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ADENOSINE DIMETHYLTRANSFERASE KsgA: BIOCHEMICAL CHARACTERIZATION OF THE PROTEIN AND ITS INTERACTION WITH THE 30S SUBUNIT

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

DTT Dithiothreitol

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

MIC Minimal inhibitory concentration

MgOAc magnesium acetate

SAM S-adenosyl-L-methionine

SAH S-asenosyl homocysteine

TCA trichloroacetic acid

Tris tris(hydroxymethyl)aminomethane

LB Luria-Bertani media

SDS PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

BME β-mercaptoethanol

HPLC High-Performance Liquid Chromatography

rRNA ribosomal ribonucleic acid

mRNA messenger ribonucleic acid

tRNA transfer ribonucleic acid

RNase Ribonuclease

snoRNA small nucleolar ribonucleic acid

MLS-B macrolide-lincosamide-streptogramin B

RNP ribonucleoproteins

snoRNP small nucleolar ribonucleoproteins

RI Reconstitution Intermediate

Abstract

ADENOSINE DIMETHYLTRANSFERASE KsgA:
BIOCHEMICAL CHARACTERIZATION OF THE PROTEIN AND ITS
INTERACTION WITH THE 30S SUBUNIT

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Major Director: Jason P. Rife, Ph.D.
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Ribosomes form the core of the protein biosynthesis machinery and are essential to life. Ribosome biogenesis is a complex cellular process involving transcription of rRNA, pre-rRNA processing, rRNA modification and simultaneous assembly of ribosomal proteins. RNA nucleotide modification is observed in all domains of life. While there is enormous conservation of ribosome structure, very few post-transcriptional rRNA modifications have been conserved throughout evolution. A notable example of such rare conservation is the dimethylation of two adjacent adenosines in the 3'-terminal helix, a highly conserved region of the small subunit rRNA. Enzymes that carry out these

dimethylations are equally conserved and are collectively known as the KsgA/Dim1 family of methyltransferases.

The first member of the family, KsgA, was identified in *E. coli* as the determinant for resistance to the aminoglycoside antibiotic Kasugamycin. Orthologs have since been described in organisms of wide spread evolutionary origins as well as in eukaryotic cellular organelles, thus underscoring the unprecedented conservation of this family of enzymes and the resultant rRNA modification. The higher evolutionary orthologs of KsgA have adopted secondary roles in ribosome biogenesis in addition to their dimethyltransferase role. The eukaryotic ortholog, Dim1, is essential for proper processing of the primary rRNA transcript. Recently, KsgA has been speculated to function as a late stage ribosome biogenesis factor and a Δ*ksgA* genotype in *E. coli* has been linked to cold sensitivity and altered ribosomal profiles.

This report focuses on the biochemical characterization of KsgA and its interaction with the 30S subunit. We have established the salt conditions required for optimal KsgA methyltransferase activity while confirming that KsgA recognizes a translationally inactive conformation of 30S subunit in vitro. Our study of the functional conservation of KsgA/Dim1 enzymes in the bacterial system revealed that KsgA and the evolutionarily higher orthologs could recognize a common ribosomal substrate. This indicates that the recognition elements of both, the protein and the small subunit, have remained largely unchanged during the course of evolution. Finally, based on our site directed mutagenesis and biochemical studies, we report that KsgA binds to structural components of 16S rRNA other than the helix containing the target nucleosides.

CHAPTER 1: Introduction

Ribosome biogenesis

The ribosome is a universal complex enzyme essential to life. Ribosomes form the core of the protein biosynthesis machinery which manufactures proteins based on the genetically encoded messages. Since this discovery nearly five decades ago, the biogenetic, biophysical and functional aspects of the ribosome have become subjects of intensive research. For decades, microbial ribosomes have been exploited therapeutically as proven targets for anti-microbial agents[1]. The work reported in this dissertation focuses on a universally conserved ribosomal RNA modification and the equally conserved family of proteins responsible for the modification.

Under favorable growth conditions, a single cell of *Escherichia coli* can contain up to 100,000 ribosome copies; accounting for nearly 40% of the dried cell mass[2, 3]. Ribosomes consist of two unequal subunits; a large subunit (50S/60S) and a small subunit (30S/40S) in prokaryotes/eukaryotes, respectively. Each subunit is composed of ~65% ribosomal RNA (rRNA) and ~35% ribosomal proteins. Ribosome biogenesis, the process by which ribosomes are synthesized, is a fundamental cellular process involving transcription of rRNA, pre-rRNA processing, rRNA modification and simultaneous assembly of ribosomal proteins[4]. The process demands a high level of regulation, coordination and energy expenditure. It is estimated that a proliferating cell devotes more

than 50% of its cellular energy for the process of ribosomal synthesis, processing, and assembly[2].

Much of our understanding of the process of ribosome biogenesis in prokaryotes comes from years of work on *E. coli*[5-8]. The pre-16S rRNA undergoes multiple processing and maturation steps as it is transcribed, to give mature 16S rRNA[9]. Several non-ribosomal factors, including RNases are involved in this process. Based upon many observations, it is thought that 30S subunit assembly occurs co-transcriptionally or during 16S rRNA maturation, and that the modification of nucleotides within 16S rRNA occurs concurrently with the process of 30S subunit assembly. However, the order of processing and subunit assembly events remains largely unknown.

In vitro studies have contributed significantly to our understanding of ribosome biogenesis. A major milestone was achieved in 1968, when Traub and Nomura demonstrated that functional ribosomal subunits could be reconstituted in vitro using only the component rRNAs and ribosomal proteins purified from *E. coli*[5]. Subsequent to this discovery, functional 30S subunits were also reconstituted using individually purified 30S subunit proteins[10], either in natural or recombinant form, and in vitro transcribed 16S rRNA[11, 12], which lacked nucleotide modifications. These reconstituted subunits have the same sedimentation behavior as natural subunits. They also function in tRNA binding and polyphenylalanine synthesis studies, although with lesser activity than the natural ribosomes[11-13]. Neverthless, in vitro assembly remains an important achievement since it enabled biochemical studies that could not have been performed otherwise.

Another major milestone that enhanced the biophysical study of ribosomes was the first high resolution crystal structure of ribosomal subunits, the small subunit from the eubacterium *Thermus thermophilus*[14] and the large subunit from the archaeon *Haloarcula marismortui*[15]. These structures were followed by that of the large subunit from the mesophilic eubacterium *Deinococcus radiodurans*[16] and that of the 70S ribosomes from *T. thermophilus*[17] and *E. coli*[18]. Later, Selmer et al. solved the structure of *T. thermophilus* 70S ribosome complexed with mRNA and tRNA, showing that the ribosome is indeed a ribozyme[19]. These structures have contributed invaluably to our understanding of the structure and function of the ribosome.

In vitro as well as in vivo experiments have demonstrated that 30S subunit assembly is highly cooperative involving sequential addition of proteins and conformational changes in the growing subunit[9]. In vitro assembly experiments have allowed construction of a bacterial 30S subunit assembly map (Figure 1), which shows that ribosomal proteins assemble cooperatively onto the pre-16S rRNA in a roughly 5' to 3' order, indicating co-transcriptional assembly in vivo[20]. The 30S subunit can structurally be divided into three regions – body (5' domain), platform (central domain) and head (3' domain). The assembly map shows that the body is assembled first, followed by the platform, with the head forming last.

The in vitro reconstitution of 30S subunit proceeds through two experimentally determined intermediates[6] (Figure 2B). At low temperatures (0 ˚C to 15 ˚C), a particle is formed which sediments at 21S. This particle has been termed RI, for Reconstitution

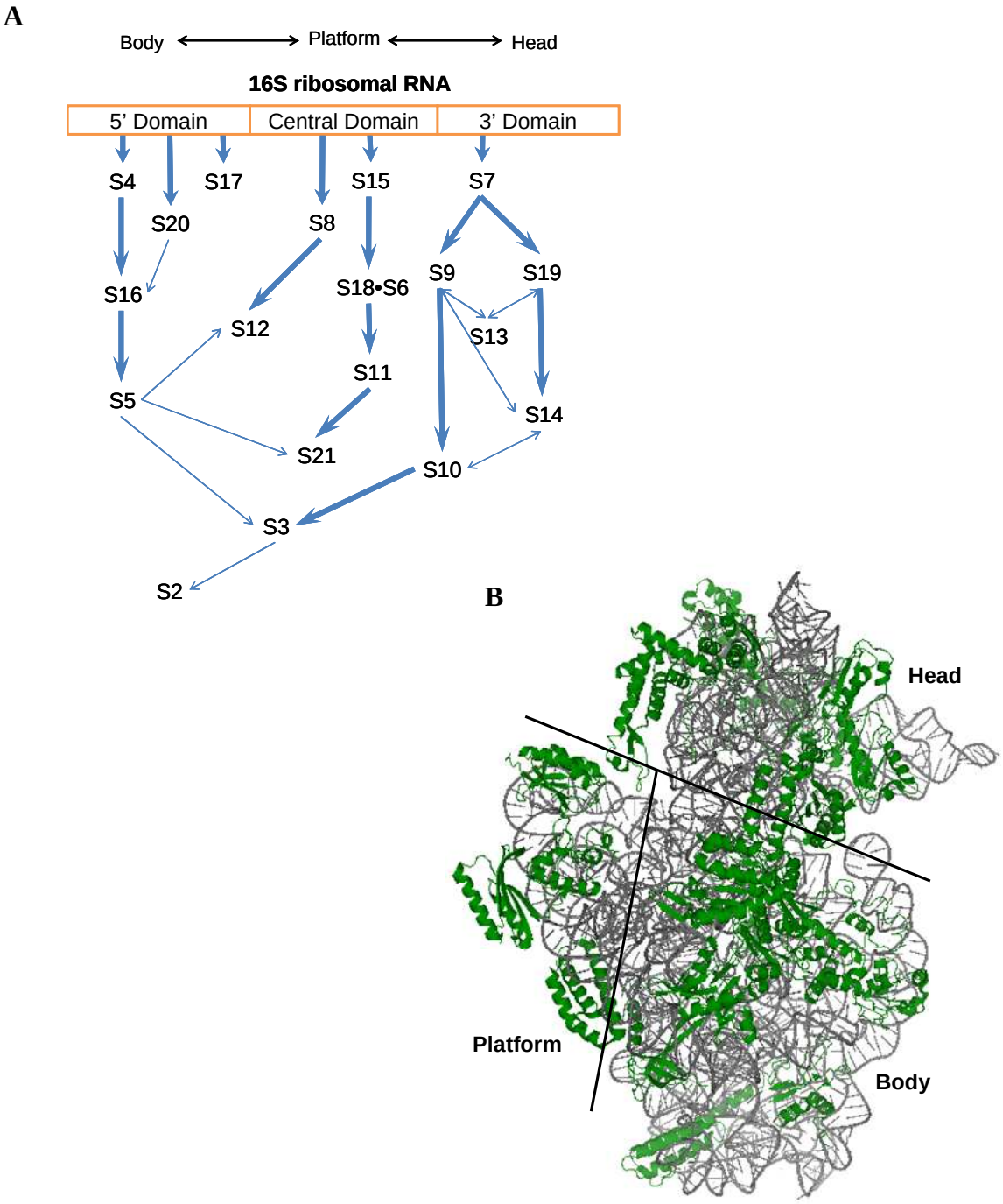


Figure 1. Assembly of the 30S subunit. (A) Assembly map (B) Crystal structure of 30S subunit. The three domains of 30S are roughly delineated and labeled. This figure was rendered using Pymol from the coordinates generated by Wimberly et al. (PDB ID 1J5E)[14].

