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Degradation of 23S rRNA in Azithromycin-treated Ribonuclease Mutants of *Escherichia coli*

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A thesis

presented to

the faculty of the Department of Biochemistry

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In partial fulfillment

of the requirements for the degree

Master of Science in Biomedical Science

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by

Jessica A. Silvers

December 2004

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Keywords: azithromycin, 50S, 23S, ribosomal RNA, ribonucleases, degradation of RNA

ABSTRACT

Degradation of 23S rRNA in Azithromycin-treated Ribonuclease Mutants of *Escherichia coli*

by

Jessica A. Silvers

Azithromycin, a macrolide antibiotic, specifically binds to the 50S ribosomal subunit of bacterial ribosomes and inhibits translation. Azithromycin also prevents 50S ribosomal subunit assembly by binding to a 50S ribosomal subunit precursor particle. When exposed to azithromycin, several ribonucleases in wild-type *Escherichia coli* cells degrade antibiotic-bound 50S precursor particles. Presumably, cells expressing one or more mutated ribonucleases will degrade the antibiotic-bound precursor less efficiently, resulting in increased sensitivity to the antibiotic. To test this, eight ribonuclease–deficient strains of *Escherichia coli* were grown in the presence or absence of azithromycin. Cell viability, growth rates, and protein synthesis rates were measured. Degradation of 23S rRNA was examined by hybridization with a 23S specific probe. Ribonuclease II and polynucleotide phosphorylase mutants demonstrated hypersensitivity to the antibiotic and showed a greater extent of 23S rRNA accumulation, suggesting that these two ribonucleases are important for 23S rRNA turnover in azithromycin-treated *Escherichia coli*.

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CONTENTS

ABBREVIATIONS

30S	…………………………………………..	small subunit of ribosome
50S	…………………………………………..	large subunit of ribosome
A_{600}	…………………………………………..	absorbance at 600 nanometers
CFU	…………………………………………..	colony forming units
CPM	…………………………………………..	counts per minute
E. coli	…………………………………………..	*Escherichia coli*
DNA	…………………………………………..	deoxyribonucleic acid
IDV	…………………………………………..	integrated density value
MIC	…………………………………………..	minimal inhibitory concentration
mRNA	…………………………………………..	messenger RNA
PAGE	…………………………………………..	polyacrylamide gel electrophoresis
PCR	…………………………………………..	polymerase chain reaction
PNP	…………………………………………..	polynucleotide phosphorylase
RPM	…………………………………………..	revolutions per minute
RNA	…………………………………………..	ribonucleic acid
rRNA	…………………………………………..	ribosomal RNA
S-buffer	…………………………………………..	subunit buffer
TB	…………………………………………..	tryptone peptone broth
TSB	…………………………………………..	tryptic soy broth
tRNA	…………………………………………..	transfer RNA

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CHAPTER 1

INTRODUCTION

Ribosomes are the cellular organelles involved in translating messenger RNA into peptides and proteins and are composed of a large and small subunit, both of which contain ribosomal RNA and proteins (Figure 1). The large subunit sediments at 50 Svedburg units (S), and is composed of 23S rRNA (2900 nucleotides), 5S rRNA (120 nucleotides), and about 33 proteins. The small subunit sediments at 30S and is composed of 16S rRNA (1500 nucleotides) and 21 proteins. The two subunits associate through a network of intermolecular bridges to form the functional bacterial ribosome that sediments at 70S (Mascaretti 2003).

The function of ribosomes is protein synthesis. Although the 30S and 50S subunits work together to accomplish this, both subunits have distinct roles. The 30S subunit mediates the interaction between mRNA and tRNA by monitoring base pairing between the codon and anticodon. The 50S subunit catalyzes peptide bond formation in the peptidyltransferase center (Mascaretti 2003).

Although similar, eukaryotic ribosomes are slightly different from prokaryotic ribosomes. The eukaryotic ribosome is larger and is composed of two subunits, the 40S subunit and 60S subunit. The 40S subunit is composed of 18S rRNA and 30 proteins, while the 60S is composed of 28S rRNA, 5.8S rRNA, and 45 proteins (Figure 1). These structural differences are sufficient that antibiotics inhibiting prokaryotic ribosomes do not affect eukaryotic ribosomes. Therefore, bacterial species within the human body can be specifically eliminated by targeting their ribosomes.

The focus of this study is azithromycin, which specifically inhibits the 50S subunit of prokaryotic ribosomes. Azithromycin is an azalide, a subclass member of the macrolide class of

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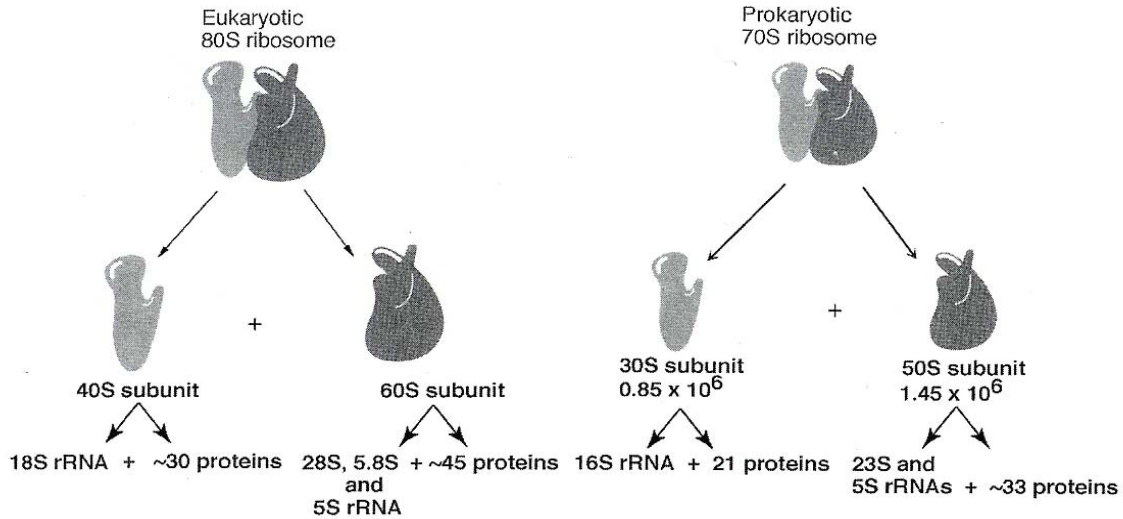
Figure 1.   Eukaryotic and Prokaryotic Ribosomal Subunits
(Mascaretti 2003)

antibiotics derived from erythromycin A.   Erythromycin A, the first macrolide antibiotic, was

discovered in 1952 as a naturally occurring compound produced by the bacterium species

Saccharopolyspora erythraea.   Since its discovery, erythromycin A has been used to treat upper

respiratory tract infections, as well as skin and soft tissue infections (Zuckerman and Kaye 1995).

Although erythromycin A is effective, it is unstable in the gastrointestinal tract and the side effects

can be severe.   Because of these issues, a number of new macrolide antibiotics with improved

microbiological and pharmacokinetic properties were produced, one of which was azithromycin.

The structures of erythromycin A and azithromycin are shown in Figure 2.   The 14-

membered ring of erythromycin A is expanded at the 9-oxime position to form azithromycin.

The result is a 15-membered ring with a tertiary amine group.   This modification to the ring

results in azithromycin being more stable in the stomach and therefore more bioavailable.   It also

increases tissue penetration and activity against certain Gram-negative bacteria such as *E. coli* and

H. influenzae (Ballow and Amsden 1992).   Azithromycin was the antibiotic used in this work

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because it is significantly more effective in *E. coli* with a minimal inhibitory concentration value of 5 μg/ml, compared to 150 μg/ml of erythromycin A.

Figure 2.   Structures of Erythromycin A and Azithromycin

In bacterial cells, azithromycin has two equivalent targets. The antibiotic binds at the entrance of the peptidyltransferase tunnel on the 50S subunit and sterically blocks peptide elongation (Mascaretti 2003). In addition, Champney (2003) has shown that azithromycin prevents 50S ribosomal subunit formation by binding to a 50S subunit assembly intermediate. The proposed model for inhibition of 50S ribosomal subunit assembly is shown in Figure 3. In the absence of antibio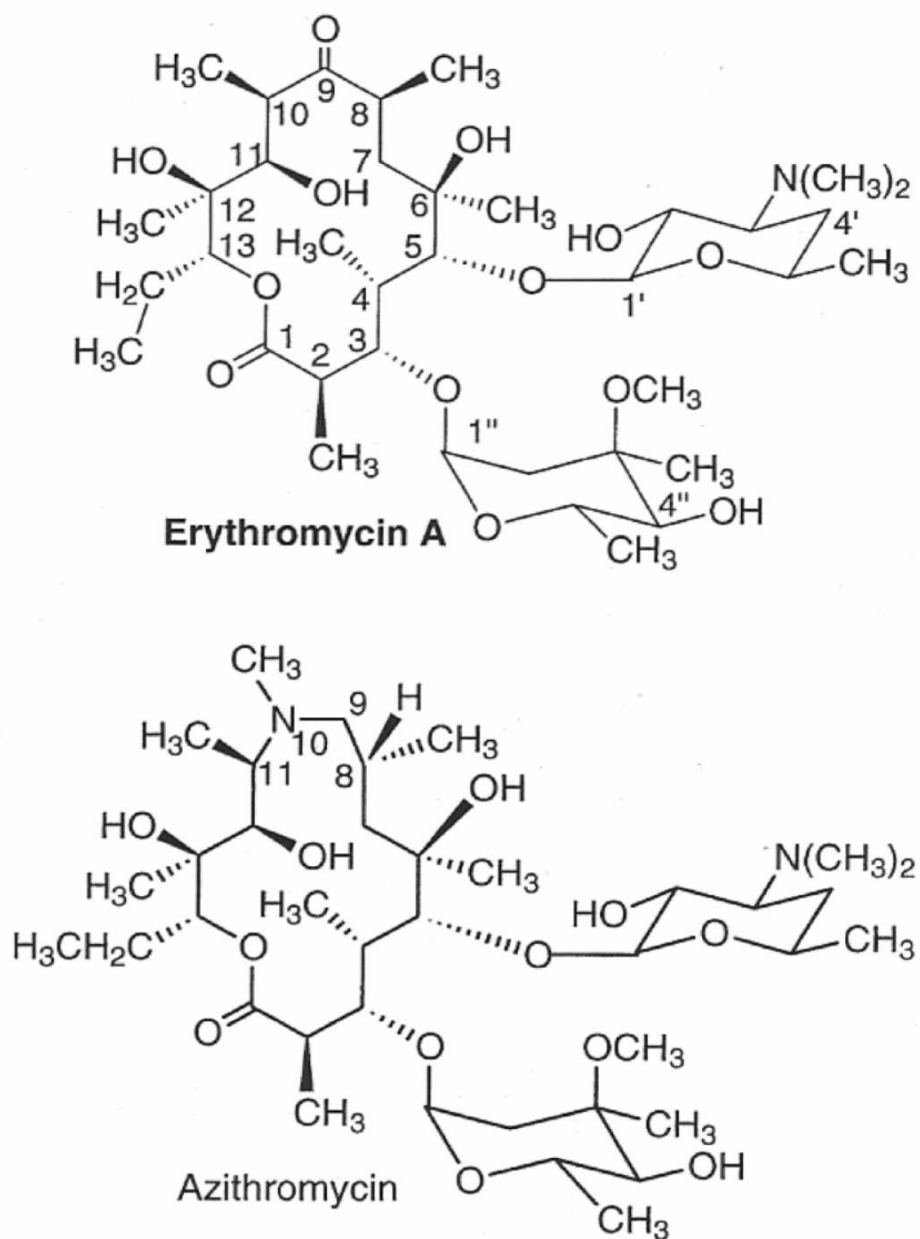tic, formation of the 50S subunit proceeds through two stable intermediate precursor particles before forming a functional 50S ribosomal subunit (Figure 3A). The first particle sediments at 32S and is composed of several ribosomal proteins, 23S rRNA, and 5S rRNA. The second particle sediments at 43S and contains several more ribosomal proteins than the 32S particle. Binding of all 33 proteins to the 23S and 5S rRNA results in a functional 50S ribosomal subunit (Osawa, et al. 1969; Nierhaus, Bordasch and Homann 1973; Lindahl 1975).

As Figure 3B shows, azithromycin can either bind to existing 50S subunits (E) or to a similar site on 50S assembly intermediates (I). When azithromycin binds to assembly intermediates, formation of the 50S subunit is disrupted by preventing a conformational change in the particle or by preventing association of certain ribosomal proteins. The result is a reduction in 50S subunit amounts and an increase in 50S intermediate particles. The stalled intermediate becomes susceptible to attack by cellular ribonucleases, which degrade the intermediate into rRNA oligonucleotides and ribosomal proteins.

