AN ABSTRACT OF THE THESIS OF

Cynthia Sue Thompson for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on May 3, 2000. Title: Oligoribonucleotides Directed at Ribosomal Protein Binding Sites as Inhibitors of Ribosomal Small Subunit Assembly and as Possible Antibiotics.

Redacted for privacy

Abstract approved: _____

Christopher K. Mathews

The effects of oligoribonucleotides of identical or similar sequences to a ribosomal protein binding site, specifically S8, on prokaryotic 30S ribosomal assembly were investigated in *Escherichia coli*. The oligoribonucleotides were expected to bind to the target protein S8 and block its incorporation into the 30S subunit, resulting in a non-functional particle. 30S assembly has been proposed to occur by similar pathways both *in vivo* and *in vitro*. If this block in assembly by the oligoribonucleotides occurs *in vitro*, it would also occur *in vivo* and could possibly lead to new modes of antibiotic therapy.

The binding site of S8 was delivered into *E. coli* through a phagemid delivery system. The S8 binding site was cloned into a PBS+ vector under control of a *lacZ* promoter. The same phagemid with the ribosomal binding site of the *lacZ* gene deleted (the Shine-Dalgarno sequence and translational start site deleted) was also cloned and both transformed into *E. coli*. Growth experiments were performed in the presence and absence of the inducer isopropylthiogalactoside (IPTG). No change in growth upon induction of the phagemids was observed. Northern blot analysis was performed on cell

lysates. By using a probe complementary to the S8 binding site and a probe 3' to the cloned S8 binding site, specific to the *lacZ* message, S8 binding site message from PBS-S8 was observed but not in amounts comparable to those of 16S rRNA. The phagemid with the deleted ribosomal binding site produced no message corresponding to either the S8 binding site or *lacZ* mRNA, possibly due to endonucleolytic attack upon the message.

Omission of protein S8 in an *in vitro* reconstitution reaction results in subunits that show an 87-98 % reduction in phenylalanine incorporation in the polyphenylalanine synthesis assay. This severe reduction of function was also used to test the hypothesis. Reconstitutions were performed in the presence of three unmodified oligoribonucleotides (CT4, CT10, CT11) identical or similar in sequence to the S8 binding site and assayed. None of the oligoribonucleotides showed a reduction of function when added at up to a 10:1 ratio of oligoribonucleotide to protein. CT11 was assayed to a 20:1 ratio of oligoribonucleotide to 30S proteins (TP30) and showed a 20 % decrease of function at that ratio. Filter-binding assays were performed to test whether the oligoribonucleotides were binding to a protein in the TP30 mixture. The oligoribonucleotides bind protein but at much higher protein concentrations than 16S rRNA, signifying a lower affinity for the oligoribonucleotides. These data suggest that the oligoribonucleotides are binding to a protein but not with high enough affinity to compete with 16S rRNA. The concentration needed to be affective as an antibiotic would be too great to be practical.

Oligoribonucleotides Directed at Ribosomal Protein Binding Sites as Inhibitors of Ribosomal Small Subunit Assembly and as Possible Antibiotics.

By

Cynthia Sue Thompson

A THESIS

Submitted to

Oregon State University

In partial fulfillment of
The requirements for the
Degree of

Doctor of Philosophy

Presented May 3, 2000
Commencement June 2000

Doctor of Philosophy thesis of Cynthia Sue Thompson presented on May 3, 2000

APPROVED:

Redacted for privacy

Major Professor, representing Biochemistry and Biophysics

Redacted for privacy

Chair of Biochemistry and Biophysics

Redacted for privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for privacy

Cynthia Sue Thompson, Author

Acknowledgments

This thesis is made possible with the help of many people. I especially would like to thank my family, my parents, Jim and Marilyn Thompson and my brother Jay for their continual support. I would like to thank all my friends who supported me throughout my adventure at OSU.

I wish to thank Dr. Chris Mathews for his support and guidance. I also wish to express my thanks to my committee: Dr. Steve Giovannoni for his assistance and insight, Dr. Sonia Anderson, Dr. Mike Penner, and Dr. Wil Gamble.

Table of Contents

Table of Contents (Continued)

List of Figures

List of Tables

Oligoribonucleotides Directed at Ribosomal Protein Binding Sites as Inhibitors of Ribosomal Small Subunit Assembly and as Possible Antibiotics

Chapter 1

Introduction

1.1 Statement of Problem

Bacteria exist almost everywhere and make up a large proportion of the biomass of this planet. Most bacteria cause little or no harm and some are useful to humans by inhibiting harmful bacteria. Some bacteria, though, are capable of causing disease in humans and some beneficial bacteria can be harmful depending on the location of the bacteria on or in the host.

Antibacterial agents are anything that can kill or inhibit the growth of bacteria. Antibacterial drugs, or antibiotics, are used in low concentrations in or on an organism to treat bacterial disease with little or no harm to the host organism. Antibiotics are also used as growth enhancers in production feed animals. Resistance to antibiotics is a phenomenon that enables some bacteria to survive the effects of antibiotics.

Antibiotic resistance is a major problem nationally and worldwide but data are insufficient to determine the full extent of the public health burden (Health, Education and Human Services, 1999). Antibiotic resistance has shown up in major pathogenic organisms including *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Neisseria gonorhoeae*, enteric pathogens, and many others (Neu, 1992). In 1997, a report was released on a 1995 clinical case of multidrug-resistant *Yersinia pestis*, the organism

responsible for bubonic plague and a species previously considered universally susceptible to antibiotics (Galimand *et al.*, 1997).

Resistance to antibiotics occurs through four major mechanisms. One is the destruction or modification of the antibiotic that renders the antibiotic inactive. Examples of this mechanism are β-lactamase and aminoglycoside modifying enzymes (Shaw *et al.*, 1993). Another mechanism of resistance is efflux of the antibiotic. An example is the tetracycline transport protein Tet A (Allard and Bertrand, 1993). Another mechanism prevents access of the antibiotic to the target due to an alteration of cell permeability to the antimicrobial agent as is sometimes the case with the quinolones (Dechene *et al.*, 1990; Poole *et al.*, 1993) and with the β-lactams (Chou *et al.*, 1993). Sometimes the resistance can take the form of alteration of the antibiotic target site. Resistance to the macrolide class of antibiotic is usually caused by a methylase that methylates an adenine residue in the peptidyl transferase domain of the 23S RNA (Ballow and Amsden, 1992). Resistance to vancomycin involves the action of nine genes that act overall to alter the peptidoglycan layer of the cell wall causing the drug to have a lower affinity for its target (Walsh, 1993).

The cost of resistance has been difficult to assess due to lack of a complete national surveillance system on infectious disease and resistant infections. Sources provide data on only part of the burden and some are based on models (Phelps, 1989). Estimates vary widely depending on whether the studies take factors into account such as indirect costs, impatient vs. outpatient costs, social costs, and the cost of premature deaths. For instance, Cohen (1992) reported that the estimated cost for treating a tuberculosis (TB) case increases from $12,000 for a susceptible strain to $180,000 for a drug-resistant

strain. But in 1995, Brown (Brown *et al.*, 1995) reported that in 1991, the cost was $2,300 for susceptible and $8,000 for drug-resistant TB. Brown reported only expenditures for outpatient TB by patient type. Therefore, an increase of three to over tenfold can be assumed for drug-susceptible vs. drug-resistant strains of bacteria. According to a 1997 survey (Health, Education and Human Services, 1999) the number of hospital discharges of patients diagnosed with and treated for infections from drug-resistant bacteria increased from 11,000 in 1994 to 43,000 in 1997. Data on deaths due to resistant bacteria are not available because resistance does not get reported on death certificates.

Public health strategies to address antimicrobial resistance include improved surveillance, at the national and international level (for an inventory of global surveillance systems on infectious disease see Harrison and Lederberg, 1998), rational use of antimicrobial drugs in both human and animal medicine as well as more prudent use of antibiotics in food production, control and guidelines to reduce the spread of infection and antibiotic resistance within the hospital and health care settings. Basic research is needed on the mechanisms of antibiotic resistance and research on rapid and reliable diagnostic tests and on vaccines. Another important strategy is research and development into new antimicrobial compounds (American Society for Microbiology Task Force, 1994).

Antibiotics currently on the market attack a relatively small number of targets (for review see Williams *et al.*, 1996). Keeping ahead of antibacterial resistance will require expanding the number of antimicrobials with novel targets and drug actions. Genomic research is already underway to probe bacterial genomes for new targets. Gene

sequencing allows for tremendous comprehension into how organisms alter themselves, biochemical pathways and transport mechanisms associated with virulence, resistance, and infectivity. Gene arrays, that allow for simultaneous study of all genes within a genome, can allow for research under different physiological conditions and the study of variations in virulence and infectivity as well as identification and study of genes associated with antimicrobial resistance (Ventor, 1998).

In March of 2000, at least two new drugs were awaiting approval from the Food and Drug Administration (FDA) (Henry, 2000). One is a completely new class of antibiotics, the oxazolidinones from Pharmacia and Upjohn. The oxazolidinones are five-membered heterocyclic ring structures that appear to act by binding the small ribosomal subunit and preventing the interaction during initiation of the small subunit, messenger RNA (mRNA), and formyl methionine. Linezolid, the first member of this class, is active against gram-positive bacteria. Another antibiotic waiting for FDA approval is gemifloxacin, a next-generation fluoroquinolone. Fluoroquinolones inhibit type II topoisomerase. Other antimicrobial drugs are under development but new classes are few.

1.2 Thesis Topic

The ribosome is the platform upon which protein synthesis occurs. The prokaryotic ribosome is a large macromolecular complex made up of both RNA and proteins, which sediments at 70S. It has two subunits, the 50S and 30S, named for their sedimentation coefficients. The 30S subunit has one RNA molecule, the 16S RNA. In *E. coli* the 16S RNA is 1542 bases long. Complexed to the 16S RNA, the 30S subunit has 21 proteins, designated S-proteins.

30S subunits can be reconstituted *in vitro* from purified 16S RNA and purified 30S proteins (TP30) (Traub and Nomura, 1969). These particles are found to be almost identical to isolated, non-reconstituted 30S particles with respect to sedimentation profile, protein composition, and functionality. Overall 30S reconstitution is stepwise, first order, and proceeds via the formula: 16S RNA + TP30 $\rightarrow$ RI (heat/Mg^{++}) $\rightarrow$ RI* $\rightarrow$ 30S. The RI particle (reconstitution intermediate) undergoes a conformational, unimolecular rearrangement to the activated RI particle (RI*), and this rearrangement is the rate limiting step. Thirteen proteins (S4, S6, S7, S8, S9, S11, S13, S15, S16, S17, S18, S19, and S20) and the 16S RNA make up the RI particle (Nomura *et al.*, 1969; Bryant *et al.*, 1974). Of these thirteen proteins, S6, S13, and S20 are found in the isolated RI particle but don't appear to be required for the RI $\rightarrow$ RI* transition (Held and Nomura, 1973). Proteins S5, S12, and possibly S19 appear to bind weakly to the RI particle at low temperature and are not found in the isolated RI particle but are needed for the temperature-dependant RI $\rightarrow$ RI* step and fully active 30S subunits. After the RI $\rightarrow$ RI* transition, the remaining 30S proteins can then bind the RI* and form active 30S subunits.

An *in vitro* 30S assembly map has been developed (Mizushima and Nomura, 1970; Held *et al.*, 1974). Proteins that bind to the 16S RNA in the absence of other proteins are called primary binding proteins. These are proteins S4, S17, S20, S8, S15, and S7. Of these, proteins S4 and S7 initiate 30S-subunit assembly in a domain-like fashion (Notwotny and Nierhaus, 1988). It has been suggested that S8 and its neighbor protein S5 play an important role in the interconnection of the two assembly domains. Proteins that require the binding of the primary proteins to the 16S RNA in order for them to bind

to the subunit are called secondary proteins. For instance, protein S8 binds to a short, double strand, imperfect helical region in domain II of the 16S RNA between bases 590-659 (*E. coli* numbering) and is a primary protein. Protein S5, a secondary protein, requires the binding of protein S8 and also S16 in order for it to bind to the subunit. Protein S3 requires the binding of protein S5 and the binding of protein S2 is facilitated by the binding of protein S3. Omitting any of the primary binding proteins from the reconstitution mixture except S20 results in subunits with diminished functionality, that sediment between 20S and 25S, and are deficient in some secondary proteins as well as the omitted primary binding protein (Nomura *et al.*, 1969).

The study of ribosome assembly *in vivo* has been studied primarily through cold sensitive mutants defective in ribosome assembly (Nashimoto *et al.*, 1971; Bryant *et al.*, 1974), pulse-labeling experiments (Lindahl, 1975), and precursor particles that have been isolated during *in vivo* assembly (Nierhaus *et al.*, 1973). One precursor particle, $p_1$30S, does not have a full complement of 30S proteins. It sediments at approximately 21S. These precursor particles isolated from different labs have similar protein profiles to the *in vitro* RI particle (Held and Nomura, 1973; Nierhaus, 1991).

The aim of this research is to inhibit complete 30S assembly *in vitro* by adding an oligoribonucleotide identical or similar in sequence to a ribosomal protein binding site, specifically S8. S8 was tested because of the small size and simplicity of the binding site on the 16S RNA. The oligoribonucleotide binds to the target protein S8 and inhibits protein S8's binding to the 16S RNA. Since the targeted protein S8 can no longer bind to the 16S RNA, some secondary proteins, namely S5, S3, and S2, also can no longer be incorporated into the 30S subunit resulting in a particle that sediments more slowly and is

non-functional (Held *et al.*, 1974; Buck *et al.*, 1991). Since 30S ribosomal subunit assembly has been proposed to occur by similar pathways both *in vitro* and *in vivo*, it is believed that if this block in assembly occurs *in vitro* it would also occur *in vivo* if methods can be devised to bring about the uptake of the inhibitory agent into bacterial cells. Oligoribonucleotides such as these (in analog form) may serve as useful antibiotics that target ribosome assembly.

1.3 Research Approach

Oligoribonucleotides do not penetrate well into bacterial cells. Therefore, to test whether introducing an oligoribonucleotide of identical or similar sequence to the S8-RNA binding site into the cell would block ribosomal assembly and inhibit cell growth, the binding site of S8 was delivered into *E. coli* through a phagemid delivery system. The S8 binding site was synthesized and inserted into a phagemid vector under control of a *lacZ* promoter by using molecular biology techniques. The same phagemid with the Shine-Dalgarno and translational start site of the *lacZ* gene deleted by the polymerase chain reaction (PCR) and recircularized was also constructed and both transformed into *E. coli*. Spectrophotometric growth experiments were performed in the presence and absence of the inducer isopropylthiogalactoside (IPTG). In order to determine whether transcription occurs from the phagemid constructs, Northern blot analysis was performed on cell lysates grown in the presence and absence of the inducer IPTG.

30S subunits can be reconstituted *in vitro* from purified 16S RNA and purified 30S ribosomal proteins. Omission of protein S8 in the reconstitution reaction results in subunits that sediment more slowly and show an 87-98 % reduction in polyphenylalanine synthesis (Nomura *et al.*, 1969; Higo *et al.*, 1973). This severe reduction of function was

used to test the hypothesis. Reconstitutions were performed in the presence and absence of unmodified oligoribonucleotides identical or similar in sequence to the S8 binding site and the reconstitution mixtures were assayed for polyU-directed polyphenylalanine incorporation. Filter-binding assays were employed to test whether the oligoribonucleotides had a lower affinity to 30S proteins in the reconstitution than the 16S rRNA to the 30S proteins.

Each of the chapters within this thesis addresses a specific topic. Chapter 2 reviews literature on the ribosome and ribosomal assembly, protein S8 and its binding site to the 16S RNA, and ribosomal component regulation. The materials and methods used in the investigations are discussed in Chapter 3. Chapter 4 presents the results of the experiments performed. Chapter 5, the final chapter, includes a discussion and analysis of the results as well as clinical implications of the experimentation.

Chapter 2

Literature Review

2.1 The Ribosome

Ribosomes are large ribonucleoprotein structures responsible for translation of the genetic code into proteins. The *E. coli* ribosome is the most studied and is a model for this organelle. The *E. coli* ribosome is made up of 38 % protein and 62 % RNA. There is now considerable evidence that the RNA is a functional component as opposed to just a structural component of the ribosome (Green and Noller, 1997). The prokaryotic ribosome sediments at 70S and has two subunits, the large subunit, or 50S, and the small subunit, the 30S. Recently, the 70S ribosome as well as the 50S and 30S subunits have been crystallized and their solutions solved to 5.5 angstroms for the 30S (Clemons *et al.*, 1999), 5.0 angstroms for the 50S (Ban *et al.*, 1999), and the 70S, containing messenger RNA and transfer RNA, to 7.8 angstroms (Cate *et al.*, 1999; Culver *et al.*, 1999). Considering the amazing complexity of the ribosome, these are amazing feats of science and will prove invaluable for the study of translation, especially when the structures are solved to higher resolutions.

The 50S subunit has two RNA molecules, the 23S and the 5S. The 5S RNA is 120 nucleotides long in *E. coli*. The 23S RNA is 2904 nucleotides long and contains numerous posttranscriptional modifications including methylations and pseudouridines. Not all of the modifications have been placed in the sequence (Baken and Ofengand, 1993). Complexed to the RNA's are approximately 34 proteins designated L1 through L36. This nomenclature is based on the proteins mobility in a two-dimensional gel

electrophoresis system (Kaltschmidt and Wittman, 1970). L8 is an aggregate of $(L7/L12)_4$*L10, L26 is identical to protein S20, and L7 and L12 are identical except for L7 is N-terminal acetylated. The molecular weights of the proteins range between 4,000 and 30,000 daltons.

The 30S subunit has one RNA, the 16S RNA. In *E. coli* it is 1542 nucleotides long. Like the 23S RNA, it too has posttranscriptional modifications including 10 methylations and one pseudouridine. Through comparitive sequence analysis, it's shown to have extensive secondary structure and tertiary interactions including two-pseudoknot structures (Figure 2.1). Complexed to the 16S RNA are 21 proteins designated S1 to S21 with a molecular weight range between 8,000 and 60,000 daltons. Most ribosomal proteins are basic proteins with S1, S6, and L7/L12 being exceptions (Kaltschmidt, 1971).

The role of the ribosome is to synthesize polypeptide chains of specified amino acids that are specified by mRNA. The ribosome has both a catalytic function and a decoding function. During decoding, the tRNA is accurately aligned to the mRNA so that correct codon-anticodon interaction takes place. The small subunit is the major contributor to the decoding site. The peptidyl transferase region of the ribosome catalyzes peptide bond formation by bringing together the aminoacyl end of the tRNA to the peptidyl-tRNA and transfers the nascent polypeptide chain to the next amino acid on the aminoacyl-tRNA. The mRNA is moved one codon relative to the ribosome and the peptidyl-tRNA translocates to the P-site. The catalytic peptidyl transferase function lies in the large subunit.

Figure 2.1. The primary and secondary structure of the 16S RNA in *E. coli* as deduced by sequence comparison analysis. Material taken from Gutell, 1996.

