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Characterization of 50S Ribosomal Subunit Assembly Inhibition in Erythromycin Treated *Escherichia coli* Cells.

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CHARACTERIZATION OF 50S RIBOSOMAL SUBUNIT ASSEMBLY INHIBITION IN ERYTHROMYCIN TREATED ESCHERICHIA COLI CELLS.

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by
Jerry E. Usary
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APPROVAL

This is to certify that the Graduate Committee of

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met on the

6th day of July, 2000.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

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ABSTRACT

CHARACTERIZATION OF 50S RIBOSOMAL SUBUNIT ASSEMBLY INHIBITION IN ERYTHROMYCIN TREATED ESCHERICHIA COLI CELLS

by

Jerry E. Usary

Erythromycin has long been recognized for its ability to inhibit protein synthesis by interfering with mRNA translation on the bacterial ribosome. We have recently shown that erythromycin also inhibits the assembly of the 50S ribosomal subunit in growing bacterial cells. The nature of this assembly inhibition has been investigated using ^{3}H-uridine pulse-chase labeling of control and erythromycin treated E. coli cells.

Subunit assembly was examined by sucrose gradient centrifugation of labeled cell lysates. Normal assembly kinetics of subunit assembly were observed in control cells at 37^{o}C. Formation of the 30S subunit was completed by 7.5 minutes and assembly of the 50S subunit was finished by 15 minutes after an unlabeled uridine chase. At 37^{o}C, in the presence of erythromycin, 30S subunit assembly was unaffected but 50S assembly was greatly reduced. When the assembly kinetics were examined at 27^{o}C, the assembly of both subunits was slowed and 30-32S precursor particle was seen to accumulate. This particle was found to bind ^{14}C-erythromycin in vivo and in vitro.

RNase E has been implicated in the normal degradation and turnover of rRNA. A RNase E- mutant accumulated substantially more precursor to the 50S subunit than did control cells at either 37^{o}C or 27^{o}C. This precursor particle was also found to bind ^{14}C-erythromycin . Specific 50S proteins and the 23S and 5S rRNAs were found in the 30S gradient region from lysates of cells grown at 27^{o}C, confirming the presence of a 50S subunit precursor co-sedimenting with 30S subunits under these conditions. The precursor particle in the RNase E^{-} mutant had a larger number of associated 50S proteins than did the precursor from SK901. These data are consistent with our model of 50S subunit inhibition by erythromycin in which a fraction of 50S precursor particles undergo normal maturation, while the remainder are eventually degraded.

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I am eternally grateful to my wife Debbie for her love and support. Debbie has been especially helpful on this project. She often came in to the lab late at night and helped me process a seemingly endless number of sucrose gradient fractions.

I am thankful to my father Joseph and mother Brenda for not giving up on me during my more difficult years. I am especially grateful to my wonderful daughter Amanda.

Finally, I would like to acknowledge the myriad philosophers and authors whose works have influenced my life by quoting two of my most favored:

Examine your beliefs.

--- Socrates

All who wander are not lost.

--- J.R.R. Tolkien

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ABBREVIATIONS

2-D	two-dimensional
Bis-Tris	bis-tris(hydroxymethyl) aminomethane
CPM	counts per minute
dH_2O	deionized water
EDTA	ethylenediaminetetraacetic acid
ERY	erythromycin
e.u.	equivalence units of ribosomal subunit proteins
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid
L Broth	Luria broth
MIC	minimal inhibitory concentration
ND	no drug (antibiotic) present
MOPS	3-(N-morpholino)propanesulfonic acid
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
T Broth	tryptone broth
TCA	trichloro-acetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TP30	total proteins from 30S subunits
TP50	total proteins from 50S subunits
TP70	total proteins from 70S ribosomes
Tris	tris(hydroxymethyl) aminomethane

CHAPTER 1
INTRODUCTION

Isolation of antibiotic resistant bacteria from clinical infections has risen dramatically in the past decades. Our arsenal of effective antibiotics is rapidly diminishing, and researchers are seeking new and more effective antimicrobials. An important step in the search is a better understanding of currently used antibiotics, both their targets and mechanisms of action. The effectiveness of some classes of antibiotics results from their specificity for bacterial ribosomes and their ability to inhibit protein synthesis during translation.

Ribosomes are the cellular organelles that perform the task of protein synthesis. In E. coli, ribosomes constitute 25% of the dried cell mass and a single cell contains about 15,000 ribosomes (Nierhaus 1982). Bacterial ribosomes are particles with a sedimentation coefficient of 70S and a molecular mass of 2.3 MDa. They are composed of 2 subunits, a 30S (small subunit) and a 50S (large subunit) as shown in Figure 1. Each of the subunits is a ribonucleoprotein complex.

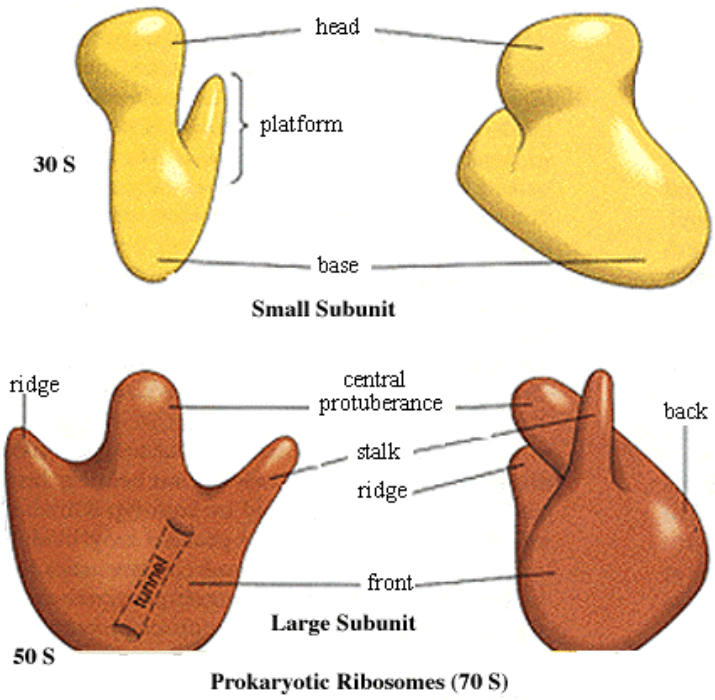
In E. coli, the 30S subunit contains a single 16S rRNA molecule (1542 nucleotides) and 21 different proteins. The 50S subunit has two rRNA molecules, a 23S (2900 nucleotides) and a 5S (120 nucleotides) along with 32 proteins (Hardy et al. 1969).

The rRNA genes in E. coli are located in seven rRNA operons in the order 16S-spacer-23S-spacer-5S. RNase III cleavage of the pre-rRNA transcript yields precursor rRNA molecules that are further trimmed by other ribonucleases into mature molecules. The rRNAs also undergo extensive post-transcriptional methylations (Nierhaus 1982).

Ribosomal proteins have a molecular weight range of 5.3 to 29.4 kDa. Many of them are small, hydrophobic, and basic with most having a pI in the range of 8.5 and 10 (Giri et al. 1984). This neutralizes the negative charges on the phosphates of the rRNA and permits close packing of the molecule.

Ribosomal proteins are designated by either an L or an S (for large or small subunit) and a number that corresponds to their migration in a two-dimensional gel system.

FIGURE 1. THE PROKARYOTIC RIBOSOME.



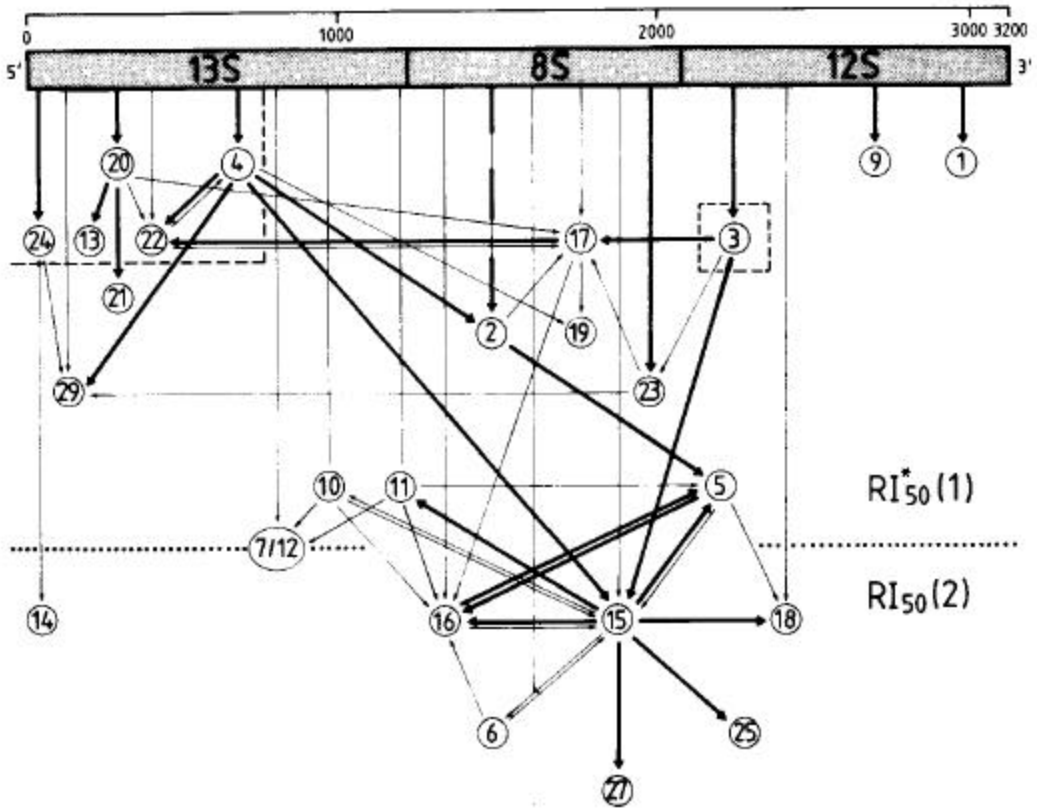
Source: http://ntri.tamuk.edu/cell/ribosomes.html, 2000.

The small subunit proteins (S1-S21) are present in 1 copy each except S6, which is present in 2 copies. The protein composition of the large subunit (L1-L34) is more complex. Despite the nomenclature, there are 32 different proteins, not 34. The spot originally identified as protein L8 is an aggregation artifact. The proteins L7 and L12 are identical except that one of the serines on the N-terminal of L7 is acetylated. The large subunit also contains one copy of each protein except for L7/L12 which is present as a dimer of dimers (four copies). In addition, S20 is the same protein as L26 (Hardy et al. 1969; Nierhaus 1982).

Assembly of ribosomes involves the sequential addition of proteins and conformational changes in the growing subunit (Nierhaus 1991). The order of addition of ribosomal proteins during in vitro reconstitution of the 50S subunit has been experimentally determined and is shown in Figure 2.

FIGURE 2. E. COLI 50S RIBOSOMAL SUBUNIT ASSEMBLY MAP.

Arrows from the rRNA point to proteins capable of binding directly to the rRNA. Thick arrows from proteins strongly facilitate the binding of the other proteins to which they point. Thin arrows form proteins weakly assist the binding of proteins to which they point. The proteins of the first reconstitution intermediate are above the dashed line and the second reconstitution intermediate are below the dashed line.



Source: Nierhaus 1980.

Stable precursor particles that arise in the course of in vivo ribosome assembly have been isolated. Two precursor particles have been characterized in the assembly process of the 30S subunit (Hayes and Hayes 1971). A precursor particle called $p_1$30S contains the 16S

rRNA, ten small subunit proteins, and has a sedimentation coefficient of 21S. A $p_2$30S particle contains all 21 small subunit proteins, but the final processing of the 16S rRNA has not occurred and extra nucleotides are present at the 5' and 3' ends.

In vivo assembly of the large subunit occurs through 3 stable intermediate precursor particles termed $p_1$50S, $p_2$50S, and $p_3$50S with sedimentation values of 32S, 43S and about 50S respectively (Osawa, et al. 1969; Nierhaus, et al. 1973; Lindahl 1975). These particles all contain 23S and 5S rRNAs and increasing subsets of proteins.

The synthesis of ribosome components is highly regulated. More ribosomes are needed to carry out additional protein synthesis when nutrient levels or environmental conditions become more favorable for growth. When conditions are limiting, it becomes energetically expensive to produce unneeded ribosomes. Additionally, it is important that the cell maintain stoichemetric production of ribosomal components (Dennis 1974; Champney 1977).

Regulation is maintained through 2 negative feedback loops which operate at both the transcriptional and translational levels (Nomura 1983). When amino acid supplies are low, deacylated tRNAs bind the 30S subunit and trigger synthesis of an allosteric factor that acts to down-regulate transcription of both the rRNA and mRNAs for the ribosomal proteins. When more nutrients become available acetylated tRNAs bind the 30S subunit, production of the stringent factor ceases, and ribosome biosynthesis resumes. Translational control occurs when there is no free rRNA to begin assembly of a new subunit. Some ribosomal proteins act as repressors by binding to the mRNAs of other ribosomal proteins and halting their translation.

Degradation of ribosomes in vivo is mediated via a multicomponent ribonucleolytic complex termed the degradosome (Bessarab et al. 1998). An important component of the degradosome is RNase E, an endonuclease that cleaves the 16S and 23S RNAs (Miczak et al. 1996). Other degradosome-associated enzymes, including polynucleotide phosphorylase and RNA helicase, continue the process of ribosome turnover. RNase E has previously been identified as an enzyme involved in mRNA and rRNA turnover but not in the processing of tRNA (Kusnher et al. 1993; Coburn and Mackie 1998).

The information for each step and conformational change in the assembly pathway lies in

the molecular components of the ribosome. The information for the secondary and tertiary structure of the ribosome is present in the primary sequences of the rRNA and proteins (Piccon et al. 1975). This is demonstrated by the fact that functional ribosomes can be reconstituted in vitro from isolated components with high efficiency (Amils et al. 1979).

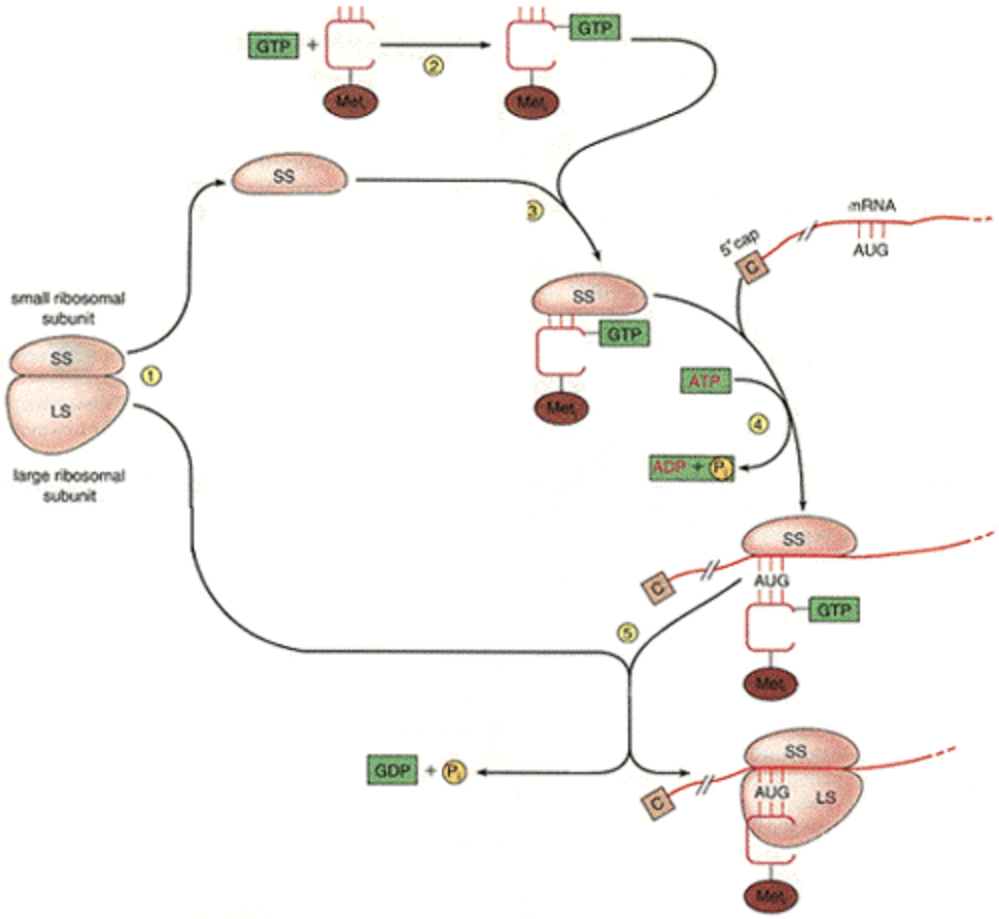
Reconstitution of the 30S subunit in vitro proceeds through only one reconstitution intermediate termed RI_{30}. The RI_{30} intermediate can be isolated if 16S RNA and TP30 (total proteins extracted from the 30S subunit) are incubated together in a reaction buffer at 0^oC (Guthrie et al. 1969). If the reaction temperature is increased to 40^oC, the RI_{30} undergoes a rate limiting conformational change to an RI^*_{30} particle and the remaining proteins are then assembled onto the forming 30S subunit (Traub et al. 1971; Nierhaus et al. 1973).

Reconstitution of the 50S subunit requires a two-step procedure using different temperatures and concentrations of magnesium. Three stable intermediate precursor particles can be isolated during the in vitro reconstitution process (Dohme and Nierhaus 1976). These have been termed RI50(1), $RI50^*(1)$, and RI50(2). The RI50(1) and $RI50^*(1)$ contain the same rRNAs and protein subset but have very different sedimentation coefficients of 33S [RI50(1)] and 41S [$RI50^*(1)$] because of a conformational change in the particle. The RI50(2) particle contains all the proteins found in the fully formed 50S subunit but is completely inactive in translation assays. A second conformational change that is both temperature and magnesium dependent results in an active 50S subunit (Osawa et al. 1969; Nierhaus 1991).

The ribosome has 4 binding sites that are used in protein synthesis. The peptidyl-tRNA binding site (P-site) holds a tRNA attached to the growing polypeptide. The aminoacyl-tRNA binding site (A-site) holds an incoming tRNA attached to an amino acid. An exit site (E-site) briefly holds the departing tRNA before it is released. The 3' end of the16S rRNA contains a binding site that base pairs with the Shine-Dalgarno recognition sequence on the mRNA.

Protein synthesis in prokaryotes proceeds through three steps, initiation, elongation, and termination. These are shown in Figures 3, 4, and 5. (For a review, see Nierhaus 1982).

FIGURE 3. INITIATION OF PROTEIN SYNTHESIS.



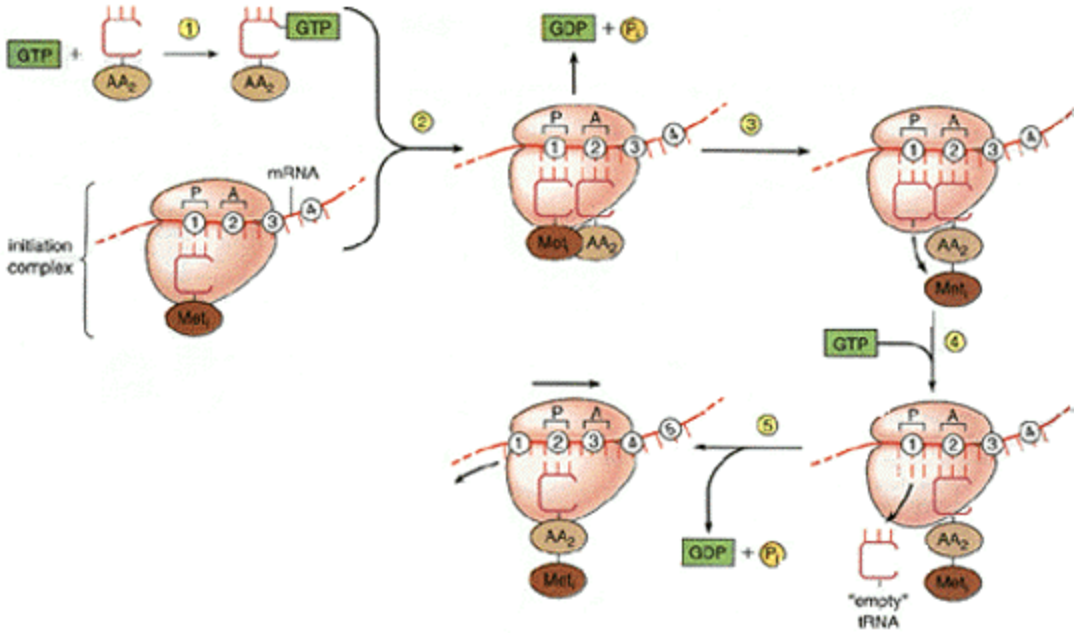
Source: http://ntri.tamuk.edu/cell/ribosomes.html, 2000.

During the initiation step, 3 initiation factor proteins (IF1, IF2, and IF3) transiently associate with the free 30S subunit. An F-met-tRNA complex attaches to the P-site of the small subunit. The small subunit attaches to the 5'-end of an mRNA and slides along it until the tRNA-F-met anticodon recognizes an AUG codon and stops. GTP is hydrolyzed, initiation factors are released, and the remaining complex binds to the 50S subunit. An aminoacyl tRNA then enters the A-site and chain elongation begins.

The elongation step is a cyclic process that is repeated for each amino acid that is added to the growing polypeptide. The next aminoacyl-tRNA is brought to the vacant A-site of

the ribosome by elongation factor EF-TU.

FIGURE 4. ELONGATION OF THE POLYPEPTIDE.

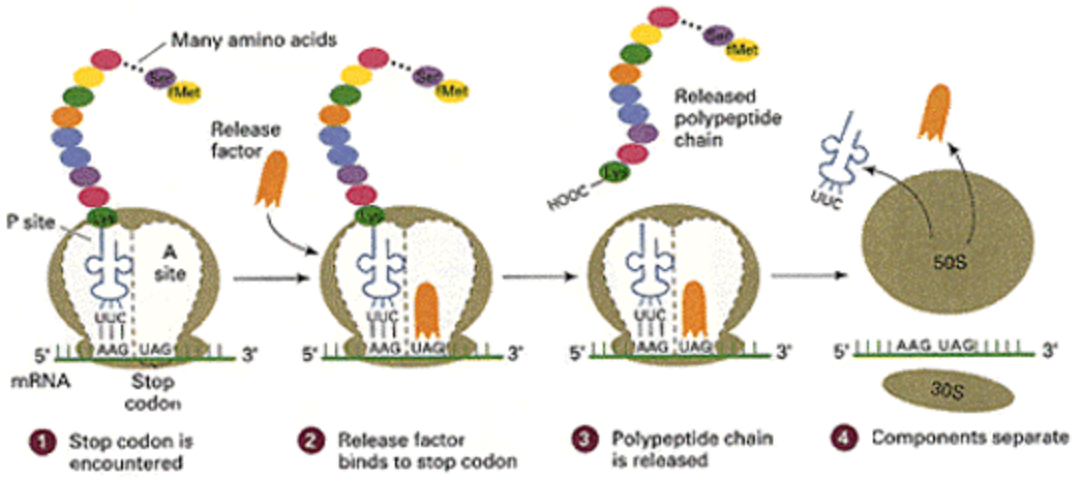


Source: http://ntri.tamuk.edu/cell/ribosomes.html, 2000.

The carboxyl-end of the nascent peptide attached to the tRNA in the P-site is detached and reattached by a peptide bond to the amino-end of the amino acid attached to the tRNA in the A-site. The protein is now one amino acid longer, but it is bound as peptidyl-tRNA in the A-site. Peptide bond formation occurs at the peptidyl transferase center on the 50S subunit. The activity is believed to be mediated by the 23S rRNA because removal of many proteins from the 50S subunit does not abolish the peptidyl transferase activity of the remaining rRNA.

The peptidyl-tRNA is then translocated from the A site to the P site and the empty tRNA moves to the E site and dissociates from the ribosome. This translocation step requires EF-G and GTP hydrolysis. During translocation, the ribosome shifts three bases along the mRNA and moves a new codon into the A-site to make room for the next incoming aminoacylated tRNA. The elongation cycle is repeated until all the amino acids that are specified by that mRNA have been added.

FIGURE 5. TERMINATION OF PROTEIN SYNTHESIS.



Source: http://ntri.tamuk.edu/cell/ribosomes.html, 2000.

Termination occurs when 1 of 3 stop codons enters the A-site on the ribosome and signals that the polypeptide is complete. Release factors cause the complex to disassociate, releasing the polypeptide chain. The ribosome detaches from the mRNA and dissociates into 30S and 50S subunits that are free to begin another round of translation (Nierhaus 1982).

Protein synthesis in bacteria occurs very rapidly. It begins as the mRNA is still being transcribed from the DNA and a single mRNA molecule can serve as template for many ribosomes translating simultaneously.

A number of antibiotics that act on the ribosome are derived from bacteria. Those obtained from the actinomycete Streptomyces include the aminoglycosides, tetracyclines, and macrolides (Cundliffe 1980). The aminoglycosides include streptomycin, kanamycin, tobramycin, and gentamicin. They act by binding to bacterial ribosomes and preventing the initiation of protein synthesis. The tetracyclines (including tetracycline, chlortetracycline, and doxycycline) are broad-spectrum antibiotics that act by blocking the binding of aminoacyl tRNA to the A-site on the ribosome. The macrolides (including erythromycin and oleandomycin) halt bacterial protein synthesis by binding to the 50S ribosomal subunit. The bound antibiotic either

inhibits elongation of the protein by peptidyl transferase or prevents translocation of the ribosome or both (Corcoran 1984).

Erythromycin and related macrolide antibiotics are effective against a variety of Gram positive and Gram negative bacteria. Erythromycin has a 14-member macrolactone ring to which an amino sugar and a neutral sugar are attached through glycosidic bonds (Nakagawa and Omura 1984). The structures of erythromycin, azithromycin and oleandomycin are shown in Figure 6.

Currently, several semi-synthetic derivatives of erythromycin such as azithromycin and clarithromycin are used to treat clinical infections. These compounds are more acid stable as they pass through the digestive tract and are effective against a broader spectrum of microbes (Whitman and Tunkel 1992; Champney 1999).

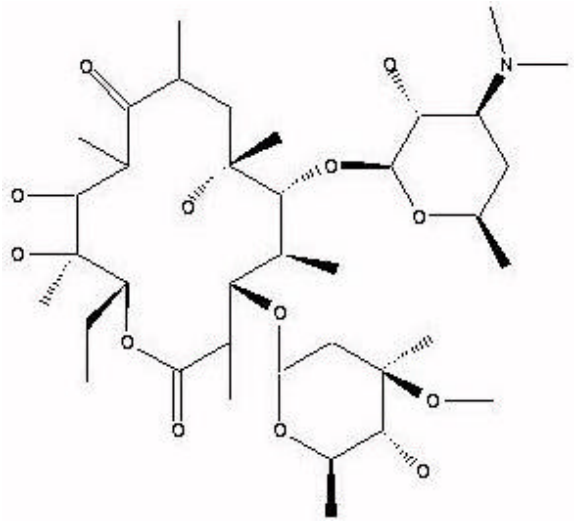
Erythromycin binds stiochimetrically with a 1:1 ratio to the 50S subunit (Pestka 1974). The binding site has been identified as the central loop of domain V in the 23S rRNA, part of the peptidyl transferase region (Douthwaite et al. 1985; Douthwaite et al. 1995). Proteins L15 and L16 also have been directly implicated in erythromycin binding (Teraoka and Nierhaus 1978).

Macrolide resistance most commonly occurs due to the action of a plasmid encoded methyltransferase (Corcoran 1984). Dimethylation of a single adenine residue at position A2058 in the 23S RNA results in 50S subunits that are unable to bind the antibiotics. Resistance to macrolides also is associated with mutational changes in large subunit proteins, particularly L4 and L22 (Teraoka and Nierhaus 1978; Chittum and Champney 1994).

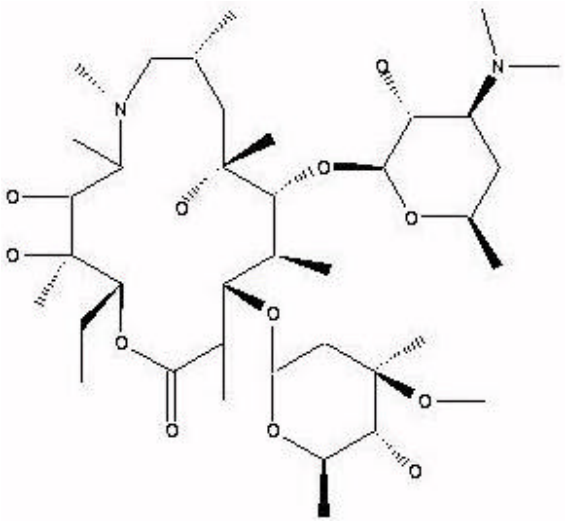
Previous investigations of macrolide activity have focused on their ability to inhibit protein synthesis (Gale et al. 1981; Moazed and Noller 1987). Our lab recently has discovered that inhibition of 50S ribosomal subunit assembly in growing cells is a second novel activity of erythromycin (Chittum and Champney 1995).

In the presence of erythromycin, the normal sequence of 50S subunit assembly is interrupted leading to a "stalled" intermediate precursor particle that becomes a target for degradation by cellular ribonucleases.

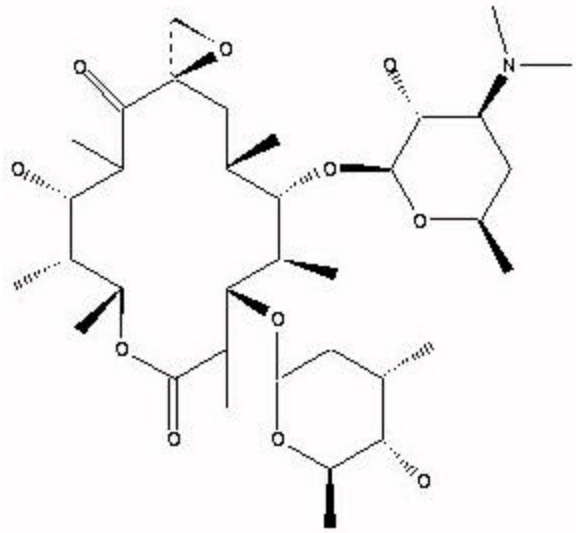
FIGURE 6. STRUCTURES OF ERYTHROMYCIN, AZITHROMYCIN AND OLEANDOMYCIN.