Previous work with erythromycin A supports this model (Usary and Champney 2001). *E. coli* cells grown with [^{14}C]-erythromycin A revealed increasing amounts of antibiotic bound particles sedimenting in the 30S gradient region. Hybridization experiments confirmed that these particles were the result of incompletely formed 50S subunits sedimenting in the 30S gradient region. Interestingly, it was shown that strain SK5665, a ribonuclease E mutant, was more inhibited by erythromycin A than the wild-type strain SK901 in terms of growth rate, protein synthesis, and 50S subunit formation. In addition, substantially more 50S precursor
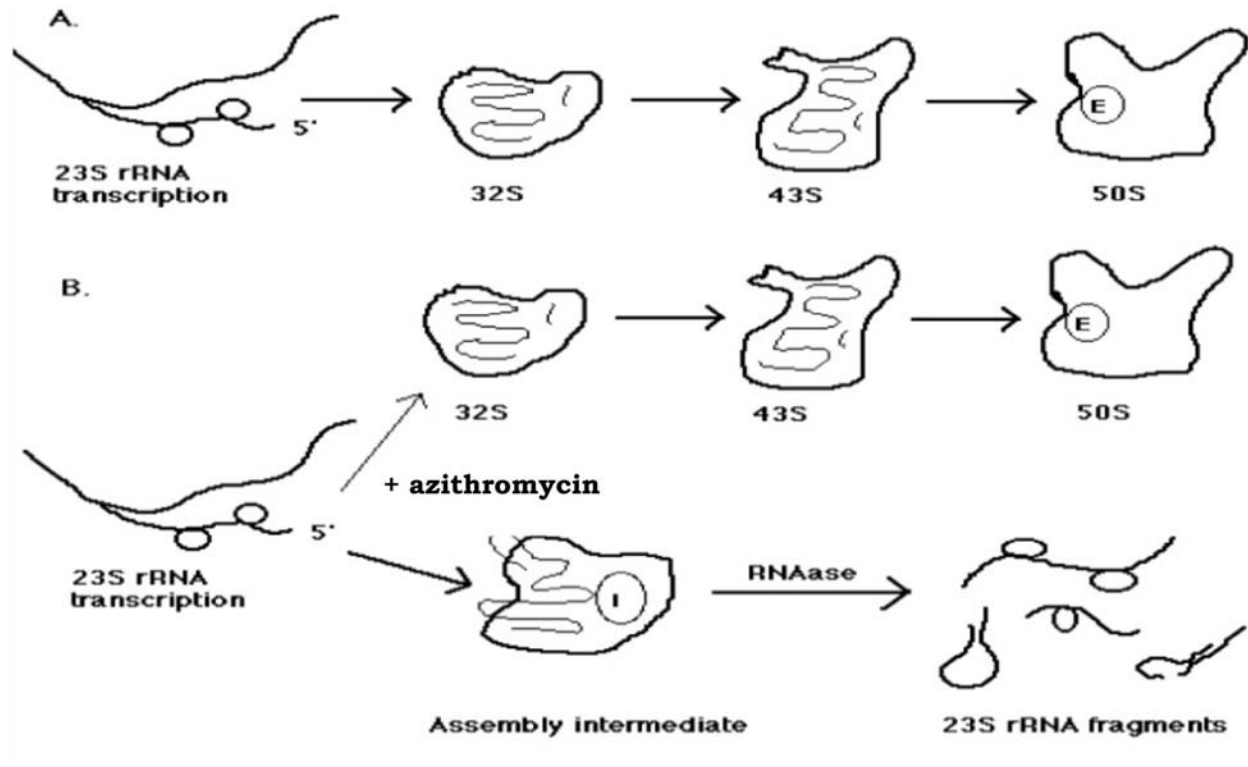
Figure 3.   Model for 50S Subunit Assembly Inhibition by Azithromycin

(A)   Normal pathway for *in vivo* synthesis of 50S ribosomal subunits

(B)   In the presence of azithromycin, assembly intermediate precursor accumulates, stalling 50S subunit assembly.   In wild-type cells, ribonucleases degrade the precursor, allowing the synthesis of new rRNA.   In ribonuclease-deficient cells, degradation of the azithromycin-bound precursor is stalled, impairing the formation of new 50S subunits, which prevents the formation of new, functional 70S ribosomes.

particle accumulated in the 30S gradient region compared to the wild-type strain. Accumulation of these particles suggests that RNase E is an important enzyme involved in 23S rRNA turnover and the inability to degrade these molecules may increase the cell's sensitivity to azithromycin.

Although the functions of several ribonucleases are becoming well established, little has been established about the degradation pathways involved in rRNA decay.   As many as 15 ribonucleases have been discovered in *E. coli* and more are thought to exist.   Most ribonucleases have redundant, overlapping functions and are classified as either exoribonucleases, which cleave nucleotides from the ends of RNA, or endoribonucleases, which cleave within an RNA molecule. Table 1 lists the functions of the major endo- and exoribonucleases found in *E.coli*.

rRNAs are stable molecules under normal conditions, but under unfavorable conditions, such as starvation, destabilizing mutations, and membrane damaging reagents, these molecules are degraded extensively (Deutscher 2003).   In addition, macrolide antibiotics have also been shown to trigger degradation of rRNA (Champney 2003).   The purpose of this study was to determine which ribonucleases are important in 23S rRNA turnover in cells exposed to azithromycin. Eight ribonuclease mutant strains were investigated (See Table 2).   The selected mutants have one or more defective ribonucleases which have been implicated in RNA turnover. Hypersensitivity to azithromycin was evaluated by examining growth properties, protein synthesis rates, and 50S subunit amounts after azithromycin exposure.   Recovery of the 50S subunit after azithromycin exposure and rRNA degradation/accumulation was also examined.   Strains with mutations in ribonucleases II and polynucleotide phosphorylase, both exoribonucleases, demonstrated hypersensitivity to azithromycin, suggesting that they are significant for the turnover of rRNA in *Escherichia coli* cells exposed to azithromycin**.**

Table 1.   Known Functions of *E. coli* Ribonucleases

ENDORIBONUCLEASES	
RNase I	Located in the periplasm and is involved in RNA scavenging; involved in RNA turnover under specific conditions
RNase III	Participates in the maturation of rRNA precursors; initiates decay of specific mRNA
RNase E	Component of degradosome; cleavage of 9S rRNA transcript; involved in mRNA decay
RNase HI	Cleaves RNA-DNA hybrids; appears to be more important in DNA metabolism
RNase P	A ribozyme involved in the maturation of tRNA 5' ends

EXORIBONUCLEASES	
RNase II	Primary function is the decay of mRNA; may protect specific mRNAs from activity of other ribonucleases
Polynucleotide phosphorylase	(PNPase) Component of degradosome; exonucleolytic degradation of mRNA fragments; other functions have been implicated
RNase PH	Primary function is tRNA 3' end maturation, similar sequence to PNPase
RNase T	Participates in the maturation of rRNA 3' ends
RNase D	Involved in the maturation of tRNA 3' ends; possible role in RNA degradation
RNase R	Exact function unknown; has 60% sequence similarity to RNase II and c-terminal RNA binding domain similar to PNPase
Oligoribonuclease	Hydrolyzes short oligoribonucleotides to 5'-mononucleotides

(Deutscher and Li 2001; Nicholson 1999; Mohanty and Kushner 2003)

CHAPTER 2

MATERIALS AND METHODS

Materials

Azithromycin was a gift from Pfizer Pharmaceuticals. Stock solutions of azithromycin were made at 10 mg/ml in methanol. Lysozyme, RNase free-DNase, thymidine, N,N,N',N'-tetramethyl-ethylenediamine, and proteinase K were purchased from Sigma Chemical Corporation. Tryptic soy broth (TSB), tryptone peptone (TB), agar, agarose, sucrose, Scintisafe Gel scintillation fluid, sodium dodecyl sulfate (SDS), 3-(N-Morpholino)-propanesulfonic acid (MOPS), trichloro-acetic acid (TCA), 20X saline-sodium citrate (SSC), sodium salicylate, formaldehyde, formamide, methanol, urea, Mirus Label-IT® biotin labeling kit, Fuji medical X-ray film, Kodak GBX developer, and fixer were purchased from Fisher. Acrylamide, bis-acrylamide, and bis-tris(hydroxymethyl) aminomethane(Bis-Tris) were purchased from Bio-Rad. Nytran SPC nylon transfer membranes were purchased from Scheicher & Schuell. The North2South® chemiluminescent nucleic acid hybridization and detection kit was purchased from Pierce. Washing & Pre-Hybridization solution and background quencher were purchased from Molecular Research Center, Inc. PCR primers were obtained from Life Technologies. The PCR Super-mix was purchased from Gibco BRL. Century Plus RNA markers were purchased from Ambion. The GF/A glass fiber filters and blotting paper were purchased from Whatman International. ^{3}H-uridine (1.0 Ci/mmol) and ^{35}S-methionine (TRANS^{35}S-LABEL 1175 Ci/mmol) were purchased from ICN Pharmaceuticals.

Cricket Graph III software was used to construct graphs and curves and for calculations. Alpha Inotech software was used to determine band intensities from hybridization films.

Bacterial Strains

Nine strains of *Escherichia coli* were examined, wild-type SK901 and eight strains with one or more mutant ribonuclease enzymes (See Table 2).

Table 2.   Strains of *Escherichia coli* Used in this Study

Name	Genotype	Reference
SK901	F⁻, malA⁻, thi⁻	(Champney 1979)
D10	Hfr H (po1), met⁻, Rnase1⁻, relA (λ⁺)	(Gesteland 1966)
N7060	F⁻, metB1, tryA451, rpsL478, rna-19, rnb-464, pnp-13	(Apirion and Watson 1975)
CA244	F⁻, pnpΔ::kan^R	(Reuven and Deutscher 1993)
SK5665	F⁻, thyA715, λ⁻, rne-1	(Babitzke et al. 1993)
SK7622	F⁻, thyA715, λ⁻, rncΔ38::kan^R	(Babitzke et al. 1993)
SK4803	gal, thi, ton, sup, hasdR4, endA, sbcB15, rnb-296	(Donovan and Kushner 1986)
SK5704[*]	F⁻, thyA715, λ⁻, pnp-7, rne-1, rnb-500, rph-1	(Arraiano Yancey and Kushner 1988)
MRE600	RNAse 1⁻	(Cammack and Wade 1965)

 *rne-1 is conditionally lethal

Media

Tryptic Soy Broth: 30 g tryptic soy broth in 1 L dH$_2$O.

Tryptic Soy Broth Plates: 1 L tryptic soy broth and 16 g agar.

Tryptone Peptone Broth: 13 g tryptone peptone and 7 g NaCl in 1 L dH$_2$O

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 NaCitrate·$(2H_2O)$ to 1 L dH$_2$O

Buffers

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

1X TBE Buffer: 90 mM tris-borate (pH 8.0), 1 mM EDTA

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

RNA Resuspension Buffer: 4 ml formamide, 1.6 ml formaldehyde, 0.8 ml 10X MOPS buffer.

RNA Running Buffer: 730 ml sterile dH$_2$O, 100 ml 10X MOPS buffer, 170 ml formaldehyde.

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

5X Neutralizing Buffer: 79.25 g Na_2HPO_4, 60.25 g NaH_2PO_4 in 1 L dH$_2$O

Formamide Hybridization Buffer: 25 ml formamide, 12.5 ml 20X SSC, 0.5 ml 10% sarkosyl, 100 µl 10% SDS, 10 ml 10% BSA, and 1.9 ml sterile dH$_2$O.

Methods

MIC Determination

The minimal inhibitory concentration (MIC) for azithromycin was determined by a dilution method as described (Champney and Burdine 1998).   Six test tubes were filled with 1 ml of TSB or TB.   Each tube received 50 µl of an overnight culture of *E. coli* cells and azithromycin over a range of concentrations.   The tubes were incubated at either 32°C or 37°C overnight and the absorbance at 600 nm was measured.   An A_{600} of 1.0 = 6.5 X 10^8 cell/ml.

Analysis of Cell Growth and Cell Viability

Cell cultures were grown in a water bath at either 37°C or 32°C in TB in the presence or absence of azithromycin. The media was supplemented with 50 μg/ml of thymine for strains SK5665, SK7622, and SK5704. The azithromycin concentration was 1.0 μg/ml for strains SK5704 and MRE600 and 2.5 μg/ml for all other strains. Growth was initiated by adding cells from an overnight culture to TB in a side arm flask. Growth rates were measured by recording the increase in cell density over time using a Klett-Summerson colorimeter. Once the cells had doubled in number twice (equivalent to a Klett reading of 80), 10 μl was removed from the growing culture and added to 1ml of 1X A-salts. A serial dilution was performed to achieve a final dilution of 10^{-5} and 10 μl was plated on square TSB agar plates (Jett et al. 1997). After 24-48 hours incubation at 32°C or 37°C, colonies from the control and drug treated sample were counted and compared to determine the effect of azithromycin on cell viability.

Analysis of the Rate of Protein Synthesis

Cell cultures were grown as described above. After two cell doublings in the presence or absence of azithromycin, ^{35}S-methionine was added to the culture at a concentration of 1 μCi/ml. Three samples of 0.4 ml were removed at 5 minute intervals after addition of the ^{35}S-methionine. Cells were precipitated in 20% TCA and collected on Whatman GF/A glass fiber filters. Radioactivity was measured by liquid scintillation counting.

Analysis of Ribosomal Subunit Assembly

All strains were grown as described above. At a Klett reading of 20, azithromycin was added to the appropriate cultures. After 10-minutes of growth, ^{3}H-uridine (1 μCi/ml) was added to the control and azithromycin treated samples. Following two cell doublings, an excess of uridine (50 μg/ml) was added to each culture to halt further isotope incorporation. After a 15-minute chase period, cells were centrifuged in a Beckman low speed centrifuge (J2-21) at 6,000

rpm for 12 minutes in a JA21 rotor.   Cells were washed with S-buffer and centrifuged again. Supernatants were discarded in the radioactive waste container and the cell pellets were stored at -70°C before cell lysis.

To examine the inhibition of subunit assembly, cell lysates were centrifuged on linear sucrose gradients.   Cell lysates were obtained by re-suspending the cell pellets in 200 μl of S-buffer and 5 μl of a 10 mg/ml solution of lysozyme.   Following a 10-minute incubation period at room temperature, cells were subjected 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. After lysis, DNA was eliminated by adding 1-2 units of RNase free DNase to each sample.   The samples were centrifuged at 6,000 rpm for 12 minutes.

The resulting supernatant was loaded on the top of 5-20% linear sucrose gradients. Gradients were made using Buchler gradient makers and 6 ml each of 5% and 20% sucrose in S-buffer.   The samples were placed in a SW40 rotor and centrifuged in a Beckman LE80K Ultracentrifuge at 39,000 rpm for 4.5 hours or 20,000 rpm for 17-18 hours.   After centrifugation, gradients were pumped through an ISCO Model UA-5 absorbance monitor, which measured the absorbance at 254 nm.   Equivalent fractions of the gradient were collected into vials and mixed with 3 ml of Scintisafe gel.   The incorporation of ^{3}H-uridine into RNA was then measured by liquid scintillation counting.