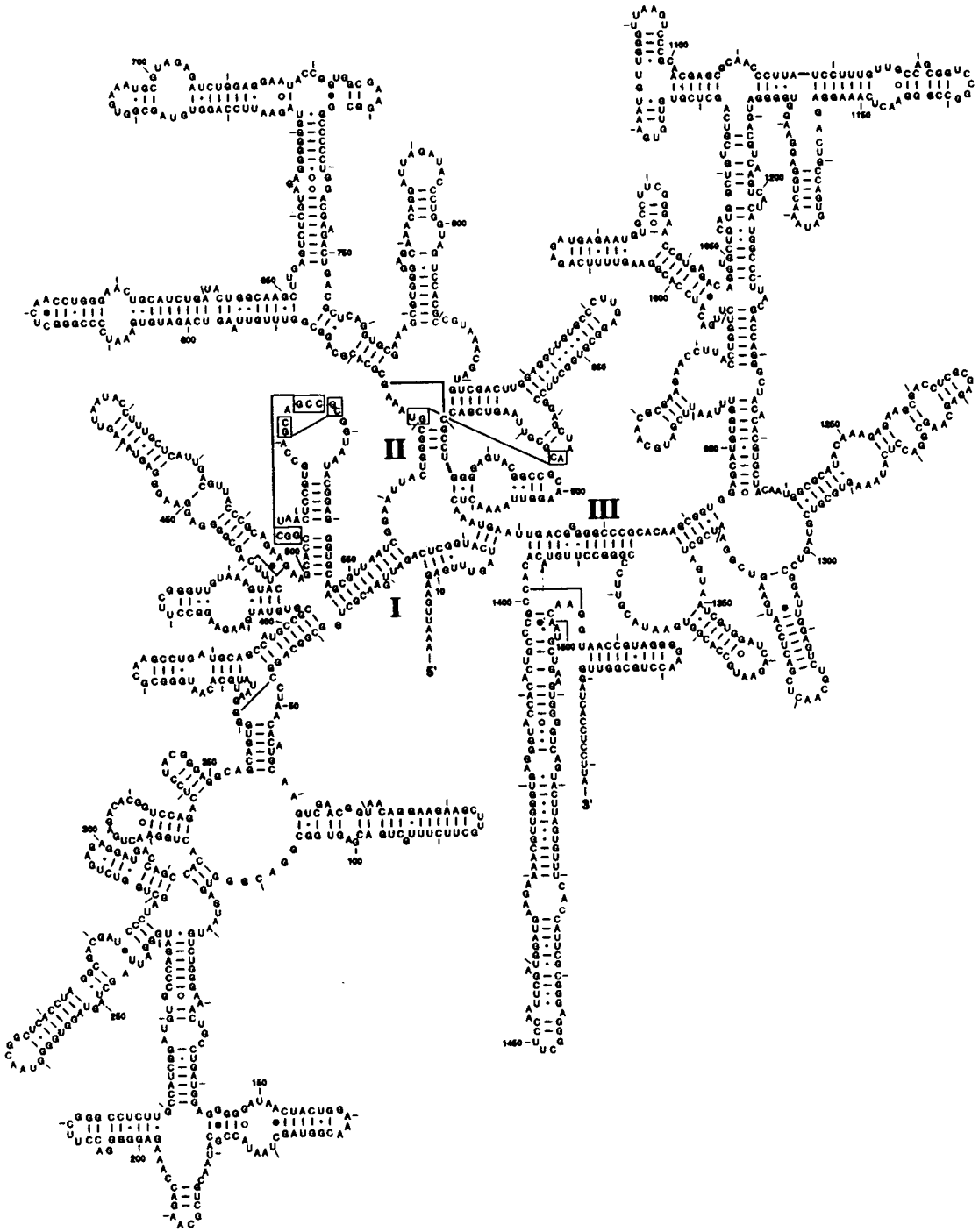


Figure 2.1

2.2 Ribosomal Assembly

2.2.1. *In vitro* 30S Small Subunit Assembly

30S subunits can be reconstituted *in vitro* from purified 16S RNA and purified 30S ribosomal proteins (Traub and Nomura, 1969; Held *et al.*, 1973). These particles are found to be almost identical to isolated 30S subunits with respect to sedimentation profile, protein composition, and several functional abilities including tRNA binding and polyphenylalanine synthesis. The reconstitution of active ribosomes demonstrates that all the information needed for assembly is contained in the molecular components. 30S subunits have been reconstituted using heat denatured RNA (Barritault, 1979) and *in vitro* transcribed RNA (Krzyzosiak *et al.*, 1987), the later signifying that the base modifications in the 16S RNA are not absolutely required for function of the small subunit.

Functionally active hybrid 30S subunits have been reconstituted using protein and RNA components from different species (Nomura *et al.*, 1968; Higo *et al.*, 1973). It was found that *Azotobacter vinelandii* and *Bacillus stearothermophilus* 16S RNA's can replace *E. coli* 16S RNA in a reconstitution using *E. coli* proteins. The reconstituted particles had the same sedimentation profile and activity as measured by the polyphenylalanine synthesis assay. The use of *Miccrococcus lysodeikticus* 16S RNA in the reconstitution resulted in variable particles (3-36 % activity), slower sedimentation profile, and formed insoluble aggregates but reconstitution of active particles using both 16S RNA and proteins from *M. lysodeikticus* was unsuccessful under the reconstitution conditions used as well. The activity of reconstituted particles using *E. coli* 16S RNA and *B. stearothermophilus* or *A. vinelandii* proteins were about half the activity of their

controls (Nomura *et al.*, 1968). Most individual *B. stearothermophilus* proteins can replace *E. coli* proteins in an *E. coli* reconstitution system. The activity is somewhat variable depending on the protein. For example, using *B. stearothermophilus* S2 in the reaction instead of *E. coli* S2 results in 62 % activity. Using *B. stearothermophilus* protein S8 instead of *E. coli* S8 results in 106 % activity. All protein replacements except S2 results in 80-100 % activity compared to their controls (S1, S6, S9, and S13 were not tested) (Higo *et al.*, 1973). Protein S8 from *Thermus thermophilus* binds the *E. coli* ribosomal RNA binding site as efficiently as protein S8 from *E. coli* (Vysotskaya *et al.*, 1994). These results taken together suggest that the fundamental structural organization of the 30S subunit is similar among eubacteria.

Efficient reconstitution of functional *E. coli* 30S subunits has occurred using a complete set of recombinant ribosomal proteins (Culver and Noller, 1999). Interestingly, efficient reconstitution occurred only if the protein components were added sequentially following either the 30S assembly map or following the order of protein assembly predicted from *in vitro* assembly kinetics (both discussed below). Reconstitution with the recombinant proteins was inefficient when performed by adding all of the ribosomal proteins together in the mixture at the same time, which is the standard method.

Overall reconstitution proceeds via the formula: 16S RNA + ribosomal proteins $\rightarrow$ RI (heat/Mg^{++}) $\rightarrow$ RI* $\rightarrow$ 30S (Traub and Nomura, 1969). Under optimal conditions, ribosome assembly is sequential, cooperative, and first order with respect to active 30S subunits. The RI particle (reconstitution intermediate) undergoes a conformational, unimolecular change to RI* (activated reconstitution intermediate) that is the rate limiting step to 30S particles. When the reaction is carried out at 30°C and analyzed using

sedimentation centrifugation, the RI particle is isolated and sediments at 21S. These particles can then be heated (40°C) in the absence of free protein (except protein S5, S12, and S19) and the new particles, RI*, sediment at 26S and can bind the remaining ribosomal proteins even at lower temperatures. Thirteen proteins (S4, S6, S7, S8, S9, S11, S13, S15, S16, S17, S18, S19, and S20) and the 16S RNA make up the isolated RI particle (Nomura *et al.*, 1969; Bryant *et al.*, 1974). Some of these proteins (S6, S9, S13 and S20) are found in the isolated RI particle but appear not required for the RI to RI* transition (Held and Nomura, 1973). Proteins S5, S12, and S19 are not found in the isolated RI particle but are required for the RI to RI* transition and full activity of the 30S subunits, supposedly due to weak binding to the RI particle. After the RI* transition, the remaining 30S ribosomal proteins are then able to bind the RI* and form fully active 30S subunits.

An *in vitro* 30S assembly map has been developed using the logic that if a protein will not bind well to RNA until another protein has bound, then the first protein follows the second protein in the assembly reaction (Figure 2.2) (Mizushima and Nomura, 1970; Held *et al.*, 1974). Proteins that bind to the 16S RNA in the absence of any other proteins are called primary binding proteins. These are S4, S17, S20, S8, S15, and S7. Of these, S4 and S7 initiate 30S subunit assembly in a domain-like fashion (Nowotny and Nierhaus, 1988). It has been suggested that S8 and S5 play and important role in the interconnection of the two assembly domains. Proteins that require the binding of the primary proteins to the 16S RNA in order for them to bind to the particle are called secondary binding proteins, the remaining are called tertiary binding proteins.

Figure 2.2. Assembly map of the 30S subunit. Arrows between proteins show facilitating effect of one protein on the binding of another to the particle. A thick arrow indicates a major facilitative effect. The arrows from the 16S RNA to S4, S17, S20, S8, S15, and S7 indicate primary binding proteins that bind directly to the 16S RNA in the absence of other proteins. S8 and S20 (arrows not shown) as well as S9 and S19 facilitate the binding of S7. S13, S16, S17, S20, S8, and/or S4 may facilitate protein S11 but which ones are unknown. Proteins above the solid line are those proteins found in the RI particle or are required for the RI to RI* transition (S5, S9, and S12 are not found in the RI particle possibly due to only weak binding to the RI particle but are required for the RI to RI* transition). Data taken from Held *et al.*, 1974.

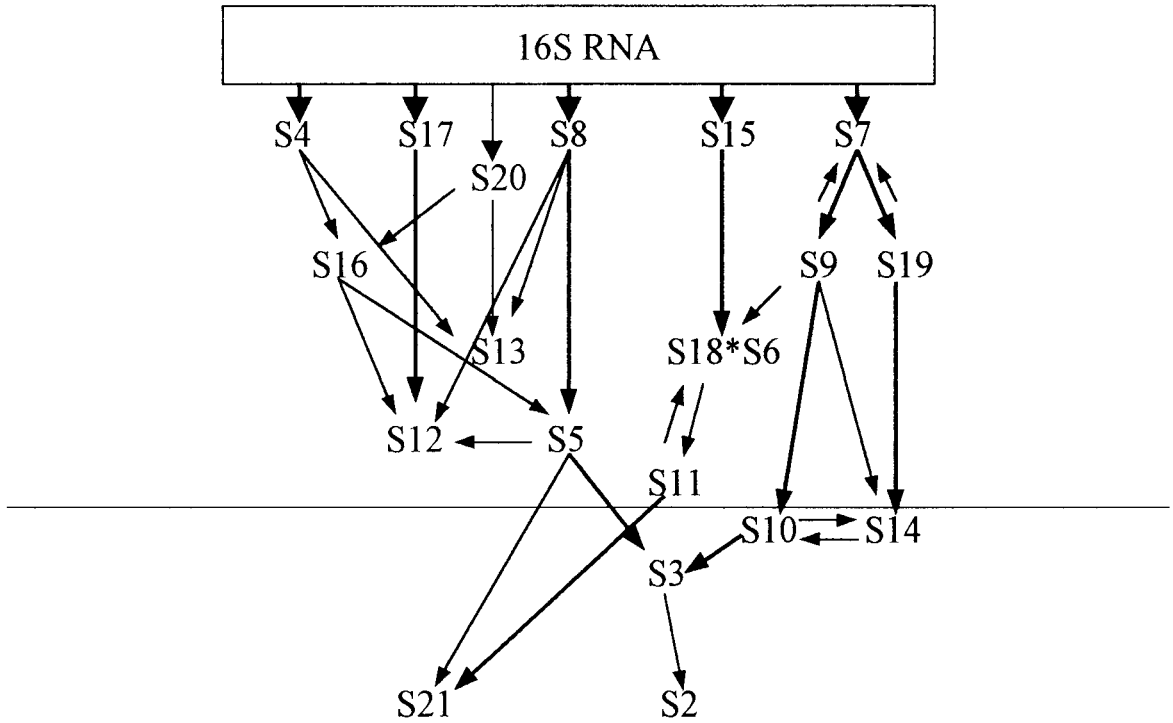

16S RNA
S4
S17
S8
S15
S7
S20
S16
S9
S19
S13
S18*S6
S12
S5
S11
S10
S14
S3
S21
S2

Figure 2.2

The dynamics of *in vitro* assembly has also been studied using the temperature dependency of the reaction (Powers *et al.*, 1993). Assembly complexes at different temperatures were chemically probed for RNA reactivity at various time points. For instance, at 30°C, reconstitution takes about 2 hours to go to completion (as opposed to a few minutes at 40°C) and intermediate stages of assembly can be monitored. Some regions of the RNA were protected almost immediately while others were protected at later stages of assembly. Some bases became more reactive initially and then non-reactive later during assembly. These experiments demonstrate that *in vitro* 30S assembly proceeds with a 5' to 3' polarity (with exception of S5 and S12 showing slow kinetics) possibly due to the coupling of ribosome assembly to transcription. The experiments provided evidence for the sequential nature of assembly by showing that bases foot-printed by late-assembly proteins show slower kinetics than residues foot-printed with early binding proteins in the assembly map, in other words, the experiments showed a close correlation to the order implied in the assembly map.

2.2.2. *In vivo* 30S Ribosomal Subunit Assembly

Precursor particles have been isolated during *in vivo* assembly (Nashimoto *et al.*, 1971; Nierhaus *et al.*, 1973; Bryant *et al.*, 1974). The study of ribosome assembly *in vivo* has been studied primarily through cold-sensitive mutants defective in ribosome assembly (Nashimoto *et al.*, 1971; Bryant *et al.*, 1974) and pulse-labeling experiments (Lindahl, 1975). One precursor particle, designated p 30S, contains all the 30S proteins but contains a precursor 16S RNA which is longer at both the 5' and 3' termini than mature 16S RNA (Hayes and Hayes, 1971). This suggests the final step in 16S rRNA processing is a late step in subunit maturation. The mature 5' end of 16S RNA is

processed when the precursor RNA is associated with ribosomal proteins (Dahlberg *et al.*, 1978). The endonuclease that cleaves the 5' end does not use free RNA as a substrate.

Other precursor particles have mature 16S RNA but do not have a full complement of 30S proteins. Since *in vitro* 30S assembly is strongly temperature dependent, Nomura's lab set out to find cold-sensitive mutants that have defects in ribosome assembly at low temperatures. A cold-sensitive mutant (Spc-49-1) has been characterized (Nashimoto *et al.*, 1971). The mutant accumulated 21S particles that had a similar protein composition to the RI particle (Table 2.1). All of the primary binding proteins are present in the mutant 21S particle. The mutation was mapped to protein S5 of the *spc* operon. Other assembly mutants also accumulate 21S particles such as Sad-410 (Nashimoto *et al.*, 1971), even though the mutation did not map in the *spc* operon. In the case of Sad-410, the protein composition of the 21S particle was found to be very similar to the Spc-49-1 21S particle (protein composition of Sad-410 was not published).

21S precursor particles have been isolated using hyperbolic gradient sedimentation (Nierhaus *et al.*, 1973). The isolated *in vivo* precursor particle is found to be similar to the *in vitro* RI particle with one main difference, S7, S9, S10, S14, and S19 which *in vitro* depend on the presence of S7, is missing from the *in vivo* precursor (Table 2.1). Kinetic studies of *in vitro* assembly have demonstrated that S7 and the others following S7 in the assembly map, are late binding proteins during assembly (Powers and Noller, 1993). The isolated *in vivo* 21S particle probably is a manifestation of the kinetics characterized *in vitro*. The correlation between the *in vivo* data and the *in vitro* data derived from these

Table 2.1. Protein content of reconstitution intermediates of the 30S subunit.+ stands for present, (+) for present in reduced amounts, - not present, (-) for not present but required for RI toRI* transition. Data are taken from Nashimoto *et al*., 1971; Held and Nomura, 1973; and Nierhaus *et al*., 1973.

Protein	RI	$p_1$30S	21s (from Spc-49-1 cells)
S1	-	+	-
S2	-	-	-
S3	-	-	-
S4	+	+	+
S5	(-)	+	-
S6	+	-	+
S7	+	-	+
S8	+	+	+
S9	+	-	(+)
S10	-	-	-
S11	+	-	-
S12	(-)	-	-
S13	+	+	+
S14	-	-	-
S15	+	+	+
S16	+	+	+
S17	+	+	+
S18	+	-	(+)
S19	(-)	-	+
S20	+	+	+
S21	-	-	-

different experiments indicates that the sequences of events of *in vitro* reconstitution are relevant to *in vivo* assembly.

2.2.3 Large Subunit 50S Assembly

Reconstitution of the 50S subunit can occur *in vitro* (Nomura and Erdman, 1970; Nierhaus and Dohme, 1974; Dohme and Nierhaus, 1976). Optimal reconstitution of *E. coli* 50S subunits occurs via a two step process, the first step consists of incubating the components at 4 mM Mg^{++} at 44°C for 20 minutes followed by incubation at 50°C at 20 mM Mg^{++} for 90 minutes (Dohme and Nierhaus, 1976). Through kinetic analysis, it was shown that 50S reconstitution occurs via the formula:

23S RNA + 5S RNA + total protein 50 (TP50) (4 mM Mg^{++}, 0°C) $\rightarrow RI_{50}(1)$ (33S)

$RI_{50}(1)$ (4 mM Mg^{++}, 44°C) $\rightarrow RI^*_{50}(1)$ (41S)

$RI^*_{50}(1)$ + TP50 (4 mM Mg^{++}, 44°C or 20 mM Mg^{++}, 50°C) $\rightarrow RI_{50}(2)$ (48S)

$RI_{50}(2)$ (20 mM Mg^{++}, 50°C) $\rightarrow$ 50S

It has three intermediates, $RI_{50}(1)$ which sediments at 33S and is present at 0°C, $RI^*_{50}(1)$, that sediments at 41S, is the essential product of the first incubation step, it cannot be by-passed in the second step, and it contains the same proteins as the $RI_{50}(1)$ despite the drastic change in sedimentation value, and $RI_{50}(2)$ which sediments at 48S. It was further demonstrated that $RI_{50}(1) \rightarrow RI^*_{50}(1)$ and $RI_{50}(2) \rightarrow 50S$ are the rate-limiting reactions for each of the steps and are unimolecular (Sieber and Nierhaus, 1978).

Using a similar technique as taken for the 30S subunit, an assembly map for the *E. coli* 50S subunit has been constructed by Nierhaus and co-workers (Röhl and Nierhaus, 1982; Röhl *et al.*, 1982; Harold and Nierhaus, 1987). The assembly map is more complex due to the multiple steps with different reaction conditions and the greater

number of components. L1, L2, L3, L4, $(L12)_4$-L10, L11, L15, L20, L23, and L24 bind directly and independently to the 23S RNA. L5, L18, and L25 bind to the 5S RNA. Of these, proteins L24 and L3 initiate 50S ribosomal assembly in a domain like fashion (Nowotny and Nierhaus, 1982). The 23S RNA and five proteins, L4, L13, L20, L22, and L24 (with L3 having a stimulatory effect) are essential for the $RI^*_{50}(1)$ formation. These proteins except L3 also bind towards the 5' end of the 23S RNA. The proteins, which bind early in assembly, also have the ability to bind 23S RNA before synthesis of the 23S RNA is complete. This coupling of rRNA synthesis and ribosomal assembly is termed assembly gradient (Sieber and Nierhaus, 1978). *In vivo* assembly starts with a short section of RNA and 5 proteins instead of the mature 23S RNA being exposed to all L-proteins as is the case with *in vitro* assembly and could explain why *in vivo* 50S assembly takes a few minutes at 37°C and 90 minutes at 50°C *in vitro* (Nierhaus, 1991).

In vivo precursor particles have also been studied for the 50S subunit. *In vivo* assembly occurs via three precursor particles: the $p_1$50S which sediments at 34S. the $p_2$50S that sediments at 43S, and the $p_3$50S which sediments 'near 50S'. The $p_3$50S precursor contains all the L-proteins, similar to the $RI_{50}(2)$ reconstitution intermediate. The protein contents of the $p_1$50S and the $p_2$50S as well as the $RI_{50}(1)$ are shown in Table 2.2. The early group of *in vitro* assembly proteins belongs to the first ten proteins in *in vivo* assembly and are present in the $p_1$50S precursor. The proteins missing from the $p_1$50S precursor which would correspond to the late assembly proteins *in vitro* do not agree as well. Assembly *in vitro* and *in vivo* start by similar pathways but diverge towards the end of the pathways (Spillman *et al.*, 1977).