A. Erythromycin.



B. Azithromycin.



C. Oleandomycin.

Further, 50S subunit assembly inhibition has been found to be as important as protein synthesis inhibition in accounting for overall growth inhibition (Champney and Burdine 1996).

It is my hypothesis that in the presence of erythromycin, the stalled 50S intermediate precursor will accumulate in growing cells and that it will bind to erythromycin. It will contain the 23S and 5S rRNAs along with only a subset of 50S subunit proteins and it will lack sufficient density to sediment into the 50S gradient region. I hypothesize that the stalled 50S subunit precursor will accumulate in greater amounts in E. coli cells deficient in a key rRNA ribonuclease. I further hypothesize that the inhibition of 50S subunit assembly by erythromycin will occur during an in vitro reconstitution of the 50S subunit.

Several methods were used to test the validity of this hypothesis. An evaluation of the kinetics of formation and degradation of the precursor was done in a K12 strain of E. coli (SK901). An RNase E temperature-sensitive mutant strain (SK5665) was used to evaluate the role of the RNase E in 50S precursor turnover (Altman et al. 1982; Babitzke et al. 1993).

To assess the ability of the 50S precursor particle to bind erythromycin, a crucial step in the drug's mechanism of action, the binding of ^{14}C-erythromycin to the precursor was measured using in vivo and in vitro studies. The presence of 23S rRNA and 5S rRNA in the 50S subunit intermediate precursor was determined by polyacrylamide gel electrophoresis and nucleic acid hybridization experiments. The 50S subunit proteins in the intermediate precursor were identified via two-dimensional gel electrophoresis.

To determine if the in vivo inhibition of 50S subunit assembly could be mimicked in vitro, reconstitution experiments using 30S and 50S subunits in the presence and absence of several antibiotics were performed. The sedimentation properties of the ribosomal subunit reconstitution products were determined via sucrose gradient centrifugation.

CHAPTER 2
MATERIALS AND METHODS

Materials

Erythromycin, oleandomycin, formaldehyde, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), thymidine, thiamin, and lysozyme were purchased from Sigma Chemical Co. The 3-(N-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES), sodium dodecyl sulfate (SDS), formamide, trichloro-acetic acid (TCA), scintisafe gel, agar, and urea were purchased from Fisher. Azithromycin was a gift from Pfizer Pharmaceuticals. Tryptone and casein were purchased from Difco Laboratories. DNAse 1 was purchased from Amresco. Lumiphos reagent and streptavidin-alkaline phosphatase conjugate were purchased from Scheichler & Schuell. The radioactive compounds ^{14}C-erythromycin (54 mCi/mM) and ^{3}H-uridine (40 Ci/mM) were purchased from New England Nuclear and the ^{35}S-methionine-cysteine mixture (1175 Ci/mM) was purchased from ICN Biomedicals. Acrylamide, bis-acrylamide, protein assay kit, and bis-tris(hydroxymethyl) aminomethane(Bis-Tris) were purchased from Bio-Rad. Proteinase K and the 5S, 16S, and 23S rRNA standards were purchased from Boehringer-Mannheim. X-ray film was purchased from Fujifilm. Polaroid film (types P/N52 and P/N55) and comassie blue was purchased from Kodak Co. PCR primers were obtained from Life Technologies. The PCR Super-Mix was purchased from Gibco BRL. The Label-It probe biotinylation kit was purchased from CPG Inc. Salmon sperm DNA was purchased from Promega. The dialysis tubing was purchased from Spectrum Medical Industries Inc. Nitrocellulose filters were purchased from Millipore. The GF/A glass fiber filters and Number 3 blotting paper were purchased from Whatman International.

Bacterial Strains

Five strains of E. coli were examined, a wild type strain SK901 (see Table 1) and 4 mutant strains with enhanced erythromycin sensitivity.

Table 1. E. coli Strains Used in this Research.

Name	Genotype	Reference
SK901	K12, F^-, $malA^-$, thi^-	(Champney 1979, 1983)
SK5665	K12, F^-, thyA715, λ^-, rne^-1	(Babitzke et al. 1993)
SK1217	K12, F^-, ts, spc^{HS}, pro^-, ery^{HS}	(Champney 1979)
SK1219	K12, F^-, ts, spc^R, pro^-, ery^{HS}	(Champney 1979)
MRE600	$rnase1^-$, ery^{HS}	(Held and Normura 1973)

Media

Luria Broth: 10 g tryptone, 10 g NaCl, 5 g yeast extract to 1 L with deionized water (dH_2O).

Luria Plates: Luria broth and 1.5% agar.

Tryptone Broth: 10 g tryptone and 10 g NaCl to 1 L with dH_2O.

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.

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

Buffers

Ribosome Buffers

R-Buffer: 10 mM Tris-HCl (pH 8.0), 10 mM Mg acetate, 50 mM NH_4Cl.

S-Buffer: 10 mM Tris-HCl (pH 8.0), 0.5 mM Mg acetate, 100 mM NH_4Cl.

Ribosome Wash 1: 10 mM Tris-HCl (pH 8.0), 2 M NH_4Cl, 10 mM Mg acetate, 10% sucrose.

Hybridization Buffers

Modified Church buffer: 1 mM EDTA, 1% casein, 7% SDS, 0.25 M Na_2HPO_4 (7 H_2O) and H_3PO_4 to adjust pH to 7.4.

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

rRNA Electrophoresis Buffers

10X TE buffer: 0.89 M Tris-HCl (pH 8.0), 25 mM EDTA.

10X MOPS buffer: 200 mM MOPS (pH 2.0), 50 mM sodium acetate, 5 mM EDTA.

Protein Electrophoresis Buffers

1st dimension sample buffer: 0.1 ml of 1 M Bis-Tris (pH 4.2), 9.6 ml of 8 M urea, 0.06 ml acetic acid, 0.1 ml mercaptoethanol and dH_2O to 10 ml.

1st dimension upper tank buffer: 10 ml 1M Bis-Tris (pH 3.8) and 3.6 ml acetic acid to 1 L with dH_2O .

1st dimension lower tank buffer: 10 ml 1 M Bis-Tris (pH 6.0) and 0.4 ml acetic acid to 1 L with dH_2O.

1st dimension separating gel solution: 4 ml 1 M Bis-Tris (pH 5.5), 0.45 ml acetic acid, 4 gm acrylamide, 132 mg bis-acrylamide and 48 gm urea to 100 ml with dH_2O.

2nd dimension separating gel solution: 180 ml glacial acetic acid (pH 4.5), 2.7 gm KOH, 180 gm acrylamide, 2.5 gm bis-acrylamide and 360 gm urea to 1 liter with dH_2O.

2nd dimension running buffer: 1.5 ml glacial acetic acid (pH 4.0) and 14 gm glycine to 1 liter with dH_2O.

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 2- mercaptoethanol.

Buffer #5: 20 mM Tris-HCl (pH 6.0), 6 M urea, 4 mM Mg acetate, 400 mM NH_4Cl, 0.2 mM EDTA and 5 mM 2- mercaptoethanol.

Buffer # 6: 20 mM Tris-HCl (pH 6.0), 20 mM Mg acetate, 400 mM NH_4Cl, 1 mM EDTA and 5 mM 2- mercaptoethanol.

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 2- mercaptoethanol.

Buffer P: 10 mM Tris-HCl (pH 6.0), 2 mM Mg acetate and 1 mM 2- mercaptoethanol.

Methods

Cell Culture

Bacterial strains were maintained at -80°C in 1ml aliquots of Luria broth (L broth) containing 20% glycerol. Cells were grown in an incubator at 32°C or 37°C after being streaked onto plates containing L broth and 1.5% agar. Individual colonies were used to inoculate 1 ml overnight cultures of L broth.

Small scale cell culture was done in a shaking water bath at 27°C or 37°C. Cells were grown in side arm flasks, and the density was monitored using a Klett-Summerson colorimeter. One hundred Klett units equals 1 unit of optical density at 600 nm or approximately $8x10^8$ cells/ml. Ten ml of growth media were inoculated with cells from an overnight culture to a Klett unit reading of 20. After growth to 80 Klett units, cells were chilled on ice and collected into 15 ml round bottom Corex tubes then centrifuged for 30 minutes at 9000 rpm (9,800 xg). The supernatant was discarded and the pellet was rinsed in 200 µl of S-buffer and stored at -80°C.

Large scale cell culture was done on a shaking platform in a Bally environmental room at 32°C. One liter of sterile L Broth was inoculated with 2.0 ml of an overnight culture. Media for strain SK5665 was supplemented with thymidine at 5 µg/ml. Cell densities were determined

by reading the absorbance at 600 nm in a Beckman model 35 spectrophotometer.

Determination of Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration for each antibiotic used was determined by a dilution method. Tubes containing 1 ml of LB were inoculated with 50 µl of E. coli cells from an overnight culture. Different concentrations of antibiotics were added to each tube and the cells were incubated overnight at 32°C. Growth was measured by reading the absorbance at 600 nm. Concentrations of each antibiotic were chosen for each strain that reduced cell growth without halting it entirely.

Sucrose Gradient Fractionation

Linear sucrose gradients (12 ml) of 5-20% sucrose in S-buffer were constructed in 14 ml Seton polyallomer centrifuge tubes. Samples of either 70S ribosomes or cell lysates were layered on top of the gradients. Tubes were centrifuged four hours at 39,000 rpm (284,000 xg) in a Beckman L5-65 or LE-80K preparative ultracentrifuge using an SW40 rotor. Gradients were fractionated through an ISCO UA-5 absorbance monitor and fractions of six drops each were collected. Standard curves for 70S ribosomes and 30S and 50S subunits were obtained using an analog-digital signal converter along with Dynamax capture and integration software (Ranin).

Isolation of 70S Ribosomes

Each cell pellet was resuspended in 5 ml R-buffer and 0.2 ml of freshly made lysozyme (2 mg/ml in R-buffer) was added. Tubes were incubated 5 minutes at room temperature, then frozen and thawed three times using a dry ice/ethanol bath.

RNase free DNase 1 (40 units) was added and the tubes were incubated at room temperature for 5 minutes. Cell debris was pelleted by centrifugation in a JA-20 rotor at 10,000 rpm (12,100 xg) for 30 minutes at 4°C. The supernatant was transferred to stainless steel tubes and spun in a Ti 50 rotor at 30,000 rpm (240,000 xg) for 30 minutes at 4°C to pellet intermediate sized cell debris. Finally, the supernatant was transferred to other stainless steel

tubes and spun at 45,000 rpm (300,000 xg) for 3 hours to pellet the crude 70S ribosomes. The pelleted ribosomes were resuspended in 0.5-1.0 ml R-buffer and the concentration was quantitated by measuring the absorbance at 260 nm (A_{260}). One optical density unit of 70S ribosomes at A_{260} = 26 pmol/ml (Stanley and Bock 1965). The ribosomes were stored at -80°C.

Washing of Crude 70S Ribosomes

The resuspended crude 70S ribosomes were layered over an equal volume of Ribosome Wash 1 in sterile stainless steel tubes. The tubes were spun for 3 hours in a Ti 50 rotor 45,000 rpm (300,000 xg) at 4°C to pellet the washed 70S ribosomes. The supernatant was discarded, and the pellets resuspended in 0.5-1.0 ml R-buffer and stored at -80°C.

Separation of 70S Ribosomes into 50S and 30S Subunits

Washed 70S ribosomes (150 A_{260} units) were loaded onto 38 ml gradients (5-20% S-buffered sucrose) and spun in an SW 27 rotor at 18,000 rpm for 18 hours (30,000 xg). Gradients were fractionated through an ISCO monitor and pools of the 30S and 50S peaks were collected separately in sterile 30 ml round bottom Corex tubes. One M Mg acetate was used to raise the concentration of magnesium ions to 20 mM and the solution was mixed with an equal volume of 100% ethanol.

After 1 hour at -20°C, the precipitated subunits were centrifuged at 10,000 rpm for 30 minutes. The resulting pellet was dried, then resuspended in a small volume of R-buffer, the concentration was quantitated by absorbance at 260 nm, and stored -80°C. One optical density unit of 30S subunits at 260 nm = 76 pmol/ml. One optical density unit of 50S subunits at 260 nm = 44 pmol/ml (Stanley and Bock 1965).

^{3}H-uridine Pulse-Chase Labeling

Cells were grown in selected medium to approximately 20 Klett units at either 27°C or 37°C. The antibiotic used for the particular experiment was added at concentrations

determined by the MIC assays. Growth was continued until approximately 80 Klett units (two cell doublings). The RNA was pulse-labeled by adding ^{3}H-uridine [5 µCi/ml], incubating for 90 seconds, and chasing with a 50X excess of unlabeled uridine [20 µg/ml]. Aliquots of cells were harvested to chilled tubes at specified time intervals. They were spun down, washed in 200-500 µl of S-buffer, re-pelleted and held at -80°C until lysis. In some experiments, ^{14}C-erythromycin was added at 75 µg/ml for SK901 or 18 µg/ml for SK5665.

Protocol for Pulse-Chase Cell Pellet Lysis

The cell pellet was thawed and resuspended in 0.2 ml of S-buffer. Five µl of lysozyme [5 µg/ul], freshly made in S-buffer, was added to the Corex tubes. The tubes were incubated for 5 minutes at room temperature, then frozen and thawed 3 times using a dry ice/ethanol bath. RNase free DNase 1 (4 units) was added and the tubes were incubated at room temperature for 5 minutes. Large cell debris was pelleted by centrifugation at 10,000 rpm (12,100 xg) for 20 minutes at 4°C and the supernatant from each pellet was applied to a sucrose gradient.

Isolation of Total rRNA from 70S Ribosomes

70S ribosomes were resuspended in 0.5 ml of R-buffer and extracted 3 times with 1.0 volume of Tris-saturated phenol made 1% in SDS. The aqueous phase containing the rRNA was then extracted with an equal volume of chloroform/isoamyl alcohol (24/1 v/v). The rRNA was precipitated by adding 2 volumes of ice-cold absolute ethanol and placing the samples at -20°C for 2 hours.

The dried 70S rRNA pellet was resuspended in a minimal volume of R-buffer (or buffer #7 if the rRNA was to be used for reconstitutions), quantitated by reading the absorbance at 260 nm and stored at -80°C. One optical density unit of total rRNA from 70S ribosomes at 260 nm contains 26 pmol each of the 23S, 16S, and 5S rRNA species (Stanley and Bock 1965).

Isolation of Proteins from Ribosomes and Subunits

One molar Mg acetate (0.1 volume) and glacial acetic acid (2 volumes) were added to

ribosomal subunits or ribosomes suspended in R-buffer. The mixture was held on ice for 1 hour to precipitate the rRNA.

After centrifugation in a JA-20 rotor at 7,500 rpm (6,800 xg) for 30 minutes, the supernatant containing the proteins was transferred to fresh tubes and acetone (5-7 volumes) was added. Samples were kept at -20°C overnight to precipitate the proteins, centrifuged again in a JA-20 rotor at 7,500 rpm (6,800 xg) for 30 minutes, and the protein pellet air dried in a 44°C incubator.

The 30S or 50S ribosomal protein pellet was resuspended in 0.5-1 ml of buffer #5, then dialyzed three times (45 minutes each) against 100 volumes of buffer #4. The 50S proteins were then dialyzed twice (45 minutes each) against 100 volumes of buffer P. Final protein concentrations were determined by using a protein assay dye reagent kit (Bio-Rad) and aliquots were stored at -80°C.

Ribosomal Subunit Reconstitution Methods

All reconstitution experiments used ribosomal subunits from strain MRE600. Reconstitution of 30S ribosomal subunits was accomplished using a single-step incubation of isolated 16S rRNA and TP30. 16S rRNA (0.75 A_{260} units) was resuspended in 10 µl of buffer # 7. 0.1 volume of buffer # 9 was added to create ionic conditions the same as buffer #4. TP30 (1.5-3 e.u. in buffer # 4) was added to the reaction along with a sufficient amount of buffer # 4 to bring the total volume to 100 µl.

Tubes were incubated in a water bath for 20 minutes at 40°C then chilled on ice. Sedimentation properties of the products were analyzed by sucrose gradient centrifugation using 5-20% R-buffered sucrose in an SW50.1 rotor. Samples were spun at 49,000 rpm (224,000 xg) for 2 hr at 4°C.

Reconstitution of 50S ribosomal subunits was accomplished using a 2-step incubation of isolated 70S rRNA and TP50. 3.8 A_{260} units of 70S rRNA was resuspended in 15 µl of buffer #7. 0.1 volume of buffer # 9 was added to create ionic conditions the same as buffer #4. Sufficient Tris-HCl, Mg acetate, NH_4Cl, EDTA, and mercaptoethanol were added to the TP50

in buffer P to change the ionic conditions to that of buffer #4. Eighty-five µl of the TP50 (5-6 e.u. in buffer #4 now) was added to the reaction along with a sufficient amount of buffer # 4 to bring the total volume to 120 µl.

The first incubation was done in a water bath at 44°C for 30 minutes. One M Mg acetate was added to increase the magnesium ion concentration to 20 mM and the tubes were transferred to a 50°C bath for 90 minutes. Sedimentation properties of the products were analyzed by sucrose gradient centrifugation using 5-20% R-buffered sucrose in an SW50.1 rotor at 49,000 rpm (224,000 xg) for 2 hours at 4°C.

Polymerase Chain Reaction (PCR)

The nucleotide sequence of the 23S rRNA was examined using the oligonucleotide analysis program Oligo (National Biosciences). Suitable primer pairs were selected for the PCR amplification of a 23S rRNA hybridization probe and were obtained from Gibco BRL Custom Primers. The selected probe was complementary to the central region of the 23S rRNA (nucleotides 1188-1334).

Fifty µl PCR reactions were constructed using 45 µl PCR SuperMix High Fidelity reagent mixture (Gibco BRL), 1 µl of plasmid pKK template DNA (6.5 ng), 1 µl (10 pmol) forward primer (sequence 5' TAG GGG AGC GTT CTG TAA G 3'), 1 µl (10 pmol) reverse primer (sequence 5' CCC GAT TAA CGT TGG AC 3'), and 2 µl dH_2O. Samples were placed in a PTC-100 programmable thermocycler (MJ Research) for thirty cycles under the following conditions: Denaturation at 94°C for 2 minutes, annealing of primers to target DNA at 55°C for 2 minutes and extension of the primers at 72°C for 1.5 minutes. To insure homogeneity of the 146 base pair PCR product, five µl from each reaction mixture were examined on a 2% agarose gel along with molecular weight markers. The 23S DNA probe was biotinylated using a Label-It 3' Biotin End Labeling Kit (CPG Inc.) following manufacturers instructions. Product labeling was checked by comparison to a prelabeled oligonucleotide provided with the kit.

rRNA Hybridization

Pulse-chase experiments were performed as described and the cell lysates were applied to sucrose gradients. Fractions were collected and 10 µl of a 10% SDS solution containing 5 µg Proteinase K were added to each tube. The tubes were then incubated at 44°C for 30 minutes. The samples were applied to a 7 x 10 cm nylon membrane with a pore size of 0.2 µm (Scheichler & Schuell) using a Bio-Dot apparatus (BioRad). After blotting, samples were cross-linked by UV irradiation at 254 nm (120 µJ/cm^2).

A hybridization incubator (Fisher-Biotech) was used to prehybridize the membrane at 65°C for 1-2 hours with 5 ml modified Church's buffer solution containing 10 µg/ml denatured herring sperm DNA (Promega). Biotin labeled probe was denatured by heating to 65°C for 15 minutes in a solution containing 50% formamide, 6% formaldehyde and 1X MOPS then quick cooled on ice. Four pmol of denatured probe per membrane was added and allowed to hybridize overnight at 65°C.

All washes and rinses were done in hybridization bottles. The membrane was rinsed with 10 ml of 5X SSC, 0.1% SDS, then washed at 65°C with 10 ml of 5X SSC, 0.1% SDS for 15 minutes, at 45°C with 10 ml of 2X SSC, 0.1% SDS for 15 minutes, and at 40°C with 10 ml of 1X SSC, 0.1% SDS for 15 minutes. Then, 0.5 ng streptavidin-alkaline phosphatase enzyme was added to 10 ml of 1X SSC, 0.1% SDS and the membrane was washed exactly 10 minutes with this solution. The membrane was rinsed twice in 10 ml 1X SSC 0.1% SDS, washed at room temperature for 15 minutes with 10 ml 1X SSC 0.1% SDS, and rinsed in sterile deionized water. Lumiphos reagent was sprayed on the membrane and it was kept in darkness for 1 hour and then exposed to Fuji RX medical X-ray film. The film was developed in Kodak GBX developer for 5 minutes, rinsed in water and fixed with Kodak GBX fixer for 5 minutes.

Polyacrylamide Gel Electrophoresis of RNA

Sucrose gradient fractions of subunits were transferred to 1.5 ml microfuge tubes and 10 µl of a 10% SDS solution containing 5 µg Proteinase K was added to each tube, which

were then incubated at 44°C for 30 minutes. One molar sodium acetate (0.1 vol.) and absolute ethanol (2 vol.) were added and tubes were kept at -20°C for 2 hours. The rRNA was pelleted by centrifugation in a microcentrifuge at 10,000 rpm (7,200 xg), rinsed with 70% ethanol and respun, then dried in a 44°C incubator.

The rRNA pellets were resuspended in 10 µl of denaturing solution (50% formamide, 6% formaldehyde and 1X MOPS), heated at 65°C for 15 minutes and quick cooled on ice. Five µl of 80% glycerin / 1% bromo-phenol blue was added and the samples were electrophoresed on a 4% / 7% polyacrylamide gel at 200V for 1.5 hours in 0.5X MOPS buffer. For fluorography, gels were soaked for 30 minutes in 1 M sodium salicylate, dried under vacuum onto Whatman Number 3 paper and exposed to X-ray film at -80°C.

Erythromycin Binding Assays

In vivo binding: Dual labeling experiments were performed as follows: ^{14}C-erythromycin at 18 µg/ml (1.2 µCi/ml) was added to growing cells at approximately 20 Klett units. At about 80 Klett units, a ^{3}H-uridine pulse-chase assay was performed as described. Cells were collected at 15 minutes post-chase, lysed, and gradient fractionated. Fractions corresponding to 30S and 50S regions were pooled separately, then the absorbance at 260 nm was read. The amounts of ^{3}H-uridine and ^{14}C-erythromycin in each pool were measured by liquid scintillation counting in a Beckman LS-3801 instrument.

In vitro binding: SK901 cells were grown at 27°C with and without erythromycin at 75 µg/ml. Ribosomal subunits and precursor particles from appropriate gradient fractions were pooled and dialyzed to remove the sucrose and unlabeled erythromycin. ^{14}C-erythromycin at 18 µg/ml (1.2 µCi/ml) was added and the pools were incubated at 37°C for 15 minutes. The subunits were captured on a Millipore 0.45 micron filter, washed 3 times with 1 ml of R buffer and counted for bound erythromycin.

Two-Dimensional Gel Electrophoresis of Ribosomal Proteins

Cells were grown in minimal media at 27°C. At about 30 Klett units, 50 µCi/ml of a

^{35}S-methionine-cysteine mixture was added and growth was allowed to continue. At 80 Klett units, cells were harvested as described, lysed and fractionated on a 5-20% sucrose gradient. Samples were collected that contained the top, 30S, and 50S gradient regions. The samples were sealed in 3,500 molecular weight cut off tubing (Spectrapor) and dialyzed overnight against three changes (500 ml each) of R-buffer. Proteins from the entire top pool or from eight A_{260} units of subunits from the 30S and 50S pools were extracted by acetic acid/acetone as described. The dried proteins were resuspended in 50 µl of 1st dimension sample buffer.

First dimension gels: Glass tubes (0.2 X 10 cm) were filled with 0.4 ml of separating gel and polymerized with 1 µl/ml TEMED and 3 µl/ml of 10% ammonium persulfate. Samples were electrophoresed 30 minutes at 60V then for 5 hours at 150 volts. In the upper tank, 250 ml of 1st dimension upper tank buffer (pH 3.8) was used. In the lower tank, 250 ml of 1st dimension lower tank buffer (pH 6.0) was used.

Second dimension gels: Gels were cast in a Hoefer SE 500 slab gel apparatus using 60 ml of two-dimensional (2-D) gel solution that had been mixed with 1.2 ml of 10% ammonium persulfate, and 300 µl of TEMED. After the first dimension run, the gels were removed from the glass tubes and polymerized atop the second dimension gels.

Electrophoresis was carried out with the cathode on top using 400 ml of 1 X 2-D running buffer in the upper tank and 1200 ml of 0.3X 2-D running buffer in the lower tank. The proteins were stacked at 40 volts for 1 hour followed by 100 volts for 13 hours.

The gels were stained a solution of 0.1% Coomassie Brilliant Blue R-250, 7.5 % acetic acid, and 50% methanol. They were destained in a mixture of 5% glacial acetic acid and 10% ethanol. After destaining, the gel slabs were photographed using a digital camera (Alpha Innotec).

For fluorography, gels were soaked for 30 minutes in 1M sodium salicylate, dried under vacuum onto Whatman Number 3 paper and exposed to X-ray film at -80^{o}C. For quantitation, gel spots were excised using a cork borer and dissolved in 0.5 ml of 30% hydrogen peroxide for 8 hours at 80^{o}C. Samples were mixed with 3 ml of Scintisafe cocktail and the radioactivity was measured in a liquid scintillation counter.

CHAPTER 3

RESULTS

Previous investigations have shown that the macrolide antibiotics erythromycin, azithromycin, and oleandomycin inhibit 50S ribosomal subunit formation in Escherichia coli, Bacillus subtilis and Staphylococcus aureus cells (Champney 1999). Further characteristics of assembly inhibition were investigated in E. coli because of the availibility of mutant strains and because the ribosomes of E. coli have been well studied (Hampl et al. 1981; Nierhaus 1982).

A wild type strain SK901 and 4 mutant strains with enhanced erythromycin sensitivity (see Tables 1 and 2) were initially examined for the effects of the three macrolide antibiotics on growth and protein synthesis rates.

Macrolide Effects on Cell Growth

Experiments were conducted to find concentrations of antibiotics that inhibited growth but did not halt it entirely. Concentrations of each antibiotic were chosen based on MIC experiments (see Table 2). The concentrations used were expected to reduce the growth rate by about one-half that of the minus antibiotic control.

Initially, wild type strain SK901 was used to assess the effect of the macrolides on the rates of growth and protein synthesis. Figure 7 shows the growth of SK901 with and without antibiotics.

Growth rates relative to the control were: erythromycin (46%), azithromycin (35%), and oleandomycin (58%). Azithromycin was a more effective inhibitor of growth than erythromycin, even at a very low concentration (2 μg/ml). Oleandomycin was the least effective under these conditions.

Figure 8 shows the results of protein synthesis inhibition experiments in SK901 by the same antibiotics at the same concentrations.

Table 2. Antibiotic Minimal Inhibitory Concentrations.

The LC_{50} equals the antibiotic concentration found to give 50% inhibition of maximal growth.

Strain	Antibiotic	LC_{50} (μg/ml)
SK901	erythromycin	80
SK901	azithromycin	0.5
SK901	oleandomycin	150
SK5665	erythromycin	20
SK5665	azithromycin	0.5
SK5665	oleandomycin	150
SK1217	erythromycin	10
SK1217	azithromycin	0.1
SK1217	oleandomycin	50
SK1219	erythromycin	20
SK1219	azithromycin	0.5
SK1219	oleandomycin	100
MRE600	erythromycin	2
MRE600	azithromycin	0.1
MRE600	oleandomycin	20

FIGURE 7. INHIBITION OF CELL GROWTH IN STRAIN SK901 BY MACROLIDE ANTIBIOTICS.

Five ml cultures of strain SK901 were grown in Tryptone broth (T broth) in 125 ml side-arm flasks at 37°C for 4 hours with either no antibiotic (□), erythromycin at 75 µg/ml (●), azithromycin at 2 µg/ml (◆), or oleandomycin at 150 µg/ml (▲).