Analysis of 50S Subunit Recovery Following Azithromycin Exposure

Strains were grown with azithromycin and labeled with 2 μCi/ml ^{3}H-uridine as described above.   At a Klett reading of 80, or when growth stopped, the cells were centrifuged at room temperature at 6,000 rpm for 12 minutes.   Cells were washed with 1ml of 32°C TB and the supernatant was discarded.   Cell pellets were resuspended in 20 ml of 32°C TB supplemented with 50 μg/ml uridine.   Growth was resumed at 32°C and 3 ml samples were removed from the culture at 5, 15, 30, 60 , 90, and 120 minutes.   Samples were centrifuged for 12 minutes at 6,000

rpm and pellets were stored at -70°C.   Cell lysis, centrifugation on 5-20% sucrose gradients, and gradient collection was performed as described above.   Radioactivity was measured by liquid scintillation counting.

RNA Analysis by Polyacrylamide Gel Electrophoresis

All strains were grown, labeled with ^{3}H-uridine, and lysed as described above.   Cell lysates were loaded on top of 5-20% sucrose gradients, centrifuged, and pumped through the ISCO absorbance monitor.   Each gradient was collected into three pools: top, 30S, and 50S. RNA was isolated from each fraction by proteinase K digestion, followed by ethanol precipitation. To digest the proteins in the gradient fractions, 10 μl of 15 mg/ml proteinase K and SDS (to a final concentration of 0.5%) was added.   Samples were mixed and incubated at 44°C for 2 hours. RNA in the samples was precipitated by adding 2 volumes of pure ethanol and 0.1 volume of 1 M Magnesium Acetate, mixing, and holding at -20°C for 1 hour.   Samples were centrifuged at 6,000 rpm for 30 minutes, the supernatant was discarded, and the samples were washed with 70% ethanol.   The RNA pellets were dried at 44°C for 15 minutes and then resuspended in 30 μl of sterile dH$_2$O.

The radiolabeled RNA isolated from each of the fractions was separated by polyacrylamide gel electrophoresis (PAGE).   RNA samples were prepared by mixing 8 μl of RNA with RNA resuspension buffer, heating at 65°C for 10-minutes, and then quickly cooling the samples on ice.   Five μl of dye (80% glycerol, 1% bromo-phenol blue) was added to the samples before electrophoresis on 8% polyacrylamide gels at 100 volts.   RNA isolated from the top gradient fractions was electrophoresed for 2 ½ hours, while RNA isolated from the 30S and 50S gradient fractions was electrophoresed for 4 ½ hours.   Following electrophoresis, the gels were soaked in 1 M sodium salicylate for 30 minutes, dried onto Whatman paper with a gel drier, and exposed to Fuji Medical X-ray film at -70°C.   Development of X-ray film involved soaking film

in Kodak GBX developer and replenisher 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.

Construction of Biotinylated 16S and 23S Specific Probes

Polymerase chain reaction (PCR) was used to amplify the 16S sequence 1174 to 1415 (241 base pair) and the 23S sequence 1183 to 1334 (146 base pair) specific probes from plasmid pKK3535 DNA.   PCR reaction mixtures contained 45 µl PCR Supermix High Fidelity reagent mixture (Gibco BRL), 1 µl of plasmid DNA (6.5 ng), 1 µl (10 pmol) of either the 16S or 23S forward primer, 1 µl (10 pmol) of either 16S or 23S reverse primer, and 2 µl sterile dH_2O. Samples were placed in a MJ Research PTC-100 programmable thermocycler for 35 cycles under the following conditions: denaturation at 94°C for 30 seconds, annealing of primers to target DNA at 57°C for 30 seconds and extension of the primers at 72°C for 30 seconds.   The PCR products were purified by extraction with an equal volume of phenol:$CHCl_3$ and precipitated with 2 volumes of pure ethanol.   The pellets were dried at 44°C for 15 minutes then resuspended in 30 µl of sterile dH_2O.   Purity of the PCR products was examined by running 1 µl on a 2% agarose gel.   The purified DNA probes were labeled with biotin using the Label-IT biotin labeling kit (Mirus) following the manufacturer's instructions.

Northern Analysis of 23S rRNA

All strains were grown, lysed, and centrifuged on 5-20% sucrose gradients as described above.   Gradients were collected into three pools: top, 30S, and 50S; and RNA was isolated as described above.   The RNA (5 µg) was mixed with 5 µl RNA resuspension buffer, heated at 60°C for 10 minutes, and then quickly cooled on ice.   After cooling, 2.5 µl of dye (80% glycerol, 1% bromo-phenol blue) was added to the samples before loading on 1% or 2% denaturing agarose gels.   Top samples ran for 2 hours, while 30S and 50S samples ran for 4 hours at 50 or 100 volts.

Following electrophoresis, RNA from the gels were blotted onto nylon membranes (Nytran) using a Turboblot apparatus. The blot system was assembled according to the manufacturer's instructions and alkaline transfer buffer was used to facilitate the transfer. Following the transfer, the membranes were neutralized in 1X neutralizing buffer and the RNA was cross-linked to the membranes using a UV oven on the optimal setting. The membranes were placed in 50 ml plastic tubes with 15 ml of 1X prehybridization solution (MRC, Inc.) and then pre-hybridized at 42°C for 30 minutes in a hybridization incubator (Fisher-Biotech). The pre-hybridization buffer was discarded and the membranes were hybridized overnight at 42°C in the hybridization incubator with 7.0 ml hybridization buffer, 0.7 ml 10X background quencher, and 4 pmol of denatured 23S or 16S probe. 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 Mirus Neutralization Buffer N1 and incubated at room temperature for 5 minutes.

Following hybridization, the membranes were washed and the probe detected using Pierce's North2South chemiluminescent hybridization and detection kit according to the manufacturer's instructions. The membrane was covered with plastic wrap on a glass plate and exposed to Fuji Medical X-ray film. Films were developed as described above.

CHAPTER 3

RESULTS

Effects of Azithromycin on Growth, Viability, and Protein Synthesis in *E. coli*

MIC determination and Growth Inhibition by Azithromycin

To examine azithromycin's inhibition of growth properties in *E. coli*, suitable growth conditions were first determined by means of MIC assays. The minimal inhibitory concentration (MIC) is the lowest concentration of the antibiotic that completely inhibits cell growth. MIC values were obtained by growing cells over a range of azithromycin concentrations and measuring the scattering of light. Figures 4 and 5 show the relationship between increasing azithromycin concentrations and decreasing number of cells in eight strains. The determined MIC values for azithromycin in the wild-type and mutant strains growing in TB and TSB are summarized in Table 3. As expected, MIC values for azithromycin are lower in *E. coli* cells grown at 32°C in TB compared to the cells grown in TSB at 37°C. Azithromycin is less effective in TSB at 37°C because of the accelerated growth rate and the enriched media. The growth rate is accelerated at higher temperatures because enzymatic reactions occur more quickly. Growing cells at 32°C in tryptone broth was advantageous in this study because growth and cellular processes such as ribosome assembly were slowed, which allowed better observation of ribosome assembly and degradation of rRNA. Cells were grown in TB at 32°C for the majority of the experiments.

Based on the MIC assays, a sub-inhibitory concentration of azithromycin was determined. A sub-inhibitory concentration slows cell growth without completely inhibiting growth of the culture. This concentration was determined to be 2.5 μg/ml for all strains except SK5704 and MRE600. Growth of these two strains was completely inhibited at this concentration, and therefore they were grown with a lower concentration of 1.0 μg/ml.

Growth rate and cell viability assays were performed to examine the inhibitory effects of azithromycin.   Table 4 summarizes the average percent increase in doubling time and average percent decrease in cell viability for all nine strains of *E. coli*.   As expected, all strains including wild-type demonstrated an increase in the doubling time and decrease in cell viability when grown with azithromycin. The doubling time for strains CA244 and SK5704 increased by 185% and 103% respectively, compared to 48% for the wild-type strain.   MRE600 was the most inhibited with an increase of 328%.   This strain is highly sensitive to azithromycin, and the sensitivity may or may not be due to the RNase I deficiency (see discussion for an explanation).

Cell viability decreased in all strains when grown with azithromycin.   Again, MRE600 was the most inhibited with a decrease of 97%, compared to 40% in the wild-type strain. SK5704 was less inhibited than the wild-type, with a decrease of 32%.

Effects of Azithromycin on the Rate of Protein Synthesis

The effect of azithromycin on the rate of protein synthesis in *E. coli* was examined by measuring the incorporation of ^{35}S-methionine into cellular proteins.   Figure 6 shows the incorporation of radioactivity over time in eight strains grown with and without azithromycin (MRE600 is not shown).   For all strains, ^{35}S-methionine was incorporated more rapidly in strains growing without azithromycin, indicating a higher rate of protein synthesis in the absence of antibiotic. The decrease in rate was greater for the mutant strains than the wild-type.   Table 5 shows the quantitative data obtained from liquid scintillation counting of samples grown with and without azithromycin.   When grown with azithromycin, the rate of synthesis in the wild-type strain dropped to 83% of the control.   Strains CA244, SK5665, and MRE600 were most inhibited, with a decline to 13%, 15%, and 11% respectively.
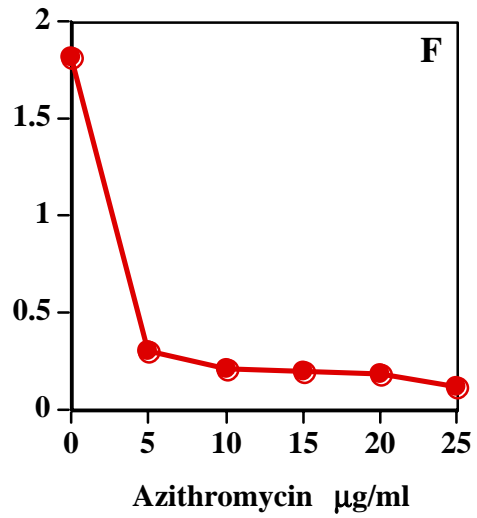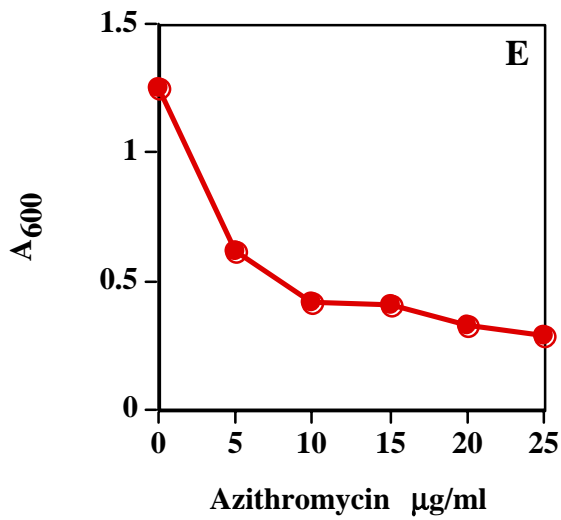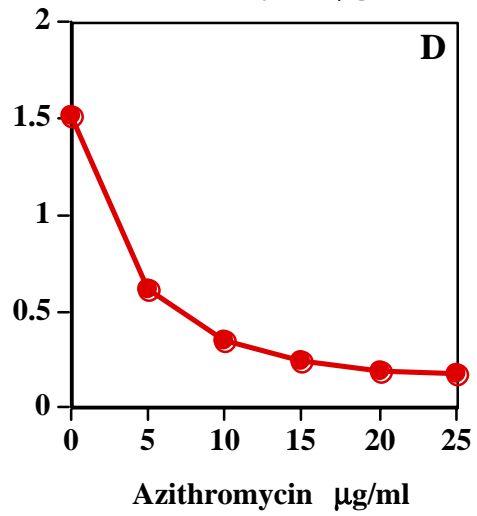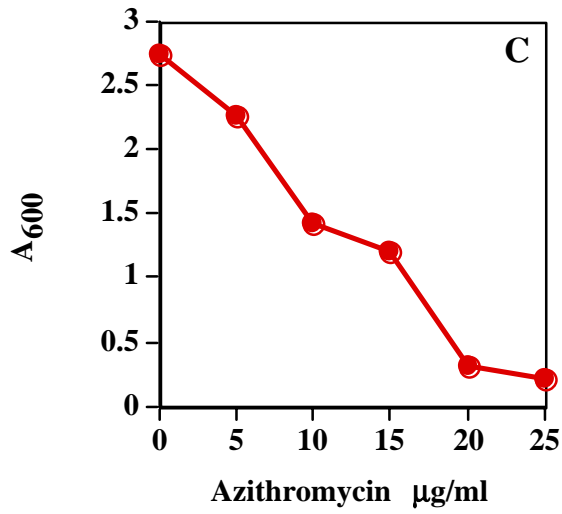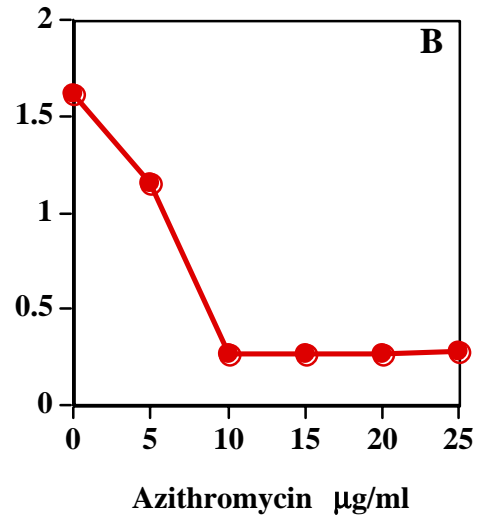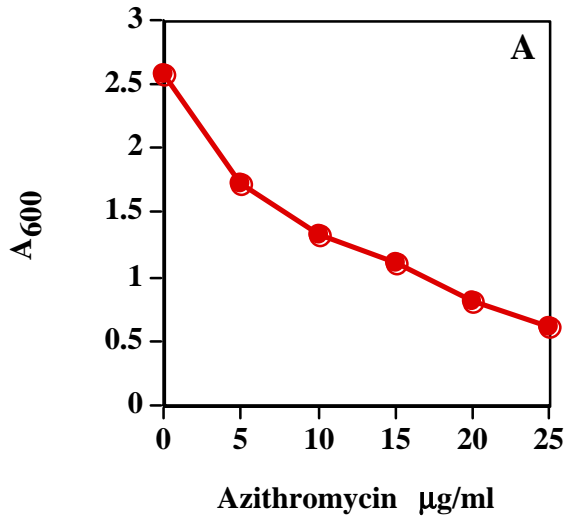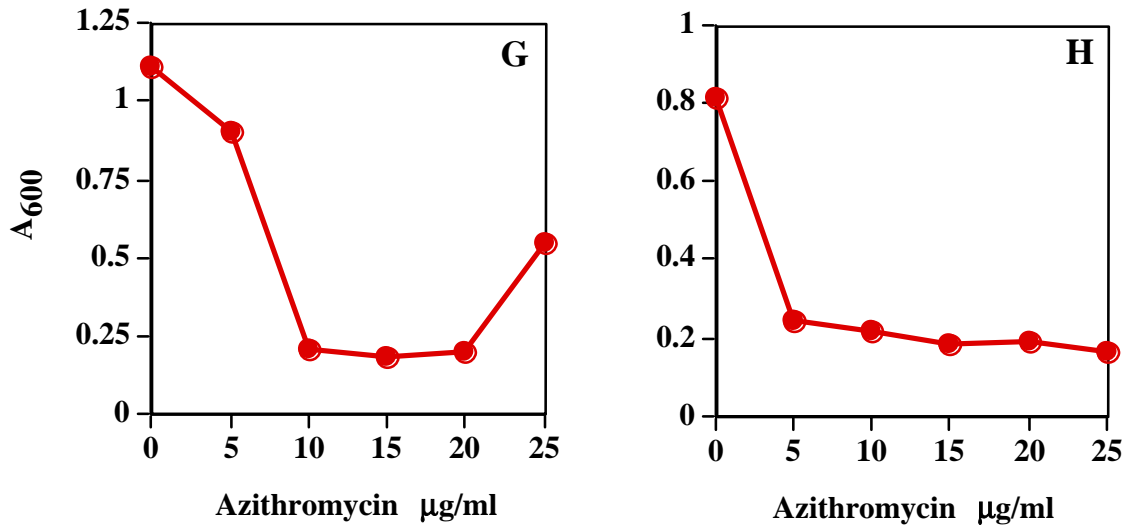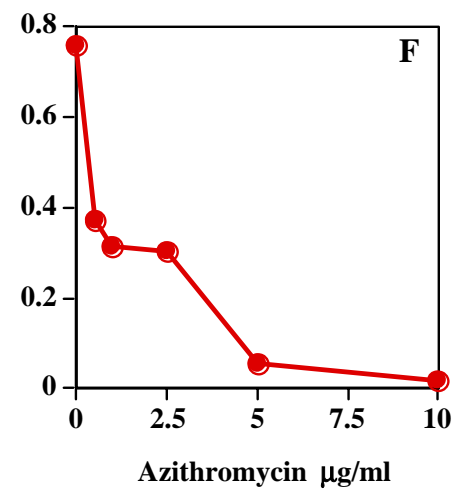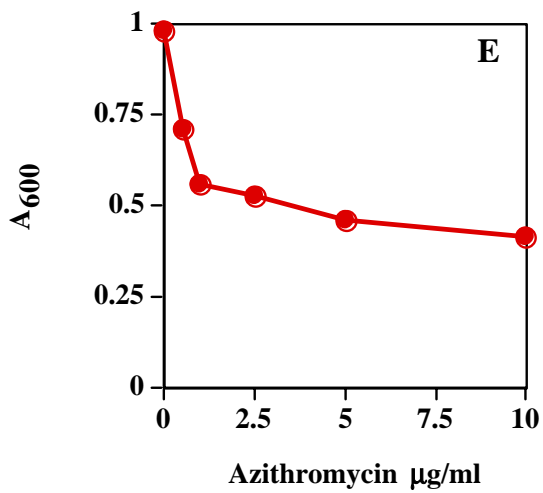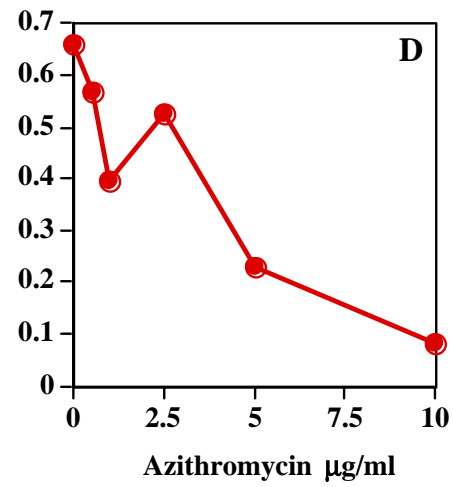
Figure 4.   MIC Assay of Azithromycin for Cells Grown in TSB at 37°C

