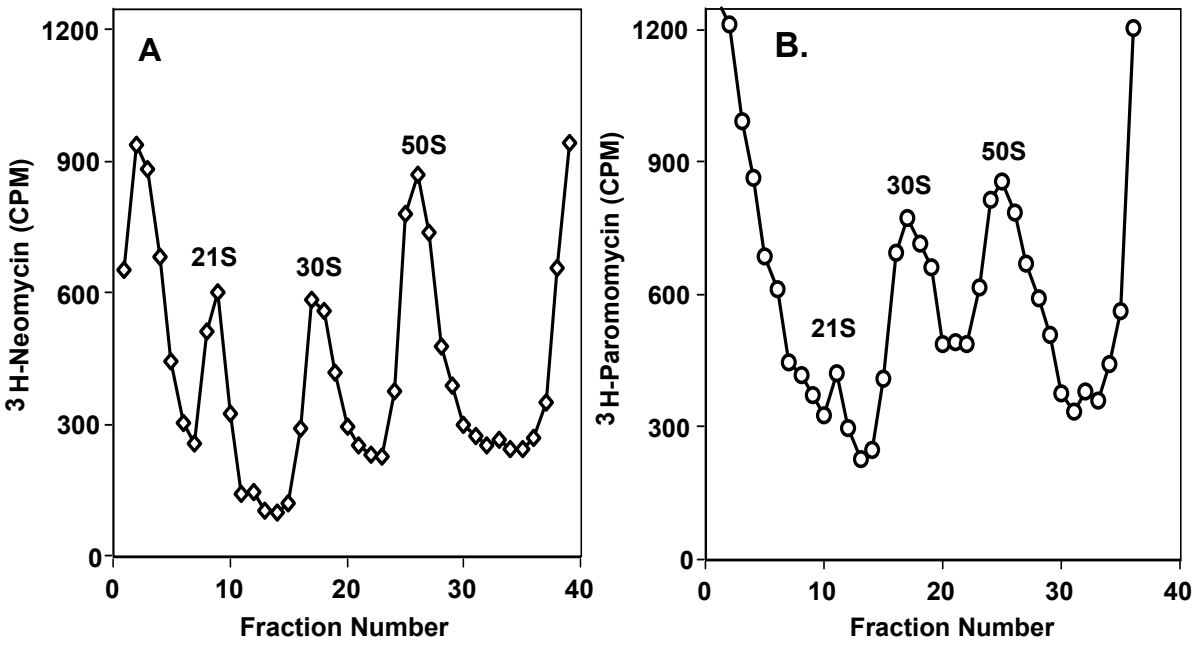


Figure 20 Aminoglycoside Binding to the *In Vivo* 30S Assembly Intermediate. Cells were grown in the presence of A.) ^{3}H-neomycin (◊) or B.)^{3}H-paromomycin (○). Non radio-labeled neomycin or paromomycin were added at 10µg/ml and streptomycin at 2µg/ml.

Filter binding assays were performed to study neomycin and paromomycin binding *in vitro*. Isolated p30S from pooled fractions (9-15) of sucrose gradients (Figure 13 A-B) were dialyzed against R buffer to remove bound antibiotic. Figure 21 shows an increased concentration dependent binding of ^{3}H-paromomycin to 50S, 30S, and p30S particles. The amount of binding correlates with the difference in size of each particle. Although binding to 50S particles was not expected, similar results were seen with paromomycin binding to streptomycin sensitive ribosomes (Lando and others 1978) and with neomycin (Dahlberg

1978; Misumi 1980). Particle binding did not reach saturation so an association constant could not be determined. Nevertheless, these results do show that the isolated p30S particle is capable of binding antibiotic with a similar affinity as 30S subunits.

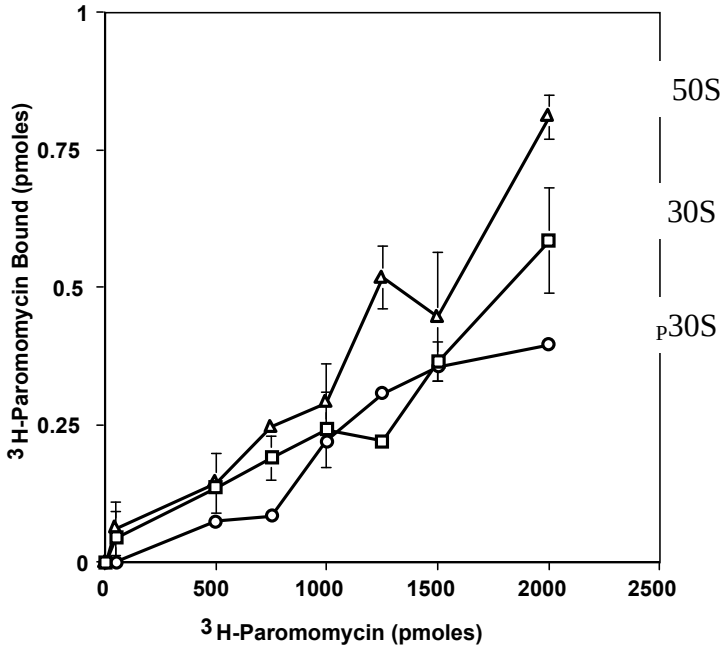


Figure 21 *In Vitro* Binding Of ^{3}H-Paromomycin to *E. coli* Ribosomal Subunits and the 21S Precursor Particle. ^{3}H-paromomycin (Moravek Biochemicals) was used. 5 pmoles of 30S (□) or 50S (Δ) ribosomal subunits or p30S (○) precursor were used. The p30S particle was isolated from cells as described in Figure 13A-B. Results are shown as SEM three determinations.

Inhibition of 30S Assembly *In Vitro*

Ribosomal reconstitution allows an in depth analysis of the functional roles of individual components in both subunit assembly and ribosome function. Studying subunit assembly *in vivo* is often difficult because genetic mutations are frequently lethal. Reconstitution can be performed using modified or mutated rRNA transcripts and recombinant proteins to examine assembly *in vivo* (Culver 2003). Therefore, 30S assembly inhibition by neomycin and paromomycin was examined using ribosomal reconstitution. Figure 22A represents the sedimentation profiles of 16S rRNA, 30S, and 50S subunits isolated from cells labeled with ^{3}H-uridine. The 21S particle (RI) was formed by reconstitution of 16S rRNA and 30S ribosomal proteins at 4°C. The fraction number corresponding to the peak for each particle was plotted versus its sedimentation coefficient and the standard in shown in Figure 22B. The sedimentation values of the intermediates produced in later reconstitution experiments were determined using the equation of the slope in Figure 22B.