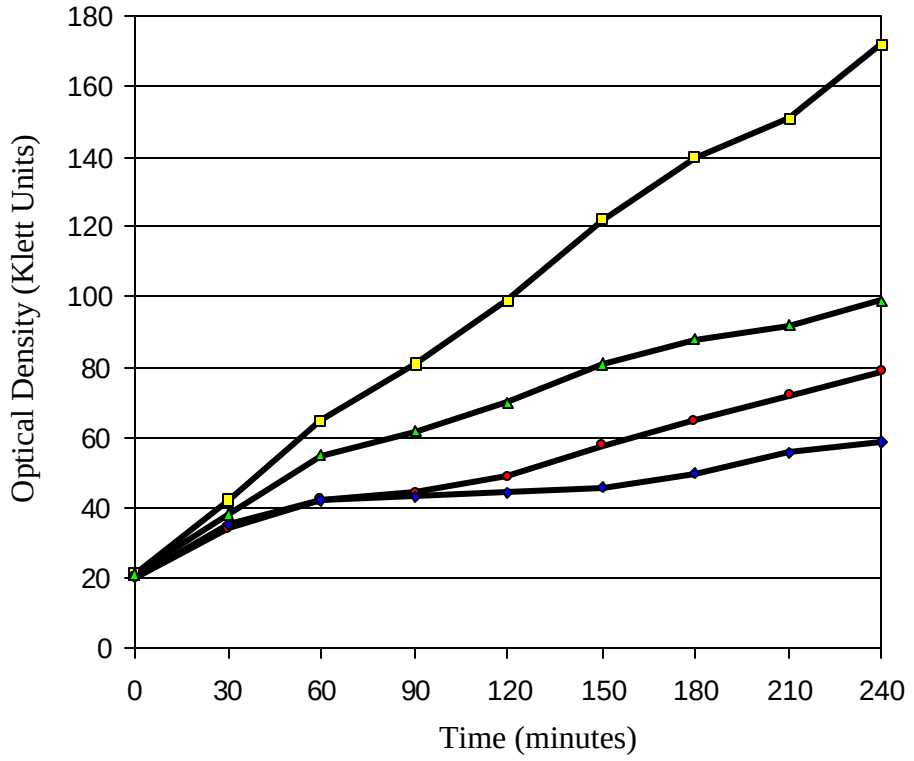
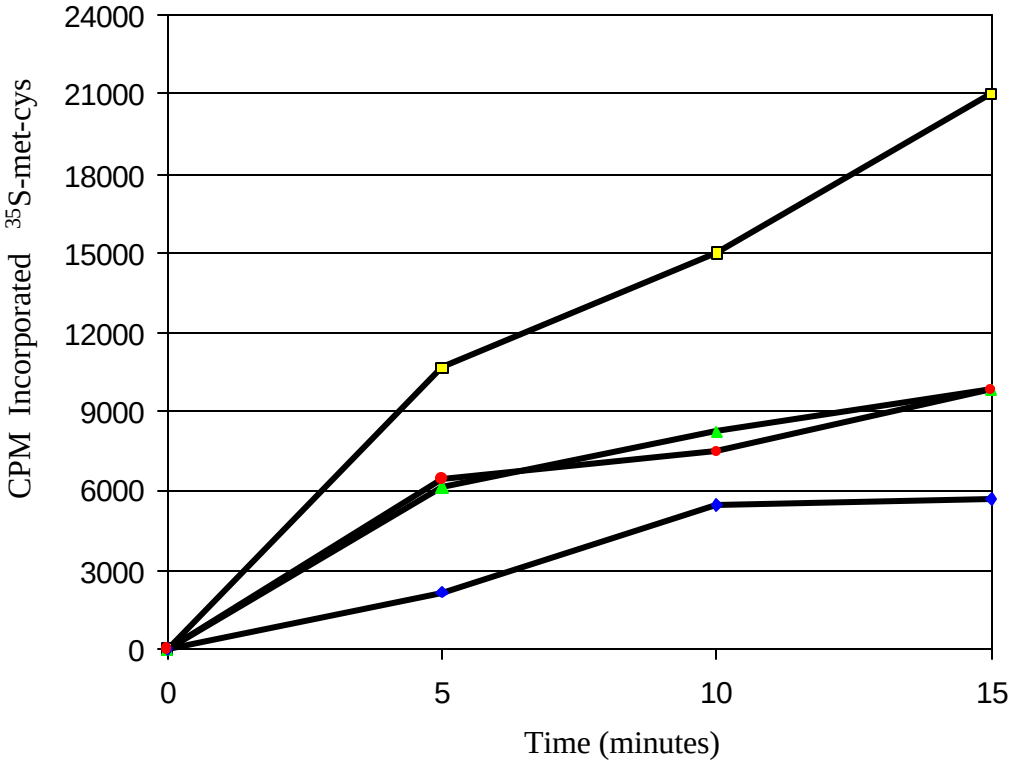


FIGURE 8. INHIBITION OF PROTEIN SYNTHESIS IN STRAIN SK901 BY MACROLIDE ANTIBIOTICS.

Strain SK901 was grown in T broth containing a ^{35}S-methionine-cysteine mixture (1 µCi/ml) at 37°C with either no antibiotic (□), erythromycin at 75 µg/ml (●), azithromycin at 2 µg/ml (◆), or oleandomycin at 150 µg/ml (▲). Samples (0.2 ml) were removed from a 5 ml culture at 5 minute intervals into 1 ml of 10% TCA. Samples were collected on GF/A filters, washed with 10% TCA and ethanol then the radioactivity was measured in 5 ml scintillation fluid.



Reduced rates of protein synthesis were observed in the cultures containing the antibiotics. Azithromycin was the most effective inhibitor of protein synthesis. Oleandomycin and erythromycin had a similar effect when the concentration of oleandomycin was twice that of

erythromycin.

A summary of the antibiotic effects on SK901 is shown in Table 3.

Table 3. Summary of Macrolide Antibiotic Effects on Growth and Protein Synthesis Rates in Strain SK901.

Condition	Antibiotic Concentration	Doubling Time	Rate of Protein Synthesis	% of Control
No Drug	0 μg/ml	50 minutes	1,333 CPM/minutes	100
Erythromycin	75 μg/ml	180 minutes	654 CPM/minutes	49
Azithromycin	2 μg/ml	400 minutes	376 CPM/minutes	28
Oleandomycin	150 μg/ml	100 minutes	655 CPM/minutes	49

Comparison of macrolide effects on growth rate (by Klett unit reading) and protein synthesis (by ^{35}S amino acid incorporation).

Four mutant E. coli strains were tested for sensitivity to erythromycin. In comparison to SK901, each of the mutant strains was found by previous work to be hypersensitive to growth inhibition by erythromycin (Held and Nomura 1973; Champney 1979; Babitzke et al. 1993).

A measure of hypersensitivity was the recovery time required to resume normal growth after erythromycin was removed from the cell culture. Figures 9 and 10 show growth curves for normal growth, inhibition by erythromycin, and erythromycin recovery in strains SK901 and MRE600 respectively.

FIGURE 9. ERYTHROMYCIN INHIBITION AND RECOVERY IN STRAIN SK901.

Strain SK901 was grown in T broth at 37°C with no antibiotic present (◆), or with erythromycin at 75 µg/ml (●). Recovery was assessed by starting a culture without antibiotic (■), then adding 75 µg/ml of erythromycin at the indicated time (★). After a period of growth inhibition, cells were collected by centrifugation, resuspended in fresh T broth (☆) and allowed to continue growth.

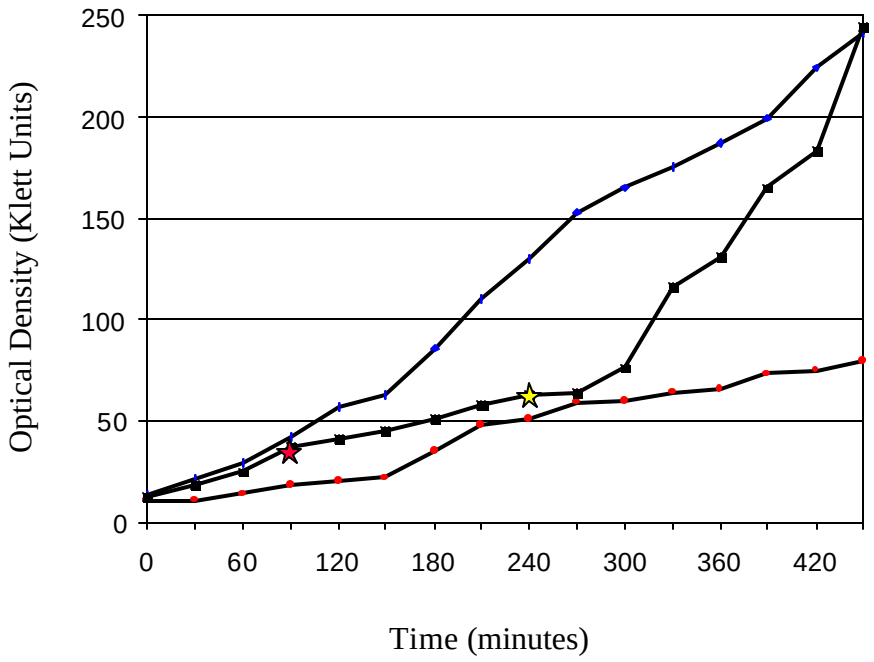
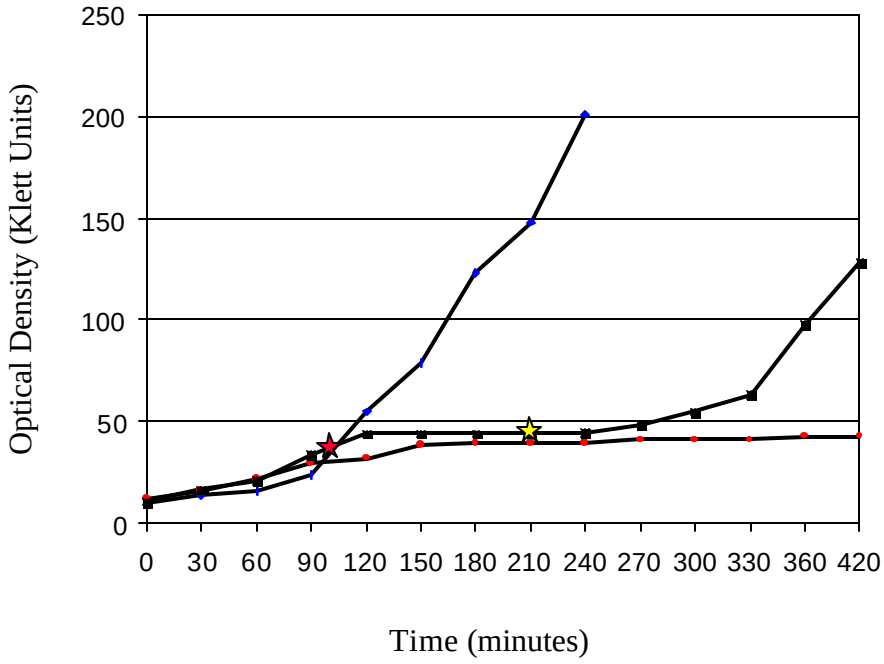


FIGURE 10. ERYTHROMYCIN INHIBITION AND RECOVERY IN STRAIN MRE600.

Strain MRE600 was grown in T broth at 37°C with no antibiotic present (◆), or with erythromycin at 2 µg/ml (●). Recovery was assessed by starting a culture without antibiotic (■), then adding 2 µg/ml of erythromycin at the indicated time (★). After a period of growth inhibition, cells were collected by centrifugation, resuspended in fresh T broth (☆) and allowed to continue growing.



Strains SK1217 and SK1219 are hypersensitive to erythromycin due to a mutation in the gene for ribosomal protein L15 (Champney, unpublished observation). Recovery from eryhtromycin inhibition in these strains is shown in Figures 11 and 12.

FIGURE 11. ERYTHROMYCIN INHIBITION AND RECOVERY IN STRAIN SK1217.

Strain SK1217 was grown in T broth at 37°C with no antibiotic present (◆), or with erythromycin at 20 µg/ml (●). Recovery was assessed by starting a culture without antibiotic (■), then adding 20 µg/ml of erythromycin at the indicated time (★). After a period of growth inhibition, cells were collected by centrifugation, resuspended in fresh T broth (☆) and allowed to continue growing.

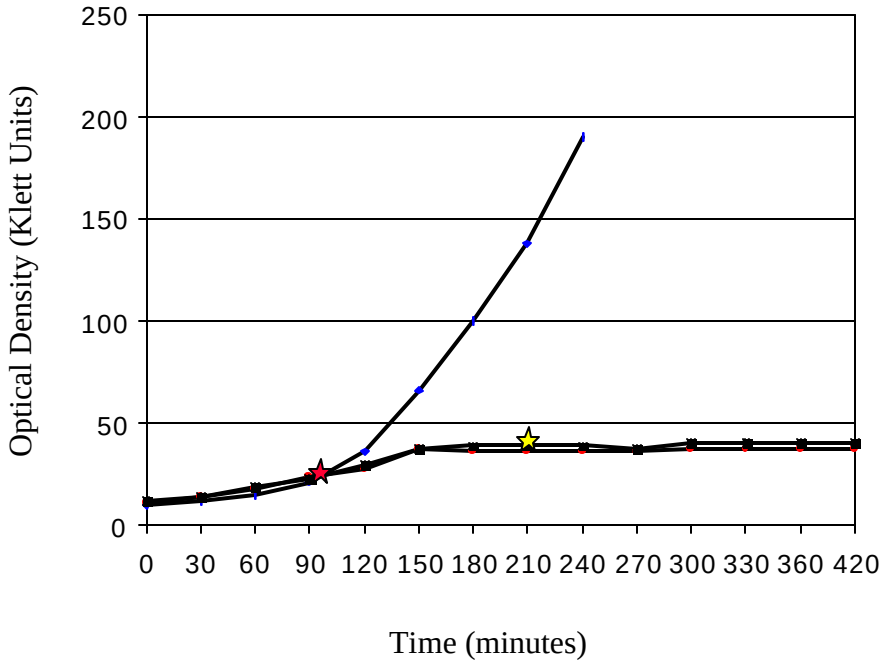
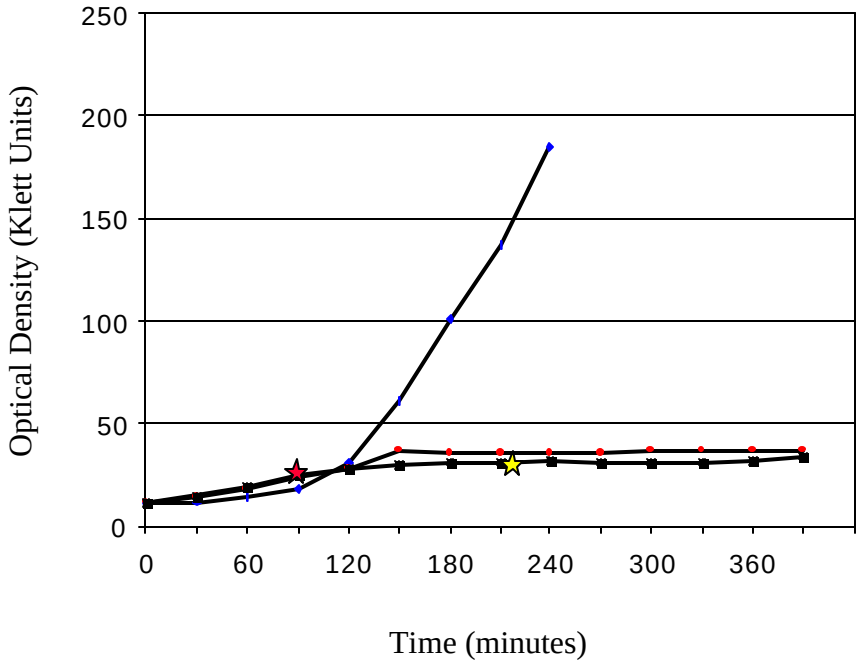


FIGURE 12. ERYTHROMYCIN INHIBITION AND RECOVERY IN STRAIN SK1219.

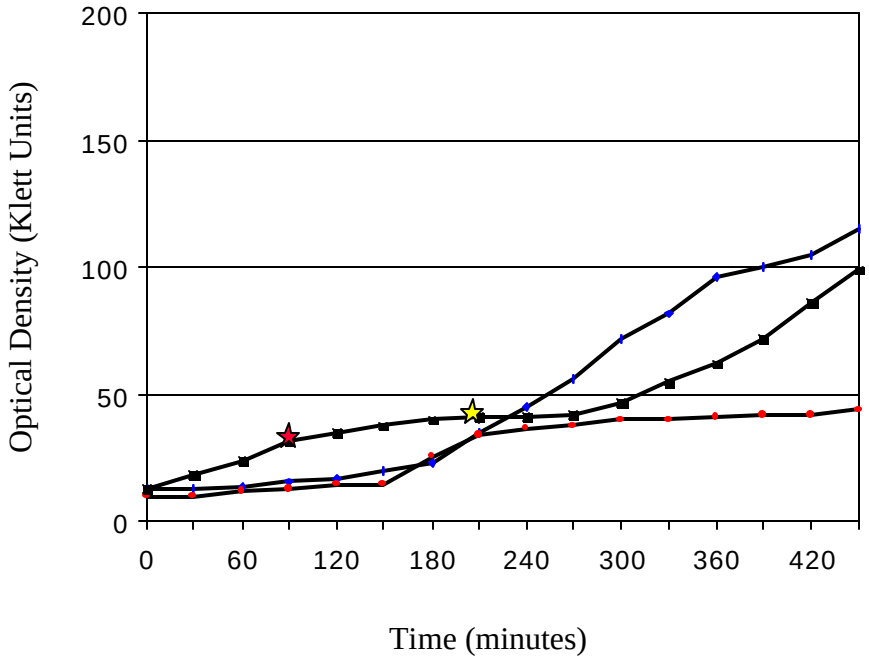
Strain SK1219 was grown in T broth at 37°C with no antibiotic present (◆), or with erythromycin at 20 µg/ml (●). Recovery was assessed by starting a culture without antibiotic (■), then adding 20 µg/ml of erythromycin at the indicated time (★). After a period of growth inhibition, cells were collected by centrifugation, resuspended in fresh T broth (☆) and allowed to continue growing.



Finally, a temperature-sensitive RNase E mutant strain SK5665 was examined because RNase E has been shown to be involved in rRNA turnover (Bessarb et al. 1998). Figure 13 shows the results for erythromycin recovery assays in this strain.

FIGURE 13. ERYTHROMYCIN INHIBITION AND RECOVERY IN STRAIN SK5665.

Strain SK5665 was grown in T broth at 37°C with no antibiotic present (◆), or with erythromycin at 18 µg/ml (●). Recovery was assessed by starting a culture without antibiotic (■), then adding 18 µg/ml of erythromycin at the indicated time (★). After a period of growth inhibition, cells were collected by centrifugation, resuspended in fresh T broth (☆) and allowed to continue growing.



A summary of the recovery times for these strains is shown in Table 4.

Table 4. Summary of Inhibition Data by Erythromycin in Five E. coli Strains.

Recovery time was the interval required by the cultures to resume a logarithmic growth phase. Strains SK1217 and SK1219 remained static.

Strain	Phenotype	Erythromycin Concentration	Recovery Time (minutes)
SK901	wild type	75 µg/ml	30
MRE600	RNase 1^-	2 µg/ml	120
SK1217	ts L15	20 µg/ml	>450
SK1219	ts L15	20 µg/ml	>450
SK5665	RNase E^-	18 µg/ml	45

Erythromycin Uptake and Efflux Assays

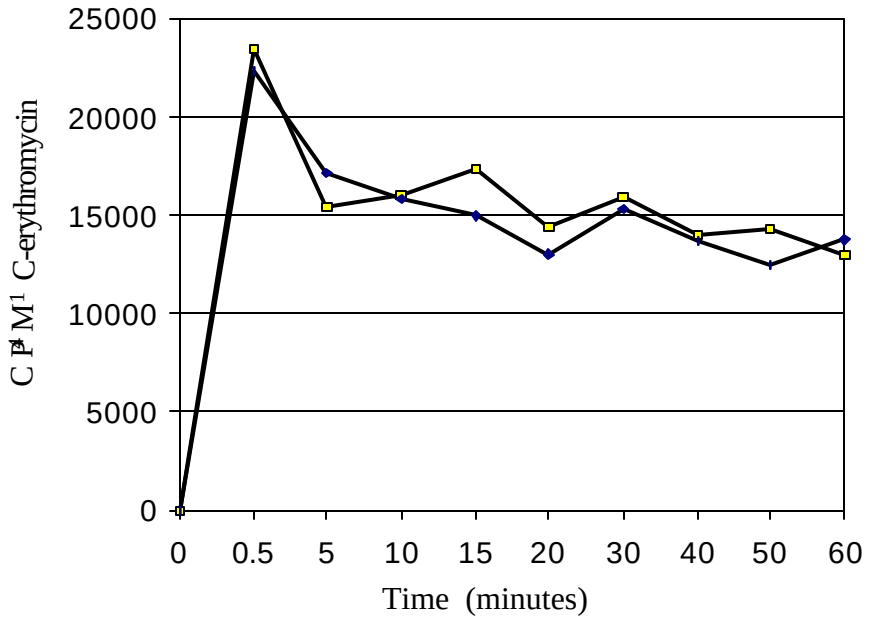
The basis for erythromycin hypersensitivity in strains SK5665, SK1217 and SK1219 is more clearly understood than that of MRE600. It is possible that the difference in erythromycin sensitivity between SK901 and MRE600 was related to the rate of erythromycin uptake or loss.

In order to compare the relative rates of uptake and loss of antibiotic, experiments were done using ^{14}C-erythromycin. Uptake was measured in strains SK901 and MRE600 that were either growing or static. Loss experiments were done in growing cells in these strains as

described. Figure 14 shows rates of erythromycin uptake into these cells. In both strains, the first sample collected contained the most erythromycin.

FIGURE 14. UPTAKE OF ^{14}C-ERYTHROMYCIN INTO GROWING CELLS.

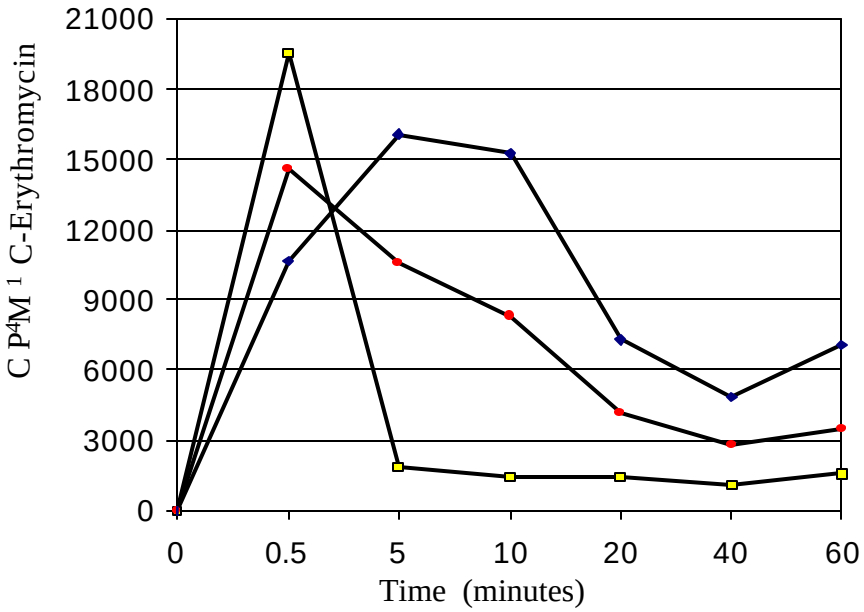
Strains SK901 (□) and MRE600 (◆) were grown in T broth at 37°C to equal cell densities. A mixture of 15 µg/ml unlabeled erythromycin and 1.5 µg/ml (0.1 µCi/ml) of ^{14}C-erythromycin was added to each culture. Samples of cells (0.2 ml) were collected at 5 minutes time intervals, captured on a filter, washed with S-buffer, and the radioactivity measured.



In an attempt to verify that the results in Figure 14 represented rapid uptake, erythromycin uptake experiments were repeated in SK901 using static cells that had been suspended in HEPES buffer for 15 minutes prior to addition of erythromycin and also by adding excess unlabeled carrier antibiotic as described. Figure 15 shows the results from these assays.

FIGURE 15. COMPETITION FOR ^{14}C-ERYTHROMYCIN BY UNLABELED ERYTHROMYCIN IN STATIC SK901 CELLS.

1.5 µg/ml of ^{14}C-erythromycin (0.1 µCi/ml) and 15 µg/ml (□) or 75 µg/ml (●) unlabeled erythromycin were added at the same time. In another experiment, the isotope was added 5 seconds after 75 µg/ml of unlabeled antibiotic (◆). Samples of 0.2 ml were collected at 5 minute intervals and captured on filters as described (Figure 14).

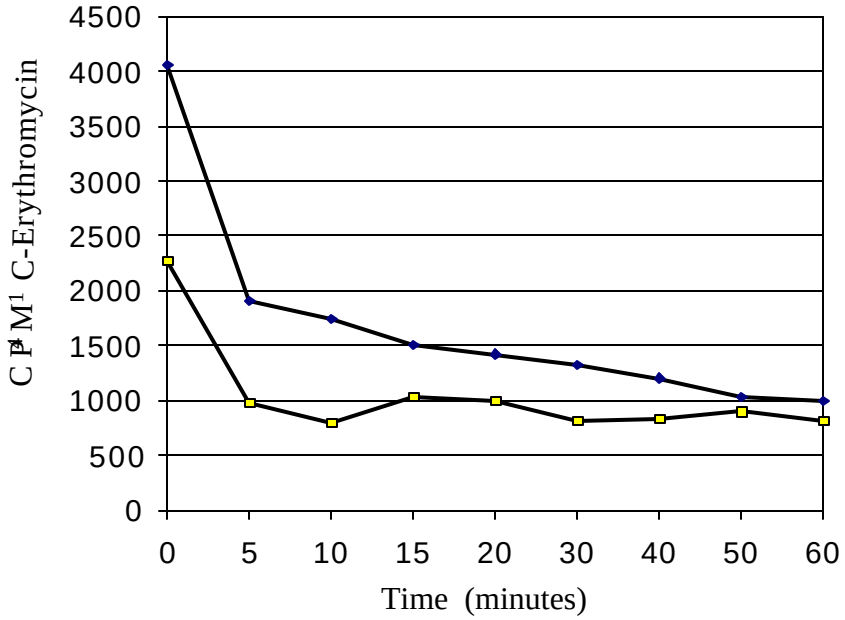


Increasing the amount of unlabeled erythromycin carrier from 15 µg/ml to 75 µg/ml resulted in a reduced rate of antibiotic loss from the cells. Addition of the isotope 5 seconds after the carrier caused the maximum uptake to be delayed by 5 minutes. At 15 µg/ml of unlabeled erythromycin, maximum loss from the cells occurred in the first 5 minutes. At 75 µg/ml of unlabeled erythromycin, maximum loss was not seen until the 40 minute time point.

A possible reason for the difference in erythromycin sensitivity between SK901 and MRE600 was reduced rates of loss as Figure 16 shows.

FIGURE 16. LOSS OF ^{14}C-ERYTHROMYCIN FROM GROWING CELLS.

Strains SK901 (□) and MRE600 (◆) were grown to equal cell densities in the presence of 15 µg/ml unlabeled erythromycin and 1.5 µg/ml of ^{14}C-erythromycin. Cells were pelleted by centrifugation and resuspended in antibiotic-free T broth. Samples of 0.2 ml were collected at 5 minute intervals and captured on filters as described (Figure 14).



After being centrifuged and resuspended in fresh T broth, the cells lost about 50% of the intercellular antibiotic in the first 5 minutes and the remainder at a slower rate. MRE600 had more erythromycin at the beginning and lost it more slowly. Erythromycin loss in other strains was very similar to SK901 (data not shown).

Pulse-Chase Analysis of Ribosomal Subunit Assembly

To see if the hypersensitivity to erythromycin in these 5 strains was reflected in 50S subunit assembly inhibition, a pulse-chase analysis of subunit assembly was conducted. This method was used to track the fate of a discrete population of rRNA molecules that were synthesized during a 90-second pulse labeling period. The rates at which the labeled rRNAs were assembled into ribosomal subunits were examined. Figure 17 shows sucrose gradient profiles of labeled subunits from SK901 following a 45-minute chase period during growth in the presence and absence of erythromycin.

After a 45-minute chase period, SK901 had incorporated most of the labeled rRNA into 30S and 50S subunits. A normal 1:1 particle stoichiometry of the ribosomal subunits was indicated in these wild type cells by the 2:1 ratio of radioactivity in the 50S:30S peaks. In the presence of erythromycin, there was a marked decrease in 50S subunit assembly and the 50S:30S ratio fell to 0.5:1 and an additional peak sedimented into the region between the 30S and 50S subunit peaks. A similar pulse-chase analysis was performed on strain MRE600 and the gradient profiles are shown in Figure 18. The inhibition of 50S subunit assembly was seen in this RNase 1^- strain at a very low erythromycin concentration. The peak in the 30S region was broader and of a lower S value compared with that of strain SK901, suggesting heterogeniety.

In Figure 19, results are shown for the pulse-chase labeling of the L15 mutant strain SK1217. The cells growing with the antibiotic present showed a highly reduced level of subunit assembly. In strain SK1217 an altered 50S:30S ratio was also apparent.

The other L15 mutant was also analyzed by the pulse-chase method and the results are shown in Figure 20. In the presence of erythromycin, strain SK1219 assembled an RNA-containing particle that sedimented between the 30S and 50S peaks.

Finally, the pulse-chase analysis of the RNase E mutant strain SK5665 revealed a major reduction in 50S subunit assembly and a large peak that sedimented into the 20S-30S region in the erythromycin-treated culture as shown in Figure 21.

FIGURE 17. SUCROSE GRADIENT PROFILE OF SUBUNITS FROM STRAIN SK901 GROWN WITH AND WITHOUT ERYTHROMYCIN.

Strain SK901 was grown at 37°C in T broth either with no antibiotic (□) or with erythromycin at 75 µg/ml (▲). The samples were pulse-labeled and chased for 45 minutes. Cell lysates were separated by centrifugation and the ^{3}H-uridine CPM in gradient fractions was measured.