E. coli cells from an overnight culture (50 μl) were placed in 1 ml of tryptic soy broth with increasing concentrations of azithromycin.   Cells were grown overnight at 37°C.   Growth density was measured using a spectrophotometer at 600 nm.   The graphs are representative of one experiment. (A) SK901 (B) N7060 (C) D10 (D) CA244 (E) SK7622 (F) SK4803 (G) SK5665 (H) SK5704
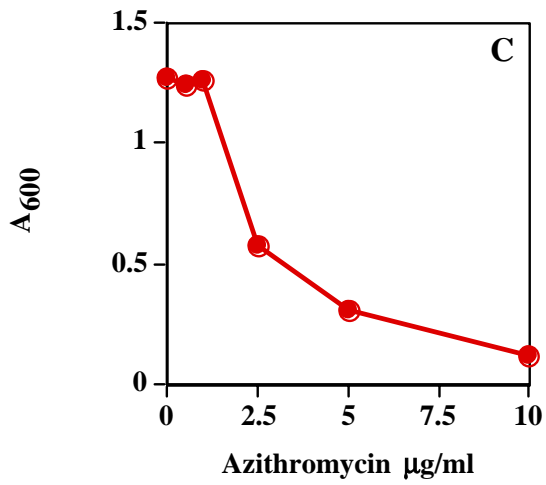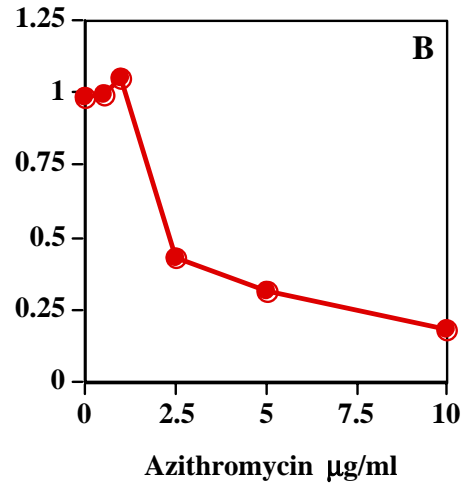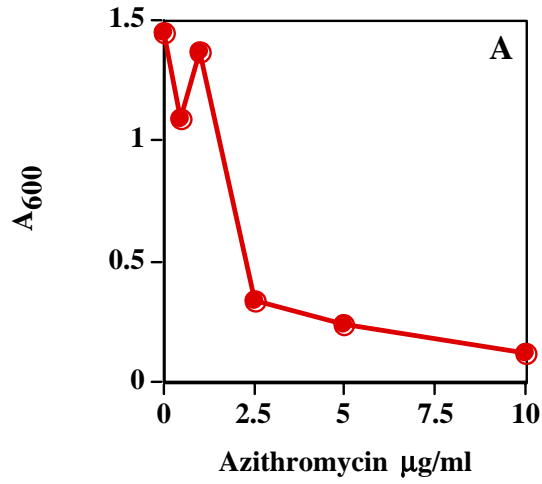
Figure 5.   MIC Assay of Azithromycin for Cells Grown in TB at 32°C

E. coli cells from an overnight culture (50 µl) were placed in 1 ml of tryptone peptone broth with increasing concentrations of azithromycin.   Cells were grown overnight at 32°C.   Growth density was measured using a spectrophotometer at 600 nm. The graphs are representative of one experiment.   (A) SK901 (B) N7060 (C) D10 (D) CA244 (E) SK7622 (F) SK4803 (G) SK5665 (H) SK5704
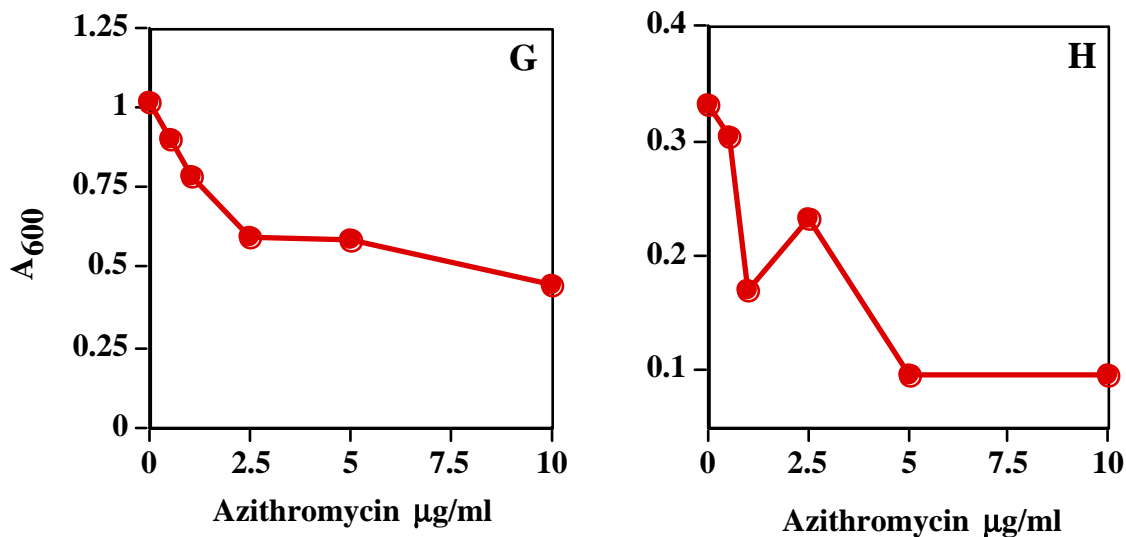
Table 3.   Minimal Inhibitory Concentration Values of Azithromycin

STRAIN	MIC OF AZITHROMYCIN (µg/ml)	
	Tryptic Soy Broth/37°C	Tryptone Broth/32°C
SK901	30.0	11.0
D10	25.0	11.0
MRE600	30.0	2.5
N7060	10.0	11.0
CA244	20.0	11.0
SK5665	10.0	11.0
SK7622	20.0	11.0
SK4803	8.0	5.0
SK5704	5.0	5.0

Minimal inhibitory concentration values of azithromycin as determined from Figures 4 and 5.

MIC experiments are performed to ascertain ideal growth conditions and antibiotic concentration.

This experiment was performed once at 37°C in TSB and once at 32°C in TB broth.

Table 4.   Inhibition of Cell Growth and Viability by Azithromycin

STRAIN	AZI	CELL VIABILITY		DOUBLING TIME	
		CFU	% DECREASE	MIN	% INCREASE
SK901	-	151		35	
	+	90	40	52	48
D10	-	168		45	
	+	55	67	63	40
MRE600	-	119		35	
	+	3	97	150	328
N7060	-	160		43	
	+	62	61	73	69
CA244	-	125		48	
	+	37	70	137	185
SK5665	-	96		52	
	+	21	78	65	25
SK7622	-	135		65	
	+	41	69	93	43
SK4803	-	40		122	
	+	15	63	157	29
SK5704	-	30		85	
	+	20	32	173	103

