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Comprehending a Molecular Conundrum: Functional Studies of Ribosomal Protein Mutants from *Salmonella enterica* Serovar Typhimurium

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1. Introduction

Of all the molecules present in the living organism, proteins are the most abundant and are often described as the most fundamental. This is a reasonable assertion, considering the enormous diversity of crucial cellular functions that are performed by proteins, which range from structural support and signal transduction to enzyme catalysis and immune defence. Given the vital nature of their function, efficient and orderly production of proteins is essential for cell fitness and survival.

In every living cell, protein synthesis or translation as it is otherwise known, is performed by complex, dynamic ribonucleoprotein particles known as ribosomes. Conversion of the genetic code into biologically active proteins is an elaborate process that relies on the availability of mRNA template, as well as amino acid substrates that are delivered to the ribosome attached to tRNA molecules. In addition, the ribosome is assisted by a number of protein factors, known as "translation factors", which serve to streamline and accelerate various sub-steps of the process. Having said that, however, all phases of the translation cycle can be performed in the absence of such factors, albeit slow, so the ribosome can be regarded as the core of translation. It manages to manufacture proteins at an incredible speed (up to 20 amino acids per second) and with very few errors (one in every 1,000 – 10,000 codons deciphered) (Kramer & Farabaugh, 2007). In *Escherichia coli (E. coli)*, ribosomes account for up to 50% of the dry mass of the cell and considering their central role in gene expression, it is not surprising that the cell devotes up to 40% of its total energy to ribosome production during rapid growth (Nierhaus, 1991; Nierhaus, 2006). The immensity of the task of the ribosome is adequately reflected in the sophistication of its structure, where the modern ribosome represents one of the largest and most complex cellular machines. Despite substantial progress in terms of genetic, biochemical and structural techniques, designed to delineate the mechanism of action of the ribosome, many aspects of protein synthesis and ribosome function remain to be elucidated.

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1.1 Bacterial ribosomes – Structure and function

Ribosomes are universally conserved and are composed of ribosomal RNA (rRNA) and ribosomal proteins (r-proteins) in all organisms. Ribosomal RNA is the major structural component of all cytoplasmic ribosomes, where it accounts for approximately two thirds of the total mass. The remaining third consists of a large number of mostly small, basic ribosomal proteins (r-proteins) that are scattered over the surface of the structure. The simplest ribosome, that of the bacterial cell, totals approximately 2.5 MDa and is over 200 Å in width (Williamson, 2009), establishing it as the largest enzymatic particle in the cell. The number of ribosomes in the cell can reach up to tens of thousands in order to cope with the increased demand for protein synthesis during periods of rapid cell division (Bremer & Dennis, 1996).

All ribosomes consist of two subunits of unequal size. In prokaryotes the smaller of the two subunits is known as the 30S and the larger subunit is known as the 50S, which are named based on their coefficient of sedimentation. The subunits exist as free, separated molecules in the cell cytoplasm when inactive but associate to form a translation-competent 70S ribosome upon the initiation of protein synthesis. The 30S is composed of one strand of 16S rRNA (1,542 nucleotides) and 21 r-proteins. As expected the 50S contains more rRNA (23S and 5S containing 2,904 and 120 nucleotides, respectively) and more protein (33 r-proteins). For many years, the detailed structure of the ribosome was elusive due to limitations of crystallographic techniques, which was compounded by the tremendous size of the particle. However, at the turn of the century, crystallographic images of ribosomal subunits at the atomic level appeared and marked a gigantic leap forward in our understanding of the structural mysteries (Ban et al., 2000; Wimberly et al., 2000). Since then detailed images of

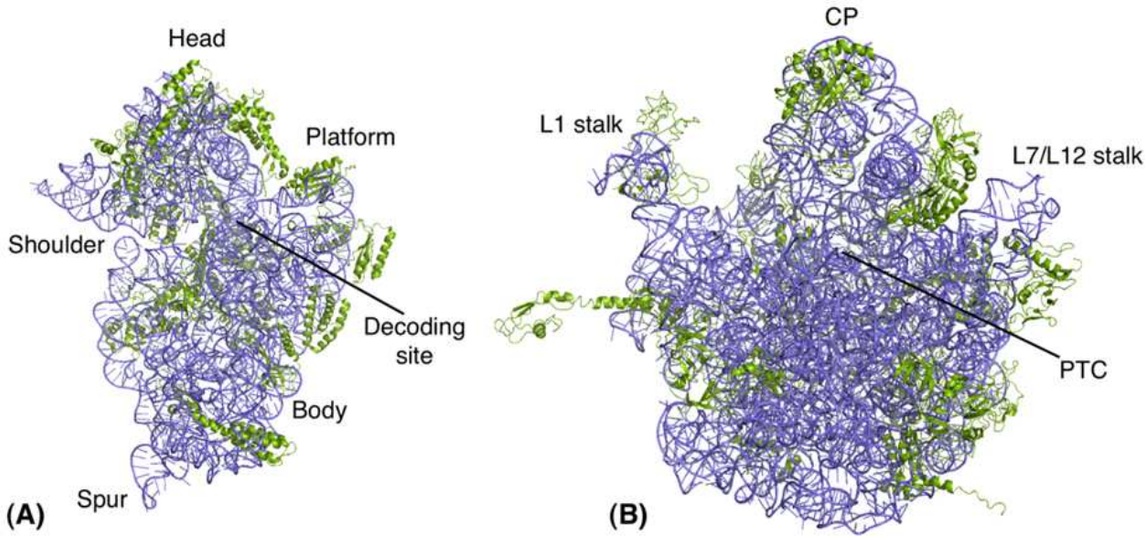


Fig. 1. **High-resolution crystal structures of the ribosomal subunits.** Tertiary structures of the bacterial 30S (A) and 50S (B) viewed from the inter-subunit face. Ribosomal RNA depicted in blue and r-proteins as green ribbons. Abbreviations are as follows: CP, central protuberance; PTC, peptidyl-transferase centre. The image was generated in PyMol (www.pymol.org) from the coordinates of the crystal structure of the *T. thermophilus* ribosome (PDB ID 2J00 & 2J01)(Selmer et al., 2006).

the full ribosome with translation ligands bound have been generated and to date the highest resolution crystal structure available is that of the *Thermus thermophilus (T. thermophilus)* 70S ribosome (Figure 1) (Selmer et al., 2006).

As clearly illustrated by the crystal structure, the two subunits are markedly different in their overall shape. The morphology of each subunit is an inherent feature of the folds adopted by each rRNA species and gives rise to a rigid large subunit and a more flexible small subunit. The 50S is a compact, compressed particle consisting of a hemispherical base with three conspicuous protuberances designated the L1 stalk, the central protuberance (CP) and the L7/L12 stalk (Figure 1B). The two rRNA strands (23S and 5S rRNAs) are highly interlaced to form a dense rRNA mesh. In contrast, the 30S is not only smaller in size, but is also less compact. The four secondary structure domains of the 16S rRNA correlate well to distinct regions of the three dimensional structure such as the head, body and platform (Figure 1A). The r-proteins are spatially dispersed at the periphery and back of the subunits, remote from the main functional sites such as the decoding centre, peptidyl-transferase centre (PTC) and the inter-subunit face. With this realisation, the controversy surrounding the basis of ribosomal enzymatic activity was finally laid to rest since the structures established that the PTC was devoid of protein and the ribosome was quickly recognised as a ribozyme (Nissen et al., 2000). In addition, high-resolution structures of the 30S revealed that decoding was primarily mediated by dynamic rearrangements of the 16S rRNA upon cognate tRNA binding at the ribosomal A-site (Ogle et al., 2003). Together, these studies propelled rRNA into the spotlight, securing its superior position as the major mediator of ribosomal function.

1.2 The ribosome at work

A detailed description of bacterial translation is beyond the scope of this text, however a brief overview is presented to facilitate a clear understanding of the experiments that will be described later. The process can be conceptually divided into four major phases as described below.

1.2.1 Initiation

Initiation begins on the 30S subunit and requires the binding of an mRNA template that carries the blueprint for the protein product. Next, selective and rapid binding of the unique, initiator tRNA (fMet-tRNA^{fMet}) to the 30S subunit takes place. This event is coordinated by the action of three initiation factors (IF1, IF2 and IF3), the principal one being the G-protein factor IF2, forming the 30S preinitiation complex. The 50S subunit is recruited and the initiation factors are released to form what is known as a 70S initiation complex (IC) that is now ready to proceed to the next phase.

1.2.2 Elongation

During elongation, the polypeptide grows as a result of the processive addition of amino acids that are delivered to the 70S ribosome as aminoacyl-tRNAs in a ternary complex with EF-Tu and GTP. Upon acceptance of the cognate substrate, rapid peptidyl transfer occurs. The tRNA-mRNA complex is re-positioned by a distance of exactly one codon to facilitate further addition of aminoacyl-tRNAs and this translocation step is stimulated by GTP

hydrolysis on EF-G. Amino-acyl tRNA selection, peptidyl transfer and translocation are repeated until a stop codon appears at the A-site.

1.2.3 Termination

In eubacteria, two release factor proteins (RF1 and RF2) recognize stop codons in a semi-specific manner and activate release of the nascent protein from the P-site tRNA to terminate translation. A third release factor RF-3, removes and recycles RF1 and RF2.

1.2.4 Ribosome recycling

During the final phase, ribosome recycling factor (RRF), in combination with EF-G are required to split the 70S into its subunits. The deacylated tRNA attached to the P-site of the 30S is ejected upon IF3 binding, the 30S can slide along the mRNA to re-initiate translation or the mRNA is also released. This event leaves the ribosome and its factors available to participate in further rounds of translation.