Table 2.2. Protein composition of *in vivo* precursor particles and *in vitro* reconstitution intermediates of the 50S subunit. Data for $RI_{50}(1)$ taken from Harold and Nierhaus, 1987 and Nierhaus *et al*., 1973 for the precursor particles, $p_1$50S and $p_2$50S. L8 is the pentamer $L10(L7/L12)_4$ and is not shown, L26 is the 30S protein S20 and is not shown. L34, L35, L36 have not been assigned.

Protein	$p_1$50S	$p_2$50S	$RI_{50}(1)$
1L	+	+	+
L2	-	-	(+)
L3	+	+	+
L4	+	+	+
L5	+	+	+
L6	-	-	-
L7/L12	(+)	+	+
L9	+	+	+
L10	+	+	+
L11	(+)	+	+
L13	+	+	(+)
L14	-	+	-
L15	-	+	+
L16	-	-	-
L17	+	+	+
L18	+	+	(+)
L19	-	+	-
L20	+	+	+
L21	+	+	+
L22	+	+	+
L23	+	+	+
L24	+	+	+
L25	+	+	-
L27	+	+	-
L28	-	-	-
L29	+	+	+
L30	+	+	-
L31	-	-	-
L32	-	-	-
L33	-	+	(+)

2.3 Small Ribosomal Subunit Protein S8 and its 16S RNA Binding Site

2.3.1 Small Ribosomal Subunit Protein S8

Ribosomal protein S8 is one of the primary ribosomal binding proteins to the 16S RNA. It binds to the 16S RNA with high affinity and in the absence of other proteins. S8 is about 130 amino acids long, depending on the species. In *E. coli*, S8 is 129 amino acids long with a molecular weight of 14,000 daltons (Allen and Wittman-Leibold, 1978). S8 is a basic protein with an isoelectric point of 9.1 in *E. coli* (Kaltschmidt, 1971). According to cross-linking data (Tindall and Aune, 1981; Lambert *et al.*, 1983) and neutron diffraction studies (Capel *et al.*, 1987), the protein neighbors of S8 are S2, S4, S5, S12, S15, and S17 in the 30S subunit.

Two crystal structures have been determined for S8, one from *Bacillus stearothermophilus* (Davies *et al.*, 1996) and one from *Thermus thermophilus* (Nevskaya *et al.*, 1998). The overall crystal structures are closely related. S8 is a globular protein and consists of two domains that are independently folded and tightly associated. The domains are connected by an interdomain loop that varies in length among species but does not display flexibility in the structures. Based on the crystal structures, there are three proposed functional sites on protein S8. One is located on the N-terminal domain and is bordered by positive potential. The site is generally conserved and is proposed to be a secondary RNA binding site that has also been suggested through chemical probe studies (Stern *et al.*, 1989) and hydroxyl radical foot-printing (Powers and Noller, 1995). Another proposed functional site is a conserved, concave hydrophobic patch flanked by two basic regions located in the C-terminal domain. It has been proposed that this site is a protein-protein interaction site and the best candidate for this interaction is protein S5.

Protein S5 has been shown to be one of the nearest neighbors to protein S8 in the ribosome (Tindall and Aune, 1981; Lambert *et al.*, 1983; Capel *et al.*, 1987). The crystal structure for S5 has also been determined (Ramakrishnan and White, 1992) and it has been shown to have a convex hydrophobic patch which is approximately equal in size and generally complementary in shape to the hydrophobic patch of S8 (Davies *et al.*, 1996).

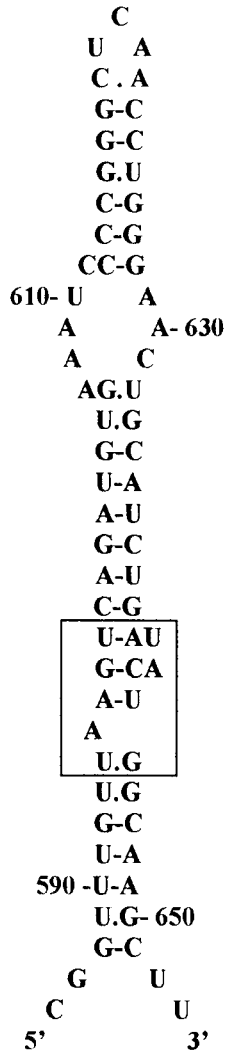
The third functional site lies in the C-terminal portion of the C-terminal domain and is proposed to be the primary RNA binding site. The crystal structures for S8 can be aligned very accurately in this region (Nevskaya *et al.*, 1998). Several residues are completely conserved including basic amino acids potentially involved in RNA phosphate backbone interactions and aromatic amino acids potentially involved in specific base interactions. Mutational experiments have been performed where the N-terminal 51 amino acids have been removed from the protein and the protein still retains specific binding albeit with lower affinity (Uma *et al.*, 1995). Mutations in the C-terminal portion of the protein resulted in the most severe impairment of binding to the main 16S RNA site and removal of the last 8 amino acids of the C-terminus resulted in a severe reduction of binding to the RNA (Wower *et al.*, 1992; Uma *et al.*, 1995).

2.3.2 The Binding Site of Protein S8 and the Binding to the 16S RNA

The primary RNA binding site of protein S8 is a short double strand imperfect helical sequence (helix 21) on the 16S RNA between nucleotides 590-650, *E. coli* numbering (Figure 2.3) (Schaup *et al.*, 1973; Ungewickell *et al.*, 1975; Zimmermann *et al.*, 1975). Through filter-binding assays, the apparent association constant for the binding of S8 to its binding site ranges between 2.7 and 5 x 10^7 M^{-1} (Schwarzbauer and

Figure 2.3. Primary and secondary structure of *E. coli* helix 21, the S8 binding site, of the 16S RNA. A. Secondary structure according to Gutell, 1996. The core S8 binding site is boxed. B. Structure of the core S8 binding site of *E. coli*. The A595•(A596•U644) base triple identified by NMR studies and supported by phylogenetic comparison is boxed. The bases involved in the base triple suggested by NMR and by hydroxyl radical studies but not supported by sequence comparison are double underscored.

A


C
U A
C . A
G-C
G-C
G.U
C-G
C-G
CC-G
610- U A
A A- 630
A C
AG.U
U.G
G-C
U-A
A-U
G-C
A-U
C-G
U-AU
G-CA
A-U
A
U.G
U.G
G-C
U-A
590 -U-A
U.G- 650
G-C
G U
C U
5' 3'

B

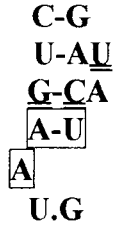

C-G
U-AU
G-CA
A-U
A
U.G

Figure 2.3

Craven, 1981; Mougel *et al.*, 1986) and 4 x 10^7 M^{-1} using a sedimentation assay (Mougel *et al.*, 1986). The size of the loop is variable (Gutell *et al.*, 1985). In eubacteria and archaebacteria the length of the loop remains mostly constant. In mitochondria the helix ranges from one base pair shorter (in plants) to completely deleted (bovine). In eukaryotic cytoplasmic 16S-like-RNA, there's a 225-nucleotide insertion (Winker and Woese,1991). The minimal binding site has been further delineated to include only a portion of helix 21, nucleotides G588 to G605 and C633 to C651 *E. coli* numbering (Mougel *et al.*, 1993; Wu *et al.*, 1994). The binding site consists of the asymmetric region C of nine nucleotides surrounded by a base-paired helix (Figure 2.3 B). Eight of the nine nucleotides are conserved (Gutell, 1993).

To elucidate the fine structure of the S8 binding site, the site has been studied extensively and by a number of methods including site directed mutagenesis, chemical footprinting, hydroxyl radical footprinting, SELEX (Moine *et al.*, 1997), and nuclear magnetic resonance spectroscopy (NMR) (Kalurachchi *et al.*, 1997; Kalurachchi and Nikonowicz, 1998). On the basis of chemical probing experiments (Mougel *et al.*, 1987) a non-canonical, U598-U641 basepair was suggested but is not supported by NMR experiments (Kalurachchi *et al.*, 1997) nor sequence comparison models (Gutell, 1996). Both NMR and SELEX (Moine *et al.*, 1997) support a base triple between U641·(G597·C643) but the base triple is not supported by comparative analysis. A base triple between A595·(A596·U644) has been identified by NMR and is also predicted by comparative phylogenetic evidence. The deletion of A595 severely retards the binding of protein S8 (Mougel *et al.*, 1993) suggesting that the formation of this structure is essential for S8 binding.

Mutational analysis has established that helices of several basepairs in length surrounding the core are needed for efficient S8 association (Wu *et al.*, 1994). According to NMR studies, protein S8 may form nonspecific contacts with based U591 and G650 at the base of the helix possibly involving the phosphate backbone. The base of the helix has also been cross-linked to protein S8 (Wower and Brimacombe, 1983). NMR has also shown that protein binding occur without significant alterations to the core RNA structure.

2.4 Ribosomal RNA (rRNA) Operons and the *spc* Operon

2.4.1. rRNA Operons

There are seven rRNA operons (designated *rrnA* through *rrnH* except there is no *rrnF*) in *E. coli*. The rRNAs encoded by them are similar. Each operon contains 16S, 23S, and 5S genes in that order (Figure 2.4 A). Between the 16S and 23S and in some operons, at the end of the transcipts, lie tRNA genes. The direction of transcription of rRNA genes matches that of replication, probably to avoid collisions with the replication machinery. The replication machinery does indeed displace RNA polymerase as it passes through the rRNA genes. The progress of the replication fork is significantly impeded when an active rRNA operon and replication are in opposite directions (French, 1992).

There are two promoter regions upstream of the 16S RNA gene in all operons and they are designated P1 and P2 (Figure 2.4 B). For all promoters, the –10 region matches exactly the consensus sequence for recognition by RNA polymerase (TATAAT) (Keener and Nomura, 1996). The –35 region varies slightly from the consensus and there is only 16 base pairs in between the two regions verses 17 base pairs for the consensus. The

Figure 2.4. Generic structure of a ribosomal operon in *E. coli*. A. The coding region for the mature rRNAs. B. The P1 promoter region expanded to show the FIS binding sites, the UP element and the core promoter region (the –35 and –10 region).

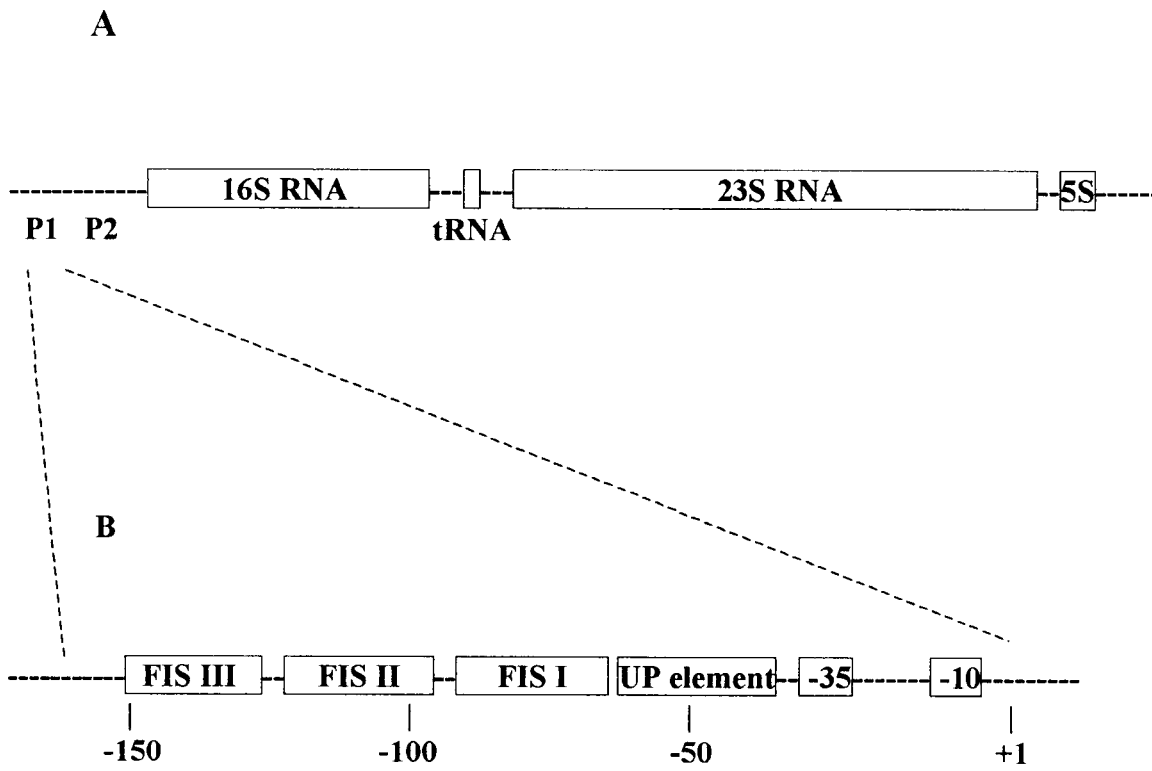

A
16S RNA
23S RNA
5S
P1
P2
tRNA
B
FIS III
FIS II
FIS I
UP element
-35
-10
-150
-100
-50
+1

Figure 2.4

addition of one base pair to the spacer region and mutations that change the –35 region to that of the consensus, greatly increase promoter activity but abolish growth–rate control of transcription. Growth rate control is characterized by the synthesis of rRNA as a consequence of growth in media of different nutritional qualities and conditions that result in a variation of growth rates. These promoter sequences have evolved for regulation and optimal growth under changing conditions as well as high activity.

The P1 promoter of the *rrnB* operon contains an upstream element (UP element) that increases *rrnB* activity by a factor of 30 in the absence of protein factors other than RNA polymerase (Ross *et al.*, 1993). It is a 20-base pair region between –40 and –60 that is AT rich. It binds the α subunit of RNA polymerase. Other P1 promoters also have sequences rich in AT in this region and probably are UP elements as well. The P1 promoter also contains three Factor for Inversion Stimulation (FIS) binding sites upstream of the UP element between bases –60 to –150 (Ross *et al.*, 1990).

Under rapid growth conditions, rRNA synthesis occurs mostly from the *rrn* P1 promoters (Sarmientos *et al.*, 1983) and is sensitive to growth-rate control and stringent control. Stringent control is characterized by the repression of rRNA synthesis and an increase in guanosine tetraphosphate during amino acid starvation. The *rrn* P2 promoters contribute little to the total rRNA transcription at high growth rates (de Boer and Nomura, 1979) but are thought to be basal contributors to rRNA synthesis. These have been shown to be insensitive to growth-rate control but are sensitive to stringent control.

2.4.2. *spc* Operon

The *spc* operon is a polycistronic message that codes for ribosomal and non-ribosomal proteins. It is localized at the classic *str* locus at 73 minutes on the *E. coli*

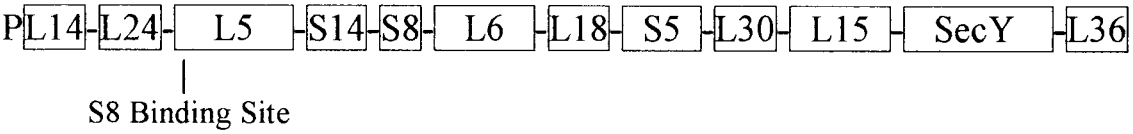
chromosome between the S10 operon and the α operon (Nomura *et al.*, 1984; Lindahl and Zengel, 1986). The order of genes in the *E. coli spc* operon is L14, L24, L5, S14, S8, L6, L18, S5, L30, L15, *secY*, and L36 (Figure 2.A) (Ceretti *et al.*, 1983; Ueguchi *et al.*, 1989). Transcription of the *spc* operon is initiated at a promoter site in the 163- basepair region between the last gene of the S10 operon (S17) and the first gene of the *spc* operon (L14) (Post *et al.*, 1978). Read through of the S10 operon into the *spc* operon has been observed (Henkin *et al.*, 1989).

Expression of the proteins of the *spc* operon is autogenously regulated post-transcriptionally by the binding of excess protein S8 to a target sequence located at the beginning of the L5 gene. This produces translational repression of L5 and through translational coupling, also represses the genes downstream to L5 (Dean *et al.*, 1981; Cerretti *et al.*, 1988). Protein S8 also inhibits the synthesis of protein L14, L24, and L5 by inducing endonucleoytic cleavage downstream of the mRNA target site followed by 3'-5' exonucleolytic degradation (Mattheakis *et al.*, 1989).

Regulation of the *spc* operon in *E. coli* is mediated by the binding of protein S8 to the target mRNA of the L5 gene (Figure 2.5). Most of the regulation element lies at the L5 AUG start site and in the gene itself (Cerretti *et al.*, 1988). Through structure-specific nuclease studies and computer-generated models, a structure for the regulatory binding site has been proposed (Figure 2.5 B). There is a primary and secondary structure similarity between the mRNA and rRNA binding sites (compare Figure 2.3 with Figure 2.5) suggesting that the binding of S8 is mediated by the same elements of the protein. There are differences in the mRNA and rRNA structures in particular, the bases C955 and U952 that are bulged in the mRNA. Deletion experiments in the mRNA and

Figure 2.5. Features of the *spc* operon. A. Transcriptional unit of the *spc* operon. Promoter is designated as P. Open reading frames of proteins are boxed. Genes are in order from left to right. The regulatory S8 binding site is marked. B. The primary and secondary structure of the S8 binding site at the translational start site of protein L5. The translational start site (AUG) is boxed. The structure is that proposed by Cerretti *et al.*, 1988. Compare with figure 2.3.

A


PL14
L24
L5
S14
S8
L6
L18
S5
L30
L15
SecY
L36
S8 Binding Site

B

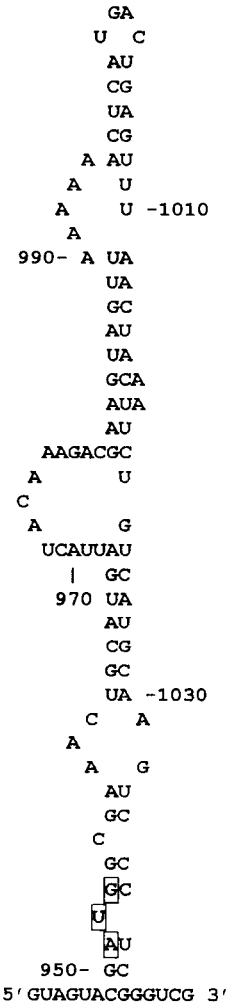

GA
U C
AU
CG
UA
CG
A AU
A U
A U -1010
A
990- A UA
UA
GC
AU
UA
GCA
AUA
AU
AAGACGC
A U
C
A G
UCAUUAU
| GC
970 UA
AU
CG
GC
UA -1030
C A
A
A G
AU
GC
C
GC
GC
U
AU
950- GC
5' GUAGUACGGGUCG 3'

Figure 2.5

insertion experiments in the rRNA have confirmed that these bulged bases modulate the strength of S8-RNA interactions. The affinity for the rRNA is approximately fivefold higher than the mRNA (Wu *et al.*, 1994).

Chapter 3

Materials and Methods

3.1. Growth Curve Studies and Northern Blot Analysis

3.1.1 Phagemid Preparation containing S8 Binding Site, PBSS842-2 and Transformation into Bacterial Cells

Oligoribonucleotides do not penetrate well into bacterial cells. Therefore, to study the *in vivo* effects of the S8-RNA binding site, a phagemid containing the S8 binding site under a *lacZ* promoter was constructed and transformed into bacterial cells as described in the following paragraphs. The S8 binding site RNA was then produced by induction of the *lacZ* gene with IPTG. Growth and RNA were then monitored.