Strains were grown with or without 2.5 µg/ml azithromycin (1.0 µg/ml for SK5704 and MRE600) at 32°C in TB.   Doubling time was determined graphically by plotting the increase in cell density of each culture as a function of time.   Cell viability was measured by plating a 10^{-5} dilution of cells and counting the colonies forming units (cfu).   The values represent the mean of three experiments, with an S.E.M. of +/- 11.4 for cell viability assays and +/- 10.9 for doubling time assays.
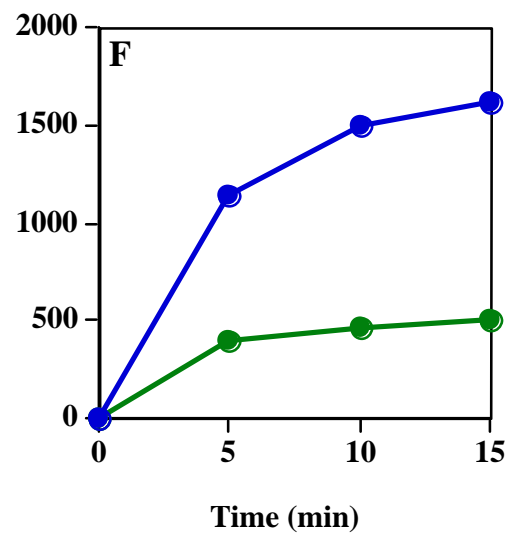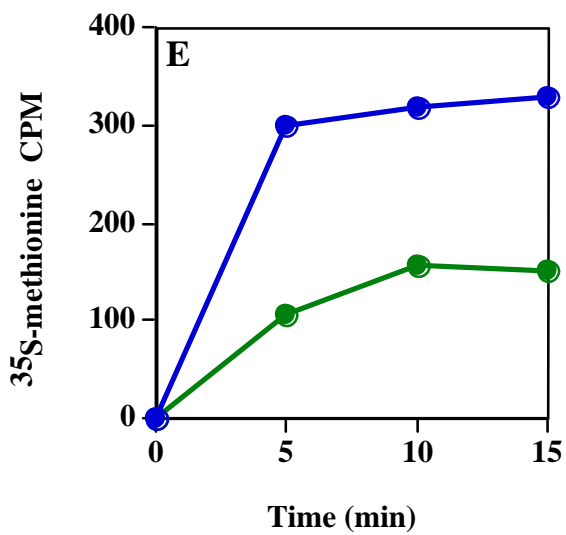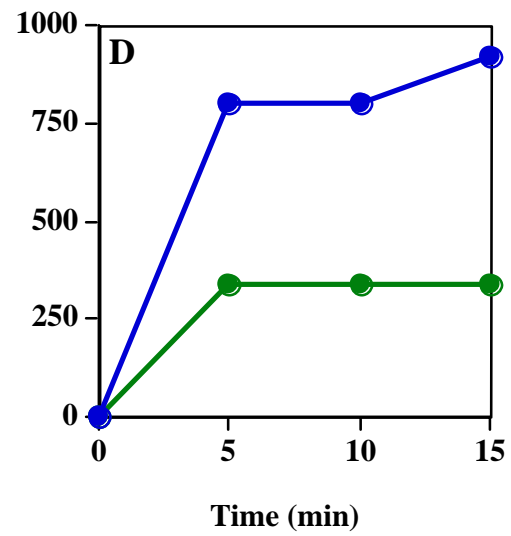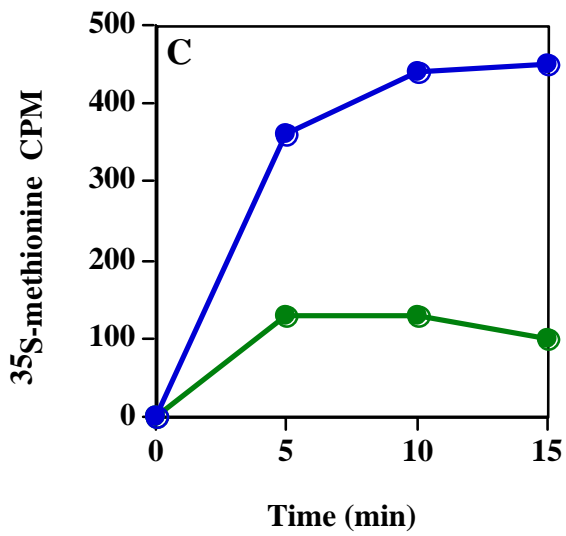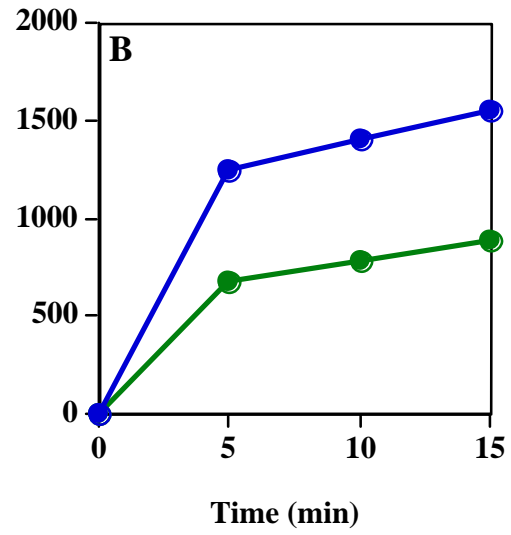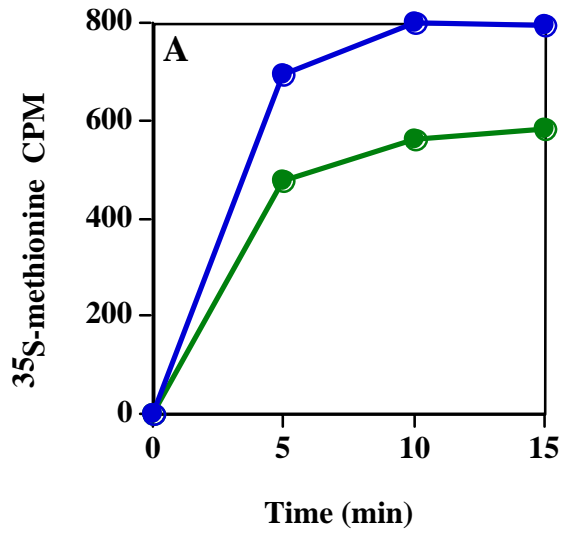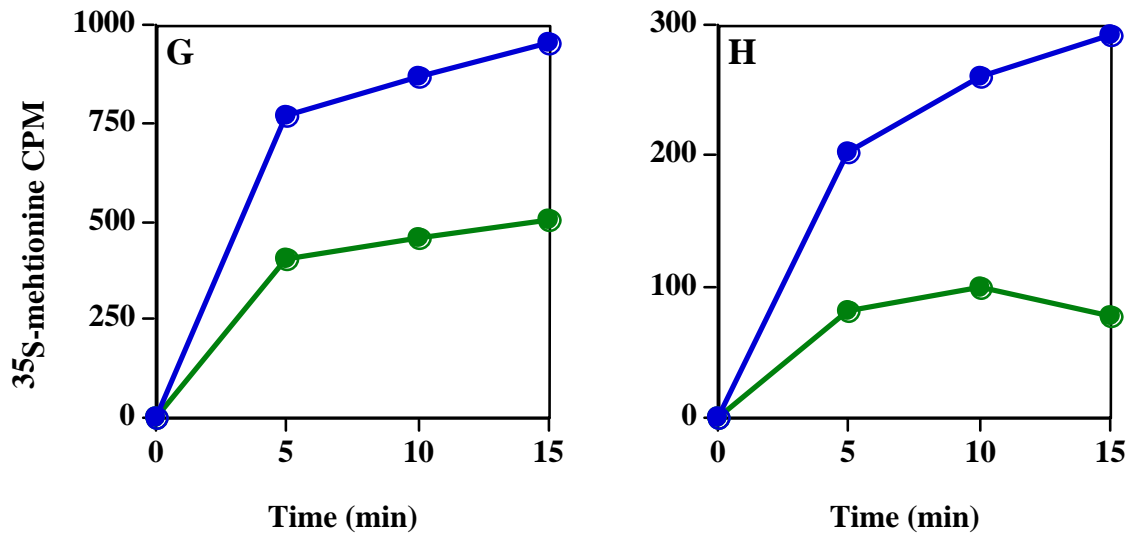
Figure 6.   Inhibition of Protein Synthesis Rate by Azithromycin

Cells were grown at 37°C in the presence (*green*) or absence (*blue*) 2.5 µg/ml of azithromycin.
Growth was measured using a Klett-Summerson colorimeter.   At a Klett reading of 80, cells
were labeled with ^{35}S-methionine (1 µCi/ml) and 400 µl samples were precipitated in 20% TCA at
5, 10, and 15 minutes after labeling.   The precipitate was collected on glass fiber filters and
measured by liquid scintillation counting. The graphs are representative of one experiment. (A)
SK901 (B) N7060 (C) D10 (D) CA244 (E) SK7622 (F) SK4803 (G) SK5665 (H) SK5704

Table 5.   Inhibition of Protein Synthesis Rate by Azithromycin

STRAIN	CONTROL (CPM)	AZITHROMYCIN (CPM)	% CONTROL
SK901	5446	4543	83
D10	5534	4252	76
N7060	14658	4286	29
MRE600	3734	418	11
CA244	5501	712	13
SK5665	10069	1511	15
SK7622	5201	2480	48
SK4803	23354	15959	68
SK5704	5622	1942	35

Strains growing with or without 2.5 µg/ml of azithromycin (1 µg/ml for SK5704 and MRE600) were labeled with ^{35}S-methionine as described in Figure 4.   Counts per minute (cpm) was determined by liquid scintillation counting.   The cpm values shown represent the 15- minute time sample and are the mean of three experiments with an S.E.M. of +/- 11.9%.

Inhibition and Recovery of 50S Subunit Assembly Following Azithromycin Exposure

Specific Inhibition of 50S Ribosomal Subunit Amounts by Azithromycin

Previous studies have shown that macrolide antibiotics have a second inhibitory target in microorganisms (Champney 2003). In addition to inhibiting translation, macrolide antibiotics specifically inhibit the formation of 50S ribosomal subunits in growing *E. coli* cells (Usary and Champney 2001). To confirm previous observations, 50S ribosomal subunit amounts were quantitated in cells growing in the presence or absence of azithromycin. Sucrose gradient profiles of ribosomal subunits labeled with ^{3}H-uridine during growth with azithromycin indicated a reduction in 50S subunit amounts in most of the strains, as expected. The reduction in RNA from the 50S region resulted in increases in RNA in the top and/or 30S region (Table 6). As Table 6 and Figure 7 show, 50S subunit assembly was most inhibited in strains SK4803, SK5704, and MRE600. In strain SK4803, 50S subunit amounts decreased from 42.2% to 18.7%, a difference of 23.5%. The RNA content in the top and 30S increased by 21.9% and 2.3%, respectively. In strain SK5704, the 50S subunit amounts decreased by 19%, while RNA in the top and 30S increased by 16% and 0.6%. The RNA increases in the top and 30S regions is assumed to be partially degraded 23S rRNA, since a previous study in our lab showed that in the presence of macrolides, antibiotic-bound 50S precursor co-sediments with the 30S subunit, and therefore increases the RNA content in this region. (Usary and Champney 2001). This experiment confirms the 50S specificity of azithromycin and also shows the differences in RNA content of each region after azithromycin exposure due to ribonuclease deficiencies.

Recovery of the 50S Ribosomal Subunit Following Azithromycin Treatment

Because azithromycin is bacteriostatic, treated cells are capable of recovery after removal of the macrolide antibiotic. Following antibiotic removal, normal 50S subunit synthesis resumes and subunit amounts eventually increase to normal levels. This assay was performed to determine

the rate of recovery of the 50S subunit and to determine if ribonuclease deficiencies affect the rate of recovery.   Figure 8 shows the percent radioactivity of RNA from the top, 30S, and 50S, as a function of time in eight of the strains (MRE600 is not shown).   As the 5-minute time sample shows, initially, the majority of the RNA is located in the top region of the gradient. In most of the strains, RNA in the top decreased as a function of time, while RNA in the 50S region increased.   The wild-type, N7060, D10, and SK5665 showed comparable results with the 50S subunit fully recovering to normal amounts within two hours.   The decrease in RNA from the top of the gradient and recovery of the 50S subunit was dramatically slower for strains SK4803 and SK5704.   The 30S remained constant, or increased slightly, in all strains as expected.