1.3 Ribosomal proteins

Despite the predominance of rRNA, the bacterial ribosome does contain an impressive array of 54 individual r-proteins. In *E. coli* and *Salmonella enterica* serovar Typhimurium, 21 proteins are associated with the small subunit (S proteins) and 33 with the large subunit (L proteins) (Wilson & Nierhaus, 2005). Among eukaryotes, the number of r-proteins increases by approximately 20-30 (Doudna & Rath, 2002). In bacteria, all r-proteins, with the exception of L7/L12, are present as a single copy per ribosome.

The overall abundance of rRNA and its direct involvement in the major tasks of the ribosome (decoding and peptidyl-transfer) have allowed the r-proteins to evade direct scrutiny and their contribution to translation is largely ill-defined. Furthermore, many of these proteins tend to be highly conserved, which forcefully calls our attention to questions regarding their functional roles (Lecompte et al., 2002). In general, they are considered important for the maintenance and stability of the overall structure of the ribosome (Cech, 2000). This assumption is largely due to their extensive contact with rRNA where many of the proteins contain extended projections that insert into the busy network of rRNA helices, prompting the idea that they probably promote or may even facilitate correct folding of the rRNA into its active conformation. However, few studies have directly addressed this issue and we are in the dark regarding the functional roles of most of the proteins. With this in mind, our research has focused on ascertaining the requirement and involvement of particular r-proteins for optimal cell growth and ribosome activity. We have posed the following questions:

1. If rRNA is responsible for the basic function of the ribosome then what, if any, are the selective advantages offered by the r-proteins? In other words, are they all actually necessary for ribosome function?
2. Is it possible to create mutants that completely lack a r-protein and mutants that carry a non-native version of the protein from another species?
3. What are the physiological consequences of such mutations with respect to cell fitness and ribosome function?

4. If such mutations confer fitness costs (i.e. slower growth), is it possible to compensate for such costs?
5. What are the mechanisms of fitness compensation that evolve in response to removal and replacement of r-proteins?

By addressing such questions we are ultimately interested in probing the complexity and robustness of the translation apparatus. To approach some useful answers to these questions, we have used *Salmonella enterica* serovar Typhimurium LT2 (hereafter referred to as *S. typhimurium*) as a model organism and introduced deletions of the genes encoding the r-proteins S20 and L1 and replaced S20, L1 and L17 with orthologous counterparts from close and phylogenetically distant relatives, including the archaeon *Sulfolobus acidocaldarius* and the eukaryote *Saccharomyces cerevisiae*.

2. Using *S. typhimurium* as a model in the ribosome field

E. coli has long served as a model organism for genetic and biochemical studies of bacterial ribosome function. However, due to the co-linearity of their genomes, *S. typhimurium* represents a convenient alternative for such studies and is preferable in our case due to a more favourable and better characterized genetic background for the purposes of genetic suppression studies (McClelland et al., 2001) (see below). Although highly similar in terms of ribosomal gene content, *S. typhimurium* does possess some unique features that are described below.

2.1 Primary sequences of rRNA and r-proteins

In terms of the basic building blocks of the ribosome, rRNA and r-proteins, the genes encoding these components are generally highly conserved between *E. coli* and *S. typhimurium*, a reflection of their close phylogenetic relationship. In both organisms, there are seven independent rRNA operons (*rrnA, rrnB, rrnC, rrnD, rrnE, rrnG* and *rrnH),* each encoding a single copy of the 16S, 23S and 5S rRNAs. The genes encoding the 16S and 5S rRNAs show strong similarities; however, more variation is detected between the genes encoding the 23S rRNA species (*rrl* genes). In particular, the primary sequences of *rrl* genes in *S. typhimurium* are between 90 and 110 nucleotides longer due to the presence of so-called intervening sequences (IVSs) that are removed during maturation (Burgin et al., 1990). Despite this discrepancy in the length of their primary 23S rRNA sequences, the mature, core sequence in *S. typhimurium* is nearly identical (97%) to that of *E. coli* (Burgin et al., 1990).

In terms of the r-proteins, a high degree of conservation is also observed upon alignment of the amino acid sequences of *S. typhimurium* LT2 proteins and *E. coli* K-12 MG1655 proteins. For the 21 r-proteins associated with the small subunit, a median similarity of 99.2% is detected with an average identity of 98.9% and a standard deviation of 0.9%. Similarly, for the 33 r-proteins associated with the large subunit, the median amino acid identity is 98.9% with an average similarity of 97.7% and a standard deviation of 3.6%. From these calculations, we can conclude that there is slightly more variation in the amino acid sequences of large subunit proteins when comparing the primary sequences of *S. typhimurium* LT2 and *E. coli* K-12 MG1655. However, overall, a high degree of conservation exists, which is not surprising given the close phylogenetic relationship.

2.2 rRNA processing during ribosome biogenesis

During transcription of the rRNA operons, the primary transcripts are processed to mature products by means of RNase cleavage and chemical modification (Kaczanowska & Ryden-Aulin, 2007). The vast majority of studies that have examined these processing events have looked at the *E. coli* system only, however it is expected that *rrn* processing in close relatives such as *S. typhimurium* should be highly similar.

Primary *rrn* transcripts of *E. coli* are initially substrates for the endoribonuclease RNase III that catalyzes separation of the rRNAs into pre-16S (17S), pre-23S and pre-5S (9S) species (Nierhaus, 2006). Final trimming of the 5′ and 3′ ends of each transcript involves a number of exonucleases and occurs in the context of pre-ribosomal particles (Deutscher, 2009). In some genera of bacteria, including *S. typhimurium*, mature 23S rRNA is not fully intact but is fragmented during processing. Experiments have revealed that the fragments arise due to RNase III-mediated removal of intervening sequences (Burgin et al., 1990). Although the resulting fragments of 23S rRNA are not re-ligated they are fully functional. Some heterogeneity exists between the seven copies of 23S rRNA genes in *S. typhimurium* LT2 with regard to the presence of these additional sequences. In the case of *rrlG* and *rrlH*, an insertion of approximately 110 nucleotides is observed in helix-25 (located at nucleotide position 550, approximately) and *rrlA, rrlB, rrlC, rrlD, rrlE* and *rrlH* contain an insertion of approximately 90 nucleotides in helix-45 (located at nucleotide position 1170, approximately). Interestingly, ribosomes of a *S. typhimurium* mutant lacking RNase III remain functional, implying that fragmentation is not required for the activity of *S. typhimurium* ribosomes (Mattatall & Sanderson, 1998). This would also suggest that it is unlikely that these extra sequences evolved to support proper functioning of *S. typhimurium* ribosomes. Their intermittent distribution among diverse genera of bacteria would suggest that they may have been acquired by horizontal gene transfer (Mattatall & Sanderson, 1996).

2.3 Unusual sucrose gradient profile of *Salmonella* ribosomes

Analysis of ribosome function *in vitro* requires the extraction of large quantities of intact ribosomes from the cell using a standard sucrose density ultracentrifugation protocol (Johansson et al., 2008). Typically, this involves preparing a pure cell lysate that is passed through two sucrose cushions to remove cell debris. Ribosomal particles (70S, 50S and 30S) are subsequently separated on a final sucrose gradient (10 – 50%) based on differences in their densities. The standard protocols available are optimised for *E. coli* and pure fractions of each ribosomal particle are easily obtainable. The high degree of similarity between *S. typhimurium* and *E. coli* might, *a priori*, lead one to expect a similar profile when the protocol is applied to *S. typhimurium*. However, in our hands, we have consistently observed a striking difference.

Looking at a typical, large-scale 70S purification profile from *E. coli* K12 strain MRE600 (Figure 2A), clean, distinct peaks are observed for each ribosomal particle, as indicated. In contrast, the 70S peak from wild-type *S. typhimurium* LT2 contains a distinct shoulder (Figure 2B). Upon examination of the protein and rRNA content of the second, smaller peak, we observed that this contains 30S particles only, indicating that the 70S and 50S peaks coincide when ribosomes are prepared from *S. typhimurium* on a large scale. This is a consistent feature of the wild-type strain, as well as all r-protein mutant strains that we have

worked with. At the present time, there is no definitive explanation for these observations but the unusual co-migration of 70S and 50S species may be related to 23S rRNA processing differences between the species, or, 70S ribosomes of *S. typhimurium* may be composed of loosely-coupled subunits that require a higher magnesium concentration for stabilization.

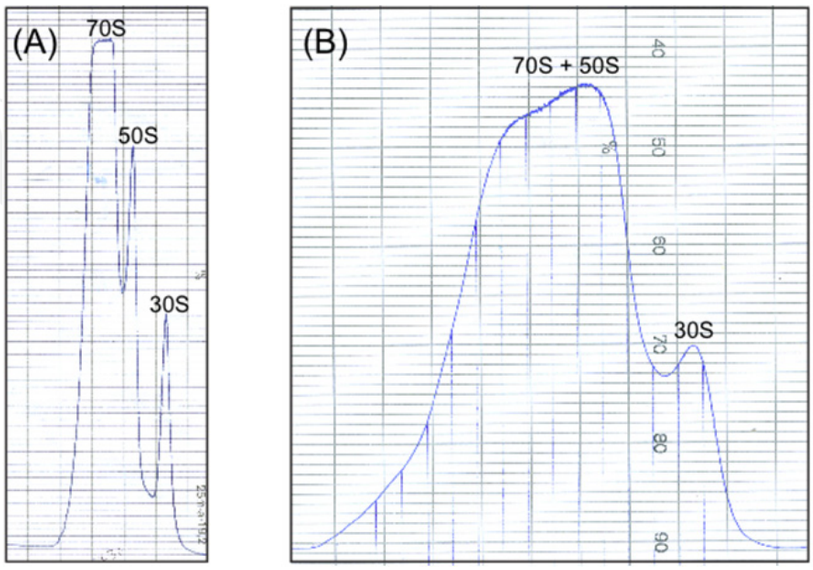


Fig. 2. **Sucrose gradient ultracentrifugation profiles obtained during 70S ribosome purification.** (A) Profile obtained from *E. coli* MRE600 showing three separate peaks for 70S, 50S and 30S particles. (B) Profile obtained from wild-type *S. typhimurium* showing a distinctive shouldered peak containing a mixture of 70S and 50S particles followed by a 30S peak.