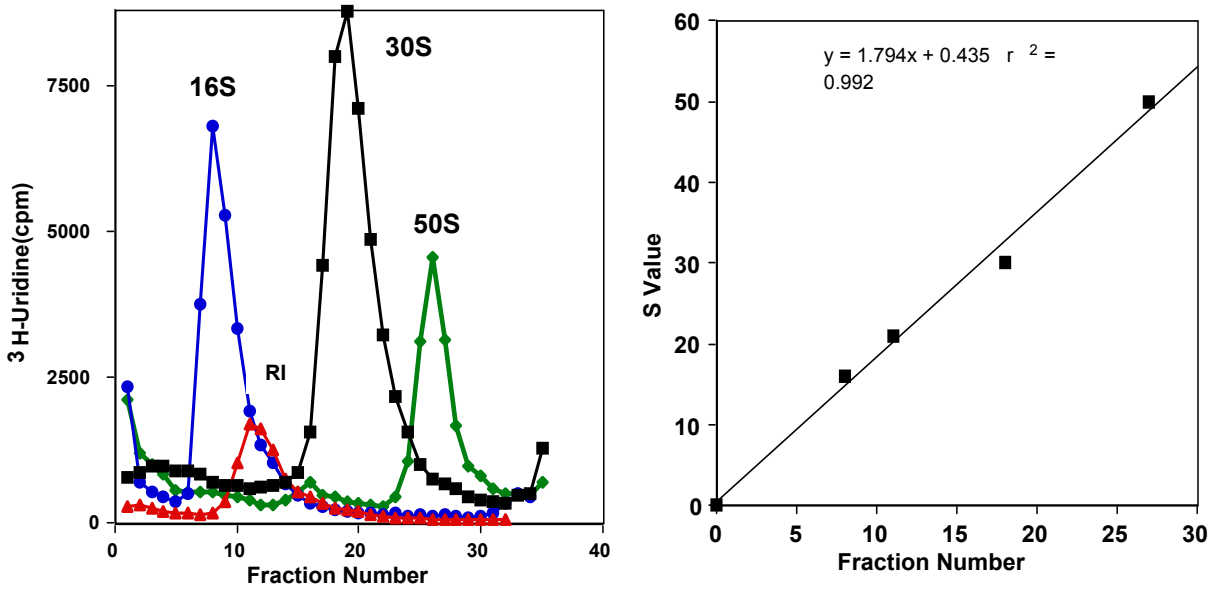


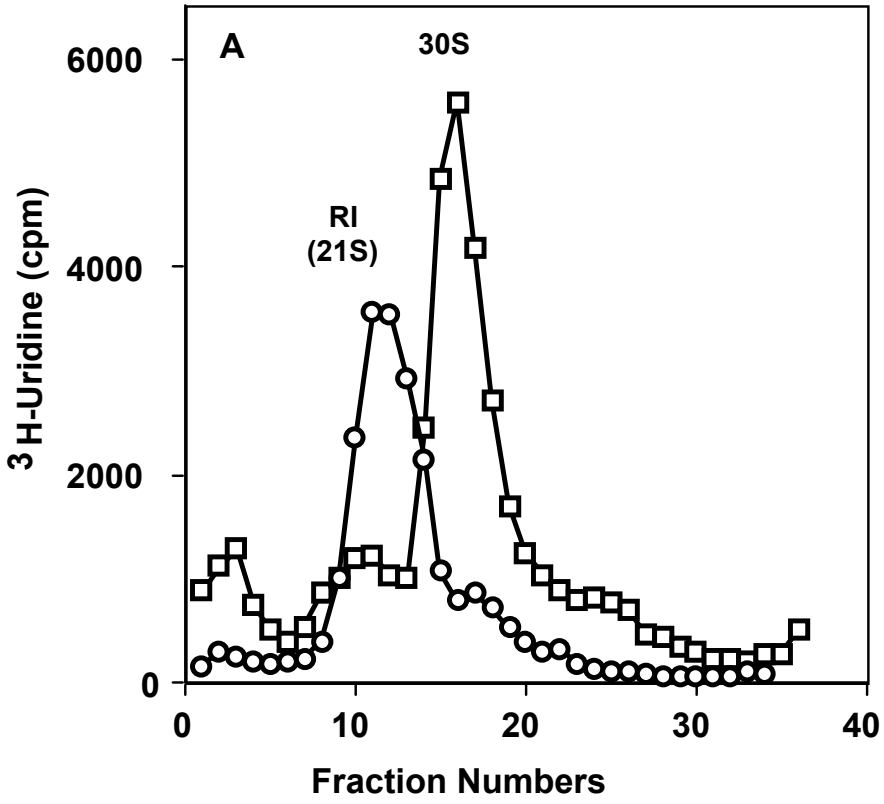
Figure 22 Sucrose Gradient Profiles and Standard Curve for Sedimentation Coefficients for Ribosomal Subunits and 16S rRNA. A.) Sucrose gradient profiles for ^{3}H-uridine labeled 16S rRNA (●), the 21S reconstitution intermediate (RI) (▲), 30S subunit (■), 50S subunit (●). The subunits and rRNA were isolated from cells and centrifuged on 5-20% S buffer sucrose gradients. B.) A standard curve showing the linearity of the sucrose gradient and sedimentation of each particle. The peak fraction from the sucrose gradient in 23A is plotted on the X-axis versus the S value of the particle (Y-axis).

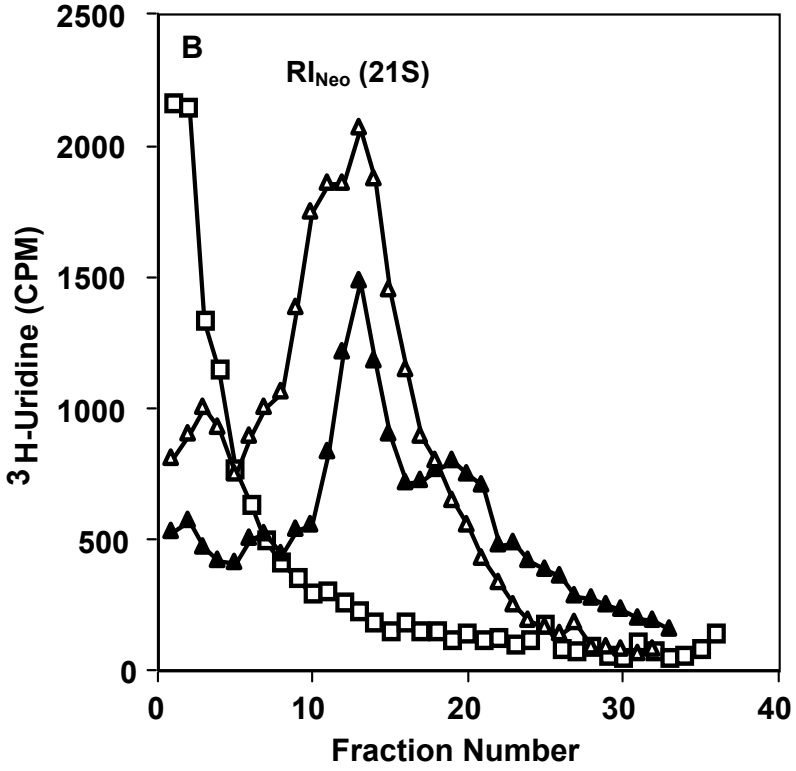
16S rRNA isolated from ^{3}H-uridine labeled cells was reconstituted with a four fold molar excess of 30S ribosomal proteins. The sucrose gradient profile in Figure 23A shows complete assembly of a 30S subunit from reconstitution performed at 40°C. The activity of the reconstituted subunits were not examined, but others have shown that reconstitution performed with the conditions