Table 7 shows the time required to degrade half the initial amount of RNA and the time required for full recovery of the 50S subunit in eight strains.   The RNA content of the top region decreased in all strains, but the rate of decay varied. Degradation was slowest in SK4803 and SK5704 with $T_{1/2}$ values of 740 and 600 minutes, respectively.   The 50S subunit, clearly inhibited in all strains, indicated varying rates of recovery, with strains SK5704 and SK4803 having the slowest rates.   These two strains, which have exoribonuclease mutations, required 650 minutes to return to normal 50S subunit levels of 28%.   This was dramatically longer than all remaining strains.
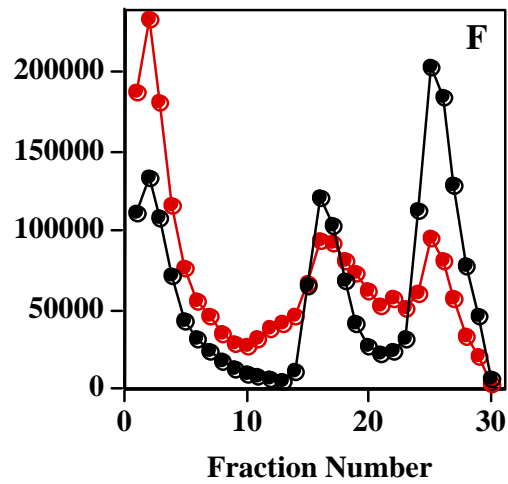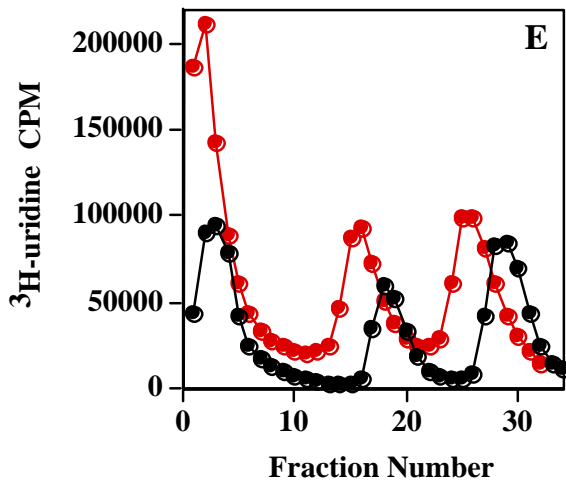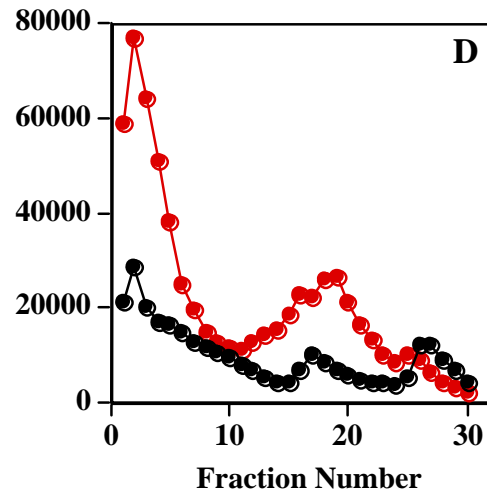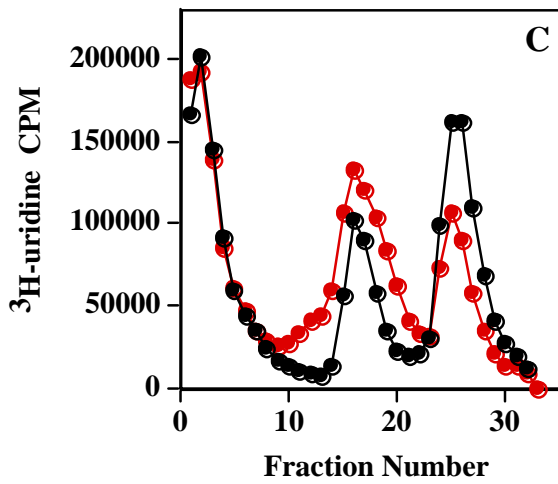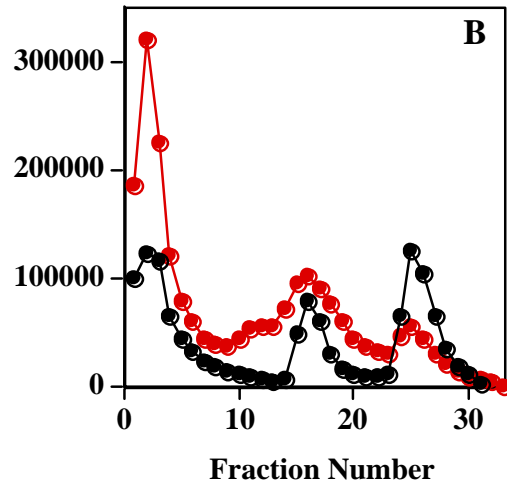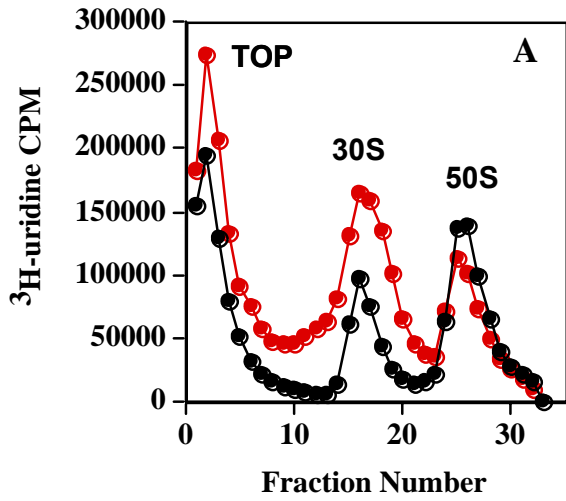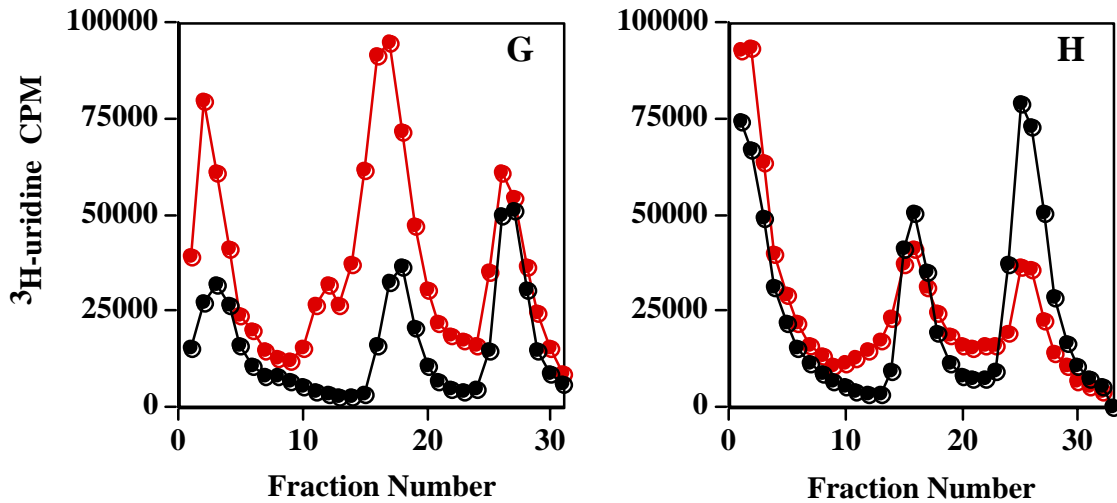
Figure 7.   Sucrose Gradient Profiles of ^{3}H-uridine Labeled Cell Lysates

Cells were grown in the presence (*red*) or absence (**black**) 2.5 µg/ml of azithromycin (1.0 µg/ml for SK5704 and MRE600) and labeled with 1 µCi/ml ^{3}H-uridine.   Cells were lysed, and lysates were centrifuged on 5-20% sucrose density gradients and fractions were collected.   Radioactivity was measured by liquid scintillation counting.   The graphs are representative of one experiment. (A) SK901 (B) N7060 (C) D10 (D) CA244 (E) SK7622 (F) SK4803 (G) SK5665 (H) SK5704

Table 6.   Inhibition of the 50S Subunit Assembly by Azithromycin

STRAIN	AZI	% TOTAL RADIOACTIVITY		
		TOP	30S	50S
SK901	-	42.7	17.7	33.2
	+	50.7	24.7	16.8
D10	-	42.5	17.3	33.8
	+	47	25.5	18.5
MRE600	-	30.6	21.0	41.1
	+	56.0	21.9	6.0
N7060	-	45	18.8	33.1
	+	63.5	22.4	11
CA244	-	63	13.7	17.3
	+	64	13.1	13.8
SK5665	-	28.8	21.9	40.8
	+	32.7	23.9	32.2
SK7622	-	42	18.7	33.8
	+	47.7	18.3	23.1
SK4803	-	32	21.4	42.2
	+	53.9	19.1	18.7
SK5704	-	37.2	20.6	36.5
	+	52.5	21.2	17.5

Total radioactivity of each gradient shown in Figure 7, was calculated and the percent
radioactivity present in each fraction, top, 30S, or 50S, was determined.   This experiment was
performed once for each strain.
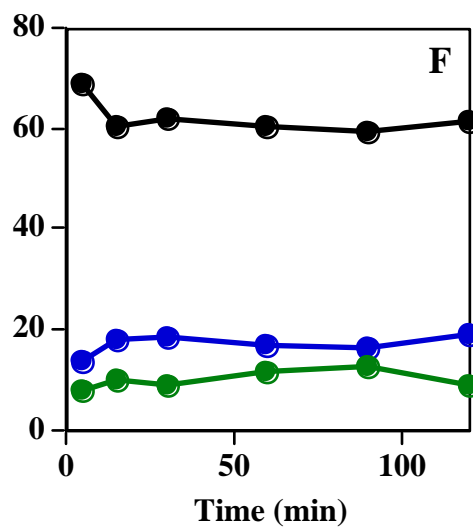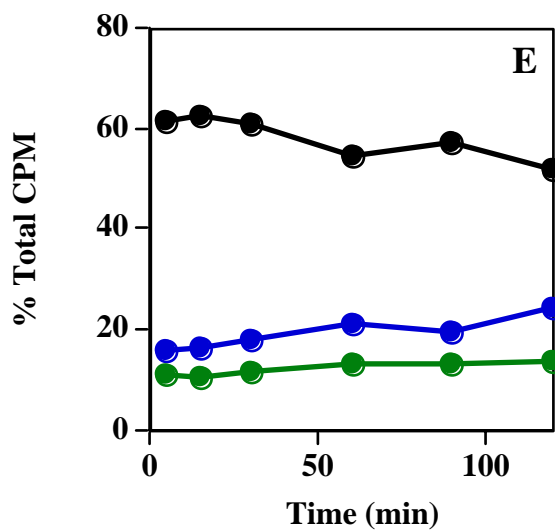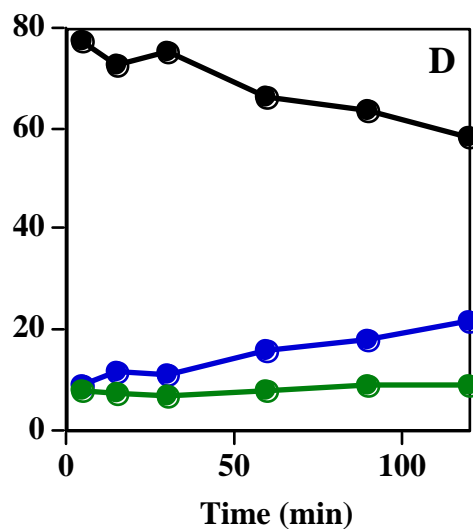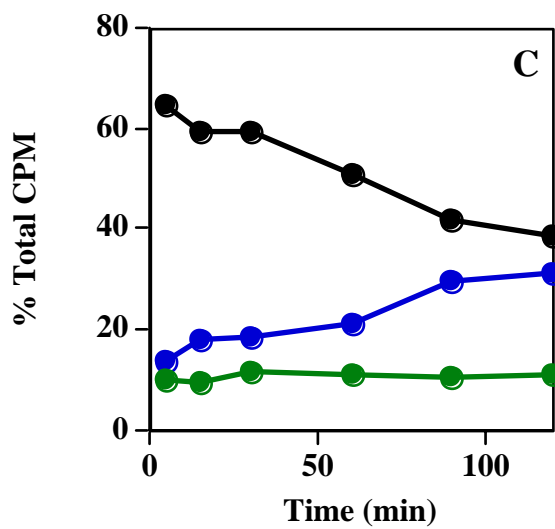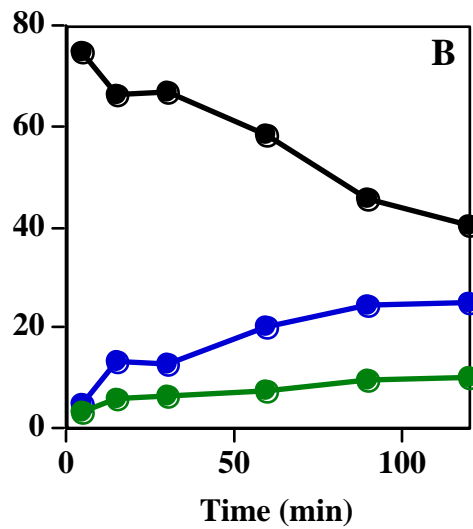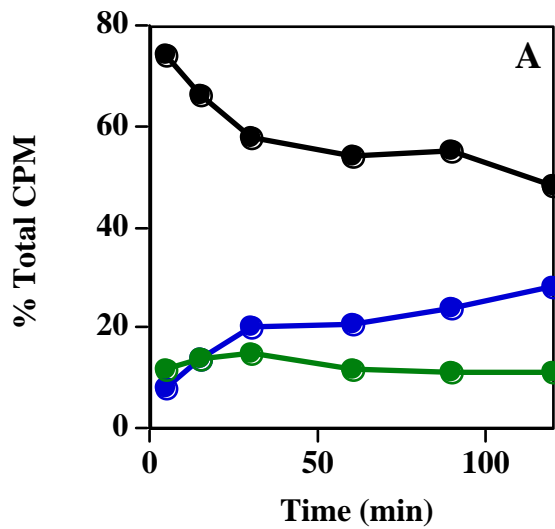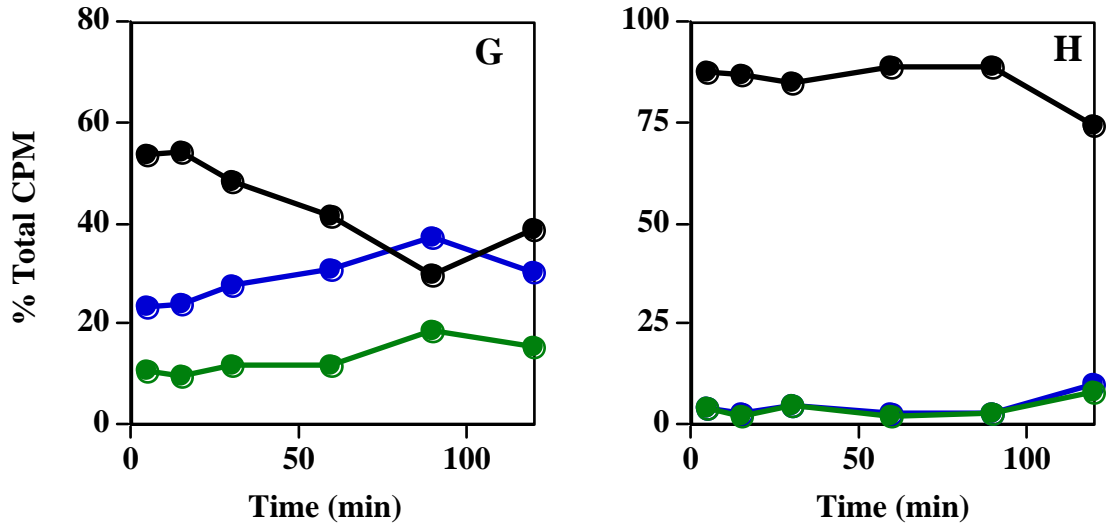
Figure 8.   Recovery of the 50S Ribosomal Subunit Following Azithromycin Removal

Cells were grown with 2.5 µg/ml of azithromycin (1.0 µg/ml for SK5704) and labeled with 2 µCi/ml ^{3}H-uridine.   After two cell doublings, cells were pelleted and washed with 32°C TB. Cells were resuspended and 3 ml samples were removed after 5, 15, 30, 60, 90, and 120 minutes. Lysed cells were centrifuged on 5-20% sucrose density gradients and fractions were collected. Radioactive counts per minute (cpm) was measured by liquid scintillation counting.   The percent radioactivity in the top is shown in **black,** the 30S subunit in **_green_**, and the 50S subunit in **_blue_**. The graphs show the average percent cpm of two experiments. (A) SK901 (B) N7060 (C) D10 (D) CA244 (E) SK7622 (F) SK4803 (G) SK5665 (H) SK5704

Table 7. Percent RNA in Each Fraction Following Azithromycin Removal

| | % Total Radioactivity | | | | | |
| | Top | | | 50S | | |
	5 min	120 min	$T_{1/2}$	5 min	120 min	Recovery Time (m)
SK901	74	48	175	8	28	120
D10	65	39	140	14	31	95
N7060	75	41	125	5	25	125
CA244	77	58	240	9	22	185
SK5665	54	39	145	24	30	40
SK7622	62	52	375	16	25	185
SK4803	69	62	740	14	19	650
SK5704	88	75	600	4	10	650

The values shown represent the total percent radioactivity for the 5 and 120 minute time sample for the Top and 50S gradient region as shown in Figure 8. The $T_{1/2}$ value is the time required to degrade half of the RNA content in the top region of the gradient. The recovery time is the time required to reach 28% total radioactivity in the 50S gradient region. $T_{1/2}$ and recovery time values were determined graphically using the best fit line of the average of two experiments.

