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Characterization of Two Temperature-sensitive Mutants of Escherichia Coli Exhibiting an Altered L22 Ribosomal Protein

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Burnette-Vick, Bonnie Aldine, Ph.D.

East Tennessee State University, 1991

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CHARACTERIZATION OF TWO TEMPERATURE-SENSITIVE MUTANTS OF *ESCHERICHIA COLI* EXHIBITING AN ALTERED L22 RIBOSOMAL PROTEIN

A Dissertation

Presented to

the Faculty of the Department of Biochemistry

James H. Quillen College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biomedical Sciences

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by

Bonnie Burnette-Vick

August 1991

APPROVAL

This is to certify that the Graduate committee of

Bonnie Burnette-Vick

met on the

Tenth day of June, 1991

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

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Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean of the Graduate School

CHARACTERIZATION OF TWO TEMPERATURE-SENSITIVE MUTANTS OF *ESCHERICHIA COLI* EXHIBITING AN ALTERED L22 RIBOSOMAL PROTEIN

by
Bonnie Burnette-Vick

Analysis of *E. coli* strains SK1047 and SK1048 have shown them to be temperature-sensitive, protein-synthesis deficient. An alteration in ribosomal protein L22 was detected in both strains using two dimensional gel electrophoresis. Protein L22 was purified from both strains by reversed phase high performance liquid chromatography and from two dimensional electrophoretic gels. Purified ribosomal protein L22 was labeled by reductive methylation and used in 23S RNA binding assays with and without ribosomal protein L4. At the permissive temperature, protein L22 from SK1047 bound less efficiently than the control while protein L22 from SK1048 bound as efficiently as the control. At the restrictive temperature, both forms of mutant protein L22 bound less efficiently than the control.

In both mutants, temperature sensitivity was mapped to the chromosomal region containing the *rplV* gene for ribosomal protein L22 using bacteriophage P1 transduction and bacteriophage λ complementation. The wild type *rplV* gene subcloned into plasmid pLF1.0 was also shown to complement temperature sensitivity. The partial diploid nature of strains complemented by λfus2 and plasmid pLF1.0 was verified when both wild type and mutant protein L22 were found on two dimensional gels. Reisolation of protein L22 from gels of λfus2 complemented cells showed that both forms of protein L22 were in equal proportion irrespective of growth temperature. Reisolation of protein L22 from gels of plasmid pLF1.0 complemented cells showed that incorporation of the mutant protein exceeded the control protein at the permissive temperature; while the reverse was seen at the restrictive temperature. Temperature-shift experiments were conducted on complemented mutant cells to determine the effect of increased gene dosage on the coordinated regulation of ribosomal protein synthesis. Mutants complemented with λfus2 exhibited normal cell growth, indicating that regulation was not effected. Cells transformed with plasmid pLF1.0 exhibited a reduction in growth possibly due to the disruption of balanced synthesis.

The wild type and both mutant *rplV* genes were amplified using polymerase chain reaction and the PCR product was sequenced using primer extension. Sequencing of DNA from both mutants revealed the codon CGC for the amino acid arginine at position 8 in the protein chain was mutated to the TGC codon for the amino acid cysteine. The wild type ribosomal protein L22 contains no cysteine residues. The mutation was confirmed by testing control and mutant protein L22 for the presence of sulfhydryls using 4,4′-dithiodipyridine. Ribosomal protein L22 isolated from both mutant strains was found to contain one cysteine sulfhydryl group.

ACKNOWLEDGMENT

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To the next generation Harold, Dumi, Lin, and Steve may your experiments work the first time.

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ABBREVIATIONS

A site	aminoacyl site
ATP	adenosine 5′-triphosphate
aro E	gene coding for dehydroshikimate reductase
B. subtilis	*Bacillus subtilis*
bp	base pair
BSA	bovine serum albumin
CTAB	0.7 M NaCl, 10% hexadecyltrimethyl ammonium bromide
DEAE	diethylaminoethyl derivative of Sephadex
d	doubling time
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EF-G	elongation factor G
EF-Ts	elongation factor Ts
EF-Tu	elongation factor Tu
EtBr	ethidium bromide
ETOH	ethanol
ETSH	2-mercaptoethanol

g	gravity
gm	grams
GTP	guanosine 5′-triphosphate
HOAC	acetic acid
HPLC	high-performance liquid chromatography
IF-1	initiation factor 1
IF-2	initiation factor 2
IF-3	initiation factor 3
k	specific growth rate constant
kb	kilobase pair
mRNA	messenger ribonucleic acid
P site	peptidyl site
PCR	polymerase chain reaction
4-PDS	4,4′-dithiodipyridine
PEG	polyethylene glycol
pmol	picomole
PPO	2,5-diphenyoxazole
R buffer	10 mM Tris-HCl pH 7.6, 10 mM $MgOAc_2$, 50 mM NH_4Cl, 0.2 mM ETSH
RF-1	release factor 1
RF-2	release factor 2
RNA	ribonucleic acid
RNase	ribonuclease
rplV	L22 ribosomal protein gene

rpsL	S12 ribosomal protein gene
rpsE	S5 ribosomal protein gene
rRNA	ribosomal ribonucleic acid
S	Svedgerg units
S-buffer	10 mM Tris-HCl pH 7.6, 0.5 mM $MgOAc_2$, 50 mM NH_4Cl, 0.2 mM ETSH
SET	20% sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA
SDS	sodium dodecyl sulfate
STEP	0.5% SDS, 50 mM Tris-HCl pH 7.5, 20 mM EDTA, 100 µg/ml proteinase K
TB	typtone broth
TBE	89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3
TCA	trichloroacetic acid
TEG	25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose
TEMED	N,N,N′,N′-tetramethylethylenediamine
TFA	trifluoroacetic acid
TM-4	20 mM Tris-HCl pH 7.6, 4 mM $MgOAc_2$, 400 mM NH_4Cl
ts	temperature sensitive
tr	temperature resistant
tRNA	transfer ribonucleic acid

Tris	tris(hydroxymethyl)aminomethane
$T_{10}E_1$	10 mM Tris-HCl pH 8.4, 1 mM EDTA
$T_1E_{0.1}$	1 mM Tris-HCl pH 8.4, 0.1 mM EDTA
UV	ultraviolet

Chapter 1

INTRODUCTION

The central dogma of molecular biology states that DNA is transcribed into RNA which is translated into protein (Crick, 1958). The last step of this process, the translation of mRNA into protein, has been the focus of much research. Early research investigated the process of translation in the gram negative bacterium Escherichia coli. Studies using this model organism have provided a core of information about translation and have begun to reveal details of the structures involved in this complex process and their functions.

Translation begins with the formation of single stranded RNA products from the transcription of genomic DNA called messenger RNA (Miller, 1973). Most E. coli messenger RNAs are polycistronic, meaning that the mRNA transcript contains the information for the synthesis of more than one protein. An examination of the structure of E. coli mRNA shows that, 7-15 bases before each AUG initiation site, there is a purine rich sequence called the Shine-Dalgarno sequence (Shine and Dalgarno, 1974). This sequence allows the mRNA to attach to the machinery for translation (i.e. the 30S ribosomal subunit) at the correct starting position.

It insures that the message is read in the 5′ to 3′ direction allowing the protein to be synthesized from the amino terminus to the carboxyl terminus. The Shine-Dalgarno sequence is followed by the coding regions for the protein to be synthesized. Each protein has its own start and stop codons.

To convert the mRNA code to the amino acid sequence of the protein, a special "adaptor molecule" has evolved. This "adaptor molecule" is transfer RNA (Schimmel et al., 1979). Transfer RNA has an anticodon loop as a part of its secondary structure which interacts specifically with three sequential nucleotides on the mRNA, collectively called a codon. Since the mRNA sequence contains four bases (A,G,U,C) read in groups of three, there are sixty-four possible codons. Three of these codons are stop codons specifying the termination of a polypeptide chain. This leaves 61 codons to specify the 20 amino acids. Therefore, most amino acids are represented by more than one codon. For this reason, the genetic code is said to be degenerate. There are a battery of different tRNA molecules, at least 55 in E. coli, such that all 20 of the amino acids can be incorporated into a growing protein. The addition of an amino acid onto a tRNA molecule has been well investigated. It involves an esterification reaction between the carboxyl group of the amino acid and a hydroxyl group on the adenosine nucleotide located at the 3′ end of tRNA. This

reaction requires an enzyme called an aminoacyl-tRNA synthetase (Schimmel, 1987). The loading is not thermodynamically favorable and occurs as a two step process. In the first step, ATP is hydrolyzed to AMP and pyrophosphate. This hydrolysis provides the energy for the formation of the activated amino acid. An activated amino acid is created when an anhydride linkage between a phosphate on the AMP nucleotide and the carboxyl residue on the amino acid is formed. This reaction occurs while both components are attached to the synthetase. In the second step, the activated amino acid is attached to the tRNA with the release of AMP. In E. coli, there are 20 different synthetases, each of which recognizes a specific amino acid and a set of transfer RNAs. The aminoacyl-tRNA synthetases are responsible for maintaining the fidelity of translation by insuring that the appropriate amino acid is loaded onto the correct tRNA. Synthetases insure specificity by recognizing the three dimensional structure of the amino acid and the three dimensional structure of the tRNA, allowing the enzyme to make correct matches.

The macromolecular complex on which the process of protein synthesis occurs is the ribosome (Kurland, 1977). Since all cells carry out protein synthesis, the ribosome is a major cellular constituent. For example, an E. coli cell contains approximately 15,000 ribosomes. The ribosome has three major functions in the process of mRNA translation.

The ribosome is responsible for selecting the appropriate region of the mRNA for beginning translation, for stable pairing of the codon and anticodon, and for the formation of the peptide bond. It is important to realize that in E. coli many ribosomes simultaneously translate a given message. The E. coli ribosome has a mass of 2.5 x 10^6 daltons and a sedimentation coefficient of 70S (Svedberg units). The 70S ribosome separates into two subunits of uneven size when the magnesium concentration is low. The larger subunit has a sedimentation coefficient of 50S (1.6 x 10^6 daltons), while the smaller subunit is 30S (0.9 x 10^6 daltons); the reason for the uneven size of the subunits must have arisen from some yet unknown functional necessity. By mass the bacterial ribosome is composed of about one-third protein and two-thirds RNA (Nierhaus, 1982). The smaller, or 30S, subunit is made up of 21 ribosomal proteins and the 16S RNA. The larger, or 50S, subunit is composed of 32 proteins and two RNA molecules, 23S and 5S.

Translation in E. coli has been divided into three stages: initiation, elongation, and termination. Initiation begins when three initiation factors (IF-1, IF-2, IF-3) interact with the 30S subunit of a ribosome (Gold, 1981). IF-3 allows the mRNA to fasten to the 30S subunit and prevents the large and small subunits from associating. The mRNA Shine-Dalgarno sequence is complementary to a sequence near the 3′ end of the 16S ribosomal RNA, allowing the

initiation codon AUG to be positioned correctly for the start of protein synthesis. Once the message is bound to the ribosome, IF-3 is lost. Loss of IF-3 is followed by IF-2-GTP transporting a special charged initiator tRNA to the start site. The binding of IF-2 is aided by IF-1. The charged initiator tRNA recognizes only the AUG start codon and carries the amino acid N-formylmethionine. The initiation step requires that all proteins are synthesized with this same amino terminal residue. With the loss of IF-3, the 50S subunit combines with the 30S initiation complex forming the 70S ribosome. This association causes the GTP associated with IF-2 to be hydrolyzed to GDP plus phosphate, followed by the release of IF-1 and IF-2. As the 70S ribosome forms, the N-formylmethionine tRNA is inserted in one of two tRNA binding sites found in the 50S subunit. The two sites in the 50S subunit are called the peptidyl (P) site and the aminoacyl (A) site. F-Met tRNA fits in the P site, while the A site awaits the coming of the charged tRNA corresponding to the next codon on the mRNA.

Elongation begins once the 70S initiation complex has formed (Spirin, 1986). It is a cyclic process by which an aminoacyl tRNA complementary with the next mRNA codon is accepted in the aminoacyl (A) site. This process is aided by elongation factor EF-Tu, which carries a GTP. EF-Tu is a carrier protein which aids in the binding of the tRNA to the A site. When the tRNA is deposited, the GTP associated with

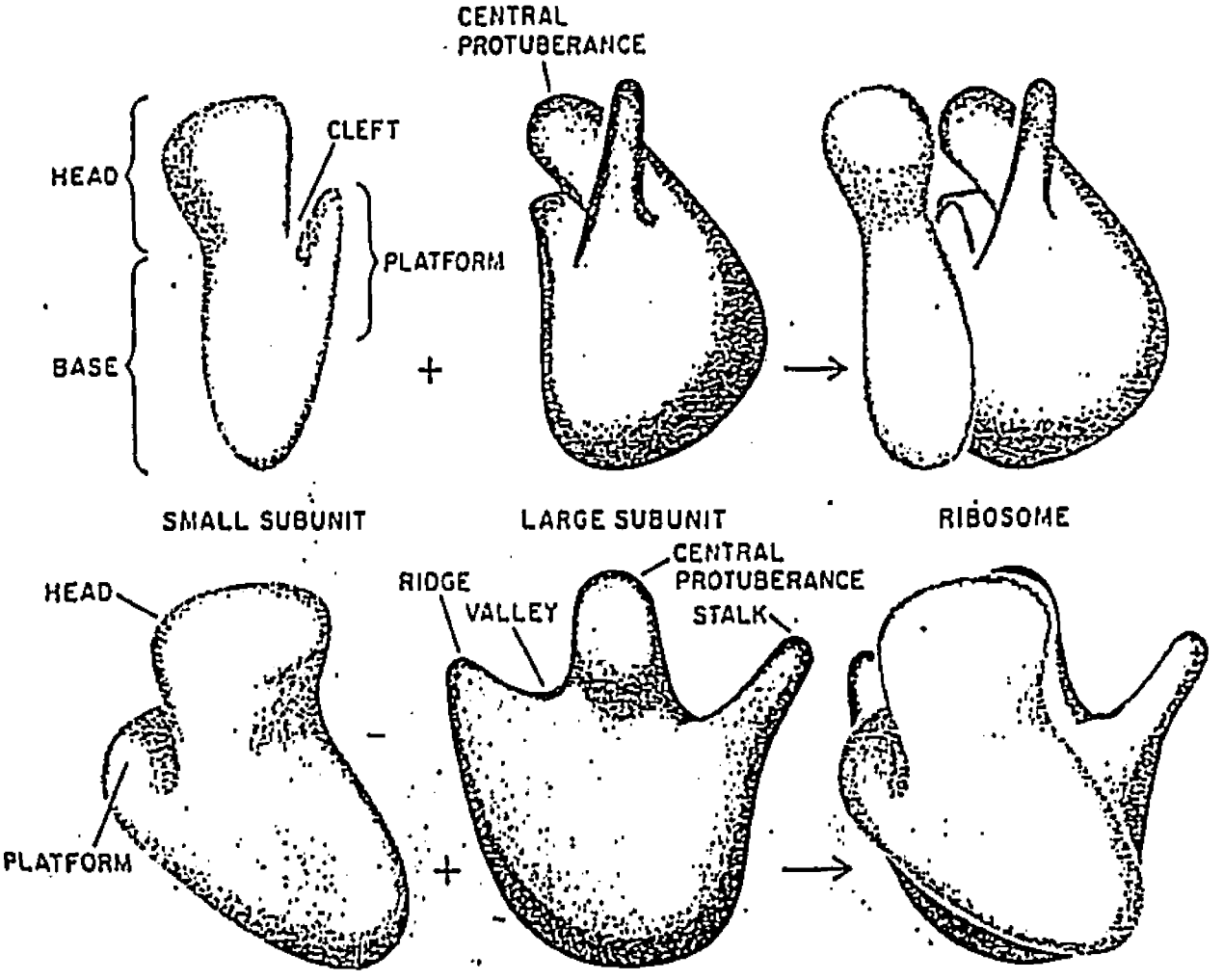
EF-Tu is hydrolyzed to GDP and phosphate causing the release of EF-Tu. The amino acid associated with a tRNA positioned in the P site and the amino acid associated with a tRNA in the A site are joined by a peptide bond. This reaction requires peptidyl transferase which is inherent to the 50S subunit (Krayevsky et al., 1979). Once the peptide bond is formed, the tRNA in the P site is released and the peptidyl-tRNA in the A site is translocated into the P site. This movement is aided by a second elongation factor, EF-G, which has an associated GTP. Elongation factor EF-Ts regenerates EF-Tu with its associated GTP from EF-Tu with GDP. The cycle is now complete and begins anew with the addition of the tRNA which is complementary to the codon occupying the A site.

Termination involves the release of the synthesized protein (Caskey et al., 1969). The termination of a protein is specified by a stop codon within the mRNA. No tRNA is complementary to the termination codon. Instead a release factor interacts with this codon and occupies the A site. E. coli contain two release factors (RF-1, RF-2) which are each specific for two stop codons. When the release factor occupies the A site the specificity of the peptidyl transferase is altered so that water is used in the bond formation. This substitution results in the formation of the carboxyl termini of the peptide. When translocation occurs the protein is released from the ribosome as the tRNA

in the P site is released. The ribosome now disassociates into its component 30S and 50S subunits. With the disassociation of the ribosome, the message may be released or may remain associated with the 30S subunit. If the message remains attached to the 30S subunit, the 30S subunit scans the message looking for a start signal for the initiation of translation. If the 30S subunit is released from the message it is free to attach to a new message.

The ribosome provides the machinery for the process of translation. Studies on the functional role of the ribosome have paralleled studies on its structure. Early investigations into the structure of the ribosome began with electron microscopy and small angle X-ray scattering, which provided a three dimensional image of the ribosome (Stoffler and Stoffler-Meilicke, 1986). From this analysis two models of the ribosome have emerged: the crown model and the kidney model, corresponding to what is believed to be a front and side view (Lake, 1981) (Figure 1). Dissection of the ribosome revealed that the small subunit was composed of three sections: one third forms the head of the subunit, the second third makes up the base, and the final third forms a side bulge or platform. The large subunit was composed of four sections referred to as a base with three projections. The central projection is called the head. To one side of the head is the L7/L12 stalk, and on the other side of the head is the L1 ridge (Lake, 1976). The 30S

Figure 1. Three Dimensional Model of the E. coli Ribosome (Lake, 1981).



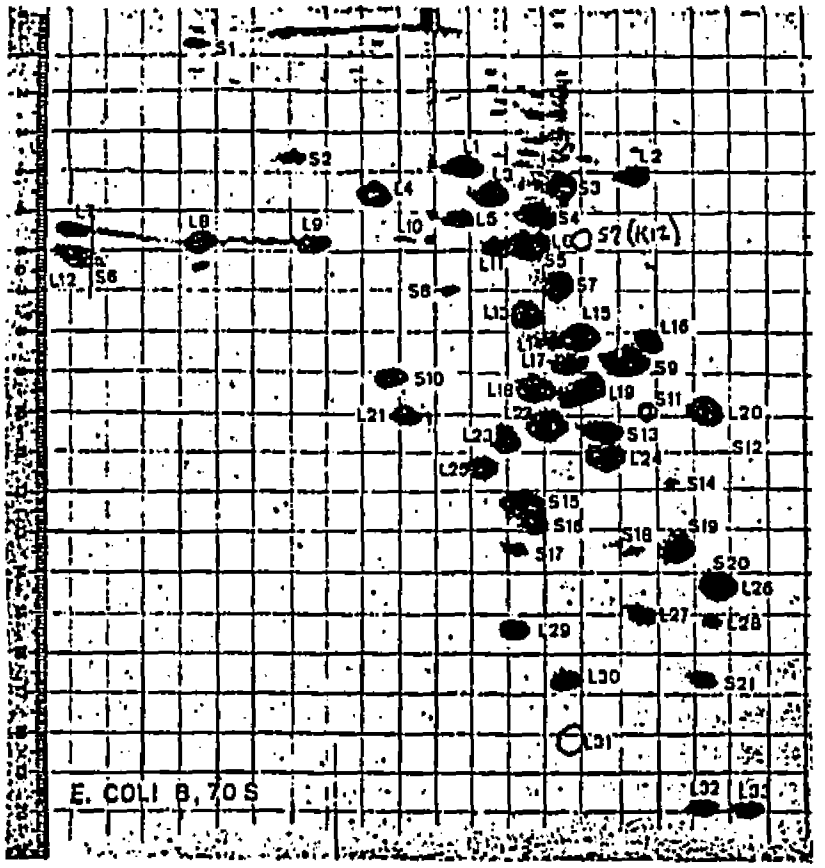
subunit has been shown to sit asymmetrically on the 50S subunit. The 30S subunit is positioned such that the head and body of the small subunit are aligned with the head of the large subunit. The cleft which forms between the 30S and 50S subunits has been named the "working region" of the ribosome. Early functional analysis showed that the ribosome contains two working domains: the translational and exit domains (Bernabeu and Lake, 1982). All of the proteins of the small subunit and most of the proteins of the large subunit have been associated with the translation domain.

Ribosomal RNA constitutes 66% of a ribosome's mass and plays a significant role in ribosome function. The sequences of the three E. coli ribosomal RNA's are known (Noller, 1984). When rRNA sequences from different organisms are compared, considerable homology is seen (Gutell et al., 1985). Therefore, it is believed that the ribosome arose early in the evolution of protein synthesis and has remained relatively unchanged. The primary structures of the rRNA's have been established. Today, research has come to focus on rRNA secondary and tertiary structure. The secondary and tertiary structures of the rRNA's have been probed using ribonuclease cleavage, chemical modification, and inter-RNA crosslinking (Brimacombe and Stiege, 1985). Sequencing of the rRNAs have revealed many regions of self complementarity which are

capable of forming double helical segments. Further analysis has shown that these complementary regions are highly conserved in evolution. It often appears that the secondary structure of the rRNA molecules is more conserved than the primary structure. Studies have shown that rRNA is involved in several processes in addition to having a role in the process of translation. Ribosomal RNA also functions in mRNA binding, antibiotic binding, and subunit association. Ribosomal RNA provides a scaffolding, allowing the correct assembly and positioning of the ribosomal proteins. Ribosomal proteins recognize a combination of primary, secondary, and tertiary rRNA structures, which allow them to attach to specific binding sites. Seventeen large subunit proteins have been shown to interact with 23S RNA, thirteen 30S proteins have been shown to bind to 16S, RNA and three large subunit proteins have been shown to associate with 5S RNA. These associations have been determined from binding, crosslinking, and reassociation assays (Nierhaus, 1982).

Kaltschmidt and Wittmann (1970a), used two dimensional electrophoresis to separate the 52 E. coli ribosomal proteins (Figure 2). With the purification of the ribosomal proteins, it became possible to determine their amino acid sequence (Wittmann, 1982). When the primary sequences of the 52 ribosomal proteins are compared, there is an absence

Figure 2. Two Dimensional Electrophoretogram of the 70S Ribosomal Proteins of E. coli B. First dimension, 4% acrylamide, pH 8.6; Second dimension, 18% acrylamide, pH 4.5. (Kaltschmidt and Wittmann, 1970a)



of significant sequence homology (Wittmann-Liebold et al., 1983). However, when the sequence of a single ribosomal protein is compared against a second taxonomic group (i.e. E. coli and B. subtilis), it appears that there are conserved regions within the protein which are crucial to the protein function (Kimura et al., 1985). The secondary and tertiary structures of the proteins have been examined using circular dichroism, proteolysis, florescence spectroscopy, and neutron scattering methods (Wittmann, 1983; Giri et al., 1984). Ribosomal proteins have a well developed secondary and tertiary structure. However, among the 52 proteins, a great variability is seen in the amount and type of these structures. Generally, ribosomal proteins are basic, globular proteins containing a high percentage of lysine and arginine residues. Ribosomal proteins are found in single copies, with the exception being the L7/L12 dimer. There are four copies of ribosomal protein L12. Two copies of ribosomal protein L12 are modified by acetylation of the amino terminus and are renamed ribosomal protein L7 (Terhorst et al., 1973). One copy of protein L7 binds to one copy of protein L12 forming the L7/L12 dimer, and there are two L7/L12 dimers within the ribosome. Immunologic studies have shown that proteins S20 and L26 on two dimensional gels are the same protein (Weber, 1972); hence, there are 53 spots on a two dimensional gel but only 52 proteins. Several of the ribosomal proteins are

modified post translationally. Proteins S5, S18, and L7 are acetylated on their amino termini (Terhorst et al., 1973; Yaguchi, 1977; Wittmann-Liebold and Greuer, 1978). Proteins S11, L11, L16, and L33 are methylated (Chen et al., 1977; Dognin and Wittmann-Liebold, 1977). The locations of the ribosomal proteins within the ribosome have been determined using immuno-electron microscopy, crosslinking, neutron scattering, and assembly studies (Moore et al., 1974). The ribosomal proteins are positioned primarily along the exterior of the folded RNA mass. Crosslinking has been used extensively to study protein-protein interactions. Distances between proteins have been calculated based on extensive neutron scattering studies (Nowotny et al., 1986). Perhaps the biggest advance in the understanding of ribosome structure came when Traub and Nomura (1968) got the components of the 30S ribosomal subunit from E. coli to assemble in vitro. Similar experiments have been carried out on the 50S subunit (Nierhaus and Dohme, 1974). Hence, an assembly map has been developed for the in vivo formation of E. coli ribosomes (Figure 3). This data has provided a better understanding of the locations, functions, and interactions of the ribosomal proteins.

The genes for all but four of the E. coli ribosomal proteins (L21, L25, L27 and L31) have been isolated and sequenced. Most of the ribosomal protein gene sequences show complete agreement with the earlier reported protein

Figure 3. Assembly Map of the E. coli Ribosomal Subunits.

A: 30S subunit- (Hochkeppel et al., 1976)
(→)= strong binding
(⇒)= weak binding
( )= binding in some preparations

B: 50S subunit- (Herold and Nierhaus, 1987)
(→)= strong binding
(→)= weak binding

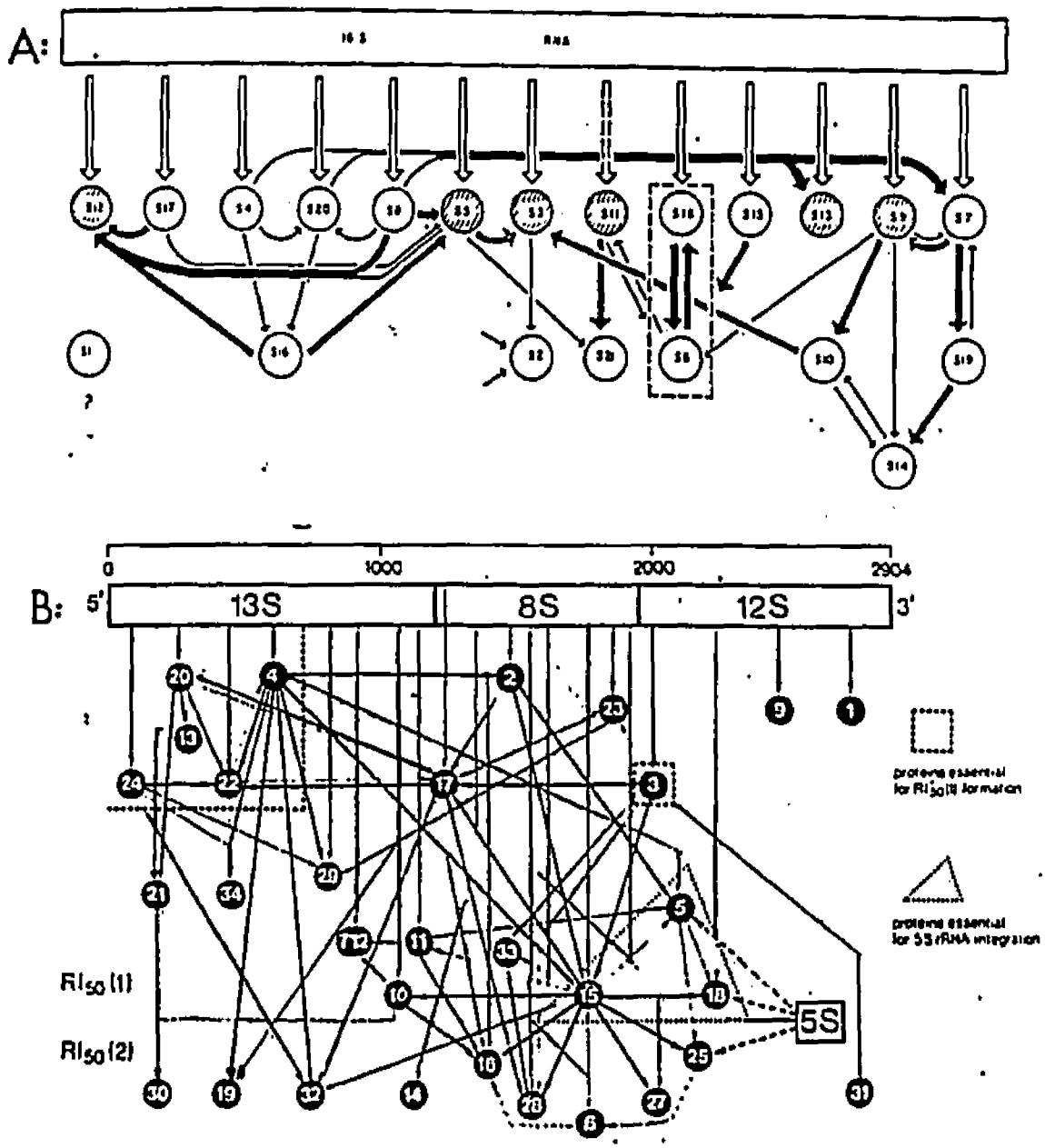
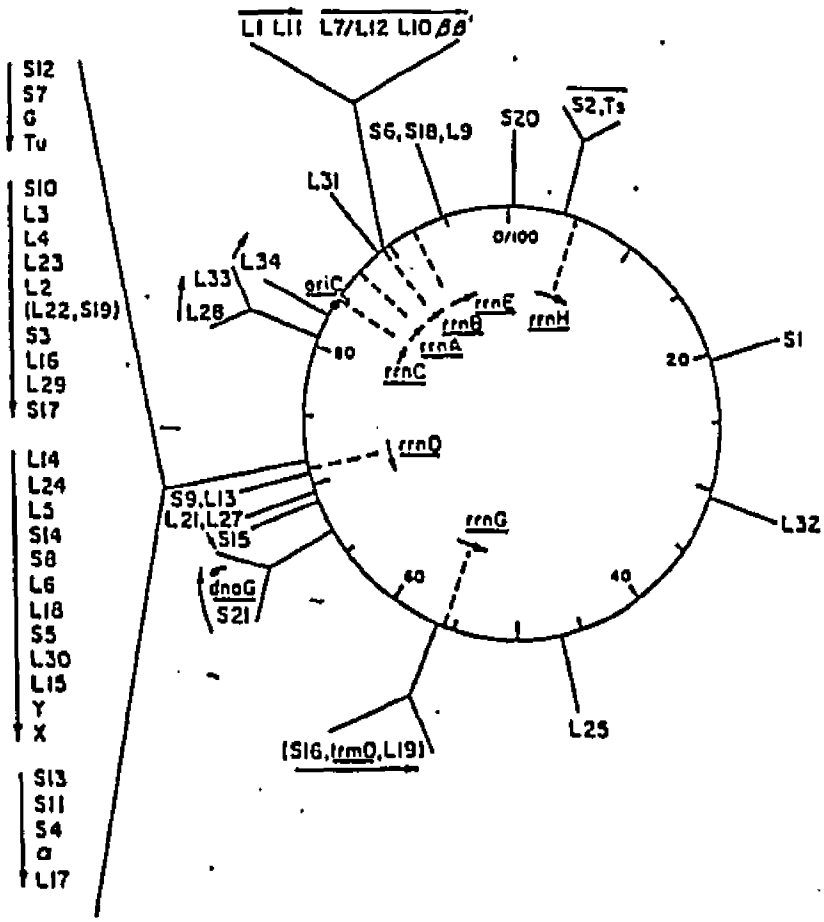


Figure 4. Genetic Map of the Translational Components of E. coli. The location in minutes, of translational component genes is shown. The major ribosomal protein gene cluster is found at 72 minutes (Isono, 1980).



sequences. Half of the ribosomal protein genes plus genes for protein synthesis factors EF-Tu and EF-G are clustered at the 72 minute region of the E. coli chromosome (Figure 4) (Fiandt et al., 1976; Isono, 1980). The remaining ribosomal protein genes are scattered throughout the E. coli chromosome. The clustered ribosomal protein genes are organized into four operons which exhibit coordinated synthesis to meet the protein synthesis requirements of the cell (Gourse et al., 1986a). The regulation of ribosomal protein formation is coupled with the regulation of rRNA synthesis. Even those components scattered at other locations in the chromosome are tied to this coordinated and balanced synthesis so that energy is not wasted. This organization permits the components required for translation to be produced in stoichiometric amounts (Gourse et al., 1986b). Regulation of protein synthesis occurs mainly at the transcriptional level using feedback inhibition, although in prokaryotes there is also control at the translational level (Nomura et al., 1980). Translational control is due primarily to ribosomal proteins being able regulate their own synthesis. Ribosomal protein genes are organized into operons which are translated as polycistronic messages. One of the proteins encoded in the operon is able to bind near the 5′ end of this polycistronic message, blocking the production of all the proteins within the operon. The ribosomal protein which binds to the 5′ end of

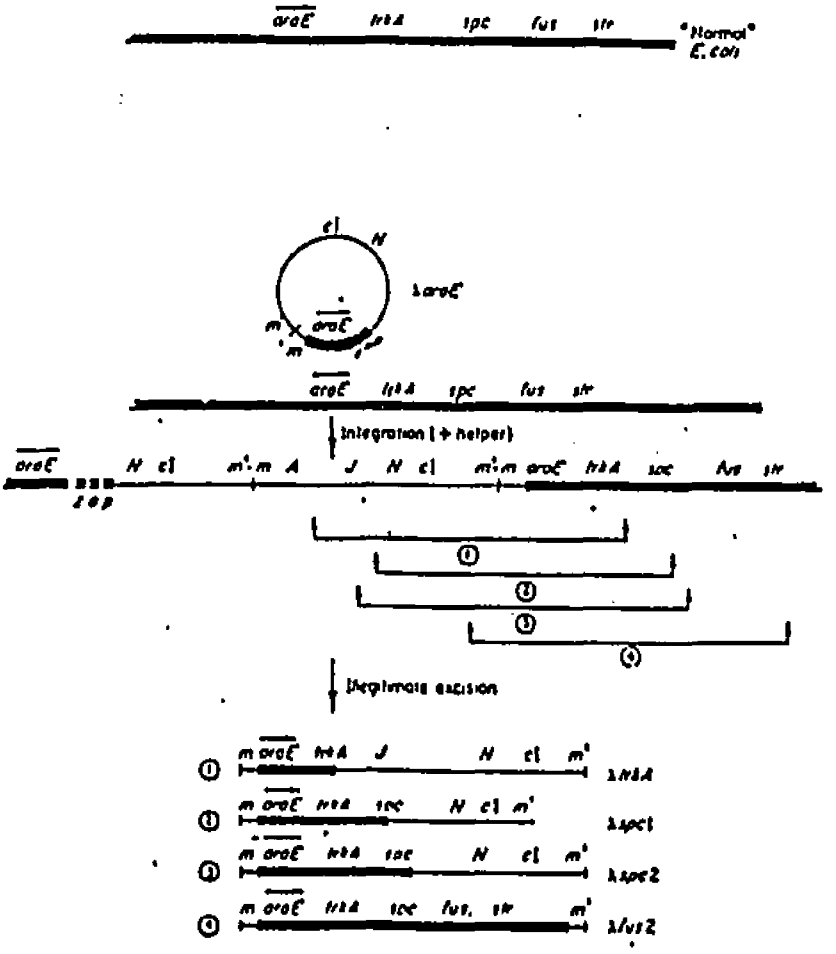
the message is also able to bind to rRNA. These proteins have a higher affinity for rRNA than for mRNA. Therefore, when rRNA is available the proteins bind to rRNA allowing assembly of the ribosome. When rRNA is in short supply these same proteins inhibit their own synthesis by binding to the mRNA from which they are synthesized. This coordinated synthesis is an excellent example of how cells have become streamlined to prevent waste.

The assignment of function to the ribosomal components has been a slow task. It is known that the large subunit operates in peptide bond formation and that the small subunit participates in the selective binding of mRNA. Further analysis has proven difficult because many of the ribosomal functions depend upon the interaction and cooperation of many components. Therefore, it has become advantageous to investigate the function of an individual component by creating mutants which show functional changes (Dabbs, 1986). By mapping the mutation a structure-function relationship often can be derived. Ribosomal antibiotic resistance was the first type of mutation characterized, followed by other types of mutants, especially temperature-sensitive and cold-sensitive mutants (Isono et al., 1980). Initially, mutants were analyzed to determine the site of the mutation. With time the characterization of these mutants also revealed information on the physiological functions of the altered component. The focus has since

shifted away from antibiotic-resistant mutants since the basis of resistance at the molecular level was difficult to understand. To study the process of protein synthesis, a class of protein-synthesis deficient mutants were needed. Parallel to the change from antibiotic resistance to protein-synthesis deficient mutants was a change from isolating spontaneous mutants to creating conditional mutants using localized and eventually site directed mutagenesis. Due to the conditional lethality associated with mutants deficient in protein synthesis, a selectable marker is required, generally either high or low temperature sensitivity. Temperature-sensitive protein-synthesis mutants have allowed the mapping and characterization of components of the process of protein synthesis. The study of temperature-sensitive mutants of protein synthesis allows analysis under two conditions, permissive temperatures with favorable growth and restrictive temperatures where the mutation becomes lethal. Most of these temperature-sensitive protein-synthesis deficient mutants have been found to be missense mutants exhibiting alterations within ribosomal proteins (Piepersberg et al., 1980).

Genetic characterization of mutant strains involves the determination of the chromosomal location of the mutation. This can be accomplished using several types of genetic mapping. Specialized lambda transducing phage have been created for use in studying genes for protein synthesis

Figure 5. Physical Map of DNA from Transducing Phages. Transducing phages λtrkA, λspc1, λspc2 and λfus2 carry different portions of the 72 minute region of the Escherichia coli chromosome (Fiandt et al., 1976).



components (Jaskunas et al., 1975, 1977a,b). These phage carry the 72 minute and 89 minute regions of the E. coli chromosome (Figure 5). Each phage contains a different, but well defined, region of the E. coli chromosome (Lindahl et al., 1976). The transducing lambda phages are capable of integrating into the host genome by generalized recombination. Phage carrying a wild type copy of the mutant gene will complement the mutation (Birge, 1981). Therefore, by complementation the mutation can be mapped to the region of the E. coli chromosome carried by the phage. A more accurate location of the mutation can be achieved using the bacteriophage P1 in transductional analysis (Miller, 1972). P1 transductional mapping involves determining the distance between the mutant loci and other neighboring genetic markers (Birge, 1981). During lysis of a P1 phage infected cell, the host chromosome is cleaved into many pieces. The phage occasionally mistakenly packages some of these chromosomal pieces using the headfull mechanism. It has been calculated that one out of every 10^7 phage particles will contain a specific gene of interest. The P1 lysate resulting from the infection can be used to reinfect cells with a known genetic background. Selection for linked movement of two genetic markers will give cotransduction frequencies which can be converted to map distances within the chromosome. Hence, the location of the mutation can be mapped to a small region of the chromosome.

The precise position and nature of the mutation can be determined by sequencing the mutant gene. Only when the change in the nucleotide sequence is determined can all the implications of the mutation be examined (Church and Kiefer-Higgins, 1988). Sequencing of the mutant gene can be achieved by isolating the mutant gene from a chromosomal digest or copying the mutant gene from the chromosome using the polymerase chain reaction (PCR) method. In PCR, sequence-specific oligonucleotides are synthesized to flank the gene region of interest. These oligonucleotides act as primers for DNA polymerase, which catalyzes a template-directed synthesis of the DNA between the primers (Saiki et al., 1988). This results in an amplification of the gene region flanked by the oligonucleotides. The amplified product can be used for further analysis, including sequencing, using primer extension.

Physical characterization of mutant strains involves the identification of an altered function in the mutants. Once a functional change has been identified, often the process can be dissected and the components of the altered system analyzed. This scrutiny continues until the mutation can be associated with an individual component. In the analysis of temperature-sensitive protein-synthesis deficient strains, the rates of translation can be determined for control and mutant strains by calculating the rate of incorporation of a labeled amino acid into a protein

directed by a specific mRNA (Champney, 1979). The rates are compared between control and mutant cells. If the rate of translation is reduced in the mutants, the components of translation, i.e the ribosomes and translation factors, can be further examined in mixing assays. Translation factors from the mutant are mixed with ribosomes from control cells and vice versa, followed by translation of message. One combination should produce an established translation rate, indicating that both components are functioning normally. The second combination should exhibit alterations in the rate of translation, which can be associated with the mutant component. If the reduction in translation is linked to the ribosomes, then the ribosome can be further analyzed. The mutation could be associated with either the large or small ribosomal subunit, as determined by a second type of mixing assay (Champney, 1979). The 30S and 50S subunits are isolated from control and mutant stains, and combinations of the subunits are tested in the translation system as above. Once the subunit containing the alteration has been identified, the subunit's components, i.e. the ribosomal proteins and rRNA, can be examined. A mutated ribosomal protein may be discovered using either two dimensional electrophoresis or reversed phase HPLC, provided the mutation causes a significant change in the charge, size, or hydrophobicity of the protein. Changes in RNA size may be seen by electrophoresis, sedimentation experiments, or with

gene probes. Changes in RNA position within the ribosome can be studied using immunoelectron microscopy. Hence, the mutation can be traced to a group of components or to an individual component of the ribosome.

The analysis of two protein-synthesis deficient mutants of E. coli provide the basis of this dissertation. Mutant strains SK1047 and SK1048 were isolated after nitrosoguanidine mutagenesis of a trp S strain (mapping at 74 minutes), with selection for revertants with linked temperature-sensitive mutations. Nitrosoguanidine is a chemical agent which can be used in localized mutagenesis. This mutagen causes linked mutational events during chromosome replication (Guerola et al., 1971). Temperature-sensitive cells were selected from the revertants (Champney, 1979). Protein-synthesis deficient mutants were picked from among the temperature-sensitive revertants.

Strains SK1047 and SK1048 were chosen for analysis when they were shown to exhibit an altered ribosomal protein L22 in two dimensional gel electrophoresis (Champney, 1980). The E. coli ribosomal protein L22 is composed of 110 amino acids and has a molecular weight of 12,227 daltons. The primary sequence of ribosomal protein L22 was determined by Wittmann-Liebold and Greuer (1980) and, like many ribosomal proteins, contains numerous basic residues (12% lysine and 6% arginine). The predicted secondary structure for L22 has revealed that it contains 28% alpha helix, 6% extended

structure, and 11% turns (Wittmann et al., 1979). Neutron scattering has shown that the radius of gyration for this protein is 21 Angstrom units. From the radius of gyration an axial ratio of 1:5 was determined, indicating that L22 is an ellipsoidal protein (Moore et al., 1974; Sturhrmann, 1976; Nowotny et al., 1986). Further study of the protein using this method indicated that the protein underwent little change in conformation when it assembled into the ribosome. Ribosomal protein L22 was located on the surface of the 50S subunit by immune electron microscopy. It is on the opposite side of the 50S subunit from the 30S subunit attachment site (Tischendorf et al., 1974). This position has been further characterized using nonradiant energy transfer, which identified ribosomal protein L22 as a component of the peptidyltransferase domain of the ribosome (Giambattista et al., 1990).