DNA homologous to the S8 binding site on the 16S RNA (bases 590-648 with bases 610-627 deleted, *E. coli* numbering) and its complement were synthesized by the Center for Gene Research and Biotechnology, Oregon State University. The sequences were 42S8-5 (5'-AGC TTT GTT AAG TCA GAT GTG AAA GAA CTG CAT CTG ATA CTG GCA ACT GCA-3') and 42S8-3 (5'-GTT GCC AGT ATC AGA TGC AGT TCT TTC AGA TCT GAC TTA ACA A-3'). They had restriction endonuclease sites synthesized on the ends so they could be ligated into the phagemid. The synthetic DNA was rehydrated and loaded onto a Sephadex G25 (Pharmacia, Piscataway, NJ) column to desalt and to remove failure sequences. One-ml fractions were collected by using a Gilson fraction collector (Middleton, WI) and were monitored spectrophotometrically at 260 nm. Fractions containing the highest A_{260} readings were pooled and dried down

overnight using a LabConco Cenrivap Concentrator (Kansas City, MS). 100 µl of autoclaved distilled water was added and the concentration was determined.

One nanomole of each strand of DNA was subjected to a polynucleotide kinase reaction (New England Biolabs, Beverly, MA). The reaction mixtures were pooled and the temperature raised to 98°C and remained above 85°C for 10 minutes to inactivate the kinase; then the strands were allowed to cool and anneal overnight.

One µg of the PBS+ phagemid (Stratagene, La Jolla, CA) was subjected to endonuclease restriction enzyme digestion using Pst I and Hind III (New England Biolabs, Beverly, MA) then subjected to filtration using a Microcon 30 (Amicon, Beverly, MA) to remove the small sequence between the restriction sites from the mixture. The efficiency of the cut phagemid was monitored by gel electrophoresis using a 1 % agarose 45 mM Tris-borate-1 mM EDTA (TBE) horizontal gel. Uncut PBS+ phagemid was used as a control. The fragments were visualized by ethidium bromide staining. The efficiency was found to be 100 % by visualization.

0.05 picomoles of cut PBS+ phagemid DNA (0.1 µg) and 0.5 picomoles of double stranded synthetic DNA were subjected to a ligation reaction (New England Biolabs, Beverly, MA). As a control, cut PBS+ phagemid DNA with no synthetic DNA was also subjected to a ligation reaction.

XL1-MRF' Blue *E. coli* (Stratagene, La Jolla, CA) was transformed with PBSS842-2 phagemid using the $CaCl_2$-heat shock method described by Sambrook *et al.* (1989a). Transformed cells were plated onto 100 x 15 mm Luria-Bertani (LB) agar plates containing 12.5 µg/ml tetracycline and 60 µg/ml ampicillin. XL1-MRF' Blue are tetracycline resistant and the PBS+ phagemid has an ampicillin resistance marker on it.

The plates were inverted and incubated overnight at 37°C. The transformed cells were then replicate plated onto 100 x 15 mm 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)-IPTG-LB agar plates containing 12.5 μg/ml tetracycline and 60 μg/ml ampicillin. Insert and non-insert-containing colonies were identified by blue-white screening; those without the insert were blue due to cleavage of X-gal by β-galactosidase. Insert-containing colonies were white due to inactivation of β-galactosidase and the inability to cleave X-gal. Select colonies were incubated overnight in LB medium containing 12.5 μg/ml tetracycline and 60 μg/ml ampicillin at 37°C with vigorous shaking (200 r.p.m. on a rotary shaker). Phagemids were isolated from the cells according to Sambrook *et al.* (1989b) and subjected to restriction enzyme analysis on a 3.0 % Metaphor agarose (FMC BioProducts, Rockland, ME) gel in TBE buffer to confirm the insert. Two types of candidates were isolated, one with a putative triple sequence insert and was named PBSS842-1 and others with a putative single sequence insert named PBSS842-2. Colonies with the putative inserts were cultured overnight in LB medium containing 12.5 μg/ml tetracycline and 60 μg/ml ampicillin at 37°C with vigorous shaking (200 r.p.m. on a rotary shaker). The phagemids were purified from cells by using a Qiagen-tip 20 anion-exchange column and plasmid purification protocol (Qiagen, Thousand Oaks, CA). Sequence analysis was performed by the Center for Gene Research and Biotechnology, Oregon State University. The cell line DH5α was also transformed with the phagemid.

3.1.2 Deletion of the Shine-Dalgarno Sequence and Translational Start Site From PBSS842-2 Phagemid with the S8 Binding Site and Transformation into Bacterial Cells

To ensure ribosomes could not bind to the message and unfold the RNA secondary structure, the Shine-Dalgarno sequence and the translational start site from the PBSS842-

2 *lacZ* gene was deleted using polymerase chain reaction (PCR) techniques. Primers lac+27 (5'-GTG TGA AAT TGT TAT CCG CT-3') and lac+58 (5'-CTC GAA ATT AAC CCT CAC TA-3') were synthesized by the Center for Gene Research and Biotechnology, Oregon State University. The primer lac+27 is complementary to coding nucleotide sequence +8 to +27 and lac+58 is complementary to +59 to +78 of the *lacZ* gene on the PBS+ phagemid. Final concentrations of the 100-µl reaction mixture were 1X buffer, 2.5 units *Taq* DNA Polymerase (Life Technologies, Gaithersburg, MD), 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 0.5 µM each primer, and 10 ng PBSS842-2 used as a template. Thermal cycling parameters are shown in Table 3.1. PCR products were resolved on a 1.0 % agarose TBE horizontal gel and visualized by ethidium bromide staining. PCR products were phenol: chloroform extracted and passed through a microcon 30 (Amicon, Beverly, MA) to remove polymerase and primers. The PCR products were then subjected to Klenow fragment (Promega, Madison, WI) reaction to generate blunt ends and ligated together with T4 DNA ligase (New England Biolabs, Beverly, MA) to generate a circular phagemid similar to PBSS842-2 but with a 31-base-pair deletion (ΔPBSS842-2).

XL1-MRF' Blue *E.coli* (Stratagene, La Jolla, CA) cultures were transformed with ΔPBSS842-2 phagemid by using the $CaCl_2$-heat shock method described by Sambrook *et al.* (1989a). Transformed cells were plated onto 100 x 15 mm-LB agar plates containing 12.5 µg/ml tetracycline and 60 µg/ml ampicillin. The plates were inverted and incubated overnight at 37°C. Select colonies were incubated overnight in LB medium containing 12.5 µg/ml tetracycline and 60 µg/ml ampicillin at 37°C with vigorous shaking (200 r.p.m. on a rotary shaker). Phagemids were isolated from the cells according to

Table 3.1. Thermocycling parameters for PCR used to delete the Shine-Dalgarno and translational start site of the *lacZ* gene from the PBS+ phagemid with S8 insert (PBSS842-2).

Cycles	Step	Temperature	Time
1	Denature Anneal Extend	94°C 62°C 72°C	3 minutes 30 seconds 90 seconds
35	Denature Anneal Extend	94°C 62°C 72°C	45 seconds 30 seconds 90 seconds
1	Extend	72°C	10 minutes

Sambrook *et al.* (1989b) and subjected to restriction enzyme analysis on 3.0 % Metaphor agarose gel in TBE buffer to confirm the deletion. A colony of cells containing the putative deletion was cultured overnight in LB medium containing 12.5 μg/ml tetracycline and 60 μg/ml ampicillin at 37°C with vigorous shaking (200 r.p.m. on a rotary shaker). The ΔPBSS842-2 phagemid was purified from cells using Qiagen-tip 20 anion-exchange column and plasmid purification protocol (Qiagen, Thousand Oaks, CA). Sequence analysis was performed by the Center for Gene Research and Biotechnology, Oregon State University.

3.1.3 Growth Curve Analysis

Cells with cloned phagemids were cultured overnight in LB medium containing 12.5 μg/ml tetracycline and 60 μg/ml ampicillin at 37°C with vigorous shaking (200 r.p.m. on a rotary shaker). These overnights were used to inoculate cultures that were grown in the presence or absence of IPTG at 200 r.p.m. on a rotary shaker. Conditions for the growth curves are shown in Table 3.2. Growth was monitored spectrophotometrically at 600 nm. Thiamine was added to the M9 medium for the XL1-MRF' cell line.

3.1.4 Northern Blot Analysis

To analyze phagemid mRNA levels, a 30-base oligonucleotide probe named lacZ was synthesized by the Center for Gene Research and Biotechnology, Oregon State University. The probe sequence: 5'-GTA AAA CGA CGG CCA GTG AAT TGT AAT ACG-3' is complementary to +164 to +194 of the *lacZ* transcriptional start site on the PBS+ phagemid. The synthesized sequence 42S8-3 complementary to the insert on the

Table 3.2. Conditions used in growth experiments.

Phagmid	Cell Type	Temperature (°C)	Media	Culture Volume (ml)	Inoculation Volume (ml)
PBSS842-1	XL1-MRF'	37	LB	50	0.5
PBSS842-1	XL1-MRF'	37	LB	50	1.0
PBSS842-2	XL1-MRF'	37	LB	50	1.0
PBS+	XL1-MRF'	37	LB	50	1.0
ΔPBSS842-2	XL1-MRF'	37	LB	50	1.0
ΔPBSS842-2	XL1-MRF'	24	M9	200	1.0
PBSS842-2	XL1-MRF'	24	M9	200	1.0
PBSS842-2	DH5α	24	M9	200	1.0
ΔPBSS842-2	DH5α	24	M9	200	1.0
ΔPBSS842-2	XL1-MRF'	24	M9	200	1.0
ΔPBSS842-2	DH5α	24	M9	200	1.0
ΔPBSS842-2	DH5α	24	M9	200	1.0
ΔPBSS842-2*	DH5α	24	M9	200	1.0
PBSS842-2	DH5α	24	M9	200	1.0
PBSS842-2*	DH5α	24	M9	200	1.0

* IPTG added at 0.4 A_{600} units.

PBSS842-2 and ΔPBSS842-2 phagemid, called the S8 probe, was also used as a probe. Following synthesis, the lacZ probe was rehydrated in diethylpyrocarbonate (DEPC)-treated autoclaved water and both probes were 5' end-radiolabeled by using $\gamma[^{32}P]$ ATP (6000 Ci/mmol, New England Nuclear, Boston, MA). The end-labeled probes were separated from free $\gamma[^{32}P]$ ATP by filtration through a Centricon 3 (Amicon, Beverly, MA) (Sambrook *et al.*, 1989c). The probes were end-labeled to a specific activity of approximately 10^8-10^9 cpm/μg.

Total RNA was extracted from XL1-MRF' *E. coli* cells according to the method of Reddy (1993). Cells were grown to 0.6 A_{600} units in LB medium with 12.5 μg/ml tetracycline and 60 μg/ml ampicillin (tetracycline only for XL1-MRF' cells with no phagemid) in the presence or absence of IPTG. The cells were shaken vigorously at 37°C. 15 ml of culture was used to isolate RNA. Concentration was determined spectrophotometrically at 260 nm. The protein content of the RNA was monitored by A_{260}/A_{280} nm ratio. Total RNA was analyzed by using a 1.0 % agarose/2.2 M fomaldehyde gel. The quality of RNA was checked by ethidium bromide staining to verify 16S and 23S bands. Gels were washed in distilled water then in 10X SSC (1.5 M NaCl, 0.15 M trisodium citrate). The RNA was transferred overnight to a Zeta-probe membrane (Biorad, Richmond, CA). The membrane was rinsed briefly, and then the RNA was fixed to the membrane by baking at 80°C for 30 minutes. The membrane was pre-hybridized overnight at 50°C in hybridization buffer [20 mM Na_2HPO_4, pH 7.2, 5X SSC, 7 % sodium dodecyl sulfate (SDS), 1X Denhardt's and 100 μg/ml denatured salmon sperm DNA]. Following pre-hybridization, the blot was hybridized for 24 hours to the 5'$[^{32}P]$ end- labeled oligonucleotide probes in hybridization buffer at 50°C. Following

hybridization, the membrane was washed twice at 50°C for 30 minutes in 3X SSC, 10X Denhardt's, 5 % SDS and 25 mM Na_2HPO_4, pH 7.5, and washed once at 50°C for 30 minutes in 1X SSC and 1.0 % SDS. The blot was then sealed in a plastic bag and autoradiographed with Dupont Cronex intensifying screens and Kodak X-Omat film at room temperature. The blots were then stripped by washing 2-3 times at 95°C in 0.1X SSC, 0.5 % SDS and the membranes were reprobed as needed.

3.2. Reconstitution Studies

3.2.1 Purification of 70S Ribosomes, Ribosomal Subunits, and S-150 Enzymes

Using a modification of the methods described by Nierhaus (1990), 70S ribosomes and ribosomal subunits were isolated. Strain D10 *E. coli* was grown in LB medium at 37°C with vigorous shaking to 0.5 A_{600} units. The cells were harvested and centrifuged at 5000 *g* for 10 minutes. Pelleted cells were stored at –80°C. All subsequent steps were performed between 0-4°C.

Cells were thawed in 1:2 (w/v) buffer 3 [20 mM Hepes pH 7.6 at 0°C, 4 mM $MgCl_2$, 30 mM NH_4Cl, 2 mM spermidine, 0.2 mM spermine, 3 mM dithiothreitol (DTT)] and centrifuged at 5000 *g* for 10 minutes. The pellet was resuspended in 1:2 (w/v) buffer 3 and deoxyribonuclease (Worthington Biochemical, Freehold, NJ) was added to 5 μg/ml. The cells were disrupted in a French press at 15,000 p.s.i. The extract was centrifuged at 10,000 *g* for 10 minutes then 30,000 *g* for 40 minutes to remove cell debris. The supernatant was centrifuged for 4 hours at 40,000 r.p.m. in a Beckman (Beckman, Palo Alto, CA) SW 55 Ti rotor to pellet the ribosomes.

To obtain S-150 enzymes the supernatant was centrifuged at 150,000 *g* for 3 hours. The upper two-thirds of the supernatant was used as S-150 enzymes. Protein concentration of the S-150 enzymes was determined as described by Bradford (1976), with bovine serum albumin as a standard.

The ribosomal pellet was resuspended in gradient buffer (0.01 M Tris pH 7.6, 0.05 M KCl, 0.3 mM $MgCl_2$, 4 mM DTT) and dialyzed overnight in 100 volumes of the same buffer. About 300 A_{260} units were subjected to zone centrifugation in 15-30 % linear sucrose gradients in gradient buffer at 27,000 r.p.m. using a Sorvall (Newtown, CT) AH-627 rotor for 14 hours. About 30 fractions were collected per gradient and aliquots of the fractions were measured spectrophotometrically at 260 nm. Fractions containing 30S and 50S subunits respectively were pooled and a 2:1 (v/v) 95 % ice-cold ethanol solution was added while stirring to precipitate the subunits. The suspensions were stored minimally overnight at –20°C and then centrifuged at 10,000 *g* for 40 minutes. The pellets were washed with ice cold 70 % ethanol, resuspended in gradient buffer and subjected again to zone centrifugation under the previous conditions. The pellets were resuspended in buffer 3 to about 300 A_{260} units/ml, clarified by low speed centrifugation, and the solutions were stored at -80°C.

3.2.2 Purification of 30S Proteins and 16S RNA

30S proteins (TP30) and 16S rRNA were isolated from purified 30S particles as previously described (Nierhaus, 1990). 30S subunits in buffer 3 (concentration of 300 A_{260} units/ml or less) were stirred with 0.1 volume of 1 M Mg acetate and 2 volumes glacial acetic acid for 45 minutes at 0°C then centrifuged at 10,000 *g* for 30 minutes. Five volumes of acetone were added to the supernatant and the mixture was stored

minimally overnight at –20°C. The suspension was centrifuged at 10,000 *g* for 30 minutes. The pellet was placed in a desiccator for about 15 minutes to remove the residual acetone then it was resuspended in buffer 5 (6 M urea, 20 mM Tris-HCl pH 7.4, 4 mM Mg acetate, 400 mM NH_4Cl, 0.2 mM EDTA, 4 mM DTT) to a concentration of about 300 equivalent A_{260} units (e.u.)/ml. It was dialyzed in buffer 5 overnight then was dialyzed 3 times against 100 volumes of buffer 4 for 45 minutes each. Concentration was determined spectrophotometrically at 230 nm using the conversion factor of 1 A_{230} unit TP30 = 8 e.u. and the solution was stored in small aliquots at –80°C.

16S rRNA was purified from 30S subunits by the addition of 0.1 volume of 10 % (w/v) SDS, 0.05 volume of 2 % (w/v) bentonite, and 1.0 volume of water-saturated phenol. The mixture was mixed for 10 minutes and then was centrifuged at 10,000 *g* for 10 minutes. 1.0 volume of water-saturated phenol was added to the upper aqueous phase and mixed for 10 minutes. The mixture was centrifuged for 10 minutes at 10,000 *g*. The upper aqueous phase was removed and 2 volumes of 95 % ice-cold ethanol were added to precipitate the RNA. The suspension was stored at –20°C overnight. The mixture was centrifuged for 45 minutes at 10,000 *g*. The pellet was washed with 70 % ice-cold ethanol to remove traces of phenol then resuspended in buffer 7 (10 mM Tris-HCl, pH 7.6 at 0°C, 4 mM Mg acetate). The concentration was determined spectrophotometrically at 260 nm, and the solution stored in small aliquots at –80°C.

3.2.3 Preparation of Oligoribonucleotides

Oligoribonucleotides CT4, CT10, and CT11 (Table 3.3) were synthesized and gel purified by Oligo Therapeutics (Wilsonville, OR). The oligoribonucleotides were

Table 3.3. Sequences of oligoribonucleotides used in reconstitution experiments.

Name	Sequence (5'-3')
16S RNA (590-648)	UUGUUAAGUCAGAUGUGAAAUCCCCGGGCUCACCUGGGAACUGCAUCUGAUACUGGCAA
CT 10*	GUUUGUUAAGUCAGAUG GCAACAUCUGAUACUGGCAAGCC
CT 11*	GGUUUGUAAGUCGAGAUG GCAACAUCUUGAAACUGCAAGCC
CT 4	CAUCUGAUACUGGCAA

* The 3' end nucleotide is a DNA.

shipped lyophilized and stored lyophilized at –80°C until ready to use. The oligoribonucleotides were rehydrated in DEPC-treated autoclaved water. The concentration was determined spectrophotometrically at 260 nm. The oligoribonucleotides were checked for purity by 5'end-labeling using T4 polynucleotide kinase and running on a 12 % polyacrylamide/6 M urea gel. 1 pmole of oligoribonucleotide was incubated with 50,000 cpm $\gamma[^{32}P]$ATP (New England Nuclear, Boston, MA), 10 units of T4 polynucleotide kinase (Sigma Chemicals, St. Louis, MI), 1X kinase buffer and DEPC-treated water to 10 µl for 1 hour at 37°C. The reaction mixtures were loaded onto a 12 % polyacrylamide denaturing gel (Sambrook, *et al.*, 1989d) and electrophoresed for 3 hours at 200V. The gel was autoradiographed by using Kodak X-Omat film. Typical exposure times were 24 hours.

3.2.4 Reconstitution of 30S Subunits

Reconstitutions of 30S subunits were performed similarly to those of Nierhaus (1990). In 50-µl reaction mixtures, 0.625 e.u. of TP30 in Buffer 4 (20 mM Tris pH 7.4 at 0°C, 4 mM Mg acetate, 400 mM NH_4Cl, 0.2 mM EDTA, 4 mM DTT), enough buffer 4 to bring the total volume to 50 µl, and 2 µl of 0.4 M Mg acetate in the presence or absence of the oligoribonucleotide. The reaction was allowed to proceed for 10 minutes at 40°C, then the mixture was put on ice. 0.625 A_{260} units of 16S rRNA in buffer 4 was added, and the reaction mixture incubated for an additional 20 minutes at 40°C, then put on ice. 40-µl aliquots (0.5 A_{260} units) were measured for activity in the polyphenylalanine synthesis assay.