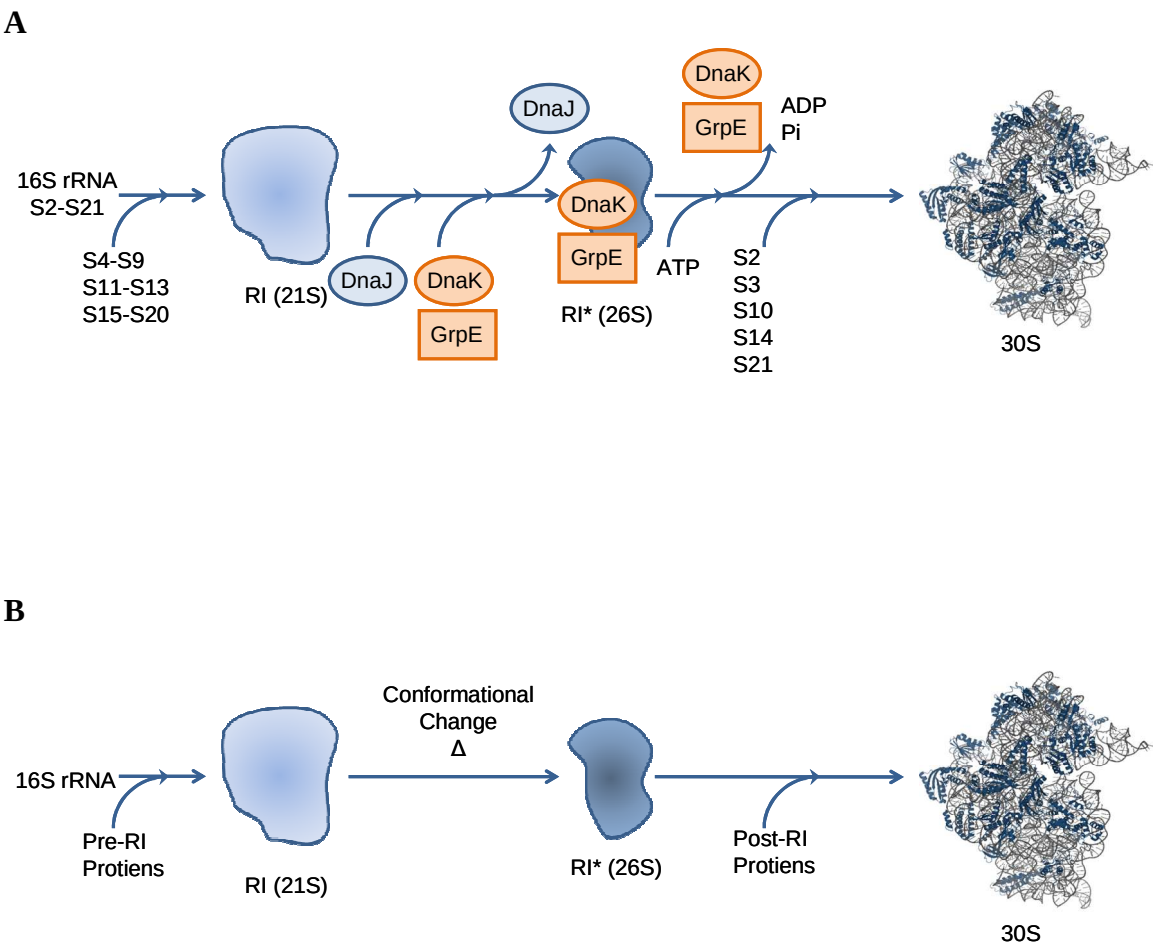


Figure 2. 30S assembly in *E. coli*. (A) In vivo assembly. The chaperon system brings about the conformational change from RI to RI*. (B) In vitro assembly. Heat can bring about the conformational change from RI to RI*.

Intermediate, and consists of 16S rRNA plus a subset of ribosomal proteins (S4-S9, S11-S13, and S15-S20). Most of these proteins are components of the body and platform. Unimolecular conformational rearrangement of RI yields RI*, a more compact particle with sedimentation coefficient of 26S, yet identical to RI in composition. The RI to RI* transition is the rate limiting step in assembly and requires either heat (42 ˚C) or the presence of a DnaK chaperone system to overcome the activation energy barrier[21] (Figure 2A). Once RI* has been formed, the remaining ribosomal proteins readily assemble on, even at low temperatures. Intermediates analogous to RI have been seen in vivo in certain cold sensitive mutants[22], indicating that the in vitro reconstitution truly resembles the 30S subunit assembly in vivo.

Although prokaryotic and eukaryotic ribosomes share similar basic architecture, ribosome biogenesis in eukaryotes is much more complex than that in prokaryotes. More than 150 factors[23], including snoRNPs, GTPases, helicase, nucleases, transport proteins, and chaperones are thought to be involved in eukaryotic ribosome biogenesis. Ribosome synthesis starts in the nucleolus, where most of the maturation also takes place. The next few steps take place in the nucleus, with the final steps being completed in the cytoplasm[24, 25] (Figure 3). 35S rRNA undergoes processing and maturation to yield the component rRNAs of the eukaryotic ribosome. As the pre-35S rRNA is being transcribed in the nucleolus, several factors including U3 snoRNP, small subunit ribosomal proteins and non ribosomal factors associate with it to form a 90S particle[26]. These particles correspond to the terminal ‘knobs’ seen in Miller chromatin spreads, a technique that

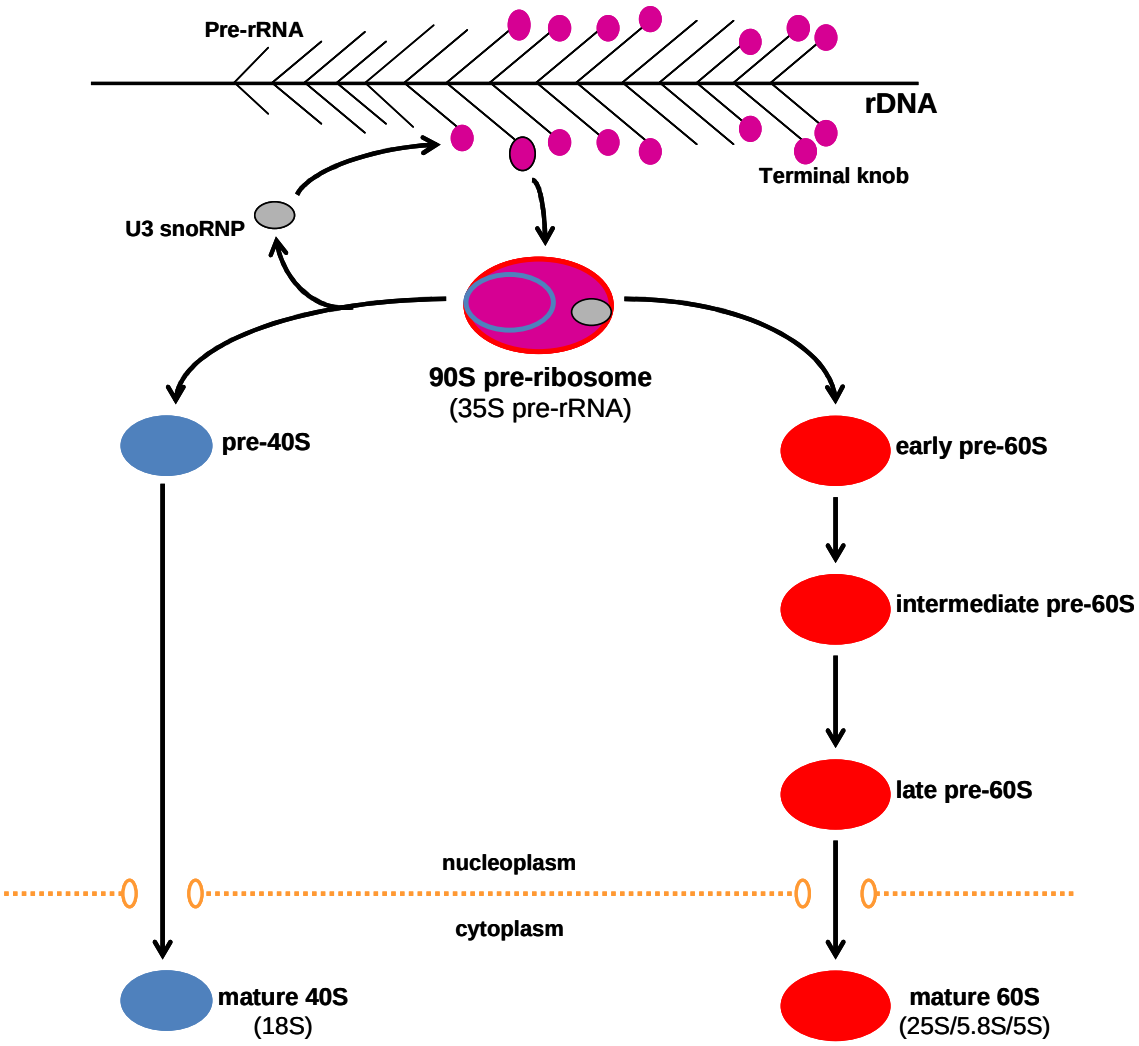


Figure 3. Schematic representation of Eukaryotic ribosome assembly. The terminal 'knobs' are shown as spheres on the transcribing rRNA.

allows observation of actively transcribing rDNA by electron microscopy[27, 28]. Next, the pre-rRNA undergoes cleavage at three sites to yield mostly complete pre-40S (small subunit) particle and a very immature pre-60S (large subunit) particle[24]. Both particles are then exported to the cytoplasm, where a few final modifications yield the mature 40S particle. The pre 60S maturation is much more complicated and requires several processing steps to yield mature 60S. The association of ribosomal proteins occurs throughout the process of maturation, but detailed assembly pathways are not known. Also, in vitro reconstitution of eukaryotic ribosomes solely from the component rRNAs and ribosomal proteins have not been achieved yet.

Ribosomal assembly in the archaeal system is not very well understood. The ribosomal protein composition is not the same in all archaeal organisms and depends on the phylogeny of the species[29]. Archaeal cells lack a nuclear membrane; however, at least one archaeal genome has been reported to contain putative homologs to nuclear and nucleolar structural genes from eukaryotes[30, 31]. Therefore, the possibility of spatio-temporal control of ribosome synthesis cannot be totally discarded. In vitro, assembly of functional arcahaeal ribosomes from component rRNAs and ribosomal proteins occurs spontaneously; however, conditions reflecting the organism's natural environment are required[32-34]. For example, thermophilic ribosomes require high temperatures[34] whereas halophilic ribosomes require high salt concentrations[35] for in vitro assembly.

Nucleotide Modification

RNA nucleotide modification is observed in all domains of life. Although the function of most rRNA nucleotide modifications remains unknown, the general view

holds that they serve to fine tune the ribosomal structure and function. Nucleotide modification occurs simultaneous to pre-rRNA processing and subunit assembly events in ribosome biogenesis. There are three major types of modifications in rRNA – conversion of uridine to pseudouridine, 2'-O-ribose methylation, and base methylations[36] (Figure 4).

Modifications in bacteria are mainly base methylations and are performed by specific enzymes, one enzyme catalyzing only one modification in most cases[37]. Contrastingly, modifications in eukaryotic ribosomes are mainly pseudouridinylations and 2'-O-ribose methylations with only a few base methylations[38]. Also, in eukaryotic cells, similar modifications are carried out by a common enzyme, guided by snoRNAs to the specific location[39]. The archaeal rRNA modification is less well characterized, and there is a great deal of variation from species to species. Modifications are mostly 2'-O-ribose methyls with only a few pseudouridines[40]. With snoRNAs guiding the modifications, the archaeal modification system appears to be more similar to that in eukaryotes[41-43].

The basic architecture of the ribosome has remained largely conserved throughout evolution[44]. The modified nucleotides are clustered within the conserved, functionally important regions of the ribosome[45]. However, no single rRNA modification has been reported to be essential for ribosome function. Collective blockade of pseudouridylations and 2'-O-methylations showed severe growth defects in *Saccharomyces cerevisiae*[46]. Similarly, eubacterial ribosomes reconstituted using in vitro