Protein L22 is an early assembly component of the 50S subunit. Protein L22 attaches to 23S RNA as a core binding protein; more specifically, ribosomal protein L22 is essential for the first conformational change required in the generation of the active 50S subunit (Roth and Nierhaus, 1980). Further analysis of the interaction between protein L22 and 23S RNA has shown that the protein interacts with domain II of the RNA. Assembly experiments have shown that protein L22 interacts directly with the 23S RNA; however, the interactions between the protein and the RNA are greatly

heightened by the addition of ribosomal protein L4 (Figure 3). Assembly maps show that the majority of ribosomal protein L22 is associated with ribosomal protein L4, which has previously attached to 23S RNA. Assembly data have further shown that ribosomal proteins L20 and L7 interact weakly with ribosomal protein L22 during subunit assembly.

Genetically, ribosomal protein L22 is coded for by the rplV gene located within the 73 minute region of the E. coli chromosome (Figure 3). The rplV gene was sequenced by Zurawski and Zurawski (1985) as part of the S10 operon. The gene sequence is in complete agreement with the protein sequence reported by Wittmann-Liebold and Greuer (1980). The S10 operon is coordinately regulated by ribosomal protein L4, which can feedback inhibit translational expression (Lindahl et al., 1983).

Research on the function of ribosomal protein L22 has shown that it forms one part of the erythromycin binding site (Arevalo et al., 1988). Wittmann et al. (1973) isolated an E. coli mutant which exhibited an electrophoretic alteration in protein L22. The altered protein L22 was associated with a resistance to high levels of erythromycin. Therefore, ribosomal protein L22 is believed to play a role in the binding of erythromycin. Ribosomal protein L22 was also found to be associated with the virginiamycin S binding site by affinity labeling (Giambattista et al., 1990). Preliminary characterization

of mutants SK1047 and SK1048 classified these mutants as temperature-sensitive and protein-synthesis deficient with the mutation lying in the large subunit (Champney, 1979). These mutants have been shown to be sensitive to erythromycin (Champney, 1979), distinguishing them from those characterized by Wittmann et al. (1973). This research describes the genetic characterization of these strains.

Chapter 2

MATERIALS AND METHODS

Sources of Materials

Adenosine 5′-triphosphate, ampicillin, all 20 L-amino acids, DEAE Sephadex, diethylpyrocarbonate, 2,5-diphenyloxazole, dithiothreitol, deoxyribonuclease I, 4,4′-dithiodipyridine, ethidium bromide, lysozyme, polyethylene glycol, ribonuclease, sodium borohydrate, and streptomycin sulfate were purchased from Sigma Chemical Corporation. Acrylamide, agarose, Biogel P-10 matrix, Bradford reagent, methylene bis-acrylamide, silver stain kit, sodium dodecyl sulfate, N,N,N′,N′-tetramethylethylenediamine, and Triton X-100 were purchased from Biorad. Acetic acid, chloroform, ethylenediaminetetracetic acid, iso-amyl alcohol, isopropanol, mercaptoethanol, and toluene were purchased from Fisher Scientific. Cesium chloride, restriction enzymes and buffers, phenol, sucrose, and urea were purchased from International Biotechnologies, Inc. Acetonitrile and spectro grade-sequanal trifluoroacetic acid were purchased from Pierce. Coomassie Brilliant Blue Stains (R-250 and G-250) were purchased from Kodak. All culture media used in this study were purchased from Difco Laboratories. Radioisotopes (^{3}H-formaldehyde and ^{35}S-dATP)

were purchased from New England Nuclear Products. DNA ligase and the polymerase chain reaction kit were purchased from Promega Corporation. Proteinase K was purchased from E.M. Biochemicals. Pyronine Y was purchased from Allied Chemical. The Sequenase 2.0 kit was purchased from United States Biochemical Corporation.

Media

Bacterial strains were stored at -80°C in 1 ml aliquots of L broth containing 10% glycerol. Cells were grown in L broth, tryptone broth (TB), or A salts solutions. L broth consists of 10 gm bacto-tryptone, 10 gm NaCl, 5 gm yeast extract, and 1.5 ml 1.0 M NaOH in 1 liter of dH_2O (Miller, 1972). Tryptone broth includes 13 gm tryptone and 7 gm NaCl in 1 liter of dH_2O. A salts solution contains per liter 10.5 gm K_2HPO_4, 4.5 gm KH_2PO_4, 1.0 gm $(NH_4)_2SO_4$, 0.5 gm sodium citrate, 0.5 ml of 1% thiamine, 1.0 ml of 20% $MgSO_4$, and 10 ml of 20% glucose (Miller, 1972). Plates were made with the same media containing 1.6% agar.

Methods

Growth Experiments

Small-scale growth experiments were done in a shaking water bath. Five milliliters of L Broth were inoculated with 50 µl of an overnight culture in a 125 ml side-arm flask. The cell density was determined using a Klett-Summerson colorimeter with a red (Number 66) filter. Large-

scale growth experiments (greater than 500 ml) were done on a shaking platform in a Bally environmental room at 32°C. Cell density was determined by reading the absorbance at 600 nm in a Beckman Model 25 spectrophotometer.

Growth Shift

Growth characteristics of mutant and control strains were tested by inoculating 5 ml of L Broth with 50 µl from a 1 ml overnight culture (1/100 dilution) in a 125 ml side-arm flask. This resulted in a Klett reading of approximately 10 (1 X 10^8 cells/ml). Flasks were placed in a shaking water bath at 32°C. When the cell density reached a Klett reading of 30 (2 X 10^8 cells/ml), they were shifted to a water bath at 42°C or 44°C and shaken for 3 hours while recording their Klett readings. For larger cell volumes, 5 ml of an overnight culture was added to 500 ml of L Broth in a 2 liter flask. Cells were set on a shaking platform in a 32°C environmental room. One milliliter samples were removed every 30 minutes to allow the absorbance at 600 nm to be determined. When the absorbance at 600 nm reached 0.2-0.3, the cultures were placed in a 42°C water bath. Cells were allowed to multiply until an absorbance at 600 nm of 1.0-1.4 was achieved.

Recovery Experiments

The ability of temperature-sensitive cells to grow after exposure to high temperature was tested by inoculating

5 ml of L Broth with 50 µl from a 1 ml overnight culture in a 50 ml side arm flask. Cells were placed in a shaking waterbath at 32°C until a Klett reading of 30 was achieved. Cultures were moved to a shaking waterbath at 42°C for approximately 60 minutes until a decline in their growth rate was observed. After exposure to high temperature, cultures were diluted to approximately 30 Klett units, by the addition of fresh L Broth, and returned to the 32°C waterbath. Cultures were shaken for several more hours while recording Klett readings.

Reversion Analysis

The reversion frequency was determined by using a variation of the Newcombe's plate spreading experiment (Birge, 1981). A 100 µl aliquot of an overnight culture was spread directly on an L Broth plate and placed in a 42°C incubator. The culture was diluted one-to-ten with L Broth and a second 100 µl aliquot plated at 42°C. A 10^{-6} dilution of the culture was made, and a third 100 µl of the 10^{-6} dilution was plated on an L Broth plate at 32°C to determine the number of cells in the overnight culture. All plates were incubated overnight and the number of colonies counted.

Isolation of λ Phage

Defective λ phages were isolated by isopycnic centrifugation in cesium chloride (Davis et al., 1980) after

heat induction of the double lysogens NO1267, NO1275, NO1328 and NO1379. Cells were grown at 32°C to an absorbance at 600 nm equal to 1.0 (2 X 10^8 cells/ml), at which time an equal volume of 52°C L Broth was added to quickly raise the temperature of the culture. The cultures were incubated at 42°C for 20 minutes and then returned to a shaking waterbath at 37°C for 3 hours. Cells were harvested by centrifugation in a JA-10 rotor at 4420 x g for 10 minutes at 4°C and resuspended in 10 ml of 20 mM Tris-HCl pH 7.6, 5 mM $MgSO_4$. Cells were lysed using 0.2 ml of chloroform. Cellular nucleic acids were hydrolyzed by the addition of DNase I at 2 µg/ml and RNase A at 200 µg/ml. The mixture was incubated 10 minutes at room temperature. The cell debris was pelleted and the supernatant containing the helper and defective phage was mixed with 3.7 gm of cesium chloride. The phage were separated by centrifugation in a SW50.1 rotor at 114000 x g for 20 hours at 20°C. Bands containing the phage were drawn off by puncturing the tubes with a syringe. The phage were dialyzed overnight against 10 mM Tris-HCl pH 7.6, 10 mM $MgSO_4$, at 4°C.

Complementation Analysis

Complementation analysis of mutant strains were done using the method of Miller (1972). Strains were grown in TB, 10 mM $MgSO_4$ and 0.1% maltose. Cells were grown to a density of approximately 2 X 10^9 cells/ml on a shaker platform at 32°C. Cells were collected using a table-top

centrifuge at 4000 x g for 3-5 minutes and were resuspended in an equal volume of 10 mM $MgSO_4$. Aliquots of 0.1 ml were mixed with 10 µl of the different λ phages: trk1, spc1, spc2, or fus2. Following a 20 minute incubation at room temperature, 30 µl of the mixture was spread on one quarter sector of an L Broth plate. In the final sector, 30 µl of the uninfected cells were plated. The L Broth plate was incubated at 42°C for 2 days. After this period, the colonies in each sector were counted.

Transductional Mapping

P1 vir phage was used in three factor crosses to map the temperature-sensitive gene locus. The procedure was done according to Miller (1972) and Silhavy et al. (1984). The genetic markers used for selection in this procedure were streptomycin resistance (rpsL) or loss of a requirement for aromatic amino acids (aroE). P1 vir lysates were constructed using 1.0 ml cultures of the SC713 donor strain grown at 32°C in L Broth with 5 mM $CaCl_2$. When the cells reached a density of 2 X 10^8 cells/ml, 20 µl of a P1 vir lysate (10^9phage/ml) was added. Cells and phage were incubated at 37°C for 20 minutes to allow infection and then incubated at 32°C until lysis. Once lysis had occurred, 0.1 ml of chloroform was added, the mixture was vortexed, and the cell debris was pelleted. The lysate was placed in a clean tube with a drop of chloroform. For transduction, 1 ml overnight cultures of recipient strains SK1047 and SK1048

were grown in the same L Broth and calcium chloride mixture. Cells were collected in a table-top centrifuge and resuspended in 0.5 ml of 5 mM $CaCl_2$ and 10 mM $MgSO_4$ solution. One tenth of a milliliter of the cell suspension was mixed with 0.1 ml of the P1 vir phage stock and placed in a 37°C waterbath for 30 minutes. A 1 ml aliquot of 10 mM $MgSO_4$ was added to the infected cells. The cells were then centrifuged as above and the supernatant discarded. The cell pellet was resuspended in 1 ml of L Broth and placed at 32°C for 60 minutes to allow phenotypic expression. Cells were centrifuged, and the pellet was resuspended in 100 µl of 10 mM $MgSO_4$. This suspension was spread onto a streptomycin plate (50 µg/ml). Colonies able to grow on the streptomycin plate were transferred onto a master plate from which replicas were made to a minimal plate at 32°C and a Luria plate at 42°C.

70S Ribosome Preparation

Ribosomes were isolated according to Traub et al. (1971). Ten milliliters of an overnight culture was used to inoculate 1 liter of L Broth. Cultures grew at 32°C until the absorbance at 600 nm reached 1.0. A sample of the culture was streaked on an L Broth plate and incubated at 32°C to check for contamination. The cells were pelleted in a Beckman Model J2-21 centrifuge using a JA-10 rotor at 8670 x g at 4°C for 20 minutes. The pellets were washed in 10 ml of R-buffer. The cell pellets were either frozen at -20°C

for storage or resuspended in 3 ml of R-buffer and placed on ice for subsequent lysis. Fresh lysozyme was added to a concentration of 30 µg/ml, followed by a 15 minute incubation at room temperature. The cells were frozen in a dry ice-ethanol bath and thawed in warm water: this was repeated 3-5 times until the cells were completely lysed. Pancreatic DNAse I (1 mg/ml in 10 mM Tris-HCl pH 7.9, 10 mM $MgSO_4$) was added to a concentration of 10 µg/ml. Lysates were allowed to stand for 10 minutes until DNA digestion was complete. The debris was pelleted by centrifugation at 12100 x g for 20 minutes at 4°C. The supernatant from the spin was layered on 5 ml of ribosome wash (10% sucrose, 2 M NH_4Cl, 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.9). Tubes were placed in a Ti50 rotor and spun at 59900 x g, for 12 hours at 4°C. The supernatant was carefully removed and discarded. One milliliter of R-buffer was added to the ribosome pellet, and the mixture was allowed to stand on ice for 2-3 hours to resuspend. Insoluble material was removed by a 2 minute microfuge spin. To determine the ribosome concentration, 2-5 µl of the ribosome solution was added to 1 ml of dH_2O. The absorbance at 260 nm and 280 nm was read. The ribosome suspension was stored in 10% glycerol at -70°C.

Isolation of Ribosomal Subunits

Ribosomal subunits were isolated by layering 150 absorbance units at 260 nm of 70S ribosomes onto 38 ml of a

15-30% sucrose gradients made with S-buffer. Gradients were centrifuged in an SW27 rotor at 43050 x g at 4°C for 17 hours. One milliliter fractions (20 drops) were collected using an Isco Fraction collector after bottom puncture of the tubes. The absorbance of the gradient fractions were determined in the spectrophotometer at 290 nm. Appropriate fractions were pooled and the magnesium level adjusted to a final concentration of 10 mM magnesium acetate. Subunits were pelleted from pooled fractions by ultracentrifugation at 4°C in a Ti50 rotor at 59900 x g for 12 hours. Pellets were resuspended and stored in R-buffer with 10% glycerol. Subunit concentration was determined by reading absorbance at 260 nm.

Extraction of Ribosomal Proteins

Ribosomal proteins were separated from ribosomal RNA by acetic acid extraction (Hardy et al., 1969). A 1/10 volume of 1 M magnesium acetate was added to the ribosome or subunit preparation. R-buffer was used to bring the volume of the mixture up to 250 µl. Two volumes of distilled HOAC were added and the solution was placed on ice for 30 minutes. The rRNA precipitate was pelleted in a Beckman J2-21 Centrifuge in a JA-20 rotor at 12100 x g for 10 minutes. The supernatant was placed in a 15 ml conical Corex test tube. The RNA pellet was washed by adding 100 µl R-buffer and 200 µl HOAC. The RNA mixture was left on ice for 20 minutes and any remaining rRNA pelleted as above. The

supernatants were combined, and 5 volumes of cold acetone (-20°C) were added. The tube was placed in the -70°C freezer for at least 3 hours to allow the ribosomal proteins to precipitate completely (Barritault et al., 1976). The suspension was centrifuged at 6800 x g in the Beckman J2-21 centrifuge with a JA-20 rotor at -10°C for 25 minutes. The supernatant was discarded and the ribosomal pellet was allowed to dry at room temperature. Proteins were stored dry at -20°C or were resuspended in 8 M urea before storage. Generally, one absorbance unit at 260 nm of ribosomes/ml yielded 20 µg of protein.

Two Dimensional Polyacrylamide Gels

Two dimensional polyacrylamide gels run as described by Howard and Traut (1973), were used for characterization of the ribosomal proteins from control and mutant strains.

First Dimension. The first dimension gel solution was pH 8.6 and contained 4% acrylamide, 0.13% N-N'methylene bis-acrylamide, 20 mM EDTA, 0.52 M boric acid, 0.4 M Tris and 6 M urea. Polymerization was initiated with 2 µl of TEMED and 5 µl of 10% ammonium persulfate per milliliter of gel solution. The first dimension upper and lower running buffer contained 6.4 mM EDTA, 0.52 M boric acid, 0.4 M Tris and was adjusted to pH 8.3 with NaOH. Gel tubes were 100 mm x 3 mm (interior diameter) and were filled with gel up to the 70 mm mark. A drop of distilled water was placed on top

of the gel to allow polymerization of the lower gel half and then removed. Protein samples were loaded at mid-gel level. Protein extracted from 30 absorbance units at 260 nm were resuspended in 50 µl of a sample buffer consisting of 8 M urea, 20% sucrose and 1X first dimension running buffer. A second 1 ml of first dimension gel solution was layered carefully on top of the sample and allowed to polymerize. Twenty microliters of a 0.1% pyronine Y in a 10% sucrose solution was used as the tracking dye. The gels were allowed to stack for one half hour at 0.5 milliamps/gel and then run seven hours at 2 milliamps/gel.

Second Dimension. The first dimension gels were removed from the glass tubes and soaked for 15 minutes in 5 ml of soaking buffer to lower the pH. The soaking buffer (pH 5.2) contained 8 M urea, 0.75 ml HOAC, and 12 mM KOH per liter. First dimension gels were positioned on freshly poured second dimension slab gel and allowed to polymerize in place. Electrophoresis in the second dimension was conducted according to the procedure of Kaltschmidt and Wittmann (1970b). The gel solution (pH 4.5) contained 18% acrylamide, 0.25% methylene bis-acrylamide, 53 ml glacial acetic acid, 48 mM KOH, 6 M urea and 5.8 ml TEMED per liter. Two hundred microliters of fresh 10% $NH_4(SO_4)_2$ was added per 10 ml of gel solution. The gel slab was 14 cm x 12 cm x 2.5 mm and held approximately 30 ml of gel solution. The 1X running buffer (pH 4.0) contained 140 gm of glycine and 15

ml of HOAC per liter. The upper reservoir buffer had a 1X concentration. The lower reservoir buffer contained 0.3 X running buffer. Pyronine Y was used as the tracking dye. Electrophoresis was carried out with the cathode on top at 40 volts for 1 hour to allow stacking of the proteins in the first dimension. After stacking, the gel was run at 100 volts for 12 hours or until the tracking dye was 1 cm from the bottom of the gel. Proteins were visualized by staining for approximately 1 hour in a solution of 0.1% Coomassie Blue R-250, 50% methanol and 7% HOAC. Gels were destained in a solution of 25% ETOH, 5% HOAC. Photographs of stained gels were taken with a Polaroid MP-4 Land camera and Polaroid type 55 film using a #8 yellow filter, f-stop 22, and a 1 second exposure.

Separation of Ribosomal Proteins using HPLC

Separation of the ribosomal proteins was achieved by using reversed-phase HPLC on a C-4 column using a procedure adapted from Ferris et al. (1984) and Cooperman et al. (1988).

System Configuration. The HPLC system consisted of two Model 510 Pumps, a Model 680 Automated Gradient controller, and a Model U6K universal liquid chromatograph injector, all from Waters Associates. Column elution was monitored for UV absorbance using a Waters Lambda-Max Model 481 LC spectrophotometer set at 215 nm. Data was compiled

using a Waters Model 730 Data Module which functioned as a printer, plotter, and integrator for the system. Fractions were collected in a Pharmacia Frac-100 Fraction Collector.

Column Information. HPLC was performed on a Pierce Chemical Company Aquapore Butyl (BU-300) Cartridge Column (4.6 mm I.D. x 10 cm). The guard column was a Pierce Poly Reversed Phase Newguard 15 x 3.2 mm, 7 micron column.

Solvent System. The aqueous solvent consisted of 0.1% HPLC/Spectro grade-Sequanal TFA in water. The organic solvent contained 0.1% TFA in HPLC/Spectro grade acetonitrile. The aqueous solvent was twice filtered through a Millipore 0.45 μm filter. Both solvents were thoroughly degassed.

Gradient Characteristics. Proteins were eluted at room temperature using a linear acetonitrile gradient at a flow rate of 0.25 ml/minute. The elution program has been defined as follows:

Time	Acetonitrile	Gradient
0-5 minutes	10%	isocratic
5-15 minutes	10%-30%	linear
15-170 minutes	30%-50%	linear
170-200 minutes	50%-60%	linear
200-210 minutes	60%-10%	linear

Sample Preparation. Three hundred micrograms of ribosomal protein was extracted as described above, allowed to dry at room temperature and resuspended in 200 μl of 66%

HOAC for injection.

Sample Collection. Fractions were collected for 2.0 minute intervals throughout the run. Fractions collected off the HPLC were lyophilized and stored dry at 10°C.

Protein Identification. Lyophilized protein fractions from selected regions of the chromatogram were resuspended in sample buffer and analyzed by one or two dimensional gel electrophoresis. Regions of the chromatogram were also analyzed using the second dimension gel system prepared as a slab gel with 20 wells. The sample buffer for the slab gels was 8 M urea, 0.1% ETSH. Slab gels were stacked for 30 minutes at 40 volts and run for 12 hours at 100 volts.

Optimizing Separation of the L22 Ribosomal Protein. From the initial separation described above protein L22 was found to elute between 40 and 41% acetonitrile. Therefore the gradient was refined to improve separation in this region. A new linear acetonitrile gradient program was established as follows:

Time	Acetonitrile	Gradient
0-5 minutes	10%	isocratic
5-15 minutes	10-30%	linear
15-70 minutes	30-40%	linear
70-90 minutes	40-41%	linear
90-110 minutes	41%	isocratic
110-150 minutes	41-50	linear
150-160 minutes	50-60%	linear
160-170 minutes	60-10%	linear

Fractions were collected as described earlier and analyzed

using the same two dimensional gel and slab gel methods.

Further Purification of the L22 Ribosomal Protein using a Second HPLC Program. From the chromatograms described above L22 was found to elute between 75 and 85 minutes. Fractions from minute 74 through minute 86 were pooled, dried and resuspended in 50 µl of 66% HOAC and rerun on the HPLC. A third gradient format was developed for rechromatography of L22:

Time	Acetonitrile	Gradient
0-5 minutes	10%	isocratic
5-15 minutes	10-30%	linear
15-70 minutes	30-39%	linear
70-80 minutes	39%	isocratic
80-90 minutes	39-40%	linear
90-110 minutes	40%	isocratic
110-150 minutes	40-50%	linear
150-160 minutes	50-10%	linear

Fractions were collected at 0.5 minute intervals and analyzed as described above. Pure ribosomal protein L22 was obtained using this program.

Extraction of Proteins from Gels

Ribosomal proteins were extracted from two dimensional gels using a procedure adopted from Bernabeu et al. (1980). The separated ribosomal protein spots were excised from the gel and chopped into small pieces. The pieces were placed in a 1.5 ml microfuge tube containing 0.5 ml of 66% HOAC and left at 4°C overnight. The supernatant was removed and combined with 0.5 ml of a slurry of 1 mg/ml DEAE Sephadex in water. The sample was mixed by inversion and spun in a

microfuge for 10 minutes. The Coomassie blue R-250 remained with the DEAE pellet. The supernatant was pulled from the tube and retained. A second 0.5 ml of dH_2O was added to the DEAE resin remaining in the tube. The sample was again mixed by inversion and spun in the microfuge. The supernatants were combined and lyophilized. The dried protein was further purified on a 10 ml P10 gel filtration column. The protein was washed through the column using 10% HOAC. One-half milliliter fractions were collected and the absorbance at 280 nm determined. Fractions containing protein were pooled and lyophilized. The dried protein was resuspended in 50 µl of 10 mM sodium phosphate pH 8.0 and 6 M urea. A Bio-Rad Protein Micro Assay was conducted to determine the amount of protein obtained from a gel core (Bradford, 1976). Generally, about 0.4 µg of protein/gel core could be extracted from the two dimensional gel system.

Isotopic Labeling of Proteins

Ribosomal proteins were labeled using a procedure modified from the works of Dottavio-Martin and Revel (1978), Jentoft and Dearborn (1979), Tack et al. (1980), and Champney (1989). Between 20 and 50 µg of ribosomal proteins were resuspended in 10 µl of phosphate-urea buffer. Two microliters of ^{3}H-formaldehyde (4 µCi/µl) was added to the protein followed by 8 µl of 6 mg/ml sodium cyanoborohydrate in 50 mM phosphate buffer pH 8.0. The mixture was incubated

at 37°C overnight. The extent of incorporation was determined by taking a 1 μl sample of the reaction mix. The sample was added to 250 μg of BSA (1 mg/ml) and precipitated with 1 ml of 50% TCA. The precipitate was collected on a GF/A glass filter, washed with 5% TCA and 95% ETOH. The dried filter was placed in a scintillation vial containing 5 ml of scintillation cocktail of 0.1% PPO in toluene. When 1 μg of protein contained approximately 1000 counts the reaction was terminated by the addition of 100 μl of 37% formaldehyde. The reaction mixture was dialyzed overnight against 10% HOAC and lyophilized. The dried protein was resuspended in reconstitution buffer (20 mM Tris-HCl pH 7.6, 4 mM magnesium acetate, 400 mM NH_4Cl, 4 mM ETSH) and was ready for binding assays. One microliter of the labeled protein was used to determine protein concentration and a second microliter was spotted on a GF/A filter and counted. From these values the specific activity (cpm/μg) was calculated.

Determining the Purity of Labeled Ribosomal Proteins.

The purity of the labeled proteins was checked by gel electrophoresis. Samples of the labeled proteins (approximately 1 μg, 1000 cpm) were placed in 20 μl of sample buffer consisting of 8 M urea, 1% ETSH. The samples were loaded into a twenty well 18% second dimension slab gel. The gel was stained with Coomassie Blue and photographed.

Isolation of Ribosomal RNA

Fifty absorbance units at 260 nm of 50S ribosomal subunits were used. For each 100 µl volume of 50S ribosomal subunits, 10 µl of 10% SDS, 5 µl of 2% Bentonite, and 150 µl of Tris pH 8.0 saturated phenol was added to a microfuge tube. The tube was inverted repeatedly for 5 minutes followed by a 10 minute spin in the microfuge. The aqueous layer was extracted twice with equal volumes of Tris saturated phenol. The RNA was precipitated overnight with two volumes of 100% ETOH. After a 20 minute microfuge spin, the RNA precipitate was washed with 2 volumes of ETOH. The RNA was resuspended in 250 µl of DEPC treated TM-4 buffer. The absorbance at 260 and 280 nm was read to determine the RNA concentration. To determine the purity of the RNA, one absorbance unit at 260 nm of RNA was run on a 1% agarose gel in TBE (pH 8.3).

Binding of Ribosomal Proteins to 23S RNA

Conditions for the binding experiments came from two sources. The binding of the protein to the RNA was conducted according to a procedure modified from Roth and Nierhaus (1980). Separation of the bound RNA-protein complex from free RNA and protein was achieved using a gel filtration procedure adapted from Stoffler et al. (1971). Binding assays were conducted at 2.5:1 and 5:1 molar ratios of the labeled ribosomal protein L22 to 23S RNA with and without the addition of protein L4. An aliquot of labeled

ribosomal protein L22 plus an equimolar amount of unlabeled L4 (if used), was incubated in DEPC treated reconstitution buffer at 32°C or 44°C for 5 minutes. An aliquot of 23S RNA in TM-4 buffer was added to the ribosomal protein followed by a 20 minute incubation at 32°C or 44°C. The protein-RNA mixture was chilled on ice for two minutes and the unbound protein was separated from the protein-RNA complexes on a Biogel A 0.5 M column (1 cm X 13.5 cm) at 4°C. Twenty 0.5 ml fractions were collected. Fractions were read at 260 nm to determine the amount of 23S RNA. Fractions were then placed in 10 ml of scintillation cocktail (666 ml tolulene, 3 gm PPO, 333 ml Triton X-100, 100 ml water) and counted in a Beckman Scintillation counter to determine protein bound to RNA and unbound protein. Twenty counts per minute were subtracted as background from all samples.

Isolation of Bacteriophage λ DNA

Purification of lambda phage was based on the method of Davis et al. (1980). DNA was extracted from both helper phage (lambda cI857S7) and defective phage. The phage was purified by CsCl centrifugation. One-half milliliter of purified phage was placed in a 1.5 ml microfuge tube along with 0.2 ml of STEP buffer and mixed by inversion. The sample was incubated at 60°C for 20 minutes. Protein was extracted by adding an equal volume of Tris buffer saturated phenol, rocking the sample and spinning it in a microfuge (8730 x g) for 5 minutes. The aqueous layer was removed

with a sterile micropipet. This layer was re-extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The pooled aqueous layers were again extracted using an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated from the upper phase by the addition of 0.1 ml of 5 M ammonium acetate and two volumes of 95% ETOH at -20°C. The sample was mixed and placed at -20°C for 20 minutes and centrifuged in the microfuge for 15 minutes to pellet the DNA. The DNA was washed with 1 ml of 70% ETOH and the pellet was dried with a speed-vacuum to remove traces of ETOH. The DNA was dissolved in 200 µl of $T_{10}E_1$ and quantitated by reading the A260 and A280 on a 5 µl aliquot sample. One absorbance unit at 260 nm was equal to 50 µg of DNA. Purity of the DNA was checked by agarose gel electrophoresis.

Preparation of Agarose Gels

Electrophoresis was carried out in a Hoefer "Minnie" Submarine Agarose Gel according to a procedure adapted from Maniatis et al. (1982). The gel solution (1%) consisted of 0.3 gm agarose and 30 ml of running buffer. The running buffer was 0.089 M Tris-borate, 0.002 M EDTA pH 8.3. The agarose solutions were brought to a boil to melt the agarose, cooled to 55°C, then poured into a mold containing a gel comb and allowed to solidify at room temperature. In some cases EtBr (0.5 µg/ml) was added to the gel solution before it was cast. The gel was run at 100 volts until the

Bromophenol Blue tracking dye reached the desired end point. Gels not containing EtBr were stained in 5 µg/ml EtBr in 10 mM $MgSO_4$ solution. DNA bands were viewed using ultraviolet light excitation of the EtBr. Gels were photographed using a Polaroid MP-4 Land camera, f-stop 4.5, one minute exposure, with a red U.V. filter #23A on Polaroid type 55 film.

Restriction Enzyme Digests

Digests of DNA by restriction enzymes were conducted according to a method adopted from Maniatis et al. (1982). DNA was digested using 10 units of restriction enzyme per microgram of DNA. Restriction enzyme buffer was added to achieve a 1X concentration, and water was added to achieve the final reaction volume. Digests were incubated at 37°C for 6-12 hours. Samples were heated in a 70°C water bath for 5 minutes to inactivate the restriction enzymes. Digests were separated on 1% agarose gels as described above.

Plasmid Isolation Procedure

Cells containing plasmid were streaked on a L Broth plate containing 50 µg/ml of ampicillin. Plasmid DNA was extracted from the bacterial cells using a miniprep procedure provided by Dr. P. Musich (unpublished). A 20 ml overnight culture was started from a single colony and was grown in L Broth with 50 µg/ml ampicillin. The cells were

collected by centrifugation at 12100 x g in a JA-20 rotor for 10 minutes. The cells were resuspended in the remaining broth by vortexing followed by the addition of 0.6 ml of SET buffer and further mixing. The cells were transferred into a 1.5 ml microfuge tube. The original tube was rinsed with 0.7 ml of SET buffer which was added to the microfuge tube. The microfuge tube was centrifuged at 8740 x g for 20 seconds. The supernatant was discarded and the pellet was vortexed in the remaining broth until creamy. The cells were resuspended in 250 µl of SET by vortexing. One-half of a milliliter of a fresh alkaline lysis solution (0.2 M NaOH, 1% SDS) was added to the mixture followed by gentle inversion of the tube. The sample was incubated at 65°C for 30 minutes with occasional inversion of the tube. Samples were returned to room temperature and 375 µl of 5 M potassium acetate solution (pH 4.8) was added and mixed by inversion. The solution was placed on ice for 20 minutes to precipitate cellular proteins and chromosomal DNA. This debris was pelleted during a 15 minute microfuge spin. The supernatant was transferred to a clean tube and again centrifuged for 10 minutes. The plasmid DNA was precipitated from the supernatant using 300 µl of a 27% PEG 8000 solution in 3.3 M NaCl. The tube was mixed by inversion and incubated on ice 2-24 hours. The plasmid DNA was pelleted during a 15 minute microfuge spin. The pellet was rinsed twice with 1 ml of 70% ETOH and vacuum dried.

The plasmid was resuspended in 200 µl of $T_{10}E_1$. A 5 µl aliquot of the DNA was electrophoresed on a 1% agarose gel allowing plasmid concentration and purity to be examined. To further purify the DNA, 200 µl of 5 M ammonium acetate, and 400 µl isopropanol were added to the DNA with mixing by inversion. The mixture was chilled for 20 minutes followed by a 15 minute centrifugation in the microfuge. The pellet was washed with 1 ml of 70% ETOH and repelleted. The plasmid DNA pellet was vacuum dried and resuspended in an appropriate volume of $T_1E_{0.1}$

Large Scale Plasmid Isolation Procedure

Large scale plasmid preparations were conducted according to the method of Ausubel et al. (1989). A 500 ml overnight culture was grown from a single colony in L Broth with 50 µg/ml ampicillin. The cells were pelleted and resuspended in 4 ml of TEG. Twenty-five milligrams of lysozyme were added and the cells were allowed to incubate at room temperature for 10 minutes. Ten milliliters of a lysis solution (0.2 M NaOH, 1% SDS) were added to the cell suspension and the mixture was gently stirred with a pipet until it became clear. The lysed cells were incubated on ice for 10 minutes. Seven and one-half milliliters of a 3 M potassium acetate solution (pH 4.8 adjusted using 88% formic acid) was added to the mixture. A large precipitate was formed by stirring with a glass pipet followed by a 10 minute incubation on ice. The precipitate was pelleted

during a 20 minute spin at 12100 x g in a JA-20 rotor at 4°C. The supernatant was transferred to a clean tube. A 0.6 ml volume of isopropanol was added to the supernatant. The tube was mixed by inversion and allowed to stand at room temperature for 10 minutes. The mixture was centrifuged for 20 minutes at 4°C. The pellet was washed with 2 ml of 70% ETOH and dried under a vacuum. The dried pellet was resuspended in 1 ml of TEG. A 2.0 ml volume of lysis solution was added to the pellet followed by a 5 minute incubation at room temperature. The addition of 1.5 ml of potassium acetate caused the formation of a second precipitate which was removed by centrifugation. The supernatant was transferred to a clean tube and treated with DNase-free pancreatic RNase at 20 μg/ml for 20 minutes at 37°C. The DNA was extracted with an equal volume of phenol. The aqueous layer was again extracted with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated in a dry ice ETOH bath by the addition of two volumes of 100% ETOH. The pellet was washed with 70% ETOH and dried under a vacuum. The pellet was resuspended in 1 ml of $T_{10}E_1$ followed by the addition of 0.8 ml of PEG solution (15% polyethylene glycol 8000, 2.5 M NaCl). The sample was incubated for 12 hours at in the refrigerator. The plasmid was harvested by centrifugation in the microfuge for 20 minutes. The precipitate was resuspended in 1 ml of $T_{10}E_1$ buffer and precipitated with two volumes of 100% ETOH. The

pellet was washed with 70% ethanol and vacuum dried. The plasmid pellet was resuspended in 250 µl of $T_1E_{0.1}$.

Elution of a DNA from Agarose Gels

Elution of a specific fragment from an agarose gel was accomplished using the method of Silhavy et al., (1984). The fragment desired was sliced from the gel, and placed in a 1.5 ml microfuge tube containing 200 µl of Tris saturated phenol pH 7.9. The sample was extensively vortexed and placed in a -70°C freezer overnight. The sample was spun in a microfuge (8730 x g) for 15 minutes to pellet the agarose. The aqueous layer was removed and placed in a clean microfuge tube. The agarose gel pellet was rinsed with 200 µl of $T_{10}E_1$, vortexed, and extracted by centrifugation for 15 minutes. The supernatant from this spin was combined with that taken earlier. The combined supernatant was extracted twice with an equal volume of Tris-saturated phenol and precipitated using absolute ETOH.

Preparation of Competent Cells

This procedure was taken from Silhavy et al., (1984). Bacterial cells were grown in L Broth to an absorbance at 600 nm of 0.2. The cells were chilled on ice for 20 minutes followed by centrifugation in the JA-10 centrifuge, at 6370 x g, 4°C. The pellet was resuspended in 200 ml of cold sterile 100 mM $CaCl_2$. The suspension was incubated on ice

for 20 minutes and repelleted as described. The cells were resuspended in 5 ml of 100 mM $CaCl_2$, made 10% in glycerol and placed at 4°C overnight. The sample was stored frozen in the -70°C freezer.

Bacterial Transformations

Transformation of plasmid with insert into competent cells was accomplished using the procedure of Silhavy et al. (1984). A 200 µl aliquot of $CaCl_2$ treated cells was thawed and the ligation mix was added. The solution was allowed to stand on ice for 1 hour, followed by a two minute heat shock at 37°C. The mixture was diluted with 1 ml of L Broth and returned to 37°C for 1 hour to allow phenotypic expression. After expression, the cells were pelleted and resuspended in 100 µl L Broth. Fifty microliters of the transformation mix was plated on an L Broth plate containing 50 µg/ml of ampicillin. The cells were allowed to grow at 37°C overnight, or 32°C when temperature sensitive cells were transformed.

Screening Transformants

Ampicillin resistant colonies were picked from the overnight transformation plate onto a second ampicillin plate. This plate was allowed to grow at 37°C for 2 to 3 hours before a replica was made to a MacConkey's agar plate to determine which colonies contained plasmid with insert. When cloning into the pUC19 plasmid, colonies which are red

in color are lactose$^+$ colonies indicating that the plamid does not contain an insert (Jenning and Beacham, 1989). White colonies are lactose- due to an insert in the plasmid interrupting the beta galactosidase gene. White colonies from the MacConkey's plates were restreaked onto ampicillin plates. From these cells, freezer stocks were made. Lactose- colonies were grown and plasmid DNA was isolated to determine the size and purity of the insert.

Small Scale Plasmid Isolation Procedure

Purification of plasmid from lactose- colonies was accomplished using the small scale alkaline lysis miniprep of Ausubel et al. (1989). A 1.5 ml culture in L Broth with 50 µg/ml ampicillin was started from a single lactose- colony and allowed to grow overnight. Cells were microcentrifuged for 20 seconds. The pellet was resuspended in 100 µl of $T_{10}E_1$ and allowed to incubate for 5 minutes at room temperature. The cells were lysed by the addition of 200 µl of lysis solution and incubated on ice for 5 minutes. A volume of 150 µl of 3 M potassium acetate solution was added causing the formation of a precipitate. The tube was inverted several times and allowed to incubate on ice for 5 minutes. The precipitate was pelleted during a 1 minute microfuge spin. The supernatant was transferred to a clean tube and the plasmid precipitated by the addition of 0.9 ml of ETOH. The precipitate incubated at room temperature for 2 minute followed by a one minute spin in the microfuge.

The plasmid pellet was washed with 70% ETOH and vacuum dried. The pellet was resuspended in 20 µl of $T_1E_{0.1}$. Five microliter aliquots were used for restriction enzyme digests with the addition of 1 µl of a 10 µg/ml RNase solution (DNase-free).

Isolation of Genomic DNA

Preparation of genomic DNA from bacterial cells was accomplished using the method of Ausubel et al. (1989). Bacterial cells were grown in 5 ml of L Broth overnight. The cells were divided into three 1.5 ml microfuge tubes. The cells were pelleted during a 2 minute microfuge spin and resuspended in 567 µl of $T_{10}E_1$. A volume of 30 µl of 10% SDS was added to the tube followed by 3 µl of a 20 mg/ml proteinase K solution. The sample was mixed by inversion and incubated at 37°C for 1 hour. One hundred microliters of 5 M NaCl plus 80 µl of CTAB solution were added to the tube which was incubated at 65°C for 10 minutes. An equal volume of cholorform:isoamyl alcohol (24:1) was added and the tube was inverted repeatedly. After a 4 minute centrifugation, the aqueous layer was transferred to a fresh tube and extracted with an equal volume of phenol:chloroform :isoamyl alcohol (25:24:1). The DNA in the upper phase was precipitated by the addition of 0.6 volumes of isopropanol. The precipitated DNA was transferred with a sealed Pasteur pipet into 1 ml of 70% ETOH and washed by inversion. The DNA was pelleted and rewashed with a second

1 ml of 70% ETOH. The DNA pellet was dried on a speed vac and resuspended in 100 µl of $T_1E_{0.1}$. The concentration and purity of the genomic DNA samples was determined by reading the absorption at 260 and 280 nm on a 1 µl sample.

Polymerase Chain Reaction

The nucleotide sequence for the S10 operon was retrieved from Genbank (Accession number X02613) (Zurawski and Zurawski, 1985). This sequence was scanned using the "Oligo" primer analysis software by Wojciech Rychlik (1990). This program searched the sequence surrounding the rplV gene for 25 base pair oligonucleotide primers for use in PCR assays. The sequences of the best forward and reverse 25 base pair oligonucleotide primers were sent to Dr. Kent Lohman (Southwest Foundation for Biomedical Research; San Antonio, TX) who synthesized these primers. The sequence of the forward primer was 5′ CGG TGG AAA GCG GAG ACA AGA AGC C 3′. The sequence of the reverse primer was 5′ ACC AGT TTT GCG TCC AGT TCA GGC T 3′. The primers were cleaned by n-butanol extraction and the concentration was determined by Dr. Phillip Musich (ETSU) who ran the primers against known DNA standards. The PCR reaction was conducted using genomic DNA isolated from the control strain, both mutant strains and λfus2, with the above primers. Enzymes, nucleotides and buffers were purchased as a kit from Promega. In a 0.5 ml microfuge tube the following ingredients were placed: 1.0 µg genomic DNA or 0.1 µg of phage DNA, 5 µl of 10X buffer (0.5

M KCl, 0.1 M Tris-HCl pH 8.4, 25 mM $MgCl_2$, 1 mg/ml gelatin, 0.1% Tween-20, 0.1% NP-40), 1 µl of dNTP mix (from 10 mM solutions), 10 pmol each of forward and reverse primers, 1 µl of Taq polymerase sequencing grade (2.5 Units) and water to make a final volume of 50 µl. Twenty-five microliters of paraffin oil was layered on top of the sample. The sample was then placed in a Precision Scientific GTC-1 thermal cycler for thirty cycles. The thermal cycler was set to denature DNA at 94°C for 2 minutes, allow annealing of primers to DNA at 55°C for 2 minutes and polymerization of DNA at 72°C for 5 minutes (Ehrlich et al., 1988). Three microliters of the PCR reaction was electrophoresed on a 1% agarose gel with molecular weight standards. Generally, only a single product of 0.9 kb was visible on agarose gels. However, when higher concentrations of the PCR product were assayed on gels, spurious bands of varying sizes were identified. The 0.9 kb PCR product was then extracted or electroeluted from the agarose gel to separate it from contaminating products, nucleotides and primers.