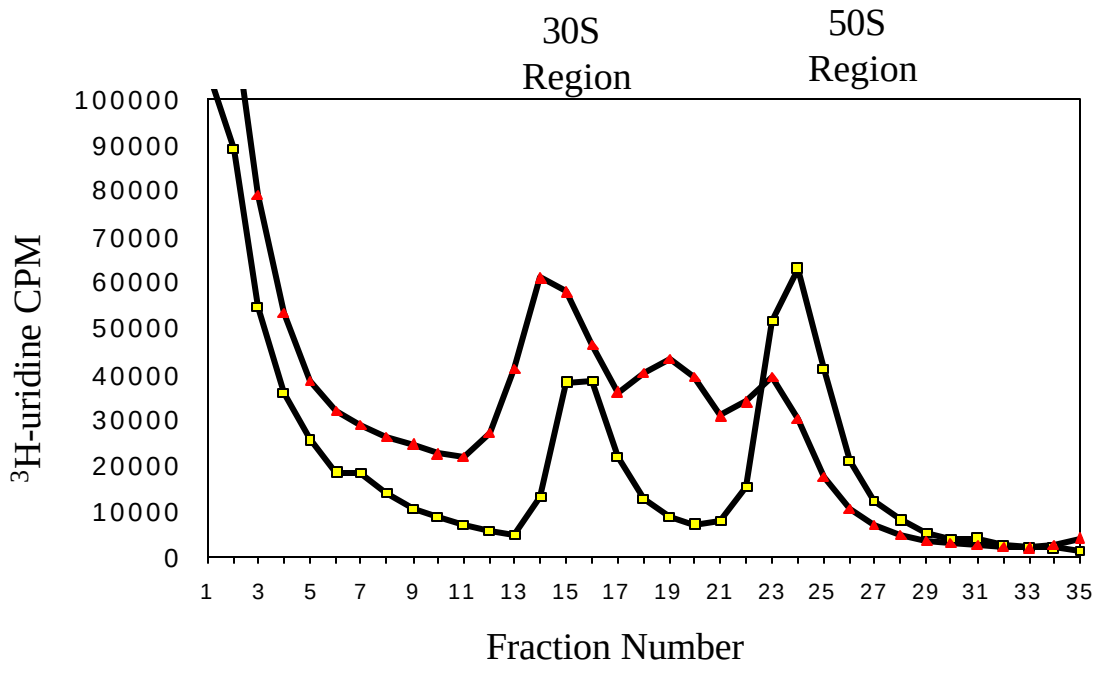


FIGURE 18. SUCROSE GRADIENT PROFILE OF SUBUNITS FROM STRAIN MRE600 GROWN WITH AND WITHOUT ERYTHROMYCIN.

Strain MRE600 was grown at 37°C in T broth either with no antibiotic (□) or with erythromycin at 2 µg/ml. The samples were pulse-labeled and chased for 45 minutes. Cell lysates were separated by centrifugation and the ^{3}H-uridine CPM in gradient fractions was measured.

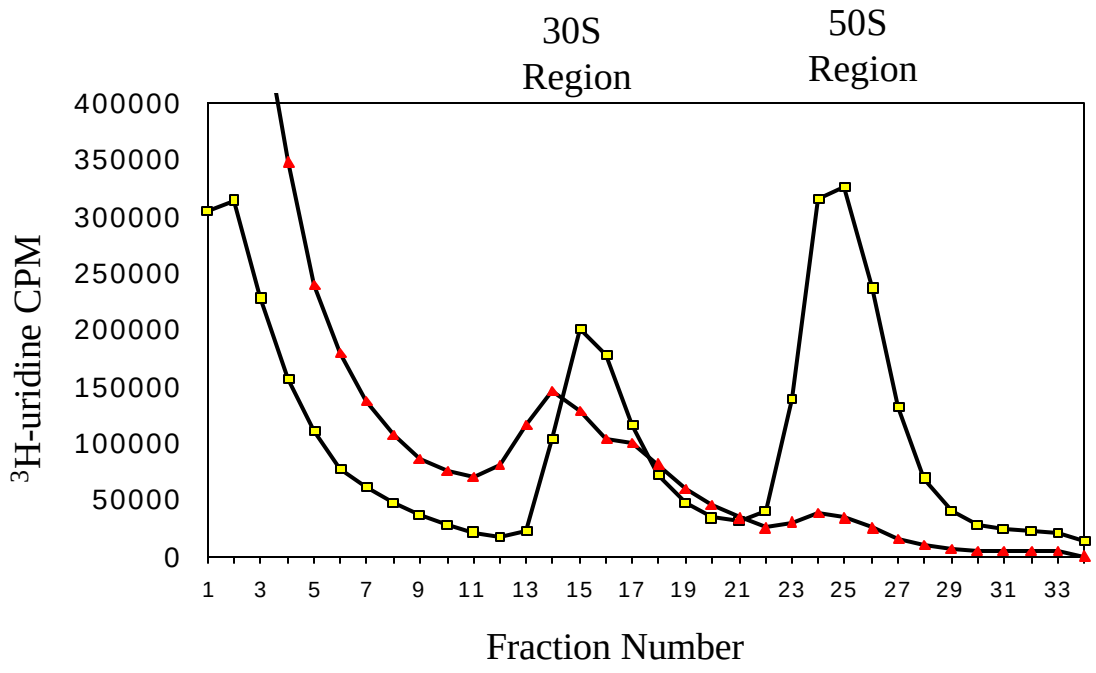


FIGURE 19. SUCROSE GRADIENT PROFILE OF SUBUNITS FROM STRAIN SK1217 GROWN WITH AND WITHOUT ERYTHROMYCIN.

Strain SK1217 was grown at 37°C in T broth either with no antibiotic (□) or with erythromycin at 20 µg/ml (▲). The samples were pulse-labeled and chased for 45 minutes. Cell lysates were separated by centrifugation and the ^{3}H-uridine CPM in gradient fractions was measured.

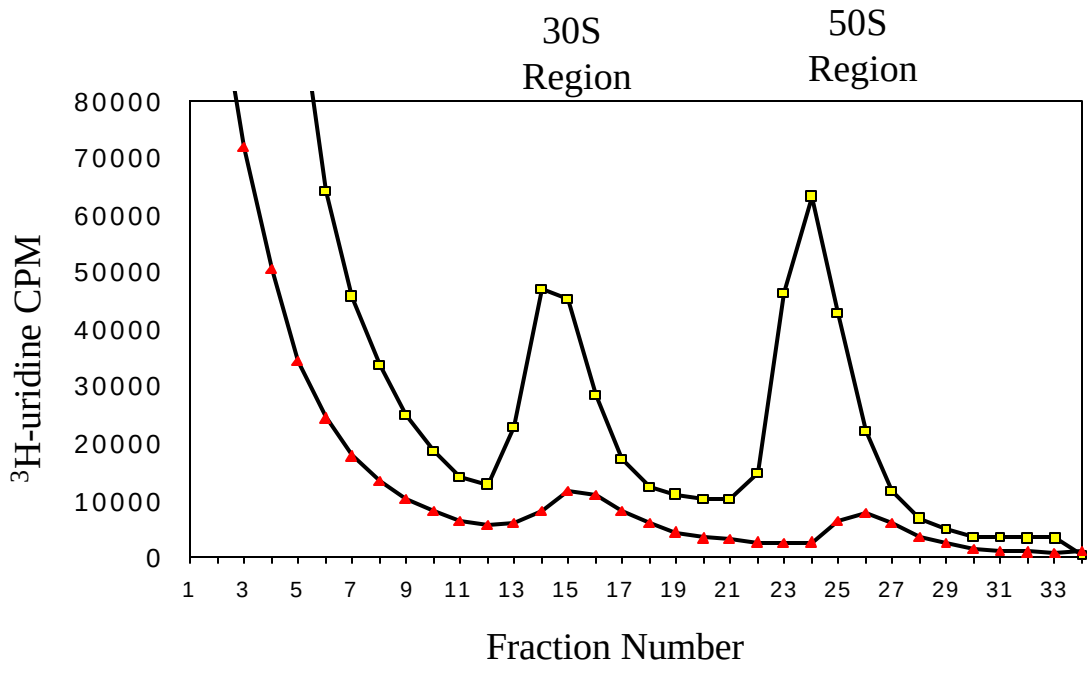


FIGURE 20. SUCROSE GRADIENT PROFILE OF SUBUNITS FROM STRAIN SK1219 GROWN WITH AND WITHOUT ERYTHROMYCIN.

Strain SK1219 was grown at 37°C in T broth either with no antibiotic (□) or with erythromycin at 20 µg/ml (▲). The samples were pulse-labeled and chased for 45 minutes. Cell lysates were separated by centrifugation and the ^{3}H-uridine CPM in gradient fractions was measured.

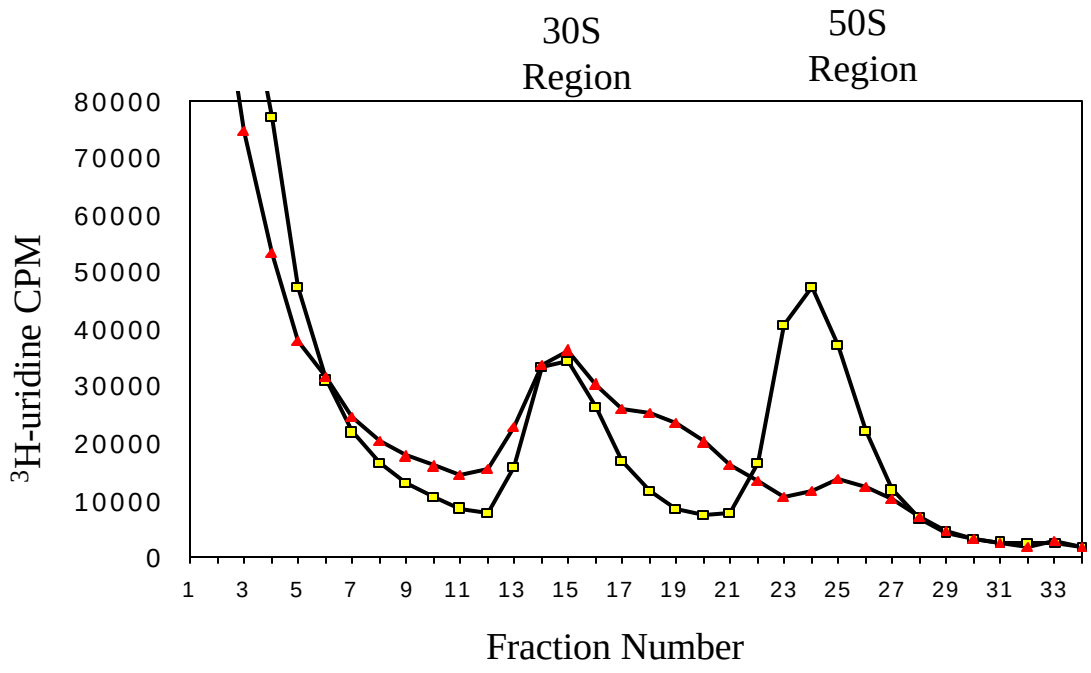
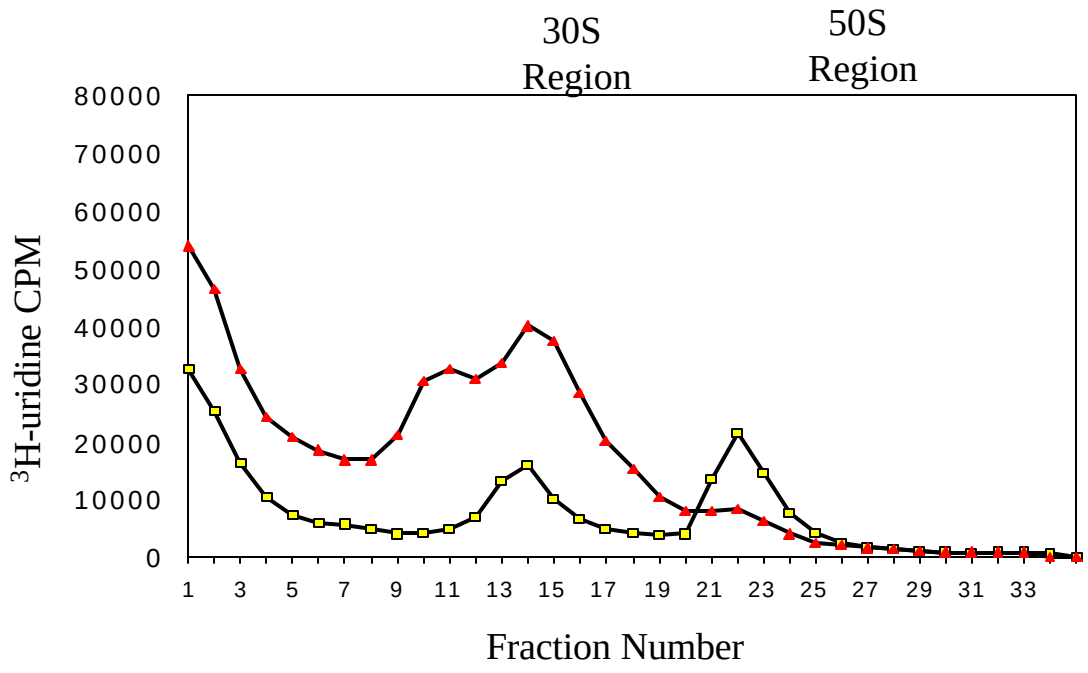


FIGURE 21. SUCROSE GRADIENT PROFILE OF SUBUNITS FROM STRAIN SK5665 GROWN WITH AND WITHOUT ERYTHROMYCIN.

Strain SK5665 was grown at 37°C in T broth either with no antibiotic (□) or with erythromycin at 18 µg/ml (▲). The samples were pulse-labeled and chased for 45 minutes. Cell lysates were separated by centrifugation and the ^{3}H-uridine CPM in gradient fractions was measured.



Assembly of the 50S subunit was inhibited and a double peak was seen in the 25-30S region of the gradient. In the absence of RNase E, there was a greater build up of precursor particle than was seen in the other strains.

A summary of the pulse-chase assembly data for the wild type and 4 mutant strains is shown in Table 5.

Table 5. Summary of Pulse-Chase Assembly Data in Five E. coli Strains.

Strain	Phenotype	[Erythromycin]	% Total Gradient CPM 30S	% Total Gradient CPM 50S	Ratio of 50S/30S	50S Subunit Reduction
SK901	wild type	0 µg/ml	12.3	17.4	1.4	
SK901	wild type	75 µg/ml	15.4	4.7	0.3	73%
MRE600	RNase 1^-	0 µg/ml	16.5	28.2	1.7	
MRE600	RNase 1^-	2 µg/ml	12.0	3.1	0.3	89%
SK1217	ts L15	0 µg/ml	8.5	10.3	1.2	
SK1217	ts L15	20 µg/ml	7.0	4.3	0.6	58%
SK1219	ts L15	0 µg/ml	10.7	14.1	1.3	
SK1219	ts L15	20 µg/ml	13.3	5.1	0.4	63%
SK5665	RNase E^-	0 µg/ml	20.4	30.0	1.5	
SK5665	RNase E^-	18 µg/ml	26.4	10.7	0.4	64%

Note that in Table 5, the 30S assembly is about the same, with and without erythromycin, in each strain. On the other hand, 50S assembly with erythromycin present was reduced by 50-80%. Thus suggests a specific effect of erythromycin on 50S subunit formation both in wild type cells and in the mutant cells examined.

Dynamics of Ribosomal Subunit Formation and Assembly Kinetics

Because of the suggested involvement of RNase E in ribosomal subunit turnover (Bessarab 1998), strains SK901 and SK5665 were chosen to characterize the dynamics of subunit formation that occurs in the presence and absence of erythromycin. Pulse-chase assays were performed at 37°C and samples were harvested at intervals of 1.5, 3, 7.5, 15, 30, and 45 minutes after the pulse of ^{3}H-uridine. Lysates were fractionated by centrifugation as described.

A second series of pulse-chase experiments were conducted at 27°C to slow the growth rate and subunit assembly time which has been shown to be proportional to the growth rate in E. coli (Lindahl 1975).

The percent of RNA in different gradient peaks from the SK901 and SK5665 pulse-chase experiments was determined by dividing the sum of the counts in each peak by the total number of counts in that gradient. These percentages were plotted against time to obtain the kinetic profiles for ribosomal subunit formation in the control and erythromycin-treated samples at 27°C and 37°C.

Figure 22 A-F is a composite of twelve pulse-chase assays showing 30S and 50S subunit assembly in SK901 at 37°C growing with and without erythromycin.

In the cells containing erythromycin, 50S subunit assembly was suppressed in the 1.5 and 3 minute samples (Figure 22 A and B). At the 7.5 minute time point (Figure 22 C), an RNA-containing particle had sedimented into the area between the 30S and 50S gradient regions. At the 15 and 30 minute time points (Figure 22 D and E), this intermediate precursor is seen between the 30S and 50S gradient regions of the erythromycin-treated samples. At the 45 minute time point (Figure 22 F), 3 distinct peaks are seen in the antibiotic treated sample.

The kinetics of the assembly process can best be observed in 3 dimensions. Dynamic profiles of these pulse-chase assays were constructed using the data from each chase period and are shown in Figure 23. Changes in color on the graphs are representative of change in ^{3}H-uridine radioactivity on the Y axis.

FIGURE 22. GRADIENT PROFILES OF RIBOSOMAL SUBUNITS FROM STRAIN SK901 PULSE-CHASE ANALYSIS.

SK901 was grown at 37°C in T broth either with no antibiotic (□) or with erythromycin at 75 µg/ml (▲), and were pulse-labeled and chased as described. Samples were taken at 1.5 minute (A), 3 minute (B), 7.5 minute (C), 15 minute (D), 30 minute (E), and 45 minute (F) chase times.

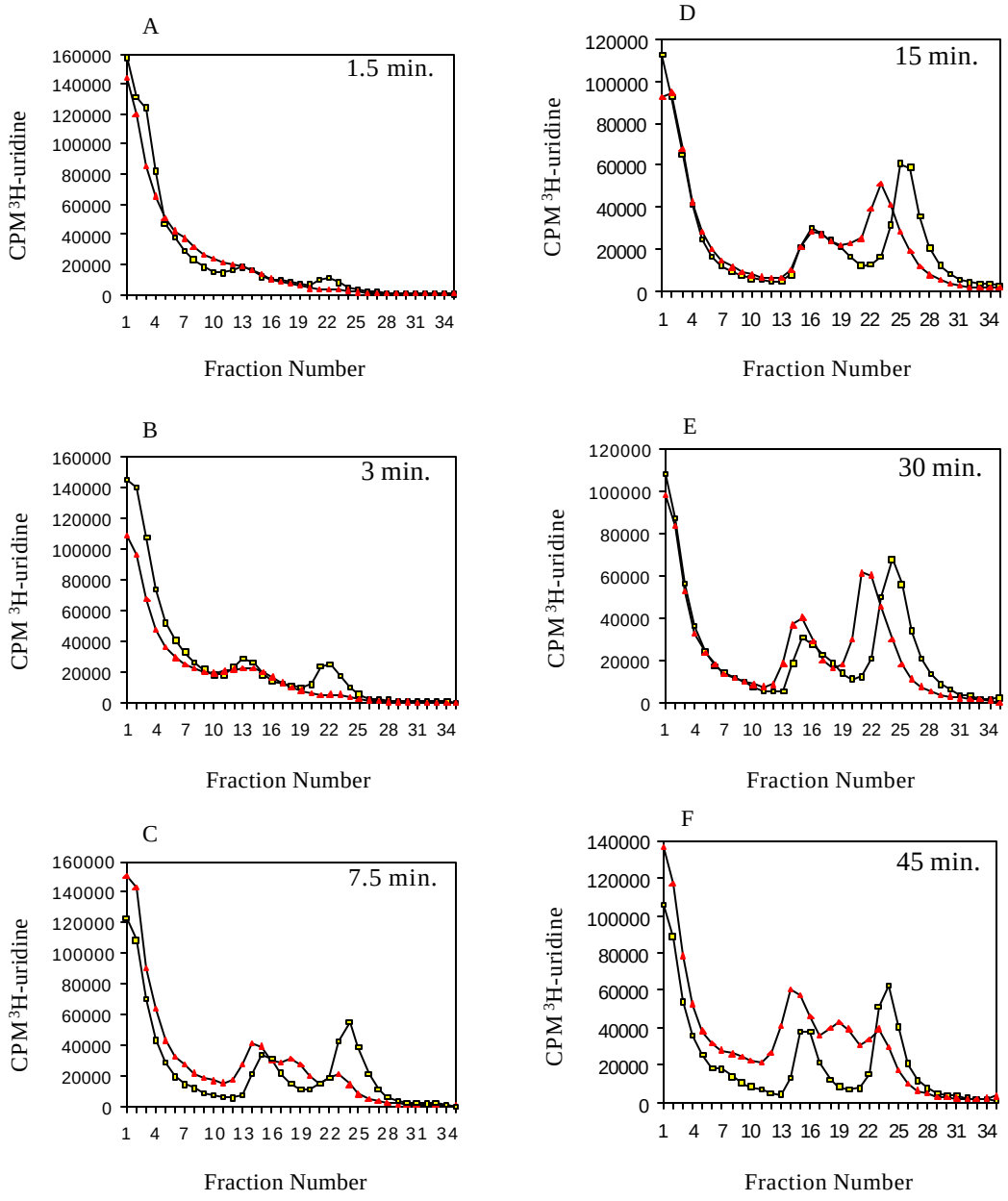
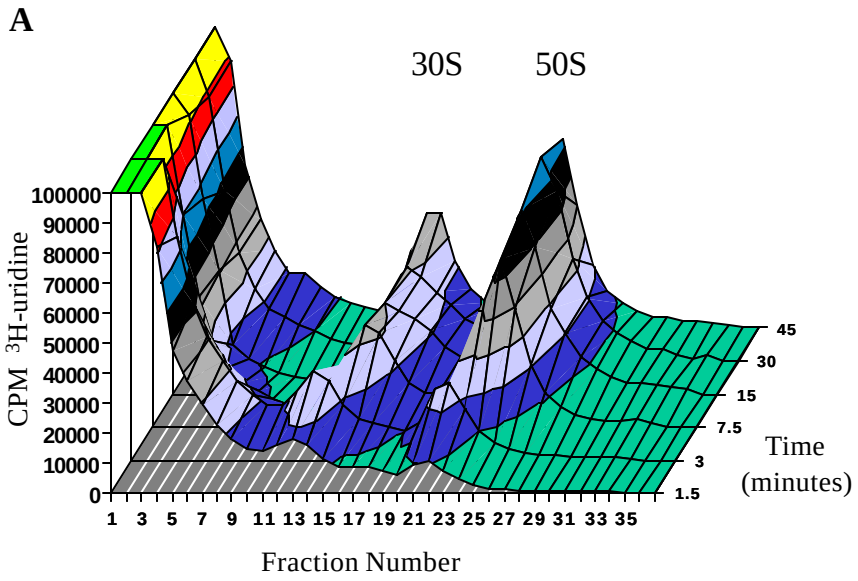
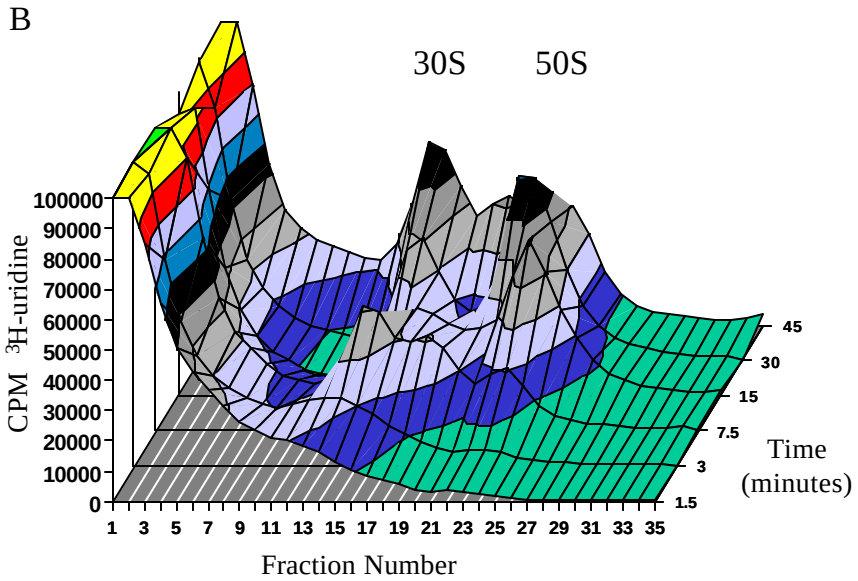


FIGURE 23. STRAIN SK901 PULSE-CHASE GRADIENT COMPOSITE AT 37°C.

Strain SK901 was grown at 37°C in T broth either with no antibiotic (A) or with erythromycin at 75 µg/ml (B). Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.





The cells grown without erythromycin (Figure 23 A) showed normal assembly of 30S and 50S subunit particles over time. In the presence of erythromycin at 75 μg/ml (Figure 23 B), a reduced 50S peak was observed and an intermediate density particle accumulated in the 40S region. The kinetics of these assembly processes are shown in Figure 24. In the SK901 culture grown at 37°C without antibiotic (Figure 24 A), assembly of the 30S subunit was complete by 7.5 minutes. The 50S subunit assembly was completed by 15 minutes. In the presence of erythromycin (Figure 24 B), 50S subunit assembly was suppressed but 30S subunit assembly appeared to be unaffected.

At the reduced temperature (27°C), SK901 subunit assembly was slowed in both the without antibiotic (Figure 25 A) and the erythromycin-treated (Figure 25 B) cultures. The kinetics of these subunit assembly processes at the reduced temperature are shown in Figure 26. In the SK901 27°C sample without antibiotic (Figure 26 A), assembly of the 30S subunit was again completed by 7.5 minutes. The 50S subunit assembly was completed by about 30 minutes. In the presence of erythromycin (Figure 26 B), 50S subunit assembly was suppressed but 30S subunit assembly seemed unaffected.

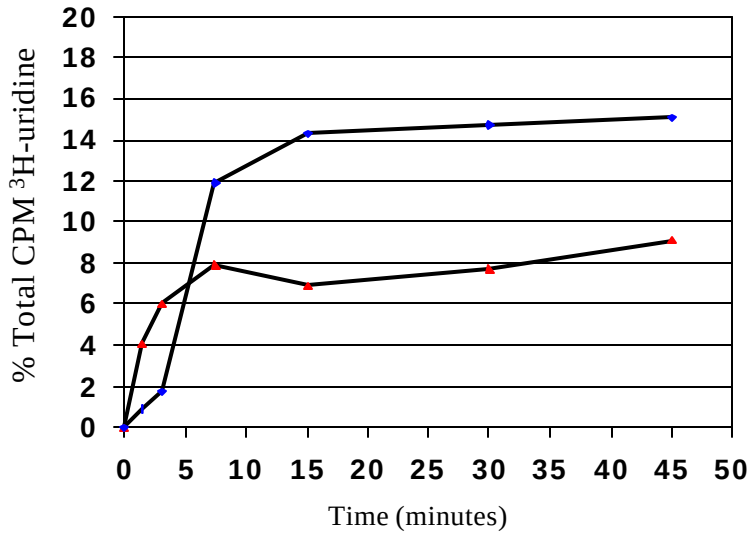
In Figure 27, the results of subunit assembly in SK5665 are shown. The SK5665 cells grown without antibiotic (Figure 27 A) showed a normal assembly sequence of 30S and 50S subunit particles over time. The accumulation of large amounts of RNA containing particle(s) in the 20-30S region of the erythromycin treated sample (Figure 27 B) was apparent. The kinetics of this assembly process at 37°C are shown in Figure 28. In the SK5665 37°C no antibiotic sample (Figure 28 A), assembly of the 30S subunit was completed by 7.5 minutes. The 50S subunit assembly was completed by 30 minutes. In the presence of erythromycin (Figure 28 B), 50S subunit assembly is extremely suppressed while 30S subunit assembly was completed by about 30 minutes.

Finally, pulse-chase analysis was repeated in SK5665 at 27°C. At the reduced temperature, SK5665 subunit assembly was slowed in cells grown without antibiotic (Figure 29 A). In the erythromycin treated sample (Figure 29 B), the increased peak is again seen in the 25S-30S region. The kinetics of this assembly process at the reduced temperature are shown in Figure 30.

FIGURE 24. RIBOSOMAL SUBUNIT ASSEMBLY KINETICS IN SK901 AT 37^{O}C.

Gradient CPM in the 30S and 50S regions from Figure 23 expressed as a percent of the total CPM. Part A shows assembly of the 30S subunits (▲) and 50S subunits (◆) of cells grown without antibiotic. Part B shows the 30S subunits (□) and 50S subunits (●) of cells grown in the presence of erythromycin at 75 µg/ml.

A



B

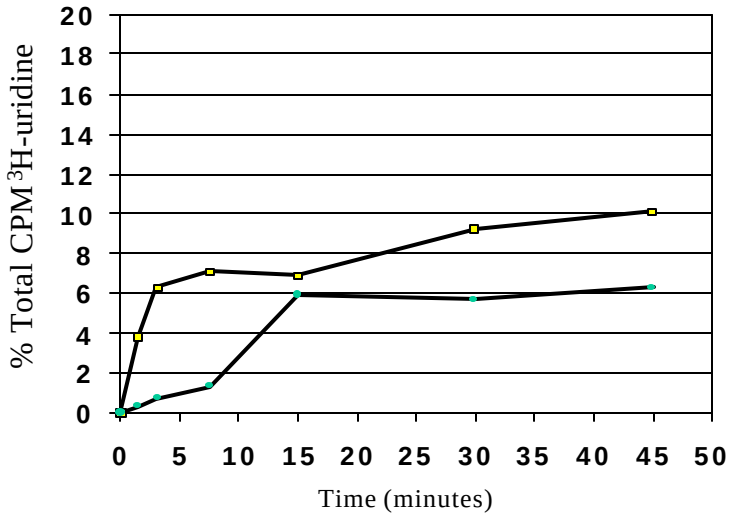


FIGURE 25. STRAIN SK901 PULSE-CHASE GRADIENT COMPOSITE AT 27°C.

Strain SK901 was grown at 27°C in T broth either with no antibiotic (A) or with erythromycin at 75 µg/ml (B). Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.