2.4 Advantages of using *S. typhimurium* as a model

E. coli is traditionally regarded as the first choice model organism for studies of ribosome function. However, it has recently become clear that the most frequently used K-12 strain has mutations in components that function in translation. Mutations in the genes *prmB* (encoding class-I release factor RF2) and *rpsG* (encoding ribosomal protein S7) represent two well-characterized examples. O' Connor et al. have reported that in all K-12 strains of *E. coli*, RF2 carries a threonine residue at position 246 as opposed to alanine, which is typically found at this position in the majority of bacterial RF2 proteins (O'Connor & Gregory, 2011). In the case of *rpsG*, a point mutation alters its canonical UGA stop codon to a leucine codon (Schaub & Hayes, 2011). Thus, the S7 protein of all K-12 strains carries an extension of 23 amino acids at its C-terminus. This protein is essential and particularly important during 30S assembly. Both of these mutations were identified as being partially responsible for the fitness cost and ribosomal perturbations associated with an *rluD* null mutation.

These studies serve to highlight the importance of using a model organism with a clean genetic background. The presence of underlying, undetected mutations complicates the matter of defining the mechanism and cause of observed phenotypes associated with a mutation of interest due to the unavoidable likelihood of synergistic and epistatic interactions. Our research has concentrated on the effects of r-protein removal and replacement in terms of bacterial fitness and ribosome function with a strong focus on suppression genetics. So,

although *E. coli* K-12 is undoubtedly the most well-studied model organism, *S. typhimurium* represents a more suitable and reliable model for the purposes of our studies.

3. Isolation and characterisation of ribosomal protein mutants

The scarcity of studies designed to examine the distinct functional roles of r-proteins, despite their high level of conservation in all three phylogenetic kingdoms, certainly warrants further experimentation aimed at understanding their contributions to cell fitness and ribosome activity. To address this issue, genetically defined *S. typhimurium* mutants were constructed in which the target proteins were either completely removed (S20 and L1) from the chromosome or replaced (S20, L1 and L17) on the chromosome with orthologous counterparts from other species.

3.1 Previous studies of r-protein mutants

The majority of the early research on mutants lacking r-proteins was initiated by the work of Dabbs and colleagues. Beginning in the mid-70s, various antibiotic selection systems were developed for the isolation of *E. coli* mutants with alterations in r-proteins (Dabbs & Wittman, 1976; Dabbs, 1978). Most of the mutants that lacked a r-protein were isolated by chemically mutagenizing antibiotic sensitive cells, selecting for antibiotic dependence followed by selection for reversion of the antibiotic dependent phenotype (Dabbs, 1979). Using such methods, *E. coli* strains were isolated lacking one or more of 16 individual r-proteins (S1, S6, S9, S13, S17, S20, L1, L11, L15, L19, L24, L27, L28, L29, L30 and L33) (Dabbs, 1991). In all of these studies, absence of each protein was only verified on the protein level by means of two-dimensional gel electrophoresis and immunological methods using antibodies specific for each missing protein. The mutants exhibited a conditional lethal phenotype and were varied in terms of growth properties. These studies were certainly pioneering in terms of showing the non-essential nature of certain r-proteins but follow-up studies designed to determine the nature of the fitness costs and ribosomal defects were hampered by the chemical mutagenesis and selection methods used for the isolation of mutants. These strains were subject to mutagenesis on a genome-wide scale and were therefore not fully defined genetically, which creates difficulty in specifically linking the observed phenotypes to absence of the protein. In the past few years, much progress has been made in the techniques used for the manipulation of bacterial genetics (Datsenko & Wanner, 2000; Sharan et al., 2009). These methods have made it possible to introduce precise deletions of targeted genes and a number of research groups have been successful in obtaining r-protein null mutants (Wower et al., 1998; Cukras & Green, 2005; Bubunenko et al., 2007; Korepanov et al., 2007). In a recent work the number of the L7/L12 proteins have been varied by deletion of the binding site of one L7/L12 dimer on the L10 protein (Mandava et al., 2011). The characterisation of such mutants has advanced our current knowledge of the functions of some of the r-proteins; unfortunately, however, these mutants are all limited to the species *E. coli.*

Attempts to produce functionally active hybrid ribosomes harboring non-native r-proteins have previously been undertaken (Liu et al., 1989; Giese & Subramanian, 1991). The majority of these studies only examined functional activity in terms of polysome formation. However, highly divergent proteins such as S18 from rye chloroplast, that shares only 35% amino acid identity with *E. coli* S18, was incorporated into 30-40% of *E. coli* monosomes and

polysomes upon expression from a plasmid (Weglohner et al., 1997). The ability of these hybrid ribosomes to form polysomes indicates that they were capable of forming functional complexes, although the extent of their functionality during specific steps of translation was not examined. In the same study, L12 from *Arabidopsis* chloroplasts was also incorporated into ribosomes but these hybrid ribosomes failed to form functional polysomes *in vivo* and were inactive in poly(U)-dependent poly(Phe) synthesis *in vitro*. In another study, the *E. coli* proteins of the GTPase-associated centre (L10, L11 and L7/L12) were replaced with their rat counterparts *in vitro* (Uchiumi et al., 2002). Although incorporated into the *E. coli* ribosome, eukaryotic elongation factors were required to support protein synthesis *in vitro*. So although divergent r-proteins can be assembled into the bacterial ribosome, proteins with defined functions, such as interactions with bacterial specific translation factors, lack function and do not preserve protein synthesis.

3.2 Construction of r-protein mutants

In our studies, mutants of *S. typhimurium* that completely lacked an r-protein, and mutants in which the native r-protein gene was replaced with closely related as well as phylogenetically distant orthologues were created. Due to its simplicity and precision, lambda red recombineering was used for the construction of all mutants (Datsenko & Wanner, 2000). For removal of S20 and L1, a kanamycin resistance cassette was PCR amplified using primers with 50 nucleotide 5' tails homologous to the flanking sequence of the target gene (*rplA* and *rpsT*). Electroporation of the linear DNA product into the cell resulted in substitution of the r-protein gene with the selectable kanamycin resistance cassette to create mutants lacking S20 (Tobin et al., 2010) and L1 (Figure 3A). A similar procedure was used for the generation of S20, L1 and L17 replacement mutants, however in this case the PCR fragment consisted of the open reading frame (ORF) of the orthologous replacement gene and an adjacent kanamycin resistance cassette (Lind et al., 2010). Homologous recombination resulted in replacement of the target r-protein ORF (*rpsT, rplA* and *rplQ*) with its orthologous ORF and the kanamycin resistance cassette (Figure 3B). Congenic wild-type control strains were also constructed and carried the kanamycin cassette adjacent to the native r-protein ORFs. In the case of both types of constructions, the native promoter and terminator sequences were preserved. Using this technique, individual mutants lacking the r-proteins S20 (ΔS20) and L1 (ΔL1) as well as replacement mutants carrying heterologous versions of S20, L1 and L17 were created.

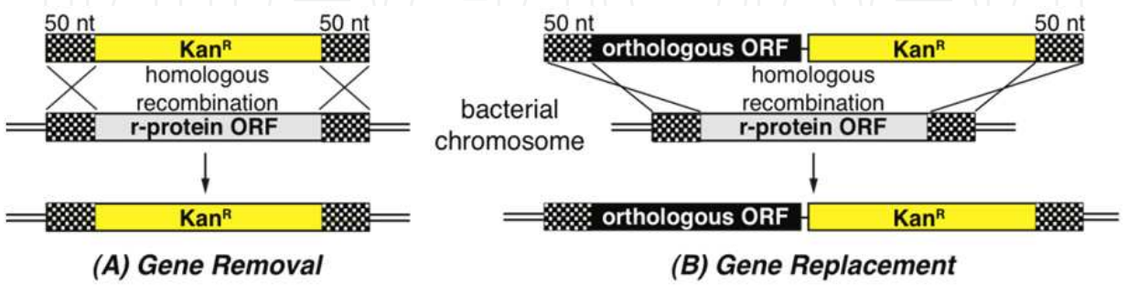


Fig. 3. **Schematic diagram representing the technique employed to generate r-protein mutants.** The recombining fragments in (A) and (B) were designed with 50 nucleotides of homology to the flanking sequences of the r-protein target genes to facilitate homologous recombination and replacement on the chromosome.

3.3 Quantification of fitness costs

In a laboratory setting, determining exponential growth rate is the most straightforward method for assaying fitness. To quantify the physiological effect of r-protein removal and replacement, we measured the doubling time of the wild-type and ribosomal mutant strains using a Bioscreen C analyzer. Relative fitness values were calculated as the growth rate of each mutant normalized to that of the wild-type reference strain used in each experiment (set to 1.0). Although relatively easy to execute, this measurement fails to evaluate fitness over the entire growth cycle and is unable to detect very small differences in fitness (sensitivity of ~ 3%). A more labour intensive method that overcomes such limitations is pair-wise competition experiments where the test strain and wild-type are genetically tagged and compete over many generations (sensitivity of ~ 0.3%). This method was used as a more sensitive fitness parameter in some of our experiments.