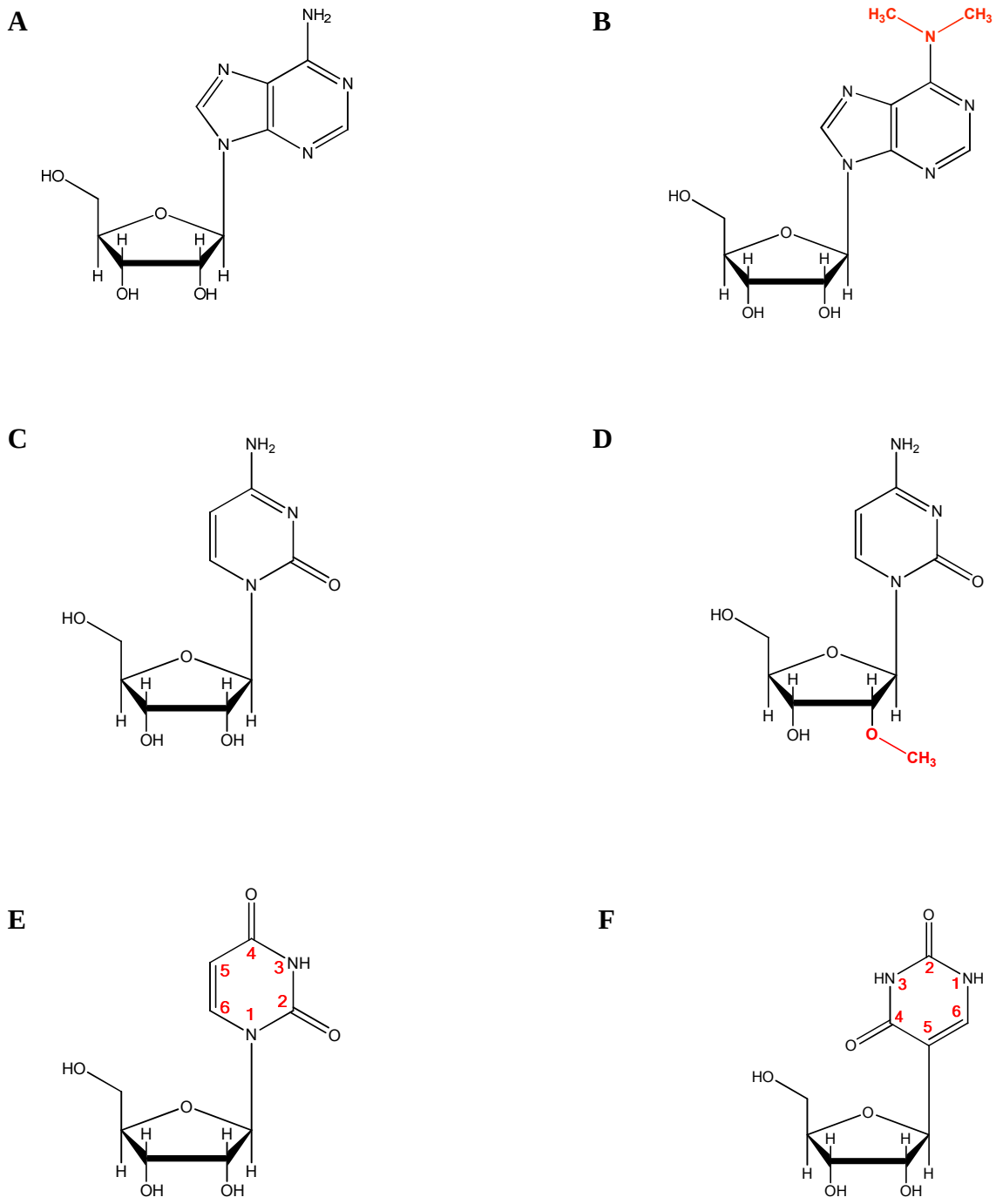
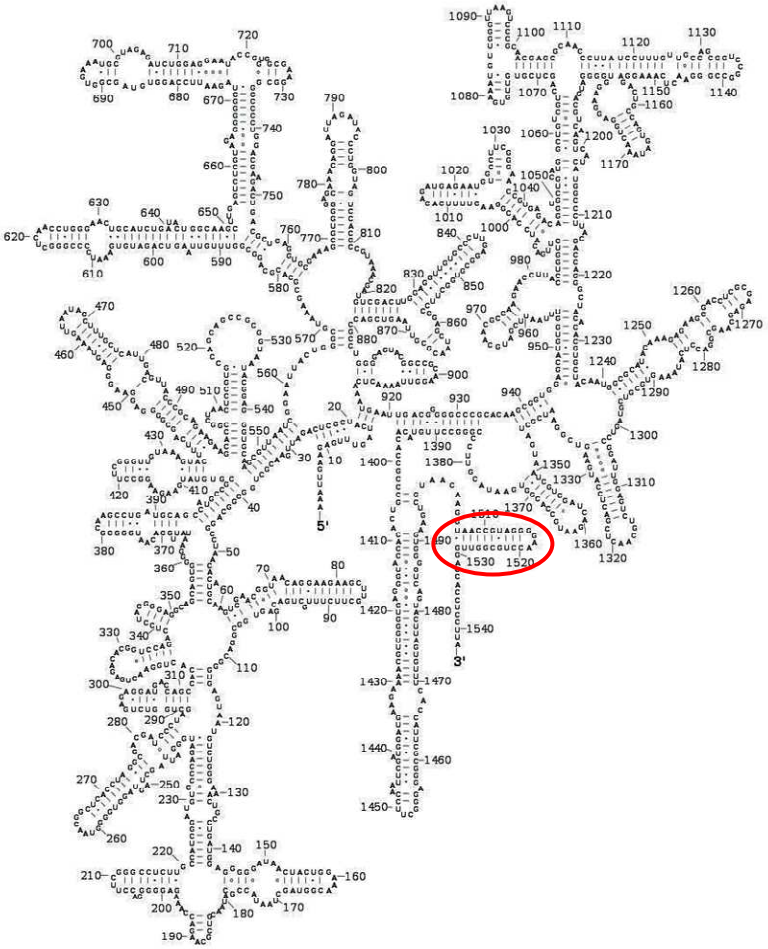


Figure 4. Nucleoside modifications. (A) Adenosine, (B) N^6, N^6-dimethyladenosine, (C) Cytidine, (D) 2'-O methylcytidine, (E) Uridine, (F) Pseudouridine

transcribed, unmodified rRNA show much less activity in peptide synthesis than ribosomes reconstituted from natural rRNA[47-49]. Thus, it seems that individual modifications are indeed involved in fine tuning of ribosome's structure and function, offering a larger benefit, collectively.

While there is enormous conservation of ribosome structure between the prokaryotic, archaeal and eukaryotic ribosomes, very few post-transcriptional rRNA modifications have been conserved throughout evolution. A notable example of such rare conservation is the dimethylation of two adjacent adenosines in the 3'-terminal helix of small subunit rRNA, A1518 and A1519 in helix 45 by *E. coli* numbering system (Figure 5). The only known exceptions are in *Saccharomyces cerevisiae* mitochondrial 12S rRNA, which has no methyl groups on the two adenosine bases[50]; *Euglena gracilis* chloroplast rRNA[51] and the 16 S rRNA of the archaebacterium *Sulfolobus solfataricus*[52], both of which have only one dimethylated adenosine base. The 3'-terminal helix, which is involved in linking the small subunit to the large subunit[53] of the ribosome and in binding to mRNA[54], is one of the most conserved regions of the small subunit[55, 56]. Enzymes that carry out the dimethylations in this highly conserved region are equally conserved and are collectively known as the KsgA/Dim1 family of methyltransferases, where KsgA is the bacterial ortholog and Dim1 is the eukaryotic ortholog.

A



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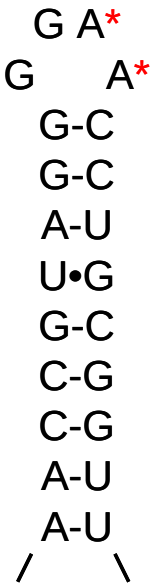

Figure 5. Dimethyladenosines in the 16S rRNA of *E. coli.* (A) Secondary structure of the *E. coli* 16S rRNA. Helix 45, where the two dimethylated adenosines are located, is circled in red. (B) Helix 45. Stars indicate modified adenosines.

KsgA/Dim1 Family of Methyltransferases

The presence and function of the KsgA/Dim1 family of enzymes have been maintained throughout all domains of life, including the eukaryotic cellular organelles. The enzyme was first described in *E. coli*, where it catalyzes the conversion of two adjacent adenosines A1518 and A1519, also conserved, in the 30S subunit rRNA to N^6, N^6-dimethyladenosines (Figure 6)[57, 58]. Orthologs have since been described in organisms of diverse origins including KsgA in the eubacteria *Bacillus stearothermophilus*[59], Dim1 in *Saccharomyces cerevisiae*[60] and *Kluyveromyces lactis*[61], and MjDim1 in the archaea *Methanocaldococcus jannaschii*[62]. Analogus methyltransferases, h-mtTFB and Pfc1, have been identified in the human mitochondria[63] and in the chloroplasts of *Arabidopsis thaliana*[64], respectively. Although KsgA/Dim1 from no other species has been tested, the wide spread evolutionary origins of the above members underscore the unprecedented conservation of this family of enzymes and the resultant rRNA modification.

KsgA (Figure 8A) is dispensable in bacteria with only modest consequences and forms the dominant mode by which bacteria become resistant to the aminoglycoside antibiotic kasugamycin[57, 65]. A KsgA knockout mutant lacks the dimethyls on A1518/A1519 and shows slower growth rate in *Escherichia coli* and reduced virulence in the human pathogen, *Yersina pseudotuberculosis*[66-68]. Recently, KsgA has been speculated to function as a late stage ribosome biogenesis factor[69].

The higher evolutionary members of this family have adopted secondary roles in ribosome biogenesis in addition to their dimethyltransferase role. Eukaryotic Dim1

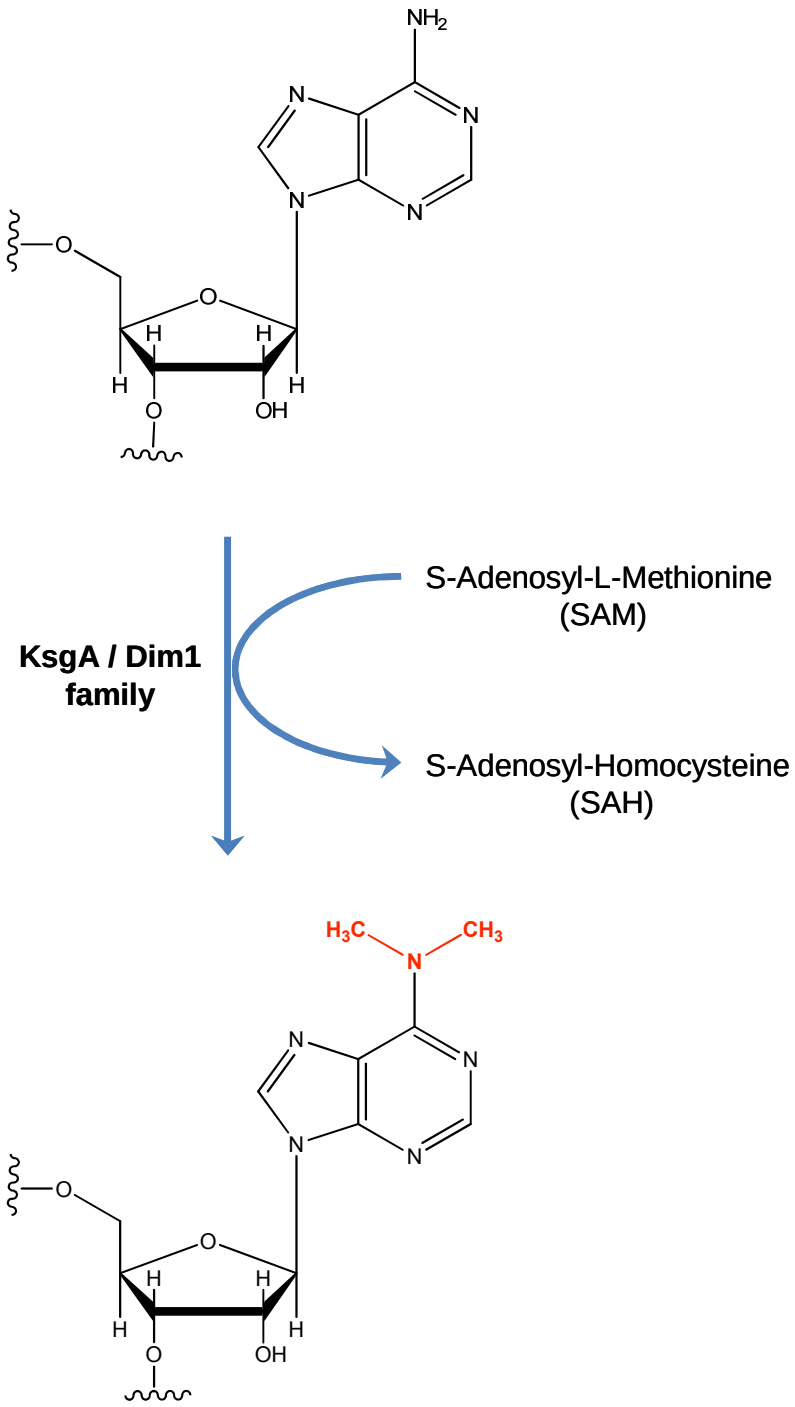

Figure 6. Conversion of adenosine to N^6, N^6-dimethyladenosine catalyzed by the KsgA/Dim1 family of methyltransferases. Two molecules of SAM are consumed per adenosine.

(Figure 8B) is a member of the processome and is essential for proper processing of the primary rRNA transcript[70]. Knockout of Dim1 in yeast is lethal[60]; loss of Dim1 in *S. cerevisiae* leads to depletion of 18S rRNA and buildup of an aberrant 22S rRNA species as a result of misprocessing[70]. Pfc1, the chloroplast ortholog, has been found to be important for proper development of chloroplasts at low temperatures[64]. Depletion of Pfc1 results in accumulation of misprocessed rRNA, suggesting a role similar to Dim1.

The human mitochondrial ortholog h-mtTFB was originally demonstrated to be a mitochondrial transcription factor[71]. h-mtTFB has also been linked to hereditary deafness associated with a polymorphic A1555G mutation in mitochondrial rRNA[72]. In some mitochondria, there are two separate mtTFB proteins, mtTFB1 and mtTFB2, which are proposed to have arisen from a gene duplication event[73]. As per the evidence, mtTFB1 is more active as a methyltransferase, while mtTFB2 is more active as a transcription factor[73, 74]. The fungi have only a single mtTFB, suggesting loss of one of the paralogs in this lineage. The *S. cerevisiae* ortholog, sc-mtTFB, serves as a transcription factor but has completely lost its methyltransferase activity[50]. sc-mtTFB lacks significant sequence homology to any of the KsgA/Dim1 enzymes and yeast mtTFBs are poorly conserved and difficult to identify via sequence homology[75].