DNA Sequencing using Primer Extension

The PCR product was sequenced using a chain-termination DNA sequencing method (Sanger et al., 1977). This procedure was conducted using materials and instructions from United States Biochemical Corporations DNA Sequencing Kit with Sequenase 2.0. The PCR product and the primers were made as described above. Annealing of the template and primer was

conducted according to the method of Ausubel et al. (1989). The annealing reaction involved the addition of 1 µg of PCR product, 1 pmol of primer (either forward or reverse), 2 µl of sequenase reaction buffer in 10 µl. The labeling mix was diluted 1:1 with water. The Sequenase 2.0 enzyme was diluted as described. One microliter of ^{35}S-dATP was used. The reactions were conducted as described in the Sequenase protocol. One-half of the sequencing reaction mix was loaded on a 5% acrylamide, 50% urea gel in 1X TBE poured using 0.4-0.8 mm wedge spacers. The sequencing buffer was 1X TBE. The gel was pre-run at 1200 volts for 60 minutes. The samples were loaded and electrophoresed for 90 minutes at 1400 volts followed by 180 minutes at 1600 volts (until the xylene cyanol dye reached the bottom of the gel). A second set of samples was then loaded and the gel was again run until the xylene cyanol reached the bottom of the gel. The gel was removed from the apparatus and soaked in 12% methanol and 10% acetic acid for 30 minutes to remove the urea. The gel was transferred onto Whatman #3 paper and dried on a gel dryer. An autoradiogram of the dried gel was prepared. The Kodak diagnostic X-OMAT AR film was exposed for 3 days at room temperature.

Confirmation of the DNA sequence by sequencing using 15 base primers. Due to difficulties encountered sequencing a fragment 926 bp long, the length of the original PCR product, new primers were designed for use with a smaller

Eco RI fragment of this PCR product. The PCR product (produced above) was cleaved using Eco RI and the fragments separated on a 1% agarose gel. A 0.5 kb fragment known to contain the rplV gene was electroeluted and precipitated. This 0.5 kb fragment was sequenced using a second set of primers. The 15 base primers for the 0.5 kb fragment were generated using the "Oligo" program and were synthesized by Dr. Kent Lohman. The sequence of the forward primer was 5' GCG ACG CTG CTG ATA 3'. The sequence of the reverse primer was 5'CCC AGG CGA ATA CCA 3'. The primers were purified using butanol extraction and resuspended in 250 µl of dH_2O. The concentration of the primers was determined by reading the absorbance at 260 nm and 280 nm on 5 µl of the primer diluted in 1 ml of dH_2O. Sequencing was conducted using materials and instructions in the United States Biochemicals DNA Sequencing Kit with Sequenase 2.0 was described above with the following modifications. The labeling mix was diluted 15 fold with water. Otherwise the reactions were conducted as described in the Sequenase protocol. Half of the reaction was loaded on a 8% acrylamide, 50% urea wedge gel in 1X TBE. The samples were loaded and electrophoresed until the xylene cyanol dye reached 30 cm from the top of the gel. An autoradiogram of the dried gel was prepared and exposed for 3 days at room temperature.

Detection of Sulfhydryl Groups

Determination of the sulfhydryl content of protein L22

was conducted according to the method of Grassetti and Murray (1967). Ribosomal protein L22 from stains SK1047, SK1048 and the control strain were isolated from two dimensional gels as described earlier and resuspended in 8 M urea. Protein concentration was determined by the Bradford assay. MS2 phage coat protein containing two cysteine residues and S. aureus V8 protease containing no cysteine residues were used as control proteins to standardize the reactions. Ten, twenty, thirty and forty micrograms of the ribosomal proteins, MS-2 phage coat protein or S. aureus V8 protease were resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.2. The protein solutions were heated to 80°C for three minutes and allowed to cool to room temperature. Twenty-five microliters of 4-PDS solution (2 mM in 1% DMSO) was added to the cooled solution. The reactions were allowed to stand at room temperature for 15 minutes to allow complete reaction of the 4-PDS with the protein. The ultraviolet absorbance at 324 nm was determined after the incubation. The absorbance at 324 nm was converted to nmol of sulfhydryl groups using an extinction coefficient of 1.98×10^4 moles/liter/cm at 324 nm for the 4-PDS derivitive (Grassetti and Murray, 1967). Molar protein concentrations were calculated using the molecular weights of 14 kilodaltons for MS2 coat protein and 12 kilodaltons for protein L22 (Sober, 1970; Wittmann-Liebold and Greuer, 1980).

Chapter 3

RESULTS

Background Information

A number of physiological and biochemical features relating to the temperature-sensitive phenotypes of strains SK1047 and SK1048 were described previously. These results have been summarized in Table 1. This data provides background information necessary for a complete description of these mutant strains and for interpretation of the results described in this dissertation.

Creation of the Mutants

Escherichia coli strain 10330, a *trp S* mutant mapping at 74 minutes (Doolittle and Yanofsky, 1968) was treated with 100 μg/ml of nitrosoguanidine. Selection was for *trp* S^+ revertants. Revertants unable to grow on replica plates at 42°C were tested to determine the linkage between the temperature-sensitive mutation and the *aro E* gene at 72 minutes by P1 phage transduction. Strains SK1047 and SK1048 were isolated in this manner (Champney, 1979).

Temperature Sensitivity and Conditional Lethality

E. coli strains SK1047 and SK1048 do not form colonies on Luria plates at 42°C. The temperature-sensitive

Table 1. Temperature-Sensitive Activities and Protein Synthesis Defects in Strains SK1047 and SK1048.

	SK901	SK1047	SK1048
Ability to form colonies at 44°C	+	-	-
Time required for 50% loss of viability at 44°C	NA	200 min	90 min
Rate of RNA synthesis based on ^{3}H-uracil incorporation	100%	12%	19%
Rate of protein synthesis based on ^{3}H-leucine uptake	100%	47%	8%
Kinetics of MS2 phage RNA directed *in vivo* protein after a 32°C to 42°C shift	increased by 42%	decreased by 30%	decreased by 84%
Kinetics of MS2 RNA directed *in vitro* protein synthesis	100%	55%	40%
Time required for 50% loss of MS2 RNA directed protein synthesis activity	258 min	97 min	137 min
Ribosomal subunit reassociation at 44°C	75%	45%	23%
MS2 RNA binding at 44°C after a preincubation	87%	76%	108%
Phe-tRNA binding to 70S ribosomes at 44°C with preincubation	100%	66%	23%
Phe-tRNA binding to 50S subunits at 44°C with preincubation	100%	40%	40%
Relative RNAase resistance of tRNA at 44°C with preincubation	98%	120%	67%

phenotype of these mutants can also be seen in liquid culture after a temperature-shift from 32°C to 42°C (Champney, 1979).

Mutant strain SK1047 showed a 50% loss of viability after 200 minutes at 44°C and mutant strain SK1048 exhibited a 50% loss of viability after 90 minutes at the same temperature (Champney, 1979).

RNA and Protein Synthesis Rates

To determine if the temperature-sensitive phenotype was effecting RNA or protein synthesis, the rates of transcription and translation were assessed. The rates were determined by measuring the incorporation of radioactively labeled uracil and leucine. The control strain was used for comparison. Strain SK1047 showed 12% of the control activity in RNA synthesis assays and 47% of the activity of the control strain in protein synthesis assays after a shift to 42°C. Strain SK1048 showed 19% of the control activity in RNA synthesis assays and 8% of the control activity in protein synthesis at 42°C (Champney, 1979). In both mutants the rates of RNA and protein synthesis were reduced when compared to the control strain.

Antibiotic Sensitivity

The mutants were tested for alterations in their sensitivity to antibiotic inhibitors of translation.

Neither mutant was changed in its sensitivity to the 30S ribosomal subunit inhibitors kanamycin, spectinomycin and streptomycin nor to the 50S subunit inhibitor erythromycin when compared with control cells (Champney, 1979).

Analysis of Subunit Reassembly

A study of ribosomal assembly probes the interactions between ribosomal RNA and ribosomal proteins. Assembly studies detect structural defects associated with temperature sensitivity. These types of studies give information on the constituents in place on the ribosome at any point during assembly. RNA was labeled with ^{3}H-uracil after a growth shift to 44°C in both control and mutant cells. The labeled cell extracts were sedimented through sucrose gradients to separate the ribosomal subunits. Gradient profiles matched those of control ribosomes, showing that there was no build up of an intermediate assembly product. From the profiles it was concluded that neither mutant strain exhibited a ribosome assembly defect (Champney, 1979).

In Vivo Protein Synthesis Rates

To determine the effect of temperature on protein synthesis, the kinetics of MS2 phage RNA directed protein synthesis was examined. Cells were infected with MS2 phage at different temperatures, treated with rifampicin to inhibit host RNA synthesis and labeled with ^{14}C leucine for

30 minutes. Labeled proteins from the cell lysates were separated by electrophoresis and autoradiographed. Densitometry was used to quantitate the amount of phage coat protein in the gel. The control strain showed a 42% increase in total coat protein synthesized at 42°C relative to 32°C. A decrease in the extent of protein synthesis was seen for the mutants with the same temperature-shift. This reduction was significant in strain SK1048 which exhibited an 84% reduction in the amount of coat protein synthesized. Strain SK1047 displayed a 30% decline over the same temperature range. Both mutant strains showed a reduced extent of *in vivo* protein synthesis when compared to control strain irrespective of growth temperature (Armstrong-Major and Champney, 1985).

In Vitro Protein Synthesis Rates

Protein synthesis assays were performed *in vitro* to further analyze translation in the mutants. The amount of protein synthesized from phage MS2 RNA was quantitated by measuring the incorporation of radiolabeled valine into coat protein. The amount of protein synthesized from the MS2 message was used to assess the effect of temperature on the ribosome activity. At 30°C, both mutant strains initiated protein synthesis at a rate comparable with the control. After incubation at 44°C, the rate of initiation diminished by 55% in SK1047 and 40% in strain SK1048 when compared to the control. The mutant ribosomes were clearly heat

sensitive in mRNA translation in vitro (Champney, 1980).

Inactivation Kinetics

The temperature lability of the altered ribosomes was examined. Ribosomes from control and mutant strains were incubated for different times at 44°C and their activity was assayed using the MS2 phage RNA translation system. Ribosomes from the mutants were shown to be heat sensitive. The time for 50% inactivation of control ribosomes was 258 minutes. This was compared with the inactivation time for mutant strain SK1047 of 97 minutes and for strain SK1048 of 137 minutes. The inactivation data further supports the ribosome as the source of temperature sensitivity in the mutants (Champney, 1980).

Ribosomal Subunit Reassociation

To examine the effect of temperature on the interaction between the large and small subunit of the ribosome, subunit reassociation tests were performed. Subunits isolated from the control strain combined to yield 75% reassembled 70S ribosomes in 20 mM magnesium acetate after incubation at 44°C. Subunits from mutant SK1047 were less active, exhibiting 45% reassociation under the same conditions. Reassociation was most strongly affected in SK1048 with only 23% of the subunits able to join together to form 70S ribosomes after heating (Champney, 1980).

mRNA Binding Assays

The ability of the mutant ribosomes to initiate protein synthesis was tested by examining their ability to bind mRNA. Ribosomes from the mutants showed no significant reduction in MS2 RNA binding at 44°C (Champney, 1980). Therefore, the initiation of protein synthesis was not effected in these mutants.

tRNA Binding Assays

To probe elongation, the binding of tRNA to the mutant ribosomes was studied. Ribosomes from both mutant strains showed reduced binding of phenylalanine tRNA at 44°C. Strain SK1047 showed a 34% reduction in phenylalanine tRNA binding and strain SK1048 showed a 77% reduction in phenylalanine tRNA binding when compared to the control (Champney, 1980).

To examine which subunit exhibited the altered phenylalanine tRNA binding, the assay was repeated using ribosomal subunits. The 30S subunits from both mutant strains were found to bind tRNA at rates comparable to the control even at temperatures of 55°C. The 50S subunits from both mutants showed a 40% reduced ability to bind tRNA at 44°C when related to the control. Therefore, the temperature-sensitive phenotype was associated with the 50S subunit of the ribosome (Champney, 1980).

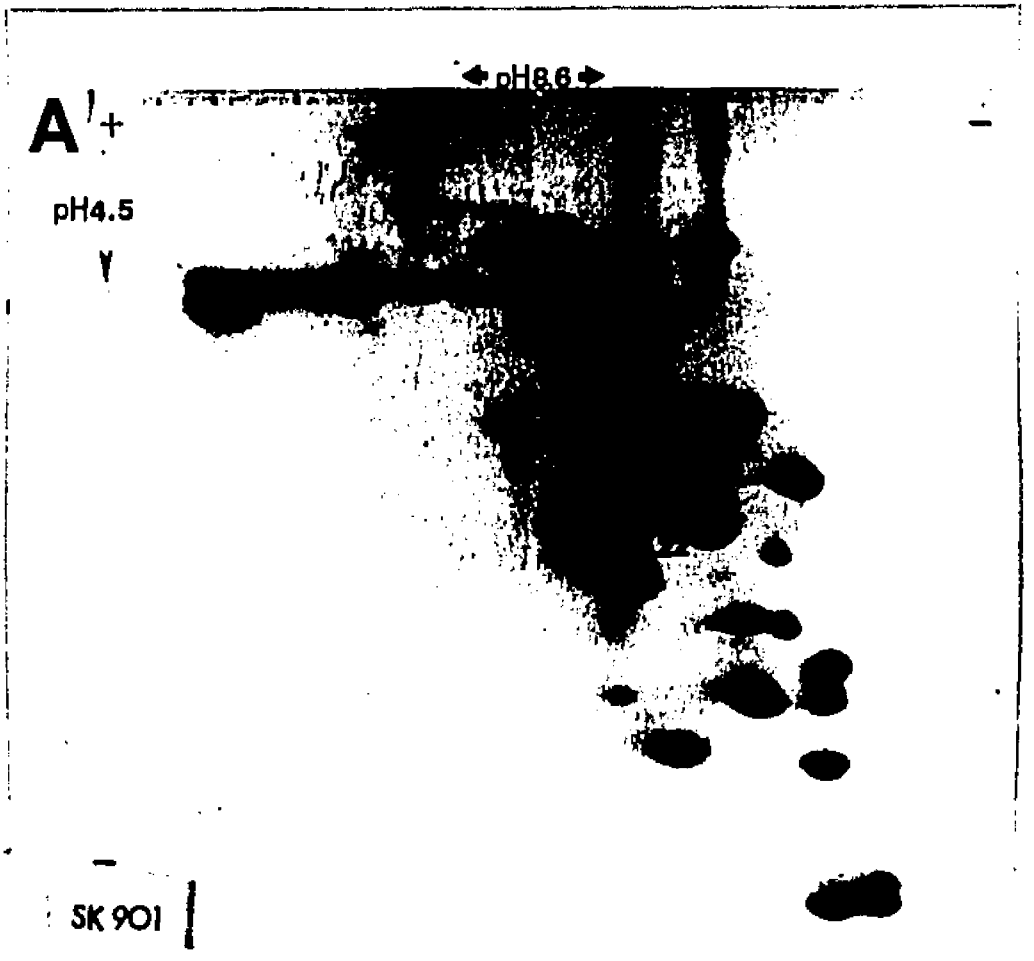
Protection Experiments

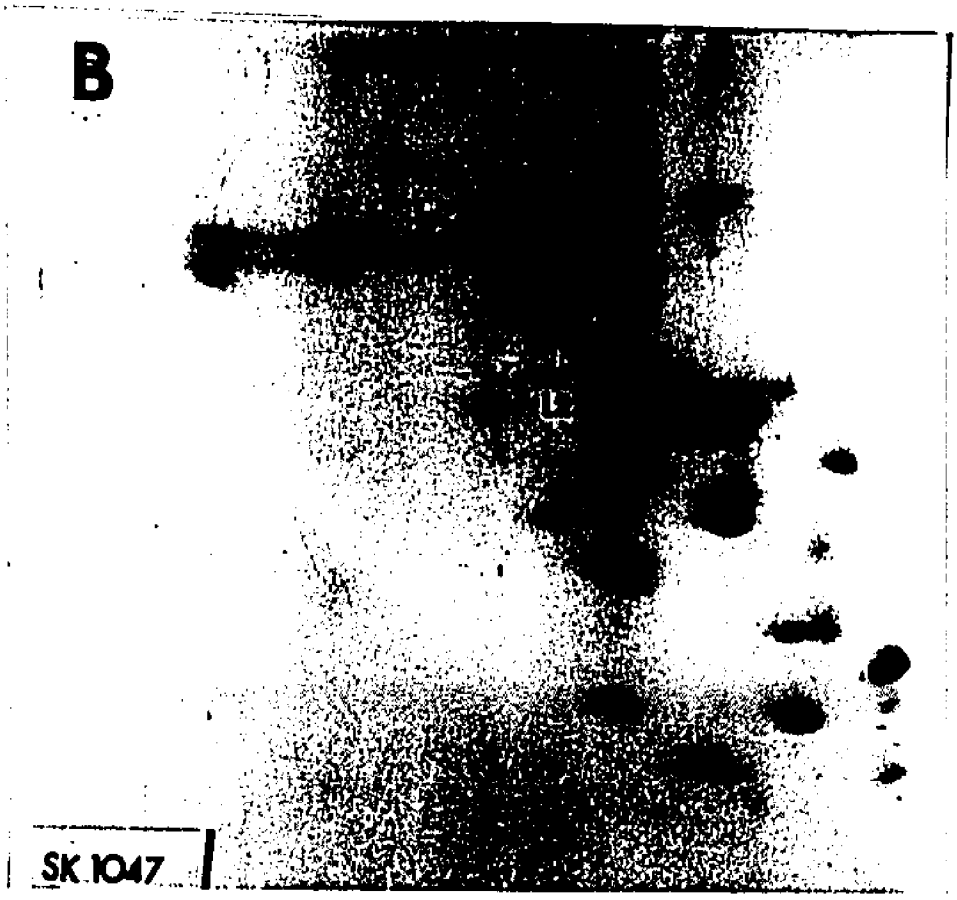
To further study interactions within the ribosome, nuclease protection experiments were conducted. Protection of bound tRNA from ribonuclease digestion is characteristic of 70S ribosomes, while tRNA bound to subunits is rapidly degraded. 50S subunits from control and mutant strains were heated to 55°C and then reassociated with 30S subunits and tRNA. Ribosomes from SK1048 assayed in this manner showed a reduced ability to protect phenylalanine tRNA (Champney, 1980). This indicated a conformational change within the ribosome as a result of exposure to heat. Ribosomes from strain SK1047 were able to protect tRNA from digestion. Therefore, the ribosomal subunit interactions were not severely altered in this strain.

Two Dimensional Gel Electrophoresis

The functional assays performed earlier suggested the 50S subunit of the ribosome as the site of the temperature-sensitive mutation. To investigate the protein composition of the ribosome, two dimensional polyacrylamide gels were used to search for possible alterations in a ribosomal protein. Gel patterns from the control strain and the mutant strains (Figure 6) were compared and ribosomal protein L22 was found to be altered in both mutant strains (Champney, 1980). The altered ribosomal protein L22 was found to exhibit a reduced mobility in the first dimension.

Figure 6. TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS OF 70S RIBOSOMAL PROTEINS. Ribosomal proteins were extracted from control and mutant strains and separated by electrophoresis using the method of Howard and Traut (1973). (A) SK901 (control); (B) SK1047; (C) SK1048. The arrows indicate the position of ribosomal protein L22 on the gels.

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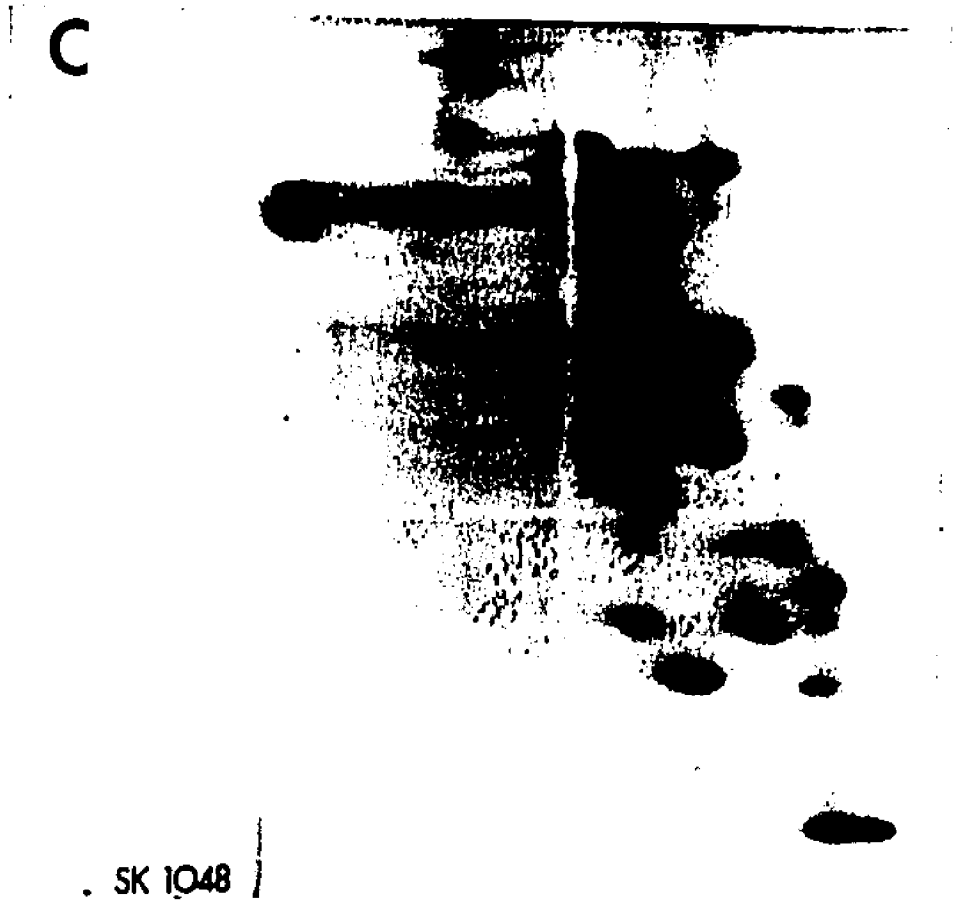
SK 1047


C
SK 1048

Experimental Results

The initial characterization of strains SK1047 and SK1048 suggested that these mutants contained an altered ribosomal protein L22 which caused them to exhibit an inhibition of growth at 44°C. There had been little prior exploration into the function of this protein within the ribosome. The present study of two mutants was designed to provide insight into the nature of the mutational alteration and the functional contribution of this protein in protein synthesis.

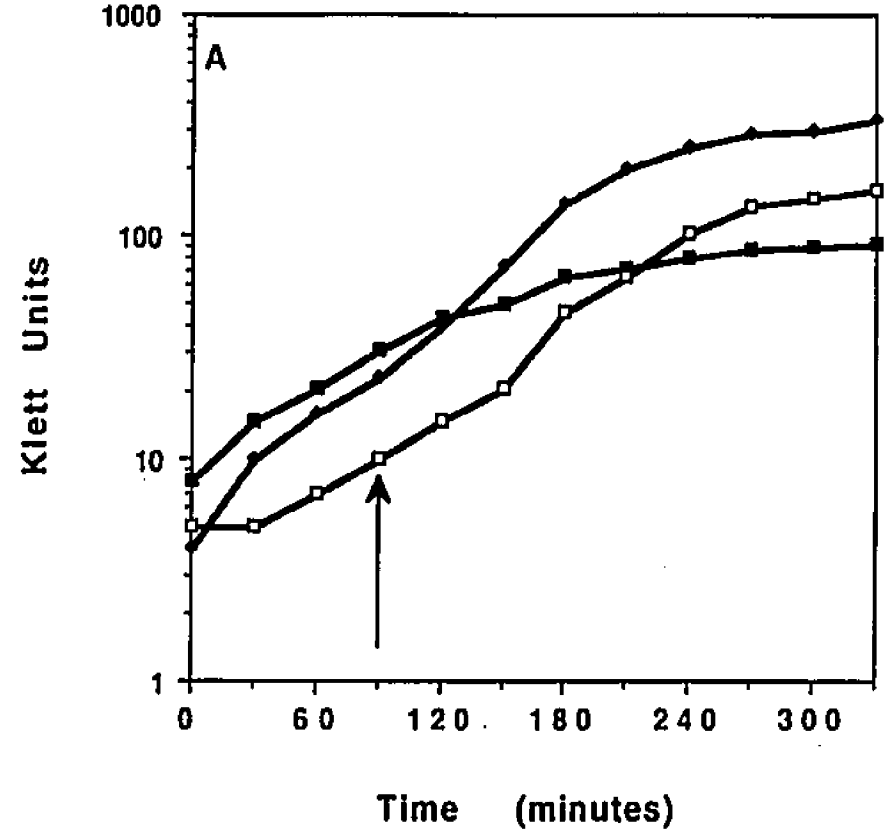
Temperature Sensitivity in the Mutant Strains

The temperature-sensitive character of the mutants was readily apparent by replica plating. A more quantitative analysis of the growth inhibition was conducted by temperature-shift and recovery experiments.

Temperature-Shift Experiments

Temperature-shift experiments were conducted on control and mutant strains. Cells were grown at 32°C until mid log phase and then held at 32°C or shifted to 44°C (Figure 7). Cells from the control strain had the fastest doubling time and reached the highest cell density irrespective of temperature (Table 2). At the permissive temperature, the mutant strains multiplied at 75% of the control rate. Control cells shifted to the restrictive temperature

Figure 7. TEMPERATURE-SHIFT EXPERIMENTS. Growth curves of the control strain and the temperature-sensitive mutant strains in L broth for the time indicated. Cultures were shifted at 90 minutes (arrow). (A) Growth curves from the control strain after a shift from 32°C to 44°C (♦), Mutant SK1047 grown at 32°C (□), and mutant strain SK1047 after a shift from 32°C to 44°C (■). (B) Growth curves from the control strain after a shift from 32°C to 44°C (♦), mutant SK1048 grown at 32°C (○), and mutant SK1048 after a shift from 32°C to 44°C (●).


A
1000
100
10
1
Klett Units
0
60
120
180
240
300
Time (minutes)

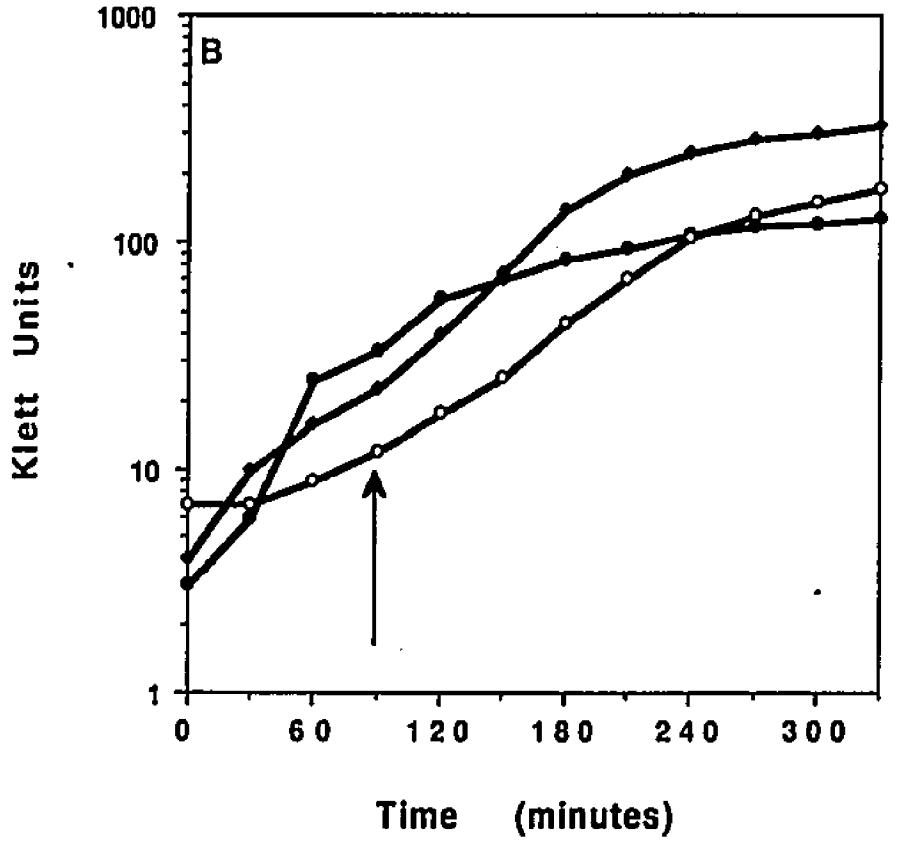

B
1000
100
10
1
Klett Units
0
60
120
180
240
300
Time (minutes)

Table 2. Temperature-Shift Experiments

	Final Cell Density (Klett units)	Specific Growth Rate Constant (k) (hr^{-1})
SK901 32°C	298	1.02
SK901 32°C -shift 44°C	332	1.02
SK1047 32°C	161	0.78
SK1047 32°C -shift 44°C	93	*
SK1048 32°C	173	0.72
SK1048 32°C -shift 44°C	130	*

Temperature-Shift Experiments. The final cell density of each culture was determined using a Klett-Summerson Colorimeter. Cultures were grown at 32°C until they obtained a density of 30 Klett units at which time cells were shifted to 44°C or left at 32°C. The specific growth rate (hr^{-1}) was calculated from a measurement of the doubling time (d) averaged from four growth estimates. [$k = 0.693/d$]

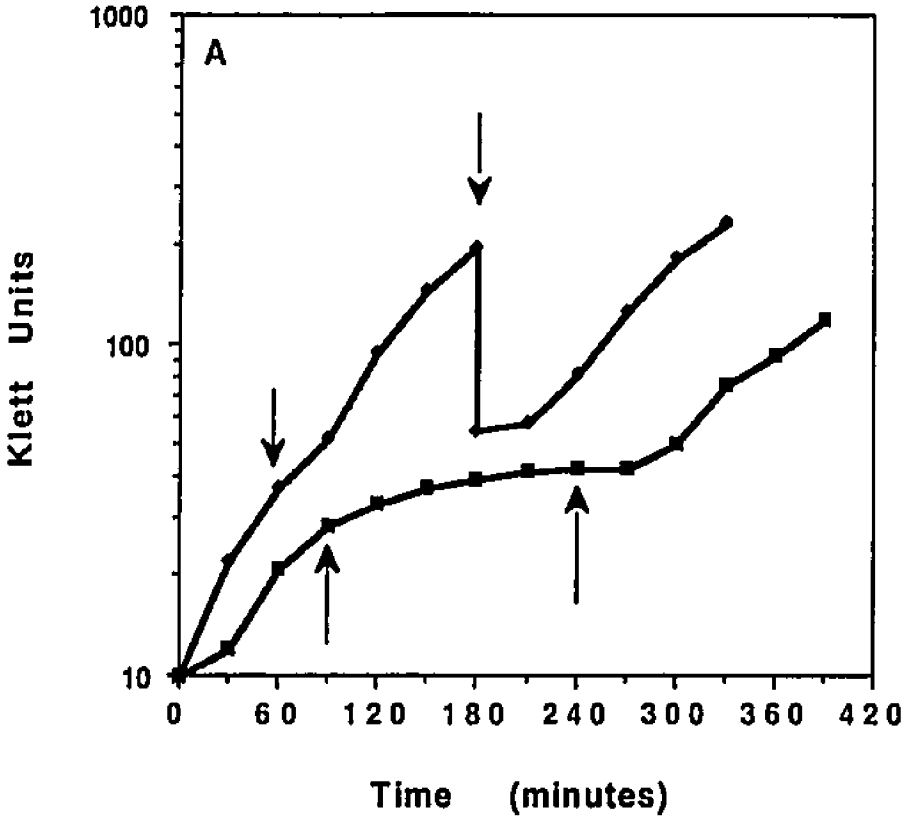
* cells ceased growing after two hours at 44°C.

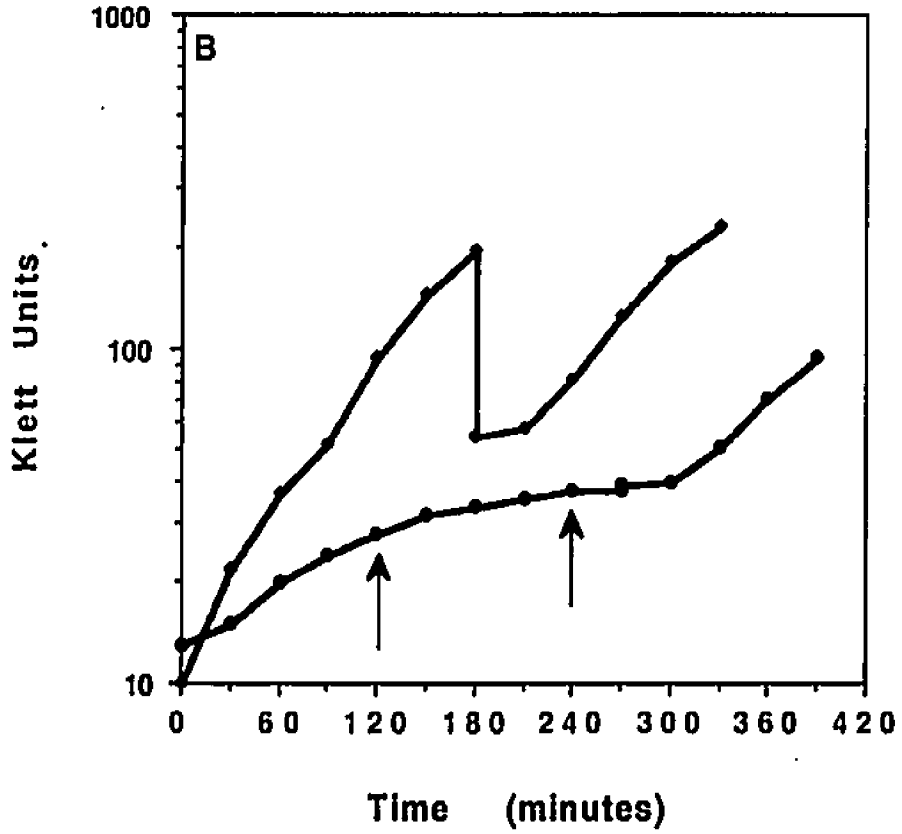
continued to grow at the same rate. Mutant cells shifted to 44°C exhibited a constantly declining cell growth rate and ceased growing after two hours. The final cell density of the mutant cultures was reduced by more than 50% compared with the control.

Growth Shift and Recovery Experiments

To examine the effects of a temperature-shift on the ability of the mutants to recover at the permissive temperature, growing cells were exposed to 44°C and then returned to 32°C in recovery experiments. Initial growth of the mutant strains was depressed when compared with the control strain growing at 32°C (Figure 8 A,B). When cultures reached midlog phase they were shifted to 44°C. At 44°C, the control strain continued to grow at a constant rate while both mutants demonstrated a reduction in cell growth. Both mutants ceased growing after two hours at the restrictive temperature. At this time, the cultures were returned to 32°C. At 180 minutes, the control culture was diluted with LB back to midlog phase since it was entering the stationary phase of cell growth. The diluted culture was returned to 32°C where it continued to double at its previous rate. No change in the growth of the control strain could be detected because of the shift to 44°C or its return to 32°C. The mutant strain SK1047 began dividing 30 minutes after the second shift returning the cultures to 32°C. Growth of mutant strain SK1048 was inhibited longer,

Figure 8. RECOVERY EXPERIMENTS. Growth shift experiments were conducted on control and mutant strains. The recovery experiments were conducted in duplicate although only a single trial is shown here. (A) Growth pattern for the control strain at 32°C, shifted to 44°C (first arrow) and returned to 32°C (second arrow) (♦). Similarly, mutant SK1047 grown at 32°C, shifted to 44°C (first arrow), and returned to 32°C (second arrow) (■). (B) Growth pattern for the control strain at 32°C, shifted to 44°C and returned to 32°C (♦). Correspondingly, mutant SK1048 grown at 32°C, shifted to 44°C (first arrow) and returned to 32°C (second arrow) (●).


A
1000
100
10
Klett Units
0
60
120
180
240
300
360
420
Time (minutes)


B
1000
100
10
Klett Units
0
60
120
180
240
300
360
420
Time (minutes)

recovering after 60 minutes at 32°C.

Reversion Analysis

Loss of the temperature-sensitive phenotype by spontaneous reversion was measured for each mutant. Reversion frequencies for the mutant strains were determined in duplicate trials by plating serial dilutions of overnight cultures at 32°C and 44°C. Temperature resistant revertants were isolated from strain SK1047 at a frequency of 1.1 X 10^{-6}. Revertants of strain SK1048 were found at a frequency of 1.5 X 10^{-6}. These frequencies are indicative of a single point mutation conferring the temperature sensitivity (Miller et al., 1972).

Reversed Phase HPLC Analysis

The altered migration of protein L22 in the mutants was very apparent by two dimensional gel analysis which separates proteins on the basis of charge and size (Figure 6). To see if the hydrophobic properties of the protein were also affected, reversed phase HPLC was used to separate the 50S subunit proteins from the control and mutant strains.

Reversed Phase Separation of Ribosomal Proteins

Total 50S ribosomal proteins from control and mutant strains were separated on a C-4 reversed phase column using an acetonitrile gradient adapted from the methods of Kerlavage et al. (1983). An enhanced separation of the

proteins eluting near protein L22 was achieved by gradually increasing the gradient from 40% to 41% over a 10 minute period followed by an isocratic elution for 20 minutes at 41% acetonitrile as suggested by Cooperman et al., (1988). Chromatography of the 33 different 50S subunit proteins resulted in 28 clearly resolved peaks (Figure 9). The identity of all 50S proteins separated by this method has been determined recently (Champney, 1990). By overlaying the control and mutant chromatograms a difference in the pattern was observed. Peaks labeled 1, 2 and 4 in Figure 9 aligned well when the chromatograms were overlayed, however, the positin of peak 3 was altered in the mutant profiles. Peak 3 eluted at approximately 80 minutes. Consequently, the 74-100 minute region of the chromatogram was analyzed to determine the identity of the ribosomal proteins eluting in this region. The proteins were identified by examining two minute fractions on polyacrylamide slab gels containing 50S ribosomal protein standards (Figure 10). The components of each fraction appeared as a series of bands which were matched to the 50S ribosomal protein standard (Figure 10B). Analysis of fractions from the control chromatogram allowed identification of the proteins eluting in each peak (Figure 10 A,B). Gel analysis showed that the 76 and 78 minute fractions corresponding to peak 1 contained ribosomal protein L23. Overlapping the elution of protein L23 was protein L29 which was found in the 78 and 80 minute

Figure 9. FRACTIONATION OF RIBOSOMAL PROTEINS USING REVERSED PHASE HPLC. Fractionation of 50S ribosomal proteins from the control strain on a C-4 reversed phase HPLC column. A sample of 400 μg of ribosomal protein was injected in 200 μl of 66% acetic acid. Proteins were eluted from the column with a 10-60% acetonitrile gradient containing an isocratic region at 41% acetonitrile to better separate proteins in the region of interest. The boxed area indicates the region of interest and the peaks examined are labeled 1 through 4. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible.

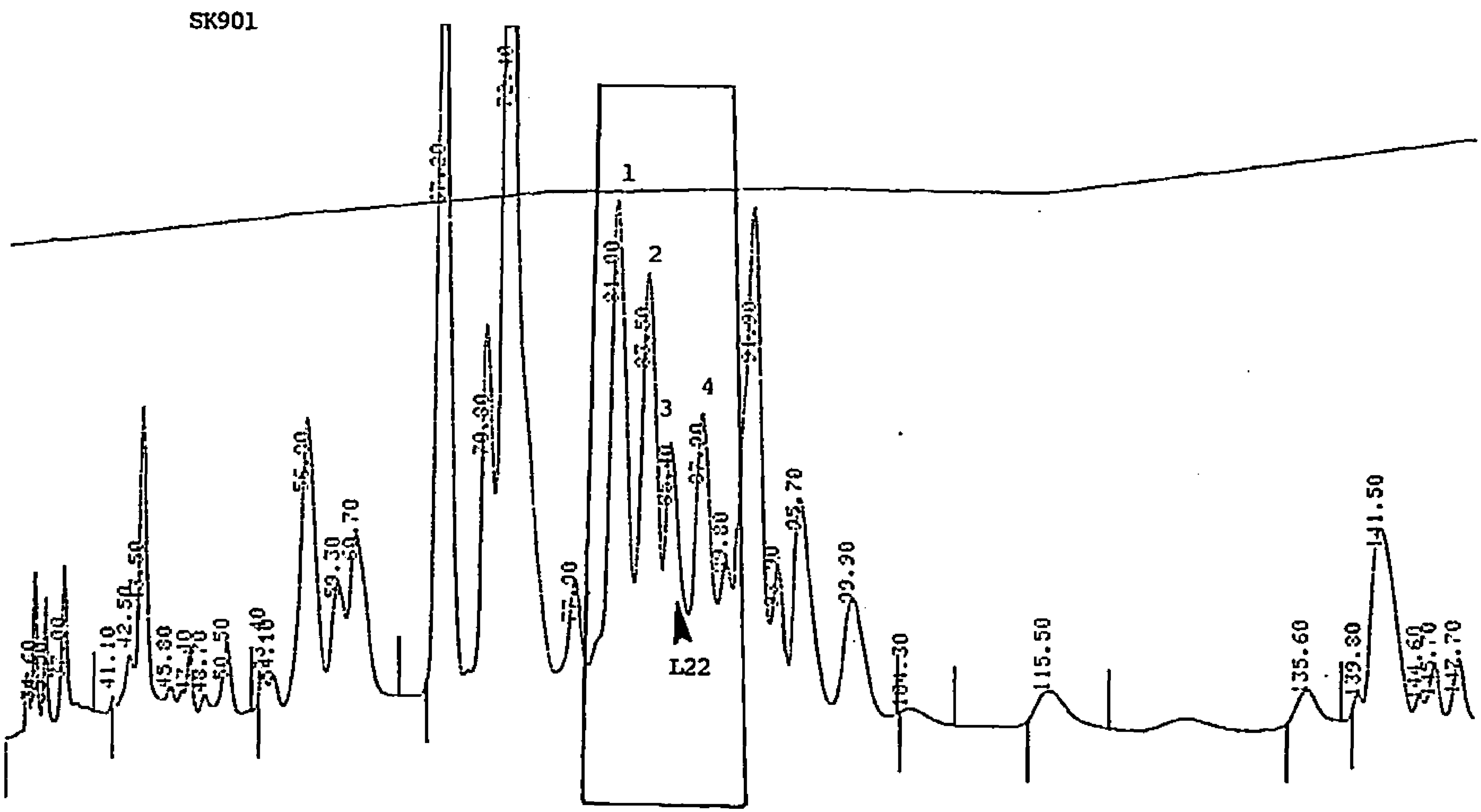
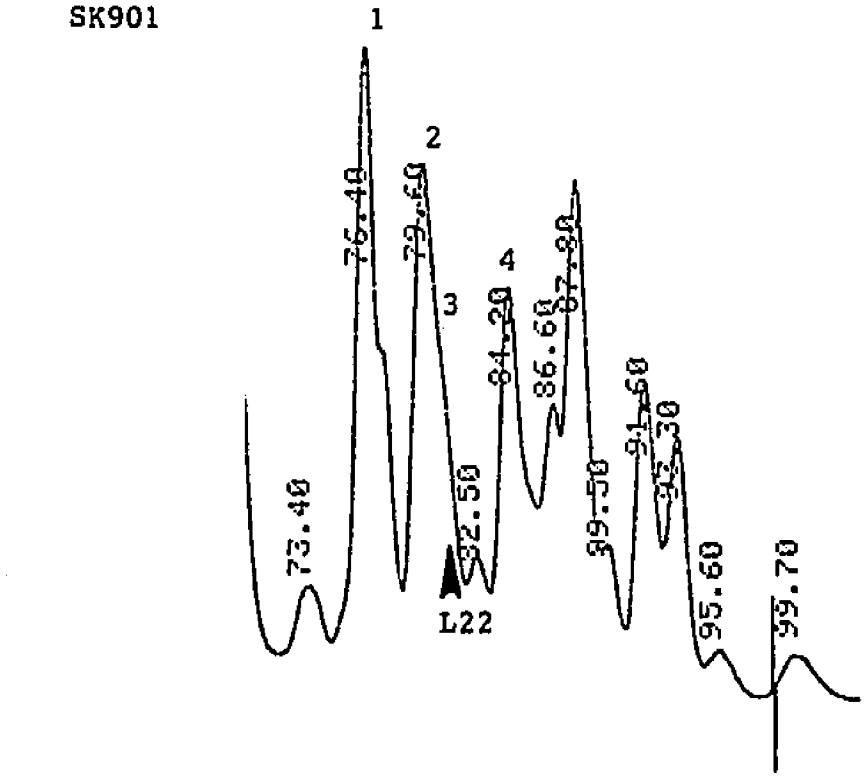
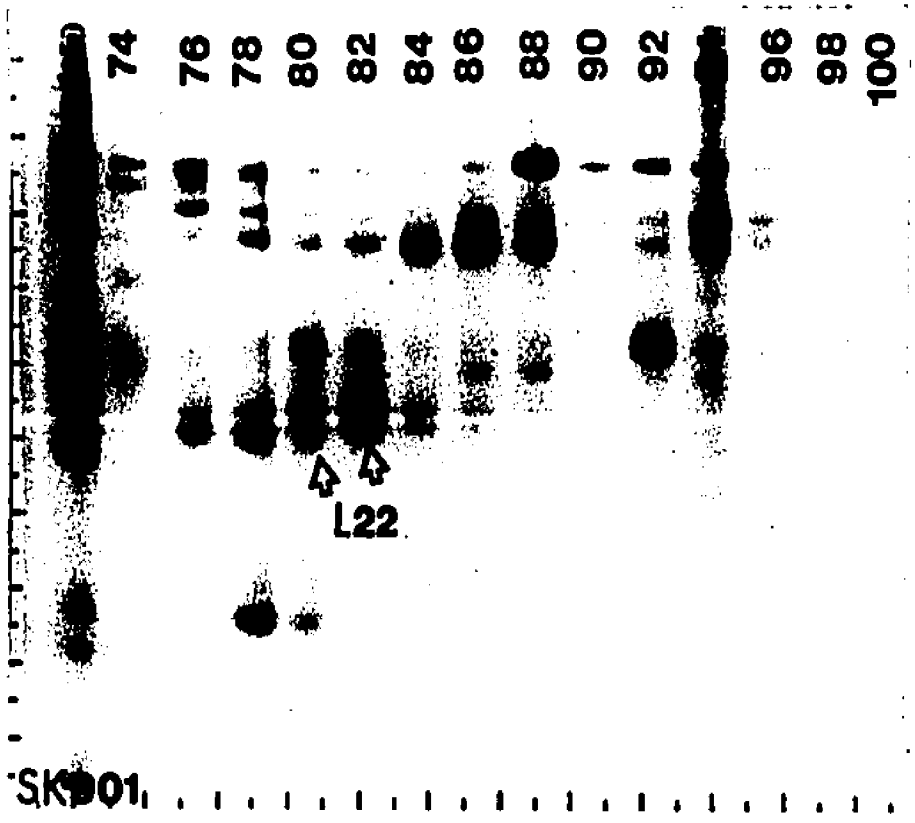

SK901
1
2
3
4
L22
41.10
95.70
99.90
115.50
135.60
141.50

Figure 10. IDENTIFICATION OF RIBOSOMAL PROTEINS IN HPLC PEAKS FROM THE CONTROL STRAIN. (A) Enlargement of the 73-100 minute region of a control HPLC chromatogram. Fractions were collected at two minute intervals during this separation. Peaks labelled 1-4 are described in the text. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible. (B) Fractions were analyzed on an 18% polyacrylamide slab gel. Components of each fraction appeared as a series of bands which were identified by matching the position of each band to the 50S standard. Lanes 1. 50S ribosomal protein standard. Lanes 2-15. Successive two minute fractions of the 74-100 minute region from the HPLC separation. Protein L22 is indicated with an arrow.