3.2.5 Polyphenylalanine Synthesis Assay

Polyphenylalanine synthesis assays were performed using a modification of Spedding (1990) and Nierhaus (1990). A volume of 40 µl containing 0.5 A_{260} units of reconstituted 30S particles in the presence or absence of oligoribonucleotides, or 40 µl of buffer 6 as a negative control, or 0.5 A_{260} units of non-reconstituted 30S particles as a positive control, was added to the polyphenylalanine synthesis assay. The ionic conditions of the assay were 20 mM Tris, pH 7.8 at 0°C, 11 mM Mg acetate, 160 mM NH_4Cl, and 4 mM β-mercaptoethanol. The assay mixture also contained 1.5 mM ATP (Boehringer Mannheim, Germany), 0.05 mM GTP (Boehringer Mannheim, Germany), 5 mM phosphoenolpyruvate (Boehringer Mannheim, Germany) and [ring-2, 6-^{3}H] Phe (50 Ci/mmole, Moravek Biochemicals, Brea, CA). Each reaction mixture had 3 µg pyruvate kinase (Boehringer Mannheim, Germany), 40 µg $tRNA^{bulk}$ *E. coli* (Boehringer Mannheim, Germany), 100 µg poly U (Boehringer Mannheim, Germany), 240 µg of S-150 enzymes, and 1.0 A_{260} unit of 50S subunits.

The reaction was allowed to proceed for 45 minutes at 30°C. 100 µl of 0.5 % Phe (Schwarz/Mann, Orangeburg, NY) was added to reduce background then 2 ml of 10 % (w/v) trichloroacetic acid (TCA) was added to precipitate the protein. The mixture was incubated for 15 minutes at 90°C to deacylate the tRNA's and to reduce background. The sample was filtered through a 13-mm Millipore GSWP 0.22-µm filter (Millipore Corp., Bedford, MA). The filter was washed with 6 ml of 5 % TCA then again with 3 ml of 5 % TCA. The filter was added to 5 ml of Aquasol (Packard Instrument Company Inc., Meriden, CT) scintillation cocktail and counted in a Beckman scintillation counter (Beckman Instruments Inc., Fullerton, CA).

3.3. Filter-Binding Studies

3.3.1 Preparation of ^{32}P-Labeled 16S rRNA and Oligoribonucleotides

To test whether the oligoribonucleotides had a lower affinity to 30S proteins in the reconstitution than the 16S rRNA to the 30S proteins, filter-binding assays were employed. One picomole of dephosphorylated 16S rRNA, synthesized CT10, and CT11 were 5' end-labeled with $\gamma[^{32}P]$ ATP (6000 Ci/mmol, New England Nuclear, Boston, MA) and T4 polynucleotide kinase (Sigma, St. Louis, MS). The labeled RNA's were separated from free $\gamma[^{32}P]$ ATP by using Centricon-3 filters (Amicon, Beverly, MA).

3.3.2 Filter-Binding Assays

Filter-binding assays were performed similar to Draper *et al.* (1988). To carry out a filter-binding titration, the RNA is renatured separately, incubated at 40°C for 15 minutes in buffer 6. Samples with protein at concentrations from zero to 2.0 μM were incubated with RNA at a total concentration of approximately 170 pM. The total reaction volume was 50 μl and the mixture was incubated for 15 minutes at 4°C in pre-siliconized centrifuge tubes (Intermountain Scientific Corp., Kaysville, UT). The reaction mixture was then filtered through a Millipore 13-mm GSWP 0.22-μm filter (Millipore Corp., MA) pre-wetted with buffer 6 and filtered under gentle suction. The filters were then placed in 5 ml of Aquasol scintillation cocktail (Packard Instruments, Meriden, CT) and counted in a Beckman LS 6000SC scintillation counter (Beckman Instruments Inc., Fullerton, CA).

Chapter 4

Results

4.1 Growth Curve Studies and Northern Blot Analysis

4.1.1. Growth Curve Studies

The PBSS842-2 phagemid was constructed by inserting a short DNA sequence into the PBS+ phagemid. Sequence analysis confirmed the presence and correct sequence of the insert within the PBSS842-2 phagemid (Figure 4.1). The ΔPBSS842-2 phagemid was constructed, through PCR, by deleting the ribosomal binding site within the *lacZ* gene of the PBSS842-2 phagemid. Sequence analysis also confirmed the correct deletion and correct insert sequence of the ΔPBSS842-2 phagemid. Upon sequencing of the PBSS842-1 phagemid, the putative tandem triple insert sequence, it was found the sequence insert was not a full tandem triple insert sequence but in fact a conglomeration of several partial sequence inserts (data not shown). The PBSS842-1 phagemid was not considered further.

Sample growth curves of *E. coli* strain XL1-MRF' with PBSS842-2, ΔPBSS842-2 and PBS+ control phagemid grown in LB medium at 37°C are shown in Figure 4.2. The growth curves are representative of growth experiments with the above phagemids using different growth conditions. The doubling times for these experiments, in the presence of IPTG and their no-IPTG control are shown in Table 4.1. There is no or little increase in doubling time upon induction with IPTG of the phagemids used in these experiments.

E. coli strain XL1-MRF' was used in these experiments because this strain is the host strain for the PBS+ phagemid. Strain XL1-MRF' is $lacI^q$, so the Lac repressor is

Figure 4.1. Sequence of the *lacZ* gene of the PBSS842-2 phagemid. The sequence starts at zero at the *lacZ* transcriptional start point. The S8 binding site insert and the ΔPBSS842-2 deletion are bold. Boxes surround the sequences of the S8 probe and lacZ probe.

5′..AATTGTCAGCGGATAACAATTTCACAC**AGGAAACAGCTATGACCATGATTACGCCAAG**CTCGAAATTA
3′...TTAACAGTCGCCTATTGTTAAAGTGTG**TCCTTTGTCGATACTGGTACTAATGCGGTTC**GAGCTTTAAT

ΔPBSS842-2 Deletion

ACCCTCACTAAAGGGAACAAAAGCT**TTGTTAAGTCAGATGTGAAAGAACTGCATCTGATACTGGCAA**CTGC
TGGGAGTGATTTCCCTTGTTTTCGA**AACAATTCAGTCTACACTTTCTTGACGTAGACTATGACCGTT**GACG

S8 Binding Site Insert
S8 Probe (boxed)

AGGTCGACTCTAGAGGATCCGCGGGTACCGAGCTCGAATTCGCCCTATAGTGAGTCGTATTACAATTCACT
TCCAGCTGAGATCTCCTAGGGGCCCATGGCTCGAGCTTAAGCGGGATATCACTCAGCATAATGTTAAGTGA

LacZ Probe

GGCCGTCGTTTTAC...3′
CCGGCAGCAAAATG...5′

Figure 4.1

Figure 4.2. Growth curve analysis of *E. coli* XL1-MRF'. 50-ml cultures were grown at 37°C in LB medium with a 1.0-ml inoculation of overnight culture. IPTG was added at the start of the analysis. A. Cells containing the PBSS842-2 phagemid. B. Cells containing the ΔPBSS842-2 phagemid. C. Cells containing the PBS+ control phagemid.

A

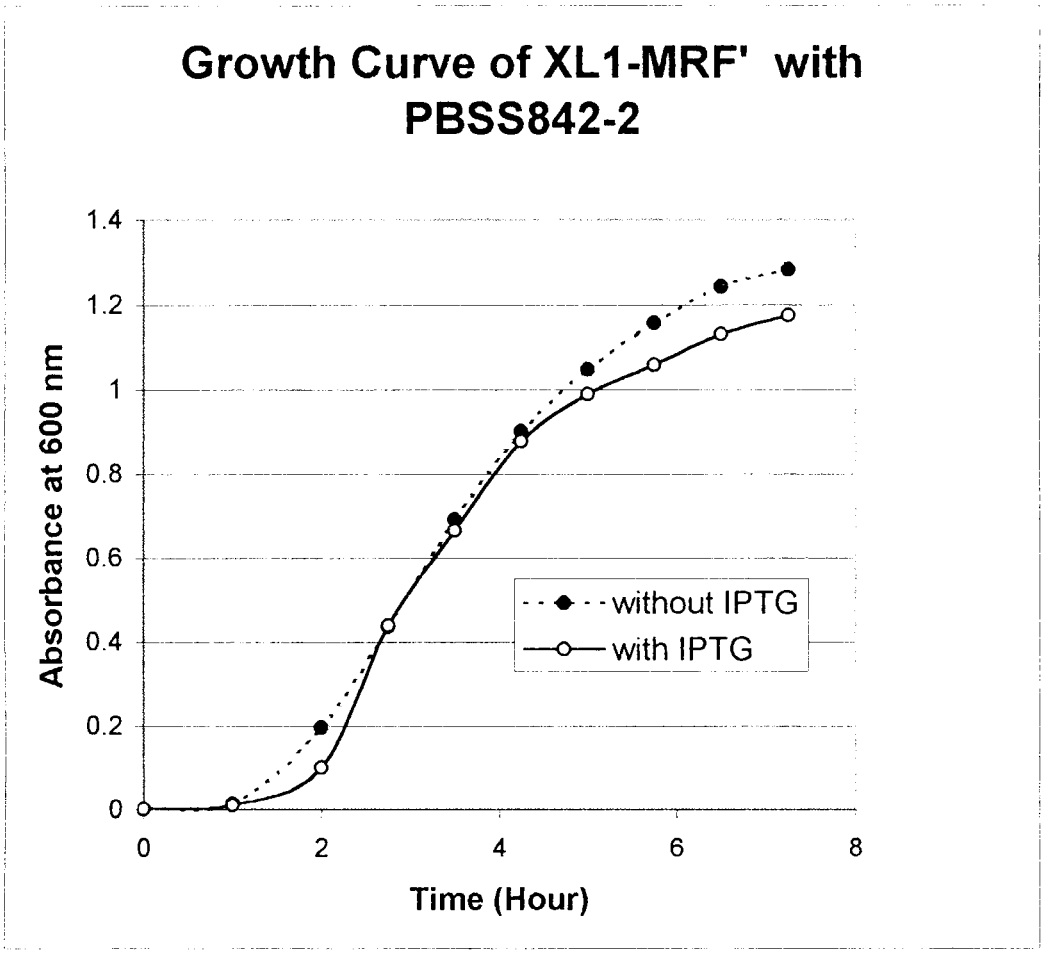

Growth Curve of XL1-MRF' with PBSS842-2
Absorbance at 600 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Time (Hour)
without IPTG
with IPTG

Figure 4.2

B

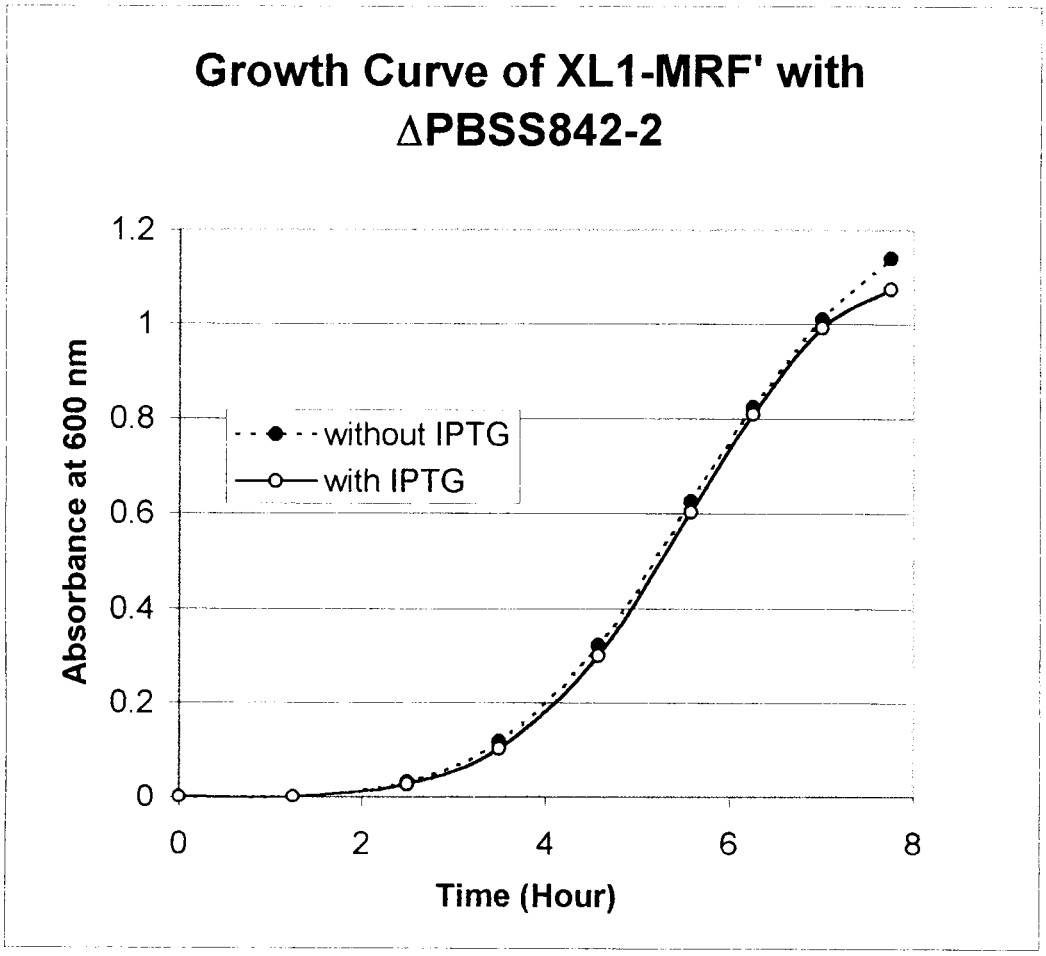


Figure 4.2 con't

C

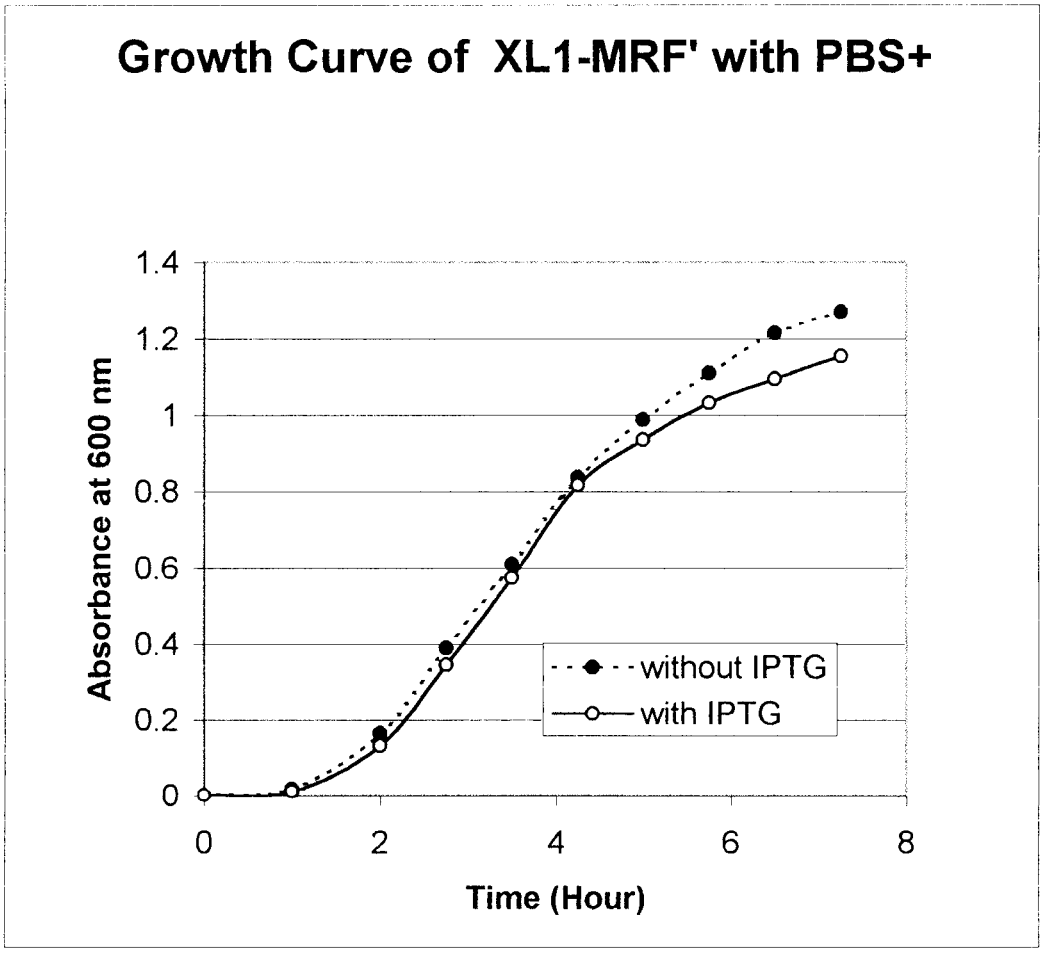


Figure 4.2 con't

Table 4.1. Doubling times of cultures used in growth experiments.

Phagemid	Cell Line	Medium	Temperature (°C)	Doubling Time no IPTG (Hour)	Doubling Time with IPTG (Hour)
PBSS842-1	XL1-MRF'	LB	37	0.64	0.64
PBSS842-1	XL1-MRF'	LB	37	0.62	0.62
PBSS842-2	XL1-MRF'	LB	37	0.79	0.79
PBSS842-2	XL1-MRF'	LB	37	0.83	0.83
PBS+	XL1-MRF'	LB	37	0.74	0.74
PBS+	XL1-MRF'	LB	37	0.74	0.76
ΔPBSS842-2	XL1-MRF'	LB	37	0.73	0.70
ΔPBSS842-2	XL1-MRF'	LB	37	0.73	0.74
ΔPBSS842-2	XL1-MRF'	M9	24	3.9	3.9
PBSS842-2	XL1-MRF'	M9	24	4.4	4.4
ΔPBSS842-2	XL1-MRF'	M9	24	3.3	3.3
ΔPBSS842-2	DH5α	M9	24	2.8	2.8
PBSS842-2	DH5α	M9	24	3.3	3.3
ΔPBSS842-2	DH5α	M9	24	3.3	3.3
ΔPBSS842-2	DH5α	M9	24	3.5	3.7
ΔPBSS842-2*	DH5α	M9	24	3.5	3.7
ΔPBSS842-2*	DH5α	M9	24	3.7	3.7
PBSS842-2	DH5α	M9	24	2.5	2.5
PBSS842-2*	DH5α	M9	24	2	2.5
PBSS842-2*	DH5α	M9	24	2.5	2.5

* IPTG added at 0.4 A_{600} units.

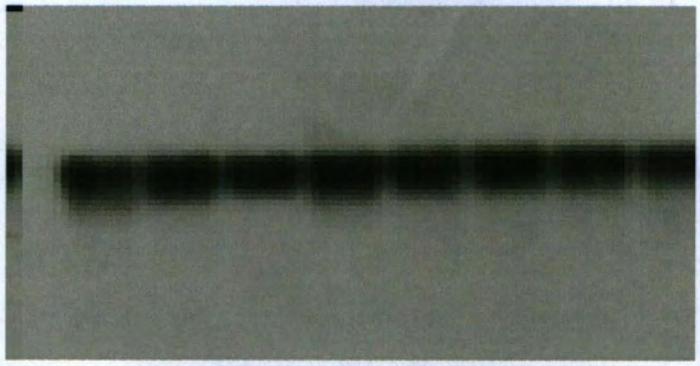
expressed in high amounts. *E. coli* strain DH5α was also transformed with PBSS842-2, ΔPBSS842-2 and PBS+ phagemids and tested in the presence and absence of the inducer IPTG. DH5α expresses the Lac repressor in normal amounts and may have shown an increase in RNA production and in doubling time upon induction of the phagemids with IPTG. Strain DH5α transformed with the PBSS842-2 and ΔPBSS842-2 phagemids also produced no or little increase in doubling time when grown in the presence of the inducer IPTG.