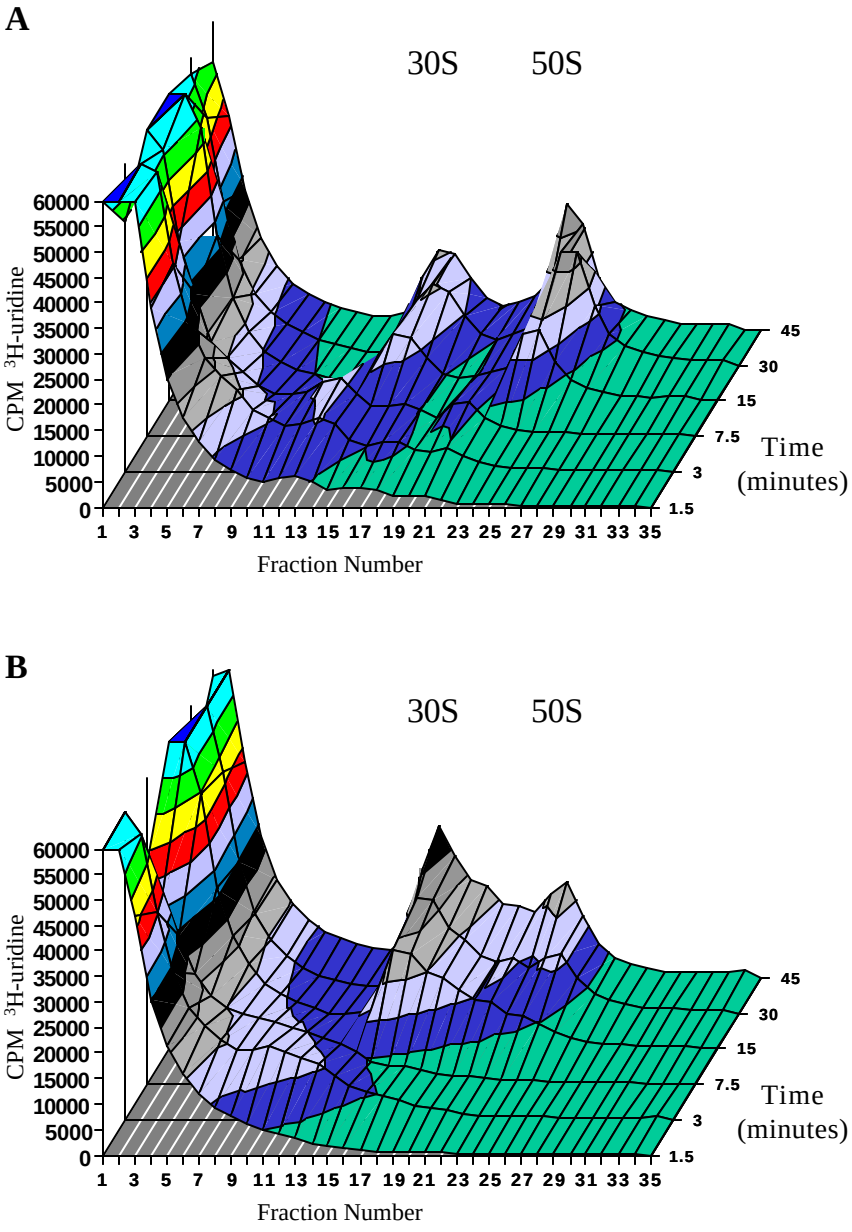
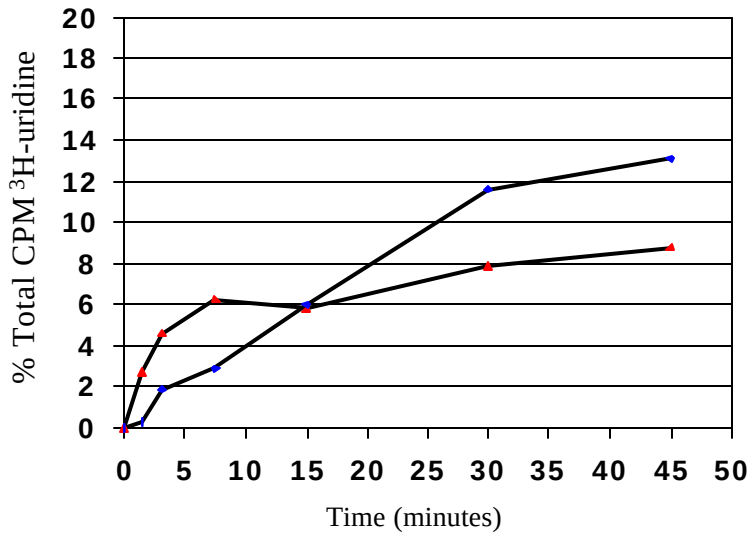


FIGURE 26. RIBOSOMAL SUBUNIT ASSEMBLY KINETICS IN SK901 AT 27^{O}C.

Gradient CPM in the 30S and 50S regions from Figure 25 expressed as a percent of the total CPM. Part A shows assembly of the 30S subunits (▲) and 50S subunits (◆) of cells grown without antibiotic. Part B shows the 30S subunits (□) and 50S subunits (●) of cells grown in the presence of erythromycin at 75 µg/ml.

A



B

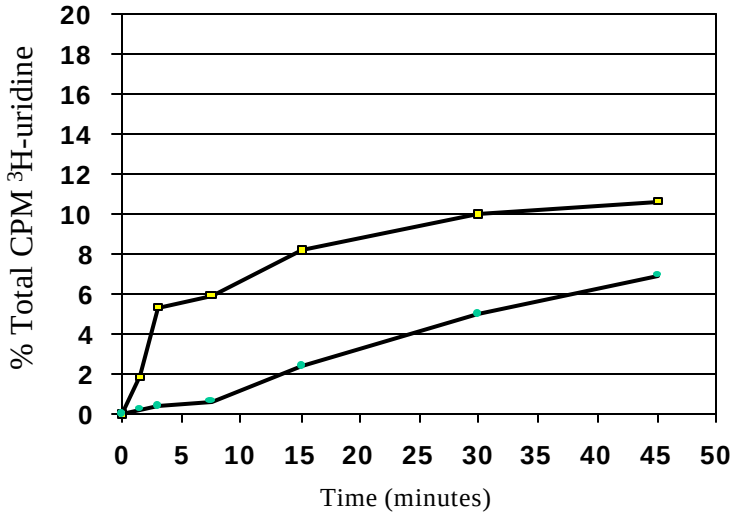


FIGURE 27. SK5665 PULSE-CHASE COMPOSITE AT 37°C.

Strain SK5665 was grown at 37°C in T broth either with no antibiotic (A) or with erythromycin at 18 µg/ml (B). Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.

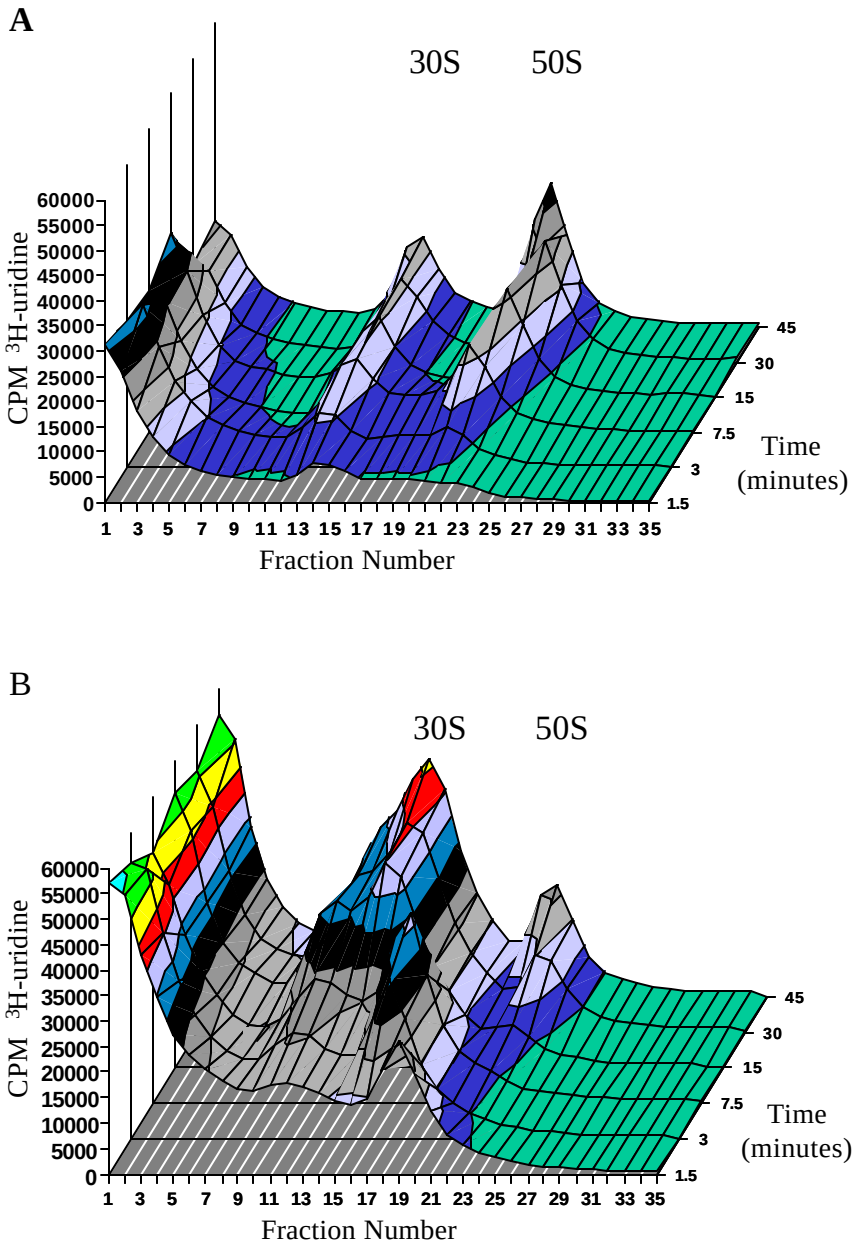
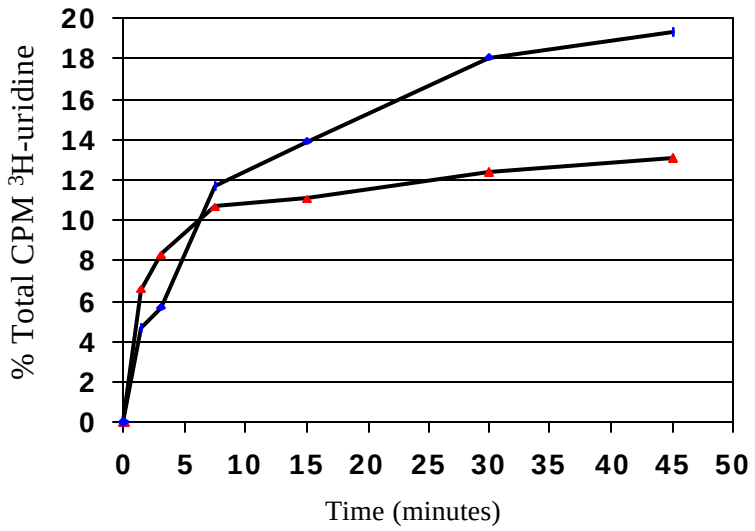


FIGURE 28. RIBOSOMAL SUBUNIT ASSEMBLY KINETICS IN SK5665 AT 37^{O}C.

Gradient CPM in the 30S and 50S regions from Figure 27 expressed as a percent of the total CPM. Part A shows assembly of the 30S subunits (▲) and 50S subunits (◆) of cells grown without antibiotic. Part B shows the 30S subunits (□) and 50S subunits (●) of cells grown in the presence of erythromycin at 18 µg/ml.

A



B

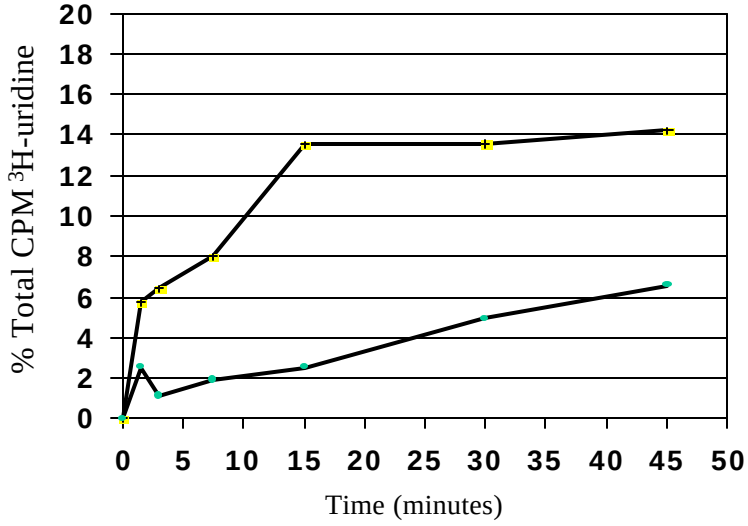


FIGURE 29. SK5665 PULSE-CHASE COMPOSITE AT 27°C.

Strain SK5665 was grown at 27°C in T broth either with no antibiotic (A) or with erythromycin at 18 µg/ml (B). Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.

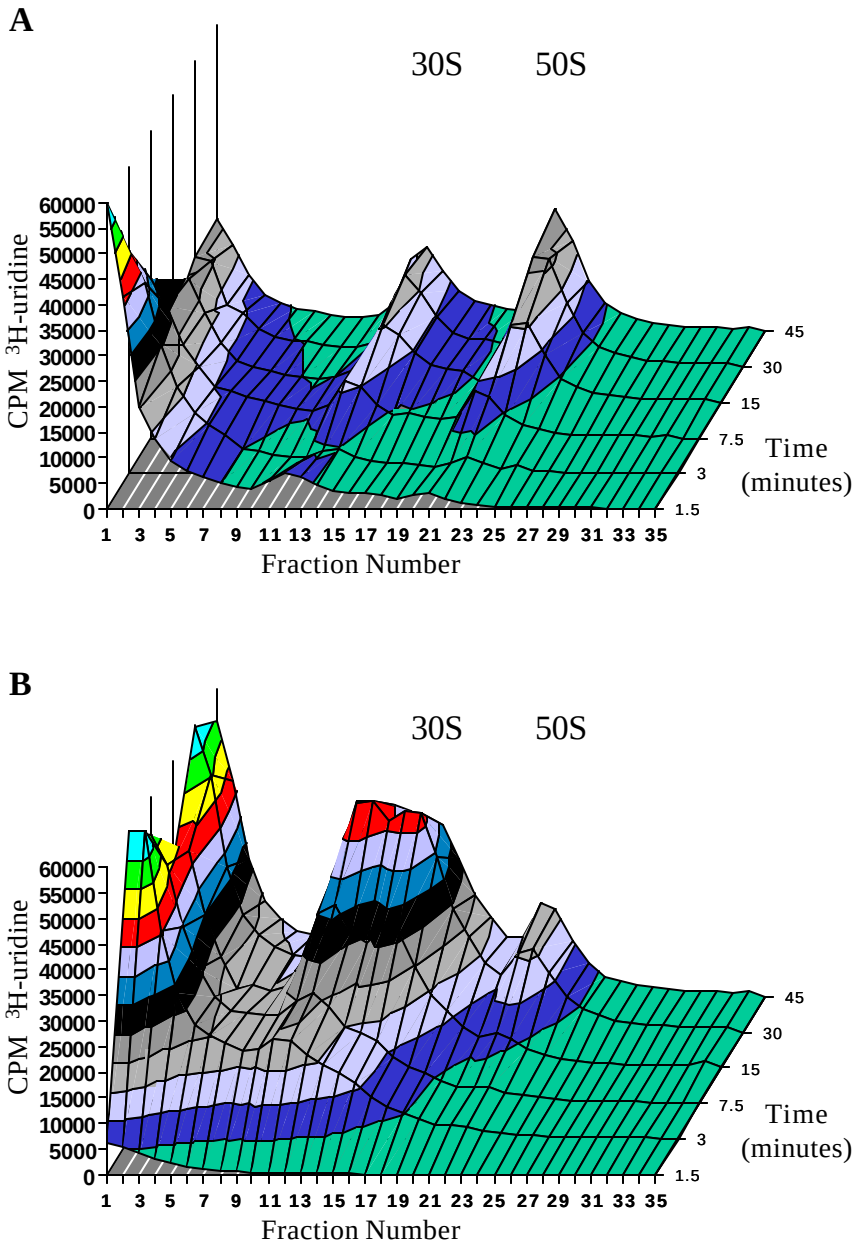
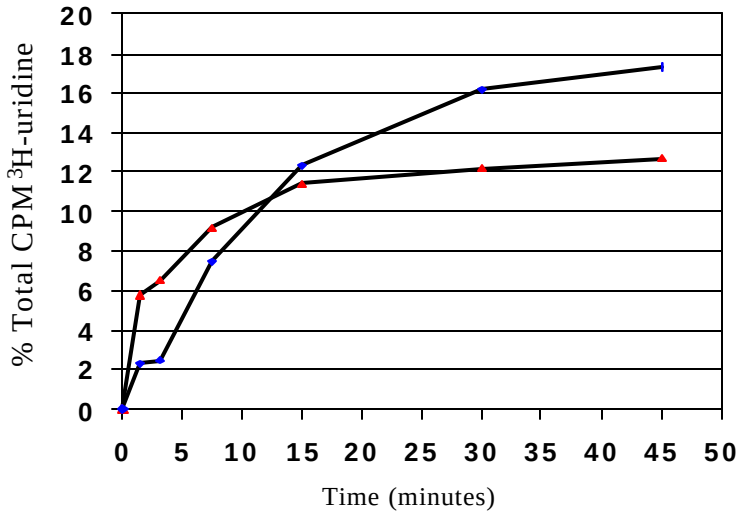


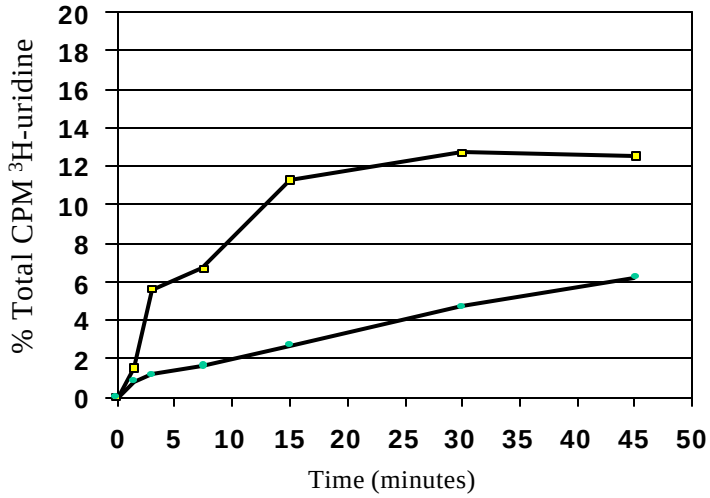
FIGURE 30. RIBOSOMAL SUBUNIT ASSEMBLY KINETICS IN SK5665 AT 27^{O}C.

Gradient CPM in the 30S and 50S regions from Figure 29 expressed as a percent of the total CPM. Part A shows assembly of the 30S subunits (▲) and 50S subunits (◆) of cells grown without antibiotic. Part B shows the 30S subunits (□) and 50S subunits (●) of cells grown in the presence of erythromycin at 18 µg/ml.

A



B



In the SK5665 27°C no antibiotic sample (Figure 30 A), assembly of the 30S subunit was completed by 15 minutes. The 50S subunit assembly was completed by 30 minutes. In the presence of erythromycin (Figure 30 B), 50S subunit assembly was suppressed but 30S subunit assembly was unaffected.

Growth of strains SK901 and SK5665 at 27°C slowed the growth rate and increased the time required for assembly and decay of ribosomal subunits and their intermediates. This provided better conditions for isolation of precursor particles from gradient pools. Table 6 shows a summary of the data from these experiments.

Table 6. Temperature Effects on Growth and Subunit Assembly in SK901 and SK5665.

				Total Gradient % CPM after			% Reduction in 50S Subunit
Strain	Temp.	Erythromycin Concentration	Doubling Time	30S	50S	50S:30S Ratio	Assembly
SK901	37°C	0 µg/ml	45 min.	6.6	14.3	2.2	---
SK901	37°C	75 µg/ml	360 min.	6.9	5.8	0.8	60 ± 8.6
SK901	27°C	0 µg/ml	90 min.	5.8	6.0	1.0	---
SK901	27°C	75 µg/ml	>480 min.	6.2	2.6	0.4	56 ± 8.7
SK5665	37°C	0 µg/ml	90 min.	11.1	11.9	1.0	---
SK5665	37°C	18 µg/ml	>480 min.	13.0	2.5	0.2	78 ± 4.7
SK5665	27°C	0 µg/ml	100 min.	9.4	7.3	0.8	---
SK5665	27°C	18 µg/ml	>480 min.	11.3	2.7	0.2	63 ± 6.3

At the reduced temperature, cell doubling times increased and the assembly of

ribosomal subunits was slowed. The decreased 50S:30S subunit ratio that occurred in the presence of erythromycin was more pronounced at the reduced temperature in strain SK901. Strain SK5665 was severely inhibited in growth and subunit assembly at both temperatures.

The pulse-chase experiments on both strains showed a clear inhibition of 50S formation and indicated a possible precursor in the 30S region of the gradients (Figures 23 B, 25 B, 27 B, and 29 B). The rRNA and the protein content of these gradient fractions were examined to look for 23S and 5S rRNA species and 50S subunit proteins.

RNA Analysis

Cells of both strains were grown at 27^{o}C in the presence of ^{3}H uridine, and subunits were separated by sucrose gradient centrifugation as described. RNA was isolated from each fraction and separated by electrophoresis. The gels were dried and radioactivity in the RNA was examined by fluorography.

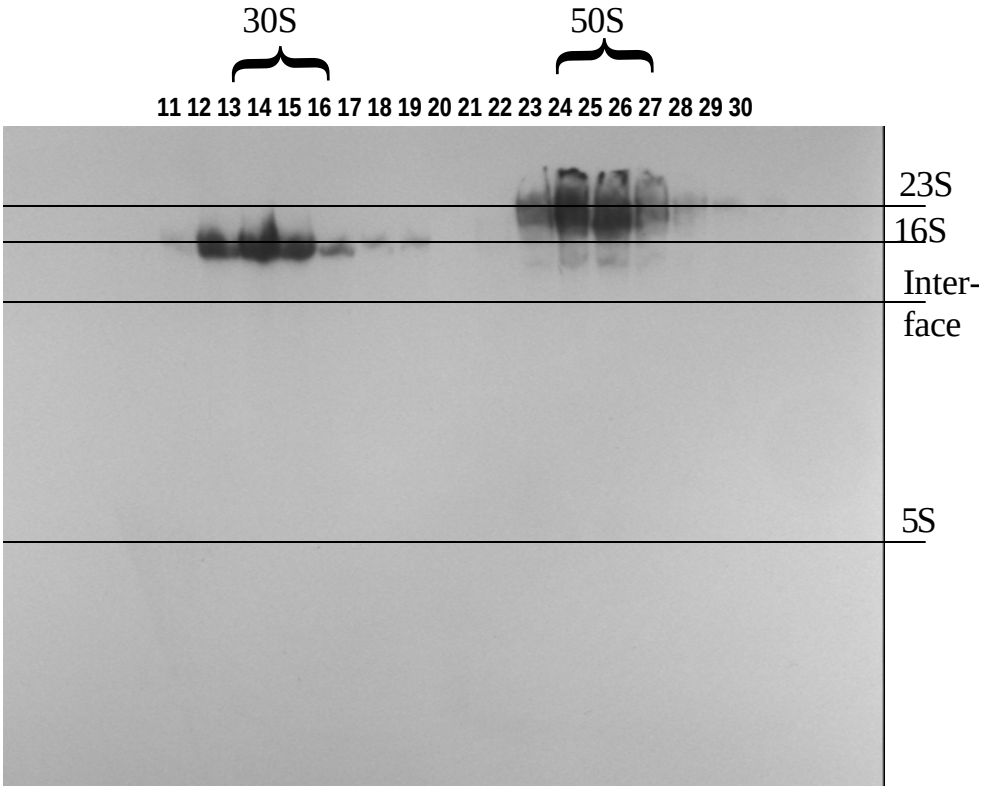
Polyacrylamide gel electrophoresis was used to determine the rRNA content of the ribosomal subunit particles assembled both without antibiotic and in the presence of erythromycin. A dual concentration of polyacrylamide was used to separate RNA from gradient fractions 11-30. The top one-third of each gel contained 4% polyacrylamide to separate the 16S and 23S rRNAs. The bottom two thirds of each gel contained 7% polyacrylamide to retard the 5S rRNA and small oligonucleotides.

Figure 31 shows the results from SK901 cells grown without antibiotic. The 16S rRNA was found in the 30S gradient region and 23S rRNA in the 50S region as expected. Fractions 20-22 did not contain appreciable amounts of RNA.

Figure 32 shows the results from SK901 cells grown in the presence of erythromycin at 75 μg/ml. 16S rRNA was found in the 30S region, but in fractions 17-22, 23S rRNA was seen also. A small amount of 5S rRNA was also seen in this region. 23S rRNA was found in the 50S region (fractions 24-28) as expected. In contrast to the without antibiotic samples, fractions 20-22 also contained dual RNA bands.

FIGURE 31. RNA FLUOROGRAM FROM SK901 CONTROL CELLS.

Gradient fractions from SK901 cells labeled with ^{3}H-uridine for 30 minutes were separated by electrophoresis on a 4%/7% polyacrylamide gel. The fluorogram was exposed to X-ray film at -80^{o}C for 3 weeks.



Breakdown products of rRNA in control and erythromycin-treated cells were studied by examining small oligoribonucleotides that accumulated in the upper sucrose gradients fraction (1-10). Figure 33 shows the results from SK901 cells with and without erythromycin. Fractions from cells grown with erythromycin (75 μg/ml) contained a large amount of small or broken RNA fragments relative to fractions from cells grown in the absence of antibiotic.

FIGURE 32. RNA FLUOROGRAM FROM SK901 CELLS GROWN WITH ERYTHROMYCIN.

Gradient fractions from SK901 cells grown with erythromycin (75 µg/ml) and labeled with ^{3}H-uridine for 30 minutes were separated by electrophoresis on a 4%/7% polyacrylamide gel. The fluorogram was exposed to X-ray film at -80^{o}C for 3 weeks.

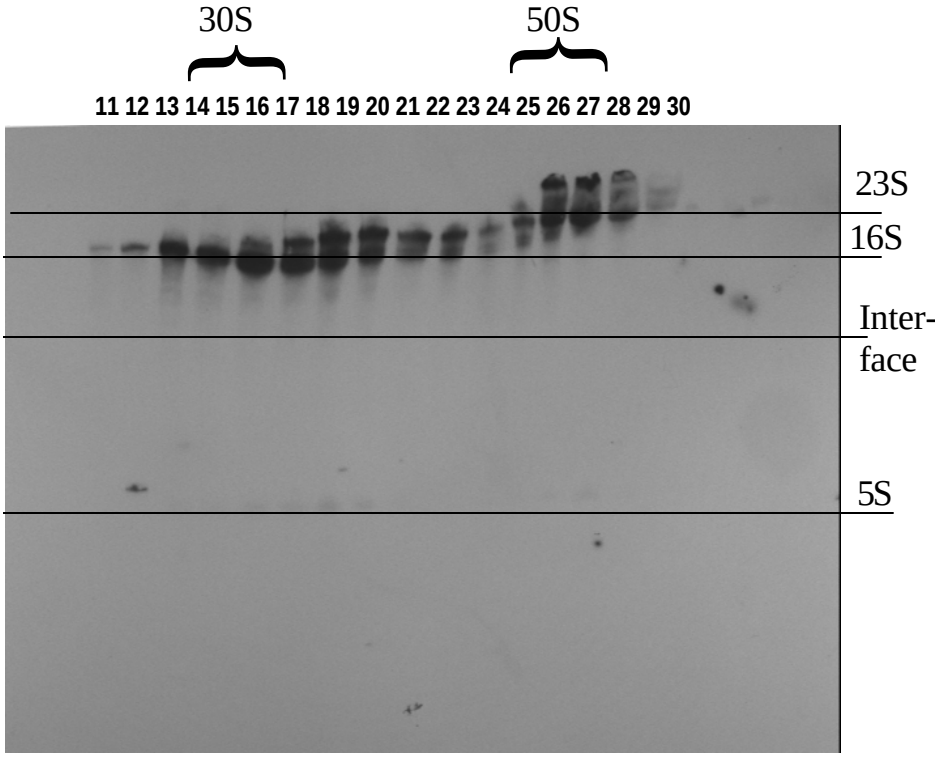
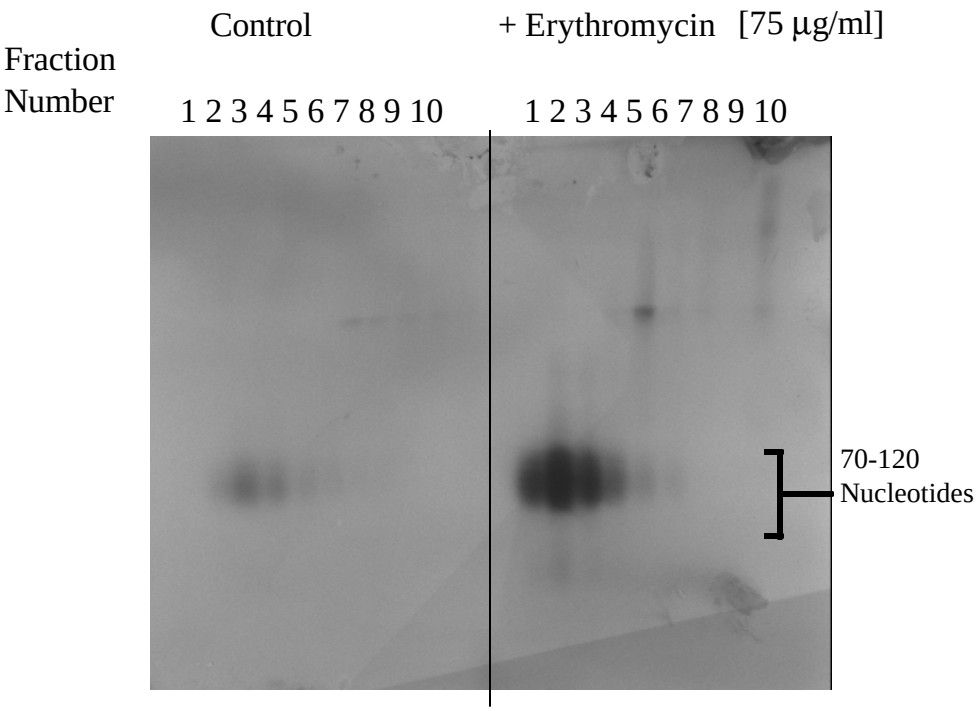


Figure 34 shows the 16S rRNA that was found in the 30S gradient region along with RNA that migrated to the gel interface. The 23S and 5S rRNAs were seen in the 50S region as expected. Fractions 20-22 contained RNA that was trapped at the gel interface.