Analysis of RNA by Electrophoresis and Hybridization

PAGE Analysis of Radiolabeled RNA

Compared to the wild-type strain, the experiments above indicate that certain strains, particularly SK4803 and SK5704, were more inhibited at identical concentrations of azithromycin. The reason for this increased sensitivity is unknown, but it may be the result of slower degradation of antibiotic bound 50S assembly intermediates. To test this, the RNA content of cells exposed to azithromycin was examined by PAGE analysis. Figure 9 shows fluorograms of radiolabeled RNA isolated from the top fraction of sucrose gradients. With the exception of disassociated transfer RNA, the control samples contain very little additional RNA, while the drug treated samples show significant amounts of RNA accumulation in a range of sizes. More RNA is present in the control sample than the azithromycin treated sample in strain SK5704. No difference in the control and azithromycin treated samples is evident in the 30S and 50S fluorograms (photos not shown), as expected. These results show that significant amounts of various sized RNA molecules accumulate in cells grown with azithromycin, which supports the hypothesis that RNA accumulation results in hypersensitivity to azithromycin.
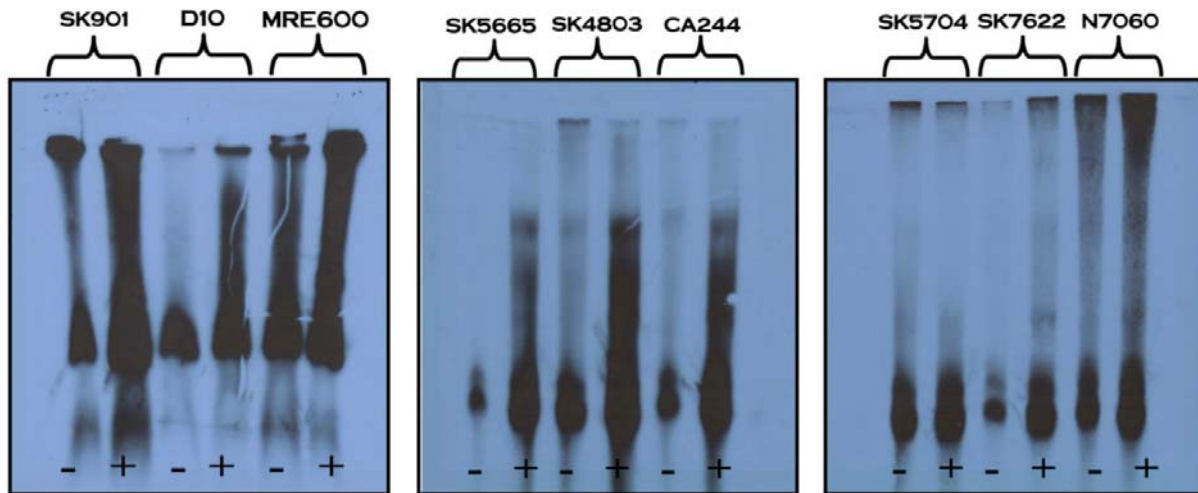
Figure 9. Fluorograms of ^{3}H-uridine Labeled RNA Separated on 8% Polyacrylamide Gels

Cells were grown in the presence (+) or absence (-) of azithromycin.   Cell lysates were

centrifuged on 5-20% sucrose gradients and Top, 30S, and 50S fractions were collected.   RNA

from each fraction was isolated and separated on 8 M urea, 8% polyacrylamide gels.   The dried

gels were exposed to X-ray film at -70°C for 7 days and then developed.   The 30S and 50S

fluorograms are not shown.   These fluorograms are representative of one experiment.

<u>Northern Hybridization Analysis of 23S rRNA Exposed to Azithromycin</u>

To confirm that the RNA accumulating in azithromycin treated cells is partially degraded 23S rRNA from the 50S ribosomal subunit, hybridization experiments were performed. Figure 10 shows Northern blots of RNA separated on 1% denaturing agarose gels hybridized with a biotinylated 23S DNA probe. In the top region (Figure 10A), partially degraded 23S rRNA is found in all strains including wild-type when grown with azithromycin. In the absence of azithromycin, partially degraded 23S rRNA does not accumulate. Comparable results were found for the 30S region (Figure 10B), although the blot shows more partially degraded 23S rRNA in strains SK4803 and CA244. In the 50S region (Figure 10C) similar amounts of 23S rRNA is found in the control and azithromycin treated samples, as expected.

To confirm the specificity of azithromycin, RNA isolated from the 30S region of the sucrose gradient was hybridized with a biotinylated 16S DNA probe (Figure 10D). Since azithromycin specifically affects the 50S subunit, no significant change in 16S rRNA levels is expected when cells are treated with the antibiotic. Figure 10D confirms no difference in 16S rRNA between the control and azithromycin treated samples.

The partially degraded 23S rRNA in the all Northern blots was quantitated using the Alphaease software program. Photos of the blots were placed in an Alpha Inotech MultiImage™ light cabinet and the total integrated density value (IDV) was determined using the spot density function. The percent total in each lane for the top, 30S, and 50S blots was determined by the software as shown in Table 8. In the top and 30S region, all strains except for SK5704 and SK7622, contained more partially degraded 23S rRNA in the azithromycin treated samples. In the top, the results were similar for most strains. Without antibiotic, 23S rRNA was essentially absent from the samples. When exposed to azithromycin, strain CA244 contained the least 23S rRNA, 2.4%, compared to 7.2% for the wild-type. MRE600 contained the greatest percent (9.8%). In the 30S blots, all strains had more partially degraded 23S rRNA

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than the wild-type.   Strain CA244 (11.0%) and SK7622 (12.2%) both contained significantly higher percentages than the remaining strains.   SK4803, SK5704, and N7060 also contained significant amounts of RNA in the 30S region, indicating the presence of incompletely assembled 23S precursor particle.   For the 50S region, insignificant differences were seen in all strains. The control and azithromycin treated samples of N7060, SK7622, and SK5704 did not differ, but the total amounts compared to the other stains were low.   For this region only, exposure of the blot containing these three strains was lighter, which resulted in lower percentages.

Northern Hybridization Analysis of 23S rRNA with RNA Size Standards

The hybridization experiments clearly indicated the presence of 23S rRNA particles of various sizes in the top and 30S regions of the sucrose gradients when treated with azithromycin. To obtain an estimate of the size of these particles in the top region, Northern analysis was repeated with biotinylated RNA size standards.   For better resolution of the RNA, 2% agarose gels were run at 40 volts. Figure 11A shows a Northern blot with the RNA size standards in lanes 1 and 12.   The standard curve (Figure 11B) was constructed on semi-log graph paper by plotting the measured migration distance of the RNA size standards versus the base size of each band. To determine the sizes of the unknown 23S rRNA bands, migration distance of each band was measured and compared to the standard curve.   Table 9 summarizes the sizes and range of the 23S rRNA particles found in the top region for all of the strains.   When treated with azithromycin, two distinct bands are present in all strains.   The larger band is around 1000 bases and the smaller is 400-700 bases.   Strains SK5704, SK4803, SK5665, and D10 have an additional, smaller RNA molecule that ranges from 260-396 bases.   This experiment intiated the characterization of the partially degraded 23S rRNA, but much more experimentation is needed to characterize the degradation products and draw any definitive conclusions.

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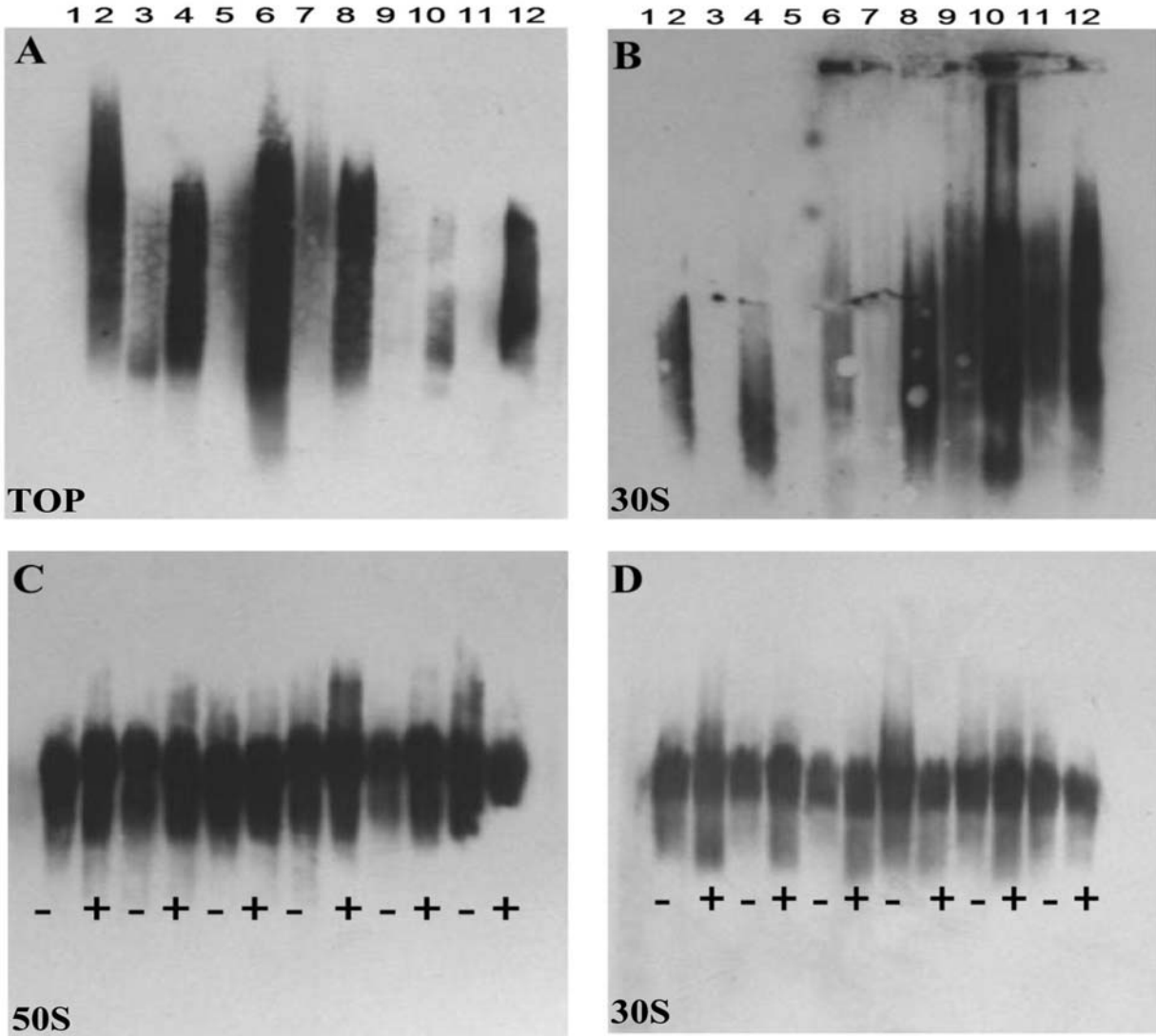
Figure 10.   Northern Analysis of 23S rRNA

Cells were grown in the presence (+) or absence (-) of azithromycin.   Cell lysates were centrifuged on 5-20% sucrose density gradients and top (A), 30S (B,D), and 50S (C) fractions were collected.   RNA was isolated from each fraction and separated on a 1% agarose gel.   A blot of the gel was hybridized with a biotin labeled 23S DNA probe to analyze 23S rRNA degradation.   (D) A biotin labeled 16S DNA probe was hybridized to RNA from the 30S region as a control.   These blots are representative of one experiment.   Lanes 1 and 2 are wild-type SK901, 3 and 4 are D10, 5 and 6 are MRE600, 7 and 8 are SK5665, 9 and 10 are CA244, and 11 and 12 are SK4803.

Table 8. Quantitation of 23S rRNA in the Top, 30S, and 50S Regions of Gradients

STRAIN	AZI	% INTEGRATED DENSITY VALUE		
		TOP	30S	50S
SK901	-	1.9	0.9	6.7
	+	7.2	3.4	7.2
D10	-	4.2	1.2	6.5
	+	7.2	3.8	6.5
MRE600	-	4.8	1.6	6.5
	+	9.8	3.3	7.1
N7060	-	4.1	2.0	2.8
	+	7.7	8.8	2.9
CA244	-	1.4	6.8	6.3
	+	2.4	11.0	6.9
SK5665	-	5.3	2.3	7.2
	+	7.4	6.6	7.9
SK7622	-	8.3	9.0	2.5
	+	7.9	12.2	3.2
SK4803	-	1.5	6.7	8.4
	+	5.0	7.9	6.0
SK5704	-	7.0	6.4	2.8
	+	7.0	6.0	2.8

Films, like those shown in Figure 10, were analyzed using the Alpha Inotech MultiImage™ light cabinet and Alphaease software.   Integrated density values were determined using the spot density function.   The areas analyzed were identical for each lane.   The values represent data from one experiment.