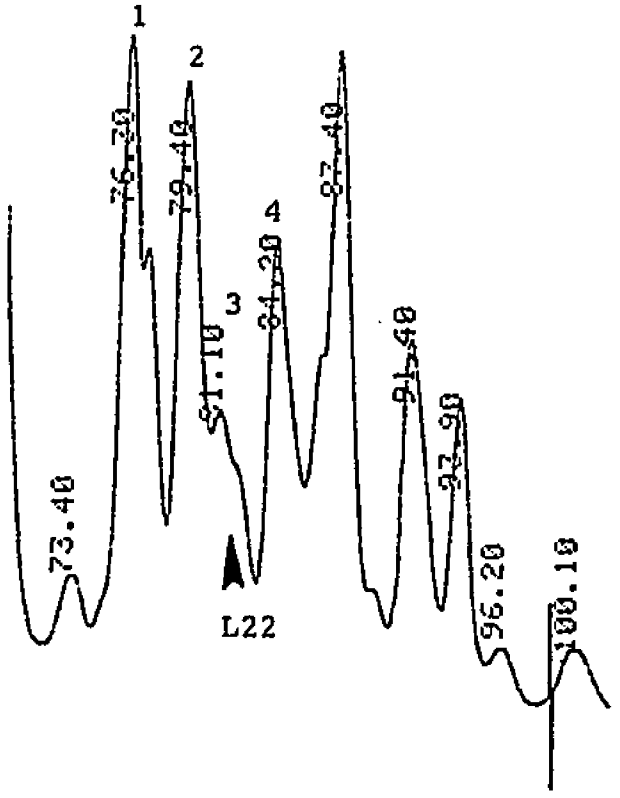

SK901
1
2
3
4
73.40
76.40
79.60
82.50
84.20
86.60
87.90
89.50
91.60
92.30
95.60
99.70
L22

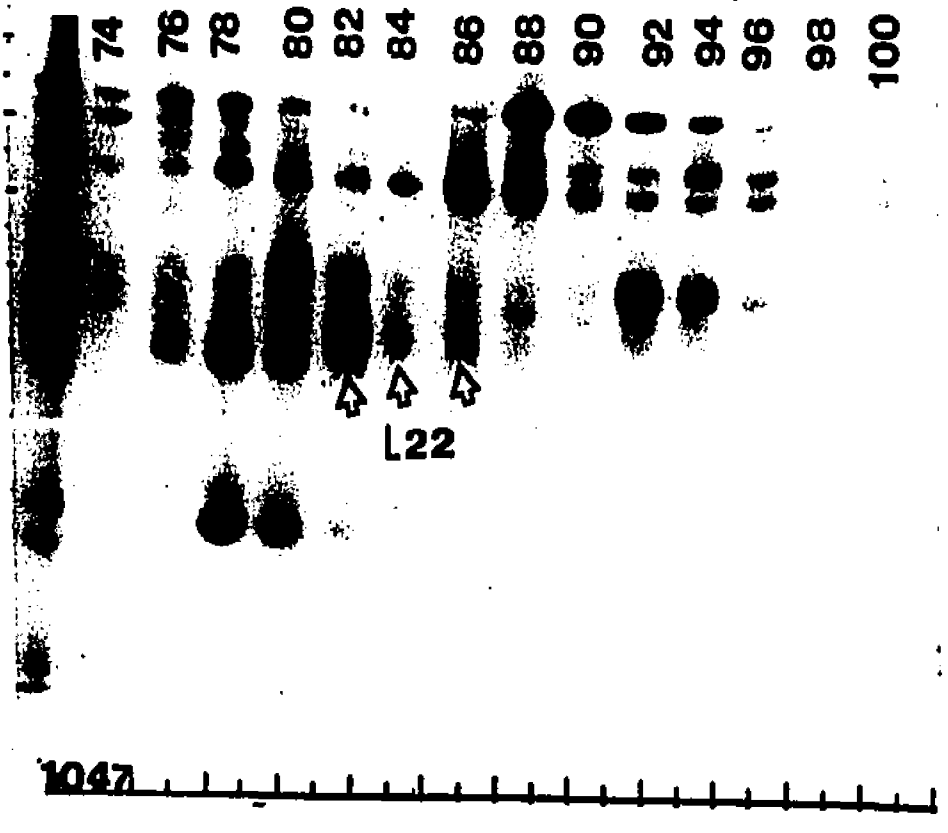

74
76
78
80
82
84
86
88
90
92
96
98
100
L22
SK901

fractions and assigned to the back shoulder of peak 1. The majority of ribosomal protein L15 was found in the 80 minute fraction and therefore this protein was assigned to peak 2 eluting at 79.6 minutes. Peak 3 was not clearly resolved on this chromatogram but instead appears as a shoulder of peak 2 due to a reduction of the resolution in this region. The elution time for peak 3 was determined by calculating the difference between the elution times of peak 2 and peak 3 on an analogous chromatogram (Figure 9). In this manner, peak 3 was predicted to elute 0.9 minutes later than peak 2 placing its elution time at 80.5 minutes. Overlapping the elution of ribosomal protein L15, was protein L22. Protein L22 was found to elute in the 80 and 82 minute fractions and was assigned to the peak 3 at 80.5 minutes. A small peak was observed at 82.5 minutes on this chromatogram. This peak contained a trace amount of ribosomal protein S5, a contaminating 30S ribosomal protein. Following protein L22, protein L11 eluted in the 84, 86 and 88 minute fractions. It was assigned to peak 4 with an elution time of 84.2 minutes. The chromatogram of 50S proteins from mutant SK1047 revealed that peak 3 eluted at 81.1 minutes (Figure 11). No other differences from the control pattern were detected in this region (peaks 1, 2 and 4). Gel analysis of fractions from this chromatogram verified the assignments made using the control strain. The only variation observed was an increased quantity of protein L22 in the later

Figure 11. IDENTIFICATION OF RIBOSOMAL PROTEINS IN HPLC PEAKS FROM STRAIN SK1047. (A) Enlargement of the 73-100 minute region of a HPLC chromatogram conducted using 50S ribosomal protein from strain SK1047. Fractions were collected at two minute intervals during this separation. Peaks labelled 1-4 are described in the text. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible. (B) Fractions were analyzed on an 18% polyacrylamide slab gel. Components of each fraction appeared as a series of bands which were identified by matching the position of each band to the 50S standard. Lane 1. 50S ribosomal protein standard. Lanes 2-15. Successive two minute fraction of the 74-100 minute region from the HPLC separation. Protein L22 is labeled with an arrow.

SK1047

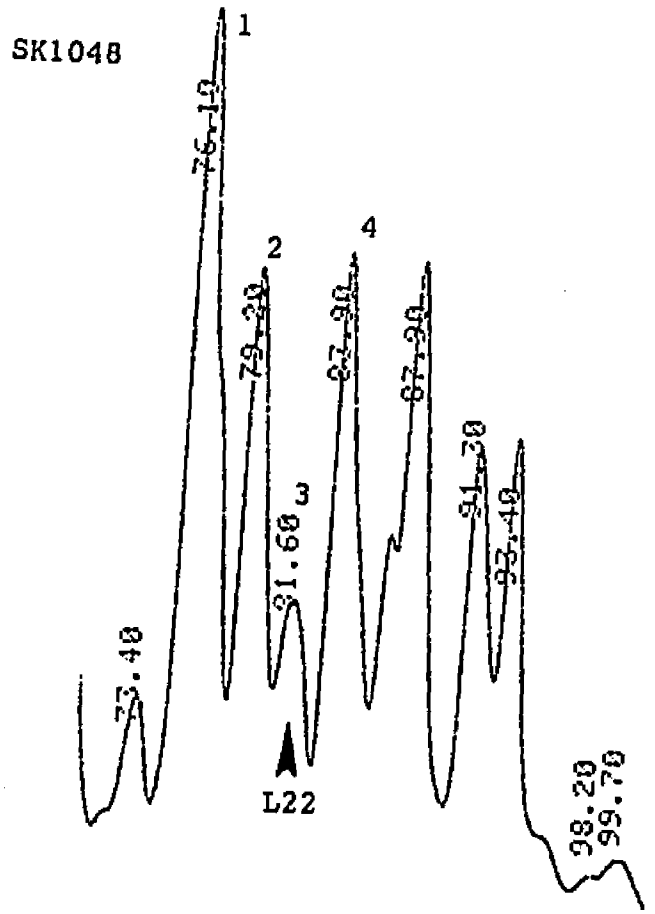

1
2
3
4
73.40
76.30
79.40
81.10
84.20
87.40
91.40
93.90
96.20
100.10
L22

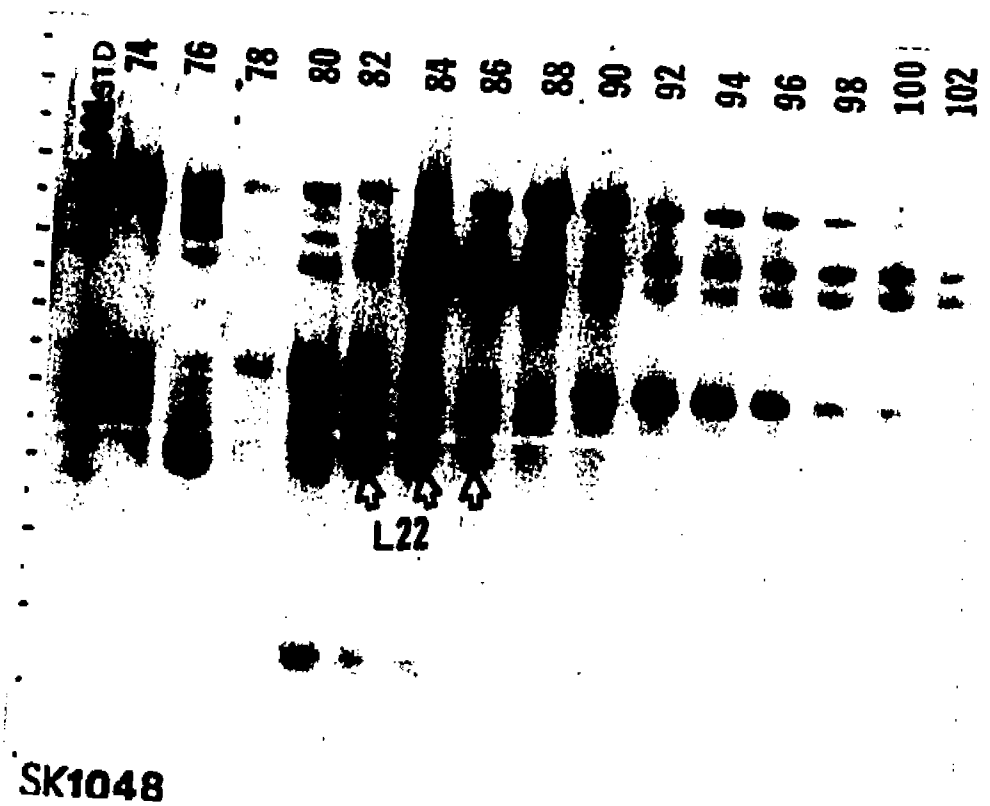

74
76
78
80
82
84
86
88
90
92
94
96
98
100
L22
1047

fractions which supported the delayed elution of this protein. On the chromatogram from mutant SK1048, peak 3 eluted at 81.6 minutes (Figure 12). Gel analysis of fractions from this chromatogram revealed an increased amount of ribosomal protein L22 in the 84 and 86 minute fractions. This defined the delayed elution of protein L22 from SK1048. The delayed elution of the protein in peak 3 on the mutant chromatograms must be the result of the mutation in ribosomal protein L22. The best comparison of the differential elution of protein L22 can be seen in Figure 13. Ribosomal protein L22 from the control strain eluted at 80.5 minutes, 0.9 minutes after protein L15. Protein L22 from SK1047 eluted at 81.1 minutes, 1.7 minutes after protein L15 and protein L22 from SK1048 eluted latest at 81.6 minutes, 2.4 minutes after protein L15. The increased retention time of protein L22 from both mutants suggested an increased hydrophobicity for the altered proteins.

An attempt was made to purify protein L22 by further modification of the HPLC elution conditions as described in the Materials and Methods section. Ribosomal proteins from the 74 to 90 minute region were pooled and separated using a second reversed phase HPLC procedure. The new program included additional isocratic elution steps to enhance the separation of the ribosomal proteins surrounding protein L22. In addition, fractions were collected every 30 seconds

Figure 12. IDENTIFICATION OF RIBOSOMAL PROTEINS IN HPLC PEAKS FROM STRAIN SK1048. (A) Enlargement of the 73-100 minute region of a HPLC chromatogram conducted using 50S ribosomal protein from strain SK1048. Fractions were collected at two minute intervals during this separation. Peaks labeled 1-4 are described in the text. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible. (B) Fractions were analyzed on an 18% polyacrylamide slab gel. Components of each fraction appeared as a series of bands which were identified by matching the position of each band to the 50S standard. Lane 1. 50S ribosomal protein standard. Lanes 2-15. Successive two minute fraction of the 74-100 minute region from the HPLC separation. Protein L22 is labeled with an arrow.

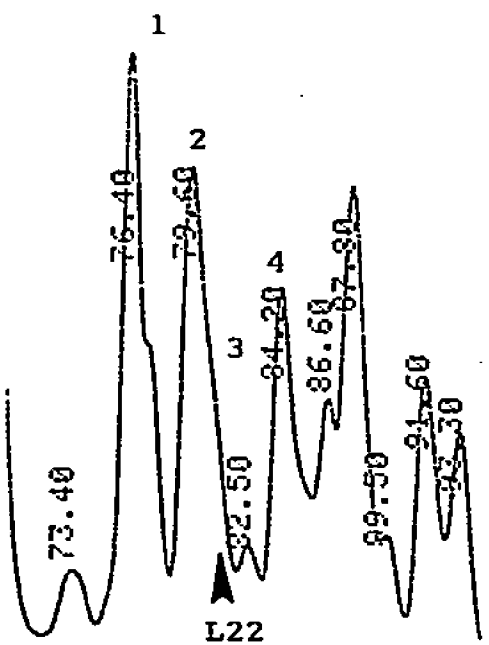

SK1048
1
2
3
4
76.10
79.30
81.60
87.30
87.30
91.30
93.40
73.40
98.20
99.70
L22


STD
74
76
78
80
82
84
86
88
90
92
94
96
98
100
102
L22

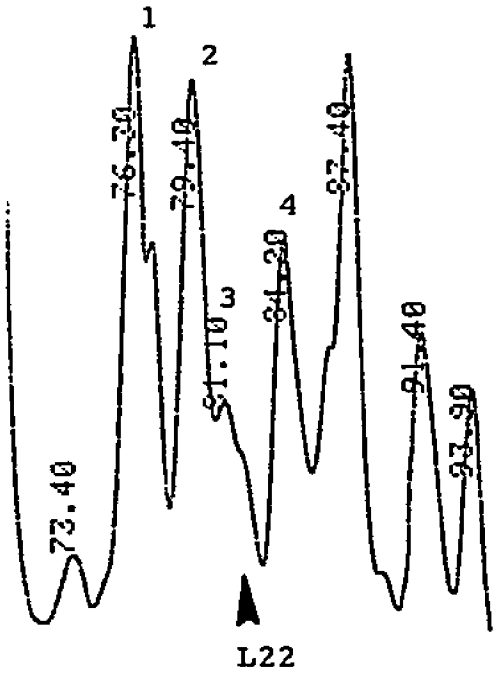
SK1048

Figure 13. COMPARISON OF CHROMATOGRAMS BETWEEN THE CONTROL AND MUTANT STRAINS. The 73-100 minute region of HPLC chromatograms describing the separation of 50S ribosomal proteins isolated from the (A) control strain (B) mutant strain SK1047 and (C) mutant strain SK1048. An arrow indicates the position of the altered peak. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible.

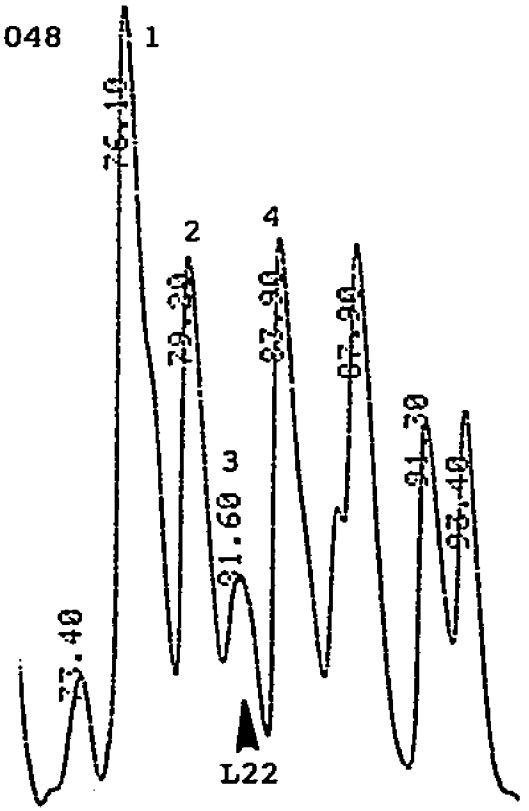
SK901



SK1047



SK1048



instead of every 2 minutes. The assignment of proteins to each peak was determined by slab gel electrophoresis as before. Peak 1 contained proteins L23 and L29 which eluted at 78.5 minutes in the control strain and 81 minutes in the mutant strains (Figure 14, 15, 16). Peak 2 containing protein L15 eluted at 83.5 minutes in the control strain and 84 minutes in the mutants. In the chromatograms from the control strain, the back shoulder of peak 2 contained ribosomal protein L22. The elution time of this protein was predicted at 84.5 minutes in the control strain. In the mutant strains, protein L22 separated from peak 2 and eluted at 86 minutes. Finally, peak 4 eluted between 88 and 89 minutes in both the mutant and control strain and was found to contain ribosomal protein L11. A comparison of chromatograms from the control and mutant strain is shown in Figure 17. Ribosomal protein L22 from the control stain eluted 1 minute after peak 2 containing ribosomal protein L15. Protein L22 from strain SK1047 eluted 2 minutes after peak 2 and protein L22 from strain SK1048 eluted 2.3 minutes after peak 2.

Figure 14. IDENTIFICATION OF RIBOSOMAL PROTEINS IN HPLC PEAKS AFTER THE RECHROMATOGRAPHY OF PROTEINS FROM THE CONTROL STRAIN. (A) Enlargement of the 76-78 minute region from the rechromatography of 70S ribosomal proteins from SK901. Fractions were collected at thirty second intervals during this separation. Peaks labeled 1-4 are described in the text. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible. (B) Fractions were analyzed on 18% polyacrylamide slab gels. Components of each fraction appeared as a series of bands which were matched to the 50S standard. Lane 1. 50S ribosomal protein standard. Lanes 2-15. Successive thirty second fractions from the 77-86 minute region of the rechromatography of selected ribosomal proteins. The position of protein L22 is indicated with an arrow.

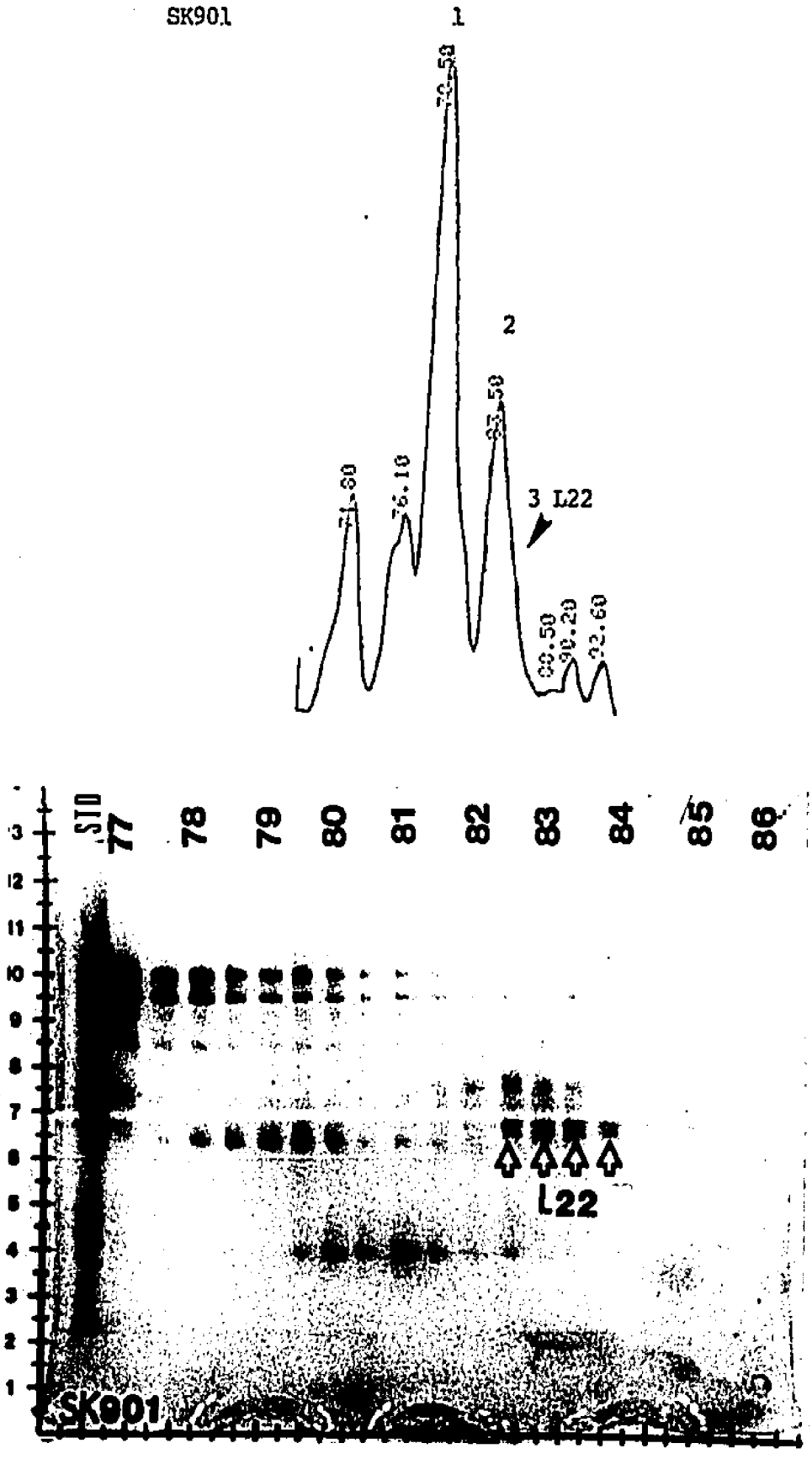
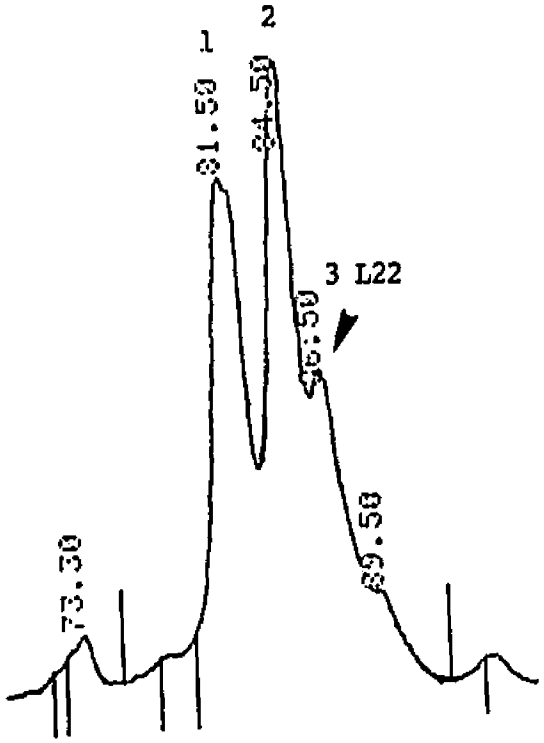

SK901
1
2
3 L22
71.80
76.10
79.50
83.50
88.50
90.20
92.60
SJ10
77
78
79
80
81
82
83
84
85
86
L22
SK001

Figure 15. IDENTIFICATION OF RIBOSOMAL PROTEINS IN HPLC PEAKS AFTER THE RECHROMATOGRAPHY OF PROTEINS FROM STRAIN SK1047. (A) Enlargement of the 76-78 minute region from the rechromatography of ribosomal proteins L23, L29, L15, L22 and L11 from SK1047. Fractions were collected at thirty second intervals during this separation. Peaks labeled 1-4 are described in the text. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible. (B) Fractions were analyzed on 18% polyacrylamide slab gels. Components of each fraction appeared as a series of bands which were matched to the 50S standard. Lane 1. 50S ribosomal protein standard. Lanes 2-15. Successive thirty second fractions from the 77-86 minute region of the rechromatography of selected ribosomal proteins. The position of protein L22 is indicated with an arrow.


1
2
81.50
84.50
3 L22
86.50
89.58
73.30

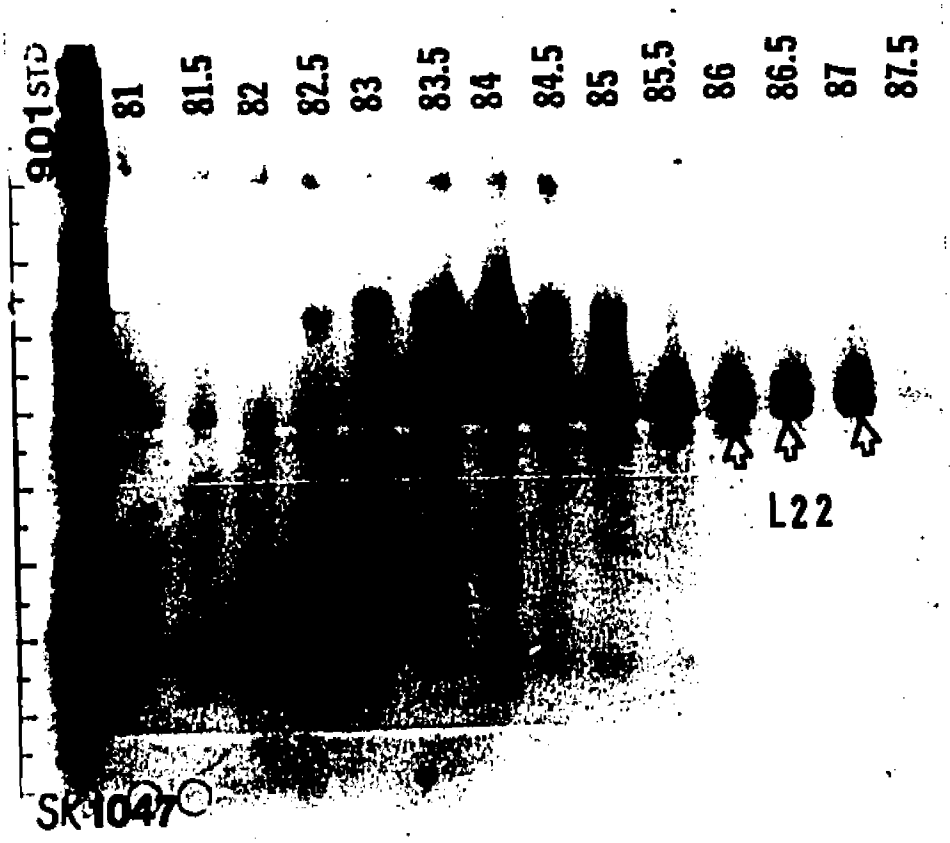
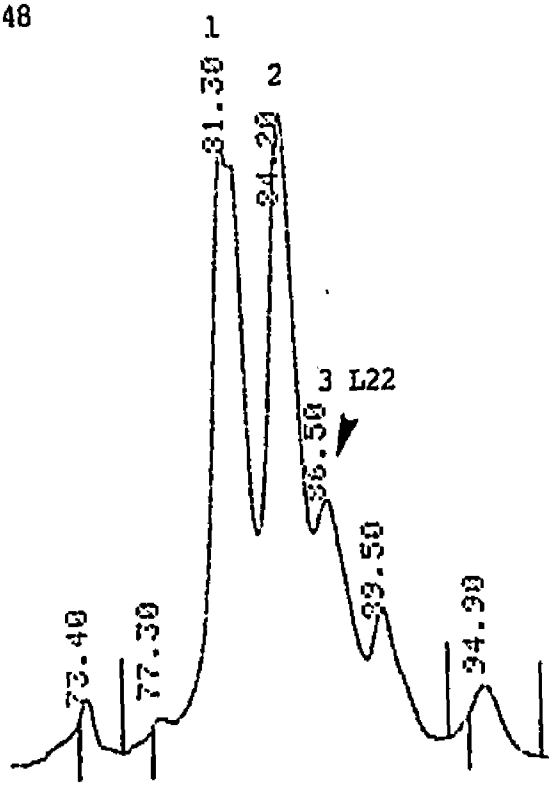

901STD
81
81.5
82
82.5
83
83.5
84
84.5
85
85.5
86
86.5
87
87.5
L22
SK1047

Figure 16. IDENTIFICATION OF RIBOSOMAL PROTEINS IN HPLC PEAKS AFTER THE RECHROMATOGRAPHY OF PROTEINS FROM STRAIN SK1048. (A) Enlargement of the 76-78 minute region from the rechromatography of ribosomal proteins L23, L29, L15, L22 and L11 from SK1048. Fractions were collected at thirty second intervals during this separation. Peaks labeled 1-4 are described in the text. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible. (B) Fractions were analyzed on 18% polyacrylamide slab gels. Components of each fraction appeared as a series of bands which were matched to the 50S standard. Lane 1. 50S ribosomal protein standard. Lanes 2-15. Successive thirty second fractions from the 77-86 minute region of the rechromatography of selected ribosomal proteins. The position of protein L22 is indicated with an arrow.

SK1048


1
2
3 L22
81.30
84.20
86.50
89.50
94.90
73.40
77.30

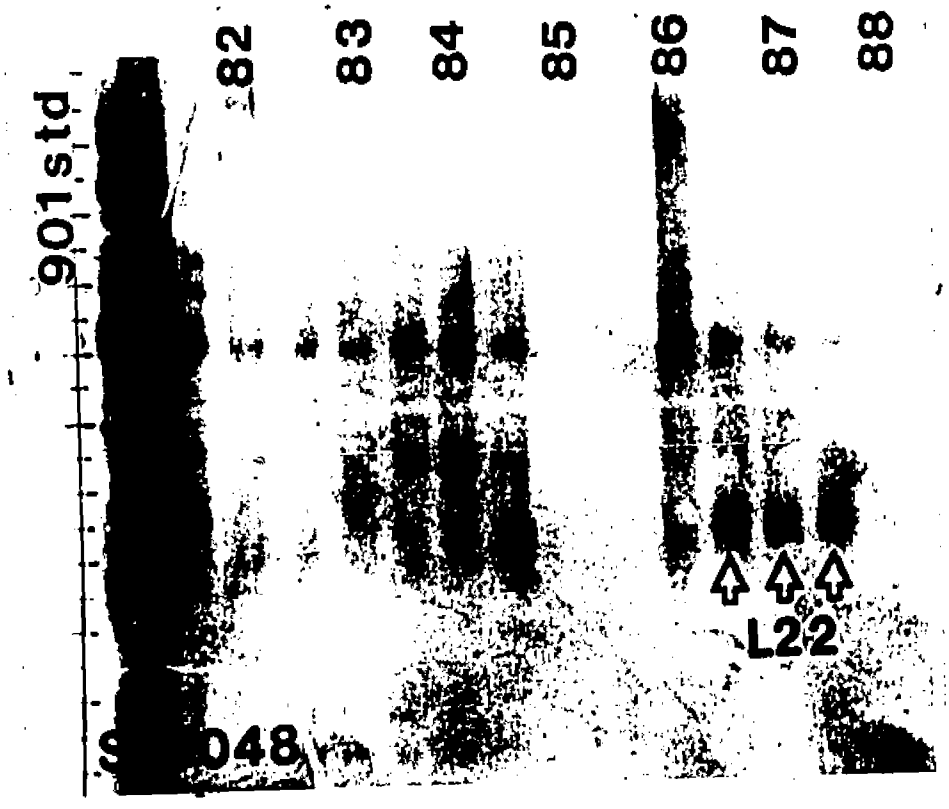
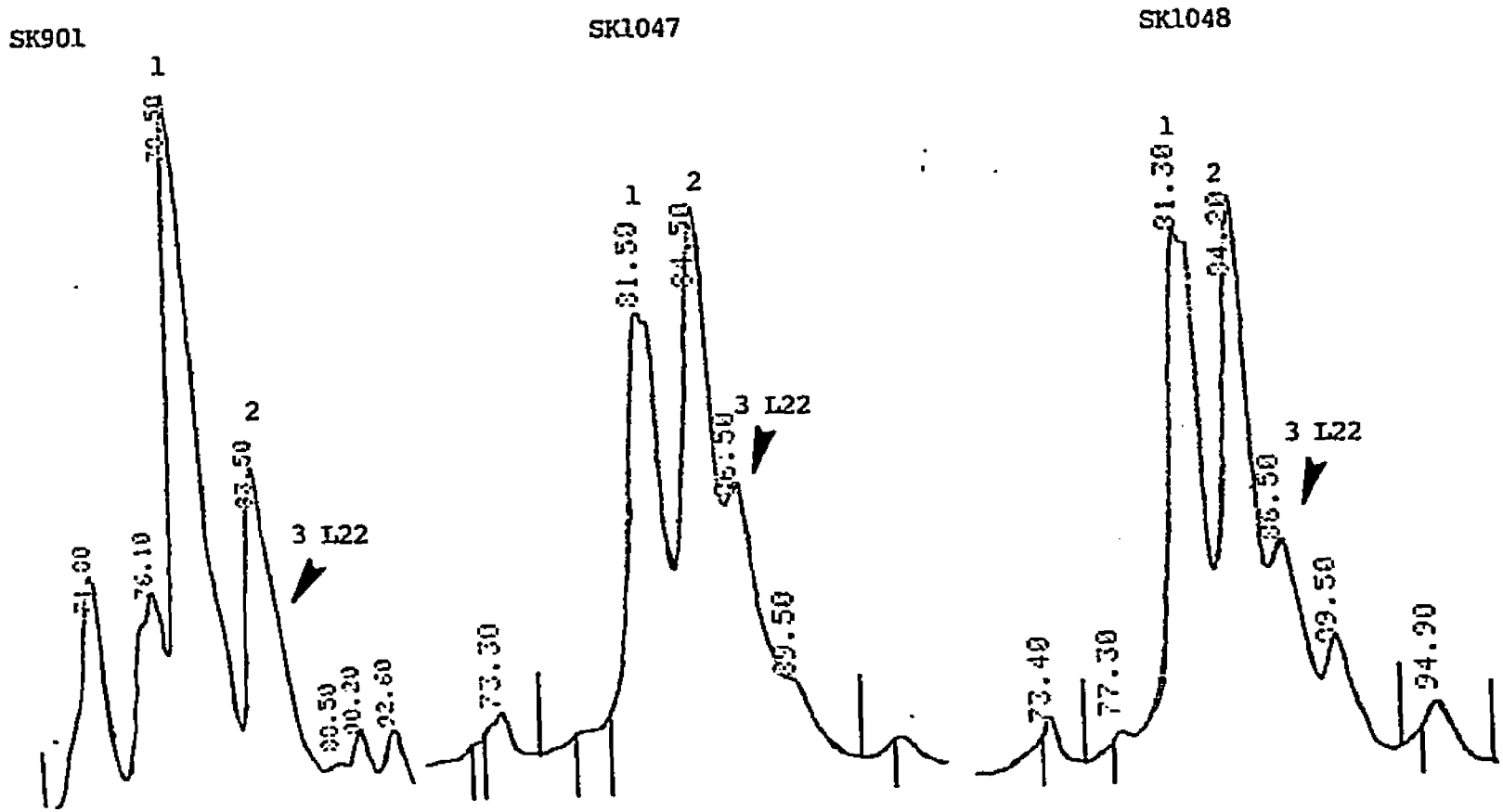

901 std
82
83
84
85
86
87
88
L22
048

Figure 17. COMPARISON OF CHROMATOGRAMS FROM THE RECHROMATOGRAPHY OF PROTEINS FROM THE CONTROL AND MUTANT STRAINS. Enhanced separation of ribosomal proteins from the 74-88 minute region was achieved using a second HPLC run. The 73-100 minute region of chromatograms describing the separation of ribosomal proteins from the (A) control strain (B) SK1047 and (C) SK1048. An arrow indicates the position of protein L22 in each chromatogram. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible.


SK901
1
71.80
76.10
79.50
2
83.50
3 L22
89.50
90.20
92.60
SK1047
73.30
1
81.50
2
84.50
3 L22
89.50
SK1048
73.40
77.30
1
81.30
2
84.20
3 L22
89.50
94.90

Genetic Analysis of Mutant Strains

Mapping of the temperature-sensitive loci to a gene on the E. coli chromosome was done to insure that the temperature-sensitive mutation was within the rplV gene for ribosomal protein L22. Mapping was conducted by two types of complementation tests using plasmid and λ phage. Mapping of the temperature-sensitive mutation was also accomplished in 3 factor genetic crosses by P1 transduction.

Bacteriophage λ Complementation

Complementation tests were carried out using specialized lambda transducing phages. Selection was for growth at 42°C indicating that the temperature-sensitive defect was complemented. Phages λfus2 and λspc2 were found to complement the temperature-sensitive mutations while λspc1 did not (Table 3). The mutation causing temperature sensitivity was therefore mapped to the chromosomal region carried by phage λspc2 not carried by λspc1 (Figure 18A). This indicated that the temperature-sensitive mutation lay within one of nine genes in the S10 operon. This region includes the rplV gene for the ribosomal protein L22.

Transductional Analysis

The thermosensitive mutation was mapped with respect to known genetic markers in bacteriophage P1 mediated transduction experiments. P1 phage lysates produced on donor strain SC713 were used to infect mutant strains SK1047

Table 3. Complementation Tests

Strain	Uninfected	λspc1	λspc2	λfus2
	Colonies in 1 ml at 42°C			
SK1047	132	99	858	1320
SK1048	33	66	1056	1914

Complementation Analysis. Overnight cultures of temperature-sensitive mutant strains SK1047 and SK1048 were infected with λ phage. Thirty microliter aliquots of the λ phage infected cultures and an uninfected control were plated in sectors of Luria plates at 42°C. Colonies capable of growth at 42°C were counted.