4.1.2. Northern Blot Analysis

Northern blot analysis was used to monitor mRNA levels from the *lacZ* gene of the ΔPBSS842-2, PBSS842-2, and PBS+ phagemid in response to induction with IPTG. *LacZ* mRNA in response to induction with IPTG along with the non-induced controls using the S8 probe is shown in Figure 4.3. The S8 probe is complementary to the S8 binding site of the 16S rRNA and the S8 binding site insert in the ΔPBSS842-2 and PBSS842-2 phagemids. 16S rRNA is a positive control band for all the lanes when using the S8 probe and is seen in each lane. No insert mRNA was demonstrated in the non-induced controls for all the cell lines within exposure time for the experiment (lanes 2, 4, 6, 8). The XL1-MRF' with the PBS+ phagemid and the XL1-MRF' with no phagemid are both negative controls for the insert mRNA when induced with IPTG. No message is seen below the 16S rRNA when induced with IPTG (lanes 5 and 7). A small amount of message is seen below the 16S rRNA in lane 3 (Figure 4.3 B) corresponding to the PBSS842-2 phagemid, with the S8 binding site insert, induced with IPTG. No message corresponding to the insert in the ΔPBSS842-2 induced with IPTG was demonstrated (lane 1).

Figure 4.3. Northern blot analysis of *E. coli* XL1-MRF' using the S8 probe. Lanes 1 and 2 are ΔPBSS842-2 induced with and without IPTG respectively. Lanes 3 and 4 are PBSS842-2 induced with and without IPTG respectively. Lanes 5 and 6 are PBS+ control induced with and without IPTG respectively. Lanes 7 and 8 are control cells with no phagemid with and without IPTG respectively. A. Blot exposed for 3 hours. B. Blot exposed for 6 hours.

A



B

Figure 4.3

LacZ mRNA levels in response to induction with IPTG using the lacZ probe are shown in Figure 4.4. The lacZ probe is complementary to the phagemid *lacZ* mRNA 3' of the insert and includes part of the multiple cloning sequence. No message was demonstrated in the non-induced controls for all cell lines (lanes 2, 4, 6, 8). No message corresponding to the ΔPBSS842-2 *lacZ* mRNA was demonstrated (lane 1). *LacZ* mRNA corresponding to the PBSS842-2 and the positive control for the lacZ probe, the PBS+ phagemid, in response to induction with IPTG was demonstrated in lanes 3 and 5 respectively.

4.2 Reconstitution Studies

30S subunits can be reconstituted *in vitro* from purified 16S rRNA and TP30 (Traub and Nomura, 1969). These reconstituted particles are found to be almost identical to isolated 30S particles in physical properties as well as functional properties, including poly U-directed polyphenylalanine synthesis. Omission of S8 from the mixture results in particles that show an 87-98 % reduction in Phe incorporation. This severe reduction of function was used to test the hypothesis since binding the oligoribonucleotide to S8 was expected to accomplish a similar effect. *In vitro* reconstitutions were performed in the presence of three unmodified oligoribonucleotides (CT10, CT11, and the control CT4). CT4 is a single-strand control oligoribonucleotide that had been shown previously not to bind protein S8 (Mougel *et al.*, 1993). CT10 and CT11 are similar in sequence to the S8 binding site on the 16S rRNA. After reconstitution, the particles were assayed for function. None of the oligoribonucleotides showed a reduction of function when added at up to a 10:1 ratio of the oligoribonucleotide to TP30 (Figure 4.5). The negative control, CT4, was assayed to a 20:1 ratio of oligoribonucleotide to TP30 and showed no reduction

Figure 4.4. Northern blot analysis of *E. coli* XL1-MRF' using the lacZ probe. The lanes are the same as those in Figure 4.3.

Figure 4.4

Figure 4.5. Polyphenylalanine synthesis functional assay of 30S ribosomal subunits reconstituted in the presence of oligoribonucleotides. 0.5 A_{260} units of 30S subunits were tested in triplicate for each ratio of oligoribonucleotide: protein. Columns represent the average of two experiments and the error bars represent the high and low of the experiments. A. Reconstitution in the presence of CT4. B. Reconstitution in the presence of CT10. C. Reconstitution in the presence of CT11.

A

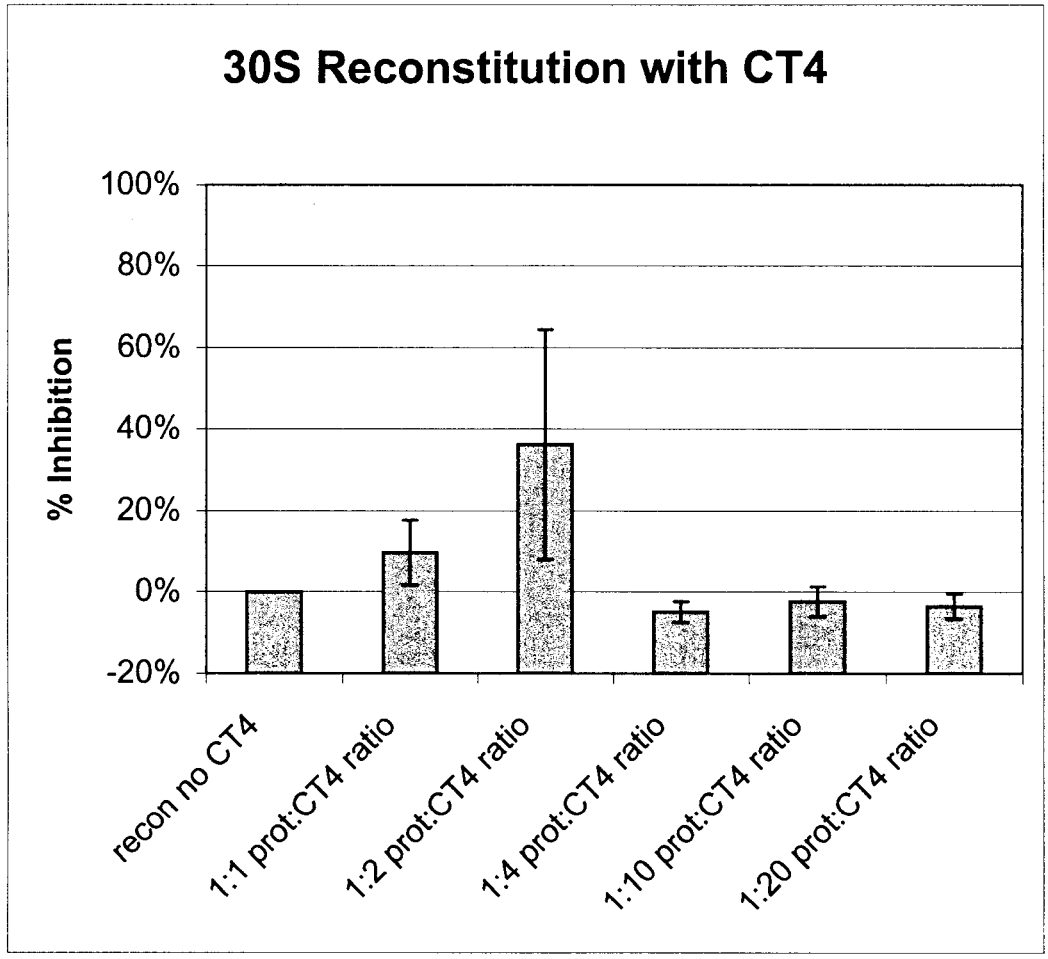

30S Reconstitution with CT4
% Inhibition
100%
80%
60%
40%
20%
0%
-20%
recon no CT4
1:1 prot:CT4 ratio
1:2 prot:CT4 ratio
1:4 prot:CT4 ratio
1:10 prot:CT4 ratio
1:20 prot:CT4 ratio

Figure 4.5

B

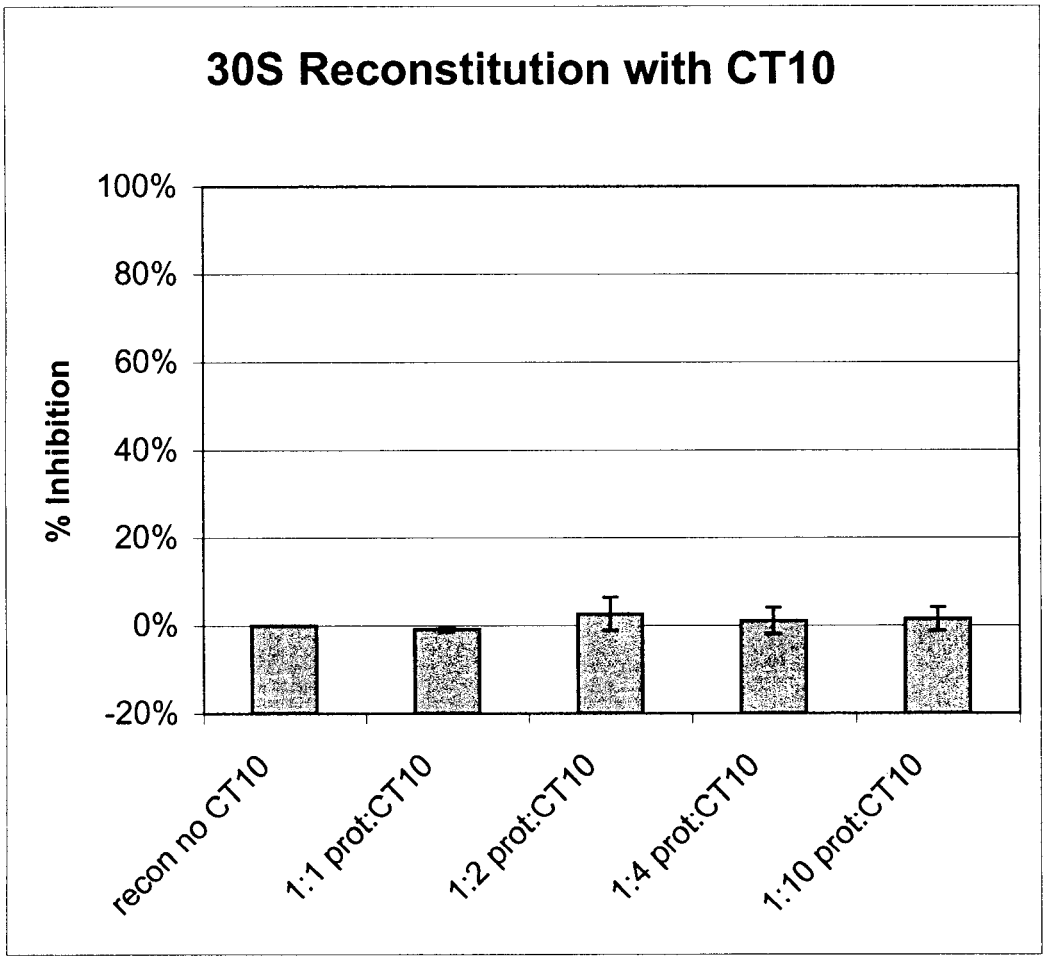
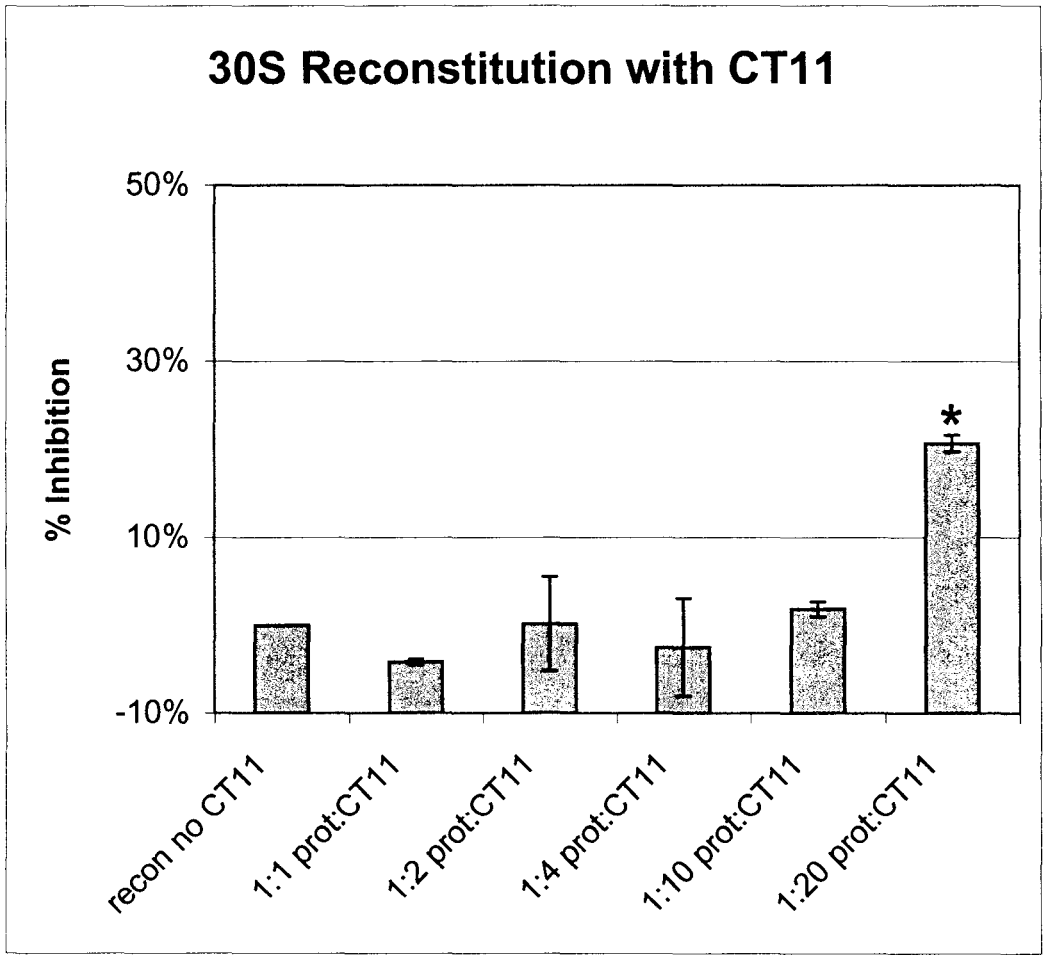


Figure 4.5 con't

C



* p-value < 0.0005

Figure 4.5 con't

of function at that level (Figure 4.5A). CT11 was also assayed to a 20:1 ratio of oligoribonucleotide to TP30 and showed a 20 % decrease of function at that ratio (one-sided p-value < 0.0005; t-test) (Figure 4.5 C).

4.3 Filter Binding Studies

Filter-binding assays were performed to test whether the oligoribonucleotides were binding to a protein in the TP30 mixture (Figure 4.6). Equal amounts of RNA were incubated with increasing amounts of TP30 then filtered under gentle suction. The non-specific retention of radioactivity on the filters averaged 15 % for the oligoribonucleotides and 26 % for the 16S RNA. For each experiment, non-specific bound radioactivity was corrected for. The yield of retention of the complex on the filter averaged 55 %. The oligoribonucleotides CT10 and CT11 reach the similar percent retentions at much higher protein concentrations than the 16S rRNA, signifying a lower affinity for the oligoribonucleotides to the proteins than the 16S rRNA to the proteins. The estimated K_d for both oligoribonucleotides is approximately five times lower than the estimated K_d for the 16S RNA.

Figure 4.6. Filter binding comparisons of oligoribonucleotides with 16S RNA. Approximately 170 picomoles of ^{32}P end-labeled RNA was incubated with increasing amounts of 30S protein at 4°C then filtered under gentle suction. A. CT10 comparison with 16S RNA. B. CT11 comparison with 16S RNA.

A

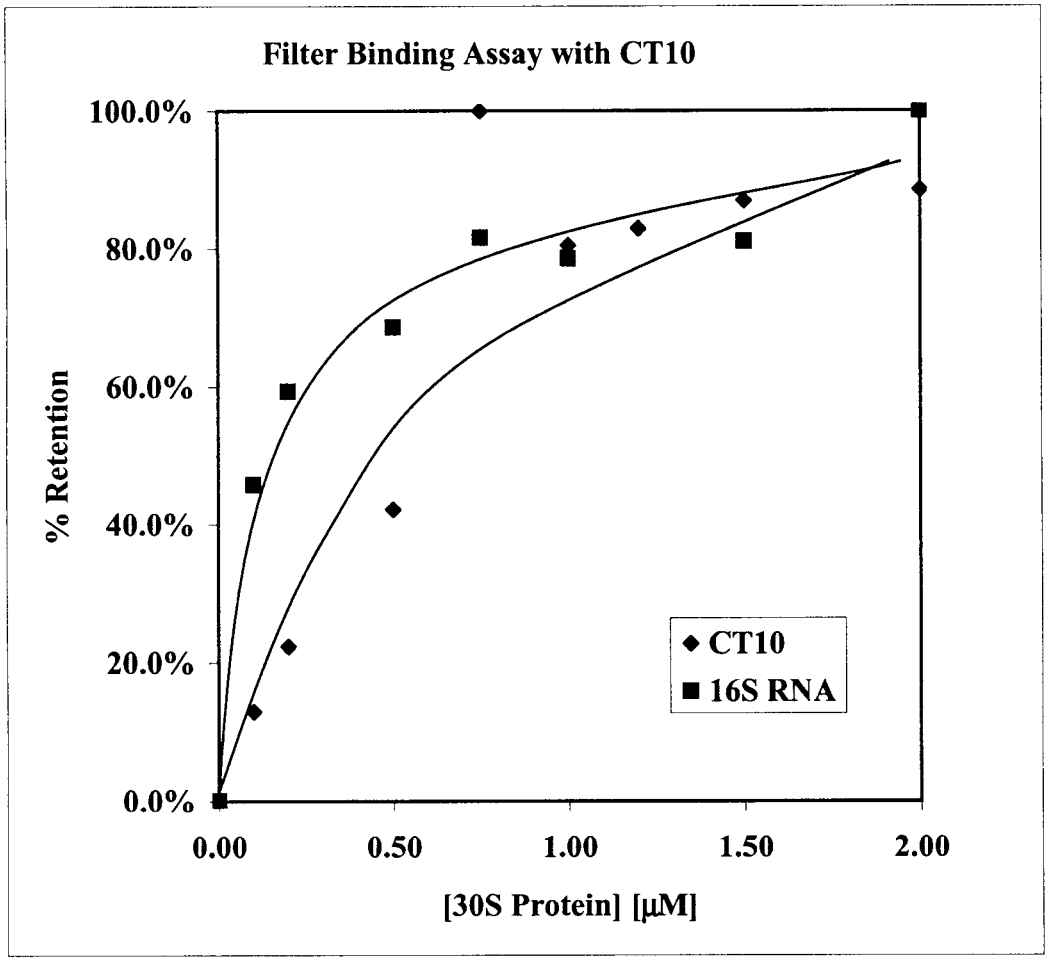

Filter Binding Assay with CT10
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
% Retention
0.00
0.50
1.00
1.50
2.00
[30S Protein] [μM]
CT10
16S RNA

Figure 4.6

B

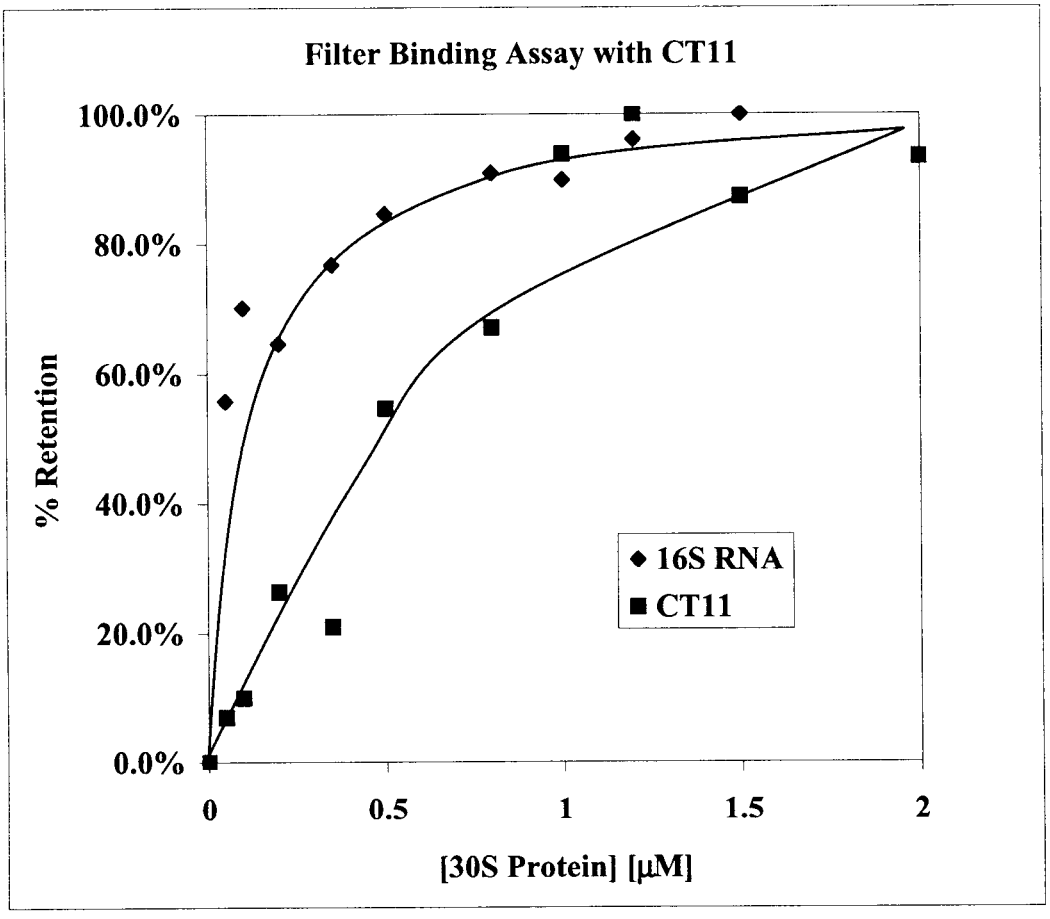


Figure 4.6 con't

Chapter 5

Discussion

5.1 Growth Experiments and Northern Blot Analysis

A difficulty in using analog oligonucleotides as antibiotics is penetration of the bacterial cell membrane. A method to study the inhibition of gene expression in bacteria has been to clone a sequence under an inducible promoter into bacteria. This has been achieved for antisense inhibition of gene expression (Parish and Stoker, 1997) and ribozyme inhibition of gene expression (Sioud and Drlica, 1991; Inokuchi *et al.*, 1994). This method of delivery was utilized in this study.