Dim1 of *S. cerevisiae* and h-mtTFB have been shown to dimethylate A1518 and A1519 of *E. coli* 30S in vivo[63, 76]. However, similar experiments by Pulicherla et al. to test for complementarity of archaeal Dim1 and bacterial KsgA in *S. cerevisiae* revealed that neither the bacterial nor the archaeal ortholog could complement for the eukaryotic Dim1[77]. This might be related to the secondary, non-methyltransferase function that

Dim1 is known to play in eukaryotic ribosome biogenesis. In the cases of both Dim1 and h-mtTFB, the methylating activity is separate from the second function; methylation-deficient mutations can be made, which leave the rRNA processing or transcription activity intact[76, 78]. Pulicherla et al. also confirmed that in *S. cerevisiae*, none of the methyl groups transferred by Dim1 are critical for growth under standard conditions and a variety of temperatures[77].

The Erm family of methyltransferases, which confers resistance to the macrolide-lincosamide-streptogramin B (MLS-B) group of antibiotics, are important paralogs of the KsgA/Dim1family[79]. These enzymes share high sequence homology with the KsgA family[80] and have most likely resulted from one or more KsgA gene duplication events[81]. The Erm enzymes transfer one or two methyl groups to a single adenosine of 23S rRNA, near the peptidyl transferase site of the 50S subunit[79].

The KsgA/Dim1 family belongs to a well-characterized group of S-adenosyl-L-methionine (SAM) dependent methyltransferases which includes RNA methyltransferases, DNA methyltransferases, protein methyltransferases and small molecule methyltransferases. Many of these enzymes have been well characterized structurally and biochemically. Enzymes of this group contain a Rossman-like fold, which consists of a central β-sheet surrounded by a variable number of α-helices, with well-defined SAM and target nucleotide binding pockets[82]. Thus, the methyltransferase function of the KsgA/Dim1 enzymes had a clear structural basis, but the structural basis for other functions remained unclear for years. Recently O'Farrell et al. carried out extensive sequence alignments and structural comparisons of the KsgA, Dim1, sc-mtTFB

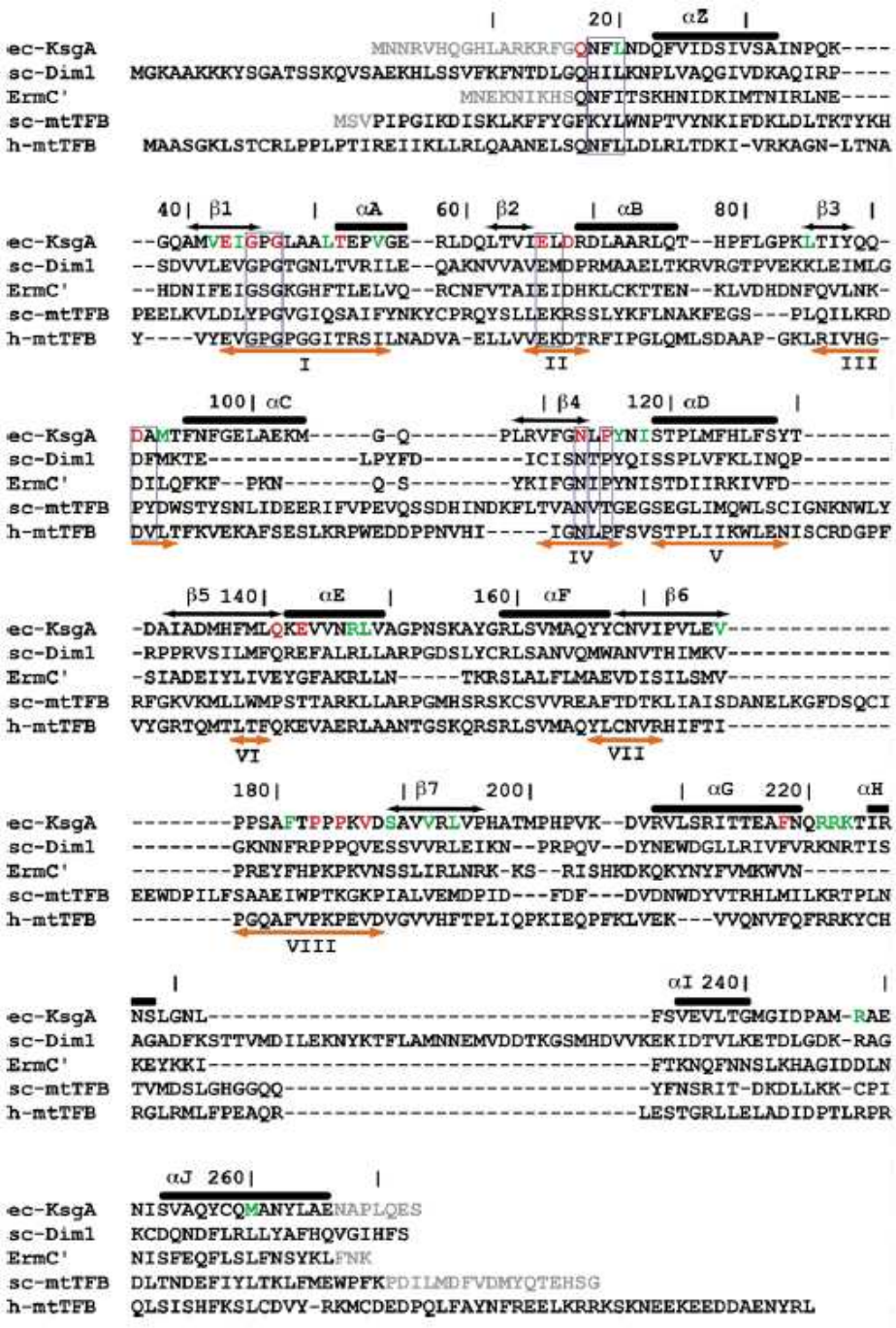

Figure 7. Sequence alignment of KsgA, Dim1, ErmC', h-mtTFB, and sc-mtTFB (GenBank accession nos. P06992, P41819, P13956, NP_057104, and P14908). Red letters designate residues that are absolutely conserved in KsgA enzymes, while green letters designate residues that are highly conserved. Figure adopted from elsewhere[84].

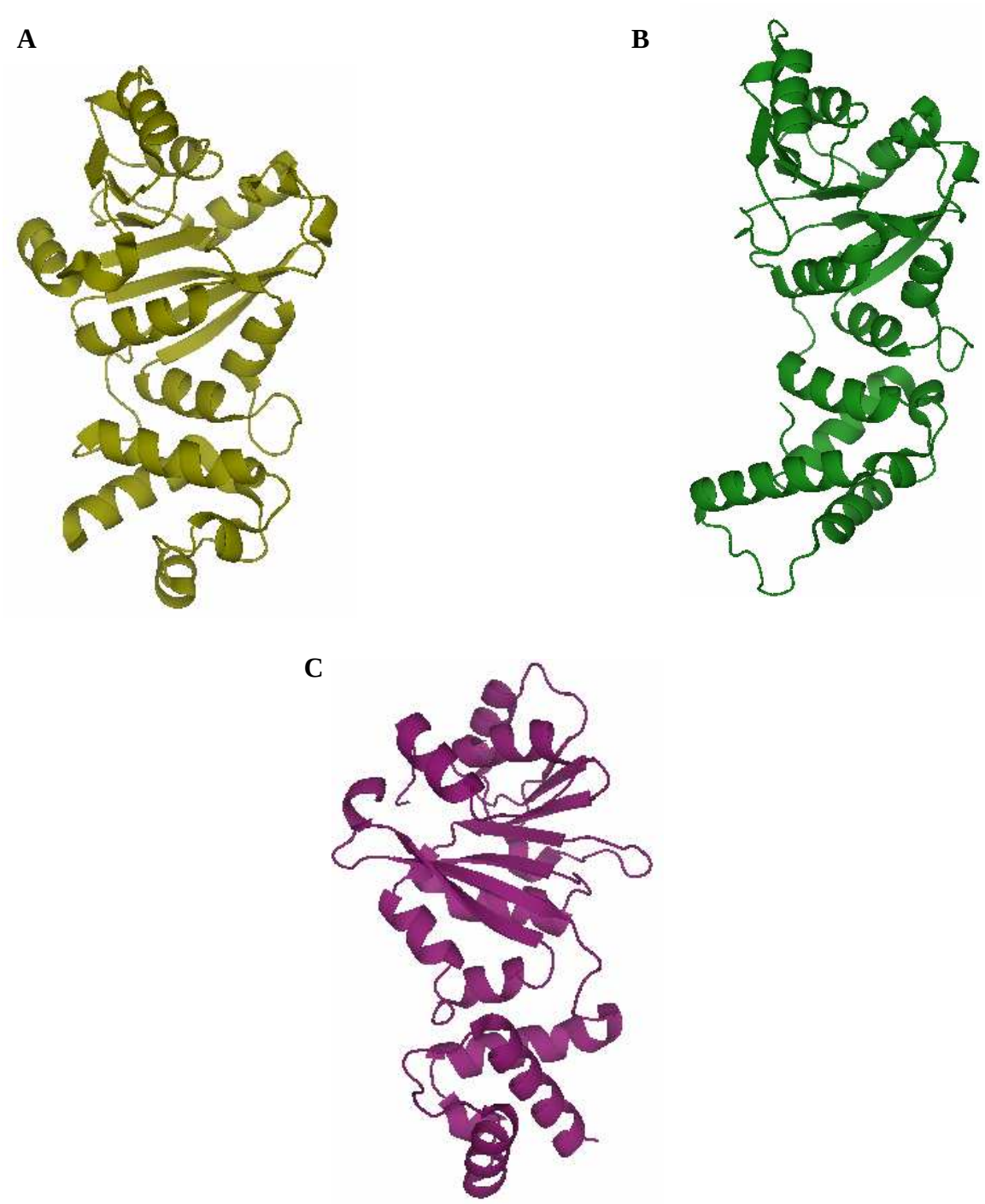

Figure 8. Crystal structures of (A) KsgA (PDB ID: 1QYR)[84](B) Dim1 (PDB ID: 1ZQ9; Dong, A.; Wu, H.; Zeng, H.; Loppnau, P.; Sundstorm, M.; Arrowsmith, C.; Edwards, A.; Bochkarev, A.; Plotnikov, A., unpublished data) (C) ErmC' (PDB ID: 1QAM)[85] All figures were generated using program Pymol.

and ErmC' enzymes and identified regions that are likely related to functions other than the common methyltransferase function (Figures 7 and 8)[83]. They reported that core regions common to the entire class of enzymes are associated with ribosome binding and therefore the rRNA methylation activity, whereas regions conserved in the subgroups are related to the non-methyltransferase functions.

KsgA

The *ksgA* gene was first described by Dahlberg et al. in 1971 as a locus that affected methylation in the 16S rRNA and determined resistance to the aminoglycoside antibiotic kasugamycin (ksg)[57]. 16S rRNA from the resistant mutant lacked dimethylation of two adenosines, A1518 and A1519, on the 3' terminal helix. Therefore, the *ksgA* locus was proposed to encode an rRNA methyltransferase. This was found to be true when an rRNA methylase activity, absent in the ksg resistant strains, was characterized in ksg sensitive strains. This methylase activity was also shown to alter the phenotype of ksg resistance by dimethylating in vitro the two adenosines in the 16S rRNA of resistant 30S subunits[65]. The methylase activity was later attributed to KsgA (Figure 8A), the product of *ksgA* gene. In the following decades KsgA has received intermittent scrutiny, uncovering facts about KsgA and its orthologs.

Although KsgA is universally conserved, it is startling that KsgA knockout is tolerated in bacteria with only modest consequences. Igarishi et al. described a kasugamycin resistant strain which grew slower than the wild type strain on certain culture media[66]. Also, the ribosomes from the resistant strain showed slower polypeptide synthesis in the in vitro assays than wild-type ribosomes. However, in this case, the translational impairment was not directly a result of the undermethylation of 16S rRNA.

The loss of modification likely affected the subunit assembly since ribosomes from the resistant strain were found to contain lower amounts of ribosomal protein S1 than those from the wild-type strain. S1 is essential for normal translation in *E. coli*[86]; ribosomes lacking the S1 protein cannot translate mRNA containing leader sequences, but are able to translate leaderless mRNAs[87]. This probably was the reason for slower polypeptide synthesis.

van Buul et al. described a *ksgA* mutant strain which displayed ribosomal ambiguity in in vitro translation assays[67]. Ribosomes purified from the mutant strain allowed leakiness of nonsense and frameshift mutations in in vitro translation assays. Although protein composition of the mutant 30S was not analyzed, given the proximity of the adenosines to the functional center of the ribosome, it seems likely that the presence or absence of the methyl groups could have a direct effect on translational fidelity. The loss of KsgA function has been shown to reduce virulence in at least one human pathogen, *Yersina pseudotuberculosis*[68].