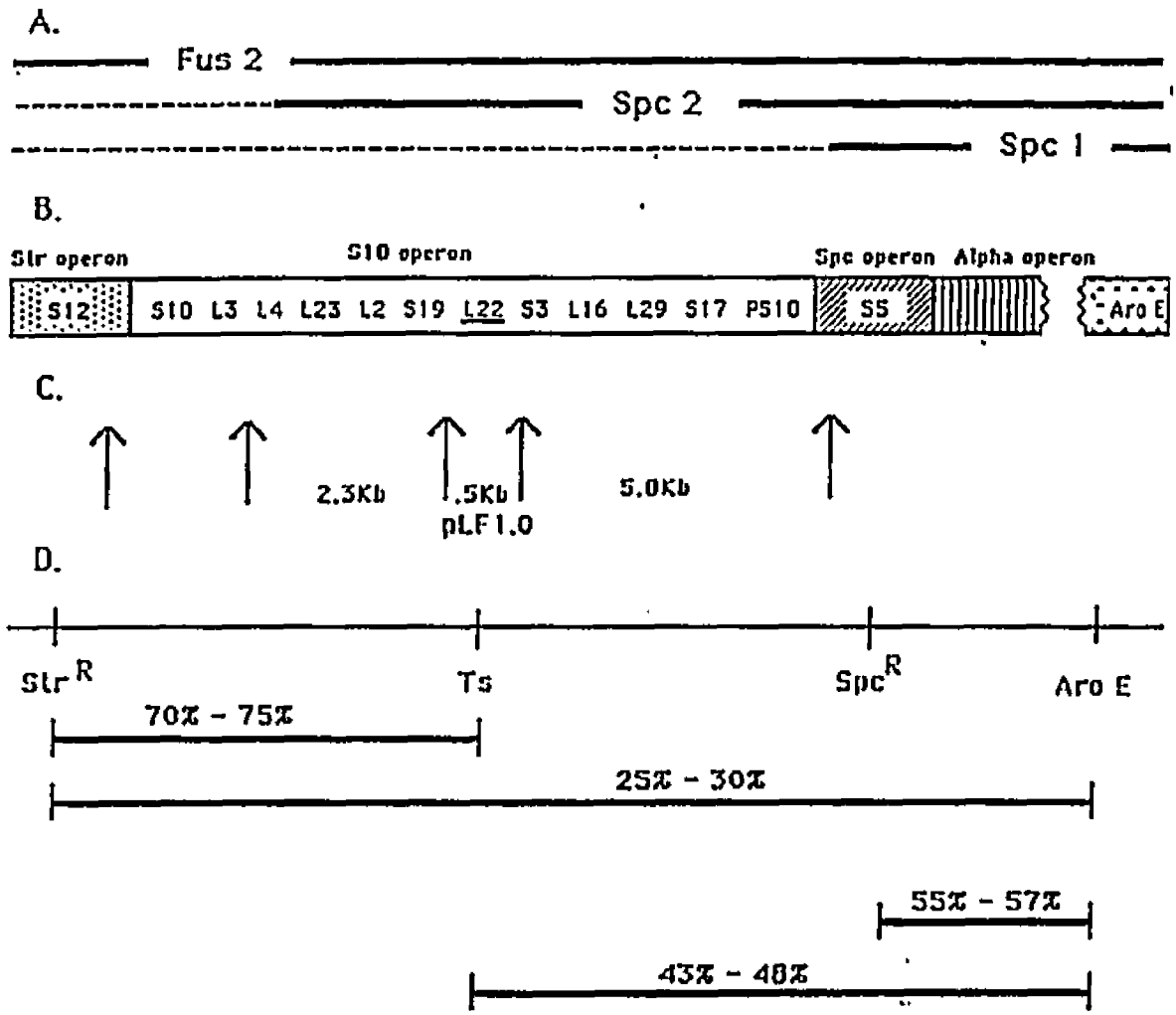
Figure 18. MAPPING THE TEMPERATURE-SENSITIVE MUTATION.

A. Region of the E. coli chromosome carried by different specialized lambda transducing bacteriophages. The solid black line indicates the extent of chromosomal DNA in the defective lambda phage.

B. Order of ribosomal protein genes in the S10 operon and the position of the other ribosomal protein gene operons in the 72 minute region of the E. coli chromosome (Lindahl et al., 1976).

C. Restriction enzyme EcoRI cleavage sites in this region of E. coli chromosome (Fiandt et al., 1976). The 0.5 kb fragment of λfus3 subcloned in plasmid pLF1.0 is indicated.

D. Location of gene markers used for transductional mapping of the temperature-sensitive mutation. The co-transduction frequencies for the selected markers in the region are indicated.


A.
Fus 2
Spc 2
Spc 1
B.
Str operon
S10 operon
Spc operon
Alpha operon
S12
S10 L3 L4 L23 L2 S19 L22 S3 L16 L29 S17 P510
S5
Aro E
C.
2.3Kb
.5Kb
pLF1.0
5.0Kb
D.
Str R
Ts
Spc R
Aro E
70% - 75%
25% - 30%
55% - 57%
43% - 48%

and SK1048. The donor strain SC713 was an aro E, Str^R, tr strain. The recipient mutant strains were aro E^+, ts, rpsL (Str^S). The initial selection was for Str^R colonies. Co-transduction of the requirement for aromatic amino acids (aro E) and temperature sensitivity (ts) were scored among Str^R transductants of both mutant strains (Table 4). Cotransduction frequencies between Str^R and ts were 74% for SK1047 and 70% for SK1048. The cotransduction frequencies between Str^R and aro E were found to be 26% for SK1047 and 30% for SK1048. The order of gene loci was determined to be aro E, ts, Str^R (Figure 18D).

Mapping was also conducted using mutants SK1047 and SK1048 as donor strains. For these crosses, strain SC711 was the recipient. Strain SC711 has a aro E, Spc^R, tr phenotype. Selection in these crosses was for aro E^+ colonies. Cotransduction of aro E with ts was 43% in SK1047 and 48% in SK1048. Cotransduction of aro E with Spc^S was 55% in SK1047 and 57% in SK1048. The order of gene loci was determined to be aro E, Spc^R, ts (Figure 18D). This data mapped the temperature-sensitive mutation to the rplV gene region.

Complementation with Plasmid pLF1.0

Precise mapping of the temperature-sensitive mutation to the rplV gene for the ribosomal protein L22 was accomplished using plasmid pLF1.0. Plasmid pLF1.0 was constructed by ligation of a 0.5 kb fragment of λfus3 DNA

Table 4. THREE FACTOR TRANSDUCTIONAL CROSSES.

A. A bacteriophage P1 lysate created on donor strain SC713 was crossed with mutant strain SK1047 and transductants were selected on a streptomycin plate at 32°C. B. A bacteriophage P1 lysate created on donor strain SC713 was crossed with mutant strain SK1048 and transductants were selected on a streptomycin plate at 32°C.

Table 4. Transductional Mapping of the Temperature Sensitive Mutation

A.				
Donor genotype:				
SC713		str^R, tr, aro E^-		
Recipient genotype:				
SK1047		str^S, ts, aro E^+		
The selected marker:		str^R		
Results of selection:		tr aro E^+		55 colonies
		tr aro E^-		19 colonies
		ts aro E^+		19 colonies
		ts aro E^-		7 colonies
		Total		100 colonies
Cotransduction frequencies:	str^R	tr	74/100	(0.74)
	str^R	aro E^-	26/100	(0.26)
B.				
Donor genotype:				
SC713		str^R, tr, aro E^-		
Recipient genotype:				
SK1048		str^S, ts, aro E^+		
The selected marker:		str^R		
Results of selection:		tr aro E^+		47 colonies
		tr aro E^-		16 colonies
		ts aro E^+		15 colonies
		ts aro E^-		11 colonies
		Total		89 colonies
Cotransduction frequencies:	str^R	tr	63/89	(0.70)
	str^R	aro E^-	27/89	(0.30)

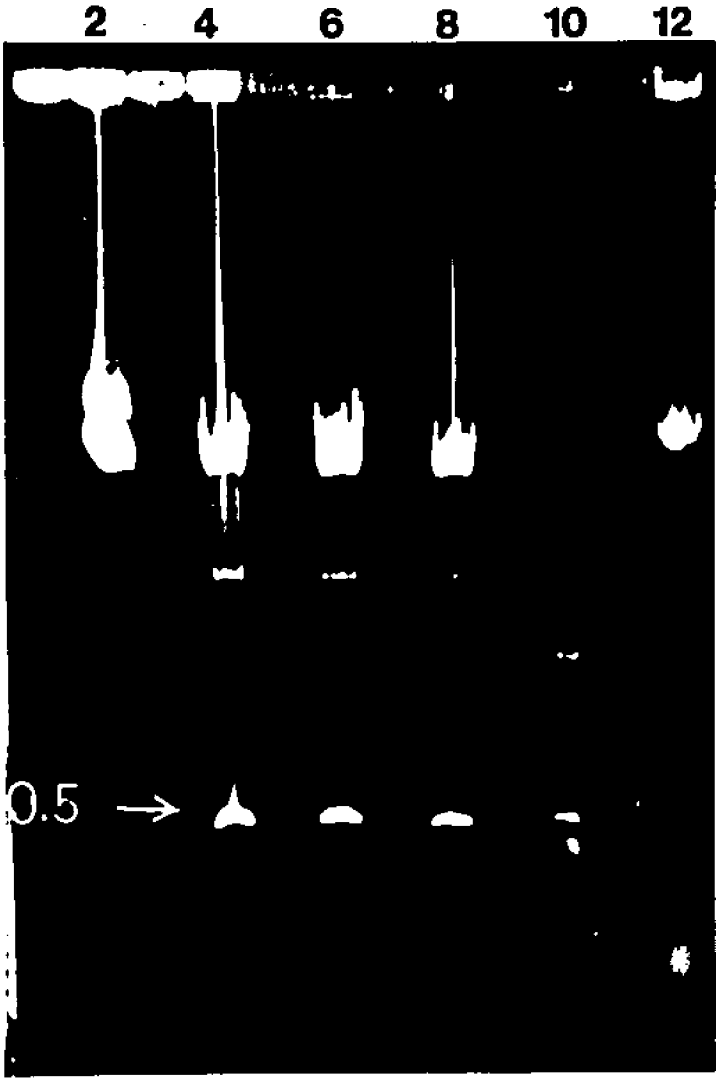
containing the rplV gene into the chloramphenicol resistance gene of pBR325 (Watson and Surzycki, 1983) (Figure 18C). The pLF1.0 plasmid was transformed into competent cells of both temperature-sensitive mutants. Cells containing plasmid were idenitified by their ability to grow at 44°C on ampicillin plates.

To verify that complementation had occurred, plasmid pLF1.0 was isolated from ampicillin resistant colonies. The isolated plasmid was further cleaved with restriction enzyme EcoRI and shown to release the 0.5 kb insert (Figure 19). Since plasmid pLF1.0 complemented the temperature-sensitive mutation in both strains, the mutation was mapped specifically to the rplV gene for ribosomal protein L22.

Characterization of Complemented Mutant Strains

Complementation with λfus2 and plasmid pLF1.0 resulted in cells which contained increased dosages of ribosomal protein genes. Complementation with λfus2 created partial diploids for the ribosomal protein genes of the S10 operon. The mutant cells contained a chromosomal copy of the S10 operon plus a second copy of the operon inserted by recombination with the defective λ phage. The two copies of the S10 operon can be distinguished since the chromosomal copy contains an altered rplV gene while the phage copy contains the wild type rplV gene. Complementation with plasmid pLF1.0 resulted in mutant cells with multiple copies of the plasmid associated rplV gene. Again, the two forms

Figure 19. REISOLATION OF PLASMID pLF1.0 FROM COMPLEMENTED STRAINS. Plasmid preparations were electrophoresed through a 1.0% agarose gel and post strained with ethidium bromide. Lane 2. Control plasmid pLF1.0 isolated from strain JM109. Lane 4. Control plasmid pLF1.0 digested with restriction enzyme EcoRI. Lane 6. Plasmid pLF1.0 isolated from complemented strain SK1047 and digested with EcoRI. Lane 8. Plasmid pLF1.0 isolated from complemented strain SK1048 and digested with EcoRI. Lane 10. The molecular weight marker was plasmid pBR322 digested with HinfI. Lane 12. Plasmid pBR322 isolated from C600.


2
4
6
8
10
12
0.5

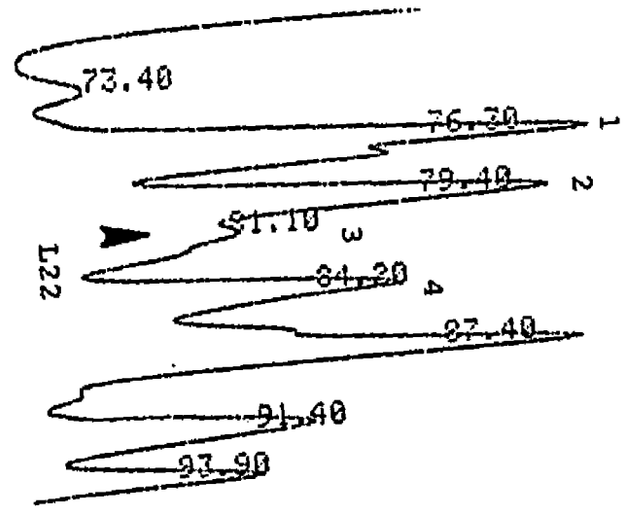
of the rplV gene can be distinguished.

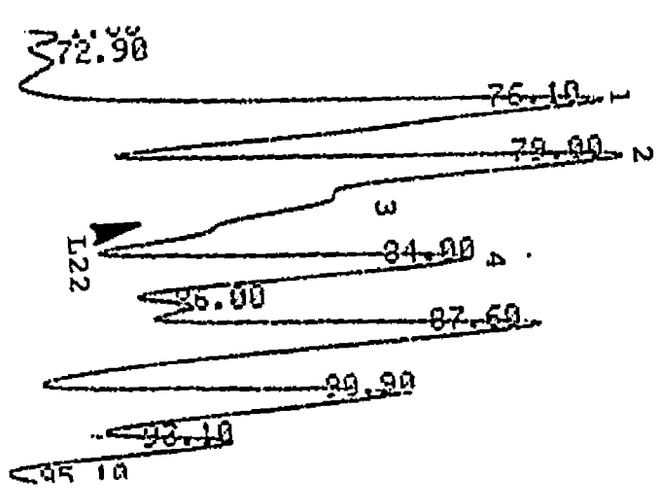
Complemented cells were selected for growth at 42°C. The mutant strains were unable to grow at the restrictive temperature due to failure of the ribosomes to function efficiently. Ribosomes from complemented mutant strains would remain functional and allow cell growth at high temperature if wild type protein L22 were synthesized from phage or plasmid genes. To examine if complemented strains produced wild type protein L22, experiments were designed to test for expression of the wild type allele in complemented strains.

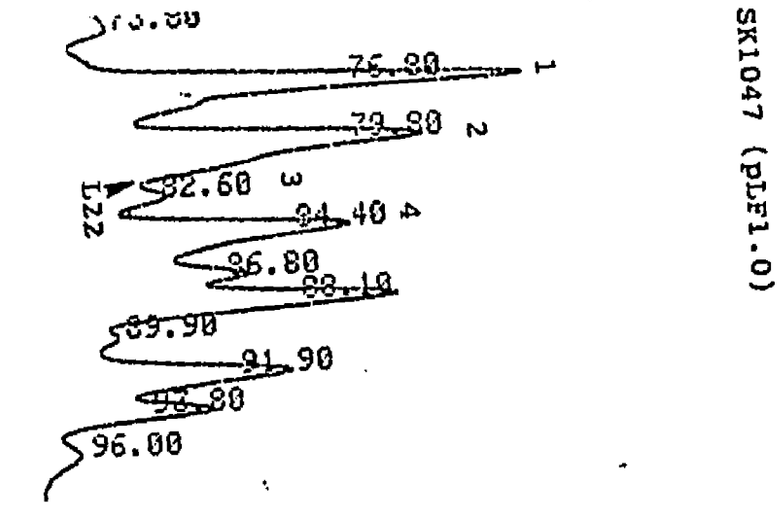
Reversed Phase HPLC Analysis of Ribosomal Proteins from λfus2 and λspc2 Complemented Strains

Transcription and translation of the phage encoded rplV gene would result in production of wild type ribosomal protein L22. Expression from the mutant rplV gene would result in production of the altered protein L22. HPLC analysis was used to determine if wild type ribosomal protein L22 was produced in the λfus2 and λspc2 complemented mutant strains. Ribosomes were isolated from these cells and total 50S ribosomal proteins were separated as described in the Materials and Methods section. The chromatogram showing the separation of ribosomal proteins from SK1047 (λfus2) clearly shows two shoulders on the back end of the 79.0 minute peak (Figure 20). The first shoulder was at 81.0 minutes while the second shoulder was at 81.6 minutes,

Figure 20. COMPARISON OF CHROMATOGRAMS FROM SK1047, SK1047 (λfus2), AND AN SK1047:CONTROL MIX. Reversed phase HPLC elution profile of 50S subunit proteins. The 73-100 minute region of chromatograms showing the separation of 50S ribosomal proteins from (A) SK1047, (B) SK1047 (λfus2) and (C) 1:1 mixture of control and mutant SK1047 50S ribosomal proteins. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducable.


SK1047
73.40
76.30
1
79.40
2
81.10
3
L22
84.30
4
87.40
91.40
93.90


SK1047 (fus2)
72.90
76.10
1
79.00
2
3
L22
84.00
4
86.00
87.60
90.90
93.10


SK1047 (pLF1.0)
75.80
1
79.80
2
L22
82.60
3
84.40
4
86.80
88.10
89.90
91.90
93.80
96.00

a difference of 0.6 minutes. Elution of the wild type protein L22 has been shown to be at 80.5 minutes and the elution of protein L22 from SK1047 was found to be at 81.1 minutes (Figure 13). This data would predict that a mixture of the two forms of protein L22 would result in two peaks separated by 0.6 minutes (Table 5). Therefore, the chromatogram from SK1047 (λfus2) is in agreement with that predicted from a mixture of the wild type and mutant SK1047 protein L22. Protein L15 was used as the reference protein to relate the run to run differences in absolute retention times of protein L22. In addition, all proteins in the peak were identified by slab gel electrophoresis as described previously.

A similar HPLC analysis was conducted for strain SK1048 (λfus2). The resulting chromatogram (Figure 21) revealed two shoulders on an 80.0 minute peak. The first shoulder peaked at 81.0 minutes and the second shoulder peaked at 82.5 minutes, a difference of 1.5 minutes. Elution of wild type protein L22 has been shown to be at 80.5 minutes and the elution time of protein L22 from SK1048 was 81.6 minutes. The elution pattern predicted for a mixture of the two forms of protein L22 would have two peaks separated by 1.1 minutes (Figure 13) (Table 5). Therefore, the chromatogram from SK1048 (λfus2) is in agreement with that predicted from a mixture of the wild type and mutant SK1048 protein L22. Similar chromatograms, exhibiting two forms

Figure 21. COMPARISON OF CHROMATOGRAMS FROM SK1048, SK1048 (λfus2) AND AN SK1048:CONTROL MIX. Reversed phase HPLC elution profile of 50S subunit proteins. The 73-100 minute region of chromatograms showing the separation of 50S ribosomal proteins from (A) SK1048, (B) SK1048 (λfus2) and (C) 1:1 mixture of control and mutant SK1048 50S ribosomal proteins. The elution of protein L22 was standardized in relation to protein L23 and L15. The standardized elution of protein L22 was reproducible.

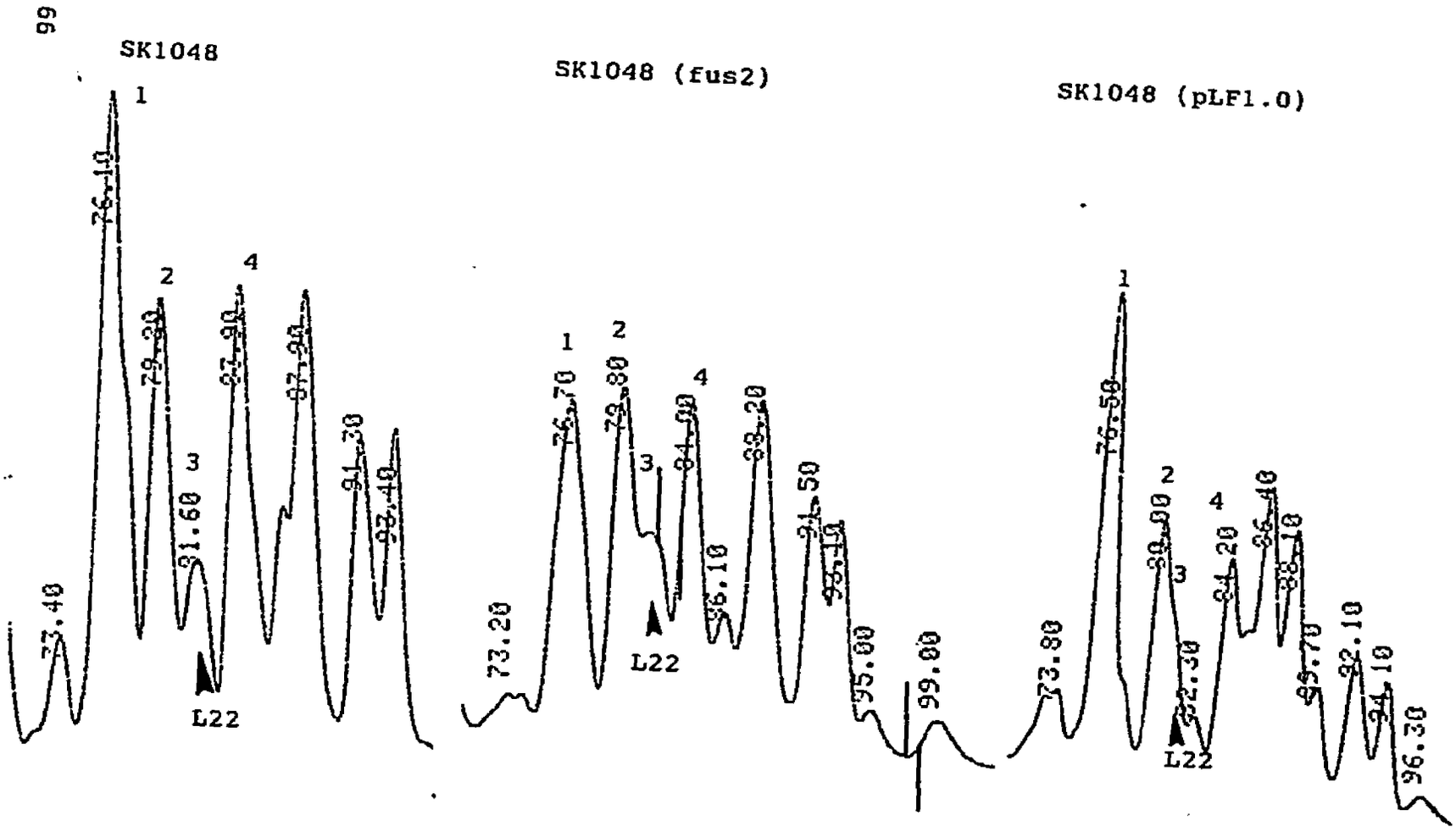

99
SK1048
SK1048 (fus2)
SK1048 (pLF1.0)
L22
L22
L22

Table 5. Retention Times of Protein L22 from Reversed Phase HPLC.

A. Retention Time in Minutes for Ribosomal Protein L15
B. Retention Time in Minutes for Wild Type Ribosomal Protein L22
C. Retention time in Minutes for Mutant Ribosomal Protein L22
D. Difference between Ribosomal Protein L15 and Wild Type Protein L22
E. Difference between Ribosomal Protein L15 and Mutant Protein L22
F. Difference between the elution of mutant and control ribosomal protein standardized to protein L15

Strain	A	B	C	D	E	F
SK901	79.6	80.5	-	0.9	-	-
SK1047	79.4	-	81.1	-	1.7	0.8
SK1047(λfus2)	79.0	81.0	81.6	2.0	2.6	0.6
SK901:SK1047 1:1	79.8	80.8	82.6	1.0	2.8	1.8
SK1048	79.2	-	81.6	-	2.4	1.5
SK1048(λfus2)	80.0	81.0	82.5	1.0	2.5	1.5
SK901:SK1048 1:1	80.0	81.0	82.3	1.0	2.3	1.3

Table 5. The elution times for the peak of protein L15 and protein L22 are taken from reverse phased HPLC chromatograms describing the separation of 50S ribosomal proteins (Figures 13, 20, 21). Identification of the position of elution of each protein has been determined by 18% polyacrylamide gel electrophoresis. The elution of the wild type and mutant forms of protein L22 was standardized by measuring its elution in relation to protein L15.

of ribosomal protein L22 were obtained when ribosomes from both mutant strains complemented with λspc2 were analyzed using HPLC (data not shown).

To mimic the complemented strains, the HPLC program was used to separate a 1:1 mixture of control and mutant 50S ribosomal protein. The patterns obtained for the control-mutant mixture was comparable to the patterns obtained for proteins from the complemented strains. Separation of the SK1047:control mixture revealed that the wild type protein L22 eluted as a back shoulder of the 79.8 minute peak. The elution time for this shoulder was determined to be 80.8 minutes which was consistent with earlier data on the elution of control protein L22 (Table 5). A second peak, with an elution time of 82.6 minutes, contained the mutant form of protein L22. The SK1048:control mix resulted in an analogous pattern. The control protein eluted as a back shoulder of an 80.0 minute peak at 81.0 minutes. The mutant protein L22 eluted in a peak at 82.3 minutes again delayed from the control protein. This provided further evidence that the complemented strains are producing two forms of ribosomal protein L22. These results are summarized in Table 5.

Two Dimensional Gel Analysis on Ribosomal Proteins from λfus Complemented Strains

To further examine the expression of the ribosomal protein genes inserted by λfus2, two dimensional gels of the

proteins from the complemented strains were studied. The mutant form of ribosomal protein L22 has been shown to run to a separate location from the wild type protein on two dimensional electrophoretic gels (Figure 6). Expression of the genes coded for by λfus2 would be evidenced by the presence of the wild type ribosomal protein L22 on these same gels. Both mutant strains complemented by λfus2 were grown under the restrictive conditions at 42°C. Ribosomal proteins were isolated from the complemented cells and separated using two dimensional electrophoresis. Two forms of ribosomal protein L22 were identified in the gels from SK1048 (λfus2) (Figure 22). The production of wild type ribosomal protein L22 from genes inserted by λfus2 was confirmed. Similar results were obtained when two dimensional gels were analyzed from SK1047 (λfus2) and from mutant strains complemented with λspc2 (data not shown).

Two Dimensional Gel Analysis on Ribosomal Proteins from plasmid pLF1.0 Complemented Strains

To determine if there was expression of the rplV gene cloned into plasmid pLF1.0, ribosomal proteins from plasmid complemented strains were examined on two dimensional gels. Ribosomal proteins were isolated from plasmid complemented mutant strains and separated using two dimensional electrophoresis. Both forms of ribosomal protein L22 were visualized on the gels containing ribosomal proteins from SK1048 (pLF1.0)(Figure 23). Gels of ribosomal proteins from

Figure 22. TWO-DIMENSIONAL GEL ELECTROPHORESIS OF RIBOSOMAL PROTEINS FROM SK1048 (λfus2). Ribosomes were isolated from cells grown at 42°C. Ribosomal proteins were separated on gels using the method of Howard and Traut (1973). Arrows identify the positions of the control and mutant forms of ribosomal protein L22.

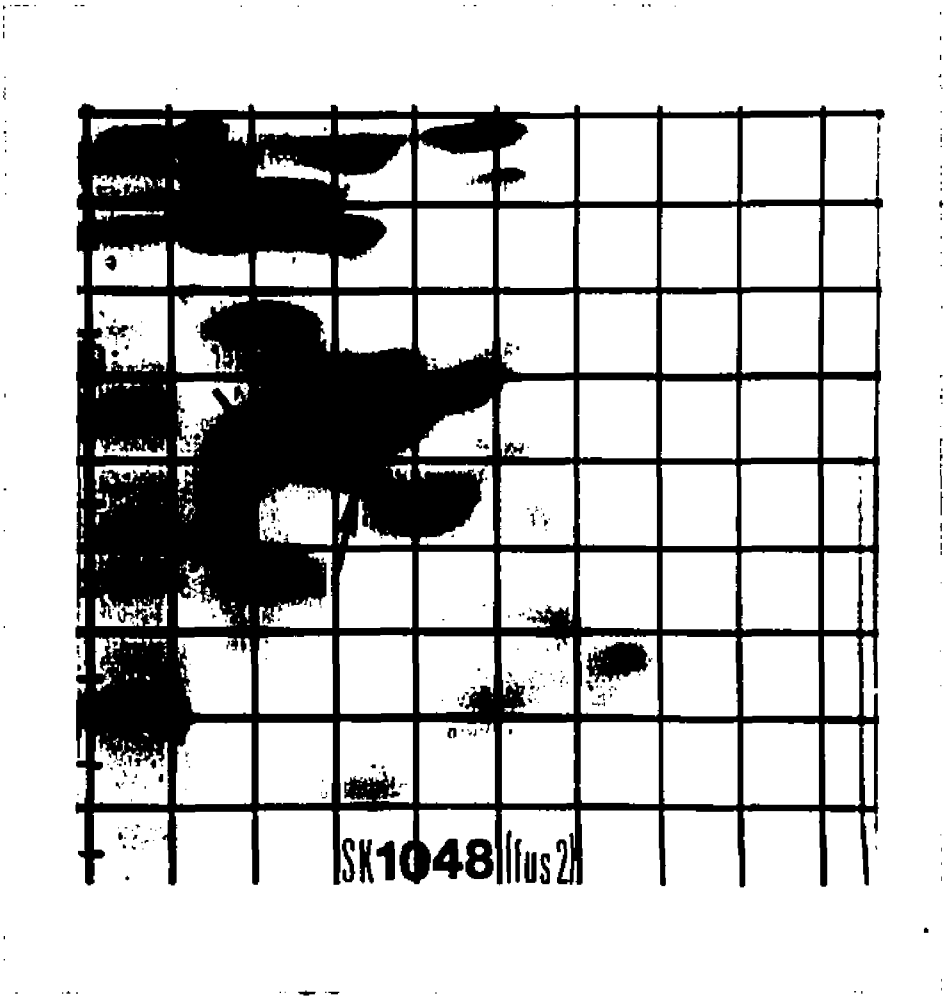
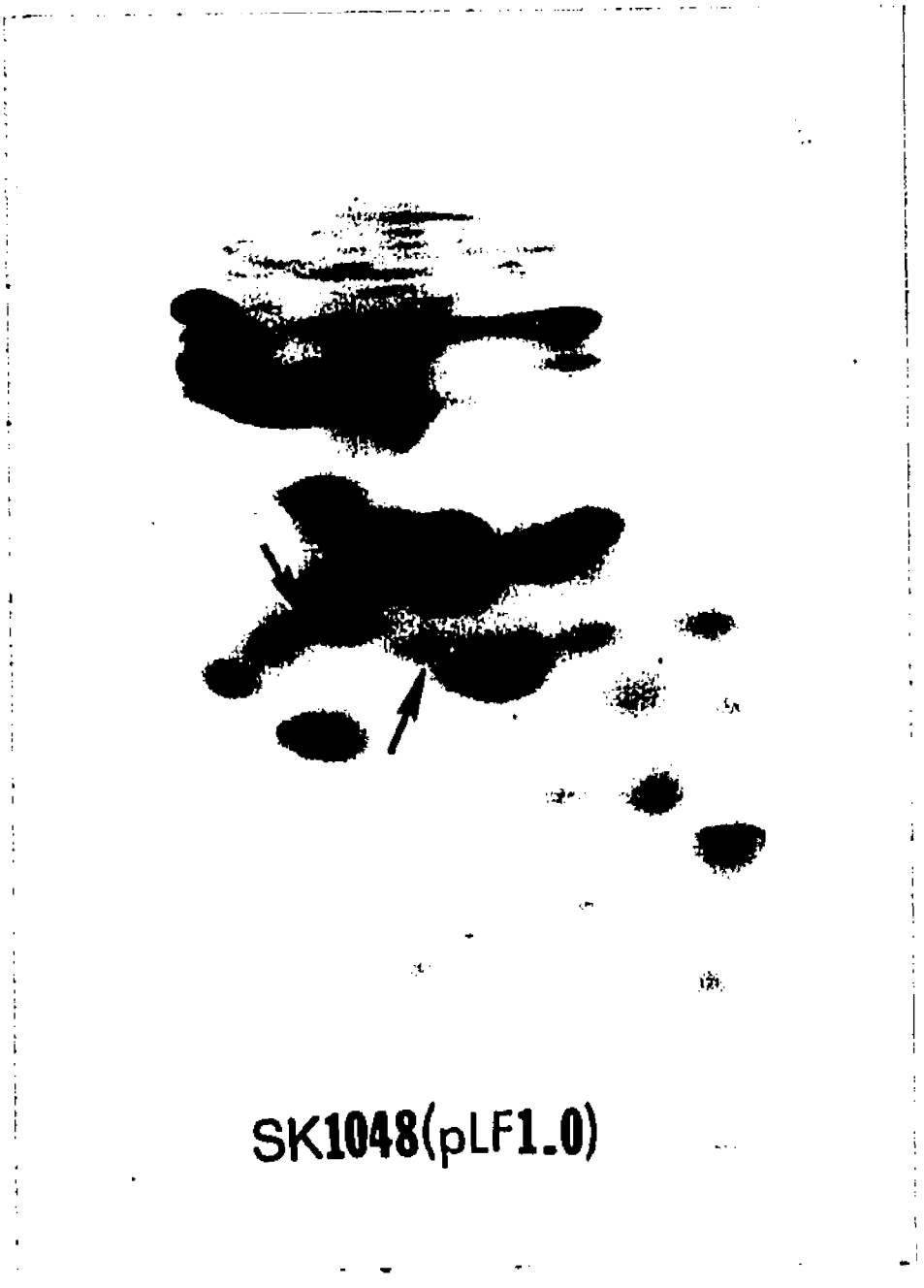


Figure 23. TWO-DIMENSIONAL GEL ELECTROPHORESIS OF RIBOSOMAL PROTEINS FROM SK1048 (pLF1.0). Ribosomes were isolated from SK1048 (pLF1.0) cells and separated using the method of Howard and Traut (1973). Arrows identify the positions of the control and mutant forms of ribosomal protein L22.



SK1047 (pLF1.0) matched those from SK1048. Therefore, strains SK1047 and SK1048 were synthesizing wild type ribosomal protein L22 from the plasmid rplV gene.

Characterization of Complemented Strains at the Permissive and Restrictive Temperatures

The appearance of wild type ribosomal protein L22 in HPLC chromatograms and on two dimensional gels indicated that there was expression from both the phage and plasmid encoded rplV genes. Further characterization of complemented mutant strains was conducted to examine gene regulation within these cells. Ribosome synthesis is coordinated and balanced. Each component is synthesized in an amount proportional to its content within the ribosome. Over expression of ribosomal components has an adverse effect on the regulation systems within these cells. Disruption of these systems interferes with cell growth and can be lethal to the cell (Nomura et al., 1986).

Expression from the phage and plasmid encoded rplV genes lent credibility to the idea that mutant strains were able to grow at 44°C because they have functional ribosomes containing wild type protein L22. If expression of the control ribosomal protein L22 permitted growth at 44°C then would expression also occur at the permissive temperature where it was not required for survival? Characterization of the complemented strains was extended to determine if expression from the wild type rplV gene was effected by

temperature. Growth shift experiments and estimates of protein L22 production were carried out.

Temperature-Shift Experiments on λfus Complemented Strains

Growth experiments were conducted at 32°C and after a temperature-shift to 44°C on control and mutant cells complemented by λfus2 (Figures 24,25). The control culture grew at a constant rate irrespective of temperature and obtained the highest cell density. The mutant cells complemented with λfus2 grown at 32°C multiplied at 90% of the rate and achieved 80% of the final density of the control strain (Table 6). Cultures complemented by λfus2 shifted to 44°C exhibited 80% of the control growth rate and reached 55% of the final density of the control strain. When the growth of complemented cultures was related to the non-complemented mutants, the complemented cells demonstrated an elevated growth rate and cell density at 32°C. At the restrictive temperature the λfus2 complemented cultures realized a 60% increase in cell density over the non-complemented cells. At the permissive temperature, a second copy of the S10 operon provided the mutant strains with the ability to obtain a growth rate and cell density comparable to the control. At the restrictive temperature, complementation with λfus2 allowed cultures to obtain a increase in cell density at a slower growth rate (Table 6).

Figure 24. TEMPERATURE-SHIFT EXPERIMENTS ON STRAINS SK901, SK1047 AND SK1047 (λfus2). Cultures in L Broth were shifted when they reached a density between 20 and 30 Klett units. The control strain SK901 after a shift from 32°C to 44°C (♦). Mutant strain SK1047 after a shift from 32°C to 44°C (-). Mutant strain SK1047 (λfus2) grown at 32°C (□). Mutant strain SK1047 (λfus2) after a shift from 32°C to 44°C (■).

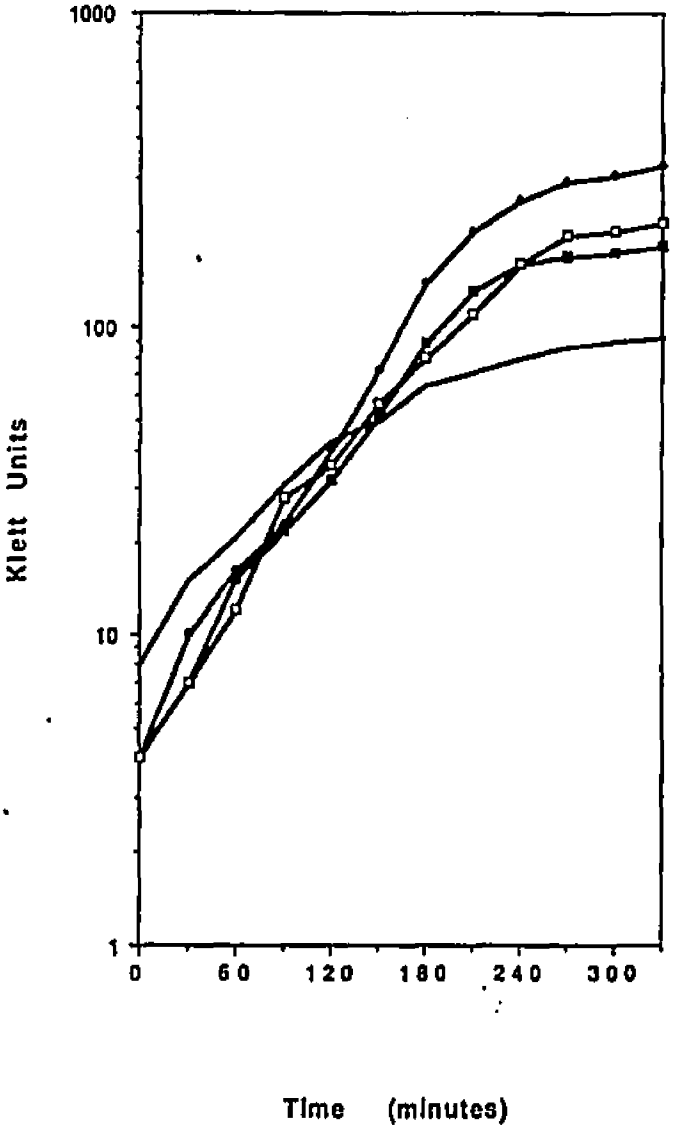


Figure 25. TEMPERATURE-SHIFT EXPERIMENTS ON STRAINS SK901, SK1048 AND SK1048 (λfus2). Cultures in L Broth were shifted when they reached a density between 20 and 30 Klett units. The control strain SK901 after a shift from 32°C to 44°C (♦). Mutant strain SK1048 after a shift from 32°C to 44°C (-). Mutant strain SK1048 (λfus2) grown at 32°C (o). Mutant strain SK1048 (λfus2) after a shift from 32°C to 44°C (●).

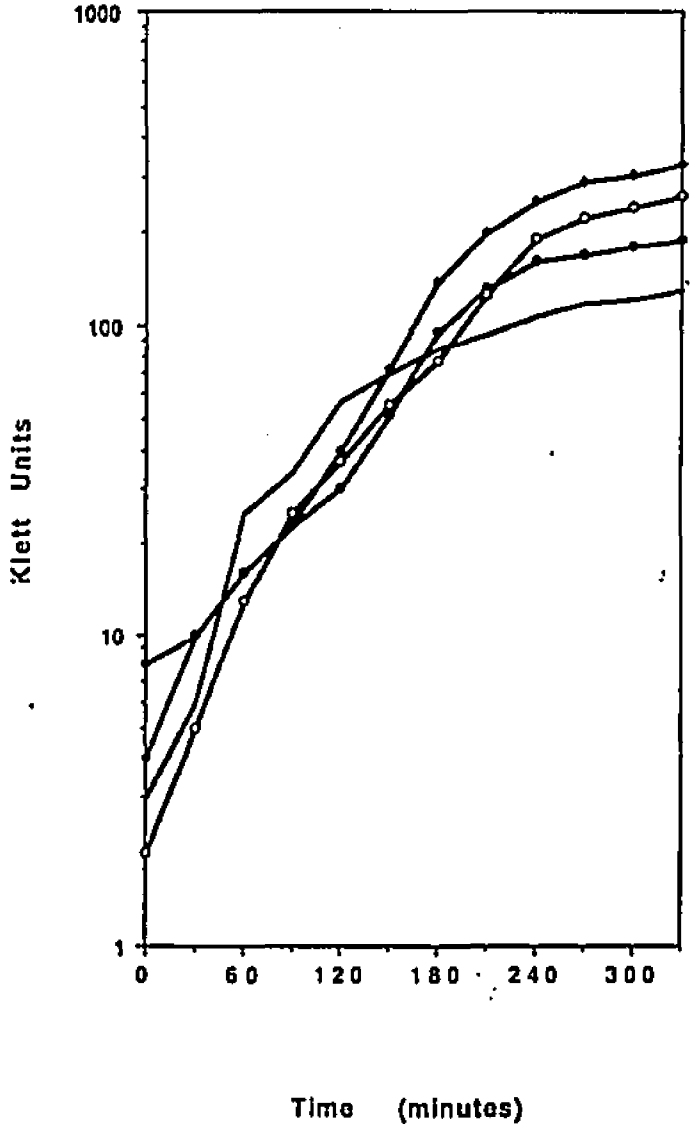


Table 6. Growth Characteristics of Mutant Strains Complemented with λfus2

	Final Cell Density (Klett units)	Specific Growth Rate Constant (k) (hr^{-1})
SK901 32°C	298	1.02
SK901 32°C -shift 44°C	332	1.02
SK1047 32°C	161	0.78
SK1047 32°C -shift 44°C	93	*
SK1047 (λfus2) 32°C	213	0.96
SK1047 (λfus2) 32°C -shift 44°C	181	0.92
SK1048 32°C	173	0.72
SK1048 32°C -shift 44°C	130	*
SK1048 (λfus2) 32°C	261	0.90
SK1048 (λfus2) 32°C -shift 44°C	190	0.86

Temperature-Shift Experiments. The growth of each culture was determined using a Klett-Summerson Colorimeter. Cultures were grown at 32°C until they obtained a density between 20 and 30 Klett units. At that time, cells were shifted to 44°C or retained at 32°C. The specific growth rate (hr^{-1}) was calculated from measurements of the doubling time (d) calculated from growth experiments. [k = 0.693/d]

* cells ceased growing after two hours 44°C.