Figure 35 shows the results from SK5665 cells grown with erythromycin (18 µg/ml). Very little 23S rRNA was found in particles dense enough to sediment into the 50S gradient region.

FIGURE 33. RNA FLUOROGRAM FROM SK901 CELLS GROWN WITH AND WITHOUT ERYTHROMYCIN FRACTIONS 1-10.

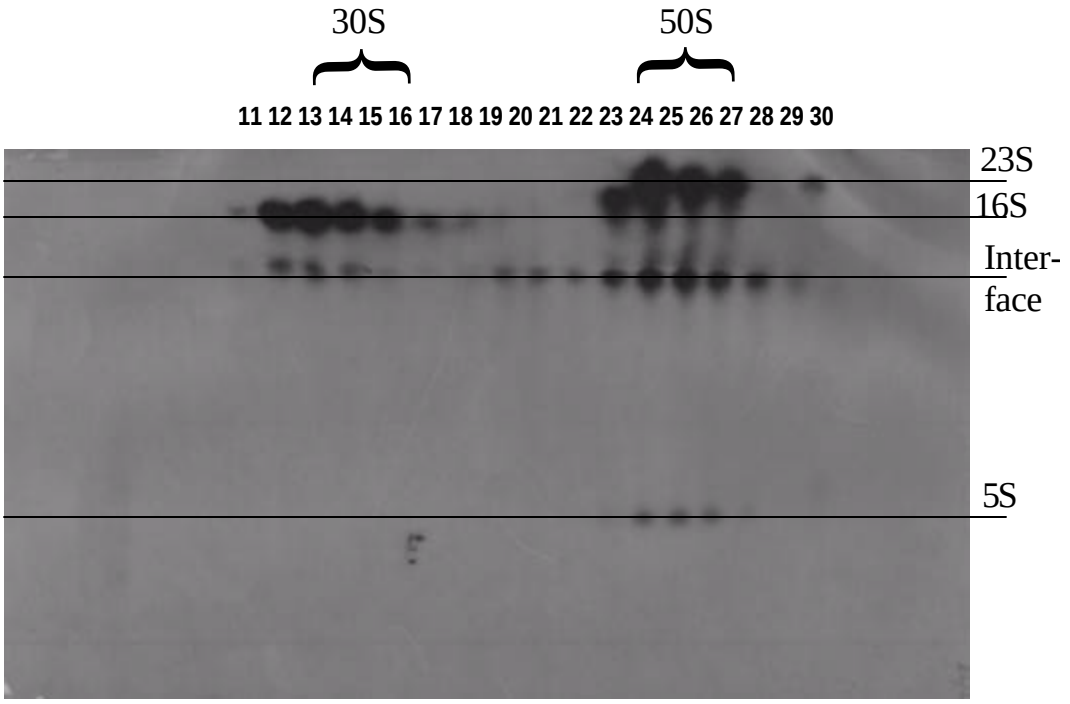
Top gradient fractions from SK901 cells grown with erythromycin (75 μg/ml) or without erythromycin were ^{3}H-uridine labeled and the RNA was separated by electrophoresis on a 7% polyacrylamide gel. The fluorogram was exposed to X-ray film at -80^{o}C for 3 weeks.



Small oligoribonucleotides that accumulated in the upper sucrose gradients fraction (1-10) were examined in SK5665. Figure 36 shows the results using cells grown with and without erythromycin. Fractions from cells grown with erythromycin (18 μg/ml) contained a large amount of small or broken RNA fragments relative to the without antibiotic cultures. A more extensive accumulation of RNA fragments was found in SK5665 compared with SK901 as figure 36 shows (compare with Figure 33). This suggests reduced turnover of rRNA in the RNase E^{-} strain.

FIGURE 34. FLUOROGRAM OF RNA FROM SK5665 CONTROL CELLS.

Gradient fractions from SK5665 cells labeled with ^{3}H-uridine for 30 minutes were separated by electrophoresis on a 4%/7% polyacrylamide gel. The fluorogram was exposed to X-ray film at -80^{o}C for 3 weeks.



A more specific identification of rRNA in the particles was performed by northern blotting and hybridization with a 23S rRNA-specific DNA probe. Figure 37 shows a comparison of slot blots done on rRNA from SK901 cells grown with and without erythromycin at 75 µg/ml. The gradient fractions from cells grown with erythromycin (front) contained most of their 23S rRNA in the 50S region. In comparison, the fractions from cells grown with erythromycin (rear) had 23S rRNA in the 30S-40S as well as the 50S gradient region.

FIGURE 35. RNA FLUOROGRAM FROM SK5665 CELLS GROWN WITH ERYTHROMYCIN.

Gradient fractions from SK5665 cells grown with erythromycin (18 µg/ml) and labeled with ^{3}H-uridine for 30 minutes were separated by electrophoresis on a 4%/7% polyacrylamide gel. The fluorogram was exposed to X-ray film at -80^{o}C for 3 weeks.

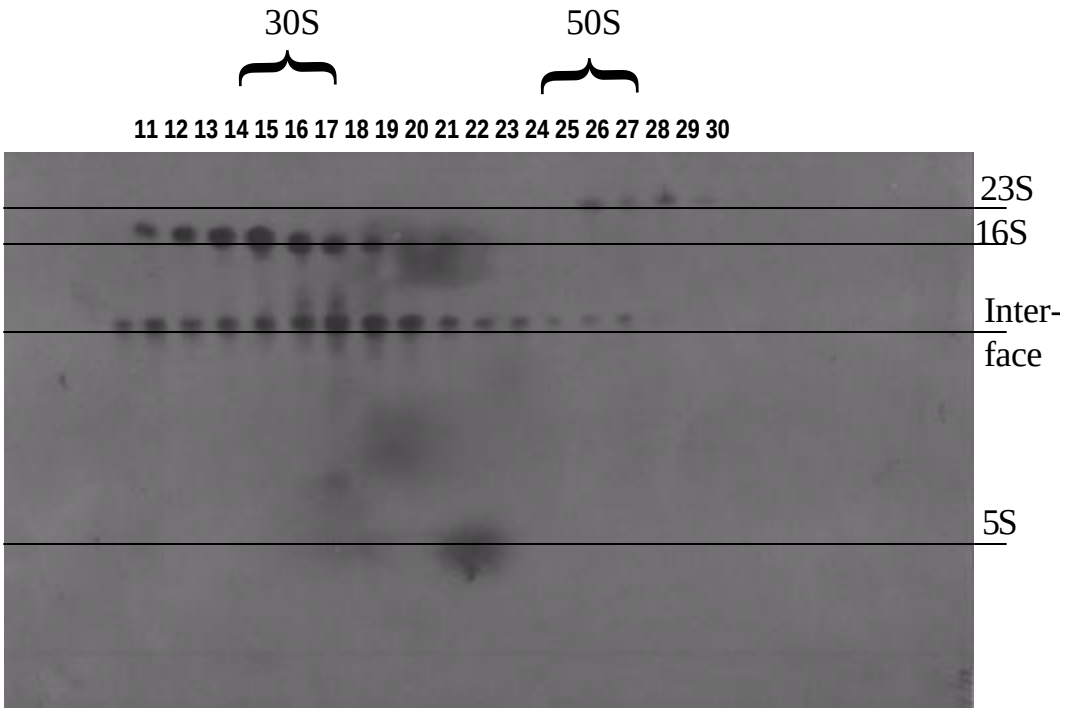


FIGURE 36. RNA FLUOROGRAM FROM SK5665 CELLS GROWN WITH AND WITHOUT ERYTHROMYCIN FRACTIONS 1-10.

Top gradient fractions from SK5665 cells grown with erythromycin (18 μg/ml) or without erythromycin were ^{3}H-uridine labeled and the RNA was separated by electrophoresis on a 7% polyacrylamide gel. The fluorogram was exposed to X-ray film at -80^{o}C for 3 weeks.

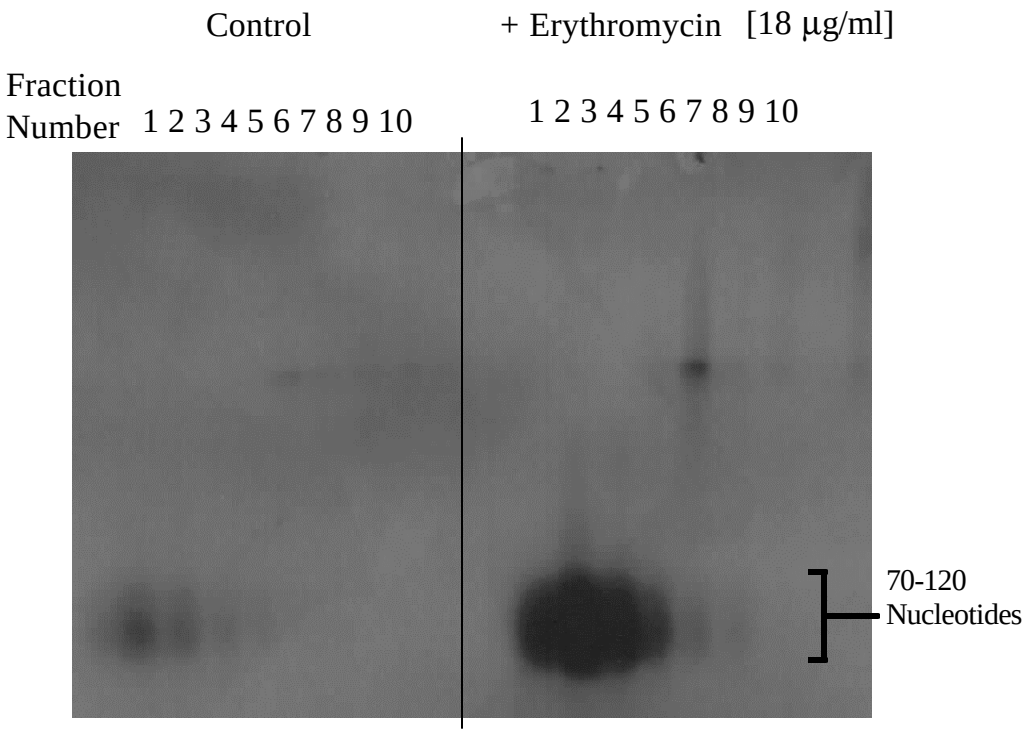
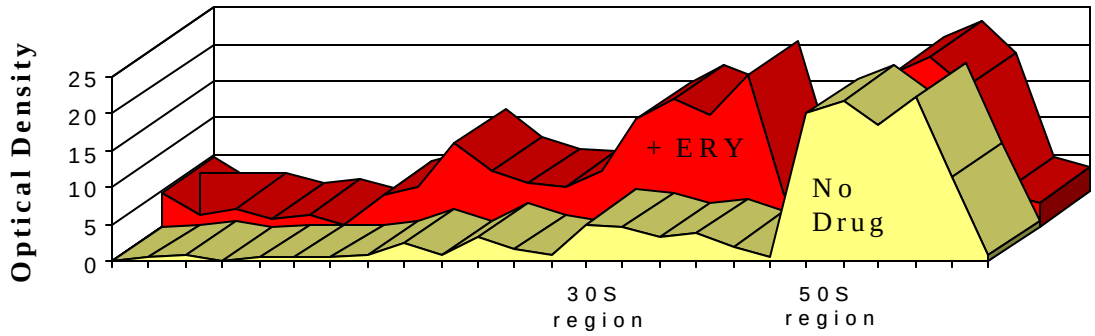


FIGURE 37. THE 23S rRNA CONTENT OF GRADIENT FRACTIONS FROM SK901 CELLS GROWN WITH AND WITHOUT ERYTHROMYCIN.

Northern hybridization to slot blots of RNA from gradient fractions of antibiotic free (front) and erythromycin-treated (rear) cells. RNA from gradient fractions was collected on a nylon membrane and hybridized with a biotinylated 146 bp DNA probe specific for 23S rRNA. X-ray film images (1 hour exposure) were captured by an Alpha Innotec imaging system and integrated to give optical density units.



Slot blots were also done on SK5665 from with erythromycin (18 µg/ml) and no antibiotic gradient fractions (27°C in T broth) and are shown in Figure 38. Hybridization to a 23S specific probe was determined by optical densitometry scanning analysis of slot blot films. Compared to the SK901 cultures, the SK5665 no antibiotic fractions (front) contained less 23S rRNA in the 50S region. The erythromycin treated fractions (rear) had a similar peak of 23S rRNA in the 30S-40S as well as the 50S gradient region.

FIGURE 38. THE 23S rRNA CONTENT OF GRADIENT FRACTIONS FROM SK5665 CELLS GROWN WITH AND WITHOUT ERYTHROMYCIN.

Northern hybridization to slot blots of RNA from gradient fractions of antibiotic free (front) and erythromycin-treated (rear) cells. RNA from gradient fractions was collected on a nylon membrane and hybridized with a biotinylated 146 bp DNA probe specific for 23S rRNA. X-ray film images (1 hour exposure) were captured by an Alpha Innotec imaging system and integrated to give optical density units.

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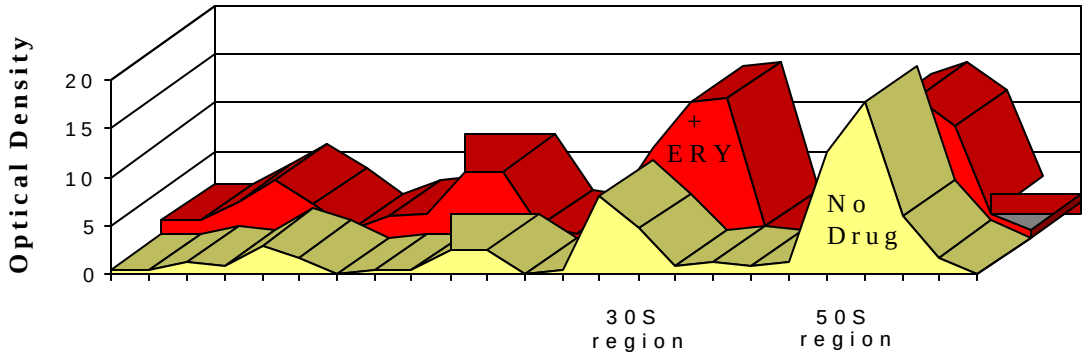


Protein Analysis

The identification of 23S and 5S rRNA in precursor particles that sedimented into the 30S region lead to an examination of the 50S proteins in these particles. Two-dimensional gel electrophoresis assays were used to discover the protein compositions of the 50S precursor particles that accumulated in the 30S gradient region in the presence of erythromycin in both

strains. First, isolated 30S and 50S proteins were electrophoresed separately to identify each of the small and large subunit proteins and to determine their relative migrations under our gel conditions. In the absence of erythromycin, 30S proteins S1-S21 were found in the gels of the 30S region and L1-L30 were found in the gels of the 50S regions of both strains.

Figures 39 and 40 show gels from strain SK901 grown with and without antibiotic present. Proteins L1-L30 from the 50S peak and S1-S21 from the 30S peak were identified. Under these conditions, the 50S precursor proteins are not apparent in the 30S pool from the erythromycin treated culture (Figure 40). In order to determine the protein composition of the 50S precursors that accumulated in the presence of erythromycin, cells were grown in minimal media at 27^{o}C with a ^{35}S-methionine-cysteine mixture with and without erythromycin. Cells were lysed and their contents separated by sucrose gradient centrifugation as described. Fractions corresponding to the 30S and 50S gradient regions were pooled and each pool was mixed with unlabeled 70S carrier ribosomes. Proteins from each of the fraction pools were extracted and separated by 2-D gel electrophoresis. Individual spots were cut from each gel, the polyacrylamide dissolved and the amount of isotope in each gel spot was quantitated by liquid scintillation counting.

In the absence of erythromycin, 30S proteins S1-S21 were found in the gels of the 30S region along with small amounts of some 50S proteins. The 50S gradient regions showed only 50S proteins under these same conditions. In the presence of erythromycin, a reduced amount of 50S proteins were found in the 50S gradient region and more 50S proteins were found in the 30S gradient pool gels. The protein composition of the 50S precursors was determined by subtracting the amount of 50S proteins that were localized in the 30S pools of cells grown in the absence of erythromycin from the 50S proteins in the 30S pools from cells grown with erythromycin. Results from these experiments using strain SK901 (Figure 41) and strain SK5665 (Figure 42) are shown.

FIGURE 39. TWO-DIMENSIONAL POLYACRYLAMIDE GELS 0F PROTEINS FROM SK901 CELLS GROWN WITHOUT ERYTHROMYCIN.

Gradient fractions from the 30S and 50S peaks of antibiotic-free (ND) cultures grown in T broth at 37°C were pooled separately and the proteins were isolated. 50S proteins L1-L30 (top) and 30S proteins S1-S21 (bottom) are shown.

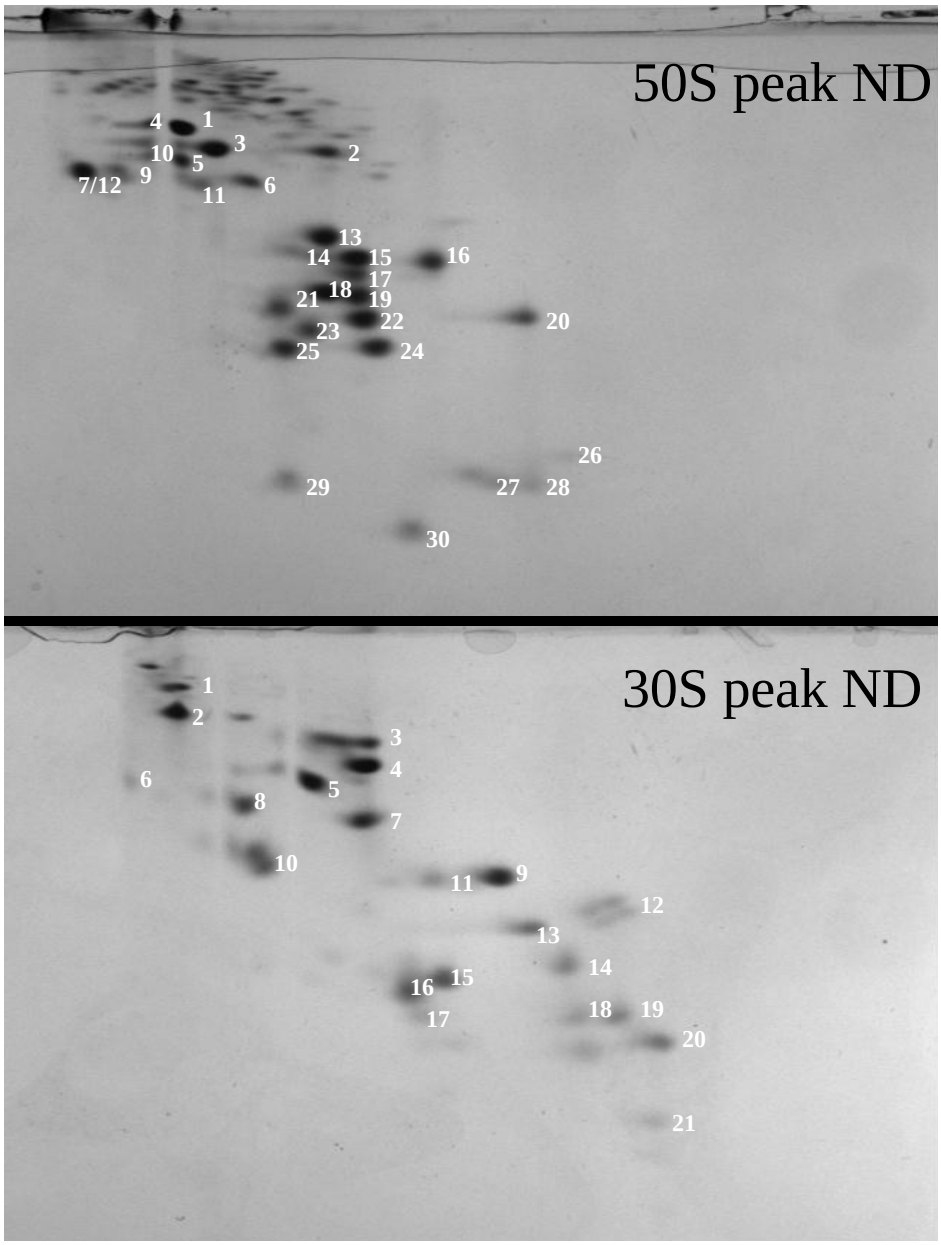


FIGURE 40. TWO-DIMENSIONAL POLYACRYLAMIDE GELS 0F PROTEINS FROM SK901 CELLS GROWN WITH ERYTHROMYCIN.

Cells grown in T broth at 37°C with erythromycin (+ERY) were lysed and fractionated. The proteins were isolated from gradient pools. 50S proteins L1-L30 (top) and 30S proteins S1-S21 (bottom) are shown.

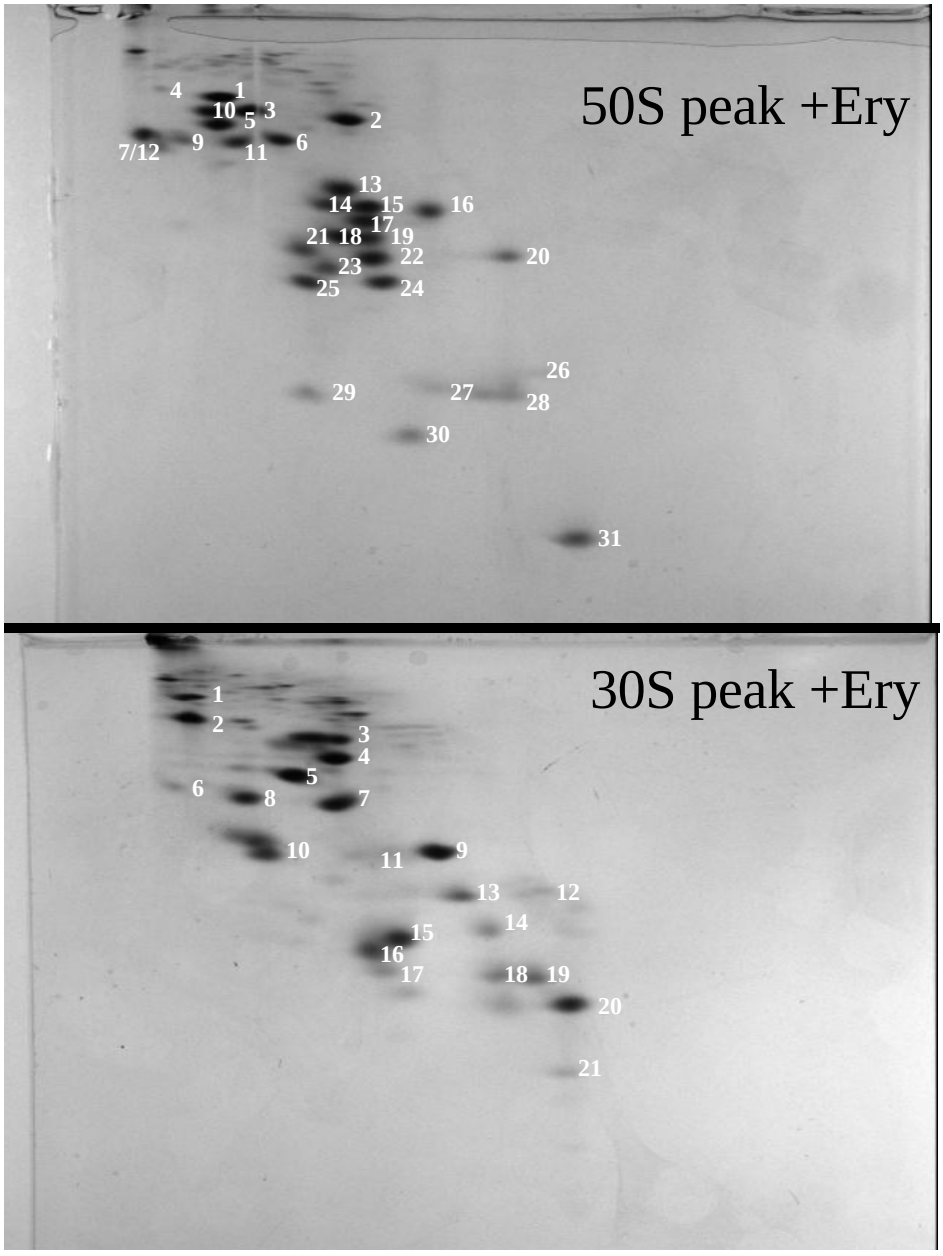


FIGURE 41. PROTEIN COMPOSITION OF THE 50S PRECURSORS THAT ACCUMULATED IN STRAIN SK901 IN THE PRESENCE OF ERYTHROMYCIN.

With the background subtracted, there were specific 50S proteins found in pooled 30S gradient fractions of SK901 cells that were labeled with ^{35}S-methionine-cysteine in the presence of erythromycin at 75 µg/ml. The number of CPM per number of sulfur containing protein residues was determined for each gel. Bar heights indicate the number of labeled amino acid residues recovered from each gel spot. The ^{35}S-CPM were normalized to 30S proteins S8 (six sulfur containing amino acid residues) and S10 (two sulfur containing amino acid residues) (Giri et al. 1984).

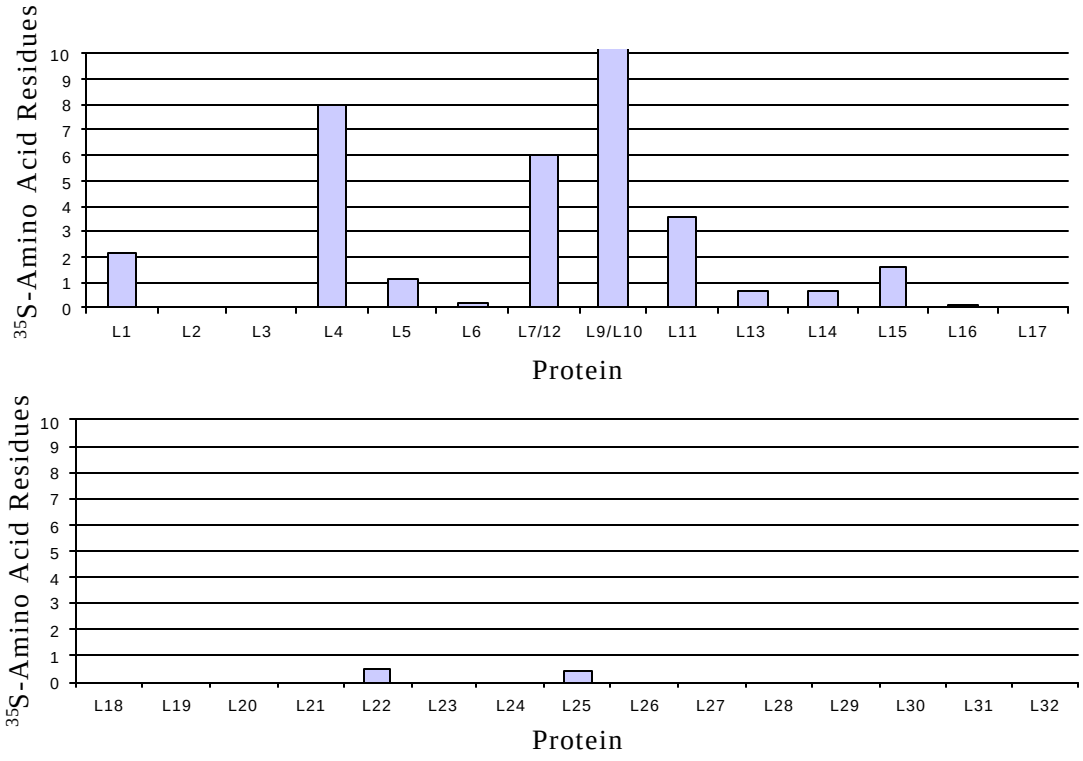
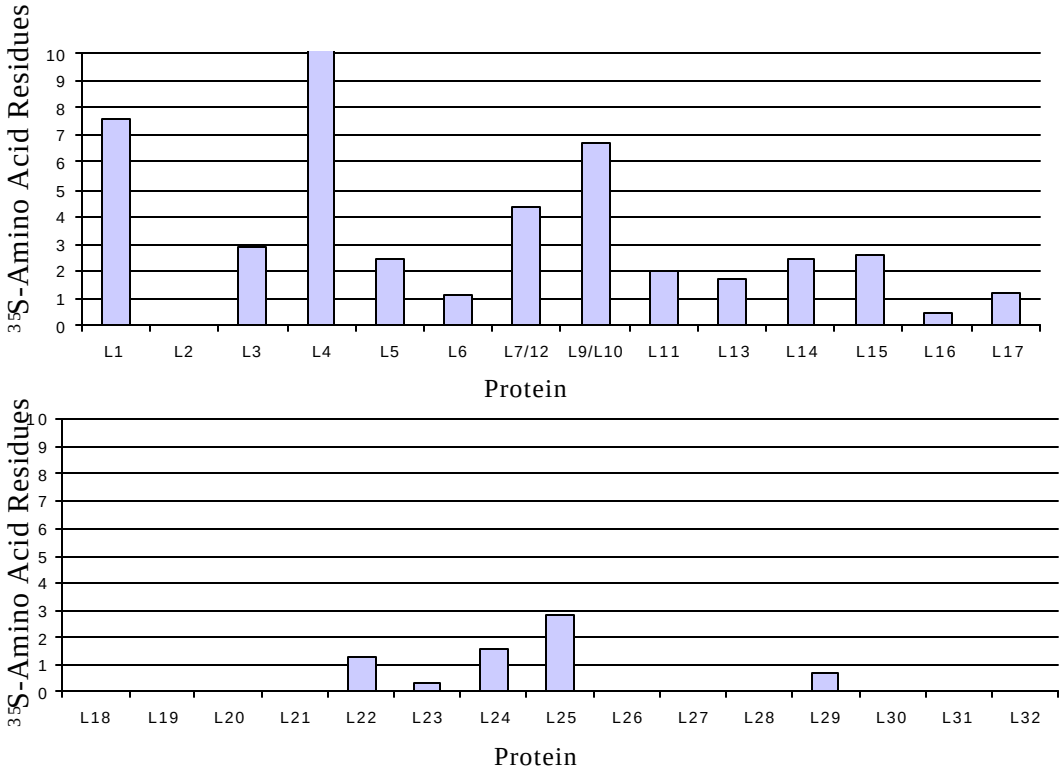


FIGURE 42. PROTEIN COMPOSITION OF THE 50S PRECURSORS THAT ACCUMULATED IN STRAIN SK5665 IN THE PRESENCE OF ERYTHROMYCIN.