3.4 Compensatory evolution

Removal and replacement of highly conserved r-proteins, that function within a central component of the cell, constitute deleterious mutations and confer fitness costs, i.e. slower growth. Because such mutants grow more slowly and therefore produce fewer progeny, they will eventually be out-grown by competitors of higher fitness and eliminated from the population. However, if the mutant can persist in the population, it has the potential to develop further mutations that suppress or compensate the fitness cost of the initial deleterious mutation. Such compensatory mutations (denoted CM) can restore fitness back to the wild-type level and effectively neutralize the deleterious effect of the initial mutation (deletion or replacement of a r-protein). Alternatively, the fitness cost may be only partially relieved by the CM, which increases fitness but not to the extent of wild-type fitness (Figure 4A). Here, we are interested in knowing if and by what mechanisms the fitness costs of L1 removal and S20, L1 and L17 replacement can be compensated. This is not only helpful in the sense that it may provide clues to the functional roles of the proteins, but it also provides a unique opportunity to reveal novel co-operative mechanisms in ribosome function.

The experiment is designed to optimize purifying selection, which reduces genetic diversity so that the population stabilizes on the most favourable (fittest) genotype. During the

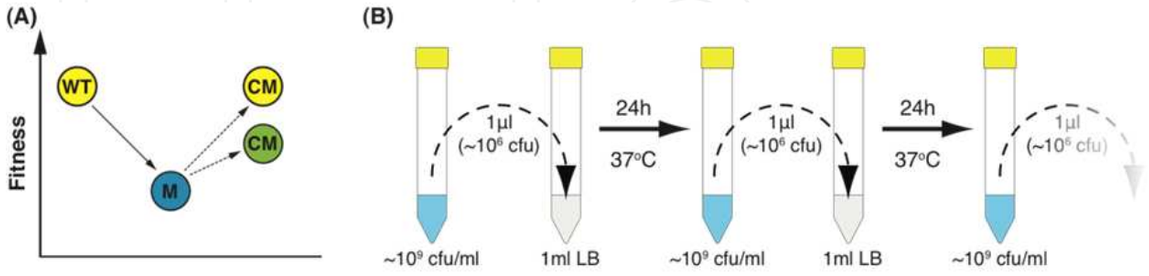


Fig. 4. **Compensatory evolution by serial passage.** (A) Schematic illustration of the principle of compensatory evolution. A deleterious mutation (M) is introduced into a wild-type population (WT) and reduces fitness. Compensatory mutations (CM) that reduce the fitness cost of the initial deleterious mutation can restore fitness completely (yellow) or partially restore fitness (green). (B) Schematic illustration of the serial passage experiment used for the isolation of compensated mutants.

evolutionary process, CMs that reduce the fitness cost conferred by loss or replacement of the r-protein become more common in successive generations. Independent lineages of the mutants are serially passaged in liquid growth medium (LB) for a number of generations. To maintain continuous population growth and to ensure that all genetic variants are represented during the entire evolutionary process, large bottlenecks of ~ 10^6 cells are used (Figure 4B). This experimental design maximises the potential to fix the compensated mutant of highest fitness. When mutants of higher fitness constitute the majority of the population, the experiment is terminated and a single colony is examined for the presence of candidate compensatory mutations that increase fitness. To verify that the identified mutations are responsible for improving fitness, genetic reconstructions are performed and fitness is quantified.

3.5 *In vivo* and *in vitro* analysis of ribosomal function

For a full appreciation of the functional roles of deleted r-proteins, both *in vivo* and *in vitro* techniques were employed to compare the activity of mutant ribosomes with their wild-type counterparts.

3.5.1 *In vivo* system

Synthesis of an easily detectable reporter protein was used here to measure the rate of polypeptide elongation and ribosomal misreading of stop codons and frameshifting. Polypeptide elongation rates of exponentially growing cultures were measured by determining the exact time taken to produce the first detectable β-galactosidase molecule following IPTG induction (Miller, 1992). As *S. typhimurium* does not harbor the *lac* operon, the normal inducible *lac* operon was supplied on an F′ factor and was transferred to strains by means of conjugation. This method was used to determine the rate of polypeptide elongation in the strains lacking r-proteins S20 and L1.

The synthesis of β-galactosidase was also used to determine the frequency of stop codon read-through and frameshifting of an L1-depleted ribosome. In this instance, strains carried an F′ factor in which the *lacI* and *lacZ* genes are fused for constitutive expression of β-galactosidase. However, the ribosome must read-through a premature stop codon (UGA_{189} or UAG_{220}) or a +1 or -1 frameshift in *lacI* to produce an active molecule of the enzyme, the activity of which is detected as described above. A read-through value is calculated based on the amount of enzyme produced by the mutated *lacIZ* construct relative to that produced in a congenic strain harbouring a non-mutated *lacIZ* construct (Bjorkman et al., 1999). A second assay, based on luciferase expression, was also employed to determine if L1 depleted ribosomes were prone to general misreading of sense codons (Kramer & Farabaugh, 2007).

3.5.2 *In vitro* system

The translation cycle is composed of a large number of intermediate sub-steps that proceed extremely fast *in vivo*. Thus, in order to specifically examine defined steps in translation, analysis of ribosome function in a controlled *in vitro* translation system is absolutely necessary. For the characterization of S20- and L1-depleted ribosomes, we have employed a

reconstituted cell-free translation system that is optimized to meet *in vivo* expectations in so far as it allows measurement of *in vitro* reactions in the millisecond range (Ehrenberg et al., 1989). Ribosomal particles and all other translation components are prepared to high purity according to standard protocols. Additionally, all experiments are performed in polymix buffer that mimics the pH and complex ionic conditions of the bacterial cell (Pettersson & Kurland, 1980). Using this system, r-protein depleted ribosomes can be accurately compared with their wild-type counterparts in all steps of the translation cycle. Although the system was developed for *E. coli*, it has previously been shown that the system can be applied to ribosomes from *S. typhimurium* (Tubulekas et al., 1991).

4. R-protein deletion mutants

Genetically defined mutants of *S. typhimurium,* lacking individual r-proteins (ΔS20 and ΔL1), represent powerful tools for the analysis of the function of these proteins in the context of translation and can also reveal their potential roles in other cellular processes. Below follows a summary of the fitness costs, compensatory mechanisms and the *in vivo* and *in vitro* analysis of the mutants lacking S20 and L1.

4.1 Removal of S20 and L1 confers substantial fitness costs

Loss of small subunit protein S20 reduced exponential growth rate approximately three-fold relative to the wild-type under standard growth conditions (LB broth at 37°C), corresponding to a relative fitness value of 0.33 (Table 1) (Tobin et al., 2010). Upon deletion of large subunit protein L1, the generation time of the mutant was reduced two-fold compared to wild-type under standard growth conditions (Table 1). This fitness cost was even more pronounced during growth at lower temperatures, indicative of a cold-sensitive phenotype. In the case of both mutants, complete restoration of the wild-type growth rate was observed upon induced expression of the deleted gene from an arabinose-inducible plasmid, verifying that the fitness costs were specifically attributable to loss of each respective protein (Figure 5A and B).

Strain	Growth Temperature	Relative Growth Rate
Wild-Type	n/a	1.0
ΔS20	37°C	0.33
ΔL1	30°C	0.36
ΔL1	37°C	0.52
ΔL1	44°C	0.58

Table 1. **Fitness costs associated with removal of S20 and L1.** The generation time of the wild-type strain is set to 1.0 and is used to normalize the growth rate of each mutant at the temperatures specified.

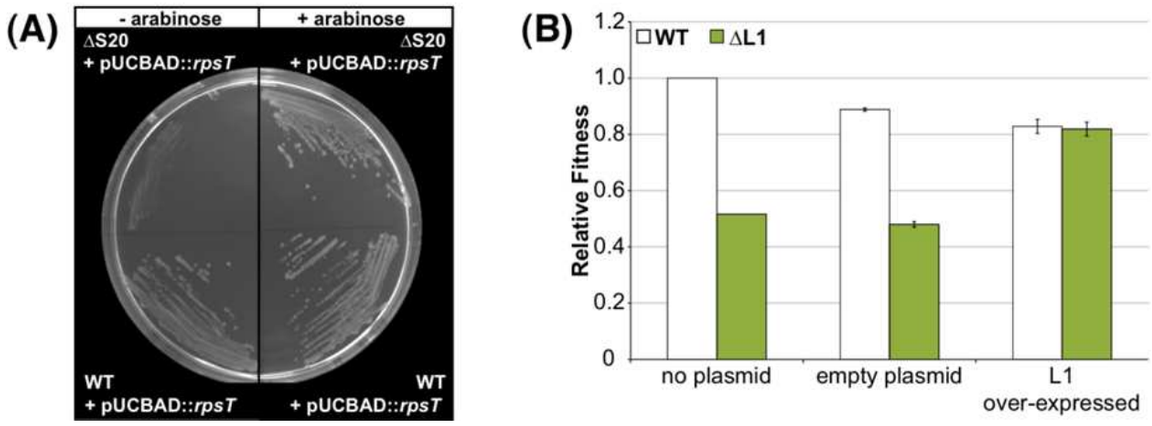


Fig. 5. **Complementation of fitness costs upon induced expression of the deleted r-protein gene from an inducible plasmid.** (A) In the absence of the arabinose inducer, the ΔS20 strain grows very poorly relative to the wild-type (left-hand side of image). In the presence of arabinose, expression of the *rpsT* gene is induced and growth of the mutant matches that of wild-type (right-hand side of image) (Tobin et al., 2010). (B) Growth rates of the ΔL1 mutant relative to the wild-type. In the presence of the arabinose inducer, *rplA* is expressed and fitness of the mutant matches that of wild-type. Error bars represent standard error of the mean.