Although the importance of the methyl groups for proper ribosome function was evident from the above works, the exact role and thus the reason for universal conservation of KsgA and the result modification remained largely elusive. Given the range of functions displayed by KsgA orthologs, it was speculated that KsgA may play a larger role in ribosome biogenesis, beyond methylation. This possibility was pointed at when Inouye et al. discovered a functional link between KsgA and Era[88-90]. They isolated a cold-sensitive Era mutation which resulted in defects in cell division and in rRNA processing. The cold-sensitive phenotype of this mutant was complemented by

overexpression of KsgA, hinting thereby that KsgA may have a role in ribosome assembly similar to that of Era, RbfA, RimM, RimN, and RsgA.

Connolly et al. recently offered a mechanistic explanation for the extreme conservation of the KsgA/Dim1 family of enzymes[69]. They propose that KsgA functions as a late stage ribosome biogenesis factor and that the methylation triggers release of KsgA from the assembling subunit, allowing it to finally mature and enter the translation cycle with the speculation that it functions as a gatekeeper. Connolly et al. also reported that a Δ*ksgA* genotype in *E. coli* is linked to cold sensitivity and altered ribosomal profiles, and that a methyltransferase-inactive form of KsgA is profoundly deleterious to cell growth and ribosome biogenesis[69]. The binding site of KsgA on the 30S ribosomal subunit is thought to overlap with that of IF3 binding site and 50S subunit association sites[91]. Xu et al. observed that addition of IF3 lead to a loss of directed cleavage of 16S rRNA by KsgA in their hydroxyl radical probing experiments[91]. This again indicates that KsgA might function as a gatekeeper, occluding these translational components from binding until an 'appropriate' assembly of structurally and functionally competent 30S subunit has occurred[91].

KsgA transfers methyl groups from four molecules of SAM to two adenosines in the 16S rRNA (Figure 6). However, the exact mechanism of methyl group transfer remains to be elucidated. There is no data concerning the number of binding events required to transfer four methyl groups or the order in which the methyl groups are transferred by KsgA to the two adenosines. In case of ErmC', there is strong evidence that dimethylation of the target adenosine occurs in two separate events[92]. Here, the enzyme

dissociates after transferring one methyl group and transfers the other in a second binding event. It has been reported that, under stringent conditions (low SAM concentration and low temperature), KsgA preferentially dimethylates the 3' proximal adenosine, suggesting some preference for ordered methylation[51]. However, if either adenosine is mutated the other is still able to be methylated, indicating that any such preference is not obligatory[93].

Substrate requirements for KsgA

So far, KsgA is the only rRNA post-transcriptional modification enzyme known to be conserved in all three domain of life as well as in eukaryotic cellular organelles[59-61, 63, 64]. The KsgA/Dim1 family bears sequence and structural semblance to a large group of S-adenosyl-L-methionine (SAM) dependent methyltransferases. ErmC' (Figure 8C), the rRNA methyltransferase involved in macrolide resistance[80] is an important paralog of KsgA. KsgA and ErmC' are closely related structurally and catalyze very similar reactions. However, substrate recognition by KsgA is subject to complex regulation that is absent from the Erm methyltransferases[84].

ErmC' can bind and methylate a 32 nucleotide RNA fragment containing the target adenosine[94]. KsgA has also been shown to bind a small RNA fragment that contains the target adenosines, but it is unable to methylate the fragment or even naked 16S[95]. KsgA remains enzymatically inactive until a partially matured small subunit has assembled[96]. The reason behind this remains unclear. One possible explanation is that KsgA is regulated allosterically; free KsgA could exist in a conformation that is competent for rRNA binding but not methylation. This hypothesis is supported by the observations that free KsgA does not bind SAM appreciably[58], and that in the crystal

structure of KsgA, the active site pocket is not large enough to accommodate two adenosine bases simultaneously[84]. Therefore, a second interaction, beyond the site of modification, between KsgA and its pre-30S substrate may be required for KsgA activation[84].

It is possible that in vivo KsgA binds to an early intermediate 16S rRNA, but only when the correct pre-30S particle is assembled, does the interaction trigger binding of SAM to KsgA and also positions the target rRNA in such a way that the two nucleotides flip into the active site successively. Dim1 and h-mtTFB have been shown to complement for KsgA function in bacteria[60, 63]. This indicates that the bacterial, eukaryotic, and organellar enzymes can recognize a common bacterial substrate, despite differences in the respective 30S maturation processes. We have extended the previous complementation studies to include an archaeal ortholog MjDim1 from the organism, *M. jannaschii* and have examined the activity of MjDim1 and Dim1 from *S. cerevisiae* (ScDim1) both in vivo and in vitro in bacterial systems (Chapter 3).

The composition of the true in vivo substrate for KsgA remains to be identified, but the study by Thammana and Held showed that in vitro, the minimal substrate requires 16S rRNA plus the ribosomal proteins S4, S6, S8, S11, S15, S16, S17, and S18, all part of the RI (21S) particle (Figure 9)[96]. However, the same study reported that subsequent addition of the proteins S3, S9, S10, S14, and S21 is refractory to KsgA activity, suggesting that dimethylation of A1518 and A1519 occurs during a specific point during ribosome assembly. According to Thammana and Held, fully formed 30S complexes are

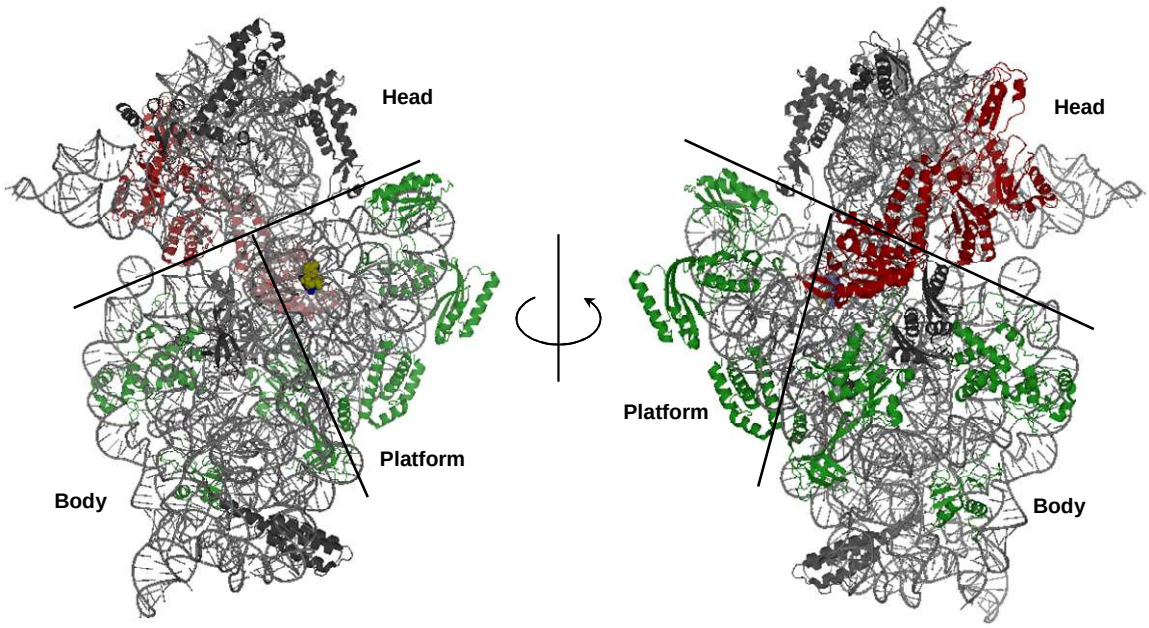

Figure 9. 30S ribosomal proteins required for and refractory to KsgA activity, according to Thammana and Held[96]. In light gray ribbon is 16S rRNA except for A1518 and A1519 of helix 45, which are yellow and blue, respectively, and in space-filling representation. In green ribbon are those proteins necessary for KsgA activity. In red ribbon are those proteins that are refractory to KsgA activity. The remaining ribosomal proteins are in dark gray ribbon. The three domains of 30S are roughly delineated and labeled. This figure was rendered using Pymol from the coordinates generated by Wimberly et al. (PDB ID 1J5E)[14].

not competent methylation substrates for the same reason. Curiously, a reevaluation of the inhibitory proteins by Poldermans et al.[58] concluded that only S21 is inhibitory to KsgA activity. They further concluded that the Thammana and Held[96] observations were the results of S21 and/or IF3 (also inhibitory to KsgA) impurities in their ribosomal RNA stocks. Also, in vivo stalled RI (21S) intermediates apparently are not methylated at A1518 and A1519[7], which suggests that methylation occurs when RI* (26S) forms or later.

Direct comparison between the results obtained by the two groups is problematic because the methylation reactions performed contained different concentrations of Mg^{2+} and NH_4^+. This is an important consideration because the conformational state of the 30S subunit is sensitive to the concentrations of monovalent and divalent cations[97, 98]. We have resolved this issue by assaying KsgA under different concentrations of monovalent and divalent cations (Chapter 2). We also report that, in vitro, KsgA does not recognize the assembly intermediate RI (21S) as a substrate, meaning that methylation occurs after RI (21S) has been formed.

Apart from regulation for substrate recognition, KsgA must also be subject to complex temporal regulation. Ten bases are methylated in *E. coli* 16S rRNA and out of them six, including A15181 and A1519, are clustered in the same immediate area. m^2G1516 is located in the loop of helix 45, very close to A15181 and A1519; m^4Cm1402, m^5C1407 and m^3U1498 are located at the base of helix 44. During the window of time between KsgA binding and release, at least two other methyltransferases are thought to bind their respective target sites, which must overlap with that of KsgA

binding site since the bases are clustered. Therefore, these processing events must require exquisite timing to ensure that the modifications are carried out correctly without the various enzymes interfering with one another.

Substrate binding to KsgA

In order to gain complete insight into the role of KsgA in ribosome biogenesis and how it interacts with other ribosome biogenesis factors or chaperons, it is extremely important to determine the binding site of KsgA on the 30S subunit. However, there seems to be a great deal of incongruity in the literature regarding this information. Initially, based on the inspection of crystal structure of KsgA, mutagenesis data of ErmC' and assumed correspondence with DNA methyltransferases, KsgA was thought to bind helix 45 along the cleft formed between its N- and C- terminal domains[84]. However, questions remained regarding this mode of binding since the KsgA crystal structure showed that the active site pocket was not large enough to accommodate two adenosine bases simultaneously, which would most likely be required for this mode of binding. Crystal structure of KsgA in complex with 30S subunit, which would possibly answer these questions, has remained unachievable for years.

Xu et al. proposed a model of KsgA-16S rRNA complex based on hydroxyl radical probing and footprinting data [91]. Their model positions KsgA such that its N-terminal interacts with the 790 loop of 16S rRNA and almost the entire protein interacts with the top portion of helix 44 in a linear fashion. This arrangement puts the target adenosines of helix 45 within close proximity to the active site of KsgA (motif VIII)[84], but KsgA never binds helix 45.

On the other hand, Tu et al. recently reported the crystal structure of *Aquifex aeolicus* KsgA bound to double stranded RNA via the cleft between its N- and C-terminal domains[99]. The RNA used in their study represents the helix 45 of *E. coli* 16S rRNA. Helix 45, according to the structure of *E. coli* 30S subunit[18], forms a hairpin structure capped by a tetraloop that contains the target adenosines methylated by KsgA. However, the KsgA-RNA crystal structure reported by this group shows a duplex formed by the association of two RNA molecules instead of a hairpin formed by a single RNA molecule. This unnatural form of RNA leaves room for skepticism on whether KsgA truly binds helix 45 in vivo. Moreover, the mode by which KsgA interacts with 16S rRNA is also different as per the above two reports. The different binding modes require dramatically different mechanistic details of KsgA. We have examined the key 16S rRNA sites implicated in binding to KsgA using site directed mutagenesis and in vitro methods (Chapter 4).

Objectives

KsgA and its orthologs represent a universally conserved family of RNA methyltransferases and have been shown to play an integral role in the biogenesis of functional ribosomes. The crystal structure of *E. coli* KsgA[84] had been solved in our laboratory at the onset of this project. Though the crystal structure greatly enhanced our understanding of the enzyme, several questions remained that demanded biochemical characterization of the protein as it associates with its substrate. The studies presented here were undertaken in order to better understand the biochemical and structural requirements for the optimal functioning of KsgA and to lay a foundation for future

investigations. In our first study, we established the salt conditions required for optimal KsgA methyltransferase activity while confirming that KsgA recognizes a translationally inactive form of 30S subunit in vitro. This information has enabled efficient in vitro assays for further characterization of KsgA/Dim1 enzymes.

Our study of the functional conservation of KsgA/Dim1 enzymes in the bacterial system revealed that KsgA and the evolutionarily higher orthologs could recognize a common ribosomal substrate. Since then, a similar complementation experiment in *S. cerevisiae* in our lab has shown that none of the orthologs could complement the eukaryotic Dim1, probably due to the non-conserved secondary, non-methyltransferase function[77].