With the background subtracted, there were specific 50S proteins found in pooled 30S gradient fractions of SK5665 cells that were labeled with ^{35}S-methionine-cysteine in the presence of erythromycin at 18 µg/ml. The number of CPM per number of sulfur containing protein residues was determined for each gel. Bar heights indicate the number of labeled amino acid residues recovered from each gel spot. The ^{35}S-CPM were normalized to 30S proteins S8 (six sulfur containing amino acid residues) and S10 (two sulfur containing amino acid residues) (Giri et al. 1984).



The summary presented in Table 7 compares the newly identified 50S subunit precursor protein content with the proteins found in two in vivo 50S assembly intermediates and an in vitro 50S reconstitution intermediate.

Table 7. Protein Compositions of 50S Ribosomal Subunit Precursors.

	In Vivo				In Vitro
	+ERY 50S Intermediate Precursor				Reconstitution
Protein	SK901	SK5665	p_1 50S	p_2 50S	RI50(1)
L1	+	+	+	+	+
L2	?	?	-	-	±
L3	-	+	+	+	+
L4	+	+	+	+	+
L5	+	+	+	+	+
L6	-	+	-	-	-
L7/12	+	+	±	+	+
L9	+	+	+	+	+
L10	+	+	+	+	+
L11	+	+	±	+	+
L13	±	+	+	+	±
L14	±	+	-	+	-
L15	+*	+*	-	+	+
L16	-	±	-	-	-
L17	-	+	+	+	+
L18	-	-	+	+	±
L19	-	-	-	+	-
L20	-	-	+	+	+
L21	-	-	+	+	+
L22	±	+	+	+	+
L23	-	±	+	+	+
L24	-	+	+	+	+
L25	±	+	+	+	-
L26	-	-	+	+	-
L27	-	-	+	+	-
L28	-	-	-	-	+
L29	-	±	+	+	-
L30	-	-	+	+	-
L31	?	?	-	-	-
L32	?	?	-	-	-

Proteins were either present (+), present in reduced amounts (±), absent (-) or not determined (?). Protein L15 (+*) was present in both 50S precursors from erythromycin treated cells.

Adapted from Traub et al. 1971; Nierhaus 1973.

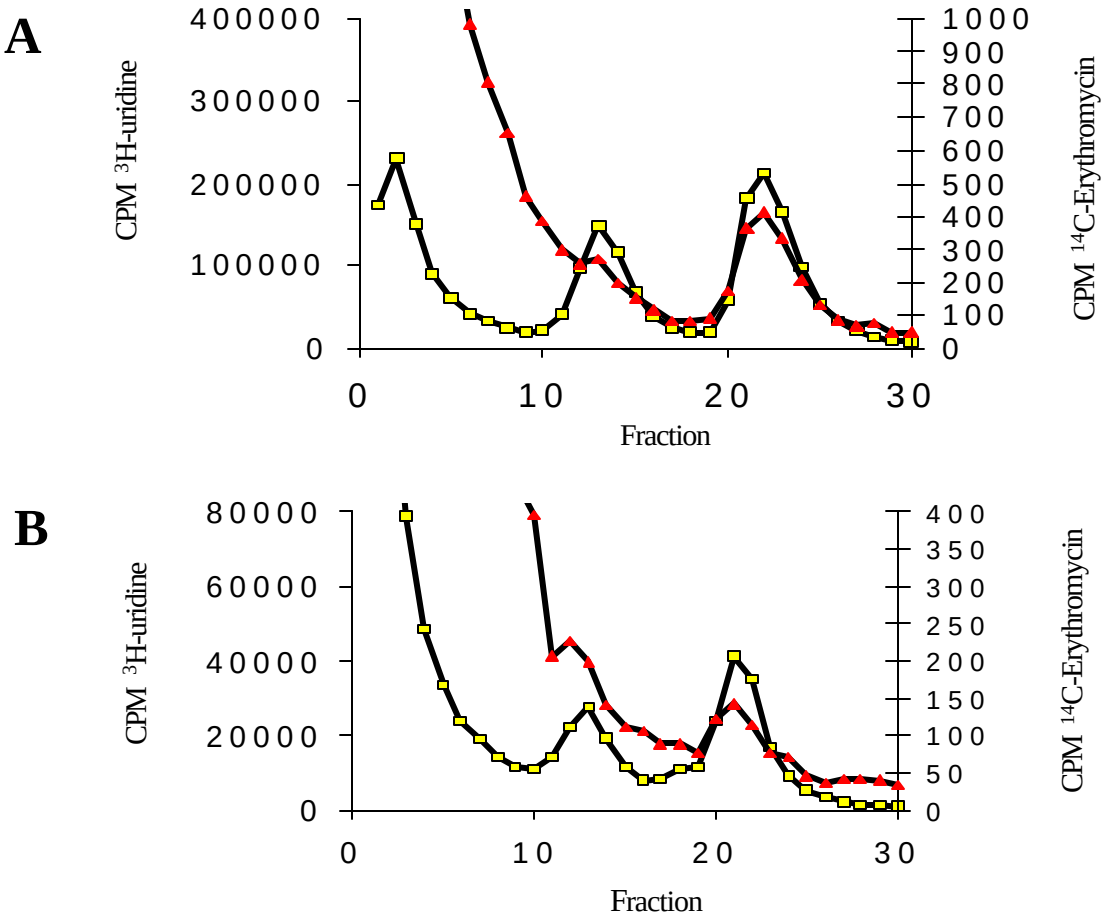
The protein composition of the stalled 50S assembly intermediate in strain SK901 was different than strain SK5665. Proteins L6, L17, and L24 were present in SK5665 but not found in SK901. Both of the stalled 50S assembly intermediates had a different protein composition than either the $p_1$50S (32S) or the $p_2$50S (42S) in vivo assembly intermediate particles that were identified by Nierhaus (1982) (see page 15). Proteins L20, L21, L26, L27 and L30 were present in both the in vivo assembly intermediate particles but were found in neither of the stalled 50S subunit assembly intermediates that accumulated in cells that were grown in the presence of erythromycin. Proteins L20, L21 and L28 were present in the first in vitro reconstitution intermediate but not in either of the stalled 50S assembly intermediates (see Figure 2).

Erythromycin Binding In Vivo

It was hypothesized that erythromycin associated with the 50S precursor particle to prevent assembly. This was tested directly by ^{14}C-erythromycin binding experiments in growing cells. These were combined with ^{3}H-uridine pulse-chase labeling to allow quantitation of 50S subunit assembly defects. In one set of experiments, ribosomal subunits were labeled by growing SK901 and SK5665 cells in the presence of ^{3}H-uridine. ^{14}C-erythromycin at 18 µg/ml was added to each strain and growth was continued for two population doublings. Cells were lysed, fractionated by sucrose gradient centrifugation, and the amount of each isotope present was determined for each gradient fraction. Both SK901 (Figure 43 A) and SK5665 (Figure 43 B) showed nearly normal 50S:30S ratios of steady-state labeled ribosomal subunits. ^{14}C-erythromycin was found in the 50S gradient regions as expected. However, there was another ^{14}C-erythromycin peak in the areas of the 30S subunits.

FIGURE 43. SUCROSE GRADIENT PROFILES OF SK901 AND SK5665 CELLS GROWN WITH ^{14}C-ERYTHROMYCIN AND ^{3}H-URIDINE AT 27°C.

Strains SK901(A) and SK5665 (B) showing ^{3}H uridine steady-state labeled ribosomal subunits (□) and ^{14}C-erythromycin (▲) bound to gradient fractions. The isotopes in the dual labeled gradient samples were measured separately by liquid scintillation counting.



To assess erythromycin binding over the course of a 45 minute chase period, pulse-chase experiments were done using both ^{3}H-uridine and ^{14}C-erythromycin at 18 µg/ml in strains SK901 and SK5665 grown in T broth at 27°C. Figure 44 shows the results from strain SK901.

The ^{3}H-uridine content (Figure 44 A) of each fraction showed a heterogenous particle

that formed in the 30S region over time. Increasing amounts of ^{14}C-erythromycin (Figure 44 B) were bound to those same gradient fractions.

In strain SK5665, most of the ^{3}H-uridine labeled rRNA (Figure 45 A) made during the pulse was found in particles that did not sediment beyond the 20-30S region. An erythromycin binding peak (Figure 45 B) was also found in the same fractions from the 20-30S region of these gradients.

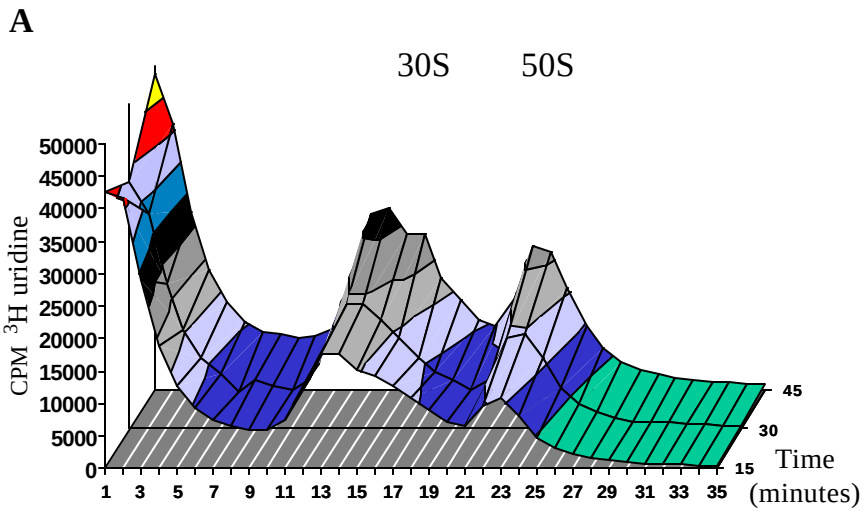
The effect of changes in erythromycin concentrations was also examined. Pulse-labeling experiments using a 30-minute pulse time were used to assess the effect of increasing concentrations of erythromycin on 50S subunit assembly and on erythromycin binding to 50S subunits and 50S subunit precursors. These were done using 12, 18, and 24 μg/ml of ^{14}C-erythromycin in strains SK901 and SK5665 grown in T broth at 27^{O}C.

Figure 46 shows that higher concentrations of erythromycin were associated with increased buildup of RNA containing particles (Figure 46 A) in the 30S gradient region of strain SK901 (Figure 46 A). The proportion of ^{14}C-erythromycin counts (Figure 46 B) bound to the 30S region also increased at higher erythromycin concentrations.

Figure 47 shows the results of similar experiments using SK5665. This strain was severely inhibited at the highest erythromycin concentration. ^{3}H-uridine counts (Figure 47 A) showed that nearly all the rRNA was incorporated into particles that did not sediment beyond the 30S region. Again, binding of ^{14}C-erythromycin (Figure 47 B) was seen in fractions from the 30S gradient region.

FIGURE 44. STRAIN SK901 COMPOSITE SUCROSE GRADIENT PROFILES OF ^{3}H-URIDINE AND ^{14}C-ERYTHROMYCIN BINDING OVER TIME.

Strain SK901 ^{3}H-uridine pulse-chase labeled ribosomal subunits (A) and counts per minute bound of ^{14}C-erythromycin (B) from the same gadient fractions. Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.



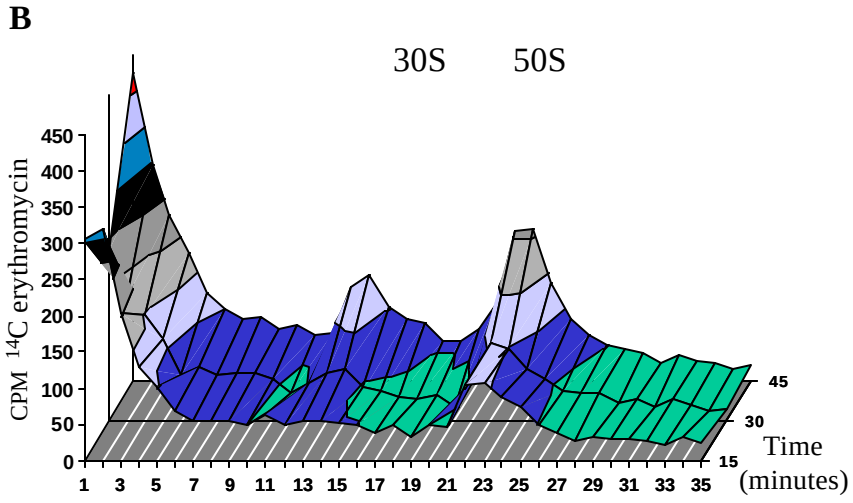
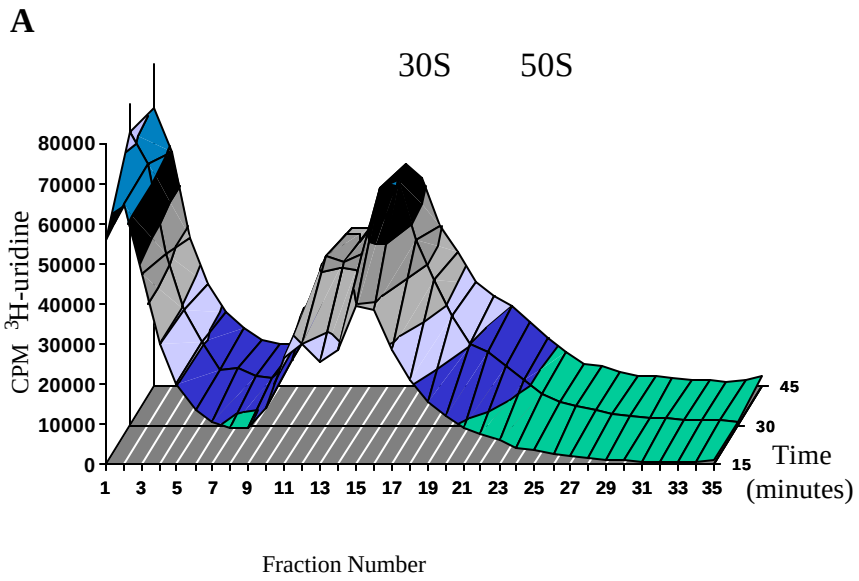


FIGURE 45. STRAIN SK5665 COMPOSITE SUCROSE GRADIENT PROFILES OF ^{3}H-URIDINE AND ^{14}C-ERYTHROMYCIN BINDING OVER TIME.

Strain SK5665 ^{3}H-uridine pulse-chase labeled subunits (A) and bound ^{14}C-erythromycin (B). Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.



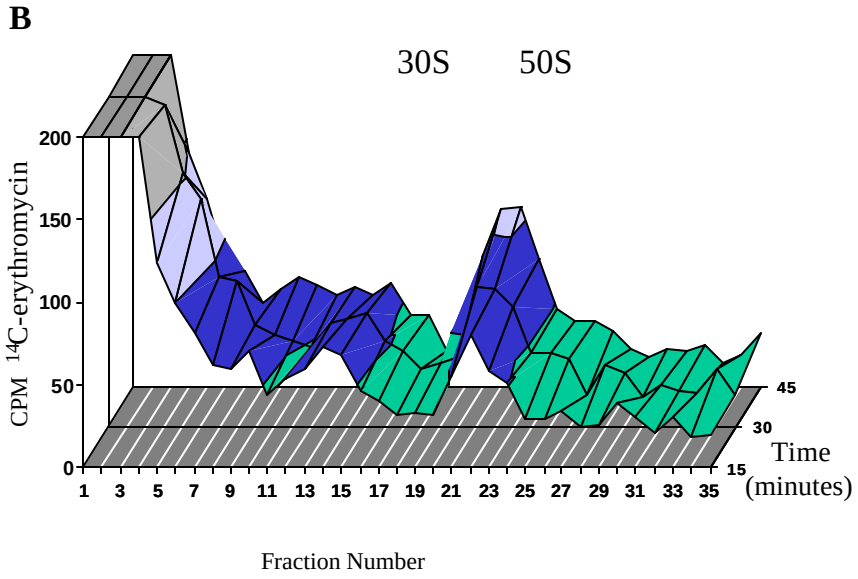
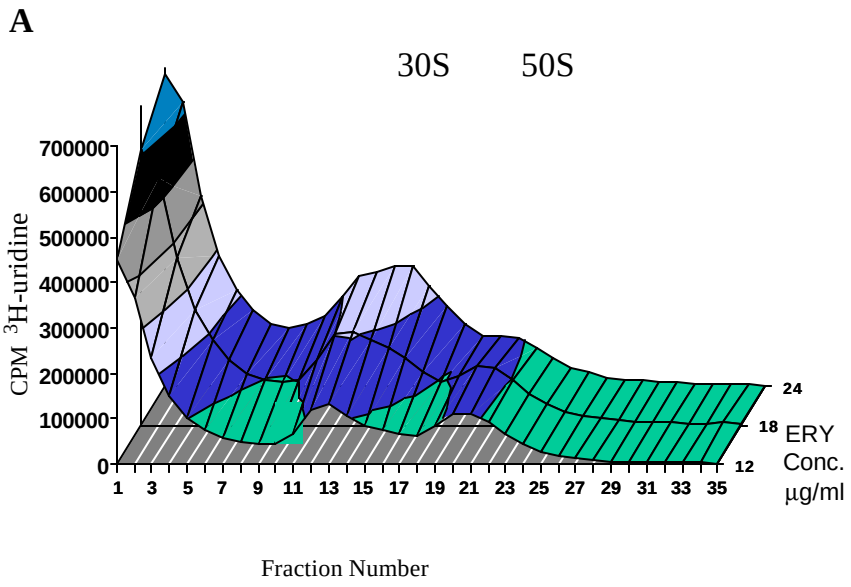


FIGURE 46. COMPOSITE OF SK901 PULSE-CHASE VS. ERYTHROMYCIN CONCENTRATION.

Sucrose gradient profiles of strain SK901 ^{3}H uridine pulse-chase labeled subunits (A) and bound ^{14}C-erythromycin (B). Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.



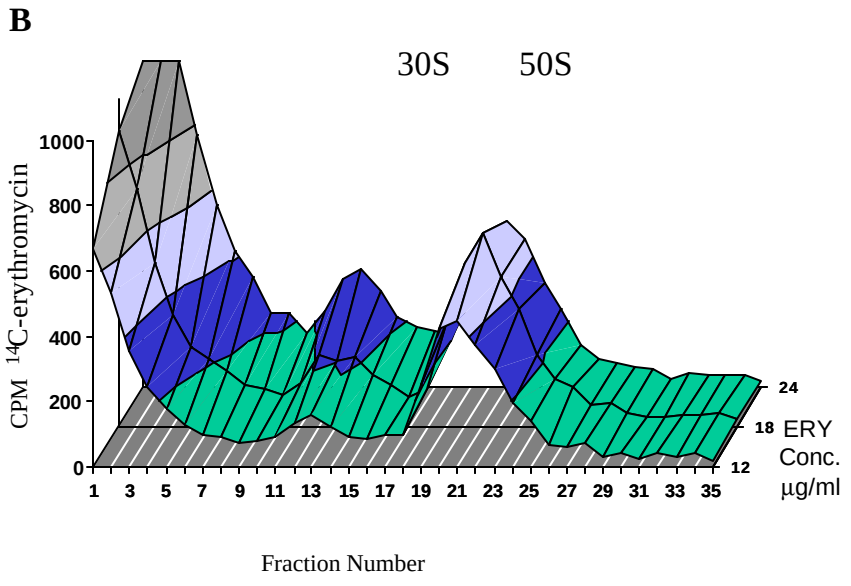
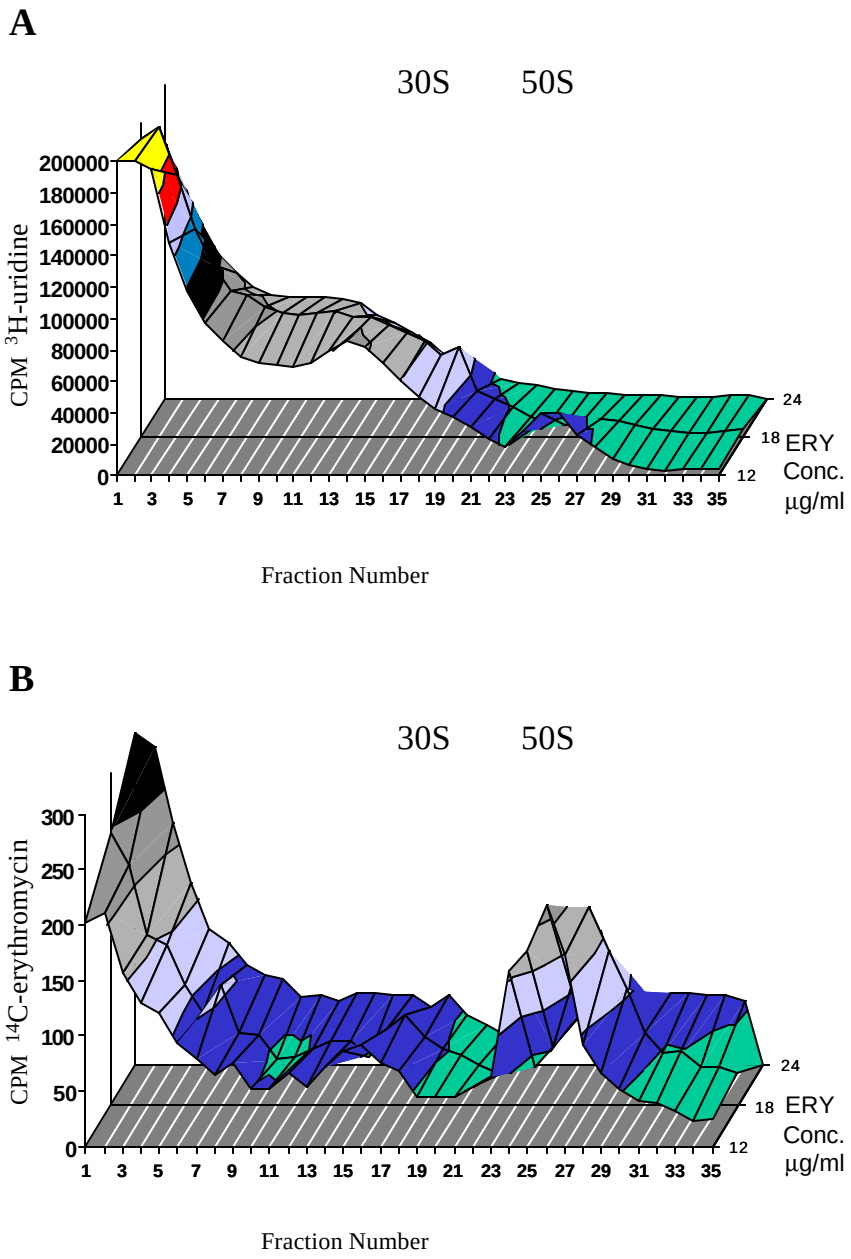


FIGURE 47. COMPOSITE OF SK5665 PULSE-CHASE VS. ERYTHROMYCIN CONCENTRATION.

Sucrose gradient profiles of strain SK5665 ^{3}H-uridine pulse-chase labeled subunits (A) and bound ^{14}C-erythromycin (B) counts per minute found in the same gradient fractions. Sucrose gradient profiles like those in Figure 22 were superimposed in this composite figure.



The data on erythromycin binding are correlated with the effects on 50S assembly (as ^{3}H uridine incorporation) in Table 8.

Table 8. Summary of ^{14}C-Erythromycin Binding In vivo vs. Time.

Strain	Time	Gradient Region	pmol Bound ERY	% of Total ^{3}H uridine CPM 30S	% of Total ^{3}H uridine CPM 50S	50S/30S Ratio
SK901	15 min. 30S	213	18.3	---	---	
SK901	30 min. 30S	226 ± 20	20.1	---	---	
SK901	45 min. 30S	462	21.5	---	---	
SK901	15 min. 50S	346	---	9.9	0.54	
SK901	30 min. 50S	332 ± 28	---	13.7	0.68	
SK901	45 min. 50S	656	---	15.2	0.71	
SK5665	15 min. 30S	248	22.5	---	---	
SK5665	30 min. 30S	249 ± 33	25.8	---	---	
SK5665	45 min. 30S	221	25.4	---	---	
SK5665	15 min. 50S	243	---	4.7	0.21	
SK5665	30 min. 50S	287 ± 13	---	6.6	0.26	
SK5665	45 min. 50S	340	---	7.6	0.29	

The influence of antibiotic concentration in both strains is shown in Table 9.

Table 9. Summary of ^{14}C-Erythromycin Binding In Vivo vs. Erythromycin Concentration.

Strain	Erythromycin	Gradient Region	pmol Bound ERY	% of Total ^{3}H-uridine CPM 30S	50S	50S/30S Ratio
SK901	12 µg/ml	30S	512	15.4	---	---
SK901	18 µg/ml	30S	799	17.2	---	---
SK901	24 µg/ml	30S	1,203	19.1	---	---
SK901	12 µg/ml	50S	1,496	---	13.8	0.9
SK901	18 µg/ml	50S	1,880	---	10.3	0.6
SK901	24 µg/ml	50S	1,678	---	7.7	0.4
SK5665	12 µg/ml	30S	325	14.4	---	---
SK5665	18 µg/ml	30S	213	15.5	---	---
SK5665	24 µg/ml	30S	254	18.5	---	---
SK5665	12 µg/ml	50S	725	---	6.0	0.4
SK5665	18 µg/ml	50S	532	---	2.0	0.1
SK5665	24 µg/ml	50S	246	---	0.9	0.05

Because of possible degradation of 23S rRNA in the 30S gradient region that might cause it to migrate in the gels along with 16S rRNA, 5S rRNA was used to more accurately quantitate the amounts of 50S precursor in the 30S gradient regions.

5S rRNA amounts were quantitated by densitometry scanning using a standard curve of known amounts run on a 7% polyacrylamide gel. These amounts were used to calculate 50S precursors and 50S subunits in gradient fractions and these results were compared with erythromycin binding in the same fractions.

Cells grown with ^{14}C-erythromycin [18μg/ml] were gradient fractionated as described. Half of each fraction was separated by electrophoresis and the amount of 5S rRNA in each fraction was quantitated. The amount of isotope in the remaining half of each fraction was determined by liquid scintillation counting. Results of these experiments are shown in Table 10. Both strains contained 5S rRNA in the 30S gradient region. Erythromycin bound to 5S rRNA containing particles in both the 30S gradient region and in the 50S region with an approximate 1:1 stoichiometry.

Table 10. 5S rRNA vs. ^{14}C-Erythromycin Binding Stoichiometry.

Strain	Gradient Region	pmol 5S rRNA	pmol Bound ^{14}C ERY	Ratio of ERY / 5S rRNA
SK901	30S	150	190	1.2
SK901	50S	216	187	0.8
SK5665	30S	212	242	1.1
SK5665	50S	211	248	1.2

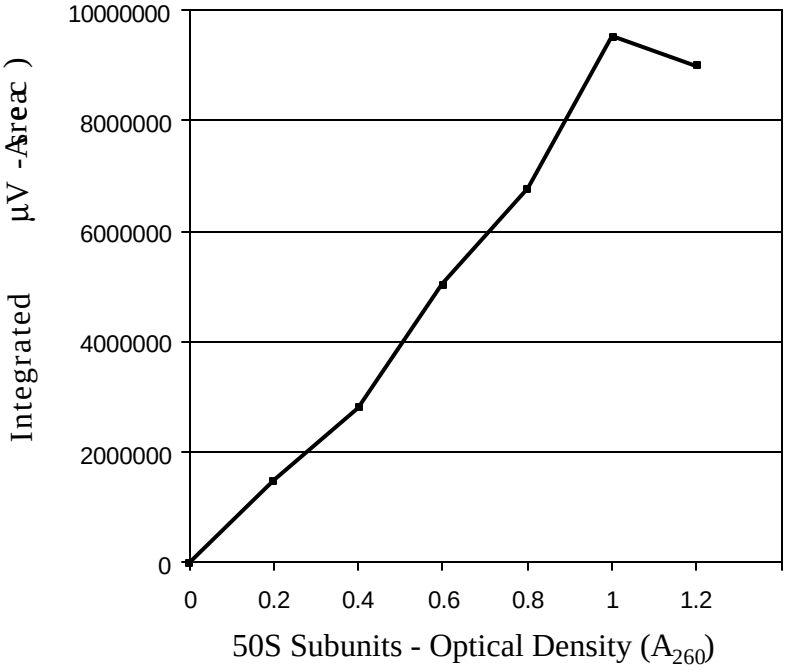
Erythromycin Binding In Vitro

Erythromycin binding to the 50S subunit precursor was compared to erythromycin binding to isolated 50S subunits to further test the binding stoichiometry. The stoichiometry was measured by standard curves relating optical density to the areas of the subunit peaks captured on Dynamax software

Isolated 50S subunits were used to determine the relationship between the quantity of subunit particles and the area under the curves obtained from Dynamax integration software and the results are shown in Figure 48.

FIGURE 48. 50S RIBOSOMAL SUBUNIT QUANTITATION USING INTEGRATED AREA FROM SUCROSE GRADIENT PROFILES.