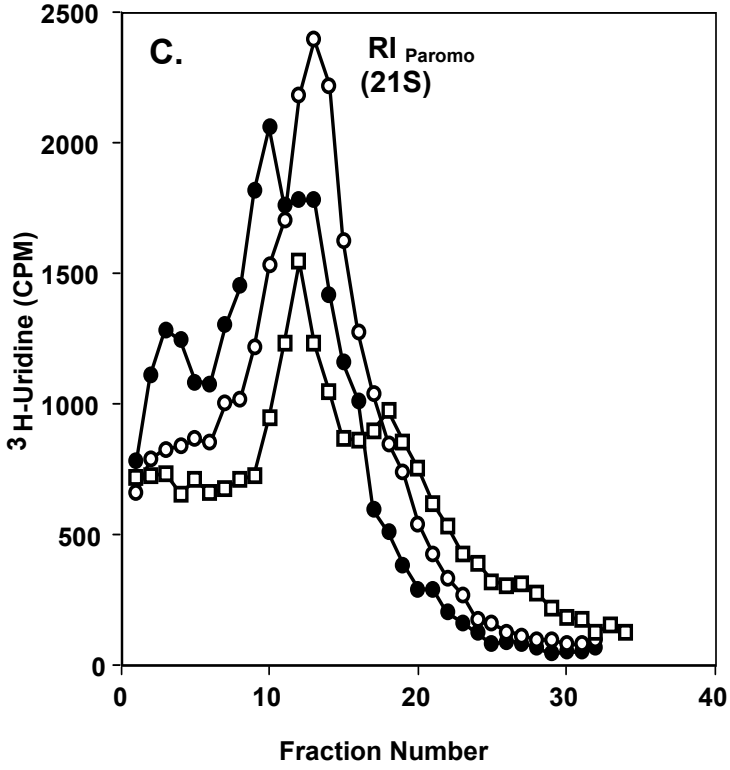
described in this study are capable of poly phenylalanine synthesis and reassociation with 50S subunits (Spedding 1990).

Traub and others have shown that reconstitution at 4°C produces a particle, RI, with a sedimentation coefficient of 21S (1971). These conditions were used to produce the RI particle in Figure 23A, which later was compared to the intermediates produced in the presence of drug. The intermediate formed at 4°C represented the maximum amount of 21S formation under the above conditions.

Figure 23B shows reconstitution of 30S subunits at 40°C with a 10,100, and 1000-fold excess of neomycin or paromomycin. Increasing concentrations of the antibiotic inhibited the reconstitution of the RNA into a 30S subunit. The intermediate produced by neomycin was RI_{neo} with a sedimentation coefficient of 21S. Reconstitution with 0.4μM neomycin did not produce an intermediate particle suggesting that the RNA and proteins were not able to assemble under these conditions. In contrast, reconstitution with paromomycin at the same concentration resulted in a 21S particle (RI_{paromo}). This result also occurred with 0.2 and 0.04μM of paromomycin.


A
30S
RI
(21S)
6000
4000
2000
0
^{3}H-Uridine (cpm)
0
10
20
30
40
Fraction Numbers


B
RI$_{Neo}$ (21S)
^{3}H-Uridine (CPM)
Fraction Number
0
500
1000
1500
2000
2500
0
10
20
30
40


C.
RI $_{Paromo}$
(21S)
^{3}H-Uridine (CPM)
Fraction Number
0
500
1000
1500
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10
20
30
40

Figure 23 Inhibition of 30S Assembly *In Vitro* by Aminoglycosides. A.) 30S reconstitution in the absence of antibiotic at 40°C (□). 0.5 A_{260} units of ^{3}H-16S rRNA and 2.0 A_{260} of total 30S ribosomal proteins were used. Reconstitution performed under the same conditions except at 4°C produced a 21S particle (RI) (○). The reconstituted particles were layered on 5-20% sucrose gradients in buffer # 7 and the profiles were determined by liquid scintillation counting following centrifugation. B.) 30S reconstitution at 40°C was performed as described in A. but in the presence of neomycin, 0.04μM (Δ), 0.2 μM (▲), and 0.4 μM (□) C.) 30S reconstitution at 40°C with 0.04μM (○), 0.2 μM (●), and 0.4 μM (□) of paromomycin.

Increasing concentration of neomycin and paromomycin resulted in a decrease in 30S subunit formation (Figure 24), suggesting that smaller amounts of RNA were able to associate into a complete particle. On the other hand, the increased amount of RI_{neo} or RI_{paromo} at the lower concentration of drug shows that a greater amount of RNA was capable of reconstituting and possibly proceeding to 30S assembly. While more precursor accumulation is seen *in vivo*, reconstitution examines RNA folding and protein association, and the factors that would limit the formation of 30S assembly.

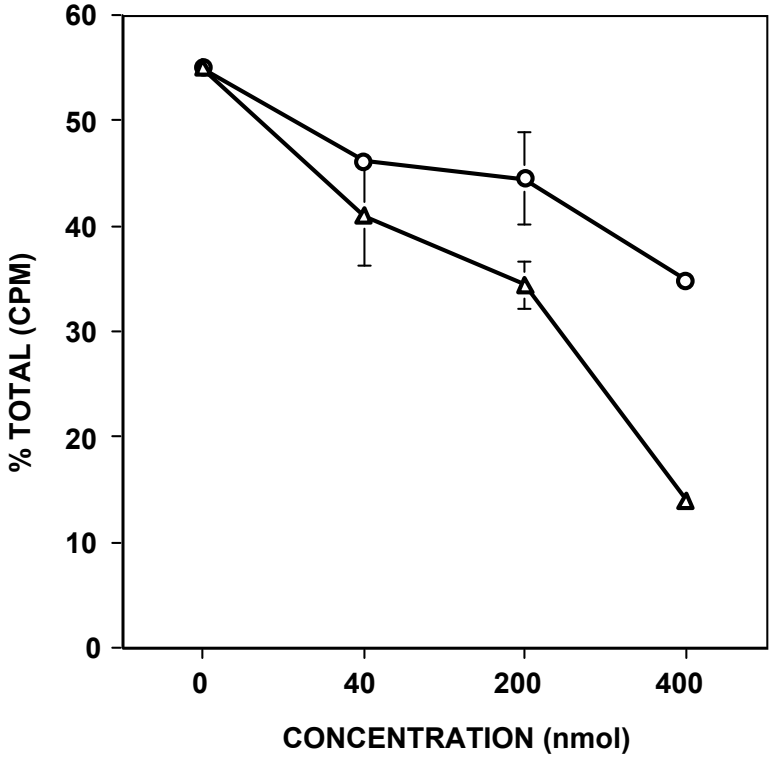

Figure 24 The Percent of 30S Formation with Increasing Concentration of Neomycin or Paromomycin. The amount of 30S formation is represented as a percent of the sum of radioactivity in the RI_{neo} and RI_{paromo} particles (fractions 9-16) and the total amount of RNA from each sucrose gradient in Figure 24. The maximum amount of precursor formed without drug is RI as shown in Figure 24A, neomycin (Δ), and paromomycin (○). Data are shown as the SEM and the mean of three experiments.