Finally, to characterize the interaction of KsgA with the 30S subunit, we carried out site-directed mutagenesis of 16S rRNA followed by biochemical analysis of purified mutant 30S subunits with KsgA. Our results support the KsgA-16S rRNA model recently proposed by Xu et al.[91]

CHAPTER 2: Characterization of Substrate

The KsgA/Dim1 family bears sequence and structural resemblance to a large group of S-adenosyl-L-methionine (SAM) dependent methyltransferases, one of which is ErmC', the rRNA methyltransferase involved in macrolide resistance[80]. KsgA and ErmC' are closely related structurally and both catalyze the transfer of two methyl groups from SAM to the target adenosines on rRNA. However, substrate recognition by KsgA is subject to complex regulation that is absent from the Erm methyltransferases[84]. ErmC' can bind and methylate a 32 nucleotide RNA fragment containing the target adenosine[94]. KsgA has also been shown to bind a small RNA fragment that contains the target adenosines, but it is unable to methylate that fragment or even naked 16S[95]. KsgA remains enzymatically inactive until a partially matured small subunit has assembled[96]. The reason behind this remains unclear.

From the crystal structure of KsgA, it is clear that the active site pocket of KsgA is not large enough to accommodate two adenosine bases simultaneously[84]. Also, SAM binds KsgA very weakly until the enzyme interacts with fully or partially formed 30S subunits[58]. Therefore, a second interaction, beyond the site of modification, between KsgA and its pre-30S substrate may be required for KsgA activation[84]. It is possible that in vivo KsgA binds to an early intermediate 16S rRNA but, only when the correct pre-30S particle is assembled, does the interaction trigger binding of SAM to KsgA and also

positions the target rRNA in such a way that the two nucleotides flip into the active site successively.

The composition of the true in vivo substrate for KsgA remains to be identified, but the study by Thammana and Held showed that in vitro, the minimal substrate requires 16S rRNA plus the ribosomal proteins S4, S6, S8, S11, S15, S16, S17, and S18[96]. However, the same study reported that subsequent addition of the proteins S3, S9, S10, S14, and S21 is refractory to KsgA activity, suggesting that dimethylation of A1518 and A1519 occurs during a specific point during ribosome assembly. Curiously, a reevaluation of the inhibitory proteins by Poldermans et al.[58] concluded that only S21 is inhibitory to KsgA activity. They further concluded that the Thammana and Held observations[96] were the results of S21 and/or IF3 (also inhibitory to KsgA) impurities in their ribosomal RNA stocks.

Direct comparison between the results obtained by the two groups is problematic because the methylation reactions performed contained different concentrations of Mg^{2+} and NH_4^+. This is an important consideration because the conformational state of the 30S subunit is sensitive to the concentrations of monovalent and divalent cations[97, 98]. Thammana and Held used high concentrations of NH_4^+ and high Mg^{2+}, which stabilize the conformation of 30S into an 'active' state. The active state is defined as the ability of 30S subunits to support translation and integral translational functions such as binding tRNA and mRNA. The buffer used by Poldermans et al.[58], which contained low NH_4^+ and intermediate Mg^{2+}, was optimized to enhance methylation of 30S particles by KsgA. Traditionally, intermediate NH_4^+ and low Mg^{2+}concentrations have been used to stabilize

30S in a translationally 'inactive' state. The buffer used by Poldermans et al. should still be able to trap the 30S in the inactive state. Despite the different reaction conditions, both groups observed that S21 is inhibitory to KsgA activity. It is possible that only S21 is truly inhibitory but the proteins S3, S9, S10 and S14 also become inhibitory at the elevated monovalent and divalent cation concentrations used by Thammana and Held. This chapter directly addresses KsgA activity as it relates to 30S conformation. We also report that, in vitro, KsgA does not recognize the assembly intermediate 21S as a substrate, meaning that methylation occurs after 21S has been formed.

Recombinant KsgA is functional

To confirm that the recombinant KsgA was active and that the 30S subunits isolated from a kasugamycin resistant *E. coli* strain were unmodified at positions A1518 and A1519 of 16S rRNA, we performed methylation activity assays using our biochemical stocks and a published protocol (Figure 10)[58]. Recombinant KsgA is able to efficiently methylate 30S subunits isolated from strains of *E. coli* resistant to kasugamycin, but not wild-type 30S subunits, indicating that the methylation function is specific for A1518 and A1519. Had non-specific methylation taken place then both 30S types would have been modified by KsgA.

KsgA recognizes 30S in an 'inactive' state

Standard protocols to isolate 30S subunits from sucrose gradients trap them in an 'inactive' state that require heat activation at 42 °C for 10min in the presence of 100mM NH_4^+ and 20mM Mg^{2+} prior to use in standard translation assays[97]. 30S subunits in very

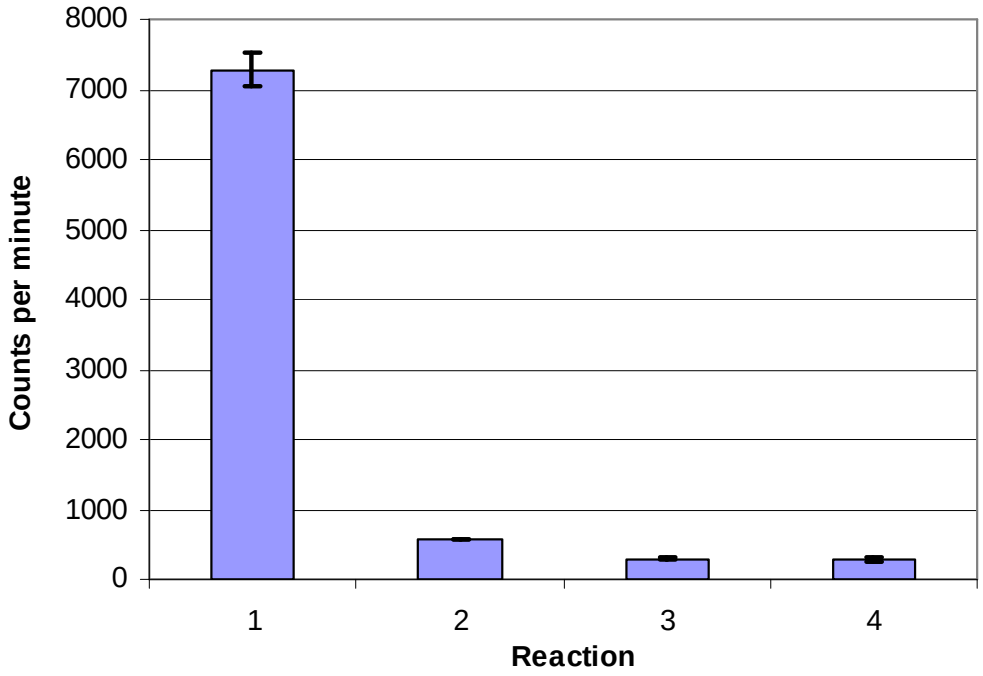

Figure 10. Methylation of 30S by recombinant KsgA. All reactions were performed for 30 min at 37 °C in Buffer I and contained ^{3}H-SAM. 1, KsgA + 30S subunits from Kasugamycin-resistant *E. coli* strain *ksgR11*. 2, KsgA + 30S from wild-type *E. coli*. 3, KsgA without any 30S subunits. 4, 30S from *ksgR11* without KsgA.

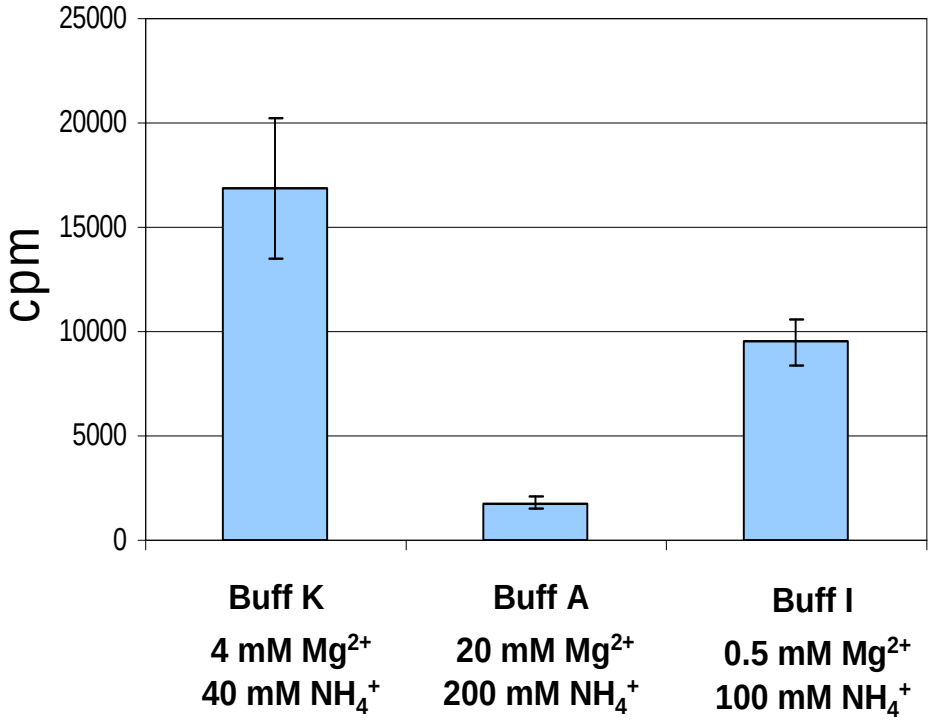

Figure 11. The effect of 30S conformation on KsgA activity. Standard activity assay in buffers K, A and I. Buffers K and I stabilize 30S subunits into a translationally inactive state while Buffer A stabilizes 30S subunits into the translationally active state. Error bars indicate plus and minus one standard deviation unit.

low NH_4^+ or low Mg^{2+} will never (or very slowly) convert to the active conformation as a result of the annealing step[98].

We directly compared conditions used by Thammana and Held (high NH_4^+ (200mM) and high Mg^{2+} (20mM) buffer), which traps 30S in the translationally active state with those used by Poldermans et al. (low NH_4^+ (40mM) and intermediate Mg^{2+} (4 mM) buffer), which trap 30S in a translationally inactive state, along with conditions traditionally used to stabilize 30S into the translationally inactive state (intermediate NH_4^+ (100mM) and low Mg^{2+} (0.5 mM) buffer; Figure 11). Most striking is the fact that under cation concentrations that stabilize 30S subunits in the translationally active state (high NH_4^+ and high Mg^{2+}) very little methylation occurs. In contrast, the two reaction conditions expected to hold 30S in an inactive state promote efficient methylation. Buffer K, which contains 4mM Mg^{2+}, is the most active with regard to KsgA activity. Poldermans et al. previously reported that KsgA activity is maximal at 4mM Mg^{2+} when NH_4^+ concentration remained low[58]. The intermediate activity observed in Buffer I is probably the result of a Mg^{2+} concentration of only 0.5mM, which is below the optimal concentration for KsgA activity. It is important to stress that a concentration of only 3mM Mg^{2+} is too low for 30S subunits to adopt the active conformation[98]. We do not know if KsgA has a direct Mg^{2+} requirement or if the suitability of 30S to serve as a substrate is altered when the Mg^{2+} concentration is changed from 0.5 to 4mM. Nevertheless, it is clear that KsgA is unable to utilize 30S subunits in the translationally active state as a substrate.

The above conclusions are supported by numerous structural data of 30S in the active and inactive conformations. In the crystal structure of the *Thermus thermophilus* 30S subunit, which is a snapshot of 30S in the active conformation, A1518 and A1519 are well buried into the groove of helix 44 and therefore sequestered from KsgA[14]. However, conversion to the inactive state exposes the same two nucleotides to the solvent[100] through domain movements that involve the platform region of 30S[101]. The structure of the GGAA tetraloop in helix 45 in context of the crystal structure and free in solution are also different[14, 102], which might also impact the reactivity of A1518 and A1519.

Does KsgA recognize an assembly intermediate of 30S?

Closer scrutiny of the proteins required for KsgA activity and those inhibitory to KsgA function argues that dimethylation of A1518 and A1519 occurs at an intermediate point during the assembly pathway of 30S. In vitro assembly of 30S is a highly cooperative process[6, 103, 104]. Early binding proteins, S4–S9, S11–S13, and S15–S20, assemble with 16S rRNA to form a relatively loose ribonucleoprotein particle with a sedimentation coefficient of the 21S particle. The remaining ribosomal proteins, S2, S3, S10, S14, S10, and S21, are unable to bind to 21S. However, heating 21S at 42 °C in vitro results in a change in 16S rRNA conformation that causes 21S to collapse into a denser particle of the same composition, but with a sedimentation value of 26S[105]. At this point the remaining ribosomal proteins readily bind to 26S, yielding active 30S. Thammana and Held reported that purified 16S rRNA plus a set of eight proteins (S4, S6,

S8, S11, S15-S18), all part of the 21S/26S particle, could serve as a minimal substrate for KsgA in vivo[96].