The PBSS842-2 and ΔPBSS842-2 (the Shine-Dalgarno and AUG start site deleted) phagemids were constructed by inserting a short DNA sequence homologous to the 16S RNA binding site of S8 under a *lacZ* promoter. Upon induction with IPTG, RNA polymerase would synthesize a mRNA that should be able to fold into the proper structure to bind protein S8. The binding of protein S8 to the introduced RNA structure would inhibit the binding of S8 to the 16S RNA. This would cause protein S8 to be unavailable during ribosomal assembly and thus produce incomplete 30S subunits unable to translate. When this mechanism is induced in the cell, the expectation would be an increase in generation time among the bacteria.

Upon induction of the various cell lines including PBS+ controls with IPTG, little or no change in growth was apparent (Table 4.1). This lack of growth inhibition occurred when using two different strains of *E. coli* grown at different temperatures and using different media.

Northern blot analysis was performed to monitor mRNA levels from the *lacZ* gene and the insert of the PBSS842-2, ΔPBSS842-2, and the PBS+ phagemids upon induction with IPTG. The S8 probe is complementary to the binding site of the 16S RNA and the binding site insert in the PBSS842-2 and ΔPBSS842-2 phagemids. A small amount of message is seen below the 16S rRNA in lane 3 of Figure 4.3 corresponding to the PBSS842-2 phagemid, with the S8 binding site induced with IPTG. No message corresponding to the insert in the ΔPBSS842-2 was demonstrated (lane 1 of Figure 4.3).

LacZ mRNA levels using the lacZ probe are shown in Figure 4.4. The lacZ probe is complementary to the phagemid *lacZ* mRNA and includes part of the part of the multiple cloning sequence. *LacZ* mRNA corresponding to the PBSS842-2 and the positive control, the PBS+ phagemid, in response to induction with IPTG, is demonstrated in lanes 3 and 5 in Figure 4.4 respectively. The levels of mRNA appear to be similar in both the control and insert phagemid. No message was demonstrated corresponding to the ΔPBSS842-2 message.

Northern blot analysis demonstrates there is message coming from the insert of the PBSS842-2 phagemid and it is similar to the S8 binding site on the 16S RNA. The amount of message is much less than the amount of 16S RNA in the cell. The small amount is not due to endonucleolytic attack due to S8 binding the insert since the PBS+ control does not have the insert and its *lacZ* message is a similar level.

The ΔPBSS842-2 phagemid was constructed so translating ribosomes could not bind to the message and inhibit folding of the insert binding site or inhibit the binding of S8 to the site. No message from the phagemid was demonstrated under the conditions of the experiment using either of the probes. One reason for this could be due to a mutation

in the leader region of the *lacZ* gene during the PCR reaction. Sequencing of the gene revealed a T →C mutation at position –36. This position in the AT-rich –35 region would produce a down mutation. One would still expect to see some message produced. Other possibilities include a decrease in half-life of the message due to removal of the Shine-Dalgarno and translational start site from the message as well as endonucleolytic cleavage 3' of the bound protein S8 if S8 is indeed bound to the site on the mRNA. S8 has been shown to regulate the synthesis of the first and second gene products of the *spc* operon (L14 and L24) by binding at the mRNA target site (similar to the S8 binding site on the 16S RNA) and inducing endonucleolytic mRNA degradation (Mattheakis *et al.*, 1989). These data together may account for the lack of message from the ΔPBSS842-2 phagemid upon induction with IPTG within the time course of the experiment.

5.2 Reconstitution Studies and Filter-Binding Analysis

In vitro reconstitution assays were employed to test whether the binding of oligoribonucleotides of similar sequence to ribosomal protein binding sites on the 16S RNA could inhibit ribosomal assembly. Since the protein S8 can no longer bind to the 16S RNA, non-functional particles would result and could be assayed for polyphenylalanine synthesis. The oligoribonucleotides CT10 and CT11 are similar to the protein S8 binding site on the 16S RNA. The sequence of the core nucleotides in CT11 was chosen because it showed the same affinity as the homologous or wild type site in SELEX experiments (Moine *et al.*, 1997). CT4 is a single strand control oligoribonucleotide similar in sequence to half of the S8 binding site that had been shown previously not to bind to protein S8 (Mougel *et al.*, 1993).

CT4 was assayed at up to a 20:1 ratio of oligoribonucleotide to TP30 and showed no reduction of function up to that level. CT10 was increased only up to a 10:1 ratio of oligoribonucleotide to TP30 and showed no inhibition of function. CT11 was assayed up to a 20:1 ratio of oligoribonucleotide to TP30 and it showed a 20 % reduction of polyphenylalanine synthesis at that level (one-sided p-value < 0.0005; t-test).

This result was unexpectedly high and filter-binding assays were employed to test whether the oligoribonucleotides could bind a protein in TP30. It was determined that CT11 and CT10 could both bind TP30 but at a lower affinity than the 16S RNA.

5.3 Analog Oligonucleotides as Antibiotics

There have been few reported efforts to use oligonucleotide analogs in bacterial systems (Jayaraman *et al.*, 1981; Miller *et al.*, 1981; Rahman *et al.*, 1991; Chrisey *et al.*, 1993; Rapaport *et al.*, 1996; Good and Nielson, 1998). Oligonucleotide targets have been mRNA, through an antisense mechanism and the anti-Shine-Dalgarno sequence on the 16S RNA.

One hurdle for use of analog oligonucleotides as antibiotics is bacterial cell membrane penetration. Good and Nielsen (1998) found that wild type *E. coli* were hardly affected by peptide nucleic acids (PNAs). A permeable strain of *E. coli* could be inhibited at micromolar levels of antisense PNAs suggesting cell uptake of PNAs is limited. Rapaport *et al.* (1996) studying the antimycobacterial activities of antisense phosphorothioates had to use ethambutol (which induces alterations in the cell wall) and covalently attached either biotin or D-cycloserine to the oligonucleotides to produce growth inhibition in *Mycobacterium smegmatis*. Rahman *et al.* (1991) used

polyethyleneglycol (molecular weight 1000) (PEG-1000) attached to methyl carbamate DNAs of three or four nucleotides in length to inhibit translation in *E. coli* D-10 cells. They also used a cell-wall-less strain of *E. coli* to elicit an effect.

Chrisey *et al.* (1993) studied the internalization of phosphorothioates by *Vibrio parahaemolyticus* by cell fractionation techniques without attempting to permeabilize the cells. The phosphorothioate was found primarily in the periplasm and peptidoglycan layer. There was also a significant amount recovered from the cytosol (up to 1 %) and more so when minimal medium was used, possibly because the bacteria maybe less discriminating when subjected to a starved environment or proteins in the rich media may bind the oligonucleotides making the oligonucleotides unavailable for uptake to the bacteria. Taken together, it is possible that advances in chemical modification of analog oligonucleotides could increase bacterial cell penetration. Chemical modifications have resulted in increased stability and uptake in eukaryotic systems and similar success should be possible for delivery into bacteria.

5.4 Other Antibiotics that Target Ribosome Assembly

There are antibiotics that inhibit ribosomal assembly suggesting that ribosomal assembly is an effective target for antibiotics. Chloramphenicol is one such antibiotic (Osawa *et al.*, 1969). Its cellular target is the binding to the 50S subunit and its action affects the orientation of aminoacyl-tRNA in a way that prevents peptide bond formation. It slows or stops protein synthesis without affecting DNA or RNA synthesis. It also inhibits ribosomal subunit synthesis through the breakdown of cooperative assembly under high rRNA to r-protein ratios (Dodd and Nomura, 1991). Growth inhibition by

chloramphenicol results in incomplete assembly of both ribosomal subunits that is a generalized effect on the translation of mRNA's for all ribosomal proteins.

Other antibiotics that affect ribosomal assembly are erythromycin and other macrolides, including the second-generation macrolides, the ketolides (Chittum and Champney, 1995; Champney and Burdine, 1995; Champney and Tober, 1998). The exact mechanism of protein synthesis inhibition by the macrolides is not well understood; they may inhibit a step in translation between initiation and elongation or affect Psite-dependent binding or dissociation of peptidyl tRNA. The molar concentration of erythromycin required to affect translation *in vitro* is significantly greater than the measured inhibitory concentrations *in vivo,* suggesting a second target in cells (Corcoran, 1984). Studies using different macrolides and ketolides in *Staphylococcus aureus* suggest that there may be related binding sites for macrolide-like compounds, one that affects translational activity, and another on a precursor large subunit particle which leads to assembly inhibition. 50S subunit formation is specifically inhibited. The breakdown of mature 50S subunits is not affected and 30S assembly is not inhibited (Chittum and Champney, 1995). Not all macrolide-like compounds are effective 50S assembly inhibitors potentially due to side groups on the molecules but the additional effect on ribosome assembly may be an important target for drug activity.

5.5 Summary

This thesis project was designed to explore the feasibility of using analog oligoribonucleotides targeting 30S ribosomal subunit assembly as possible antibiotics. The initial approach attempted to bypass the cell's lack of permeability to

oligonucleotides by cloning the site into a vector and transforming it into cells. The lack of growth inhibition upon induction of the phagemid to produce the binding site, and the difficulty in interpreting the events occurring in the cell leading to the lack of inhibition, lead to the pursuit of answering the question through an *in vitro* system.

The aim of the *in vitro* set of experiments was to initially bind unmodified oligoribonucleotides to TP30 in an *in vitro* reconstitution system and test the disruption of assembly through the inhibition of function in the polyphenylalanine synthesis assay. If an oligoribonucleotide tested positive, that is, the inhibition of ribosome function at low concentrations, then a modified oligoribonucleotide of the same sequence would have been tested similarly *in vitro*.

One of the oligoribonucleotides tested (CT11) was found to inhibit function but only at high concentrations. This lead to the test of whether the oligoribonucleotides were in fact binding protein in the reconstitution mixture. It was determined that the oligoribonucleotides were indeed binding protein in the mixture but with a lower affinity than the proteins bind to 16S RNA. 16S RNA is a very abundant molecule in the cell. Under optimal growth conditions, 16S RNA is rapidly produced from the seven rRNA operons. This leads to the overall conclusion that this set of oligoribonucleotides tested can bind TP30 and inhibit ribosome assembly but at concentrations that would be too great to be clinically useful.

Bibliography

Allard, J.D., and Bertrand, K.P. 1993. Sequence of a Class E. tetracycline resistance gene from *Escherichia coli* and comparison of related tetracycline efflux proteins. *Journal of Bacteriology* 175(14): 4554-4560.

Allen, G., and Wittman-Liebold, B. 1978. The amino acid sequence of the ribosomal protein S8 of *Escherichia coli. Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie* 359: 1509-1525.

American Society of Microbiology (ASM) Taskforce on Antibiotic Resistance. 1995. The report is based on a workshop, "Antibiotic Resistance: Current Status and Future Directions," held on July 6, 1994 at the ASM headquarters in Washington DC. Available: http://www.asmusa.org/pasrc/reports.htm [Apr. 14, 2000].

Bakin, A., and Ofengand, J. 1993. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyl transferase center: analysis by the application of a new sequencing technique. *Biochemistry* 32: 9754-9762.

Ballow, C.H., and Amsden, G.W. 1992. Azithromycin: the first azalide antibiotic. *The Annals of Pharmacotherapy* 26: 1253-1261.

Ban, N., Nissen, P., Hansen, J., Capel, M., Moore, P.B., and Steitz, T.A. 1999. Placement of protein and RNA structures into a 5 Å-resolution map of the 50S ribosomal subunit. *Nature* 400: 841-847.

Barritault, H.D., Guerin, M.F., and Hayes, D.H. 1979. Reconstitution of active 30-S subunits *in vitro* using heat-denatured 16-S rRNA. *European Journal of Biochemistry* 98: 567-579.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 86: 142-146.

Brown, R.E., Miller, B., Taylor, W.R., Palmer, C., Bosco, L., Nicola, R.M., Zelinger, J., and Simpson, K. 1995. *Archives of Internal Medicine* 155: 1595-1600.

Bryant, R.E., Fujisawa, T., and Sypherd, P.S. 1974. Ribosomal proteins and ribonucleic acids of ribosome maturation mutants of *Escherichia coli. Biochemistry* 13(10): 2110-2114.

Buck, M.A., Perrault, A.R., and Cooperman, B.S. 1991. The protein composition of reconstituted 30S ribosomal subunits: the effects of single protein omission. *Biochimie* 73: 769-775.

Capel, M.S., Engelman, D.M., Freeborn, B.R., Kjeldgaard, M., Langer, J.A., Ramakrishnan, V., Schindler, D.G., Schneider, D.K., Schoenborn, B.P., Sillers, I.-Y., Yabuki, S., and Moore, P.B. 1987. A complete mapping of the proteins in the small ribosomal subunit of *Escherichia coli. Science* 238: 1403-1406.

Cate, J.H., Yusupov, M.M., Yusupov, G.Z., Earnest, T.N., and Noller, H.F. 1999. X-ray crystal structures of 70S ribosome functional complexes. *Science* 285: 2095-2104.

Ceretti, D.P., Dean, D., Davis, G.R., Bedwell, D.M., and Nomura, M. 1983. The *spc* ribosomal protein operon of *Escherichia coli*: sequence and cotranscription of the ribosomal protein genes and a protein export gene. *Nucleic Acids Research* 11: 2599-2616.

Cerretti, D.P., Mattheakis, L.C., Kearney, K.R., Vu, L., and Nomura, M. 1988. Translational regulation of the *spc* operon in *Escherichia coli*: identification and structural analysis of the target site for S8 repressor protein. 1988. *Journal of Molecular Biology* 204: 309-329.

Champney, W.S., and Burdine, R. 1995. Macrolide antibiotics inhibit 50S ribosomal subunit assembly in *Bacillus subtilis* and *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy* 39: 2141-2144.

Champney, W.S., and Tober, C.L. 1998. Inhibition of translation of 50S ribosomal subunit formations in *Staphylococcus aureus* cells by 11 different ketolide antibiotics. *Current Microbiology* 37: 418-425.

Chittum, H.S., and Champney, W.S. 1995. Erythromycin inhibits the assembly of the large ribosomal subunit in growing *Escherichia coli* cells. *Current Microbiology* 30: 273-279.

Chou, J.H., Greenberg, J.T., and Demple, B. 1993. Posttranscriptional repression of *Escherichia coli* OmpF protein in response to redox stress: Positive control of the *micF* antisense RNA by the *soxRS* locus. *Journal of Bacteriology* 174(4): 1026-1031.

Chrisey, L.A., Walz, S.E., Pazirandeh, M., and Campbell, J.R. 1993. Internalization of oligodeoxyribonucleotides by *Vibrio parahaemolyticus. Antisense Research and Development* 3: 367-381.

Clemons Jr., W.M., May, J.L.C., Wimberly, B.T., McCutcheon, J.P., Capel, M.S., and Ramakrishnan, V. 1999. Structure of a bacterial 30S ribosomal subunit at 5.5 Å resolution. *Nature* 400: 833-840.

Cohen, M.L. 1992. Epidemiology of drug resistance: implications for a post-antimicrobial era. *Science* 257: 1050-1055.

Corcoran, J.W. 1984. Mode of action and resistance mechanisms of macrolides. In *Macrolide Antibiotics,* ed. S. Omura, 231-259. Orlando, Fla: Academic Press.

Culver, G.M., Cate, J.H., Yusupova, G.Z., Yusupov, M.M., and Noller, H.F. 1999. Indentification of an RNA-protein bridge spanning the ribosomal subunit interface. *Science* 285: 2133-2135.

Culver, G.M., and Noller, H.F. 1999. Efficient reconstitution of functional *Escherichia coli* 30S ribosomal subunits from a complete set of recombinant small subunit ribosomal proteins. *RNA* 5: 832-843.

Dahlberg, A.E., Dahlberg, J.E., Lund, E., Tokimatsu, H., Rabson, A.B., Calvert, P.C., Reynolds, F., and Zahalak, M. 1978. Processing of the 5' end of *Escherichia coli* 16S ribosomal RNA. *Proceedings of the National Academy of Science U.S.A.* 75(8): 3578-3602.

Davies, C., Ramakrishnan, V., and White, S.W. 1996. Structural evidence for specific S8-RNA and S8-protein interactions within the 30S ribosomal subunit: ribosomal protein S8 from *Bacillus stearothermophilus* at 1.9 Å resolution. *Structure* 4: 1093-1104.

Dean, D., Yates, J.L., and Nomura, M. 1981. *Eschrichia coli* ribosomal protein S8 feedback regulates part of *spc* operon. *Nature* 289: 89-91.

de Boer, H., and Nomura, M. 1979. *In vivo* transcription of rRNA operons in *Escherichia coli* initiates with purine nucleoside triphosphates at the first promoter and with CTP at the second promoter. *The Journal of Biological Chemistry* 254: 5609-5612.

Dechene, M., Leying, H., and Cullman, W. 1990. Role of the outer membrane for quinolone resistance in enterobacteria. *Chemotherapy* 36: 13-23.

Dodd, J., and Nomura, M. 1991. Lack of complete cooperativity of ribosome assembly *in vitro* and its possible relevance to *in vivo* ribosome assembly and the regulation of ribosomal gene expression. *Biochimie* 73: 757-767.

Dohme, F., and Nierhaus, K.H. 1976. Total reconstitution and assembly of 50S subunits from *Escherichia coli* ribosomes *in vitro. Journal of Molecular Biology* 107: 585-599.

Draper, D.E., Deckman, I.C., and Vartikar, J.V. 1988. Physical studies of ribosomal protein-RNA interactions. In *Methods in Enzymology,* Volume 164, eds. H.F. Noller, Jr. and K. Moldave, 203-220. San Diego, Academic Press.