Temperature-Shift Experiments on Plasmid pLF1.0 Complemented Strains

Similar temperature-shift experiments were conducted at 32°C and 44°C on mutant cells complemented by plasmid pLF1.0 (Figures 26,27). The control strain multiplied at a constant rate and obtained the highest cell density (Table 7). Cultures complemented with plasmid pLF1.0 grown at 32°C achieved a growth rate of 90% of the control and reached a cell density about one half of the control cells. Cultures shifted to 42°C exhibited a growth rate reduced by 60% and reached a density about 40% of that achieved by the control cells. The plasmid pLF1.0 complemented cells were able to grow at 42°C at a slow, continuous rate compared to the non-complemented parents which ceased growing at this temperature. When compared to the control strain and the lambda phage complemented strains the plasmid complemented cultures grown at high temperature were depressed both in their rate of growth and in their final cell densities. Plasmid complementation affected growth more than complementation using lambda phage. This was determined by comparing values in Tables 6 and 7.

Two Dimensional Gels on Ribosomal Proteins from λfus2 Complemented Strains at 32°C and 42°C

It has been established that cells complemented with

Figure 26. TEMPERATURE-SHIFT EXPERIMENTS ON STRAINS SK901, SK1047 AND SK1047 (pLF1.0). The control strain and mutant strain SK1047 were grown in L Broth. Mutant strain SK1047 (pLF1.0) was grown in L Broth with 50 μg/ml of ampicillin. Cultures were shifted when they reached a density between 20 and 30 Klett units. The control strain SK901 after a shift from 32°C to 44°C (♦). Mutant strain SK1047 after a shift from 32°C to 44°C (-). Mutant strain SK1047 (pLF1.0) grown at 32°C (□). Mutant strain SK1047 (pLF1.0) after a shift from 32°C to 44°C (■).

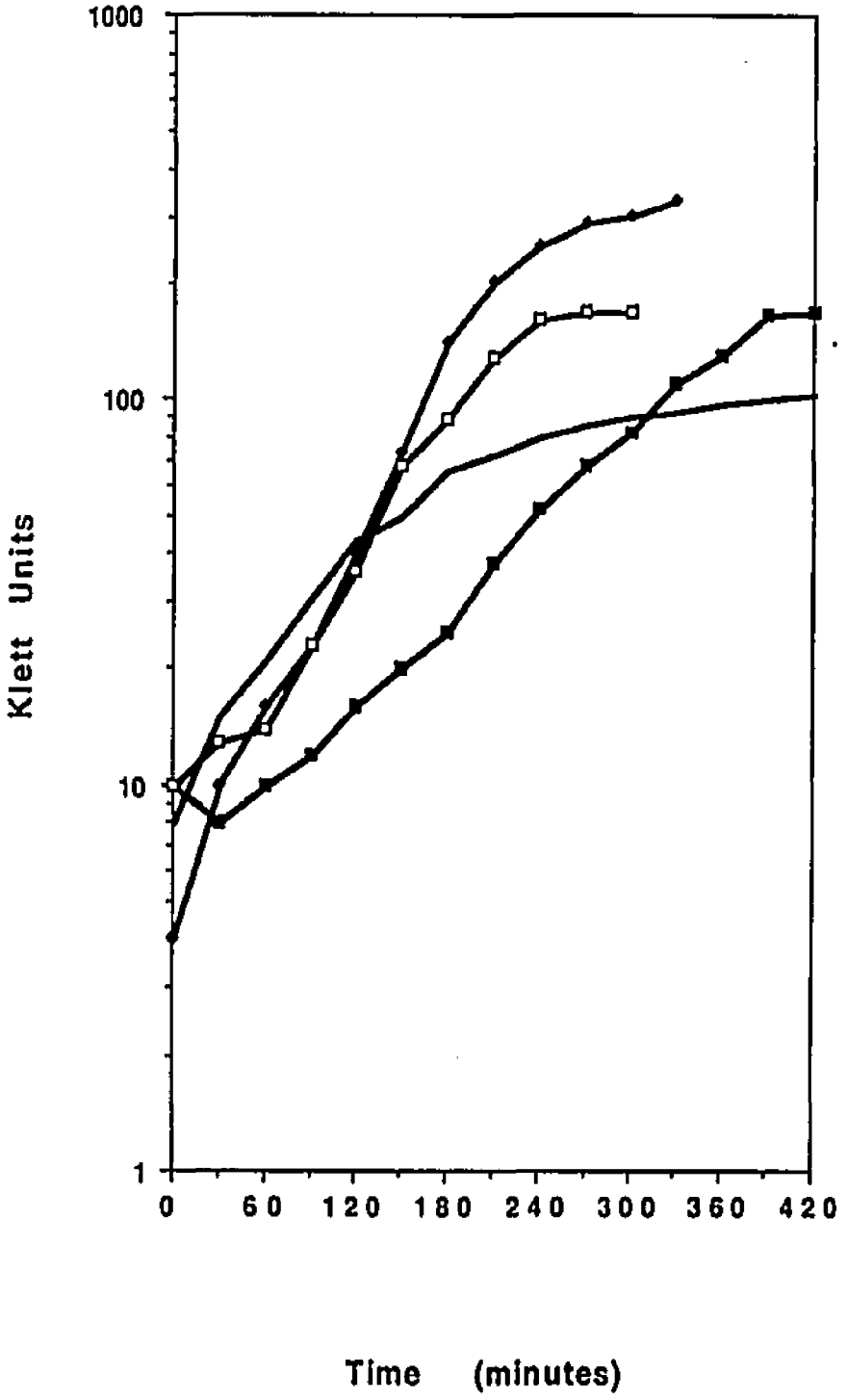

1000
100
10
1
Klett Units
0
60
120
180
240
300
360
420
Time (minutes)

Figure 27. TEMPERATURE-SHIFT EXPERIMENTS ON STRAINS SK901, SK1048 AND SK1048 (pLF1.0). The control strain and mutant strain SK1048 were grown in L Broth. Mutant strain SK1048 (pLF1.0) was grown in L Broth with 50 μg/ml of ampicillin. Cultures were shifted when they reached a density between 20 and 30 Klett units. The control strain SK901 after a shift from 32°C to 44°C (♦). Mutant strain SK1048 after a shift from 32°C to 44°C (-). Mutant strain SK1048 (pLF1.0) grown at 32°C (O). Mutant strain SK1048 (pLF1.0) after a shift from 32°C to 44°C (●).

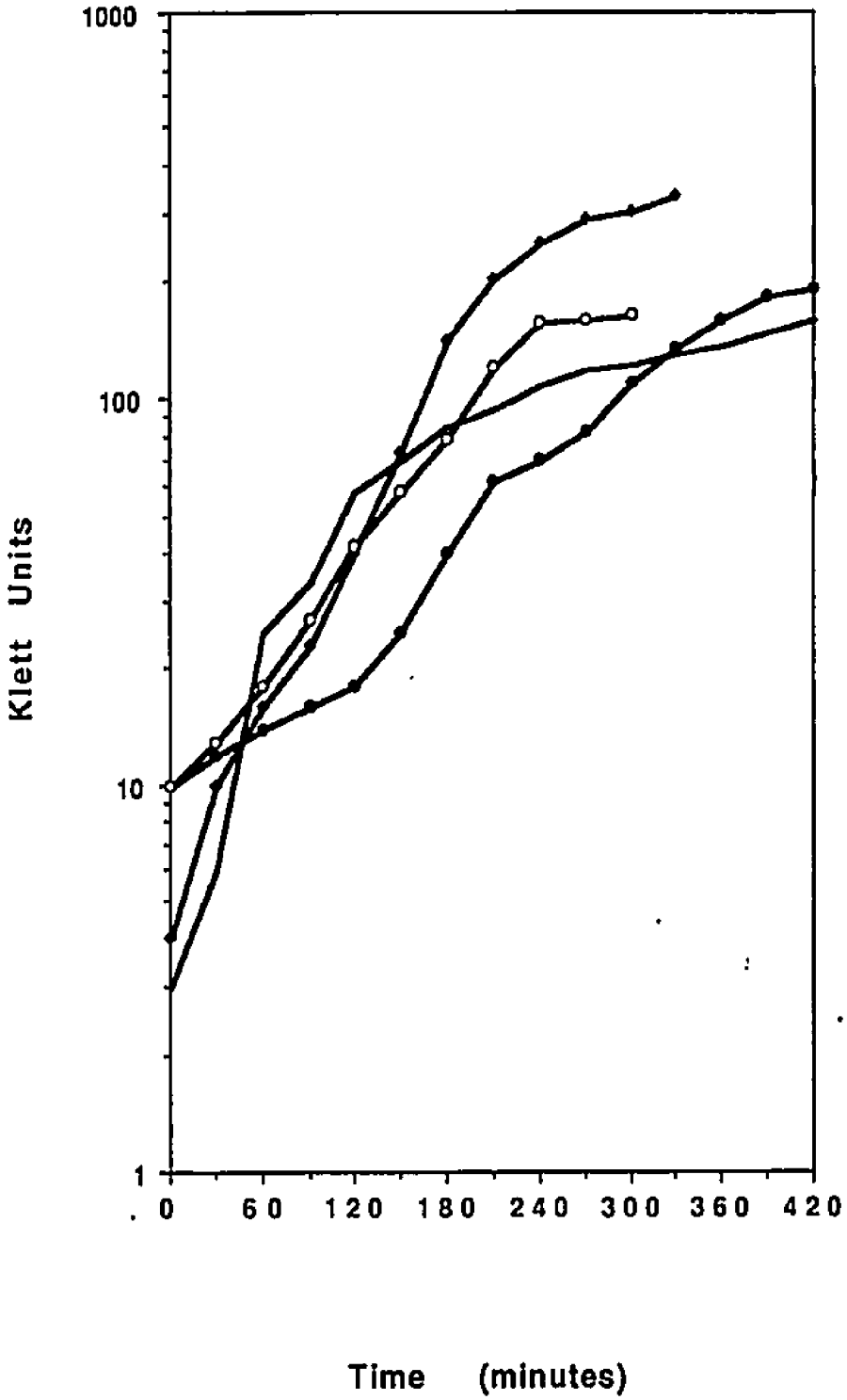

1000
100
10
1
Klett Units
0
60
120
180
240
300
360
420
Time (minutes)

Table 7. Growth Characteristics of Mutant Strains Complemented with Plasmid pLF1.0

	Final Cell Density (Klett units)	Specific Growth Rate Constant (k) (hr^{-1})
SK901 32°C	298	1.02
SK901 32°C -shift 44°C	332	1.02
SK1047 32°C	161	0.78
SK1047 32°C -shift 44°C	93	*
SK1047 (pLF1.0) 32°C	170	0.92
SK1047 (pLF1.0) 32°C -shift 44°C	110	0.45
SK1048 32°C	173	0.72
SK1048 32°C -shift 44°C	130	*
SK1048 (pLF1.0) 32°C	163	0.90
SK1048 (pLF1.0) 32°C -shift 44°C	135	0.38

Temperature-Shift Experiments. The growth of each culture was determined using a Klett-Summerson Colorimeter. Cultures were grown at 32°C until they obtained a density between 20 and 30 Klett units. At that time, cells were shifted to 44°C or retained at 32°C. The specific growth rate (hr^{-1}) was calculated from measurements of the doubling time (d) calculated from growth experiments. [$k = 0.693/d$]

* cells ceased growing after two hours 44°C.

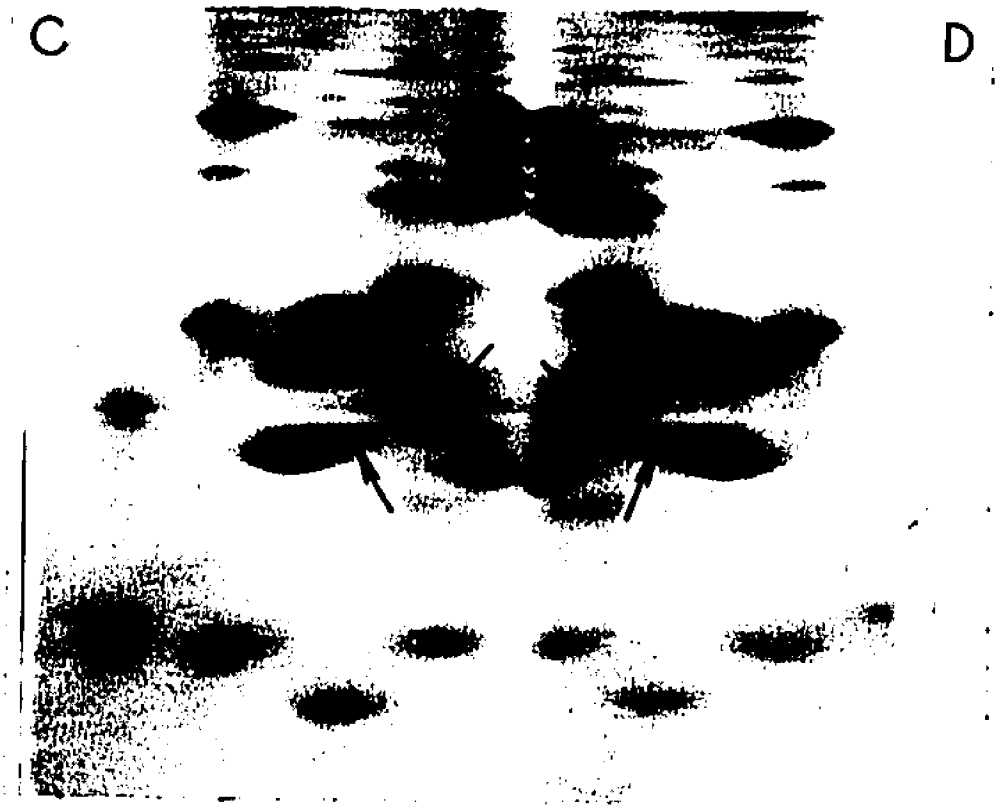
λfus2 grown at 42°C produced two forms of ribosomal protein L22. To examine expression of this protein at both the permissive and restrictive temperatures, parallel experiments were conducted. Mutant strains complemented with λfus2 were grown at 32°C or shifted to 42°C. Ribosomes were isolated from cells grown under both conditions. Equal amounts of ribosomal proteins were separated using two dimensional gel electrophoresis (Figure 28). Two dimensional gels were scanned for the presence of the control and mutant forms of ribosomal protein L22. Both the mutant and the control form of ribosomal protein L22 were identified in gels from the complemented strains grown at each temperature (Figure 28). Expression of the phage encoded genes took place both at 32°C and at 42°C.

Two Dimensional Gels on Ribosomal Proteins from Plasmid pLF1.0 Complemented Strains at 32°C and 42°C.

It has been established that cells complemented with plasmid pLF1.0 produced two forms of ribosomal protein L22. To examine expression from the plasmid at 32°C and at 42°C, experiments were conducted in duplicate. Mutant strains complemented with plasmid pLF1.0 were grown at 32°C or shifted to 42°C and ribosomes were isolated from each class of cells. Ribosomal proteins were separated using two dimensional gel electrophoresis (Figure 29). Two dimensional gels were scanned for the presence of the

Figure 28. TWO-DIMENSIONAL GEL ELECTROPHORESIS OF RIBOSOMAL PROTEIN FROM MUTANT STRAINS COMPLEMENTED WITH λFUS2 AT 32°C and 42°C. (A) Ribosomal proteins were extracted from SK1047 (λfus2) cells grown at 32°C and separated using the two dimensional gel system. (B) Ribosomal proteins were extracted from SK1047 (λfus2) cells grown at 42°C and separated using the two dimensional gel system. (C) Ribosomal proteins were extracted from SK1048 (λfus2) cells grown at 32°C and separated using the two dimensional gel system. (D) Ribosomal proteins were extracted from SK1048 (λfus2) cells grown at 42°C and separated using the two dimensional gel system. Arrows identify the positions of the control and mutant forms of ribosomal protein L22. Only the basic region (lower half) of each gel is shown.


A
B


C
D

control and mutant forms of ribosomal protein L22. Both forms of the protein were identified in gels from the complemented strains at both temperatures (Figure 29). Expression from the plasmid occurred at both 32°C and at 42°C.

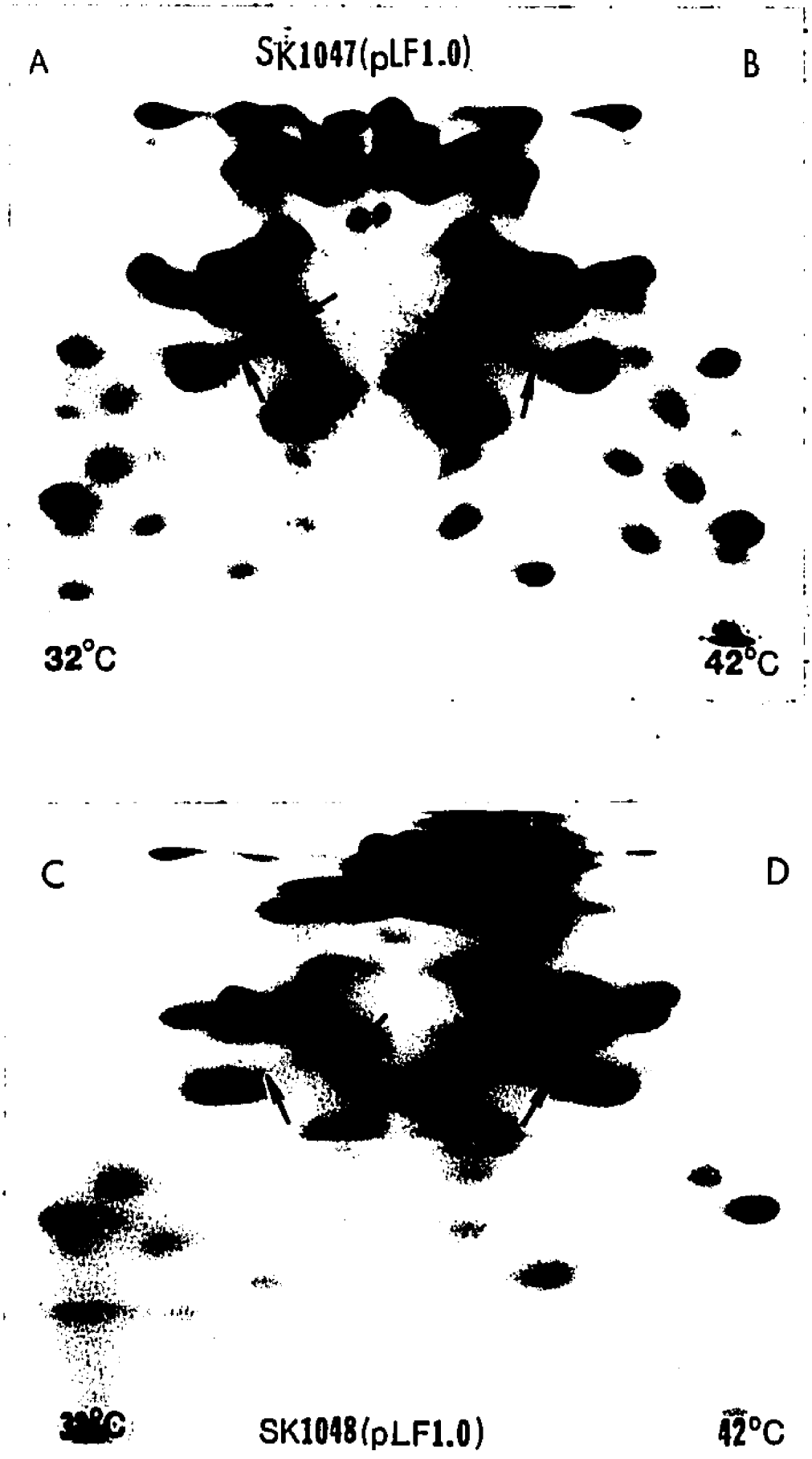
Quantitation of Protein L22 Production in Complemented Mutant Strains

The appearance of control ribosomal protein L22 at both the permissive and restrictive temperature indicated that there was some level of expression from the phage and plasmid encoded rplV genes irrespective of temperature. To determine if the amount of protein produced from these genes varied with temperature, a quantitative estimate was made of the relative assembly of the wild type and mutant forms of ribosomal protein L22 into 50S subunits. The amount of control and mutant protein L22 incorporated was determined both at 32°C and at 42°C for comparison.

Quantitation of λfus2 Complemented Strains

To quantitate the expression of the control ribosomal protein L22 produced in complemented strains, the amount of the mutant and the wild type ribosomal protein L22 assembled into ribosomes was measured. Both forms of ribosomal protein L22 were isolated from gels of proteins from mutant strains complemented with λfus2, as shown in Figure 28. The method used to recover pure ribosomal proteins from two

Figure 29. TWO-DIMENSIONAL GEL ELECTROPHORESIS OF RIBOSOMAL PROTEINS FROM MUTANT STRAINS COMPLEMENTED WITH PLASMID pLF1.0 AT 32°C AND 42°C. (A) Ribosomal proteins were extracted from the control strain and separated using the two dimensional gel system for comparison. (B) Ribosomal proteins were extracted from SK1047 (pLF1.0) cells grown at 42°C and separated using the two dimensional gel system. (C) Ribosomal proteins were extracted from SK1048 (pLF1.0) cells grown at 32°C and separated using the two dimensional gel system. (D) Ribosomal proteins were extracted from SK1048 (pLF1.0) cells grown at 42°C and separated using the two dimensional gel system. Arrows identify the positions of the control and mutant forms of ribosomal protein L22. Only the basic regions (lower half) of each gel is shown.


A
SK1047(pLF1.0)
B
32°C
42°C
C
D
SK1048(pLF1.0)
42°C

dimensional gel cores is shown in Figure 30. Protein recovery was studied in relation to two other ribosomal proteins, L3 and L29. These proteins were chosen because they are expressed from the S10 operon and subject to the same regulatory controls as protein L22 (Figure 18B). For more quantitative recovery, gels were overloaded with 70S or 50S ribosomal proteins. Reisolation of ribosomal proteins L3, L22 and L29 from the control and both mutants indicated that the amount of protein L22 in the 50S subunit was equivalent to the amount of proteins L3 and L29 (Table 8A). In complemented strains, the relative amount of both forms of protein L22 was measured (Table 8B). The protein ratios were determined at the permissive and restrictive temperatures. Analysis of λfus2 complemented strains showed that about 50% of protein L22 assembled into ribosomes was mutant protein and the remainder was the wild type form. No significant alteration in the assimilation of mutant ribosomal protein L22 into ribosomes was detected with the shift in temperature.

Quantitation of plasmid pLF1.0 Complemented Strains

Complementation using plasmid pLF1.0 involved maintaining multiple copies of the plasmid in the mutant cells. Each plasmid copy contains a wild type rplV gene which when expressed produces control ribosomal protein L22. Mutant cells complemented with the plasmid exhibited two

Figure 30. ISOLATION OF RIBOSOMAL PROTEINS FROM GEL CORES. A representation of the procedure used for extracting ribosomal protein from a core excised from a two dimensional gel. A more complete description of the procedure is presented in the Materials and Methods.

Reisolation of Ribosomal Proteins from 2-D Gels

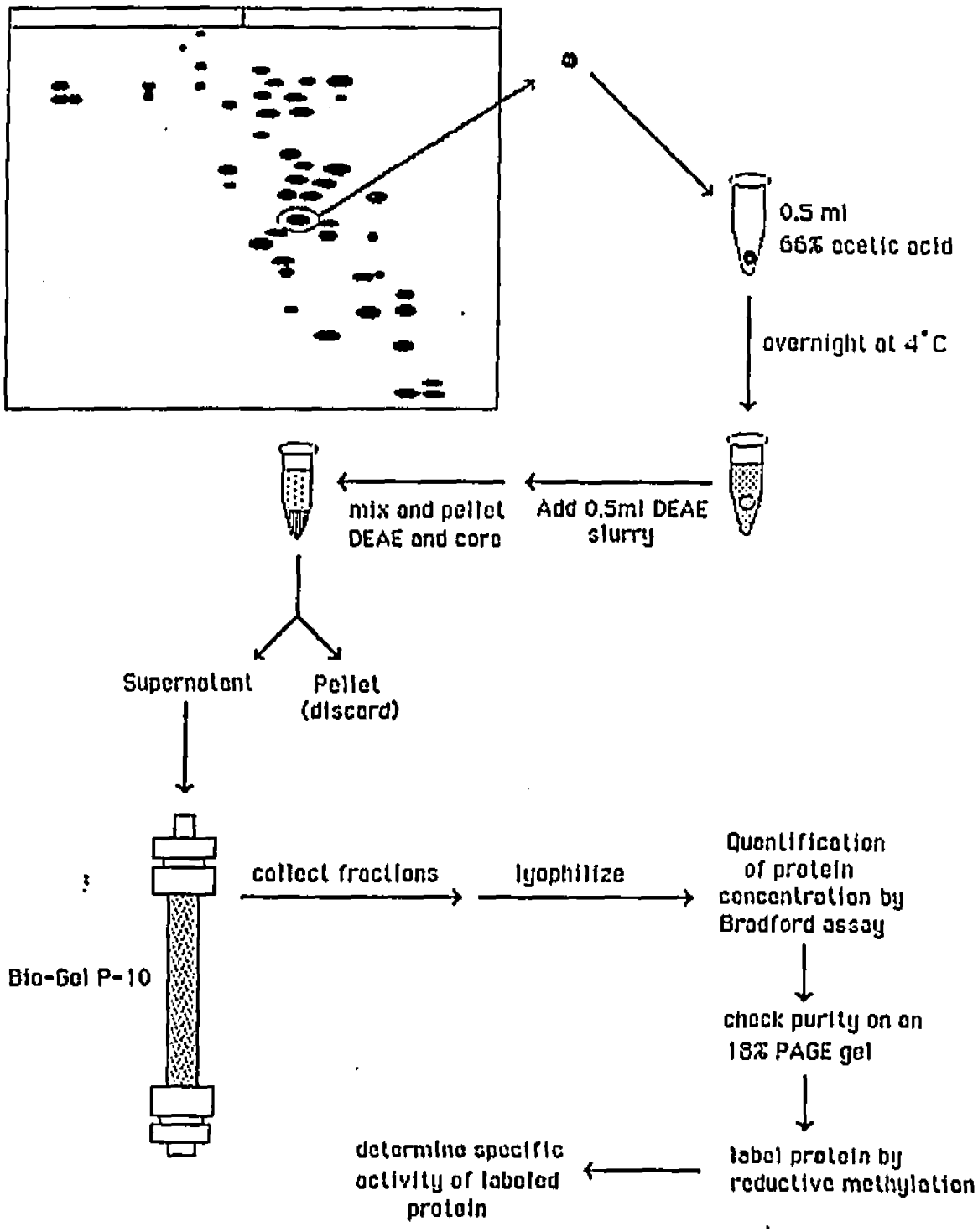


Table 8. EXPRESSION STUDIES IN COMPLEMENTED MUTANT STRAINS. (A) Comparison of the amount of ribosomal protein L22 in micromoles isolated from gel cores to the total amount of proteins L3, L22 and L29. Microgram amounts were converted to micromoles for comparison using molecular weights of 22,258 daltons for ribosomal protein L3 (Muranova et. al., 1978), 12,227 daltons for ribosomal protein L22 (Wittman-Liebold and Greuer, 1980) and 7,274 daltons for ribosomal protein L29 (Bitar, 1975). (B) Comparison of the percent of the total protein L22 that was found in the wild type and mutant form, compared with the total L3, L22 and L29 production. Results represent the quantitation of protein reisolated from a single set of two dimensional gels.

Table 8. Expression Studies in Complemented Mutant Strains

A

Sample	I Relative amount of Protein L22	II Ratio to SK901
SK901-32°C	0.33	1.00
SK1047-32°C	0.40	1.21
SK1048-32°C	0.35	1.06

$I=L22_{total} \backslash (L3+L22_{total}+L29)$

$II=L22_{mutant} \backslash L22_{control}$

B

	Ribosomal Distribution of protein L22: Wild Type	Mutant
SK1047-32°C	0	100%
SK1047 (λfus2)-32°C	54%	46%
SK1047 (λfus2)-42°C	50%	50%
SK1047 (pLF1.0)-32°C	45%	55%
SK1047 (pLF1.0)-42°C	71%	29%
SK1048-32°C	0	100%
SK1048 (λfus2)-32°C	50%	50%
SK1048 (λfus2)-42°C	43%	57%
SK1048 (pLF1.0)-32°C	42%	58%
SK1048 (pLF1.0)-42°C	65%	35%

forms of protein L22 on two dimensional gels (Figure 29). Reisolation and quantitation of each form of protein L22 was studied in relation to proteins L3 and L29 (Table 8) as described above. An excess of mutant protein L22 was assembled into ribosomes at 32°C in both complemented mutant strains. Ribosomal incorporation of the plasmid coded wild type protein L22 was elevated in cells grown at 42°C (Table 8B). Incorporation of the mutant L22 ribosomal protein decreased to one-third of the total L22 with increasing growth temperature.

Functional Studies

Ribosomal protein L22 is a core binding protein in the assembly of the large subunit (Herold and Nierhaus, 1987). Binding between protein L22 and 23S RNA is enhanced in the presence of protein L4 (Nierhaus, 1980). Therefore, an important functional test of isolated ribosomal protein L22 was its ability to bind to 23S rRNA.

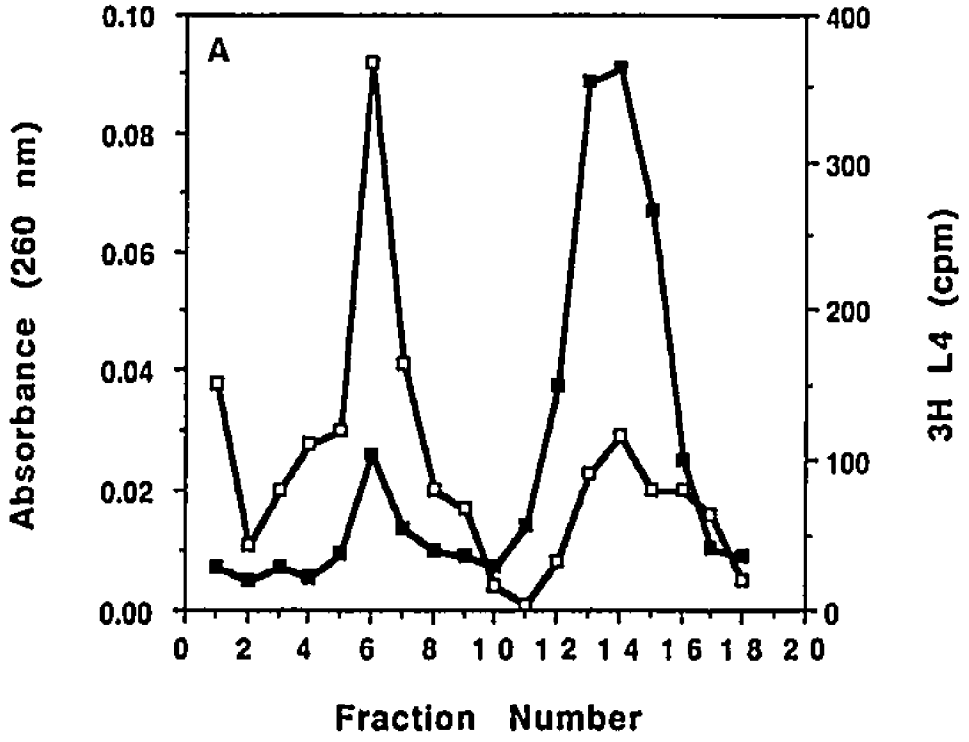
Binding of Protein L22 to 23S RNA

Prior assays have shown that ribosomes isolated from the mutant strains exhibited different functional characteristics compared to each other and to the control strain (Table 1). To examine functional differences between the mutant forms of protein L22, binding assays were conducted using ^{3}H-labeled protein L22 and control 23S RNA. Proteins L4 and L22 were purified from two dimensional gels

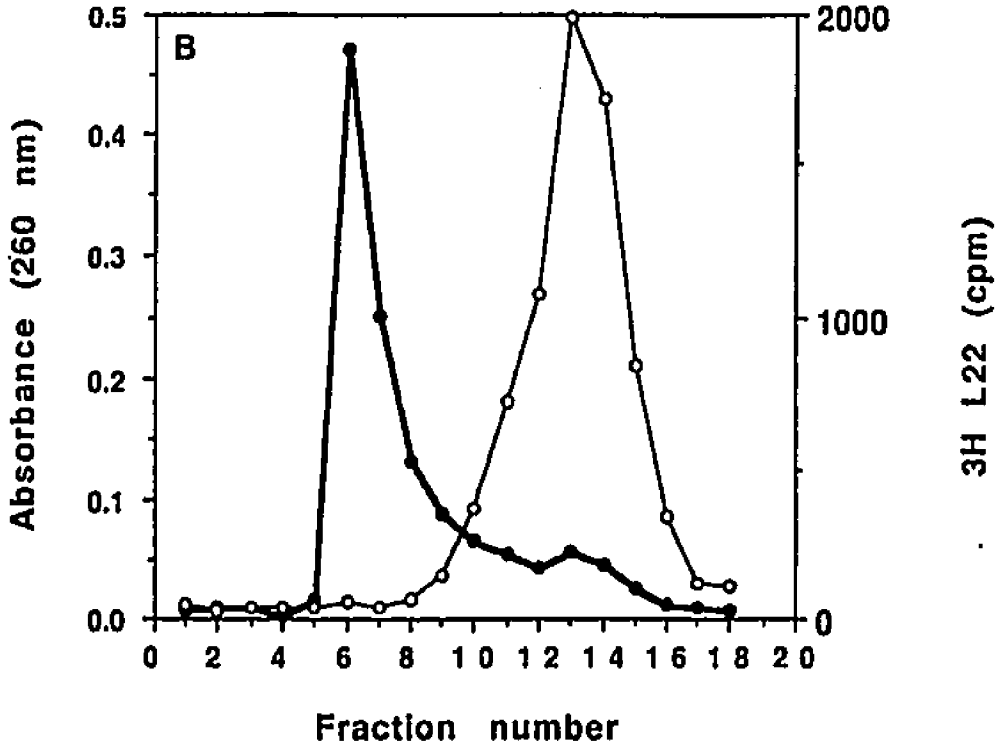
according to the method described in Figure 30. The protein was labeled by reductive methylation and the specific activity determined. In studying the association between ribosomal protein L4 and 23S RNA, a 5:1 molar excess of protein to 23S RNA was used. In these assays, 1 picomole of protein L4 was found to bind to 1 picomole of 23S RNA (Figure 31A). Thus, the binding of ^{3}H-L4 to 23S RNA using this system reproduced the work of Nierhaus (1980). The association of the wild type and mutant forms of ribosomal protein L22 with 23S RNA were tested in assays analogous to those with protein L4. Protein L22 was found to bind weakly to 23S RNA, as previously shown by Nierhaus (1980) (Figure 31B). The ratio of binding was calculated to be between 0.3 and 0.02 picomole of protein L22 per picomole of RNA. To facilitate protein L22 binding, assays were performed in the presence of unlabeled protein L4. Assembly experiments have shown that protein L22 binds to protein L4 which is attached to 23S RNA (Herold and Nierhaus, 1987). In the presence of protein L4, the binding of both control and mutant ribosomal protein L22 was enhanced. Experiments were conducted using 2.5 picomoles of protein L22, 2.5 picomoles of protein L4 and 1 picomole of 23S RNA at both 32°C and 44°C. Examination of binding at both temperatures was conducted to ascertain if binding was temperature-sensitive (Table 9). Ribosomal protein L22 isolated from the control strain bound to 23S RNA in the presence of L4 both at 32°C and 44°C (Figure 32).

Figure 31. BINDING OF RIBOSOMAL PROTEINS L4 AND L22 TO 23S RNA. A 5 fold molar excess of ^{3}H-labelled protein was incubated with 23S RNA in 250 µl of TM-4 buffer for 20 minutes. The complexes were separated from unbound protein and RNA on a Bio-gel A 0.5 m column at 4°C in the same buffer. One-half milliliter fractions were collected, read for absorbance at 260 nm and then counted for ^{3}H-labelled protein. (A) The binding of ribosomal protein L4 to 23S RNA. Absorbance units of RNA were measured (□). The radioactivity in ^{3}H-labelled protein was counted (■). (B) The binding of wild type ribosomal protein L22 to 23S RNA. The absorbance units of RNA were measured (●). The radioactivity of ^{3}H-labelled protein was counted (○).

Ribosomal Protein L4 binding to 23S RNA



Ribosomal Protein L22 binding to 23S RNA



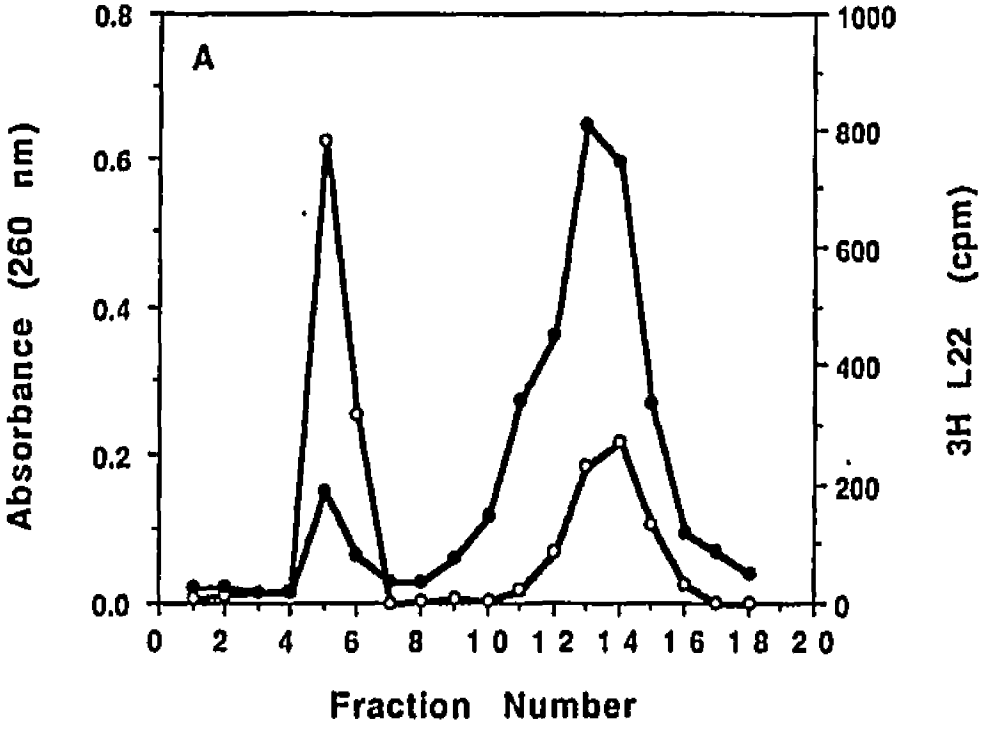
The binding was found to be reduced at the higher temperature. Protein L22 isolated from mutant SK1047 was unable to associate with RNA at high temperature and bound RNA poorly at low temperature (Figure 33). Protein L22 isolated from strain SK1048 associated with 23S rRNA to a greater extent than the control; approaching the 1:1 stoichiometry achieved in the ribosome (Figure 34). However, at high temperature there was a 30% loss of binding. The specificity of binding was examined in tests performed using ribosomal protein L22 and 16S RNA (Figure 35). These assays revealed a small amount of non-specific association between ribosomal protein L22 and 16S RNA, 0.06 picomoles of protein L22 associated with 1 picomole of 16S RNA. This non-specific binding became important when examining the association of protein L22 from strain SK1047 with 23S RNA. Essentially no specific binding occurred between SK1047 protein L22 and 23S RNA.

Sequencing of the rplV Gene

The mutation causing temperature sensitivity has been mapped to the rplV gene. To fully understand the nature of the change in ribosomal protein L22, sequencing of the mutant gene was undertaken. Only when the change in the nucleotide sequence is determined can all the implications of the mutation be examined.

Figure 32. BINDING OF WILD TYPE RIBOSOMAL PROTEIN L22 TO 23S RNA. The association of ^{3}H-labelled protein L22 with 23S RNA in the presence of protein L4. A 2.5 fold molar excess of ^{3}H-labelled protein L22 and unlabeled protein L4 were incubated with 23S RNA in 250 µl of TM-4 buffer for 20 minutes at either 32°C or 44°C. The complexes were separated from unbound protein and RNA on a Bio-gel A 0.5 m column at 4°C in TM-4 buffer. One-half milliliter fractions were collected, read for absorbance at 260 nm (O)and then counted for ^{3}H-labelled protein (●). (A) The binding of control ribosomal protein L22 to 23S RNA at 32°C. (B) The binding of control ribosomal protein L22 to 23S RNA at 44°C.