Although 21S and 26S particles were identified in vitro, counterparts are present in vivo [22, 106, 107]. Based on the fact that the pre-30S assembly intermediates 21S and 26S both contain the eight proteins important to KsgA activity, one would predict that in vivo either the 21S or the 26S (or both) conformation is a substrate for KsgA. However, analysis of nuclease digests of in vivo pre-30S particles, which are analogous to the in vitro 21S particle, indicate that the dimethyladenosines are not yet present, which led the authors of that work to conclude that methylation at A1518 and A1519 had not yet occurred[7]. If KsgA recognizes a specific assembly state of 30S in vivo, then that assemblage appears to come after 21S has formed.

The lack of methylation in the in vivo 21S particle presents a conundrum. The 21S intermediate contains all eight ribosomal proteins shown to be essential for efficient methylation in vitro. On this basis we would expect 21S to be a viable substrate in vitro. Therefore, we asked if KsgA could methylate 21S particles generated by in vitro reconstitution. At 40 °C, reconstitution of 16S rRNA and total proteins of 30S (TP30) proceeds to completion i.e. 30S, while at and below 15 °C, it is stalled at 21S[6, 103]. 16S rRNA unmodified at positions A1518 and A1519 was reconstituted with TP30 at 40 °C and 15 °C. These reconstitution reactions were then assayed for KsgA activity at the respective temperatures. Purified natural 30S unmodified at A1518 and A1519 was tested as control.

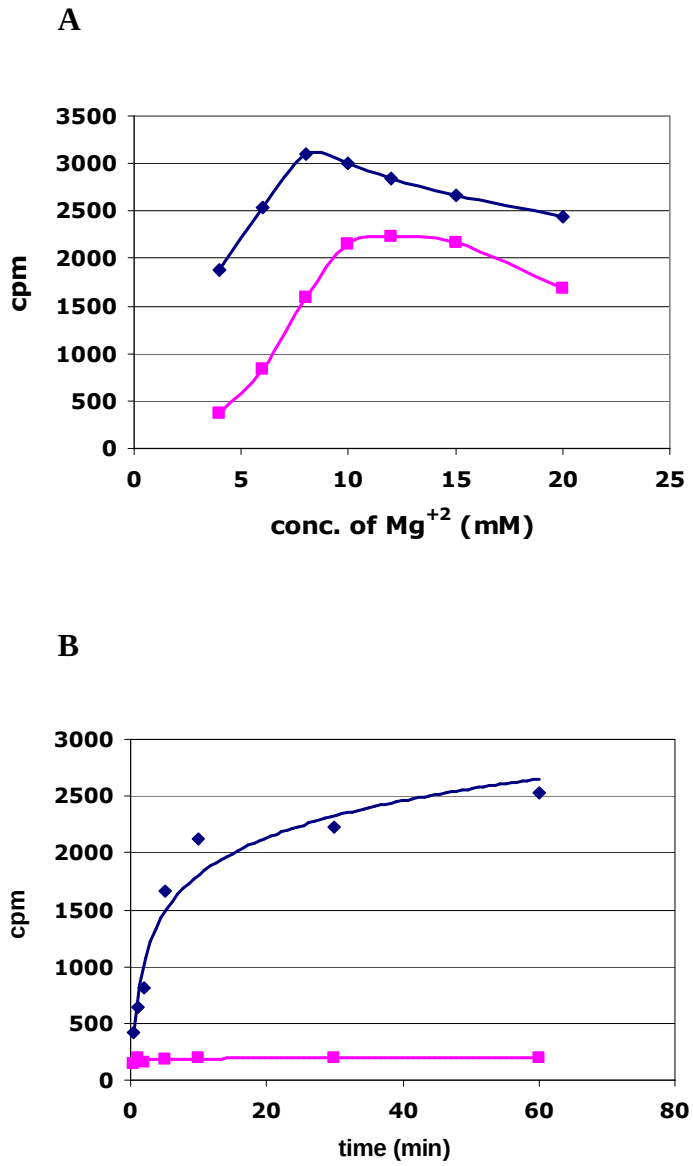

Figure 12. (A) Magnesium dependence assay at 40 °C to determine the optimal Mg^{+2} concentration for KsgA activity under the reconstitution salt conditions (B) In vitro time course assay at 15 °C to assess the methylation of 21S particles by KsgA. The Mg^{+2} concentration in these reaction was 10mM, as found optimal in (A). In both, (A) and (B), purified natural 30S subunits were used as a control. Diamonds indicate the control assays containing natural 30S subunits, whereas squares indicate assays containing the reconstitution product.

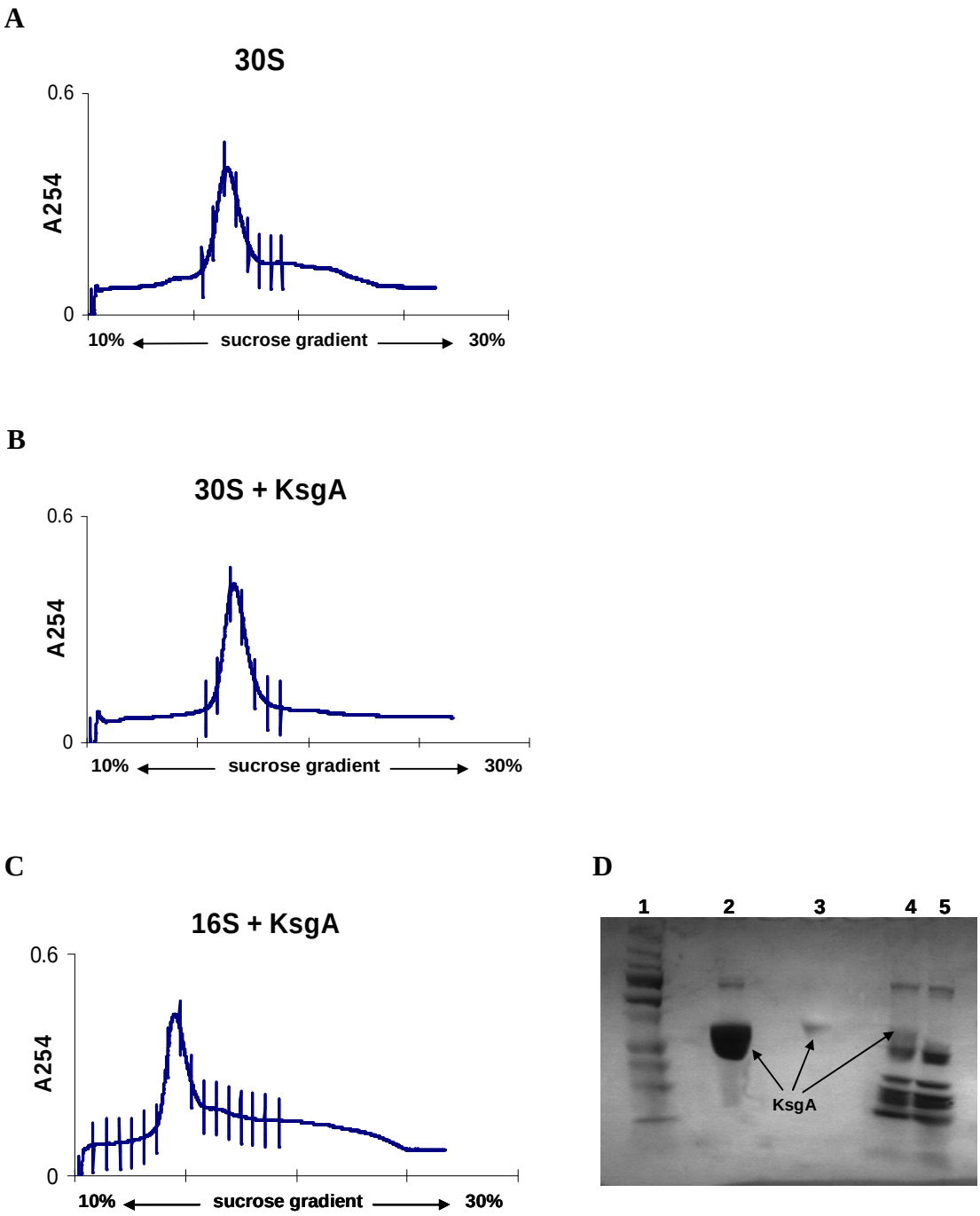

Figure 13. (A), (B) and (C) represent the sucrose gradient profiles of 16S rRNA + KsgA, 30S + KsgA, and 30S, respectively. (D) SDS PAGE analysis of the peak collected from (A), (B) and (C), where 1- Protein Standards, 2- KsgA (control), 3- 16S rRNA + KsgA, 4- 30S + KsgA and 5- 30S.

We found that natural 30S subunits are substrates to KsgA at both temperatures, however, only those reconstitution reactions that were incubated at 40 °C show methylation (Figure 12). Therefore, it appears that KsgA requires something more folded than 21S, which is known to be a loose assemblage relative to more mature particles like 26S and 30S. Hence, our in vitro analysis is consistent with the observation that KsgA doesn't act before the formation of in vivo 21S intermediates. The identification of the core eight ribosomal proteins was made by single and bulk omission reconstitutions using 16S rRNA and different set of ribosomal proteins. All of these reconstitutions were done at 42 °C, which means that a 21S-like particle was never formed. Therefore, the core eight proteins are required but not sufficient for KsgA function. High temperature annealing is also required.

Ribosome biogenesis occurs co-transcriptionally, which means that there is never free 16S rRNA available for ribosome assembly. Nevertheless, it is of interest to understand just how immature assembly can support KsgA binding, though not necessarily catalytic function. Therefore, we incubated 16S rRNA, which is known not to be a substrate, with KsgA in 1:5 ratio at 37 °C for 20 min before running it on a 10-30% sucrose density gradient. As control, 30S+KsgA and 30S alone were also treated similarly. Fractions containing 16S rRNA in each experiment were collected and concentrated. The presence of KsgA was assayed by SDS PAGE analysis. The KsgA/16S rRNA complex was clearly strong enough to survive a sucrose gradient. Therefore, it appears binding isn't the only critical factor for function. Obviously, overall 30S and partial 30S subunit assembly is essential.

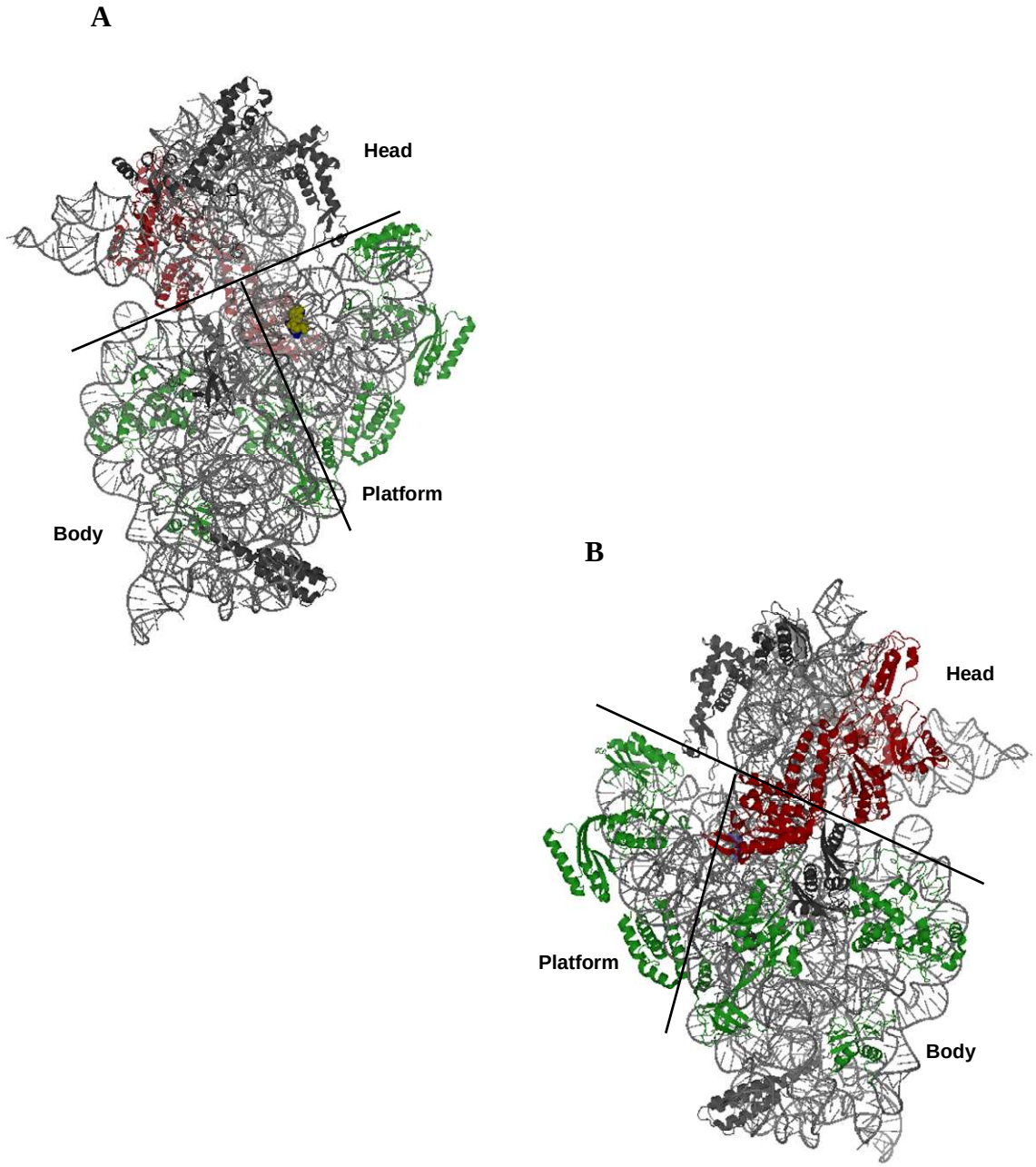

Figure 14. Crystal structure of the 30S subunit. In light gray ribbon is 16S rRNA except for the pair of apical adenosines of helix 45, which are yellow and blue, respectively, and are in space-filling representation. In green ribbon are those proteins necessary for KsgA activity. In red ribbon are those proteins that are refractory to KsgA activity. The remaining ribosomal proteins are in dark gray ribbon. The three domains of 30S are roughly delineated and labeled. (A) The subunit interface view of 30S. (B) The solvent exposed view of 30S. These figures were rendered using Pymol from the coordinates generated by Wimberly et al. (PDB ID 1J5E)[14].