French, S. 1992. Consequences of replication fork movement through transcriptional units *in vivo. Science* 258: 1362-1365.

Galimand, M., Guiyoule, A., Gerbaud, G., Rasoamanana, B., Chanteau, S., Carniel, E., and Courvalin, P. 1997. Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. *The New England Journal of Medicine* 337(10): 677-680.

Good, L., and Nielsen, P.E. 1998. Antisense inhibition of gene expression in bacteria by PNA targeted mRNA. *Nature Biotechnology* 16: 355-358.

Green, R., and Noller, H.F. 1997. Ribosomes and translation. *Annual Review of Biochemistry* 66: 679-716.

Gutell. R.R. 1993. Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucleic Acids Research* 21(13): 3051-3054.

Gutell, R. R. 1996. Comparative sequence analysis and the structure of 16S and 23S rRNA. In *Ribosomal RNA: Structure, Evolution, Processing, and Function in Protein Biosynthesis, eds.* Robert A. Zimmerman and Albert E. Dahlberg, 111-128. Boca Raton, Fl: CRC Press.

Gutell, R.R., Weiser, B., Woese, C.R., and Noller, H.F. 1985. Comparative anatomy of 16-S-like ribosomal RNA. *Progress in Nucleic Acid Research* 32: 155-211.

Harold, M., and Nierhaus, K.H. 1987. Incorporation of six additional proteins to complete the assembly map of the 50S subunit from *Escherichia coli* ribosomes. *Journal of Biological Chemistry* 262: 8826-8833.

Harrison, P.F., and Lederberg, J. editors. 1998. *Antimicrobial Resistance: Issues and Options*, 75-93. Washington, D.C.: National Academy Press.

Hayes. F., and Hayes, D.H. 1971. Biosynthesis of ribosomes in *E. coli* I. – Properties of ribosomal precursor particles and their RNA components. *Biochimie* 53: 369-382.

Health, Education, and Human Services. 1999. *Antimicrobial resistance: data to assess public health threat from resistant bacteria are limited.* (Publication No. RCED-99-132). Washington , DC: Government Accounting Office.

Held, W.A., Ballou, B., Mizushima, S., and Nomura, M. 1974. Assembly mapping of 30S ribsomal proteins from *Escherichia coli. The Journal of Biological Chemistry* 249(10): 3103-3111.

Held, W.A., Mizushima, S., and Nomura, M. 1973. Reconstitution of *Escherichia coli* 30S ribosomal subunits from purified molecular components. *Journal of Biological Chemistry* 248: 5720-5730.

Held, W. A., and Nomura, M. 1973. Rate-determining step in the reconstitution of *Escherichia coli* 30S ribosomal subunits. *Biochemistry* 12(17): 3273-3281.

Henkin, T.M., Moon, S.H., Mattheakis, L.C., and Nomura, M. 1989. Cloning and analysis of the *spc* ribosomal protein operon of *Bacillus subtilis*: comparison with the *spc* operon of *Escherichia coli. Nucleic Acids Research* 17: 7469-7486.

Henry, C.M. 2000. Antibiotic resistance. *Chemical & Engineering News* 78(10):41.

Higo, K., Held, W., Kahan, L., and Nomura, M. 1973. Functional correspondence between 30S ribosomal proteins of *Escherichia coli* and *Bacillus stearothermophilus. Proceedings of the National Academy of Sciences, U.S.A.* 70: 944-948.

Inokuchi, Y., Yumama, N., Hirashima, A., Nishikawa, S., Ohkawa, J., and Taira, K. 1994. A hammerhead ribozyme inhibits the proliferation of an RNA coliphage SP in *Escherichia coli. Journal of Biological Chemistry* 269(15): 11361-11366.

Jayaraman, K., McParland, K., Miller, P., and T'so, P.O.P. 1981. Selective inhibition of *Escherichia coli* protein synthesis and growth by nonionic oligonucleotides complementary to the 3' end of 16S rRNA. *Proceedings of the National Academy of Science U.S.A.* 78: 1537-1541.

Kaltschmidt, E. 1971. Ribosomal proteins. XIV. Isoelectric points of ribosomal proteins of *E. coli* as determined by two-dimensional polyacrylamide gel electrophoresis. *Analytical Biochemistry* 43: 25-31.

Kaltschmidt, E., and Wittman, H.G., 1970. Number of proteins in small and large ribosomal subunits of *Escherichia coli* as determined by two-dimensional electrophoresis. *Proceedings of the National Academy of Sciences U.S.A.* 67: 1276-1282.

Kalurachchi, K., and Nikonowicz, E.P. 1998. NMR determination of the binding site for ribosomal protein S8 from *Eschrichia coli* 16S rRNA. *Journal of Molecular Biology 280: 639-654*

Kalurachchi, K., Uma, K., Zimmerman, R.A., and Nikonowicz, E.P. 1997. Structural features of the binding site for ribosomal protein S8 in *Escherichia coli* 16S rRNA defined using NMR spectroscopy. *Proceedings of the National Academy of Science, U.S.A.* 94: 2139-2144.

Keener, J., and Nomura, M. 1996. Regulation of ribosome systhesis. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, ed. Frederick C. Neidhardt, 2nd edn. 1417-1431. Washington, DC: ASM Press.

Krzyzosiak, W., Denman, R., Nurse, K., Hellmann, W., Boublik, M., Gehrke, C.W., Agris, P.F., and Ofengand, J. 1987. *In vitro* synthesis of 16S ribosomal RNA containing single base changes and assembly into a functional 30S ribosome. *Biochemistry* 26: 2353-2364.

Lambert, J.M., Boileau, G., Cover, J.A., and Traut, R.R. 1983. Cross-links between ribosomal proteins of 30S subunits in 70S tight couples and in 30S subunits. *Biochemistry* 22: 3913-3920.

Lindahl, L. 1975. Intermediates and time kinetics of the *in vivo* assembly of *Escherichia coli* ribosomes. *Journal of Molecular Biology* 92: 15-37.

Lindahl, L., and Zengel, J.M. 1986. Ribosomal genes in *Escherichia coli. Annual Review of Genetics* 20: 297-332.

Mattheakis, L., Vu, L., Sor, F., and Nomura, M. 1989. Retroregulation of the synthesis of ribosomal proteins L14 and L24 by feedback repressor S8 in *Escherichia coli. Proceedings of the National Academy of Science U.S.A.* 86: 448-459.

Miller, P.S., McParland, K.B., Jayaraman, K., and T'so, P.O.P. 1981. Biochemical and biological effects of nonionic nucleic acid methyl phosphonates. *Biochemistry* 20: 1874-1880.

Mizushima, S., and Nomura, M. 1970. Assembly mapping of 30S ribosomal proteins from *E. coli. Nature* 226: 1214-1218.

Moine, H., Cachia, C. Westhof, E., Ehresmann, B., and Ehresmann, C. 1997. The RNA binding site of S8 ribosomal protein of *Escherichia coli*: selex and hydroxyl radical probing studies. RNA 3: 255-268.

Mougel, M., Allmang, C., Eyermann, F., Cachia, C., Ehresmann, B., and Ehresmann, C. 1993. Minimal 16S rRNA binding site and role of conserved nucleotides in *Escherichia coli* ribosomal protein S8 recognition. *European Journal of Biochemistry* 215: 787-92.

Mougel, M., Ehresmann, B., and Ehresmann, C. 1986. Binding of *Escherichia coli* ribosomal protein S8 and 16S rRNA: kinetic and thermodynamic characterization. *Biochemistry* 25: 2756-2765.

Mougel, M., Eyermann, F., Westhof, E., Romby, P., Expert-Bezançon, A., Ebel, J.-P., Ehresmann, B., and Ehresmann, C. 1987. Binding of *Escherichia coli* ribosomal protein S8 and 16S rRNA. A model for the interaction and the tertiary structure of the RNA binding site. *Journal of Molecular Biology* 198: 91-107.

Nashimoto, H., Held, W., Kaltschmidt, E., and Nomura, M. 1971. Structure and function of bacterial ribosomes XII. Accumulation of 21S particles by some cold-sensitive mutants of *Escherichia coli. Journal of Molecular Biology* 62: 121-138.

Neu, H.C. 1992. The crisis in antibiotic resistance. *Science* 257: 1064-1073.

Nevskaya, N., Tishchenko, S., Nikulin, A., Al-Karadagh, S., Liljas, A., Ehresmann, B., Ehresmann, C., Garber, M., and Nikonov, S. 1998. Crystal structure of ribosomal protein S8 from *Thermus thermophilus* reveals a high degree of structural conservation of a specific RNA binding site. *Journal of Molecular Biology* 279: 233-244.

Nierhaus, K.H. 1991. The assembly of prokaryotic ribosomes. *Biochimie* 73: 739-755.

Nierhaus, K.H. 1990. Reconstitution of ribosomes. In *Ribosomes and Protein Synthesis: A Practical Approach,* ed. G . Spedding, 161-190. New York: IRL Press.

Nierhaus, K.H., and Dohme, F. 1974. Total reconstitution of functionally active 50S ribosomal subunits from *Escherichia coli. Proceedings of the National Academy of Science U.S.A.* 71: 4713-4717.

Nierhaus, K.H., Bordasch, K., and Homann, H.E. 1973. Ribosomal proteins XLIII. *In vivo* assembly of *Escherichia coli* ribosomal proteins. *Journal of Molecular Biology* 74: 587-597.

Nomura, M., and Erdmann, V.A. 1970. Reconstitution of 50S ribosomal subunits from dissociated molecular components. *Nature* 228: 744-748.

Nomura, M., Gourse, R., and Baughman, G. 1984. Regulation of the synthesis of ribosomes and ribosomal components. *Annual Review of Biochemistry* 53: 75-117.

Nomura, M., Mizushima, S., Ozaki, M., Traub, P. and Lowry, C.V. 1969. Structure and function of ribosomes and their molecular components. *Cold Spring Harbor Symposium on Quantitative Biology* 34: 49-61.

Nomura, M., Traub, P., and Bechmann, H. 1968. Hybrid 30S ribosomal particles reconstituted from components of different bacterial origins. *Nature* 219: 793-799.

Nowotny, V., and Nierhaus, K.H. 1988. Assembly of the 30S subunit from *Escherichia coli* ribosomes occurs via two assembly domains which are initiated by S4 and S7. *Biochemistry* 27: 7051-7055.

Nowotny, V., and Nierhaus, K.H. 1982. Initiator proteins for the assembly of the 50S subunit from *Escherichia coli* ribosomes. *Proceedings of the National Academy of Science U.S.A.* 79: 7238-7242.

Osawa, S., Otaka, E., Itoh, T., and Fukui, T. 1969. Biosynthesis of 50S ribosomal subunit in *Escherichia coli. Journal of Molecular Biology* 40: 321-351.

Parish, T., and Stoker, N.G. 1997. Development and use of a conditional antisense mutagenesis system in mycobacteria. *FEMS Microbiological Letters* 154(1): 151-157.

Phelps, C.E. 1989. Bug/Drug resistance: Sometimes less is more. *Medical Care.* 27(2): 194-203.

Poole, K., Krebes, K. McNally, C., and Neshat, S. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *Journal of Bacteriology* 175: 7363-7372.

Post, L.E., Arfsten, A.E., Reusser, F., and Nomura, M. 1978. DNA sequences of promoter regions for the *str* and *spc* ribosomal protein operons in *E. coli*. *Cell* 15: 215-229.

Powers, T., Daubresse, G., and Noller, H.F. 1993. Dynamics of *in vitro* assembly of 16S rRNA into 30S ribosomal subunits. *Journal of Molecular Biology* 232: 362-374.

Rahman, M.A., Summerton, J., Foster, E., Cunningham, K., Stirchak, E., Weller, D., and Schaup, H.W. 1991. Antibacterial activity and inhibition of protein synthesis in *Escherichia coli* by antisense DNA analysis. *Antisense Research and Development* l: 319-327.

Ramakrishnan, V., and White, S.W. 1992. The structure of ribosomal protein S5 reveals sites of interation with 16S RNA. *Nature* 358: 768-771.

Rapaport, E., Levina, A., Metelev, V., and Zoumecnik, PC. 1996. Antimycobacterial activities of antisense oligodeoxynucleotide phosphorothioates in drug-resistant strains. *Proceedings of the National Academy of Science U.S.A.* 93: 709-713.

Reddy, K.J. 1993. Rapid isolation of RNA from gram-negative bacteria. In *Current Protocols in Molecular Biology*, eds. F. M. Ausubel, J.G. Seidman, J.A. Smith, and K. Struhl., 4.4.4-4.4.7. New York: John Wiley and Sons.

Röhl, R. and Nierhaus, K.H. 1982. Assembly map of the large subunit (50S) of *Escherichia coli* ribosomes. *Proceedings of the National Academy of Science U.S.A.* 79: 729-733.

Röhl, R., Roth, H.E., and Nierhaus, K.H. 1982. Inter-protein dependences during assembly of the 50S subunit from *Escherichia coli* ribosomes. *Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie* 363: 143-157.

Ross, W., Gosinle, R.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K., and Gourse, R.L. 1993. A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. *Science* 262-1407.1413.

Ross, W., Thompson, J.F., Newland, J.T., and Gourse, R.L. 1990. *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. *The EMBO Journal* 9 (11): 3733-3742.

Sambrook, J., Fritsch, E.F., and Maniatus, T. 1989a. Protocol II: Fresh competent *E. coli* prepared using calcium chloride. In *Molecular Cloning. A Laboratory Manual,* 2nd edn., 1.82-1.84. New York: Cold Spring Harbor Laboratory Press.

Sambrook, J., Fritsch, E.F., and Maniatus, T. 1989b. Small-scale preparation of plasmid DNA. In *Molecular Cloning. A Laboratory Manual,* 2nd edn., 1.25-1.28. New York: Cold Spring Harbor Laboratory Press.

Sambrook, J., Fritsch, E.F., and Maniatus, T. 1989c. Labeling of synthetic oligonucleotides by phosphorylation with bacteriophage T4 polynucleotide kinase. In *Molecular Cloning. A Laboratory Manual,* 2nd edn., 11.31-11.32. New York: Cold Springs Harbor Laboratory Press.

Sambrook, J., Fritsch, E.F., and Maniatus, T. 1989d. Recovery of synthetic oligonucleotides by electrophonesis through a denaturing polyacrylamid gel. In *Molecular Cloning. A Laboratory Manual,* 2nd edn., 11.23-11.28. New York: Cold Spring Harbor Laboratory Press.

Sarmientos, P., Contente, S., Chinali, G., and Cashel, M. 1983. Ribosomal RNA operon promoters P1 and P2 show different regulatory responses. In *Gene Expression: Proceedings of a Cetos-UCLA Symposium* held at Park City, Utah, March 26 – April 1, 1983, eds. D.H. Hamer and M.J. Rosenberg, 65-74. New York: Alan R. Liss, Inc.

Schaup, H.W., Sogin, M.L., Kurland, C.G., and Woese, C.R. 1973. Localization of a binding site for ribosomal protein S8 within the 16S ribosomal ribonucleic acid of *Escherichia coli*. *Journal of Bacteriology* 115: 82-87.

Schwarzbauer, J., and Craven, G.R. 1981. Apparent association constants for *E. coli* ribosomal proteins S4, S7, S8, S15, S17 and S20 binding to 16S RNA. *Nucleic Acids Research* 9: 2223-2237.

Shaw, K.J., Rather, P.N., Hare, R.S., and Miller, G.H. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews* 57: 138-163.

Sieber, G., and Nierhaus, K.H. 1978. Kinetic and thermodynamic parameters of the assembly *in vitro* of the large subunit from *Escherichia coli* ribosomes. *Biochemistry* 17: 3505-3511.

Sioud, M., and Drlica, K. 1991. Prevention of human immunodeficiency virus type 1 integrase expression in *Escherichia coli* by a ribozyme. *Proceedings of the National Academy of Science USA* 88: 7303-7307.

Spedding, G., 1990. Isolation and analysis of ribosomes from prokaryotes, eukaroytes, and organelles. In *Ribosomes and Protein Synthesis: A Practical Approach*, ed. G. Spedding, 1-30. New York: IRL Press.

Spillman, S., Dohme, F., and Nierhaus, K.H. 1977. Assembly *in vitro* of the 50S subunit from *Escherichia coli* ribosomes: proteins essential for the first heat-dependent conformational change. *Journal of Molecular Biology* 115: 513-523.

Stern, S., Powers, T., Changchien, L.-M., and Noller, H.F. 1989. RNA-protein interactions in 30S ribosomal subunits: folding and function of 16S rRNA. *Science* 244: 783-790.

Tindall, S.H., and Aune, K.C. 1981. Assessment by sedimentation equilibrium analysis of a heterologous macromolecular interaction in the presence of self-association: interaction of S5 with S8. *Biochemistry* 20: 4861-4866.

Traub, P., and Nomura, M. 1969. Structure and function of *Escherichia coli* ribosomes. *Journal of Molecular Biology* 40: 391-413.

Ueguchi, C., Wittekind, M., Nomura, M., Akiyama, Y., and Ito, K. 1989. The *secY-rpmJ* region of the *spc* ribosomal protein operon in *Escherichia coli*: structural alterations affecting *secY* expression. *Molecular and General Genetics* 217:1-5.

Uma, K., Nikonowicz, E.P., Kaluarachchi, K., Wu, H., Wower, I.K., and Zimmerman, R.A. 1995. Structural characterization of *Escherichia coli* ribosomal protein S8 and its binding site in 16S ribosomal RNA. *Nucleic Acids Symposium Series 33*: 8-10.

Ungewickell, E., Garrett, R., Ehresmann, C., Steigler, P., and Fellner, P. 1975. An investigation of the 16-S RNA binding sites of ribosomal proteins S4, S8, S15 and S20 from *Escherichia coli. European Journal of Biochemistry* 51: 165-180.

Ventor, C. 1998. How can the sciences help? In *Antimicrobial Resistance: Issues and Options*, eds. P.F. Harrison and J. Lederberg, 26-27. Washington, D.C.: National Academy Press.

Vysotskaya, N., Tischenko, S., Garber, M., Kern, D., Mougel, M., Ehresmann, C., and Ehresmann, B. 1994. The ribosomal protein S8 from *Thermus thermophilus* VK1: sequencing of the gene, overexpression of the protein in *Escherichia coli* and interaction with rRNA. *European Journal of Biochemistry* 223: 437-445.

Walsh, C.T. 1993. Vancomycin resistance: Decoding the molecular logic. *Science* 261: 308-309.

Williams, A.D., Lambert, P.A., and Singleton, P. 1996. *Antimicrobial Drug Action.* London: Bios Scientific Publishers.

Winker, S. and Woese, C.R., 1991. A definition of the domains *archaea, bacteria* and *eucarya* in terms of small subunit ribosomal RNA characteristics. *Systematic and Applied Microbiology* 14: 305-310.

Wower, I., and Brimacombe, R. 1983. The localization of multiple sites on 16S RNA which are cross-linked to proteins S7 and S8 in *Escherichia coli* 30S ribosomal subunits by treatment with 2-iminothiolane. *Nucleic Acids Research* 11: 1419-1437.

Wower, I., Kowaleski, M.P., Sears, L.E., and Zimmerman, R.A. 1992. Mutagensis of ribosomal protein S8 from *Escherichai coli*: defects in regulation of the *spc* operon. *Journal of Bacteriology* 174: 1213-1221.

Wu, H., Jiang, L., and Zimmerman, R.A. 1994. The binding site for ribosomal protein S8 in 16S rRNA and *spc* mRNA from *Escherichia coli*: minimum structural requirements and the effects of single bulged bases on S8 RNA interaction. *Nucleic Acids Research* 22: 1687-1695.

Zimmerman, R.A., Mackie, G.A., Muto, A., Garrett, R.A., Ungewickell, E., Ehresmann, C., Stiegler, P., Ebel, J.-P., and Fellner, P. 1975. Location and characteristics of ribosomal protein binding sites in the 16S RNA of *Escherichia coli. Nucleic Acids Research* 2: 279-300.