SK901 - 32°C



SK901 - 44°C

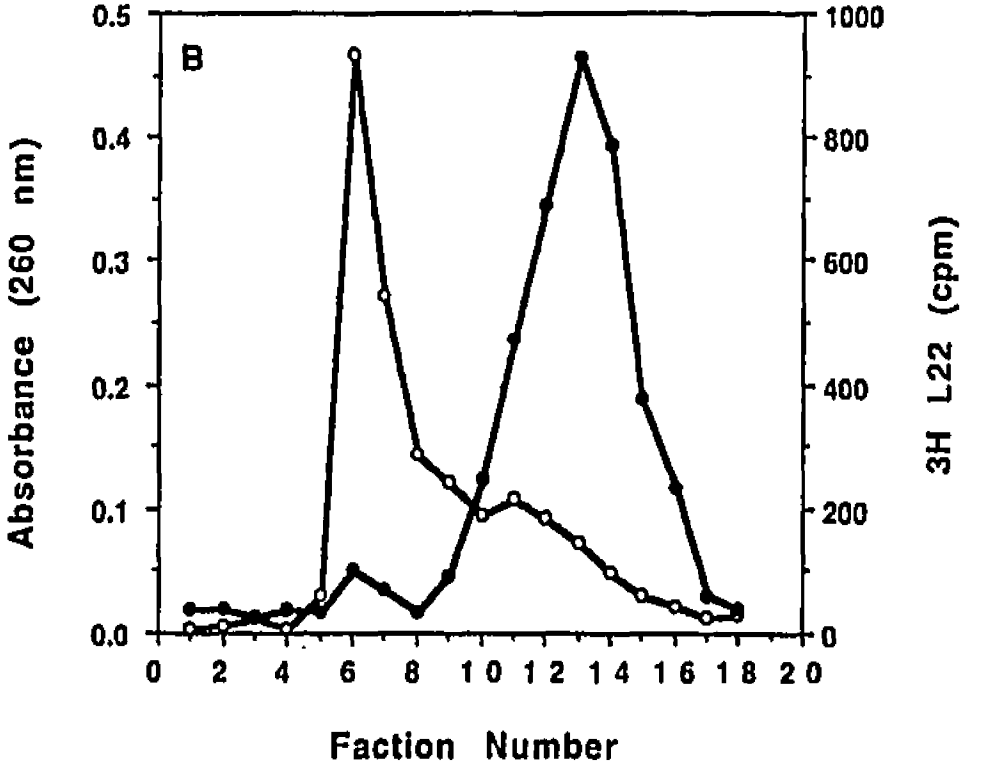
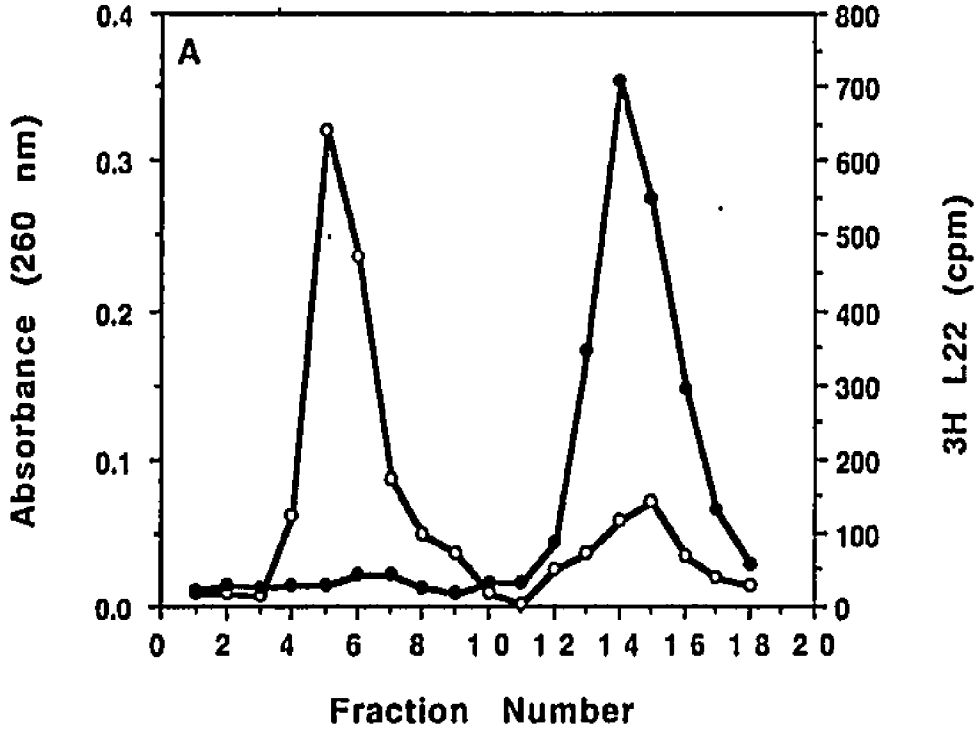


Figure 33. BINDING OF RIBOSOMAL PROTEIN L22 FROM SK1047 TO 23S RNA. The association of ^{3}H-labelled protein L22 with 23S RNA in the presence of protein L4. A 2.5 fold molar excess of ^{3}H-labelled protein L22 and unlabeled protein L4 were incubated with 23S RNA in 250 µl of TM-4 buffer for 20 minutes at either 32°C or 44°C. The complexes were separated from unbound protein and RNA on a Bio-gel A 0.5 m column at 4°C in TM-4 buffer. One-half milliliter fractions were collected, read for absorbance at 260 nm (O)and then counted for ^{3}H-labelled protein (●). (A) The binding of ribosomal protein L22 from SK1047 to 23S RNA at 32°C. (B) The binding of ribosomal protein L22 from SK1047 to 23S RNA at 44°C.

SK1047 - 32°C



SK1047 - 44°C

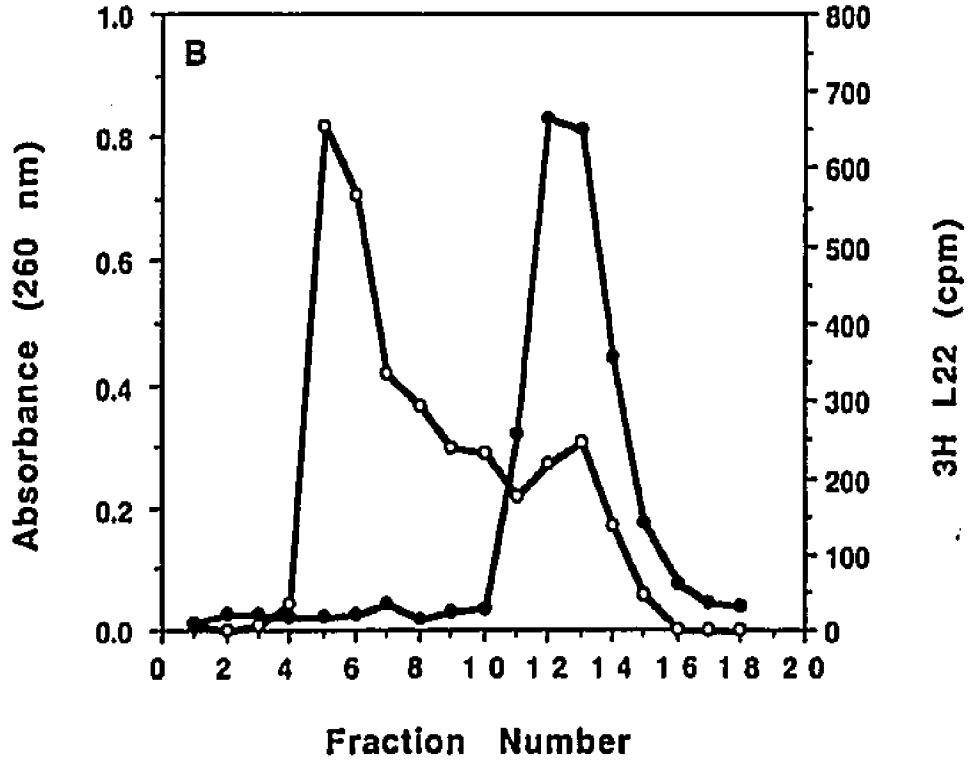


Figure 34. BINDING OF RIBOSOMAL PROTEIN L22 FROM SK1048 TO 23S RNA. The association of ^{3}H-labelled protein L22 with 23S RNA in the presence of protein L4. A 2.5 fold molar excess of ^{3}H-labelled protein L22 and unlabeled protein L4 were incubated with 23S RNA in 250 µl of TM-4 buffer for 20 minutes at either 32°C or 44°C. The complexes were separated from unbound protein and RNA on a Bio-gel A 0.5 m column at 4°C in TM-4 buffer. One-half milliliter fractions were collected, read for absorbance at 260 nm (O)and then counted for ^{3}H-labelled protein (●). (A) The binding of ribosomal protein L22 from SK1048 to 23S RNA at 32°C. (B) The binding of ribosomal protein L22 from SK1048 to 23S RNA at 44°C.

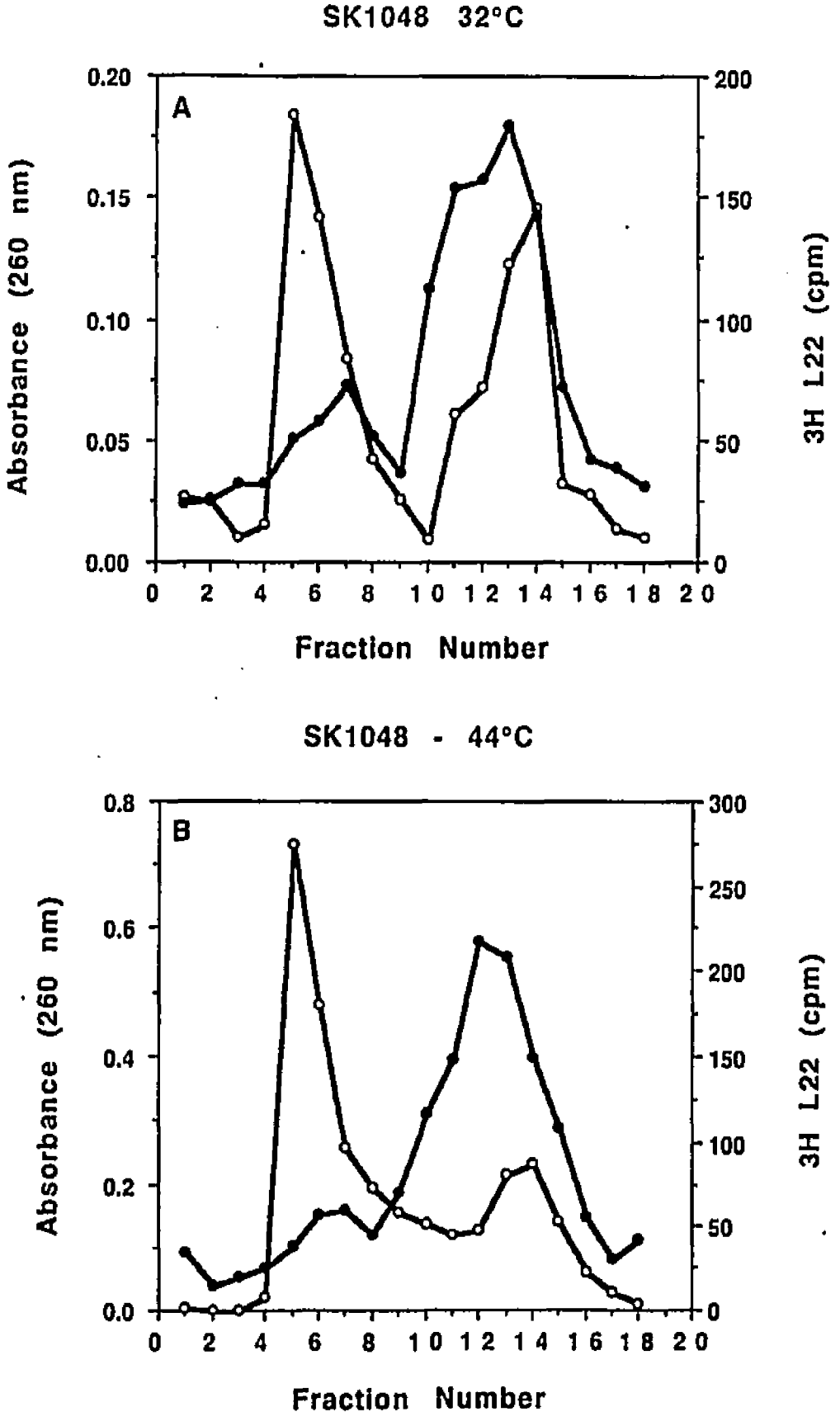

SK1048 32°C
A
Absorbance (260 nm)
3H L22 (cpm)
Fraction Number
SK1048 - 44°C
B
Absorbance (260 nm)
3H L22 (cpm)
Fraction Number

Figure 35. BINDING OF RIBOSOMAL PROTEIN L22 TO 16S RNA. To determine nonspecific binding the association of ^{3}H-labelled protein L22 with 16S RNA in the presence of protein L4 was measured. A 2.5 molar fold excess of ^{3}H-labelled protein L22 and unlabeled protein L4 were incubated with 16S RNA in 250 μl of TM-4 buffer for 20 minutes at 32°C. The complexes were separated from unbound protein and RNA on a Bio-gel A 0.5 m column at 4°C in TM-4 buffer. One-half milliliter fractions were collected, read for absorbance at 260 nm (▲) and then counted for ^{3}H-labelled protein (△).

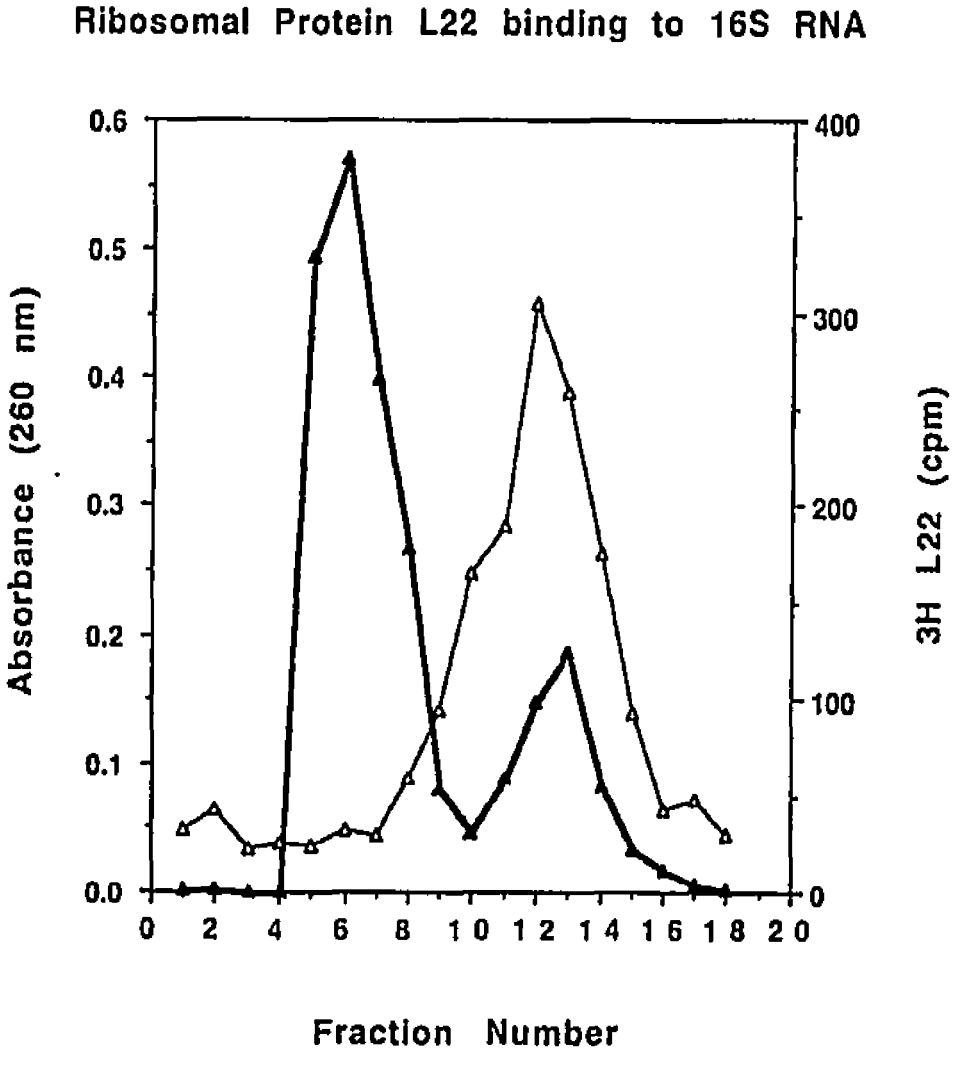


Table 9. Binding of Ribosomal Protein L22 to 23S RNA

Source of protein L22	Incubation Temperature	^{3}H-L22/ 23S RNA	% Binding at 44°C	Binding relative to SK901
SK901	32°C	0.42 (.01)		
SK901	44°C	0.27 (.015)	64%	100%
SK1047	32°C	0.07 (.02)		
SK1047	44°C	0.03 (.005)	43%	11%
SK1048	32°C	0.68 (.005)		
SK1048	44°C	0.20 (.045)	29%	74%

Binding Assays. The binding of ribosomal protein L22 to 23S RNA was conducted in the presence of ribosomal protein L4. Binding assays were conducted at 2.5:1 molar ratios of each protein to 23S rRNA. The proteins were bound to 23S RNA according to a procedure from Roth and Nierhaus (1980). Separation of the bound protein-RNA complex from the free RNA and protein was achieved using a gel filtration procedure from Stoffler et al. (1971). Binding data represents the average of two experiments with the standard error of the mean in parenthesis.

Amplification of the rplV Gene

The rplV gene for ribosomal protein L22 was copied from the E. coli chromosome using the polymerase chain reaction. PCR reactions were conducted on genomic DNA purified from the control strain and mutant strains. Twenty-five base oligonucleotides were synthesized, which flanked the rplV gene. These oligonucleotides acted as primers allowing a thermostable Taq polymerase to synthesize multiple copies of the 926 base pair region defined by the primers. Separation of the PCR reaction product on an agarose gel showed there were some spurious products of the amplification reaction for strain SK1047 while only a single product was found for SK1048 (Figure 36). The size of the PCR product was confirmed by its relation to molecular weight markers. The 926 bp fragment was purified for sequencing by electroelution from the agarose gel.

Sequencing of the rplV gene

The PCR product was used as template for sequencing using primer extension. The sequence of the control strain SK901 was found to match that published by Zurawski and Zurawski (1985). Sequencing of mutant strains SK1047 and SK1048 revealed that mutagenesis had caused a transition of a cytosine to a thymine at nucleotide 22 (position 3235 in the Zurawski and Zurawski sequence) in the rplV gene sequence (Figure 37). Further analysis of this change

Figure 36. PURIFICATION OF THE PCR PRODUCT. Three microliters of the PCR reaction product was electrophoresed through a 1.0% agarose gel and post stained with EtBr. Lane 1. The molecular weight marker was plasmid pBR322 DNA digested with HinfI. Lane 2-4. 3 µl from the PCR reaction using genomic DNA from SK1047, tubes 1, 2, 3. Lanes 5-8. 3 µl from the PCR reaction using genomic DNA from SK1048, tubes 1-4.

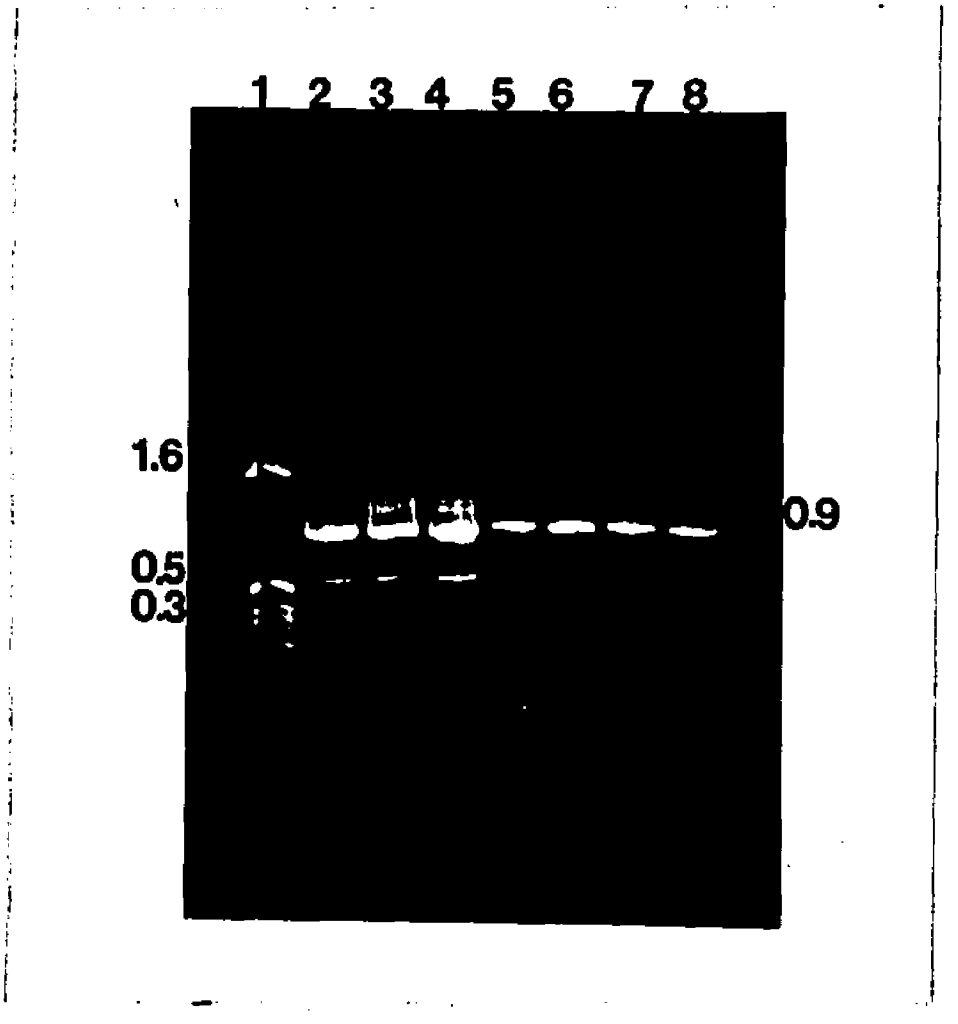


Figure 37. TRANSLATION OF SEQUENCES FROM STRAINS SK1047 AND SK1048. The nucleotide sequence of the rplV gene and the amino acid sequence of ribosomal protein L22. The three letter abbreviations for each amino acid in the sequence is shown below the nucleotide sequence. The numbers on the right of the figure indicate the nucleotide position in relation to the PCR product which was used as the template for sequencing. The 25-mer oligonuleotide primers used for PCR are underlined. A box highlights the position of the 15-mer oligonucleotide forward sequencing primer (L22-F-2) and the reverse sequencing primer (L22-R-2). A third box highlights the mutation identified in the nucleotide sequence of the rplV gene for ribosomal protein L22 in strains SK1047 and SK1048. When the amino acid sequence is derived from the mutant rplV gene an arginine residue is replaced by a cysteine residue at position 8 in the mutant protein.

CGGTCGAAAG CCGAGACAAG AAGCCCCTGC GCACTTGGTC CCGTCGTTCA 50

ACGATCTTTC CTAACATGAT CCGTTTGACC ATCGCTGTCC ATAATCGTCG 100

TCAGCACGTT CCCGTATTTG TAACCCACGA AATCGTTCGT CACAAACTGG 150

L22-F-2

GTGAATTCGC ACCGACTCGT ACTTATCGCG GCCACGCTGC TGATAAAAAA 200

GCGAAGAAGA AATAAGGTAG GACGAAGAGA TCGAAACTAT CGCTAAACAT 250

Met GluThrIle AlaLysHis

T
*
CGCCATGCTC GTTCTTCTCC TCAGAAGGTT CCCCTTGTTG CTCACCTGAT 300
ArgHisAlaArg SerSerAla GlnLysVal ArgLeuValAla AspLeuIle
*
Cys

TCGCCGTAAG AAAGTGTCGC ACGCTCTCGA TATTTTGACC TACACCAACA 350
ArgGlyLys LysValSerGln AlaLeuAsp IleLeuThr TyrThrAsnLys

AGAAAGCCGC TGTACTGGTC AAGAAAGTTC TGGAATCTGC CATTGCTAAC 400
LysAlaAla ValLeuVal LysLysValLeu GluSerAla IleAlaAsn

GCTGAACACA ACGATCCCGC TGACATTGAC GATCTGAAAG TTACGAAAAT 450
AlaGluHisAsn AspGlyAla AspIleAsp AspLeuLysVal ThrLysIle

TTTCGTAGAC GAACCCCCGA GCCTGAAGCG CATTATCCCG CGTCGCAAAG 500
PheValAsp GluGlyProSer MetLysArg IleMetPro ArgAlaLysGly

GTCGTGCAGA TCGCATCCTG AAGCGCACCA GCCACATCAC TGTCGTTGTG 550
ArgAlaAsp ArgIleLeu LysArgThrSer HisIleThr ValValVal

TCCGATCGCT GAGACTCTCG AGACTAGCAA TCCGTCAGAA AGTACATCCT 600
SerAspArg End

L22-R-2

AATCGTATTC GCCTCCGTAT TGTAAAACCA TCGAACTCTA CCTCGTTTCC 650

GAACACCAAA GAATTCCCTG ACAACCTCGA CAGCGATTTT AAAGTACGTC 700

AGTACCTCAC TAACGAACTG CCTAAACCGT CCGTATCTCG TATCGTTATC 750

CACCGTCCCG CTAAGACCAT CCGTGTAACC ATTCACACTG CTCGCCCCCG 800

TATCGTTATC CGTAAAAAAG GTGAAGACGT AGAAAAACTG CGTAACGTCG 850

TAGCCGACAT CGCTGGCGTT CCTGCACAGA TCAACATCGC CGAAGTTCGT 900

AAGCCTGAAC TGGACGCAAA ACTGGT 926

revealed that the codon CGC in the wild type sequence was now changed to the codon TGC. Translation of the wild type gene revealed that the CGC codon coded for the amino acid arginine. In the mutants, the TGC codon now coded for the amino acid cysteine. The mutation resulted in the substitution of a cysteine for an arginine in the mutant protein. The transition of cytosine to thymine was the only change observed in the rplV genes from SK1047 and SK1048.

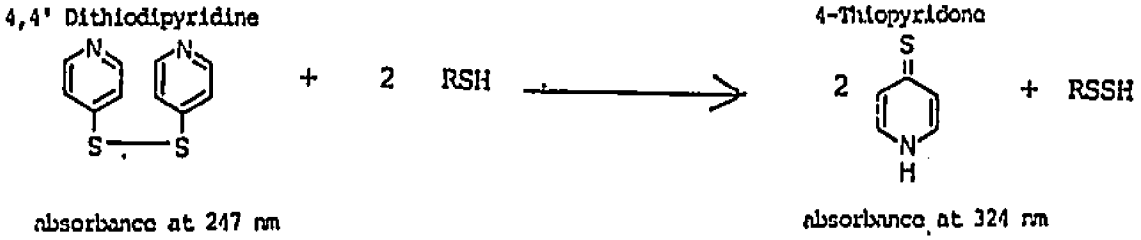
Cysteine determination in the Mutant Strains

The wild type ribosomal protein L22 contains no cysteine residues. To verify the sequence change, ribosomal protein L22 was tested for the presence of the cysteine sulfhydryl group using 4-4' dithiodipyridine. The reaction conditions were adapted from those described by Grassetti and Murray (1966). Proteins were heated to 80°C, to denature the protein, then cooled to room temperature and reacted with the 4-PDS. The presence of cysteine residues was detected by measuring the increased absorbance at 324 nm. The assays were standardized using MS2 phage coat protein and S. aureus V8 protease. The MS2 coat protein was chosen because it contained two cysteine residues. The S. aureus protease was studied because it contained no cysteine residues (Sober, 1970). Control and mutant forms of ribosomal protein L22 were purified from two dimensional gels by the method described previously. The proteins were

reacted with the 4-PDS reagent and the absorbance at 324 nm was recorded. Plotting absorbance at 324 nm versus micrograms of protein showed that mutant protein L22 and MS2 coat protein reacted with the reagent while control protein L22 and S. aureus V8 protease did not (Figure 38). The extinction coefficient of 4-4' PDS (1.98×10^4) was used to convert the absorbance units into nanomoles of cysteine. Micrograms of protein were converted to nanomoles using the following molecular weights: MS2 coat protein = 14 kilodaltons (Sober, 1970), protein L22 = 12 kilodaltons (Wittmann-Liebold and Greuer, 1980). A plot of this data (Figure 39A) showed that each mutant protein L22 contained one cysteine. The slope of the MS2 protein titration was adjusted to correct for the 80% reactivity of the MS-2 protein under these conditions. The slope was adjusted to equal the slope expected for a protein containing 2

Figure 38. DETERMINATION OF CYSTEINE WITHIN RIBOSOMAL PROTEIN L22. (A) Equation describing the reactivity of 4-PDS with sulfhydryl groups. (B) Samples containing 10, 20, 30 and 40 μg of SK1047 ribosomal protein L22 (■), SK1048 ribosomal protein L22 (O), MS2 coat protein (▲) and S. aureus V8 protease (□) were dissolved in 0.05 M phosphate buffer pH 7.2. The samples were heated to 80°C, cooled and reacted with the 4-PDS solution. The absorbance at 324 nm was read against water. A correction was applied for a 4-PDS blank.

A.


4,4' Dithiodipyridine
+ 2 RSH
4-Thiopyridone
2
+ RSSH
absorbance at 247 nm
absorbance at 324 nm

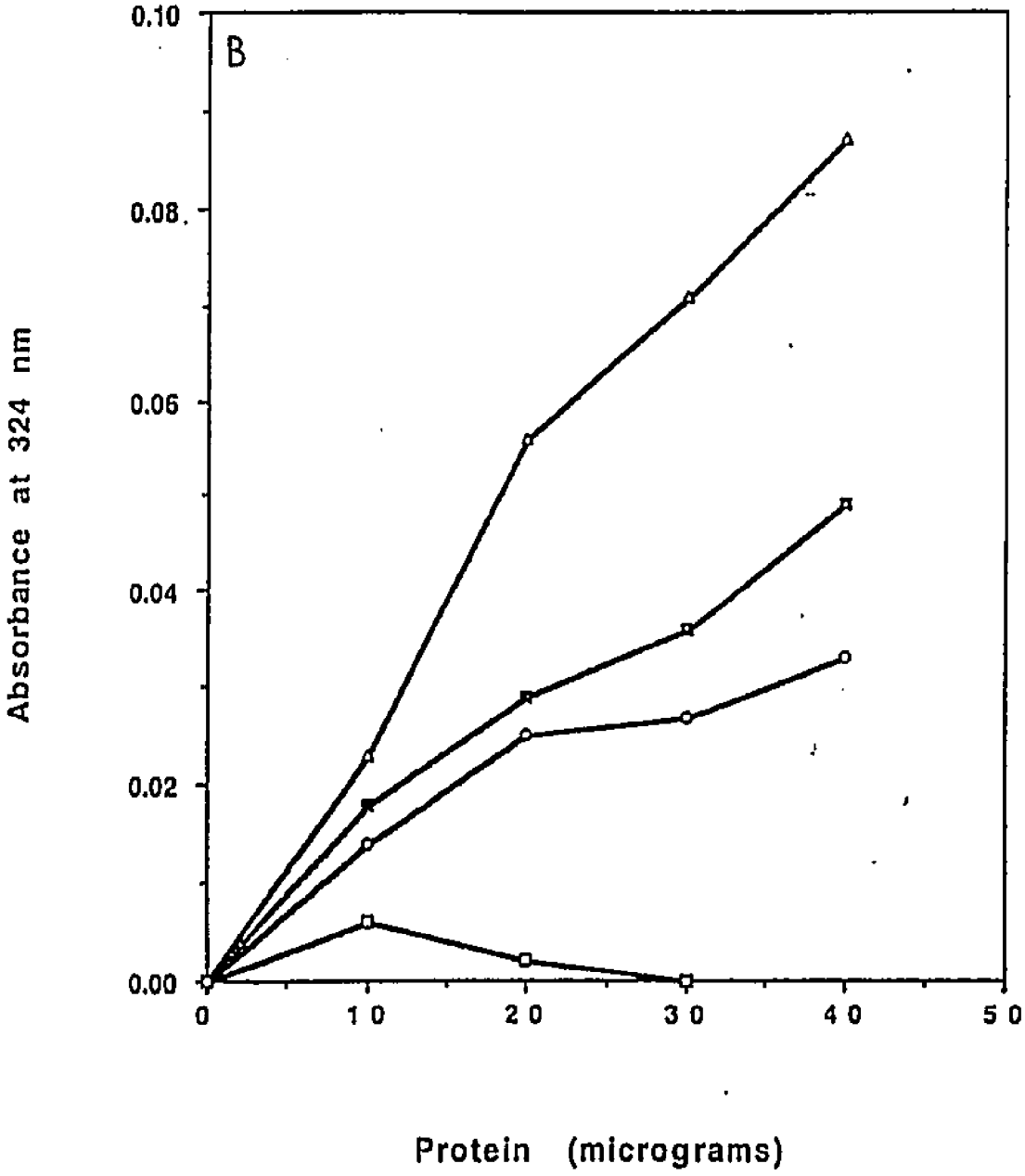
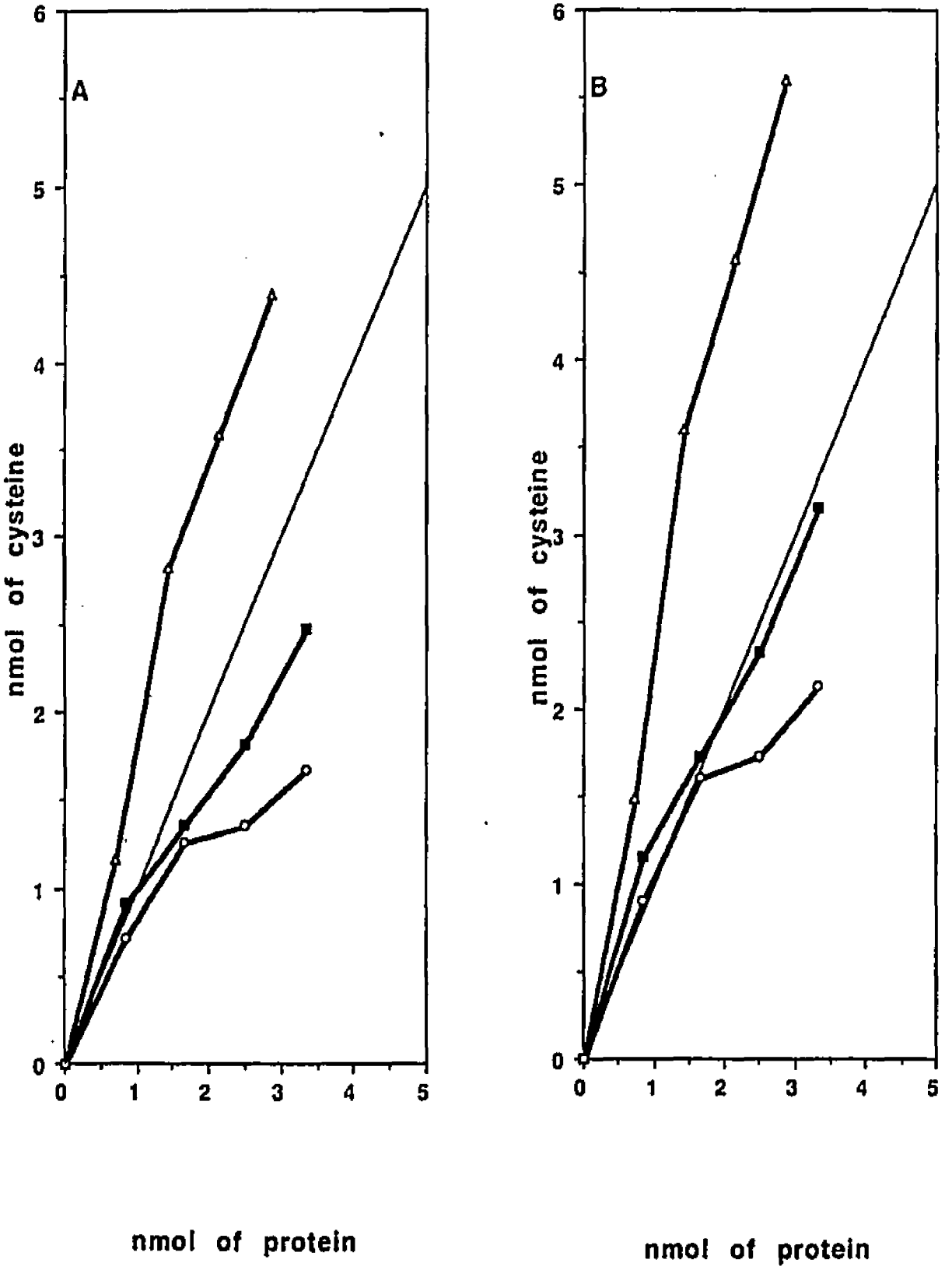

B
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
Absorbance at 324 nm
Protein (micrograms)

Figure 39. DETERMINATION OF THE STOICHIOMETRY OF CYSTEINE IN DIFFERENT PROTEIN SAMPLES. The MS2 coat protein (Δ), ribosomal protein L22 from strain SK1047 (■) and ribosomal protein from strain SK1048 (O) were evaluated to determine the number of cysteine residues present in each protein. (A) The cysteine content per mole of protein was calculated from the slope of the lines using the molar extinction coefficient of 4-PDS and the molecular weights of the different proteins. (B) The slope of the MS2 coat protein titration was adjusted to match that expected for a protein containing two cysteine residues. The correction factor was used to adjust the slope of the lines describing the cysteine content of ribosomal protein L22 from the mutant strains. The reactivity of protein containing one cysteine is added for compaision (-).


A
B
nmol of cysteine
nmol of protein
0
1
2
3
4
5
6

cysteine residues. The data from the mutants was adjusted by this same 20% factor (Figure 39B). With this correction, protein L22 isolated from strains SK1047 and SK1048 were found to contain a single cysteine residue.

Chapter 4

DISCUSSION

Ribosomes are the macromolecular complexes on which protein synthesis occurs. The best characterized ribosome, namely that of E. coli, consists of 3 RNA species and 52 ribosomal proteins (Wittmann, 1979). Structural studies on the E. coli ribosome have been extensive. All of the ribosomal proteins have been purified and characterized by chemical, physical and immunological methods. The primary sequences of 47 of the ribosomal protein genes are published (Hardesty et al., 1986). Similarly, the primary sequences of the three RNA species are known (Noller, 1984). Functional studies have revealed that ribosomal activities occur due to the interaction of many components. This has made it difficult to determine the function of the individual ribosomal constituents. The new techniques of molecular biology are presently being applied to the ribosomal RNA sequences to extend knowledge about their structure and function (Noller et al., 1990). With the emphasis on rRNA structure and function, research on the ribosomal proteins has substantially diminished. It is important to study the function of ribosomal proteins along with rRNA to achieve a comprehensive analysis of ribosome function (Nomura, 1990).

The isolation of mutants has proven to be useful tool

for obtaining information about the structure and function of ribosomal proteins (Dabbs, 1986). Initially, antibiotic resistant mutants were characterized to determine the location of the ribosomal protein genes on the E. coli chromosome (Piepersberg et al., 1980). However, antibiotic resistance mutants exhibited alterations in only a small number of ribosomal proteins. To advance the mapping studies, generalized mutagenesis of the E. coli chromosome was used to provide mutants in every ribosomal protein. The results of these studies were published in a series of papers by Isono and his colleagues from 1976 to 1980. These mutants helped fix the chromosomal location of many of the ribosomal protein genes. Eventually, the study of mutants was shifted to gain information on ribosomal protein function. Antibiotic resistant mutants were not useful in these studies as the basis of resistance at the molecular level was difficult to understand. Mutants created by generalized mutagenesis were also not useful since these mutants did not always show functional changes. Instead, a new class of protein synthesis deficient mutants were created using localized mutagenesis of the 72 minute region of the E. coli chromosome (Champney, 1979). This region was selected because it contained a cluster of ribosomal protein genes. These mutants were temperature-sensitive. With the advances in DNA sequencing, the exact nature of the temperature-sensitive mutation can be easily determined.

temperature-sensitive mutation can be easily determined. Therefore, by sequencing the mutation and by characterizing the protein synthesis deficiency, a structure and function relationship can be determined. If large numbers of mutants in the same ribosomal protein gene can be isolated then gene mapping in terms of function becomes possible.

This dissertation described the characterization of two heat sensitive, protein synthesis deficient mutants. The mutagen used in preparation of these mutants was nitrosoguanidine. Nitrosoguanidine has been shown to be effective in generalized mutagenesis for obtaining mutants with alterations in ribosomal protein genes (Isono and Isono, 1978). Nitrosoguanidine induces multiple mutations primarily at the replication forks (Guerola et al., 1971). It has been shown that mutations resulting from nitrosoguanidine mutagenesis do not always map to a discrete region of the bacterial chromosome as expected from its mechanism of action. Instead, the mutational sites can be spread over several minutes of the chromosome (Isono and Krauss, 1976). Localized mutagenesis was conducted on a trp S mutant mapping at 74 minutes on the E. coli chromosome. Initial selection was for trp S^+ revertants. This was followed by screening for temperature sensitivity. A collection of temperature-sensitive, trp S^+ revertants were further screened for those which were protein synthesis deficient (Champney, 1979). Temperature sensitivity in

bacterial chromosome by P1 transduction.

Strains SK1047 and SK1048 were chosen from among the collection of temperature-sensitive protein synthesis deficient mutants because both exhibited an electrophoretic alteration in ribosomal protein L22 on two dimensional gels (Champney, 1980). The alteration of protein L22 implicated this protein as the site of the temperaturesensitive mutation. The altered migration of protein L22 on the gels must have resulted from a mutation changing the overall charge and/or size of the protein. Protein L22, from both strains, exhibited a reduced mobility during electrophoresis in the first dimension at pH 8.6. This reduced mobility indicated that the mutant forms of protein L22 had a more negative net charge than the wild type protein at this pH. A more negative charge would result if there was the loss of a basic residue or the gain of an acidic amino acid.

Functional characterization of mutant strains SK1047 and SK1048 revealed that the mutants were temperature sensitive for protein synthesis both <u>in vitro</u> and <u>in vivo</u>. Non-ribosomal proteins and factors were tested and shown to be functioning at rates comparable with the control. By contrast, mutant ribosome function was clearly altered. Ribosomal subunits were analyzed, and the 50S subunit was found to be the site of temperature sensitivity. The temperature-sensitive nature of these strains was related to a decrease in 50S subunit activities at restrictive

temperatures in vitro (Champney 1979, 1980) and in vivo (Armstrong-Major and Champney, 1985). This correlated with the alteration in the large subunit protein L22. In different functional assays, ribosomes from SK1047 and SK1048 showed similar but not identical translational defects. Strain SK1048 was generally found to be more temperature-sensitive than strain SK1047 in these tests.

Genetic characterization of both mutant strains revealed that they reverted to growth at 44°C at a frequency consistent with a point mutation conferring the temperature-sensitive phenotype (Miller, 1972). Lambda phage complementation was used to map the temperature-sensitive mutation to a region of 9 genes on the S10 operon. This region of the chromosome contained the rplV gene for ribosomal protein L22. Temperature sensitivity was mapped in relation to known genetic markers in three factor crosses using phage P1 transduction. The suggested map order was aroE (a requirement for aromatic amino acids), rpsE (spectinomycin resistance), ts (temperature sensitivity), and rpsL (streptomycin resistance). Equivalent cotransduction frequencies were found in both mutant strains. Again, the region of the S10 operon containing the rplV gene for protein L22 was implicated.

Definitive mapping of the temperature-sensitive mutation was conducted using plasmid pLF1.0 (Watson and Surzycki, 1983). Plasmid pLF1.0 contains a 0.5 kb EcoRI

fragment of λfus DNA which contains only the wild type rplV gene. This fragment was cloned into the EcoRI site of plasmid pBR325. Plasmid pBR325 is a derivative of pBR322, a common cloning vector (Bolivar et al., 1978). Plasmid pBR325 was made by isolating the chloramphenicol resistance gene as a HaeII fragment from a P1 transducing phage. The fragment was ligated to EcoRI generated linears of pBR322 which had been treated with S1 nuclease. This ligation destroyed the EcoRI site of plasmid pBR322. However, the chloramphenicol resistance gene contains a new EcoRI site. The EcoRI fragment from λfus2 was ligated into the EcoRI site in the chloramphenicol gene inactivating it. Mutant cells transformed with plasmid pLF1.0 were capable of growth at the restrictive temperature. Since, plasmid pLF1.0 contained only the wild type rplV gene and complemented temperature sensitivity, the temperature-sensitive mutation was restricted to the rplV gene.

The exact nature of the temperature-sensitive mutation was determined by sequencing the mutatant rplV genes from SK1047 and SK1048. The genes were amplified from the E. coli chromosome using the polymerase chain reaction (PCR) (Saiki et al., 1988). The PCR product was sequenced using the Sanger method of enzymatic chain termination by dideoxynucleotides (Sanger et al., 1977). The sequence of the control rplV gene was identical to the sequence published by Zurawski and Zurawski (1985). The mutant rplV

genes, from both mutants, contained a transition at position 22 in the nucleotide sequence. A cytosine to thymine substitution was found in both mutant rplV genes. This substitution caused the CGC codon for the amino acid arginine to be replaced by the TGC codon for cysteine. Therefore, the arginine residue at position 8 in the wild type protein was replaced by a cysteine in protein L22 from both mutant strains.