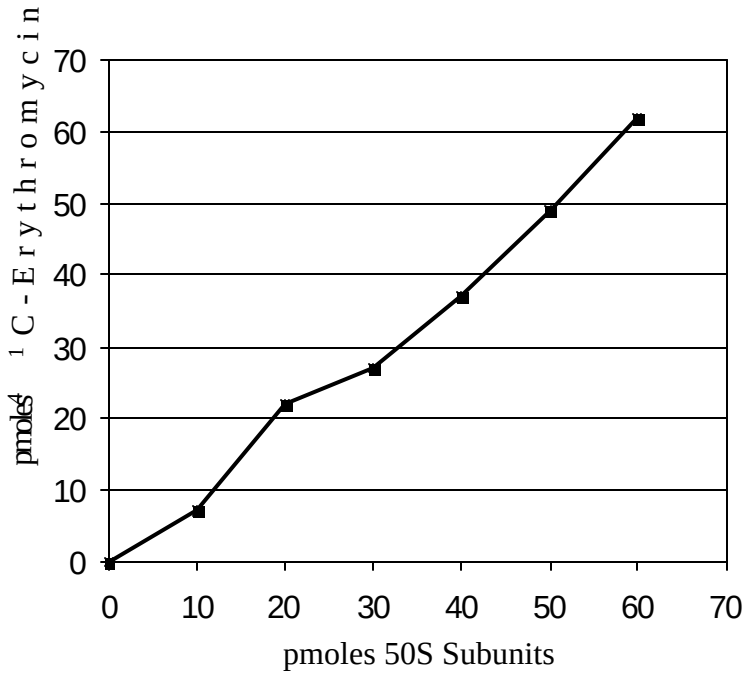
Isolated 50S subunits were quantitated by optical density and centrifuged on sucrose gradients using the same method described for cell lysates. Gradient profiles were captured using Dynamax integration software and the areas of the 50S peaks were recorded.



The relationship between the Dynamax integrated area and 50S subunits (OD) was determined to be: area = 8,214,013(OD) + 19,601. This equation allowed a determination of the amount of 50S subunits present in sucrose gradients using the captured Dynamax profiles. One optical density unit of 50S subunits at 260 nm = 44 pmol/ml. Figure 49 shows the results of filter binding assays testing the in vitro binding of erythromycin to isolated 50S subunits.

FIGURE 49. IN VITRO BINDING OF ^{14}C-ERYTHROMYCIN TO ISOLATED 50S SUBUNITS.

Different amounts of 50S subunits were incubated at 37°C for 20 minutes with 0.1 µCi of ^{14}C-erythromycin. The 50S subunits in each sample were captured on GF/A filters, rinsed with 3 ml of S-buffer and the CPM of bound ^{14}C-erythromycin was determined by liquid scintillation counting.



Filter binding assays using ^{14}C-erythromycin were performed on isolated ribosomal subunit samples. They showed a stoichiometric 1:1 binding ratio between erythromycin and 50S subunit molecules. No binding above background levels was seen between erythromycin and 30S subunits (data not shown).

To test precursor binding stoichiometry in vitro, strain SK901 was grown in T broth at 37°C, with and without unlabeled erythromycin. Strain SK5665 was examined similarly but using 18 μg/ml of erythromycin. Sucrose gradient fractions corresponding to the 30S-40S and the 50S peaks were pooled, dialyzed and incubated with ^{14}C-erythromycin as described. Filter binding assays were used to determine the amount of isotope that bound to each gradient pool and the results are shown in Table 11. Precursor particles recovered from erythromycin inhibited cells bound as much erythromycin as 50S subunits, showing a similar 1:1 binding stoichiometry.

Table 11. In vitro Erythromycin Binding to Pooled Gradient Fractions.

Strain	Pooled Gradient Peak	Cells grown in unlabeled [ERY]	Recovered pmol rRNA	Bound pmol ^{14}C ERY	RNA/ ERY Ratio
SK901	30S-40S	None	9.9	0.5	0.05
SK901	50S	None	8.8	6.5	0.74
SK901	30S-40S	18 μg/ml	7.9-13.7	4.0	0.3-0.5
SK901	50S	18 μg/ml	5.7	3.5	0.61
SK5665	30S-40S	None	12.2	0.6	0.05
SK5665	50S	None	9.7	6.2	0.64
SK5665	30S-40S	18 μg/ml	8.8-15.2	5.1	0.3-0.6
SK5665	50S	18 μg/ml	4.8	3.0	0.63

Cells grown without antibiotic yielded 30S-40S pools that did not bind as much ^{14}C-erythromycin in vitro as the 50S pools did. When cells were grown in the presence of unlabeled erythromycin, both the 30S-40S and the 50S pools contained molecules that bound to ^{14}C-erythromycin in in vitro experiments.

The exact amount of pmoles of rRNA in the 30S-40S gradient region could not be determined because our data has shown this area to contain both 30S and precursor 50S subunits. One optical density unit of 30S subunits at 260 nm = 76 pmol/ml and one optical density unit of 50S subunits at 260 nm = 44 pmol/ml, so a range of values is given. However, excess binding of ^{14}C-erythromycin to 30S gradient pools was observed in the samples that had been grown in the presence of erythromycin compared to the 30S gradient pools from cells grown without antibiotic.

Reconstitution of Ribosomal Subunits

An attempt was made to examine the effect of antibiotics on ribosomal subunit formation in vitro, using the method of subunit reconstitution. Normal 30S and 50S particles were formed under standard reconstitution conditions.

Additionally, predicted reconstitution intermediate particles were isolated from 30S and 50S reconstitution reactions when the reaction was kept at 0^{o}C. The in vitro reconstitution of 30S subunits was not inhibited by the presence of any of the antibiotics tested.

The in vitro reconstitution using 50S subunits showed no inhibition in the presence of erythromycin or of three other macrolide antibiotics tested (see Figure 50). This is in contrast to the demonstrated in vivo assembly effect of these antibiotics on 50S subunits.

A number of antibiotics were tested for effects on both 30S and 50S subunit reconstitution. The data are shown in Tables 12 and 13.

FIGURE 50. RECONSTITUTION OF 50S RIBOSOMAL SUBUNITS.

Total rRNA and 50S subunit proteins from MRE600 were used to reconstitute 50S subunits in vitro. Reconstitutions done with no antibiotic (A) or with erythromycin at a 10 fold molar excess over the amount of rRNA (B) yielded similar results when analyzed by sucrose gradient sedimentation and compared to standards (C).

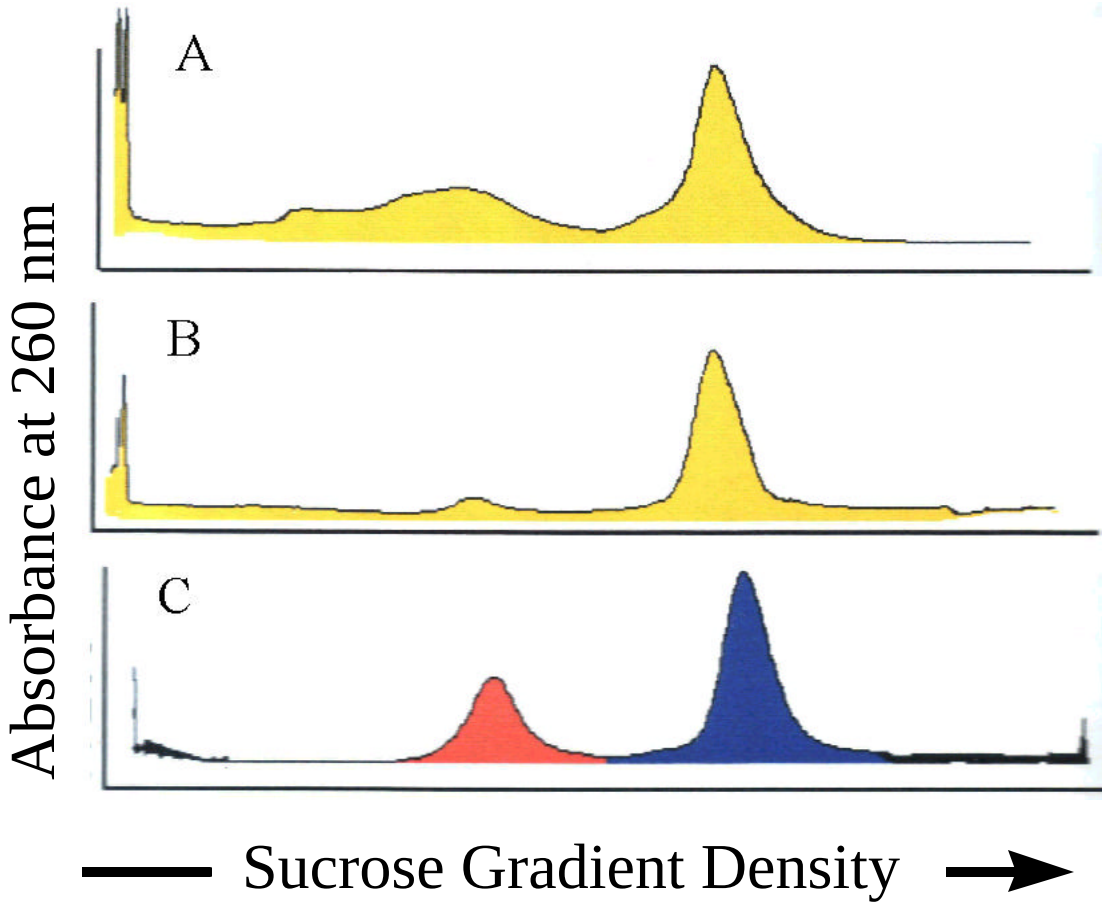


Table 12. Antibiotics Used in 30S Subunit Reconstitutions.

Reconstitutions were done in the presence of the antibiotics listed by adding rRNA, antibiotic, and then proteins to each reaction. Inhibition of assembly of the 30S subunit was not observed under these reconstitution conditions.

Antibiotic	Subunit Normally Affected in Translation	Molar Ratio Drug/RNA	Effect on 30S Reconstitution
Erythromycin	50S	1X	None
Chloramphenicol	50S	1X	None
Lincomycin	50S	1X	None
Puromycin	50S	1X	None
Spiramycin	50S	1X	None
Thiostrepton	50S	1X	None
Geneticin	30S	1X	None
Gentamycin A	30S	1X	None
Hygromycin B	30S	1X	None
Canamycin Sulfate	30S	1X	None
Neamine	30S	1X	None
Neomycin	30S	1X	None
Spectinomycin	30S	1X	None
Streptomycin	30S	1X	None
Tetracycline	30S	1X	None
Tobramycin	30S	1X	None

Table 13. Antibiotics Used in 50S Subunit Reconstitutions.

Reconstitutions were done in the presence of the antibiotics listed by adding rRNA, antibiotic, and then proteins to each reaction. In addition, erythromycin was tested by adding the antibiotic to the reaction first and also by incubating the rRNA with the antibiotic at 37°C for 15 minutes prior to adding the proteins. Inhibition of assembly of either ribosomal subunit was not observed under these reconstitution conditions.

Antibiotic	Subunit Normally Affected in Translation	Molar Ratio Drug/rRNA	Effect on 50S Reconstitution
Erythromycin	50S	1X	None
Azithromycin	50S	1X	None
Clarithromycin	50S	1X	None
Oleandomycin	50S	1X	None
Erythromycin	50S	10X	None
Azithromycin	50S	10X	None
Clarithromycin	50S	10X	None
Oleandomycin	50S	10X	None

CHAPTER 4
DISCUSSION

This inquiry into the nature of the effects of erythromycin and related macrolides on E. coli cells has lead to the identification and partial characterization of the 50S ribosomal subunit intermediate particle that accumulates as a result of assembly inhibition of the 50S subunit by erythromycin.

Prior Studies

Erythromycin and related antibiotics have been shown to inhibit 50S subunit function in translation and its mechanism has been well investigated (Corcoran 1984), with regard to the erythromycin binding site and mechanism of action. Much less is known about macrolide effects on 50S subunit assembly. Macrolide antibiotics have been shown to inhibit 50S subunit assembly in E. coli (Chittum and Champney 1995), S. aureus and B. subtilis (Champney and Burdine 1998) cells.

Recent studies (Champney 1999) have compared the effects of macrolides on the growth of E. coli and S. aureus cells. Azithromycin was a potent inhibitor of 50S assembly in both cell types at low concentrations. Lower erythromycin concentrations were needed to inhibit 50S assembly in S. aureus than in E. coli . Oleandomycin, the least effective of these antibiotics, caused only a small amount of 50S assembly inhibition in S. aureus and almost none in E. coli when used at high concentrations. With the exception of erythromycin being more effective against S. aureus, these data on E. coli are consistent with our results from assays on growth and in vivo protein synthesis. Another macrolide, clarithromycin, was found to be more effective than erythromycin at inhibiting 50S subunit assembly and had a greater effect on E. coli than S. aureus cells.

Structural differences among these antibiotics are the most likely reason for their differences in effects on growing cells (see Figure 6). Oleandomycin is a naturally occurring

derivative of erythromycin, possessing a similar 14-membered macrolactone ring, but with an epoxy group at C8 instead of the methyl group (Nakagawa and Omura, 1984). Azithromycin has a semi-synthetic 15-membered ring in which a ketone group has been replaced by a methylated nitrogen. This modification reduces the susceptibility of the azithromycin ring to acid hydrolysis (Kirst and Sides 1989; Kirst 1993). Another acid stable macrolide, clarithromycin, is a 6 methoxy derivative of erythromycin (Whitman and Tunkel 1992).

Studies on the relationship between the structure and function of several macrolide and related ketolide antibiotics in S. aureus showed that the macrolides roxithromycin and clarithromycin inhibited protein synthesis to a greater extent than they inhibited 50S subunit assembly. A derivative of clarithromycin (11,12-carbonate-3 deoxy-clarithromycin) was found to have the inverse effect, inhibiting 50S subunit assembly more than protein synthesis (Champney and Tober 1999). Other macrolides with specific structure changes are currently being examined to more precisely define the features responsible for their activities.

The ketolides are a second generation of macrolides in which the cladinose sugar at C3 of the ring has been replaced by a ketone group (Nakagawa and Omura 1984). Eleven of these compounds were found to be potent inhibitors of growth and 50S subunit assembly, even in an ermC erythromycin-resistant strain of S. aureus, suggesting that the ketolides are not inducers of the methylase gene expression (Champney and Tober 1998).

Recently, assembly inhibition of the 50S subunit has been reported in S. aureus cells by 16-membered macrolides, lincomycin and clindamycin, streptogramin B (but not streptogramin A), as well as by a polysaccharide antibiotic evernimicin (Champney and Tober 2000 A; Champney and Tober 2000 B).

Recovery from Erythromycin Effects

Recovery from the growth inhibiting effects of erythromycin varied between the strains tested. The RNase E^- mutant SK5665 took 45 minutes to resume logarithmic growth after removal of the antibiotic, compared to 30 minutes required by the wild type. This suggests that

RNase E mediated rRNA turnover may be important in overcoming macrolide effects. The RNase 1$^-$ mutant MRE600 required 2 hours to resume logarithmic growth. This was surprising, because RNase 1 is not recognized as an enzyme involved in the mediation of ribosomal assembly or turnover (Altman et al 1982). Erythromycin hypersensitivity in MRE600 might either be due to stronger antibiotic binding to the ribosomes in this strain, or due to differences in erythromycin uptake or loss rates.

The temperature sensitive L15 mutants SK1217 and SK1219 did not resume any measurable growth after the removal of the antibiotic. This may reflect either a tight binding of the drug to the ribosome or an unknown activity of the antibiotic that rendered these mutant ribosomes non-functional. Although the nature of the L15 gene to L15 protein mutation in these strains has not been well characterized (Champney 1979). L15 is an important erythromycin binding protein in 50S subunits (Teraoka and Nierhaus 1978).

Uptake and Efflux of Erythromycin

Entry of the antibiotic into cells is a prerequisite for directly affecting ribosomes. (Farmer, Li, and Hancock 1992). In the antibiotic uptake experiments using ^{14}C-erythromycin in growing cells, it was anticipated that uptake would be gradual. Instead, the first sample taken at time zero immediately after addition of the isotope contained the highest amount of radioactivity. After about five minutes, an equilibrium was reached and the amount of isotope in subsequent samples stabilized. One explanation for these observations is that the erythromycin entered the cells rapidly and then began to be actively transported out of them.

Further evidence for the rapid uptake of erythromycin in static cells was obtained both by increasing the amount of unlabeled antibiotic and by adding the unlabeled erythromycin 5 seconds after the isotope. In each instance, the uptake of isotope was slowed.

The erythromycin loss experiments showed a pattern similar to the uptake experiments. Cells grown in labeled erythromycin lost half the isotope within 5 minutes of resuspension in fresh T broth. By about 15 minutes, the counts had stabilized and this may represent

erythromycin that was bound to ribosomes and not available as a substrate for export. Strain MRE600 was hypersensitive to erythromycin and would not grow in T broth at antibiotic concentrations above 10 μg/ml. This may be related to its reduced capacity for efflux of the antibiotic compared to the other strains. Because of incomplete characterization of the mutations in strains MRE600, SK1217, and SK1219, strains SK901 and SK5665 were selected for further study and analysis.

Pulse-Chase Analysis

Previous work in our lab had suggested that accumulation and turnover of stalled 50S subunit precursor particles occurred in erythromycin-treated cells (for a review, see Champney 1999). These investigations used a pulse-chase labeling method to study the kinetic processes of ribosomal subunit assembly.

In order to determine whether stalled 50S assembly intermediates or fragments of degraded 50S subunits accumulated in erythromycin treated cells, pulse-chase assays were performed. The pulse-chase assays revealed decreased rates of assembly of 50S subunits in both strains tested as well as varying levels of accumulation of a stalled 50S precursor assembly intermediate in the 30S gradient region of erythromycin-treated cells. Dynamic analysis of the pulse-chase assays over time indicated that the stalled 50S precursor accumulated to a greater degree in the RNase E^- mutant SK5665 than in the wild type SK901. Reduction of the growth temperature from 37°C to 27°C resulted in slowed ribosomal subunit assembly in both strains and resulted in an increase in 50S precursor relative to the amount of fully formed 50S subunits that were assembled during the chase period.

RNA Analysis

To further characterize the 50S precursor particle(s), gradient fractions were assessed for both rRNA and protein content. Fluorograms from gel electrophoresis of rRNA from cells grown with erythromycin revealed 23S and 5S rRNA species within the 30S gradient region. Attempts to quantitate the precursor particle based on the amount of 23S rRNA were impeded by RNA breakdown during cell lysis and during handling of the samples. Cleavage of the 23S rRNA at a sensitive site near its center often results in a pair of nucleotides that migrate on gels along with the 16S rRNA (Winkler 1979). Gel electrophoresis and fluorography of cells grown with ^{3}H uridine showed that top fractions from antibiotic treated cells contained much more RNA than did the non- antibiotic treated cultures. This RNA was not present as intact 23S rRNA, but as smaller oligonucleotides. Champney and Burdine (1996) found that the inhibition of 50S subunit assembly was directly proportional to accumulation of oligoribonucleotides in erythromycin treated S. aureus cells.

Slot blot hybridization experiments confirmed that the both the top fractions and fractions from the 30S gradient region contained more 23S rRNA than did those same fractions from cells grown without antibiotics. Previous experiments (Champney and Burdine 1998) in S. aureus cells had revealed a direct relationship between loss of rRNA from 50S subunits and its accumulation as oligoribonucleotides in macrolide-treated cells.

Fractions at the top of the sucrose gradients did not contain appreciable amounts of free ribosomal proteins. This is not surprising, because ribosomal protein synthesis is highly regulated and pools of free ribosomal proteins are not maintained in E. coli (Gupta and Singh 1972; Marvaldi et al. 1974). Overexpression of ribosomal proteins has been shown to be highly toxic in these cells (Fallon et al. 1979).

Although the gradient positions of the 30S subunit and the 50S assembly intermediates overlapped, we were able to distinguish one from the other based on 23S rRNA hybridization, the presence of 5S rRNA and their unique protein compositions. Hybridization of a 23S rRNA-specific probe to gradient fractions using a slot blot technique was a more specific

method for differentiating 23S rRNA in 50S precursor particles from that of 16S rRNA in 30S subunits.

Role of RNase E

Strain SK5665 has a mutant ribonuclease E enzyme that is inactivated at reduced or elevated temperatures (Kushner, et al. 1993). RNase E processes 9S pre-rRNA into mature 5S rRNA and controls the degradation of E. coli mRNAs. In these cells, RNase E is part of a multi-component complex called the degradosome that includes polynucleotide phosphorylase along with several other enzymes (Miczak et al. 1996; Coburn and Mackie 1998). When RNA oligonucleotides from the degradosome were examined (Bessarab et al. 1998), they were found to be primarily fragments of 16S and 23S rRNA. This indicates that the RNase E-containing degradosome mediates the turnover of mature rRNAs as well as mRNAs.

At 27°C, strain SK5665 accumulated nearly twice as much 50S precursor than at 37°C. This accumulation may be due to the inactivation of the temperature-sensitive ribonuclease E. The loss of this endonuclease may impair the normal degradation and turnover of the stalled 50S assembly intermediates.

Protein Analysis

Analysis of the protein compositions of the 50S assembly intermediates was done to determine whether the particle that serves as a substrate for erythromycin might be similar to $p_1$50S, $p_2$50S, or RI50(1) 50S precursors that have been described (Nierhaus, et al 1973; Dohme and Nierhaus 1976). Each of these particles contain 23S and 5S rRNAs but their protein compositions are different (see Table 7).

Our results for the presence of early assembly proteins are consistent with the particles previously described except for the absence of L3 in SK901 and the presence of L6 in SK5665. The protein L2 may have been present, but the spot believed to be L2 was misidentified in our gels. More proteins were found in the SK5665 precursor than the one from SK901. The SK5665 50S precursor tended to sediment into the 25S-30S gradient region whereas the SK901 50S precursor was more often found in the 30S-40S region. Because the higher molecular weight SK5665 particle was less dense, it may reflect an abnormal conformation of the assembling subunit. The 50S assembly intermediate may have been more stable in the absence of RNase E.

Photoaffinity labeling experiments have been performed (Tejedor and Ballesta 1985) to identify the proteins associated with the erythromycin binding site of the 50S subunit. Labeled photoreactive erythromycin was bound to intact 50S subunits and proteins L2, L27, and L28 covalently bound to the antibiotic upon irradiation with UV light. The presence of protein L2 was not determined in our experiments. Protein L27 was not found in the 50S precursor particle in either strain SK901 or SK5665 but was reported in both the $p_1$50S and $p_2$50S in vivo precursors. Protein L28 was not present in any of the in vivo 50S assembly particles, including the ones isolated from cells grown with erythromycin. L28 was present, however, in the RI50(1) in vitro reconstitution intermediate.

Additional photoaffinity labeling (Arevalo et al. 1988) found that L22 and L15 also reacted with the labeled erythromycin. The location of these proteins on the subunit supports the model of erythromycin acting at the peptidyl transferase P site. Protein L15 was found in the 50S intermediate assembly precursors of both SK901 and SK5665 strains grown in the presence of erythromycin. Protein L15 was not present in the first in vivo assembly intermediate ($p_1$50S) but was found in the second in vivo assembly intermediate ($p_2$50S).

When Nierhaus and Teraoka (1978) tested isolated single ribosomal proteins for erythromycin binding ability, only L15 was able to bind the antibiotic. Although L15 and L22 were present in the assembly intermediates characterized in this work, L27 and L28 were not. These results suggest that the binding site for assembly inhibition may not contain the same

protein subset as does the binding site for translation inhibition in the fully formed 50S subunit. If the antibiotic binds L15 of the assembling subunit, it may sterically interfere with the addition of other proteins to that area (Franceschi and Nierhaus 1990). Alternatively, erythromycin may bind to the assembling 50S subunit at a novel site that exists only transiently and its potential to bind there may be lost upon the addition of subsequent proteins.

Erythromycin rapidly and non-covalently binds E. coli 50S ribosomal subunits with a 1:1 stoichiometry (Tanaka and Teroka 1966; Mao and Putterman 1969). The binding is very stable (1.0×10^{-8}M at 24°C) and it is specific for the 50S but not the 30S subunit. The presence of ^{14}C-erythromycin bound to particles that sedimented along with the 30S subunit is evidence that the cells contained appreciable amounts of incompletely or improperly assembled 50S subunits. The stoichiometry of erythromycin binding to the 50S subunit precursor was also found to be 1:1 (see Table 10). Because of 23S rRNA deterioration problems, analysis of the 5S rRNA was done and, labeled components of gradient fractions showed an approximate 1:1 binding ratio between the 50S assembly intermediate precursor and the antibiotic.

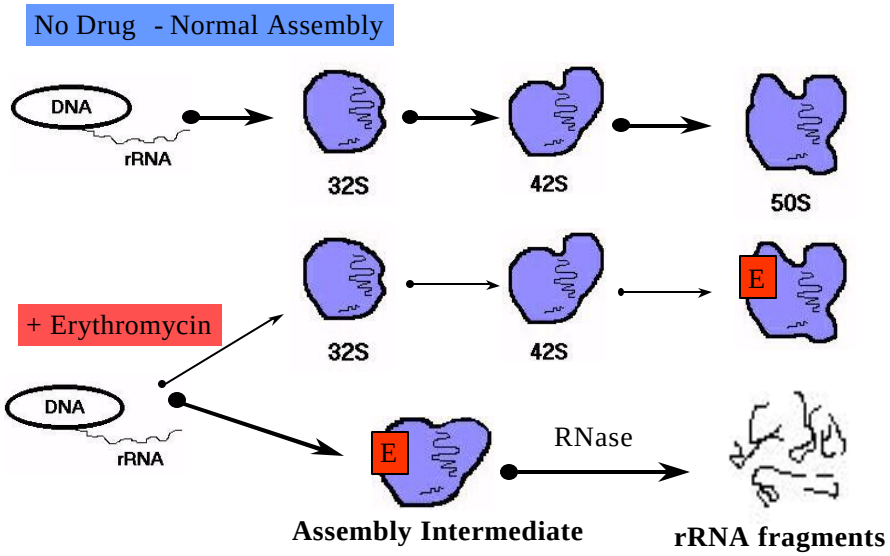
Pulse-chase gradient analysis of strain SK901 using ^{14}C-erythromycin revealed increasing amounts of isotope binding over time to 50S subunits as well as to particles that were only dense enough to sediment near the 30S gradient region. Strain SK5665 showed enhanced isotope binding over time in the same gradient fractions.

Similar pulse-chase experiments using variable concentrations of ^{14}C-erythromycin showed increased amounts of 50S precursor accumulation in the 30S region that bound increasing amounts of ^{14}C-erythromycin. However, these concentrations of erythromycin were not sufficient to produce differences in the amount of assembly of normally sedimenting 50S subunits in SK901.

Strain SK5665 had large amounts of erythromycin-binding 50S precursor in the 30S region at all three antibiotic concentrations. The assembly of fully-formed 50S subunits in SK5665 decreased dramatically as the antibiotic concentration increased. These differences in subunit assembly likely are a reflection of the difference in erythromycin sensitivity between these strains, presumably related to the function of RNase E.

In vitro analysis of erythromycin binding to particles recovered from sucrose gradients showed that after dialysis to remove the erythromycin, particles from the 30S and 50S gradient regions were able to bind erythromycin by filter binding assay (Table 11).

FIGURE 51. MODEL OF 50S SUBUNIT ASSEMBLY INHIBITION BY ERYTHROMYCIN.



Based on the results from this work, we propose a model of the 50S assembly inhibition process as shown in Figure 51. Assembly of the 50S subunit without the antibiotic present proceeds through two normal intermediate precursors that contain different protein subsets and both the 23S and 5S rRNAs (Nierhaus et al. 1973). In the presence of erythromycin, a portion of the forming 50S subunits escape assembly inhibition and become normal 50S subunits. These are then capable of binding to erythromycin and being affected in translation. According to our model, a subset of the developing 50S subunits bind to erythromycin and fail to become fully formed. These stalled assembly intermediates then become substrates for ribonucleases like RNase E which degrade the particle.

Several of the results obtained from these investigations lend support to the hypothesis stated in the introduction. The 23S and 5S rRNA species were found in the 50S assembly intermediate precursor particle (see Figure 37, Figure 38, and Table 10). 50S ribosomal proteins were found in the 50S precursor (see Figure 41 and Figure 42). The 50S precursor bound to erythromycin with the same stoichiometry as fully formed 50S subunits (see Table 9, Table 10, and Table 11). In addition, increased build up of the 50S precursor was seen in an RNase E mutant (see Figure 21, Figure 27, and Figure 29).

The portion of the hypothesis which stated that the in vivo assembly inhibition effect would be observed in an in vitro reconstitution assay was refuted. Reconstitution of ribosomal subunits in vitro was successful despite the presence of antibiotics. Properly sedimenting 30S and 50S subunits were reconstituted from their nucleic acid and protein components using methods modified from Dohme and Nierhaus (1976) and Amils et al., (1979). The reconstitution methods used different ionic conditions and temperatures than are required by cells for assembly of subunits. In addition, in vivo assembly of ribosomal subunits begins before the rRNAs are completely processed by nucleases or are methylated.

Future investigations should include the examination of 50S assembly inhibition in other organisms, particularly in potential pathogens such as Staphylococcus and Streptococcus strains. Isolation and characterization of mutant strains that are either resistant or hypersensitive to the 50S assembly inhibition effects of the macrolides will also prove useful in elucidating the molecular processes involved. In addition, examination of the erythromycin binding affinity and kinetics of the intermediate precursor particle will prove insightful. Techniques such as isothermal titration calorimitry can provide rapid and accurate binding assays requiring only minute amounts of the labile and difficult to isolate precursor particles.

This research involved characterization of a process that has applications for development of more effective antimicrobial compounds. Understanding the biochemical interactions responsible for antibiotic actions is an important prerequisite to deciding which modifications in antibiotic structure might enhance their activities. Further research is needed to characterize the nature of the structure-function relationships responsible for antibiotic activity. The information obtained may lead to the development of a new arsenal of rationally designed

antibiotics, ones capable of meeting the challenge of emerging antibiotic resistance. In contrast to the bacteriostatic effect of inhibiting protein synthesis, an antibiotic that binds preferentially to the 50S precursor could prove to be a highly bactericidal compound effectively halting assembly of ribosomes in the targeted cells.

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