4.2 Compensatory mutations that mitigate the costs of L1 deletion

Twelve independent lineages of the ΔL1 mutant were evolved for approximately 300 generations to determine if and by what mechanisms compensation could be achieved. Upon isolation of faster-growing mutants, four were chosen at random and subject to whole-genome sequencing to identify candidate compensatory mutations responsible for suppressing the fitness cost associated with loss of L1. A single nucleotide substitution in the *rplB* gene and a small deletion in the *rplS* gene were detected. These mutations resulted in the amino acid substitution E194K in large subunit protein L2 (encoded by *rplB*) and loss of the Leu codon at position 100 of L19 (referred to as *rplS* ΔL100) (Figure 6A). Using a different ΔL1 ancestral strain, the amino acid substitution P102L in the *rplN* gene (encoding large subunit protein L14) was also identified (Figure 6A). Strain reconstructions confirmed that each individual mutation was necessary and sufficient for fitness improvement and all three increased growth rate to a similar extent. In any combination, all three compensatory mutations demonstrated negative epistasis in the ΔL1 background. In the wild-type strain, each individual mutation reduced fitness and this became even more pronounced when the mutations were combined (Figure 6B).

4.3 Phenotypes of previous L1 deletion mutants

Two independent *E. coli* knockout mutants of L1 were previously isolated as spontaneous revertants of chemically mutagenized spectinomycin- and kasugamycin-dependent strains (Dabbs, 1977; Dabbs, 1980). Examination of the activity of ribosomes purified from these strains suggested that absence of L1 reduced the *in vitro* production of polypeptide by 50%, corresponding to an approximate 50% reduction in growth rate (Subramanian & Dabbs, 1980). To probe further into the ribosomal deficiencies, a follow-up study examined the

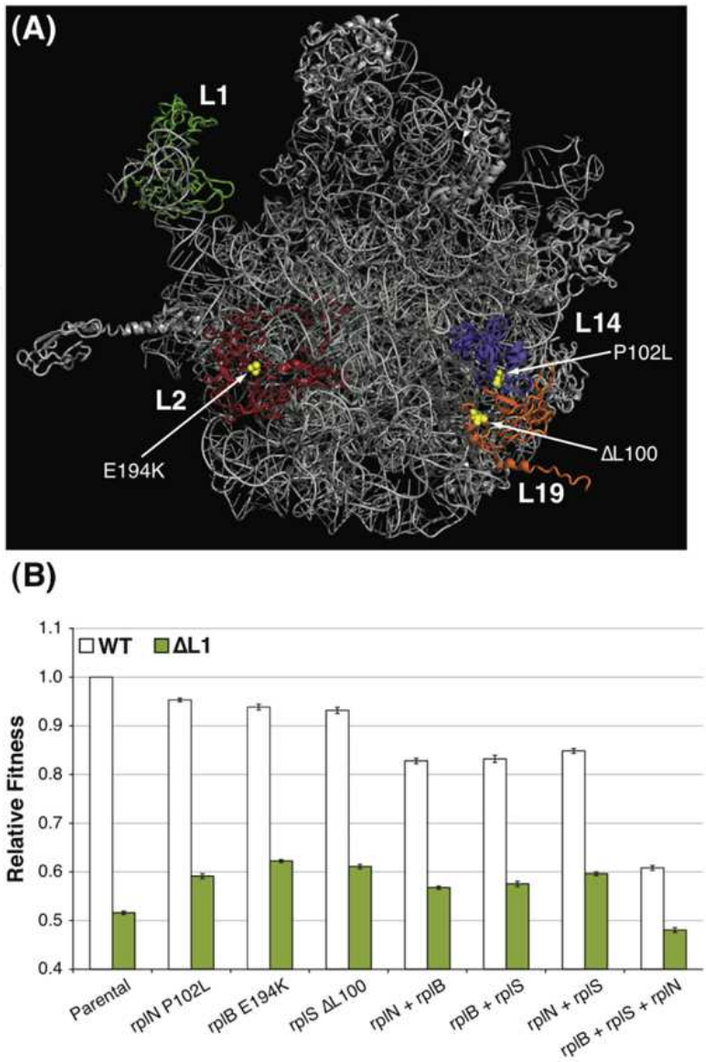


Fig. 6. **Compensation for the fitness costs of L1 deletion.** (A) Crystal structure of the *T. thermophilus* 50S subunit showing the localization of compensatory mutations (yellow spheres) in the r-proteins L2 (red), L14 (purple) and L19 (orange). Position of the L1 protein is shown in green. All other rRNA and protein residues are depicted in grey. The image was created in PyMol (www.pymol.org) using the coordinates of the *T. thermophilus* 50S subunit structure (PDB ID 2J01) (Selmer et al., 2006). (B) Genetic reconstructions and fitness effects of compensatory mutations in the ΔL1 (green bars) and wild-type (white bars) backgrounds. Relative fitness was calculated as the growth rate of each strain as a fraction of the wild-type growth rate. Error bars represent standard error of the mean.

activity of L1-depleted ribosomes using a number of partial reactions. It was found that both the binding of N-acetyl-Phe-tRNA to the P-site and stimulation of EF-G dependent GTP hydrolysis in the presence of tRNA and mRNA were significantly compromised with these ribosomes (Sander, 1983). Another L1 deletion mutant was isolated in an independent study as a spontaneous revertant of a chemically mutagenized erythromycin-dependent strain (Wild, 1988). This strain produced an excess of free ribosomal subunits and fewer 70S

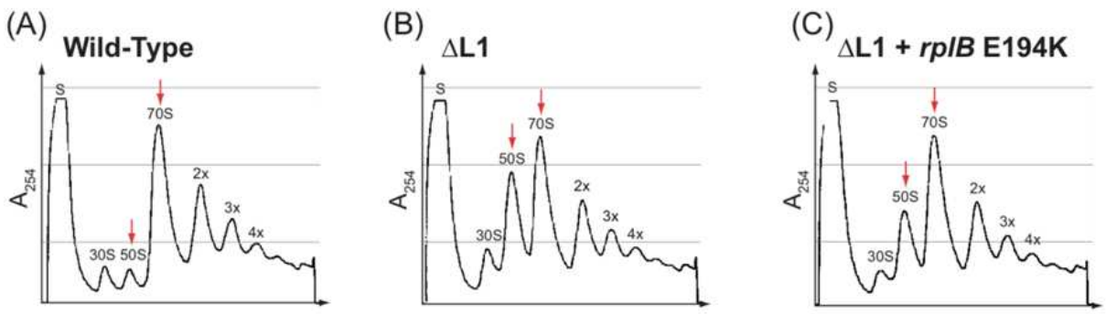
ribosomes when the sedimentation profile of ribosomes was examined. In addition, a genetically defined *rplA* deletion strain has been constructed in *E. coli* (Baba et al., 2006), but at the time of writing no details of the phenotype of this mutant are known.

4.3.1 Misreading phenotype

The proposed involvement of L1 in regulating release of the E-site tRNA and the coupling of decoding accuracy at the A-site to occupation of the E-site (Wilson & Nierhaus, 2006) prompted us to investigate the decoding properties of the L1-depleted ribosome. By means of β-galactosidase assays (described in 3.5.1), the level of frameshifting and stop codon readthrough was measured *in vivo*. The only significant difference between the wild-type and mutant was detected with respect to UGA readthrough, which was increased approximately three to five-fold in the case of the ΔL1 mutant, independent of codon context. Misreading of sense codons was also examined although no significant differences were detected, suggesting that L1 plays a minor role, at most, in decoding at the A-site. Furthermore, none of the identified compensatory mutations altered readthrough of UGA in the ΔL1 background.

4.3.2 Polysome analysis

It was previously shown that L1 has RNA chaperone activity (Ameres et al., 2007), so a role for L1 in ribosome biogenesis is possible. To investigate the distribution of different ribosomal particles in the cell, polysome profiles were examined and compared to the profile obtained from wild-type cells (Figure 7). The most striking observation of the mutant



Strain	Peak heights of ribosomal particles relative to sum of all peak heights[a] 30S	50S	70S	2x	3x	4x	Total polysomes[b]	Total 70S particles[c]
Wild-Type	0.08	0.07	0.37	0.22	0.15	0.11	0.48	0.85
ΔL1	0.11	0.23	0.29	0.16	0.11	0.09	0.36	0.65
ΔL1+ *rplB* E194K (CM)	0.09	0.19	0.32	0.18	0.12	0.09	0.39	0.71

Fig. 7. **Polysome profiles obtained following immediate translational arrest in log-phase.** Distribution of ribosomal particles in the wild-type (A), ΔL1 mutant (B) and the ΔL1 mutant with the compensatory mutation (CM) in *rplB* (encoding L2). Each peak corresponds to each different particle as labelled and polysomes are labelled as 2x, 3x and 4x. [a] The table shows average peak heights for each particle relative to the the sum of all peak heights and were based on at least three independent profiles. [b] Total polysomes were calculated as the sum of 2x, 3x and 4x peak heights. [c] Total 70S particles were calculated as the sum of 70S, 2x, 3x and 4x peak heights.

profile (Figure 7B) was the large excess of free 50S subunits, which was increased three-fold compared to the wild-type (Figure 7A). Furthermore, this increase in free subunits was biased specifically towards the 50S as the amount of free 30S was only slightly increased. Thus, removal of L1 leads to an imbalance in formation of the ribosomal subunits. This increase in free subunits occurred at the expense of functional 70S complexes, which were reduced from 0.85 in the wild-type to 0.65 in the mutant. However, the ratio of 70S monosomes to polysomes was the same for the mutant and wild-type, indicating that those 70S ribosomes that were formed could translate as efficiently as the wild-type. Upon overexpression of L1, these anomalies in ribosomal particle partitioning were removed, specifically identifying loss of L1 as the cause of the altered profile. The reduction of 70S complexes as monosomes and polysomes is likely to be responsible for the slow growth rate of the ΔL1 mutant as the translational capacity of the strain is likely to be substantially compromised. Interestingly, the E194K amino acid substitution in L2 conferred a small but reproducible reduction in the proportion of free ribosomal subunits and a consequent increase in the fraction of active 70S complexes (Figure 7C).