CHAPTER 4

DISCUSSION

This work supports earlier findings that showed a number of antibiotics that inhibit translation in cells also prevent ribosomal subunit assembly. Previously, erythromycin was found to inhibit ribosome assembly in *E.coli* in a fashion similar to the present studies using neomycin and paromomycin. A 50S precursor particle sedimenting in the 30S region of sucrose gradients from erythromycin treated cells contained 23S rRNA and bound antibiotic both *in vivo* and *in vitro* on the 50S subunit. In addition, the 50S precursor contained 18 of the 34 ribosomal proteins (Usary and Champney 2001). The translational inhibitors neomycin and paromomycin prevent 30S subunit assembly in a similar manner to what was observed for 50S assembly.

The intent of this work was to explore the features 30S assembly inhibition by these antibiotics. The *in vivo* precursor particle that accumulated in the presence of neomycin and paromomycin was characterized by identifying the protein composition, rRNA content, and antibiotic binding. Both antibiotics were capable of binding to the p30S assembly intermediate with neomycin having a greater affinity. Hybridization analysis confirmed that the 21S particle that bound both antibiotics was composed of both 16S and precursor 17S rRNA, showing that it was truly a stalled intermediate of the 30S subunit. Furthermore, the ribosomal protein composition of this intermediate was identified.

Neomycin and Paromomycin Binding to the 30S Assembly Intermediate

Binding assays revealed that the p30S intermediate and mature 30S subunits bound neomycin and paromomycin (Figures 19-21). This result was expected because that paromomycin binds to specific nucleotides of 16S rRNA *in vitro*, and that this interaction is important for antibiotic activity (Fourmy and others 1996,1998; Recht and others 1999). Furthermore, paromomycin binding induced a conformational change in the A site stabilizing RNA-RNA interactions (Fourmy 1999, Barbieri 2007). Footprinting analysis also revealed considerable protection of nucleotides in the A site when neomycin and paromomycin were bound to protein-free 16S rRNA (Purhoit and Stern 1994). These observations suggest possible interactions of the antibiotics with RNA before the native 30S conformation is stabilized (Fourmy and others 1996, 1998). Such findings support the hypothesis that the antibiotics inhibit assembly by interacting with 16S RNA as the subunit forms.

In the binding studies using radio labeled antibiotics, neomycin bound to particles with a greater affinity. The total amount of radioactivity in the p30S region of the gradient increased by 25% for cells treated with ^{3}H-neomycin (Figure 20) compared to ^{3}H-paromomycin. Initial studies on assembly inhibition had shown that in both *S.aureus* and *E.coli*, there was a similar reduction in growth rate and 30S formation with neomycin than with paromomycin (Mehta and Champney 2002, 2003). The two antibiotics are similar in structure, but the difference in one functional group can affect binding to RNA. Specificity of

aminoglycosides for the A site is a result of eight direct contacts between the conserved neamine core, but each antibiotic can make additional contacts within the A site, increasing specificity. Crystal structures of aminoglycosides complexed with A site RNA revealed that the number of antibiotic molecules occupying the A site were dependent on the number of rings and positive charges (Francois and others 2005). Kinetic analysis from several other groups also revealed that neomycin binds with a greater affinity to the A site than does paromomycin and other aminoglycosides (Benveniste and Davies 1973; Francois and others 2005).

One possible explanation for the increased binding of neomycin over paromomycin may be due to the amino group in neomycin, which increases the net charge to +6. This may facilitate an additional contact with the negatively charged RNA, relative to a hydroxyl group at C'6 of ring I in paromomycin. While this may contribute to antibiotic binding, a more plausible explanation could involve the position of the additional positive charge. Ring I establishes initial antibiotic and RNA contacts by forming a pseudo base pair with A1408. This allows the neamine core to stack above base pairs A1409:U1491 and A1406:U1495. These specific contacts mediate the “flipping out” of adenines 1492 and 1493, the structural rearrangement that induces misreading (Ogle and others 2002, 2003; Westhof and Vicens 2003).

Studies have shown that mutations in nucleotides of the A site that bind aminoglycosides distinctly affect those with an OH group versus NH_3 (Pfister and

others 2005). Mutations of A1409 and A1491 in 16S RNA revealed that aminoglycosides with an OH at position C'6 developed increased resistance and interrupted stacking with A1409:A1491. Although neomycin and paromomycin were able to base pair with A1408, the NH^{3+} group was able to compensate for the stacking disruption. Moreover, of the aminoglycosides examined, neomycin exhibited the greatest affinity for the mutant ribosomes. Others have suggested that the three additional hydrogen bonds formed with neomycin, as well as the energy difference resulting from the charged NH^{3+} hydrogen bond, contribute to this association (Pilch and others 2003).

Binding to the 50S Subunit

Many of these features might explain neomycin and paromomycin binding to the 50S subunit (Figure 20-21). Although the literature supports that neomycin and paromomycin are specific for the 30S subunit, binding was observed to the large subunit both *in vivo* and *in vitro*. Studies by Lando and others described similar binding of paromomycin to 50S subunits *in vitro* (1978). Also antibiotic competition assays between kanamycin and neomycin resulted in reduced kanamycin binding to 50S and 30S subunits by 90%. This was not observed with streptomycin, which is not specific for the ribosomal A site (Misumi and others 1978). Electrophoretic mobility shift assays of ribosome cell lysates treated with neomycin showed an increased mobility of both 30S and 50S particles, suggesting the drug was capable of binding to and causing structural arrangements of both particles (Dahlberg and others 1977).

These 50S interactions are not surprising considering the promiscuous nature of these antibiotics. Aminoglycosides are capable of binding to several RNA motifs such as RNA aptamers, the HIV Rev Response element, and group I introns (Ashen and others 1991, Zapp and others 1993). Their flexibility around the glycosidic bonds and positive charge makes them suitable candidates for interaction with a myriad of RNA molecules (Tor 2006). Thus, these investigations highlight a new exploratory feature for these drugs. However, these data are not sufficient to allow any specific conclusions, but it must be noted that they have no specific effect on 50S assembly and do not stimulate turnover of 23S RNA (Figure 15) (Mehta and Champney 2002, 2003).

Ribosomal RNA Analysis

Analysis of rRNA from the stalled intermediates in cells treated with neomycin and paromomycin revealed an accumulation of 16S rRNA in the 21S precursor region of the gradient (Figure 14B-C). This further substantiates findings that the antibiotic binds specifically to the 16S rRNA of the assembly intermediate. The presence of precursor RNA in this particle indicates that neomycin and paromomycin are indeed inhibiting the maturation of 30S subunits. Similar results have been seen in cells defective in a number of ribosomal assembly factors such as chaperones and GTPases (Charollias 2003). A temperature sensitive mutation of the heat shock protein DnaK in *E.coli* caused defects in the later stages of ribosome biogenesis. This was evident by an accumulation of unprocessed forms of 16S and 23S rRNA from 21S, 32S, and

45S ribosomal intermediates. Era, a GTPase binding protein essential for cell growth in *E.coli*, binds to 30S subunits as well as 16S rRNA *in vitro*. Depletion of the protein resulted in an accumulation of precursor 16S rRNA and a decrease in 70S ribosome formation (Sayed and others 1999). Cells containing mutations in several other GTPases, such as RsgA and YrdC, also resulted in an accumulation of immature 16S rRNA (Himeno and others 2004; Kaczanowska and Aulin 2005). Altogether, these ribosomal factors assist in the RNA conformational rearrangements necessary for subunit formation. In the absence of these factors, assembly is incomplete as is evident by 21S precursor and immature rRNA accumulation. Interestingly these results show that aminoglycosides produce similar assembly defects.