A

1 2 3 4 5 6 7 8 9 10 11 12

1000 b →

500 b →

100 b →

S - + - + - + - + - + S
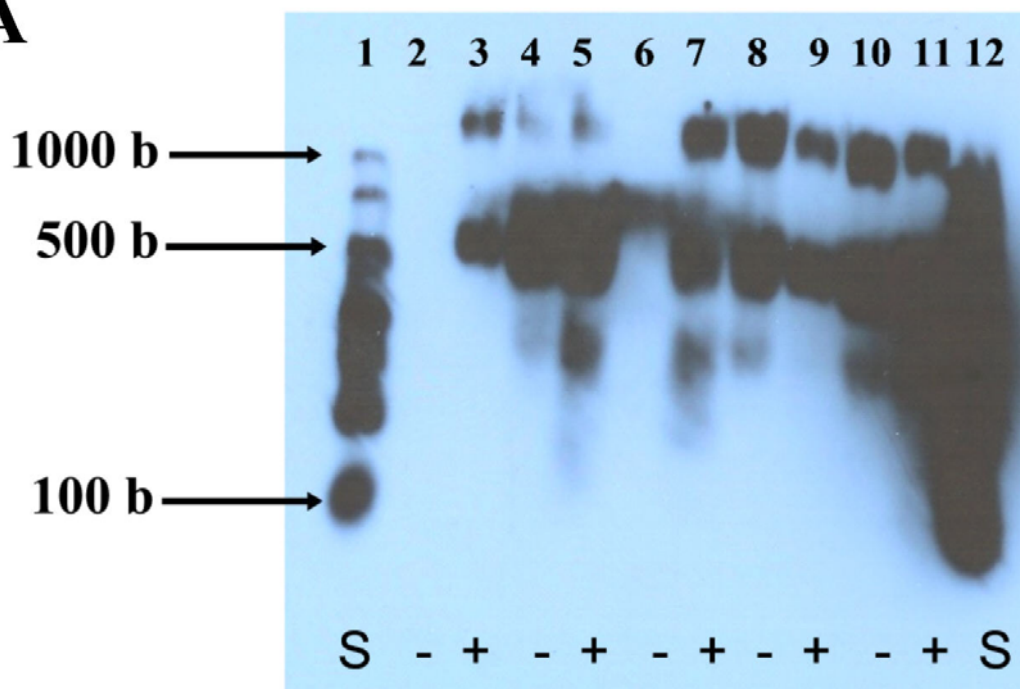
B
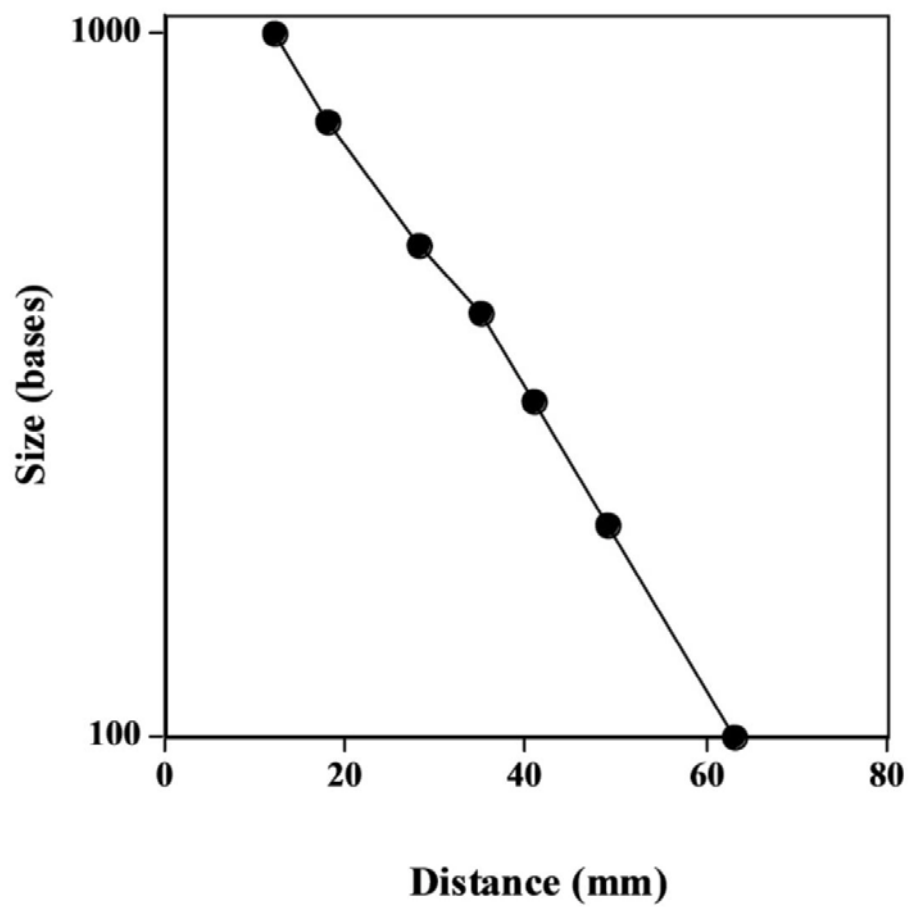
Figure 11.   Northern Analysis of 23S rRNA with RNA Size Standards

A.   Cells were grown and RNA was separated into three fractions.   RNA isolated from the top fractions was separated at 40 volts on a 2% agarose gel with biotinylated RNA size standards (Ambion's Century Marker Plus).   A blot of the gel was hybridized with a biotin labeled 23S DNA probe to determine the sizes of the 23S rRNA bands.   Lanes 1 and 12 are the biotin labeled RNA size standards.   This Figure is representative of one experiment.   Lanes 2 and 3 are wild-type SK901, 4 and 5 are D10, 6 and 7 are SK5665, 8 and 9 are CA244, 10 and 11 are SK4803.

B.   A standard curve was constructed by plotting migration distance of the size standards versus the base size of each band on semi-log graph paper.

Table 9.   Size Range of 23S rRNA in the Top Region of the Gradient

STRAIN	SIZE OF 23 rRNA BANDS (BASES)		
	AZI	SIZE (± bases)	
SK901	-	None detected	
	+	1150	585 (± 85)
D10	-	630 (± 200)	
	+	1150	630 (± 200) 300 (± 50)
N7060	-	None detected	
	+	750	
CA244	-	1075 (± 175) 540 (± 140) 293 (± 12)	
	+	1015 (± 85) 495 (± 95)	
SK5665	-	695 (± 105)	
	+	1075 (± 175) 565 (± 165) 275 (± 30)	
SK7622	-	1250 700 (± 50)	
	+	1250 700 (± 50)	
SK4803	-	933 (± 142) 460 (± 130) 260 (± 35)	
	+	953 (± 122) 408 (± 183)	
SK5704	-	1250 700 (± 50)	
	+	1225 (± 125) 685 (± 85) 396 (± 55)	

CHAPTER 4

DISCUSSION

The mechanism of translational inhibition by macrolide antibiotics in bacterial cells is well established.   Macrolides bind to a site on 23S rRNA, at the entrance of the peptidyltransferase tunnel, and sterically block peptide elongation (Mascaretti 2003; Cate et al. 1999).   Recently, numerous studies have identified a second, equivalent target in bacterial cells.   In addition to inhibiting translation, macrolide antibiotics bind to a 50S subunit intermediate and stop the assembly process.   This new target has been confirmed in several bacterial species including *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus subtillis*, and *Haemophilus influenzae* (Champney and Burdine 1995; Chittum and Champney 1995; Champney 2003; Mabe et al 2004).   A detailed study by Usary led to the identification and partial characterization of the 50S ribosomal intermediate particle that accumulates as a result of assembly inhibition of the 50S subunit by erythromycin A (Usary and Champney 2001).   This work implied that the 50S precursor particle, when bound to erythromycin A, is targeted for degradation by ribonucleases and also suggested that cells deficient in ribonucleases may exhibit increased sensitivity to macrolide antibiotics.   The present study confirms that cells deficient in certain ribonucleases, particularly exoribonucleases, exhibit hypersensitivity to azithromycin and demonstrate reduced turnover of 23S precursor rRNA when exposed to azithromycin.

Azithromycin inhibited growth and viability in all nine strains of *E.coli,* as expected. However, the extent of inhibition was not equivalent in all of the strains.   With very few exceptions, strains with exoribonuclease deficiencies were inhibited to a greater extent than the endoribonuclease mutants.   The MIC values show that strains SK4803 and SK5704 growing in TB at 32°C were inhibited at 5μg/ml, less than half the concentration for all remaining strains. For most of the assays, these two strains showed comparable results and were the most inhibited by azithromycin.   An exception to this was seen in the rate of protein synthesis for strain SK4803.

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Both SK4803 and SK5704 have a mutation in ribonuclease II.   SK5704 has an additional mutation in PNPase.

Strain CA244, which also has an exoribonuclease mutation, had a lower MIC compared to the wild-type when grown in TSB at 37°C, but had an equivalent MIC when grown in TB at 32°C.   However, the growth rate for CA244 was dramatically inhibited by azithromycin, with a doubling time increase of 185%.   The rate of protein synthesis was also significantly affected in this strain, with the rate being only 13% of the control.

Strain SK5665, an RNase E mutant, was notably inhibited in the rate of protein synthesis, with a decrease to only 15% of the control.   Despite this, cell viability and growth rate was not particularly inhibited.   Strains D10, N7060, and SK7622 were slightly more inhibited by azithromycin in the growth and protein synthesis rate assays than the wild-type but much less inhibited than strains SK4803, SK5704, and CA244.

Strain MRE600 is a ribonuclease I defective strain, but unlike D10 (also a ribonuclease I mutant), MRE600 showed hypersensitivity to azithromycin.   The apparent reason for this contradiction is that MRE600 has other genetic defects in addition to the ribonuclease I mutation. It is not a K-12 strain like the other strains in this study, and ribosomal protein S7 is abnormal, which most likely explains the observed hypersensitivity (Sun and Traut 1973). The observed hypersensitivity is most likely the result of other genetic mutations, and not the ribonuclease I mutation.   Although this observation is significant, the data for MRE600 were not considered relative to this study.

The 50S subunit inhibition and recovery assays, again, indicated SK5704 and SK4803 to be more sensitive to azithromycin than the other strains examined.   Although the sucrose gradient profiles showed a specific decrease in 50S subunit amounts in all strains, the 50S subunit decreased most dramatically in SK5704 and SK4803, from 36.5% to 17.5% and 42.2% to 18.7% respectively.   Recovery assays of the 50S ribosomal subunit correlated with the sucrose gradient profiles.   As expected, strains SK4803 and SK5704 are slowest to recover from the

inhibitory effects of azithromycin.   In fact, these two mutants do not fully recover within 2 hours of removal of azithromycin and the rate of decay of RNA in the top of the gradient is much slower.   All other strains, except SK7622, a ribonuclease III mutant, fully recover within or before 2 hours, similar to the wild-type.

The conclusion from these assays is that CA244, SK4803, and SK5704 are hypersensitive to azithromycin.   These strains have mutations in PNPase and ribonuclease II, both of which are exoribonucleases.   The apparent reason for this hypersensitivity, as predicted from the 50S subunit inhibition model, is particle accumulation.   Reduced turnover of nonfunctional 50S precursor results in RNA accumulation in the cell and thus promotes hypersensitivity to azithromycin.   This hypothesis was confirmed by gel electrophoresis and hybridization assays of RNA from azithromycin treated cells.   A preliminary experiment involving electrophoresis of ^{3}H-uridine labeled RNA indicated that substantial amounts of RNA was found in cells after azithromycin treatment, compared to non-drug controls.

To confirm that the accumulated RNA was from the 50S subunit, hybridizations with a 23S specific DNA probe were performed.   The results from these experiments clearly show that fragments of 23S rRNA were found in the 30S and top region of the gradient, whereas virtually none is found in the absence of the antibiotic.   A majority of the fragmented 23S rRNA was found in the top gradient region for the strains other than SK4803 and CA244.   For strains SK4803 and CA244, as Figure 10B shows, significantly more fragmented 23S rRNA was found in the 30S region, particularly for CA244, indicating the presence of larger fragments of 23S rRNA in these cells.   This might be expected, because these cells lack exoribonucleases and therefore cannot effectively degrade the RNA into mononucleotides.   For strain SK5704, both the PAGE analysis and hybridization experiments show that RNA accumulates in similar amounts in the presence and absence of azithromycin.   Apparently, the lack of three ribonucleases results in incomplete degradation of RNA molecules and therefore RNA accumulates even in the absence of antibiotic.   This would explain the low MIC and lower dosage requirements of this strain.

The cells simply cannot function properly with excessive accumulation of fragmented RNA and consequently, addition of azithromycin results in cell death.

The results for strain N7060 show an interesting contradiction to the results found for SK4803. N7060, a double-mutant strain, did not demonstrate notable hypersensitivity when grown with azithromycin even though it contains a mutation in ribonuclease II, the same mutation found in SK4803. In addition, N7060 lacks a functional RNase I, and as a result, the sensitivity of this mutant should have been equal to or greater than that found for SK4803. This was not the case. The most likely explanation for this observation is an ineffective mutation in the ribonuclease II gene. The results for N7060 were very similar to D10, a single mutant lacking ribonuclease I, suggesting that the ribonuclease II was still at least partially functional. Considering this, hypersensitivity would not necessarily be expected.

The results of this work strongly implicate the exoribonucleases RNase II and PNPase to be important enzymes in turnover of azithromycin exposed 23S rRNA. Fragmented 23S rRNA accumulates in cells lacking either enzyme when exposed to azithromycin, while very little RNA accumulates in the absence of the antibiotic. Under favorable conditions, ribosomal RNA is very stable and accounts for nearly 80% of the RNA in *E. coli* cells (Deutscher 2003). Degradation of these stable molecules does not occur unless unfavorable conditions, such as starvation or antibiotic, are present. Currently, little has been established about degradation and turnover pathways of stable RNAs. This work provides evidence for the importance of exoribonucleases in this process.

The significance of RNase E in azithromycin sensitivity and 23S rRNA turnover remains unclear. Although a previous study has implicated an important role in 23S precursor degradation, in this study, growth rate, cell viability, and 50S subunit recovery were marginally inhibited by azithromycin, suggesting unimportance of this enzyme. Further studies with this enzyme and other ribonucleases such as oligoribonuclease (an exoribonuclease) should provide clarification on the processes involved in azithromycin induced 23S rRNA turnover.

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