4.4 L1 plays a major role in ribosome biogenesis

A number of *in vivo* and *in vitro* methods were used in this study to evaluate the role of L1 in ribosome function. Based on the results of these assays, we propose that large subunit protein L1 is crucial for promoting balanced formation of the ribosomal subunits in *S. typhimurium*. This defect is further supported by the observed cold sensitivity of the ΔL1 mutant, a feature that is often associated with aberrant ribosome biogenesis (Dammel & Noller, 1993; Bubunenko et al., 2006). It is not yet clear why the production of free ribosomal subunits is disproportionate, but a few alternative explanations are feasible. L1 has known RNA chaperone activity so the protein may be directly involved in biogenesis of the small subunit; however, no evidence is currently available to support this suggestion. The effect of L1 deletion on 30S production may be more indirect. It is conceivable that in the absence of L1, 50S subunits are more prone to misassembly than the wild-type. This, in turn, may perturb 30S assembly via sequestering of accessory factors required for the biogenesis of both subunits, for example. Alternatively, if the 30S subunits lack properly assembled association partners, final maturation of the 30S may be impaired and/or the 30S subunits may be more susceptible to the action of degradative RNases as previously suggested (Deutscher, 2009). Although only one of the three confirmed compensatory mutations (*rplB* E194K) displayed a detectable improvement in ribosomal particle partitioning (Figure 7C), the remaining two (*rplN* P102L and *rplS* ΔLeu) occur in proteins directly involved in formation of bridging contacts required for 70S complex formation (Selmer et al., 2006). Thus, it would appear as though these two compensatory mutations may alter subunit interactions and possibly increase 70S production. Furthermore, additional compensatory mutations in the genes *rpsM* (encoding S13 that forms bridges B1a and B1b with the 50S subunit) and *engA* (encoding the 50S biogenesis factor Der) provide more evidence that L1 plays an important role in ribosome biogenesis.

Along with helices 76-78 of the 23S rRNA, L1 occupies a highly mobile region of the 50S subunit known as the L1 stalk. Crystal structures of the 50S and 70S revealed that this stalk can adopt both an "open" and "closed" conformation, suggestive of an underlying functional role in the release of deacylated tRNA from the E-site thereby alluding to a role

for this region in the translocation step of polypeptide elongation (Harms et al., 2001; Yusupov et al., 2001). These observations sparked a number of structural-based investigations aimed at investigating L1 stalk dynamics which all indicate that this region is involved in modulating decaylated tRNA transit and release during translocation (Valle et al., 2003; Fei et al., 2008; Munro et al., 2010; Trabuco et al., 2010). Thus, to determine the activity of L1 depleted ribosomes in the elongation step of translation, a number of *in vivo* and *in vitro* assays are currently underway.

4.5 Phenotype of S20 deletion mutant

Knockout mutants of S20 have been isolated previously as antibiotic dependent revertants and as a suppressor of nonsense codons (Dabbs, 1978; Dabbs, 1979; Ryden-Aulin et al., 1993). Both types of mutants displayed initiation defects in terms of initiator tRNA binding (Gotz et al., 1990) and subunit association (Gotz et al., 1989; Ryden-Aulin et al., 1993). In another study, an S20 knockout mutant was obtained as a suppressor of erythromycin dependence and produced precursor 30S particles and fewer 70S complexes, as measured by density ultracentrifugation (Wild, 1988). More recently, the recombineering approach has been used to isolate genetically defined, in-frame deletions of *rpsT* in *E. coli,* but data to describe the phenotypic effects of these deletions is currently unavailable (Baba et al., 2006; Bubunenko et al., 2007).

4.5.1 Polypeptide formation *in vivo*

The rate at which the ΔS20 mutant forms polypeptide *in vivo* was compared to the wild-type strain using the β-galactosidase assay. Similar to the results of the ΔL1 mutant, no defect in the rate of polypeptide chain elongation was detected, however a reproducible reduction in the rate of accumulation of synthesized protein was evident (Tobin et al., 2010). Considering that S20 is a designated 30S subunit protein and given the results of previous studies, we hypothesized that the reduced rate of protein accumulation was likely the result of impaired initiation. A reduction in the frequency of mRNA initiation would directly decrease the number of rounds of translation per mRNA and reduce the yield of protein production. Thus, the activity of S20-depleted 30S subunits were compared to those obtained from the wild-type, using a number of *in vitro* methods designed to measure each distinct step of translation initiation.

4.5.2 Reduced mRNA binding *in vitro*

The rate of template binding was measured using a radioactively labeled mRNA with a strong Shine-Dalgarno (SD) sequence. In both the presence and absence of initiator tRNA and the initiation factors, the rate of mRNA binding to ΔS20 30S subunits was reduced approximately 3.5-fold and maximum binding required 40 minutes compared to only 10 minutes for the wild-type. Since similar rates of mRNA binding were observed with and without the initiation ligands, a primary impairment in mRNA binding could be concluded. Reconstitution with purified S20 restored the wild-type mRNA binding phenotype and established that defective mRNA association was a direct consequence of S20 removal (Figure 8A). In addition, titration of mRNA revealed that a substantial fraction (~ 25%) of

the S20-depleted 30S subunits were completely inactive in the binding of mRNA (Figure 8B). Since the rate of fMet-tRNA^{fMet} binding emulated that of mRNA and was therefore used to detect the level of mRNA bound to 30S subunits in this assay, the possibility of unstable initiator tRNA binding could not be entirely excluded as the underlying cause of incomplete ΔS20 30S occupancy with mRNA. Thus the stability of initiator tRNA binding was measured by its rate of dissociation from 30S pre-initiation complexes. The results demonstrated that dissociation of fMet-tRNA^{fMet} occurred at the same rate for the mutant and wild-type. Hence, we could conclude that reduced initiator tRNA binding was a secondary, consequential effect of a primary impairment in mRNA binding (Tobin et al., 2010).

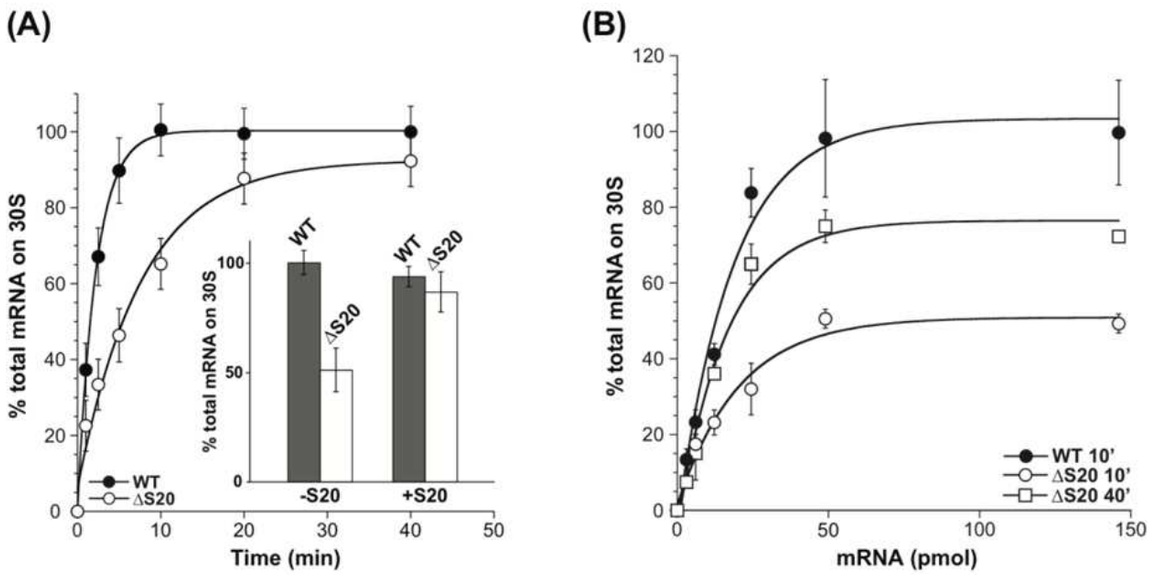


Fig. 8. **Activity of wild-type and S20-depleted 30S subunits in mRNA binding.** (A) The rate of mRNA binding to the mutant 30S subunit is reduced by a factor of approximately 3.5. Upon reconstitution with purified S20 protein, this impairment is removed (insert). (B) Upon titration with mRNA, a considerable fraction of the mutant 30S subunits failed to associate with mRNA. (Tobin et al., 2010)

4.5.3 Defective subunit association

The kinetics of 50S association to wild-type and S20-depleted 30S subunits was also measured in a stopped-flow instrument using light scattering. This assay demonstrated that naked ΔS20 30S subunits were severely impaired in association with wild-type 50S subunits and no improvement was observed when the 50S was added in excess (Figure 9A). However, a pronounced improvement (from ≤ 20% to ~ 40%) in the association capacity of the mutant 30S was observed upon pre-incubation with mRNA, initiator tRNA and the initiation factors. Similar to the results of mRNA binding, subunit association was further improved upon extension of the ΔS20 30S pre-incubation time with initiation components to 40 minutes (Figure 9B) (Tobin et al., 2010).