Northern hybridization analysis revealed differences in RNA processing in the presence of both antibiotics. Antibiotic treatment had a greater effect on maturation of the 5' end (Figure 15-16). This was surprising considering the polarity of transcription. The differences in 5' and 3' precursor observed in these studies may reflect the importance of the 5' precursor during assembly. Beascon and Wagner revealed that 5' leader sequences are critical during the early stages of assembly. Their kinetic analysis showed that the leader sequences formed transient interactions with the 5' domain of 16S RNA, and deletions in this region resulted in major assembly defects. They concluded that the 5' leader was needed to prevent premature folding of 16S RNA during assembly (1999).

Ribonuclease III was shown to cleave RNA sequences at the 5' end of 17S rRNA but only after the addition of ribosomal proteins, suggesting that the final maturation of 16S rRNA depends on the formation of the ribonulceoprotein complex (Srivastava 1990). In addition, formation of the RI intermediate largely consists of arrangements in the 5' and central domains therefore these regions are probably protected by RNA-RNA or protein interactions. On the other hand, the 3' domain consisting of minor RNA conformations is readily accessible and is sufficient for RNase cleavage.

This, however, does not sufficiently explain RNA degradation observed in the presence of both antibiotics. While the 3' end is more accessible as assembly proceeds, degradation of the 3' end is probable in abnormal cells given that exonuclease activity begins at the 3' end. Thus, greater amounts of 5' precursor would be present compared to 3' precursor RNA.

The difference in amounts of 5' or 3' precursor may also reflect the affinity or neomycin and paromomycin for the p30S intermediate. Treatment with paromomycin resulted in larger amounts of 3' precursor (Figure 16). The decreased affinity of paromomycin for the p30S intermediate would result in faster dissociation from the particle making it less susceptible to RNA degradation. Exploration of this claim requires further examination of RNA processing. Nevertheless, these results support the idea that aminoglycosides perturb assembly by affecting RNA conformational changes needed to form mature functional 30S subunits.

Ribosomal Protein Analysis

The precursor particle from antibiotic treated cells contained a different protein composition compared with the natural 21S (p30S) particle (Nomura 1973). The dissimilarity could indicate how these antibiotics affect protein-RNA conformations during assembly. In the p30S particle tertiary proteins S2, S3, S10, S14, and S21 are missing in addition to three secondary proteins. In contrast, the paromomycin stalled intermediate included S3, S10, and S14, and neomycin produced an intermediate with tertiary proteins S10 and S14 (Figure 25). *In vitro* association of these proteins can only occur following an energy dependent rearrangement of the RNA in the precursor from 21S to 26S. This transition creates functional binding sites for the tertiary proteins as well as the 30S subunit. The presence of S3, S10, and S14 in the antibiotic intermediate could suggest that some structural features akin to the 30S may be present in the 21S particle. In addition, protein binding of S3, S10, and S14 is interdependent and forms a hydrophobic core within the RNA, a feature that could stabilize antibiotic interactions.

Analysis of 21S intermediates from chaperone deficient cells also revealed differences in protein composition. Ribosomal proteins S1, S14, and S21 were absent in 21S particles isolated from cells missing ObgE (Sato and others 2005). In the absence of chaperone DnaK, 21S intermediates did not contain S3, S10, S14, S21, S2, and S5 (El Hage and Alix 2004). These variations indicate that not all p30S precursors are identical in spite of having the same S value, and that

they differ in protein content, indicating flexibility in the formation of 21S intermediates. In addition, multiple pathways may exist to form a functional 30S subunit and initial stages may allow flexibility in the order of protein folding or association. However, the 21S and 26S particles have been identified to be the most critical particles (reviewed in Culver 2003).

The absence of S8 in the precursors resulting from antibiotic treatment further supports the hypothesis that different structural arrangements occur during assembly. A primary binding protein, S8 stabilizes the central domain and provides binding sites for S6 and S18. Ribosomal protein S8 is also required for subsequent binding of S5 and S12, which are absent in the $p30S_{neo}$ and $p30S_{paromo}$ intermediates (Figure 25). The absence of this protein suggests that neomycin and paromomycin may indirectly affect formation of the central domain or that an alternate mode for its formation exists. In the absence of primary protein S15, 30S subunits were able to assemble *in vivo*. However, under suboptimal conditions or *in vitro*, subunits failed to assemble. Chemical probing and primer extension of mutant subunits revealed a similar protection pattern with wild type cells suggesting that the structure was the same. S15 forms binding sites for secondary proteins, but Orr and others showed that Mg^{2+} can cause similar changes in RNA structure to facilitate S15 binding (1998). Aminoglycoside binding rearranges the massive 30S structure, moving its head (5' domain) towards its body (3' domain), so it is not likely that compensatory rearrangements can occur during assembly. This may actually allow the drug to

be more accessible to the RNA during assembly, potentially locking the RNA in a conformation that precludes the addition of other proteins.