To verify the DNA sequencing data, protein L22 from both mutants was tested for the presence of the amino acid cysteine using 4,4′ dithiodipyridine (Grassetti and Murray, 1966). Wild type ribosomal protein L22 contains no cysteine. Consistent with this, the wild type protein L22 was found not to react with the 4,4′ dithiodipyridine. Protein L22 isolated from strain SK1047 and SK1048 reacted with the 4,4′ dithiodipyridine with the stoichiometry of a protein containing one cysteine. The DNA sequencing data was therefore confirmed at the protein level.

The substitution of a cysteine for an arginine would be expected to produce changes in the charge and size of ribosomal protein L22. Arginine has a more extended side chain than does cysteine. The basic nature of the arginine side chain results in this amino acid being positively charged at physiological pH. Because arginine is charged at physiological pH, it can participate in electrostatic interactions with neighboring charged molecules. The basic

nature of this amino acid also makes it strongly polar. Arginine is probably positioned on the exterior surface of protein L22. In this position, the amino acid can be hydrated by the surrounding aqueous environment. In comparison, cysteine has a smaller sulfur containing side chain. The sulfhydryl group of cysteine ionizes at pH 8.3. When ionized, a second oxidation can occur between cysteine residues resulting in the formation of a disulfide bond. Since there are no other cysteine residues in protein L22, the possibility of intrachain disulfide linkages must be ruled out. Protein L17 has been shown to bind to protein L22 in assembly studies. This protein contains one cysteine residue which could participate with protein L22 in an interchain disulfide linkage. Assembly studies have also shown that proteins L2, L10 and L11 are positioned in the neighborhood of protein L22 (Herold and Nierhaus, 1987). These proteins contain cysteine residues which may participate in the formation of disulfide bonds with the mutant protein L22.

At physiological pH, cysteine is uncharged. Therefore, cysteine would not participate in electrostatic interactions as arginine would. The cysteine residue would probably fold such that this residue is shielded from water. Cysteine would seek to be positioned on the interior of the protein while arginine would remain on the exterior. Figure 40 shows the Edmundson wheel predicted for wild type ribosomal

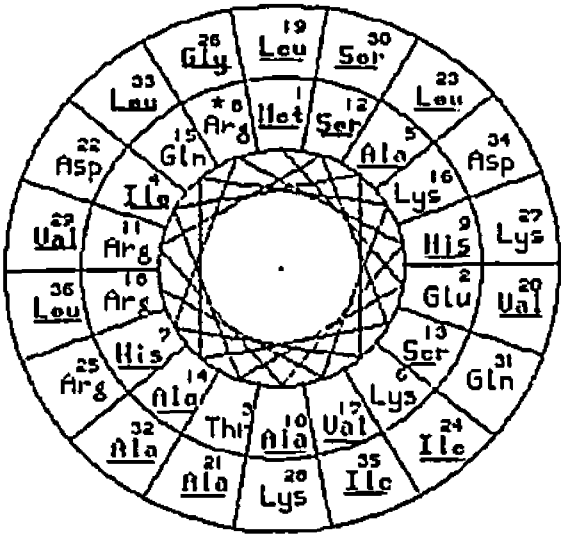
protein L22 (section A) and for the mutant ribosomal protein L22 (section B). This wheel was generated using HIBO PROSIS protein analysis software system. It uses the method reported by Schiffer and Edmundson (1967). The arginine to cysteine substitution caused a change in the Edmundson wheel predicted for protein L22. The wheel predicts that arginine will exist on the external surface of the protein while cysteine will exist on the internal part of the protein. The wheel also predicts the arginine to cysteine substitution causes an alteration in the mean hydrophobicity of the protein. Also, the hydrophobic moment for the control protein is twice that of the mutant protein. The substitution of the cysteine for arginine produces a larger cluster of hydrophobic residues. The program predicts that the wild type protein will fold such that eight hydrophobic residues are clustered together. In the mutant protein the patch has been enlarged with the incorporation of the cysteine residue. Since the folding of a protein is driven by water, the substitution of an arginine to cysteine most likely causes a change in the folding of protein L22. Secondary structure predictions generated using this program indicated that the substitution would not effect the alpha helix region of secondary structure at the beginning of protein L22. These predictions were based on the methods of Chou and Fasman (1978).

Figure 40. EDMUNDSON WHEEL FOR WILD TYPE AND MUTANT PROTEIN L22. HIBIO PROSIS protein analysis software was used to generate Edmundson wheel predictions for:

A. Wild type protein L22

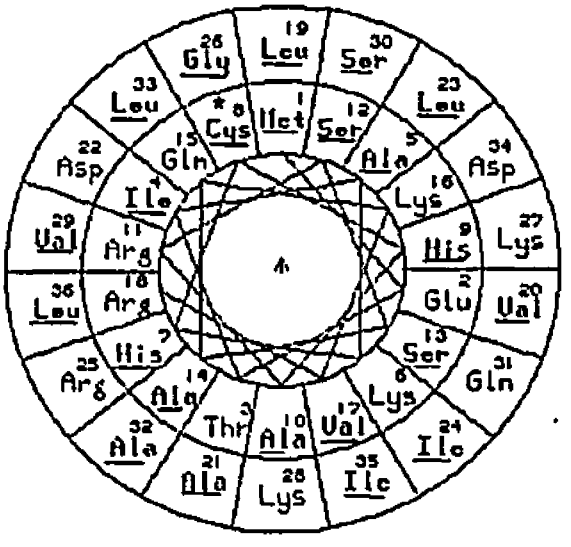
B. Mutant protein L22

EDMUNDSON WHEEL PREDICTED FOR WILD TYPE RIBOSOMAL PROTEIN L22



Hydrophobic residues	
Hydrophilic residues	
Mean hydrophobicity:	-0.10
Hydrophobic moment :	0.07

EDMUNDSON WHEEL PREDICTED FOR MUTANT RIBOSOMAL PROTEIN L22



Hydrophobic residues	
Hydrophilic residues	
Mean hydrophobicity:	-0.09
Hydrophobic moment :	0.14

The local environment surrounding an amino acid will influence the impact of the amino acid on the protein structure. On both sides of the mutated amino acid are histidine residues. Histidine is a basic amino acid with a large imidazole ring. With this amino acid positioned on either side of the arginine, the protein would contain a stretch of polar amino acids. This region of concentrated charge would have an increased potential for electrostatic interactions. The concentrated charge would also increase the probability that the protein would fold such that these amino acids are located on the exterior of the protein where they can interact with water. Repulsion between the members of this stretch of positively charged amino acids would be expected. The substitution of the cysteine for an arginine will interrupt this stretch of basic amino acids. Because of the size of the histidine residues, the cysteine may be shielded reducing its ability to react. Cysteine possesses a smaller side chain and may exist in a pocket between the two histidine residues. This pocket may support an environment different from the milieu. If the pH of the pocket becomes 8.3, the cysteine could become ionized,then oxidized. The presence of the cysteine instead of arginine would reduce the repulsion between the two histidines. This would be especially true if the cysteine were oxidized. The oxidized form of cysteine may share protons with the

histidine. If this occurred, it could decrease the ability of the histidine to participate in electrostatic interactions. If the cysteine is not oxidized it is still uncharged and therefore would serve to reduce the electrostatic interactions.

Strains SK1047 and SK1048 were chosen for further analysis because both were shown to contain a mutant ribosomal protein L22. The mutant protein L22 exhibited a changed migration in two dimensional electrophoretic gels. Analysis of the position of ribosomal protein L22 on electrophoretograms from SK1047 and SK1048 showed that the mutant protein from both strains exhibited a reduced mobility in the first dimension of the Howard and Traut (1973) gel system (Champney, 1980). This change indicated that the mutant protein had a more net negative charge than the wild type protein. The first dimension of this system involves separation of ribosomal proteins in 4% polyacrylamide at pH 8.6. At pH 8.6, the wild type protein L22 would contain an arginine residue (pKa 12.6) which carried a positive charge. In the mutant form of protein L22, the arginine is replaced by a cysteine. The cysteine side chain has a pKa of 8.3 and would therefore be deprotonated at pH 8.6. Therefore, the cysteine residue would carry a negative charge. The substitution of cysteine for arginine would result in a charge change from an amino acid with a +1 charge to one with a -1 charge. The mutant

protein L22 would be more negative than the wild type protein L22, as predicted. In this system, the wild type protein with the positively charged arginine would migrate further toward the cathode than the mutant protein L22. Therefore, the increased negative charge on mutant protein L22 would cause a reduced mobility of the protein in the first dimension of the two dimensional system. In the second dimension of this system, proteins are separated in 18% polyacrylamide at pH 4.5. The wild type protein L22 contains an arginine which would be positively charged at pH 4.5 while the mutant protein L22 contains a cysteine which is neutral at this pH. The replacement of the +1 charged arginine with neutral cysteine would also result in an altered migration of the protein in the second dimension. The wild type protein would be expected to migrate slightly further towards the cathode in the second dimension than the mutant protein. The change in protein L22 migration in the second dimension would not be as severe as that observed in the first dimension because the change in charge is not as great and the density of the polyacrylamide is 4.5 times greater. The altered migration of protein L22 in the second dimension was difficult to detect on gels with proteins from SK1047 and SK1048, but it can be seen in gels from complemented forms of the mutant strains. In the complemented mutant strains, two forms of ribosomal protein L22 are incorporated into ribosomes and both the mutant and

wild type protein L22 can be identified on electrophoretograms. These gels clearly show that the mutant protein L22 has a reduced mobility in both the first and second dimensions when compared to the wild type protein.

Purification of ribosomal protein L22 was accomplished using reversed phase high performance liquid chromatography according to procedures adopted from Ferris et al. (1984) and Cooperman et al. (1988). Reversed phase HPLC separates proteins on the basis of hydrophobicity and chain length. Wild type protein L22 was found to elute earlier than the mutant forms of protein L22. The delayed elution of the mutant forms of protein L22 indicated that the mutant protein was retained longer on the column and was therefore more hydrophobic than the wild type protein. Separation on the reversed phase column was at pH 2.0. At this pH, the amino acid cysteine would be neutral while the amino acid arginine would be positively charged. Cysteine, since it is neutral, would be more hydrophobic than arginine which is charged. Guo et al. (1986) calculated retention coefficients for polyamino acid peptides. These retention coefficients were predictions based on the separation of peptides of known composition. At pH 2.0, the addition of a cysteine residue to a standardized peptide was predicted to cause the retention time to be increased by 2.6 minutes. In a similar manner, the addition of an arginine caused a

decrease in retention by 0.6 minutes. Since reversed phase chromatography separates proteins based on hydrophobicity and chain length, the size of the protein must also be considered. A method of predicting the elution time of ribosomal proteins from a C-4 reversed phase column has been determined by Champney (1990) based on the work of Guo et al. (1986). Using this method, the retention times of all of the E. coli ribosomal proteins were predicted and shown to be in agreement with the experimental retention times. The retention time for the control and mutant form of protein L22 was calculated using this method. The predicted elution time for wild type ribosomal protein L22 was 102.9 minutes. The altered protein, with a cysteine residue instead of an arginine, had a predicted elution time of 103.7 minutes. From these values, the mutant form of protein L22 was predicted to elute 0.8 minutes after the wild type protein. The observed elution of protein L22 was standardized against the elution of protein L15 to allow comparison of control and mutant chromatograms. Protein L22 from strain SK1047 was shown to elute 1.0 minutes after the wild type protein while protein L22 from strain SK1048 eluted 1.4 minutes after the wild type protein L22. The observed and predicted retention times for mutant protein L22 are consistent with the arginine to cysteine substitution. The 0.4 minute difference between the elution of protein L22 from SK1047 versus SK1048 may be due to the

difference in precisely determining the exact location of the proteins using the HPLC.

Assembly studies have shown that protein L22 interacts weakly with 23S RNA. However, interactions between protein L22 and 23S RNA are greatly enhanced in the presence of protein L4 (Herold and Nierhaus, 1987). Binding assays were conducted to determine the ability of protein L22 isolated from the mutant strains to bind to 23S RNA with and without protein L4. As described by Herold and Nierhaus, protein L22 from the control and mutant strain was found to bind to 23S RNA poorly and the binding was greatly enhanced by the presence of ribosomal protein L4. Binding of ribosomal proteins to RNA relies in part on the electrostatic interaction between positively charged residues on the protein and the negatively charged phosphate group of the RNA. The wild type protein L22 contains an arginine at position 8 in the protein. This positively charged amino acid with its surrounding histidine residues would be capable of strong electrostatic interaction with the negatively charged phosphate groups of the RNA. By replacing the arginine with a cysteine, the positively charged amino acid is replaced by a neutral amino acid. The neutral cysteine would not be expected to interact as well with the negatively charged phosphates. The local environment around the cysteine may serve to reduce the effect of this substitution. The cysteine may be shielded

by the histidine residues, allowing wild type binding. However, if cysteine becomes oxidized, it is capable of sharing protons with the histidine residues. Hence, the positive charge in this region may be wiped out and no interaction with the 23S RNA would be possible. This could explain a decrease in the binding of protein L22.

Binding assays were conducted using protein isolated from two dimensional electrophoretic gels. The isolation procedure involved an acetic acid extraction, column purification and lyophilization. Purification was followed by labeling using reductive methylation. This harsh treatment of the proteins would be expected to render some portion of the sample non-functional. Only 50% of the wild type protein bound to 23S RNA at the permissive temperature and the amount was further reduced at the restrictive temperature. Mutant protein L22 from SK1047 was unable to bind to 23S RNA irrespective of temperature. The most likely explanation for this is that the reisolation techniques caused the majority of this protein to be non-functional in binding assays. The binding of protein L22 from SK1048 varied between the permissive and restrictive temperature. The reduction in binding suggests that there was a change in the protein with a change in temperature. A conformational change within protein L22 in response to an increase in temperature was proposed after a series of tRNA binding experiments (Champney, 1980). Ribosomes have the

ability to protect tRNA from digestion by RNase. However, ribosomes from SK1048 at high temperature were unable to protect tRNA from digestion, suggesting an alteration in the structure or confirmation of these ribosomes at high temperature. Conformational changes in ribosomes from SK1048 were also suggested by the observation that the ribosomal subunits exhibited a severe reduction in their ability to reassociate at high temperature, while re-association was comparable with the control at the permissive temperature (Champney, 1980). Other factors which may have effected binding include the interaction of protein L4 with protein L22.

Strains SK1047 and SK1048 were not identical in earlier physiological and biochemical characterization of these mutants. Differences in the function of ribosomes from SK1047 and SK1048 could be best explained by an additional mutation not related to the temperature sensitivity of L22. Nitrosoguanidine mutagenesis is known to produce linked mutational events. We have already identified mutations in the 74 minute region of the chromosome causing the trp S gene to revert and in the rplV gene for ribosomal protein L22 causing temperature sensitivity. It is possible that nitrosoguanidine created a third mutation in this region of the chromosome. Additional changes in this region would most likely affect a ribosomal protein gene. However, without a selectable marker additional mutations would be

difficult to detect. If another mutation exists it does not cause a detectable change in a ribosomal protein, as seen on two dimensional electrophoretic gels or on reversed phase HPLC. Therefore, the mutation would not cause a significant change in the charge, size or hydrophobicity of any of the ribosomal proteins as detected by the above methods. It is, however, possible to have an alteration in a ribosomal protein which would not be detected by these separation methods. If an additional mutation was responsible for the difference in phenotype between SK1047 an SK1048, it could be within SK1047, within SK1048, or within both strains. The mutation could be a suppressor mutation, which decreases temperature sensitivity in mutant SK1047, or the mutation could cause a secondary change which aggravated temperature sensitivity in SK1048. In either case, the mutation would most likely occur in a protein which interacts with protein L22. The conformational change observed within ribosomes from SK1048 make this strain more suspect than SK1047.

Another example of an arginine to cysteine mutational change in an E. coli ribosomal protein is known. Bollen et al., (1973) and Kahan et al., (1973) described the characterization of strain 258ts. Strain 258ts was produced by nitrosoguanidine mutagenesis of E. coli strain CR341. The mutant strain was shown to be temperature-sensitive and protein synthesis deficient. Initial characterization of strain 258ts showed that the ribosome was the cause of

thermosensitivity. Ribosomal proteins from 258ts were analyzed using two dimensional gel electrophoresis, according to Kaltschmidt and Wittmann, (1970b). Electrophoretograms from this strain revealed that 30S subunit protein S18 was altered in this mutant strain. The mutant protein S18 exhibited a reduced mobility in both the first and second dimensions. A comparison of the migration of the altered and wild type protein was further demonstrated in gels from meroploids. Characterization of strain 258ts indicated that there were two additional temperature-sensitive mutations which were independent of the change in the S18 protein. It is interesting to note that not all the mutations caused by nitrosoguanidine were linked to temperature sensitivity. This supports the idea that additional mutations not related to temperature sensitivity may exist in strains SK1047 and SK1048. The study of the amino acid composition of the wild type and mutant protein revealed that the mutational alteration involved the substitution of cysteine for arginine in mutant protein S18. The presence of cysteine in the mutant protein was confirmed by modifying the protein using ethyleneimine which reacts with the cysteine causing a change in the mobility of the protein on electrophoretic gels.

The study of strain 258ts provides an interesting comparison to the study of strains SK1047 and SK1048. All three mutants were made by nitrosoguanidine mutagenesis. In

all three cases, the nitrosoguanidine caused multiple mutational events which were scattered over several minutes of the bacterial chromosome. Strains SK1047, SK1048 and 258ts were shown to be temperature-sensitive, protein-synthesis deficient. In all three cases, the alteration in a ribosomal protein was detected using two dimensional gel electrophoresis. The result of the nitrosoguanidine mutagenesis was the substitution of a cysteine residue for an arginine residue in protein S18 from strain 258ts and in protein L22 from SK1047 and SK1048 proving that nitrosoguanidine can cause this type of alteration. The two dimensional gel system of Kaltschmidt and Wittmann (1970b), used for the characterization of protein S18, is comparable to that of Howard and Traut (1973), used for the characterization of protein L22, with a slight difference in the first dimension running buffer. The migration of mutant protein S18 from strain 258ts supported the two dimensional gel data on protein L22 from SK1047 and SK1048. Mutant protein S18 and mutant protein L22 showed a reduced mobility in both the first and second dimensions as the result of the substitution of a cysteine for arginine. Since strain 258ts was not sequenced, the exact nature of the mutation is unknown. According to codon usage tables there are four codons which specify the amino acid arginine; CGU, CGC, CGA, and CGG. There are two codons which specify cysteine; UGU and UGC. The first nucleotide in the arginine codon,

cytosine, must be changed to thymine in order to have an arginine to cysteine substitution. The second nucleotide in the codon, guanine, can not be changed if a cysteine to arginine substitution is to result. Due to the "wobble" seen in the third codon position this position may or may not be altered. If the third position is an adenine or guanine, then a change to thymine or cytosine would be required. The mechanism of action of nitrosoguanidine predicts that it causes single base pair alterations in a region. Therefore, the only point mutation which would result in an arginine to cysteine substitution is the replacement of the cytosine with thymine in the first position of the codon as seen in strains SK1047 and SK1048. Characterization of strain 258ts gives further support to the data reported on strains SK1047 and SK1048.

Ribosomal protein L22 has been implicated in erythromycin resistance along with protein L4. Mutant N281 was a spontaneous erythromycin resistant mutant characterized by Wittmann et al (1973). Erythromycin resistance was shown to be associated with protein L22 in transduction experiments which mapped erythromycin resistance to the 72 minute region containing the rplV gene for protein L22. Ribosomes from mutant N281 have been shown to bind erythromycin to an extent comparable to the control. It has been speculated that erythromycin resistance occurs because the alteration in ribosomal protein L22 interferes

with the effect of the antibiotic on the ribosomal active site, therefore causing an ineffective action of the drug. If this is the mode of action, then an alteration in ribosomal protein L22 would be expected. Ribosomal proteins from mutant N281 were examined on two dimensional gels using the method of Kaltschmidt and Wittmann (1970b). The electrophoretograms revealed a clear change in the 50S protein L22. The altered protein L22 exhibited a reduced mobility in the first dimension. Therefore, the mutant protein L22 had a more net negative charge than wild type protein L22. Mutant strains SK1047 and SK1048 have also shown an altered ribosomal protein L22 with a reduced mobility. The alteration in protein L22 from N281 resulted in this protein having an even greater net negative charge than protein L22 from SK1047 and SK1048. Therefore, the change in protein L22 from N281 was not identical to that seen in SK1047 and SK1048. Phenotypically these mutants also differ. Mutant strains SK1047 and SK1048 have been shown to be erythromycin sensitive while mutant N281 was found to be erythromycin resistant. Therefore, the mutation in N281 must be in a different region of the rplV gene than that characterized for SK1047 and SK1048. Sequencing of erythromycin resistant mutants like N281 may provide the basis for erythromycin resistance of protein L22.

Protein L22 was implicated in the erythromycin binding site in bacterial ribosomes in work done by Arevalo et al.

(1988). This study used isotopically labeled erythromycin derivatives which bound covalently to bacterial ribosomes. Protein L22 was shown to be the primary protein labeled by several erythromycin derivatives. Protein L22 has also been implicated in the binding of virginiamycin S (Giambattista et al., 1990). This antibiotic inhibits peptide bond synthesis through a molecular mechanism which is similar to that of erythromycin. The location of the virginiamycin S binding site on bacterial ribosomes has been established by affinity labeling studies. Ribosomal proteins L18 and L22 were found to be associated with the binding site in this investigation. In mapping the virginiamycin binding site, protein L22 was also mapped to the peptidyl transferase domain of the ribosome. This suggests that protein L22 may play an active role in the formation of peptide bonds. An alteration in protein L22 may effect the ability of the ribosome to form peptide bonds hence effecting cell growth. Growth of the temperature-sensitive mutant strains is reduced at the permissive temperature and growth ceases after two hours at the restrictive temperature. The inhibition of growth has been shown to be due to a decrease in the function of the ribosome at the permissive but especially at the restrictive temperature (Champney, 1980). The decrease in ribosome function may be linked to ineffective peptidyl transferase activity because of the alteration in protein L22. At the permissive temperature in

the mutant strains, the alteration in protein L22 would reduce but not abolish peptidyl transferase activity. At the restrictive temperature, the ability of protein L22 to participate in the peptidyl transferase complex may be further reduced or terminated, causing the cells to stop growing.

Temperature shift studies have shown that strain SK1047 does not stop growing as rapidly as strain SK1048 at the restrictive temperature (Champney, 1979). This indicates that peptide bond formation in SK1047 may be reduced to a very low level and therefore, the growth rate declines slowly instead of ceasing. For strain SK1048, the growth inhibition is more severe indicating that peptidyl transferase activity may cease altogether at the restrictive temperature causing a sharper cessation of growth. In SK1048, there is evidence that at the restrictive temperature a change in confirmation occurs within protein L22 causing a change within the ribosome. All of the protein L22 synthesized at the high temperature would have this configurational change. Incorporation of altered L22 into ribosomes would result in dysfunctional ribosomes. The ribosomes' inability to function may result from the cessation of the peptidyl transferase activity. The mutants are able to grow immediately after the shift to the restrictive temperature because the ribosomes which were synthesized during growth at the permissive temperature

continue to function before being reduced in activity and number by cell division. Growth ceases when the ribosomes produced during growth at the permissive temperature are inactivated.

To further study the mutants ability to grow after exposure to the restrictive temperature recovery assays were conducted. In recovery analysis, mutant strains were grown at the permissive temperature until mid log phase, shifted to the restrictive temperature until cell growth ceased and then returned to the permissive temperature. Mutant strain SK1047 was able to begin growth within 30 minutes after the return to the permissive temperature, while strain SK1048 took 60 minutes to recover. SK1047 was able to grow more quickly on return to the permissive temperature than SK1048. This indicates a greater ribosomal inactivation in SK1048 cultures when exposed to high temperature. This recovery data was supported by earlier studies which showed that ribosomes from SK1047 lost 50% of their activity in 200 minutes while ribosomes from SK1048 were 50% inactivated in only 90 minutes at the restrictive temperature (Champney, 1979).

In SK1048, a change in ribosomal protein L22 at the restrictive temperature presumably renders the protein non-functional. This would mean that all of the ribosomes containing protein L22 generated at high temperature would be dysfunctional. At the restrictive temperature, cell

death would occur when the ribosomes produced during the initial period of cell growth are completely inactivated. When returned to the permissive temperature, growth could occur only in living cells. These cells are living because they contain functional ribosomes produced during the initial growth period. Therefore, exposure to the restrictive temperature must be for a short period, such that a few functional ribosomes exist. Functional ribosomes are required to produce new protein L22 upon return to the permissive temperature. Ribosomal protein L22 produced at the permissive temperature is functional and allows the assembly of functional ribosomes causing an exponential increase in cell growth.

In SK1047, the activity of the peptidyl transferase may be reduced to a negligible amount at the restrictive temperature, therefore ribosome function would be severely reduced. However, this reduced activity will keep the cells alive longer at the restrictive temperature. Cell death would not occur as quickly in SK1047 as in SK1048. Cell death in strain SK1047 would require an extended exposure to the restrictive temperature. Therefore, when cells from SK1047 are returned to the permissive temperature an increased number of cells are viable. This may account for the shorter recovery period.

There are special problems associated with the overexpression of ribosomal proteins in E. coli. Ribosomal

constituents are produced in the stoichiometric amounts required for assembly. To accomplish this, the ribosomal protein genes are organized into operons which exhibit feedback inhibition at the translational level (Nomura et al., 1980). This method of regulation prevents free pools of ribosomal proteins from accumulating. It is believed that because of the tight regulation of ribosomal constituents attempts to clone ribosomal protein genes into many expression vectors are plagued with problems (Ranakrishnan and Gerchman, 1991). These problems were seen with attempts to subclone the rplV gene for protein L22.

From sequence analysis, the rplV gene for ribosomal protein L22 was found to be located in a 0.5 kb EcoRI fragment (Zurawski and Zurawski, 1985). This 0.5 kb fragment was isolated from λfus2 DNA and cloned into pBR322. An attempt was made to ligate the 0.5 kb fragment into the EcoRI site of plasmids pUC19U and pTZ19U, both common expression vectors. The insert was found to be ligated into the EcoRI site in both plasmids. The ligation mix was transformed into competent cells from strain JM109 and ampicillin resistant colonies were selected. The ampicillin resistant colonies were screened on MacConkey agar, and a small percentage of lactose negative colonies were detected. Hence, cells containing plasmid with insert were identified. When cells containing plasmid with insert were grown in liquid culture the insert was lost from the plasmid at a

high frequency. Streaking these colonies on other ampicillin plates also resulted in the loss of the insert from the plasmid. The insert did not remain stable in plasmid pUC19U nor in plasmid pTZ19U. Repeated attempts to subclone this insert from λfus2, from λspc2 or from the PCR product were not successful. Most likely the loss of the insert occurred during the replication of cells containing the plasmid. This led to the conclusion that there was a problem with expression of the cloned insert. Expression of the rplV gene in the plasmid, in addition to chromosomal expression, would result in an overproduction of protein L22. Overexpression of ribosomal proteins has been shown to be lethal to cells (Nomura et al., 1980). As a mechanism to prevent overexpression, the host strain may have eliminated the insert from the plasmid to prevent the toxic build up of protein L22.

The 0.5 kb fragment was isolated from EcoRI digests of λfus 3 DNA and inserted in plasmid pBR325 (Watson and Surzycki, 1983). The construct named pLF1.0 has been stable in growth on plates and in liquid media. Recently, Ramakrishnan and Gerchman (1991) reported that their attempts at subcloning ribosomal protein genes from B. stearothermophilus were unsuccessful until they used tightly regulated expression vectors. Therefore, the problems encountered in subcloning this 0.5 kb insert seemed to lie in the choice of plasmid. The insert is cloned into the

chloramphenicol resistance gene of pBR325. The amount of expression from the chloramphenicol gene in this construct has not been reported. However, stringent expression of the insert is most likely.

The effect of increased ribosomal protein gene dosages in the mutant strains became of interest during attempts at subcloning. Complementation with λfus2 resulted in mutant cells which contain two copies of the S10 ribosomal protein operon. Each operon should be regulated by feedback inhibition from one of its components. Expression from the two copies of the S10 operon in the mutant stains could result in overproduction of all the genes on this operon. One of the gene products, protein L4 has the ability to bind to the polycistronic mRNA leader sequence and prevent its translation. Protein L4 is capable of binding to the mRNA leader sequences of both the chromosomal and the λfus2 derived RNA messages so that toxicity from over production does not occur. The cell tolerates complementation with λfus2 because it has a method of preventing protein overexpression. Conversely, complementation with plasmid pLF1.0 resulted in mutant cells containing multiple copies of only the rplV gene. Complementation with this plasmid results in the overproduction of a single ribosomal protein. The cell is unable to regulate production of this protein since regulation is on the leader sequence of the polycistronic

mRNA. To stop protein L22 overproduction from the plasmid, the cell may lose the insert from the plasmid.

Overexpression of ribosomal genes has been shown to be toxic to the cell. A simple method of measuring the toxicity of this expression on the cell was needed. Cell growth has been used as an indicator in determining toxicity in complemented cells (Ramarkrishnan and Gerchman, 1991). Growth curves from mutant strains complemented with λfus2 at the permissive and at the restrictive temperatures indicated that these cells grew at 90% of the control rate and achieved a cell density comparable to the control cells. However, the growth rate and final cell density of phage complemented cells was increased significantly over the non-complemented temperature-sensitive cells which only grew at 75% of the control rate. Complementation with λfus2 increased the growth rate of the mutant cells to make them equivalent to wild type cells. Regulation of gene expression should be normal in these cells, since two intact operons were present. Each operon copy should be regulated with equal efficiency. Expression of protein L22 would be such that half of the protein L22 produced would be mutant and the other half would be wild type. When protein L22 was incorporated into ribosomes, half of each form will be found. With only 50% mutant ribosomes in the complemented cells, the growth rate of the complemented mutant cells should be improved by 50% over the non-complemented parent

strains. The mutant strains exhibited a 25% reduction in growth rate at the permissive temperature. The complemented mutant strains exhibited only a 10% reduction in growth rate; therefore, the growth rate was improved by 15% or roughly one-half of the reduction seen in the non-complemented parents.

Complementation with plasmid pLF1.0 caused a 60% reduction in the growth rate of mutant strains at the restrictive temperature compared with wild type cells. This indicated that protein L22 production from the plasmid encoded rplV gene increased with an increase in temperature. The increased production of wild type protein L22 at the higher temperature would be required for cell survival. The suboptimal growth rate presumably occurs because of the added burden of replicating the plasmid and eliminating the extra protein L22 produced. At the permissive temperature, the growth rate was reduced by 10% compared to the control. This indicated that there was less expression from the plasmid at the permissive temperature than at the restrictive temperature.

Complemented cells were selected for growth at the restrictive temperature. The mutant strains are normally unable to grow at the restrictive temperature due to failure of their ribosomes to function because of the altered protein L22. Complemented cells grew at the restrictive temperature indicating that there were functional ribosomes

formed from the incorporation of the wild type protein L22 produced from the phage or plasmid genes. To determine if there was expression from these genes at the restrictive temperature, ribosomal proteins from cells complemented by λfus2 and plasmid pLF1.0 were analyzed. The chromosomal copy of the S10 operon contains an altered rplV producing mutant ribosomal protein L22. The phage copy of the S10 operon carries the wild type genes from which normal protein L22 can be produced. On two dimensional electrophoretic gels, expression from the two sources could be distinguished. Reversed phase HPLC was also used to confirm that there was incorporation of control and mutant protein L22 into ribosomes from λfus2 complemented cells at the permissive temperature. A similar situation exists in complementation with plasmid pLF1.0. Expression from the phage and plasmid genes was determined by looking for the incorporation of both forms of protein L22 into ribosomes isolated from complemented stains. Two dimensional electrophoretograms showed both forms of protein L22. This confirmed the production of wild type protein L22 in the λfus2 complemented strains at the restrictive temperature. Expression from the plasmid rplV gene was also demonstrated by this method.

To gain further insight into the amount of each form of protein L22 assembled into ribosomes at the permissive and restrictive temperature, the amounts of each type of protein

incorporated into ribosomes were measured. Separation of the proteins from the complemented strains allowed recovery of the wild type and the mutant forms of protein L22 from the same gel. Standardization of protein L22 recovery was achieved by comparing the molar amount of protein L22 to the amount of proteins L3 and L29 eluted from the same gel. Ribosomal proteins L3 and L29 were chosen because their genes lie on either side of the gene for protein L22 in the S10 operon and they are distinct spots on two dimensional gels. In the wild type and the temperature-sensitive mutant cells the amount of protein L22 production was equivalent with that of proteins L3 and L29. Therefore, the incorporation of protein L22 into the ribosome was equal to the incorporation of the two other ribosomal proteins produced from the S10 operon.

Incorporation of both the control and mutant forms of protein L22 in the λfus2 complemented strains was examined. Equal amounts of the ribosomal protein from the mutant strains complemented with λfus2 were isolated from cells grown at the permissive and restrictive temperature. In the λfus2 complemented strains at the permissive temperature, the amount of mutant protein L22 incorporated into ribosomes was found to be equal to the amount of wild type protein L22 incorporated. The same was true at the restrictive temperature. This lent further credibility to the idea that both S10 operons were transcribed at equal frequencies.

Incorporation of both the control and mutant forms of protein L22 in the plasmid pLF1.0 complemented strains was also examined. At the permissive temperature, incorporation of the mutant protein L22 was found to be in slight excess of the wild type protein L22. This indicates that there was slightly less expression from the plasmid than from the chromosomal copy at the low temperature. At the restrictive temperature, incorporation of the wild type protein L22 exceeded the mutant protein L22 by two fold. This provided evidence that there was an increase in expression and incorporation from the plasmid. The increased expression was predicted to occur at the restrictive temperature because it was required for survival. At the permissive temperature, where expression was not required for survival, there did not seem to be as much wild type protein L22 incorporated into the ribosome indicating that there was less of this protein available for incorporation. At the restrictive temperature, more of the wild type protein L22 was incorporated indicating an increased production of this protein with an increase in temperature.

These assays were based on the incorporation of protein L22 into the ribosomes and rely on the fact that there is no selective pressure to incorporate the wild type protein L22 over the mutant protein L22. To determine actual expression from the phage and plasmid rplV gene, studies of the amount of mRNA produced would needed. Since the mRNA for the wild

type protein differs from the mutant protein by only one nucleotide, this would make detection of the two forms of mRNA difficult. A probe, specific for a small region of the mRNA overlapping the site of the mutation, would allow detection of the wild type mRNA from the mutant RNA. This would provide a definitive measure of expression from the two forms of the gene.

The substitution of cysteine for arginine at position 8 in protein L22 altered the ability of protein L22 to bind to 23S RNA. Site directed mutagenesis could be used to create a series of mutants in this region. The arginine could be replaced by a lysine, increasing the basic nature of the region and perhaps causing an increased binding. The arginine could also be replaced by an acidic amino acid like glutamate, making the region more acidic. It would also be possible to use this method to determine the importance of the histidine residues which surround this position.

To continue mapping the rplV gene for protein L22 in terms of function, sequencing of the erythromycin resistant mutant strain like N281 (Wittmann et al., 1973) would allow detection of where the change to erythromycin resistance lies in the rplV gene. Since the primers designed for the amplification of the rplV gene are available, sequencing of these mutants should not be difficult. If erythromycin resistance results from a mutation in a different region of the rplV gene, then mapping the gene in terms of function

becomes possible. Selection and sequencing of virginiamycin resistance mutants would indicate whether the erythromycin binding site and the virginiamycin binding site are in the same region of the rplV gene.

Proteins L4 and L22 have been shown to interact with domain II of the 23S rRNA (Chen-Schmeisser and Garrett, 1976). Erythromycin resistance has also been associated with this region of RNA and with protein L22 (Douthwaite et al., 1985). Domain II of the 23S rRNA interacts with the central loop of domain V of the 23S rRNA. A second class of erythromycin resistant mutants have been isolated with changes in the central loop of domain V. Erythromycin resistance, therefore, requires the interaction of protein L4, protein L22, Domain II of the 23S RNA, and Domain V of the 23S RNA. All of these interaction could be investigated by crosslinking experiments.

Domain V of the 23S rRNA provides the center of the peptidyl transferase site. Therefore, peptidyl transferase activity and erythromycin resistance are tied to the same ribosomal RNA region. Protein L22 was an affinity labeled member of the peptidyl transferase center (Giambattista et al., 1990). Protein L22 was also implicated in erythromycin resistance. Therefore, it appears that protein L22 interacts with domain II of the 23S RNA at or near the region which interacts with domain V. Further, Walleczek et al., (1989) have shown that protein L22 crosslinks with

protein L18, another member of the peptidyl transferase domain. An investigation into the ability of the mutant protein L22 to interact with L18 as well as 23S RNA could shed light on the numerous interactions in this region. A three dimensional model could be constructed to examine how this protein interacts with the RNA and neighboring proteins like L4 and L18. Finally, crosslinking between protein L22 and 23S RNA could also yield information on the role of protein L22 in the EF-G-dependant GTPase center. This area of protein L22 function has not yet been investigated (Thompson et al., 1988; Egebjerg et al., 1989).

Further studies on mutants SK1047 and SK1048 could involve selection of erythromycin and perhaps virginiamycin resistant forms of these strains. This would be accomplished by streaking the strains on plates with increasing concentrations of erythromycin or virginiamycin. It would also be possible to transduce in the erythromycin resistance from the strains characterized by Wittmann et al (1973). However, by transducing the rplV gene from the erythromycin mutants into the temperature-sensitive mutants there is a good chance that the temperature-sensitive mutation would be lost. Physical characterization of strains SK1047 and SK1048 are extensive. Characterization of derivatives from these strains may yield more information about the function of protein L22. New areas of research on these mutants would build on the 23S rRNA binding studies

and research into the role of protein L22 in peptidyl transferase activity.

This work demonstrated the utility of using well defined mutants in single ribosomal proteins to investigate the structure and function of specific proteins in ribosomal subunits.

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APPENDIX

STRAIN LIST

STRAIN NUMBER AND NAME	GENOTYPE AND CHARACTERISTICS	REFERENCE
I. *Escherichia coli* stocks:		
AB2834	F⁻ aroE353 mal⁻ $T6^R$	Pittard & Wallace,1966
C600	F⁻ thr-1 leu6 thi-1 supE44 lacY1 tonA21	Bachmann,1972
JM109	endA1 relA1 syrA96 thi hsdR17 (rk^-mk^+) relA1 supE44 Δ(Lac pro) [F'traD36proABlacI^q Z ΔM15]	Hanahan,1983
NO1267	*trk*A 401 *kdp*ABC5 spc^R str^R fus^R(λdspc1) (λcI857 S7) lac⁻	Jaskunas et. al.,1975
NO1275	(λdtrk) (λcI857 S7)	Jaskunas et. al.,1975
NO1328	(λdspc2) (λcI857 S7)	Jaskunas et. al.,1975
NO1379	*trk*A 401 *kdp*ABC5 spc^R str^R fus^R recA (λfus2 str^R) (λcI857 S7 b515 b519 xis 6)	Jaskunas et. al.,1977
NO1380	(λfus3) (λcI857 S7 b515 b519 xis 6)	Jaskunas et. al.,1977
SC711	spc^R aroE⁻ [P1(JC5029) x AT2472 spc selection]	Champney lab stock
SC713	str^R spc^R aroE24 [P1(MC4100 x SC711, str selection]	Champney lab stock
SK901	F⁻(aroE⁺ from AB2834), malA⁻ thi⁻	Kushner et. al.,1977
SK1047	F⁻ *ts* pro⁻ (L22 change)	Champney,1979
SK1048	F⁻ *ts* (L22 change)	Champney,1979
W3110	trpS 10330	Doolittle & Yanofsky, 1968

II. Bacteriophage lambda stocks:

P1 vir	clear plaque, generalized transducing phage	J. Scott (gift)
λcI857 S7	temperature-inducible helper phage	Jaskunas et al. 1975
λdtrk	defective phage (trk^+ $aroE^+$)	Jaskunas et al. 1975
λdspc1	defective phage ($aroE^+$ spc^S)	Jaskunas et al. 1975
λdspc2	defective phage ($aroE^+$ spc^S)	Jaskunas et al. 1975
λdfus2	defective phage ($aroE^+$ spc^S str^R fus^S)	Jaskunas et al. 1975
λdfus3	defective phage ($aroE^+$ spc^S str^S fus^S)	Jaskunas et al. 1975

III. Plasmid stocks:

pBR322	amp^R tet^R; (4363 bp)	Sutcliffe, 1978
pBR325	amp^R tet^R chl^R; (6000 bp)	Bolivar et al. 1978
pUC19	amp^R lacI lacp lacZ′; 2686 bp	Vieira & Messing, 1982
pLF1.0	1% Eco RI fragment of λfus3 subcloned into pBR325 (containing the rplV gene)	Watson & Surzycki, 1983
pTZ19U	amp^R lacz′; 2863 bp	Mead, 1986

CURRICULUM VITA

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Personal Data: Birth: July 6,1964
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Education: James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee; Ph.D., Biochemistry, 1991

The College of William and Mary, College of Arts and Science, Williamsburg, Virginia; B.S., Biology 1986

Technical Experience: Experience in protein purification, chromatographic techinques, HPLC, peptide mapping and variety of protein gel electrophoresis. Extensive training in the chemistry and biochemistry of nucleic acids (DNA and RNA), and protein - nucleic acid interactions. Techniques in molecular biology including isolation and preparation of DNA (genomic, plasmid and bacteriophage) and RNA, cloning techniques, polymerase chain reaction and DNA sequencing.

Professional Experience: Graduate Assistant 1987-1991. Department of Biochemistry, James H. Quillen College of Medicine, East Tennessee State University.

Instructor and Supervisor 1988-1991. Prematriculation Education Program, James H. Quillen College of Medicine, East Tennessee State University.

Tutor of Biochemistry and Cell Biology 1989-1990. Quillen College of Medicine, East Tennessee State University.

Teacher of Science 1986-1987. Newburgh Central School District, Newburgh, New York.

Memberships:
Association for Women in Science
Association for the Advancement of Science
Gamma Beta Phi
Omicron Delta Kappa
Sigma Xi

Honors:
First Place Graduate Division 1991, James H. Quillen College of Medicine, 7th Annual Research Forum, East Tennessee State University

Receipient of Biomedical Research Support Grant (BRSG), 1989-1990

Second Place Student Research Competition 1989 Southeastern Section of Society for Experimental Biology and Medicine

Committee Service at ETSU:
Dean's Luncheon Committee, 1989-1991
Research Forum Task Force, 1989-1990
Student Medical Government Association, 1989-1990
President, Graduate Student Association, 1989-1990

Publications:
Manuscript in preparation, B. Burnette-Vick, W.S. Champney & P.R. Musich,"A Temperature-Sensitive Mutant of Escherichia coli with an Alteraton in Ribosomal Protein L22."

B. Burnette-Vick and W. S. Champney. A Temperature Sensitive Mutant Affecting the Structure and Function of Escherichia coli Ribosomal Protein L22. Meeting: Synthesis of Ribosomes, Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

B. Burnette and W. Scott Champney. Characterization of Two Temperature Sensitive Mutants of Escherichia coli Exhibiting an Altered L22 Ribosomal Protein. American Society of Biochemistry and Molecular Biology and American Association of Immunologists, New Orleans, Louisiana, Abstract # 1799 (1990)

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