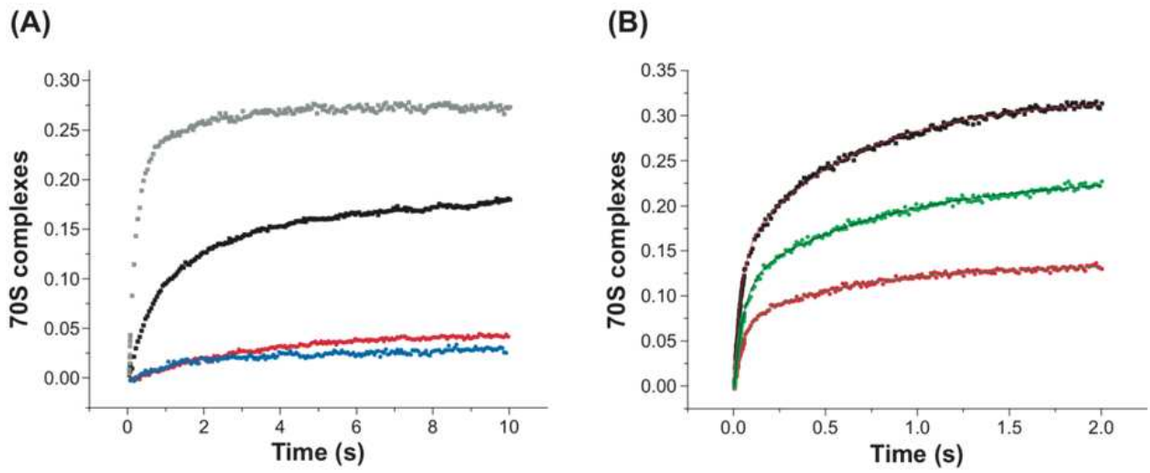


Fig. 9. **Kinetics of subunit association measured by light scattering.** (A) In the absence of the initiation ligands and when the ratio of 30S to 50S is 1:1, very few mutant 70S complexes are formed (red circles) compared to wild-type (black squares). The addition of 5x more 50S to the reaction (wild-type, grey squares; ΔS20, blue circles), did not increase 70S formation in the case of the mutant. (B) Association kinetics in the presence of mRNA, initiator tRNA, IF1 and IF2 following pre-incubation for 10 minutes (wild-type, black squares; ΔS20, red circles) and 40 minutes (ΔS20, green circles). (Tobin et al., 2010)

4.6 S20 is required for correct structural positioning of h44

Defects in the rate and extent of mRNA binding, as well as the poor association capacity of S20-depleted 30S subunits are likely to account for the reduced rate of protein accumulation observed *in vivo* and the prolonged generation time of the ΔS20 mutant. These impairments were puzzling as we could not reconcile them with the topographical location of S20 in mature 30S particles. The protein (Figure 10) is located at the base of the body of the 30S subunit, distal to the mRNA binding channel and subunit interface. However S20 has been shown to interact with helix 44 of the 16S rRNA during 30S assembly and in mature 30S particles (Brodersen et al., 2002; Dutca & Culver, 2008). High-resolution crystal structures have shown that this helix stretches across the entire length of the 30S body and forms part of the A- and P- sites where mRNA binding occurs (Schluenzen et al., 2000) and it also forms many bridging contacts with the 50S subunit (Selmer et al., 2006). Hence, upon removal of S20, h44 most likely adopts a suboptimal structural position that impairs the binding of mRNA and inhibits association with the 50S subunit (Tobin et al., 2010). Since prolonged incubation with the initiation components concealed these defects to some degree, it is likely that these 30S ligands promote a structural rearrangement of h44 that permits more stable binding of mRNA and facilitates docking of the 50S subunit.

5. R-protein replacement mutants

Besides the knowledge that can be gained regarding the essentiality and putative roles of r-proteins by examining genetically defined deletion mutants, r-protein replacement studies offer a unique opportunity to ask some more general questions. Using lambda red recombineering, the ORFs of S20, L1 and L17 were replaced with both closely related and

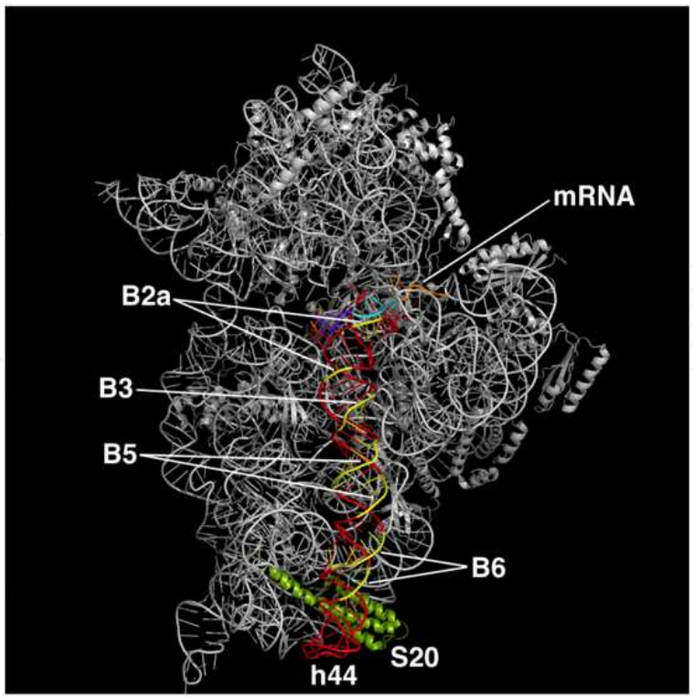


Fig. 10. **Structure of the 30S subunit highlighting the proposed involvement of S20 in mRNA binding and subunit association.** S20 (green ribbon) interacts with and possibly stabilizes h44 (red) in an orientation that is optimized for mRNA (orange; A-site, purple; P-site, cyan) binding and interactions with the 50S subunit (bridges B2a, B3, B5 and B6 depicted in yellow) (Tobin et al., 2010). All other rRNA and protein residues are depicted in grey. The image was created in PyMol (www.pymol.org) from the coordinates of the *T. thermophilus* 30S subunit (PDB ID 2J00) (Selmer et al., 2006).

highly divergent orthologues on the chromosome of *S. typhimurium* (Lind et al., 2010). Such hybrid mutants address fundamental evolutionary questions regarding translational robustness and the plasticity of the *bone fide* ribosomal components. Highly sensitive fitness measurements were performed to directly examine the costs of replacement and to indirectly determine the functional capacity of hybrid ribosomes. In addition, compensatory evolution of replacement mutants with large fitness costs revealed a general mechanism for suppressing loss of functionality when a divergent protein is imported into the ribosome.

5.1 Modest fitness costs upon r-protein replacement

Considering the highly conserved nature of r-proteins and their involvement in one of the key processes of gene expression, their removal was expected to confer substantial reductions in cell fitness. The physiological effects of r-protein replacement however, was more difficult to predict, although it was reasonable to expect relatively weaker fitness costs, even if the non-native protein lacked a high degree of homology to the host protein. The non-native replacement proteins ranged from close relatives, such as those from other proteobacteria *E. coli* and *Klebsiella pneumoniae,* to phylogenetically distant orthologues such as those from the eukaryote *Saccharomyces cerevisiae* and the archaeon *Sulfolobus acidocaldarius* (Lind et al., 2010). Exponential growth rates were measured in four different media where the doubling time of

the wild-type strain varied from 20 – 120 minutes and the growth rates of hybrid strains were expressed relative to this (Figure 11A showing L1 replacement mutants only). In the case of the S20 replacement mutants, all strains had higher fitness than the null mutant, irrespective of the growth medium used and even when amino acid identity fell to only 32%. Similarly, the fitness of the majority of L1 replacement mutants was only modestly reduced relative to wild-type, although more substantial costs were evident when the amino acid identity of the homologue reached as low as 20-30% (Figure 11A). Since L17 is an essential r-protein, comparisons to a null mutant were not feasible, however, a substantial fitness reduction relative to the wild-type, was only observed when amino acid identity of the orthologue fell below 50%. Interestingly, higher fitness of certain L17 hybrid mutants relative to wild-type was also evident. A general trend that emerged was a reduction in relative fitness as phylogenetic distance increased, however the costs of replacement for the more homologous proteins were diminutive in comparison to the costs of removal.