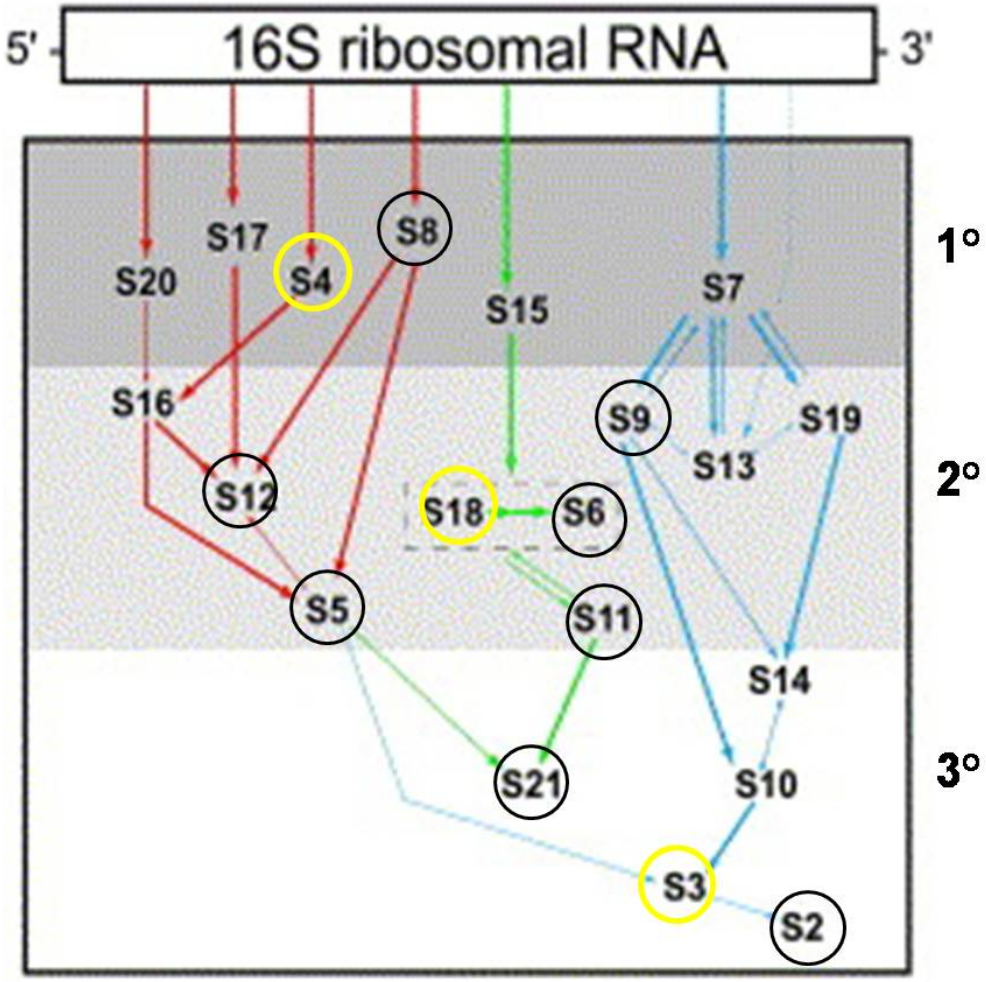

Figure 25 Ribosomal Proteins Missing in the p30S Particles from Antibiotic Treated Cells. Proteins circled in black were absent in the p30neo and p30Sparomo assembly intermediates. The absence of proteins circled in yellow was dependent on the antibiotic used (Table 1).

Structural Comparison of Assembly Intermediates

The results of this work have provided insight on the features of 30S ribosomal subunit assembly inhibition by neomycin and paromomycin in identifying the ribosomal RNA and protein content of the precursor particle. The data show that neomycin and paromomycin can impair this process producing a 21S intermediate (Figure 9). However, the extent of assembly inhibition is equal to the extent of protein synthesis inhibition (Champney 2003), implying a heterogeneous population may exist.

Structural data show that in normal cells that RNA undergoes successive changes in structure at each intermediate step. More than 50% of the changes observed during the 16S to 21S transition occur in the 5' and central domains. While assembly proceeds with transcriptional polarity, minor changes in the 3' domain take place in this early stage. A continuation of protection in certain domains or an incremental change in reactivity of 11 nucleotides during all three stages represents the dynamics of this concerted process (Holmes and Culver 2005).

The data presented here show that these assembly pathways are not congruent and that other features may exist for antibiotic binding to the stalled intermediate. In normal assembly, the decoding center forms subsequent to the 21S to 26S transition. Included in this stage is rearrangement of the 530-stem loop that forms the central psuedoknot connecting the head, body, and platform.

The addition of tertiary proteins in the last assembly step (26S to 30S) results in accommodation of sites formed in the previous step and formation of the decoding pocket. In addition, protections of nucleotides C1399-G1504, which are adjacent to the decoding region, occur during this stage (Holmes and Culver 2004). We have identified an intermediate particle which includes three of the tertiary proteins responsible for these late assembly transitions but sediments at 21S. These differences may enhance drug binding during assembly and indicate important features of a novel target for aminoglycosides in cells.

REFERENCES

Adilakshmi T, Ramaswamy P, and Woodson SA. 2005. Protein-independent folding pathway of the 16S rRNA 5' domain. J.Mol.Biol. 351(3):508-519.

Anderson RM. 1999. The pandemic of antibiotic resistance. Nature Medicine 5(45):147-149.

Ashen U, Davies J, and Schroeder R. 1991. Antibiotic inhibition of group I ribozyme function. Nature. 353(6342):368-370.

Barbieri CM, Kaul KM, Bozza-Hingos M, Zhao F, Tor Y, Hermann T, and Pilch DS. 2007. Defining the molecular forces that determine the impact of neomycin on bacterial protein synthesis: importance of the 2'-amino functionality. Antimicrob. Agents Chemother. 51(5):1760-1769.

Besancon W. and Wagner R. 1999. Characterization of transient RNA-RNA interactions important for the facilitated structure formation of bacterial ribosomal 16S RNA. Nucleic Acid Research. 27(22):4353-4362.

Beauclerk AA and Cundliffe E. 1987. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. J.Mol.Biol. 193(4):661-671.

Benveniste R and Davies J. 1973. Structure-activity relationships among the aminoglycoside antibiotics: Roles of hydroxyl and amino groups. Antimicrob Agents Chemother. 4(4):402-409.

Blount KF, Zhao F, Hermann T, and Tor Y 2005. Conformational constraint as a

means for understanding RNA-aminoglycoside specificity. J. Am. Chem. Soc. 127(27):9818-9829.

Broderson DE, Clemons WM, Jr., Carter AP, Wimberly BT, and Ramakrishnan V. 2002. Crystal structure of the 30S ribosomal subunit from *Themus thermophilus*: structure of the proteins and their interaction with 16S RNA. J. Mol. Bio. 316(3):725-768.

Bubunenko M, Korepanov A, Court DL, Jagannathan I, Dickinson D, Chaudhuri BR, Garber MB, and Culver GM. 2006. 30S ribosomal subunits can be assembled in vivo without primary binding ribosomal protein S15. *RNA* 12(7):1229–1239.

Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, and 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. 1989. Reductive methods for isotopic labeling of antibiotics. Anal Biochem 181(1):90-95.

Champney, W.S. 2003. Bacterial ribosomal subunit assembly is an antibiotic target. Current Topics in Medicinal Chemistry 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.

Chu DT, Plattner JJ, and Katz L. 1996. New directions in antibacterial research. J Med Chem 39(20):3853-3874.

Culver, G. 2003. Assembly of the 30S ribosomal subunit. Biopolymers 68(2):234-249.

Dahlberg, AE, Horodyski F, and Keller P. 1978. Interaction of neomycin with ribosomes and ribosomal ribonucleic acid. Antimicrob Agent Chemo 13(2):331-339.

Davies J, Gorini L, and Davis BD. 1965. Misreading of RNA codewords induced by aminoglycoside antibiotics. Mol Pharmacol 1(1):93-106.

Davies J and Davis BD. 1968. Misreading of ribonucleic acid code words induced by aminoglycoside antibiotics. The effect of drug concentration. *J Biol Chem* 243(12):3312–3316.

Deutscher, M.P. 2003. Degradation of stable RNA in bacteria. *J Biol Chem* 278(8):45041-45044.

El Hage A and Alix JH. 2004. Authentic precursors to ribosomal subunits accumulate in *Escherichia coli* in the absence of functional DnaK chaperone. Mol Microbol 51(1):189-201.

Faber C, Sticht H, Schweimer K, and Rosch P. 2000. Structural rearrangements of HIV-1 Tat-responsive RNA upon binding of neomycin B. J Biol Chem *275*(27):20660-20666.

Farrell RE. 1993. RNA Methodologies. San Diego (CA): Academic Press. 133 p.

Fourmy D, Recht MI, Blanchard SC, and Puglisi JD. 1996. Structure of the A site of *Escherichia coli* 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science 274(5291):1367-1371.

Fourmy D, Yoshizawa S, and Puglisi JD. 1998. Paromomycin binding induces a local conformational change in the A-site of 16S rRNA. J Mol Bio 277(2): 345.

Francois B, Russell R, Murray JB, Aboul-ela F, Masquida B, Vicens Q, and Westhof E. 2006. Crystal structure of complexes between aminoglycosides and decoding A site oligonucleotides: role of the number of rings and positive charges in the specific binding leading to miscoding. Nucleic Acids Research 33(17):5677-5690.

Gesteland RF. 1966. Isolation and characterization of ribonuclease I mutants of *Escherichia coli.* J Mol Biol 16(1):67-84.

Geyl D, Bock A, and Isono K. 1981. An improved method for two dimensional gel electrophoresis: analysis of mutationally altered ribosomal proteins of *Escherichia coli.* Mol Gen Genet 181(3):309-312.

Giri L, Hill WE, and Whittmann HG. 1984 Ribosomal proteins. Advances in Protein Chemistry. New York: Academic Press, pp. 1-78.

Hamacher K, Trylska J, and McCammon JA 2006. Dependency map of proteins

in the small ribosomal subunit. PLoS Comput Biol 2(2):80-87.

Hansen JL, Ippolito JA, Ban N, Nissen P, Moore PB, and Steitz TA. 2002. The structures of four macrolide antibiotics bound to the large ribosomal subunit. Molecular Cell 10(1):117-128.

Hansen JL, Moore PB, and Steitz TA. 2003. Structures of five antibiotics bound at the peptidyl transferase center of the large ribosomal subunit. J. Mol. Biol. 330(5):1061-1075.

Held WA, Mizushima S, and Nomura M. 1973. Reconstitution of *Escherichia coli* 30S ribosomal subunits from purified molecular components. J Biol Chem 218(5):5720–5730.

Held WA, Ballou B, Mizushima S, and Nomura M. 1974. Assembly mapping of 30S ribosomal proteins from *Escherichia coli.* Further studies. J Biol Chem 249(10):3103–3111.

Herman, T. 2005. Drugs targeting the ribosome. Curr Opin Struct Biol 15(3): 355-366.

Himeno H, Hanawa-Suetsugu K, Kimura T, Takagi K, Sugiyama W, Shirata S, and Mikami T. 2004. A novel GTPase activated by the small subunit of ribosome. Nucleic Acids Research 32(17):5303-5309.

Hirokawa G, Kaji H, and Kaji A. 2007. Inhibition of anti-association activity of translation initiation factor 3 by paromomycin. Antimicrob Agents Chemother 51(1):175-180.

Holmes KL and Culver GM. 2004. Mapping structural differences between 30S

ribosomal subunit assembly intermediates. Nature Structural Molecular Biology 11(2):179-186.

Holmes KL and Culver GM. 2005. Analysis of conformational changes in 16S rRNA during the course of 30S subunit assembly. J Mol Bio 354(2):340-357.

Huang L, Massa L, and Karle J. 2007. Drug target interaction energies by the kernel energy method in aminoglycoside drugs and ribosomal A site RNA targets. Proc Natl Acad Sci USA 104(11):4261-4266.

Jana D and Deb JK. 2006. Molecular understanding of aminoglycoside action and resistance. Appl Microbiol Biotechnol 70(2):140-150.

Jiang L, Majumdar WH, Jaishree TJ, Weijun X, and Patel DJ. 1999. Saccharide–RNA recognition in a complex formed between neomycin B and an RNA aptamer. Structure 7(7):817-827.

Kaczanowska M. and Ryden-Aulin M. 2005 The YrdC protein-a putative ribosome maturation factor. Biochimica et Biophysica Acta 1727(2):87-96.

Kaul M, Barbieri CM, and Pilch DS. 2006. Dynamics of aminoglycoside-rRNA interactions J Am Chem Soc 128(4):1261-1271.

Kaul M, Barbieri CM, and Pilch DS. 2006. Aminoglycoside-induced reduction in nucleotide mobility at the ribosomal RNA A-site as a potentially key determinant of antibacterial activity. J Am Chem Soc 128(4):1261-1271.

Klostermeier D, Sears P, Wong C, Millar DP, and Williamson JR. 2004. A three-

florophore FRET assay for high throughput screening of small-molecule inhibitors of ribosome assembly. Nucleic Acids Research. 32(9):2707-2715.

Kotra LP, Haddad J, and Mobashery S. 2000. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrobal Agents Chemother. 44(12):3249-3256.

Lando D, Cousin M, Ojasoo T, and Raynaud J. 1976. Paromomycin and dihydrostreptomycin binding to *Escherichia coli* ribosomes. Eur J Biochem 66(3):597-606.

Lerner CG and Beutel BA. 2002. Antibacterial drug discovery in the post-genomic era. Current Drug Targets-Infectious Disorders 2(2):109-119.

Mangili A, Bica I, Snydman DR, and Hamer DH. 2005. Daptomycin resistant, methicillin-resistant *Staphylococcus aureus* bacteremia. Clin Infect Dis 40(7):1058–1060.

Mack DP, Galan AA, Halim NS, Heldsinger A, Loo JA, Moreland DW, Sannes-Lowery KA, Sharmeen L, Truong HN, and Czarnik AW. 1997. Discovery of selective, small-molecule inhibitors of RNA complexes—1. The tat protein/TAR RNA complexes required for HIV-1 transcription Bioorg Med Chem 5(6):1173-1184.

Mehta R and Champney S. 2002. 30S ribosomal subunit assembly is a target for inhibition by aminoglycoside antibiotics in *Escherichia coli*. Antimicrobial

Agents Chemother 46(5):1546-1549.

Mehta R and Champney S. 2003. Neomycin and paromomycin inhibit 30S ribosomal subunit assembly in *Staphylococcus aureus*. Current Microbiology. 47(3):237-243.

Mizushima, S. and Nomura, M. 1970. Assembly mapping of 30S ribosomal proteins in *E. coli*. Nature 226(5252):1214–1218.

Miller JH. 1972. Experiments in Molecular Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mills SD 2006. When will the genomics investment pay off for antibacterial discovery? *Biochemical Pharmacology* 71(9):1096-1102.

Misumi M, Nishimura T, Komai T, and Tanaka N. 1978. Interaction of kanamycin and related antibiotics with the large subunit of ribosomes and the inhibition of translocation. *Biochem Biophys Comm* 84(2):358-365.

Moore PB. 2001. The ribosome at atomic resolution. Biochemistry 40(11):3243-3250.

Moore PB and Steitz TA. 2002. The involvement of RNA in ribosome function. Nature 418(6894):229-234.

Nashimoto H, Held W, Kaltschmidt E, and Nomura M. 1971. Structure and function of bacterial ribosomes. XII. Accumulation of 21S particles by some cold-sensitive mutants of *E.coli*. J Mol Biol 62(1):121-138.

Nicholson AW. 1999. Function, mechanism, and regulation of bacterial

ribonucleases. FEMS Micro Rev 23(3):371-390.

Nierhaus KH. 1991. The assembly of prokaryotic ribosomes. Biochimie 73(6): 739-755.

Noller HF. 1991. Ribosomal RNA and translation. Annu Rev Biochem 60 (12): 191-227.

Nomura M. 1973. Assembly of bacterial ribosomes. Science 179(6): 863-873.

Nomura MJ. 1974. The 30S Ribosomal Subunit. Supramol Struct 249(9):163–165.

Norrby SR, Nord CE, and Finch R. 2005. Lack of development of new antimicrobial drugs: a potential serious threat to public health. Lancet Infect Dis 5(2):115–119.

Ogle JM, Brodersen DE, Clemons WM, Tarry MJ, Carter AP, and Ramakrishnan V. 2001. Recognition of cognate transfer RNA by the 30S ribosomal subunit. Science 292(5518):897-902.