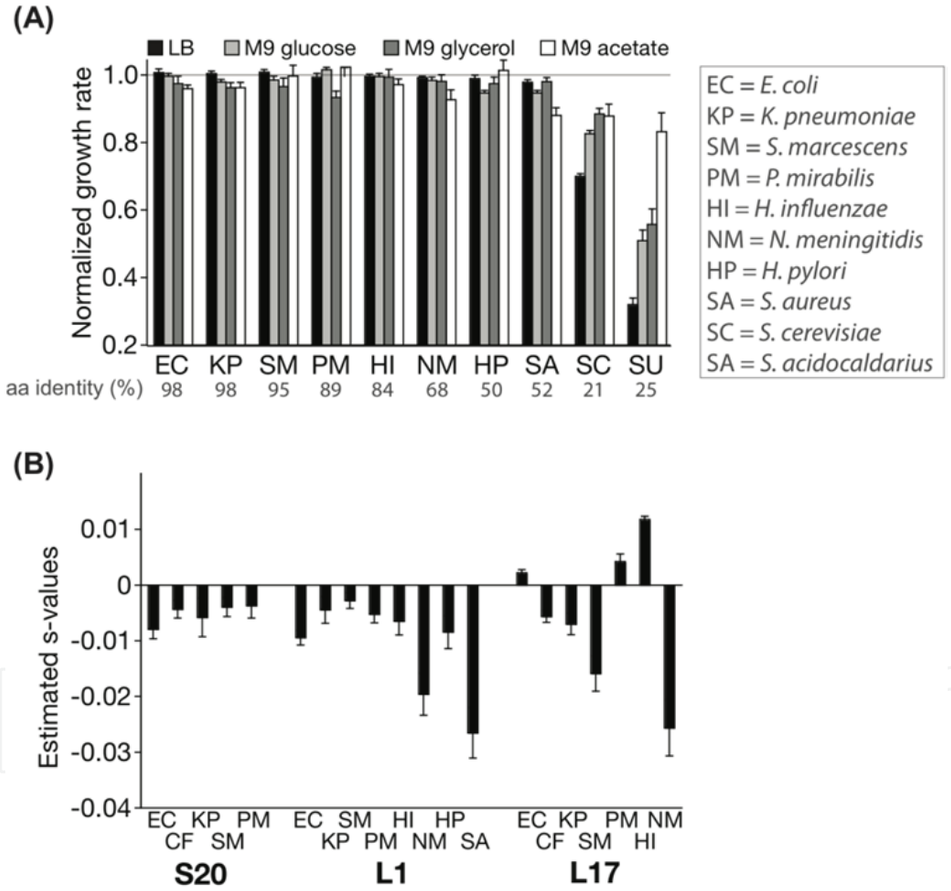


Fig. 11. **Fitness costs associated with replacement of r-proteins.** (A) Exponential growth rates of L1 replacement mutants in LB and minimal M9 medium with glucose, glycerol or acetate as carbon source. The growth rate of each mutant was normalized to that of a congenic wild-type strain in each respective medium (set to 1.0). (B) Selection coefficients associated with non-native S20, L1 and L17 r-proteins, estimated by means of pair-wise competitive growth with a congenic wild-type strain. Error bars in (A) and (B) represent standard error of the mean (SEM).

For those replacement mutants that displayed very small fitness costs as measured by exponential growth, pair-wise competition experiments were performed with the wild-type for a more sensitive estimate of the fitness costs (Lind et al., 2010). Using this method, we found that the majority of transfers were deleterious. In Figure 11B, the selection coefficient associated with each non-native protein is shown, which, in most cases, was less than or equal to -0.01, meaning that most of the imported genes conferred costs of 1% or less. Modelling was also performed to determine if such weak fitness costs represent an effective barrier for fixation of these proteins in nature via horizontal gene transfer. The results suggest that the fixation probability of such modestly counter-selected alleles is virtually nil in large natural populations of bacteria (Lind et al., 2010).

5.2 High functional conservation of r-proteins

Since the rate of bacterial growth is the product of the cellular ribosome concentration, times the rate of ribosome function (Bremer & Dennis, 1996), the fitness assays also provide an indirect measure of the translational capacity of the hybrid ribosomes. Given the generally larger fitness costs associated with complete loss of function in the null mutants, we were certain that the majority of non-native proteins improved fitness and thus appear to be at least semi-functional. Even though the replacement genes were often highly divergent in terms of base composition, codon usage and amino acid identity, overall, the fitness costs were relatively weak and significant reductions in fitness were only observed when the amino acid identity fell below 50% or so. So, although some of these proteins evolved in highly divergent organisms, they appear to function extraordinarily well in their non-native context. For example, L1 from *Helicobacter pylori* only shares 50% amino acid identity with the native *S. typhimurium* protein; the cost of replacement, however, is extremely small (approximately 1%). This clearly demonstrates the robustness of the ribosome and protein synthesis in general, and it also suggests that functional constraints are highly conserved between these proteins.

5.3 Increased dosage rescues suboptimal r-proteins

Those replacement mutants that harboured fitness costs of 10% or greater were subject to compensatory evolution to determine if and by what mechanisms fitness compensation could be achieved. After between 40 – 250 generations of growth, the S20 mutant (carrying the *H. influenzae* orthologue) and the L1 mutant (carrying the *S. cerevisiae* orthologue) showed increased fitness and were examined for an increase in the copy number of the non-native gene by Southern hybridization (Lind et al., 2010). Indeed, a two- to three-fold increase was detected in some of the lineages and corresponded to increased expression of the orthologous protein when Western blots were performed. To verify that overproduction of the non-native protein constitutes a general mechanism of fitness compensation, the orthologous proteins were expressed from an inducible high-copy number plasmid and fitness was measured. Exponential growth rates confirmed that for the majority of replacement mutants, increased dosage of the non-native protein conferred higher fitness (Figure 12). Thus, insufficient expression of the divergent protein (possibly due to unstable mRNA/protein), or a requirement for more of the protein to restore proper ribosome function and/or assembly, are two likely scenarios that could be at least partially accountable for the fitness costs of r-protein replacement. Such imbalances in the

stoichiometry of interacting cellular components has previously been suggested to confer deleterious effects (Papp et al., 2003).

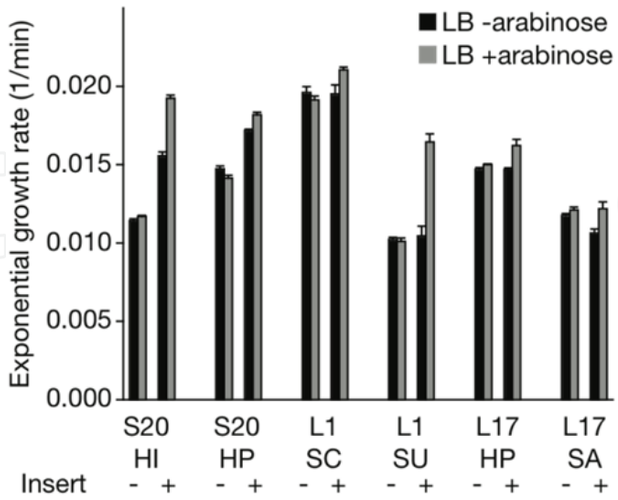


Fig. 12. **Growth compensation of r-protein replacement mutants via increased dosage of the non-native protein.** Exponential growth rates of mutants harbouring the arabinose inducible, high-copy number plasmid with (+) and without (-) the non-native gene. Abbreviations for divergent proteins from each respective species are the same as in Figure 11. Error bars represent SEM.

6. Concluding remarks and future perspectives

The ability to ascribe discrete functional roles to distinct components of the ribosome is essential to improve our understanding of how translation works and is a vital step towards furthering our knowledge of how this magnificent machine has evolved to the complex structure we see today in modern cells. The most fundamental aspect of the studies described herein, is directed towards resolving ribosome complexity to some degree in terms of the selective contributions made by certain r-proteins. The major tasks of the ribosome are RNA-mediated; however, the results of this study highlight the importance of the r-proteins for optimal function. Upon loss of either S20 or L1, we see that the cell is indeed viable; however, the substantial reductions in fitness reflect the requirement of the proteins for maintenance of proper ribosome function. In the case of both deletion mutants, the impairments in function appear to stem from perturbations in the higher order structure of the rRNA, suggesting, that both L1 and S20 are important in stabilizing the overall morphology of the subunits. Upon loss of S20, mRNA binding is severely impaired and association of the mutant 30S species with 50S subunits is drastically reduced. Both of these defects appear to occur as secondary effects caused by a primary disturbance in 16S rRNA structure; namely, distortion of the penultimate helix, h44. Similarly, in the absence of L1, an excess of free 50S subunits is formed in the cell, likely due to subunit misassembly in an L1-depleted environment. Moreover, the majority of compensatory mutations selected as a response to L1 removal were mapped to other r-proteins dispersed at various sites of the small and large subunit, suggesting that they may amend the structural perturbations to some degree. The variation in the topographical distribution of the compensatory mutations

also highlights the high degree of co-operativity inherent to the ribosome. Thus, in both cases, it seems that loss of the protein indirectly perturbs protein synthesis via alterations in the overall morphology of the ribosome. Perhaps this is not altogether surprising, considering that both proteins are primary rRNA binders and are thus considered particularly important for initiating global folding of the major rRNA domains. One major strength and unique feature of the studies described herein, is our attempt to explain the observed fitness costs in terms of the costs to ribosome function detected by our *in vitro* system. Unfortunately, very few studies have attempted to bridge the crucial gap between both sets of data. This is a considerable challenge, given the inherent complexity of the ribosome and the enormous level of co-operativity within the structure itself and at the cellular level. It is, however, an essential step towards capturing a complete picture of the mechanism of translation.

In contrast to the substantial costs conferred by r-protein removal, in general, replacement of such proteins with orthologous counterparts from other species had only modest deleterious effects on fitness. This finding implies that the non-native proteins are capable of functioning in their new environment, despite having evolved in divergent species, clearly demonstrating the plasticity and robustness of the translation apparatus. This may suggest conservation in the overall shape of the proteins or at least those residues that interact with the target sites of the ribosome, akin to structural mimics of the native proteins. Overall the results emphasize the superiority of functional requirement over conservation of the primary sequence of amino acids, lending support to the suggestion that the major function of the r-proteins is to maintain the structural integrity of the ribosome.

Future work in this area should concentrate on studies designed to delineate the specific roles of other r-proteins during translation. With a large array of approximately fifty proteins, the task is challenging. However, recent advances in genetic engineering has allowed us to construct precise deletions of various r-proteins, and the replacement approach offers a new avenue for dissecting the roles of essential proteins. By examining the function of hybrid ribosomes *in vitro*, distinct steps of the translation cycle can be evaluated and the contributions made by essential r-proteins could be exposed. The findings of these studies also highlight the usefulness of compensatory evolution in terms of revealing otherwise concealed cooperative mechanisms for partially restoring ribosome function.

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