Ogle JM, Murphy FV, Tarry MJ. Ramakrishnan V. 2002. Selection of tRNA by the ribosome requires a transition from an open to a closed form. Cell 111(5):721-732.

Ogle JM, Carter AP, and Ramakrishnan V. 2003. Insights into the decoding mechanism from recent ribosome structures. TIBS 28(5):259-266.

Orr JW, Hagerman PJ, and Williamson JR. 1998. Protein and Mg(2+)-induced conformational changes in the S15 binding site

of 16 S ribosomal RNA. J Mol Biol 275(3):453–464.

Overbye KM and Barrett JF. 2005. Antibiotics: where did we go wrong? Drug Discov Today 10(1):45–52.

Pape T, Wintermeyer W, and Rodnina MV. 2000. Conformational switch in the decoding region of 16S rRNA during aminoacyl-tRNA selection on the ribosome. Nature Structural Biology 7(2):104-107.

Pfister P, Hobbie S, Brull C, Corti N, Vasella A, Westhof E, and Bottget E. (2005) Mutagenesis of 16S rRNA C1409-G1491 base-pair differentiates between 6'OH and 6'NH_3^+. J Mol Bio 346(2):467-475.

Pilch DS, Kaul M, Barbieri CM, Kerrigan JE. 2003. Thermodynamics of aminoglycoside–rRNA recognition. Biopolymers 70(1):58–79.

Powers T, Daubresse G, and Noller HF. 1993. Dynamics of in vitro assembly of 16S rRNA into 30S ribosomal subunits. J Mol Biol 232(2):362–374.

Powers T, and Noller HF. 1995. Hydroxy radical footprinting of ribosomal proteins on 16S rRNA. *RNA* 1(2):194-209.

Purohit P and Stern S. 1994 Interactions of a small RNA with ligands antibiotic subunit. Nature 370(6491):659-662.

Recht, MI, Douthwaite S, Dahlquist KD, and Puglisi JD. 1999. Effect of mutations in the A site of 16S RNA on aminoglycoside antibiotic-ribosome interaction. J Mol Bio 286(1):33-43.

Roets E, Adams E, Muriithi I, and Hoogmartens J. 1995. Determination of the relative amounts of the B and C components of neomycin by thin-layer

chromotagraphy using florescence detection. J Chromatography 696(1): 131-138.

Sato A, Kobayashi G, Hayashi H, Yoshida H, Wada A, Maeda M, Hiraga S, Takeyasu K, and Wada C. 2005. The GTP binding protein Obg homolog ObgE is involved in ribosome maturation. Gene to Cells 10(5):393-408.

Schlunzen F, Zarivach R, Harms J, Bashan A, and Albrecht R. 2001. Structural basis for the interaction of antibiotics with the peptidyltransferase centre in eubacteria. Nature 413(6858):814-821.

Schroeder R, Waldsich C, and Wank H. 2000. Modulation of RNA function by aminoglycoside antibiotics. EMBO J 19(1):1-9.

Schroeder R. and Wallis MG. 2001. RNA binding antibiotics. Austin(TX): Landes Bioscience. 11 p.

Silvers J and Champney S. 2005. Accumulation and turnover of 23S ribosomal RNA in azithromycin-inhibited ribonuclease mutant strains of *Escherichia coli*. Arch Microbiol 184(1):66-77.

Spedding G. 1990. Ribosomes and protein synthesis a practical approach. Oxford (England) Oxford University Press. 169 p.

Spellberg B, Powers JH, Brass EP, Miller LG, and Edwards JE. 2004. Trends in antimicrobial drug development:implications for the future. Clin Infect Dis 38(9):1279–1286.

Srivastava AK, and Schlesinger D. 1990 Mechanism and regulation of bacterial ribosomal RNA processing. Annu Rev Microbiol 44:105-129.

Stage TK, Hertel KJ, and Uhlenbeck OC. 1995. Inhibition of the hammerhead ribozyme by neomycin. RNA. 1(1):95-101.

Stagg SM, Mears JA, and Harvey SC. 2003. A structural model for the assembly of the 30S subunit of the ribosome. J Mol Biol 328(1):49–61.

Stern, S, Powers, T, Changchien, LM, and Noller, HF. 1989. RNA-protein interactions in 30S ribosomal subunits: folding and function of 16S rRNA *Science* 244(4906):783–790.

Tor Y. 2006. The ribosomal A site as an inspiration for the design of RNA binders. Biochimie 88(8):1045-1051.

Toutchstone J, Dobbins M. 1983. Practice of Thin Layer Chromatography. New York (NY): Wiley-Interscience. 405 p.

Traub P, and Nomura, M. 1968. Structure and function of *E.coli* ribosomes. V. Reconstitution of functionally active 30S ribosomal particles from RNA and proteins. Proc Natl Acad Sci USA 59(3):777–784.

Traub P and Nomura M. 1969. Structure and function of *Escherichia coli* ribosomes. VI. Mechanism of assembly of 30S ribosomes studied in vitro. J Mol Biol 40(3):931-938.

Usary J and Champney S. 2001. Erythromycin inhibition of 50S ribosomal subunit formation in *Escherichia coli* cells. Molecular Microbiology 40(4): 951-962.

Varani L, Spillantini MG, Goedert M, and Varani G. 2000. Structural basis for recognition of the RNA major groove in the tau exon 10 splicing regulatory element by aminoglycoside antibiotics. Nucleic Acids Res. *28*(3):710-719.

Vaiana AC, Westhof E, and Auffinger P. 2006. A molecular dynamics simulation study of an aminoglycoside/A-site RNA complex: conformational and hydration patterns. Biochimie 88(8):1061–1073.

Vicens Q and Westhof E. 2001. Crystal structure of paromomycin docked into the eubacterial ribosomal decoding a site**.** Structure 9(8):647-658.

Vicens Q and Westhof E. 2003. RNA as a drug target: the case of aminoglycosides. Chembiochem 4(10):1018-1023.

Weitzmann CJ, Cunningham PR, Nurse K, and Ofengand J. 1993. Chemical evidence for domain assembly of the *Escherichia coli* 30S ribosome. FAESB J. 7(1):177-180.

Wilcox SK, Cavey GS, and Pearson JD. 2001. Single Ribosomal Protein Mutations in Antibiotic-Resistant Bacteria Analyzed by Mass Spectrometry Antimicrobial Agents and Chemotherapy 45(11):3046–3055.

Wimberly B, Ditlev B, William MC, Morgan-Warren RJ, Carter AP, Vonrhein C, Hartsch T, and Ramakrishnan V. 2000. Structure of the 30S ribosomal subunit. *Nature* 407(6802):327-339.

Wong CH, Hendrix M, Priestly ES, and Greenberg WA. 1998. Specificity of aminoglycoside antibiotics for the A site of the decoding region of

ribosomal RNA. Chem Biol 5(7):397-406.

Zapp, ML, Stern S, and Green MR. 1993. Small molecules that selectively block RNA binding of HIV-1 Rev protein inhibit Rev function and viral production. Cell 74(6):969-978.

Zhao F, Zhao Q, Blount KF, Han Q, Tor Y, and Hermann T. 2005. Molecular recognition of RNA by neomycin and a restricted neomycin derivative Angew Chem Int Ed 44(33):5329 –5334.

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