Our results indicate that KsgA does bind naked 16S rRNA (Figure 13) and can thus bind very immature particles. KsgA also binds 30S subunit in the inactive state[108]. Therefore, KsgA is likely to be able to bind the intermediate 21S particle, which then means that KsgA does not recognize 21S as a substrate for methylation, in vitro as well.

Interaction of KsgA with ribosomal proteins is indirect

Inspection of where on 30S the proteins that activate KsgA function and the proteins that inhibit KsgA bind reveals that they are principally split between different domains of the 30S subunit[14]. The activating proteins are located within the platform and body while the inhibitory proteins reside within the head, with the exception of the platform-bound S21 (Figure 14). Importantly, none of the proteins from either set are spatially close to helix 45, which means that none are close to where KsgA must bind. Therefore, it is reasonable to imagine that KsgA is activated or inhibited indirectly, probably through formation of alternate 30S conformers. With regard to the activating proteins, single omission experiments report that some KsgA activity remains when all but S8 are individually omitted [96]. This implies that none of the proteins directly activate KsgA, otherwise their omission would abolish all activity. On the other hand, the indispensable protein S8 is so far from helix 45 that it is inconceivable that it could directly interact with KsgA to activate its enzymatic function.

Conclusions

Based on the existing literature and the data reported here, we conclude that the discrepancies between Thammana and Held[96] and Poldermans et al.[58] observations can be explained by the occurrence of a conformational change that occurs as late binding

proteins assemble onto the nascent 30S under conditions of high Mg^{2+} and NH_4^+. Before the head proteins bind, pre-30S is unable to adopt the 'active' conformation rejected by KsgA under all buffer conditions. But as the head proteins bind, a conformational shift occurs under the buffer conditions used by Thammana and Held that leads to a pre-30S and 30S that cannot be methylated by KsgA. However, this conformational shift does not occur under the buffer conditions that stabilize 30S into the translationally inactive state (intermediate NH_4^+ and low Mg^{2+}), and KsgA can methylate such 30S.

Also, our data indicates that although KsgA binds naked 16S rRNA in vitro, it does not recognize as substrate the 21S particle that contains all the eight proteins essential for KsgA activity. Also, the dimethyladenosines are not present in the in vivo pre-30S particles analogous to the in vitro 21S particles[7]. Therefore, if KsgA recognizes a specific assembly state of 30S, it comes after 21S has formed. This leaves a very narrow window of opportunity for KsgA to methylate, since once 26S forms, the inhibitory ribosomal proteins readily assemble on[96]. This is true unless in vivo 30S passes through a conformation analogous to the in vitro inactive state discussed above, something that has not been observed in vivo but cannot be discounted as a possibility. At the very least it appears that the enzymatic action of KsgA is tightly controlled during ribosome biogenesis, which may have importance to the overall biological role of KsgA in *E. coli* and to the corresponding enzymes in other organisms. Deciphering the events of KsgA involvement in ribosome biogenesis will teach us more about in vivo ribosome assembly, because the two processes are linked. Furthermore, the strong conservation between the KsgA/Dim1 proteins in bacteria, archaea, and eukaryotes suggests that there are common

assembly milestones for all ribosomes. Identification of those putative common milestones is critical to our understanding of ribosome biogenesis in all domains of life.

Experimental

Preparation of KsgA

Recombinant KsgA was obtained as previously described[109]. Briefly, the *ksgA* gene was amplified from *E. coli* genomic DNA using PCR (forward primer: 5' - ATCGCCCATATGATGAATAATCGAGTCCACCAGG; reverse primer: 5' ATTATGCTCGAGTTAACTCTCCTGCAAAGGCG) and cloned into a pET15b expression vector to include an N-terminal polyhistidine tag (pJPR1). BL21 *E. coli* cells transformed with pJPR1 were grown to an OD600 of 0.6 in the presence of ampicillin and induced with 1 mM IPTG (Sigma-Aldrich). After 4 hours at 37 °C, cells were harvested by centrifugation. Pellets were resuspended in lysis buffer (50 mM $NaPO_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0), broken with two passages through an Emulsiflex cell breaker (Avestin), and centrifuged to remove cell debris. Cleared lysate was loaded onto a HiTrap Chelating column (Amersham) equilibrated with 0.1M $NiSO_4$, washed twice with increasing amounts of imidazole (wash buffer 1: 50 mM $NaPO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0; wash buffer 2: 50 mM $NaPO_4$, 300 mM NaCl, 50 mM imidazole, pH 8.0), and the protein eluted with elution buffer (50 mM $NaPO_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0). Protein concentrations were estimated using the Bradford method. Purified protein was dialyzed into buffer containing 50mM Tris, pH 7.4, 400mM NH_4Cl, and 6mM β-mercaptoethanol and stored in 10% glycerol at -80 °C.

Preparation of submethylated 30S ribosomal subunits

To obtain 30S subunits unmethylated at A1518 and A1519 a standard procedure of selecting for strains of *E. coli* resistant (>600 μg/ml) to the antibiotic kasugamycin was followed[110]. Individual ribosomal subunits from one such strain (*ksgR11*) were obtained via ultracentrifugation across a sucrose gradient using standard techniques[111]. As a control, 30S particles fully methylated at A1518 and A1519 were obtained from wild-type *E. coli* using the same isolation protocol. Aliquots of pure 30S subunits (wild-type and submethylated) were dialyzed into buffer A, I, or K and stored at -80 °C. Buffers: (A) 20mM Tris–HCl, pH 7.4, 200mM NH_4Cl, 20mM $MgCl_2$, 20mM DTT; (I) 10mM Tris–HCl, pH 7.4, 100 mM NH_4Cl, 0.5mM $MgCl_2$, 1mM 2-mercaptoethanol; (K) 40mM Tris–HCl, pH 7.4, 40mM NH_4Cl, 4mM MgOAc, 6mM β-mercaptoethanol.

In vitro activity assay of KsgA

The assay performed here is a slight variation of the one developed by Poldermans et al.[58]. All reactions were done in 50 μl volumes containing buffer A, I, or K, 0.02mM ^{3}H-methyl-SAM (780 cpm/pmol), 7 pmol of 30S, and 7 ng of KsgA. Reactions proceeded for 30min at 37 °C, then the contents were deposited onto DE81 filter paper (Whatman), washed twice with cold 5% trichloroacetic acid, and washed with cold ethanol. Air-dried filters were placed into scintillation fluid and counted.

In vitro activity assay of reconstituted 30S and 21S particles

16S rRNA was prepared as previously described by Moazed et al.[112] and TP30 was prepared as described by Nierhaus[113]. Buffers used for reconstitution were Rec A (20mM K^+-HEPES (pH 7.5), 0 to 20 mM $MgCl_2$, 330 mM KCl and 6mM BME) and Rec

A-. Rec A- is RecA buffer minus KCl. Reconstitution was done using 1:1.5 molar ratio of 16S rRNA to TP30. A standard activity assay reaction contained 20 pmol 16S rRNA, 30 pmol TP30, 20 pmol KsgA and 0.02 mM ^{3}H-methyl-SAM (780 cpm/pmol). For the control reactions, 16S rRNA and TP30 were replaced by 20 pmol purified natural 30S. Since TP30 is stored in a buffer containing 1M KCl, the KCl concentration of the reaction mixture was adjusted to 330 mM using RecA and RecA- buffers.

Mg^{+2} dependence assay was performed at 40 °C to determine the optimal Mg^{+2} concentration for KsgA activity under the reconstitution salt conditions. For this assay, 16S rRNA and TP30 were mixed in RecA buffer (concentration of KCl was adjusted with RecA- buffer) containing 4, 6, 8, 10, 12, 15 or 20mM $MgCl_2$, and incubated at 42 °C for 15 min before shifting the reactions to 40 °C. As a control, the same procedure was followed for natural 30S. After 10 min incubation at 40 °C, KsgA and SAM were added, and the reactions were allowed to proceed for 60 min. At the end of 60 min, the reactions were quenched, deposited on filter paper, washed, dried and counted as described above.

KsgA showed maximum activity at 10 mM $MgCl_2$. To test 21S as a substrate for KsgA, a time course activity assay was performed at 15 °C, at which temperature the reconstitution is stalled at 21S. All the reaction buffers and components were maintained on ice. Assay procedure was similar to that described above, except that for this assay 16S rRNA and TP30 were mixed in RecA buffer (KCl concentration adjusted with RecA-) containing 10mM $MgCl_2$, and incubated at 15 °C for 15 min before adding KsgA and SAM. As a control, the same experiment was performed on natural 30S.

CHAPTER 3: Functional Conservation

The presence and function of the KsgA/Dim1 family of enzymes have been maintained throughout all domains of life, including the eukaryotic cellular organelles[58-61, 63, 64]. Exceptions are rare[50-52]. However, KsgA is dispensable in bacteria with only modest consequences and forms the dominant mode by which bacteria become resistant to the aminoglycoside antibiotic kasugamycin[57, 65]. A KsgA knockout mutant shows slower growth rate in Escherichia coli and reduced virulence in the human pathogen, *Yersina pseudotuberculosis*[66-68].

The higher evolutionary members of this family have adopted secondary roles in ribosome biogenesis in addition to their dimethyltransferase role. Eukaryotic Dim1 is a member of the processome and is essential for proper processing of the primary rRNA transcript[70]. The human mitochondrial ortholog h-mtTFB, which is responsible for transferring methyl groups to the mitochondrial 12S rRNA, was originally demonstrated to be mitochondrial transcription factor[63, 71]. h-mtTFB has also been linked to hereditary deafness associated with a polymorphic A1555G mutation in mitochondrial rRNA[72]. In the cases of both Dim1 and h-mtTFB, the methylating activity is separate from the second function; methylation-deficient mutations can be made, which leave the rRNA processing or transcription activity intact[76, 78]. Pulicherla et al. recently confirmed that in *Saccharomyces cerevisiae*, none of the methyl groups transferred by Dim1 are critical for

growth under standard conditions and a variety of temperatures[77]. The key question that thus lingers is that why is the dimethyltransferase function still present and conserved universally.

KsgA is able to methylate fully formed 30S subunits, although these subunits must be in a translationally inactive conformation to support methylation[108]. However, the true in vivo substrate for KsgA is unknown. A minimal in vitro substrate has been found to consist of 16S rRNA plus a subset of ribosomal proteins[96].This suggests that KsgA methylates a discrete intermediate in the 30S assembly pathway. Dim1 and h-mtTFB have been shown to complement for KsgA function in bacteria[60, 63]. This indicates that the bacterial, eukaryotic, and organellar enzymes can recognize a common bacterial substrate, despite differences in the respective 30S maturation processes.

We have extended the previous complementation studies to include an archaeal ortholog MjDim1 from the organism, *M. jannaschii*. We have examined the activity of MjDim1 and Dim1 from *S. cerevisiae* (ScDim1) both in vivo and in vitro in bacterial systems. Our results indicate that the core methyltransferase activity of this family of enzymes, including recognition of a complex substrate, has changed little since the last ancestor. It also suggests that the basic structural components of the small ribosomal subunit, required for recognition by the enzymes have remained conserved. Although dispensable, the methyltransferase activity of the KsgA/Dim1 family of enzymes must have a role important enough to justify its preservation throughout the course of evolution.

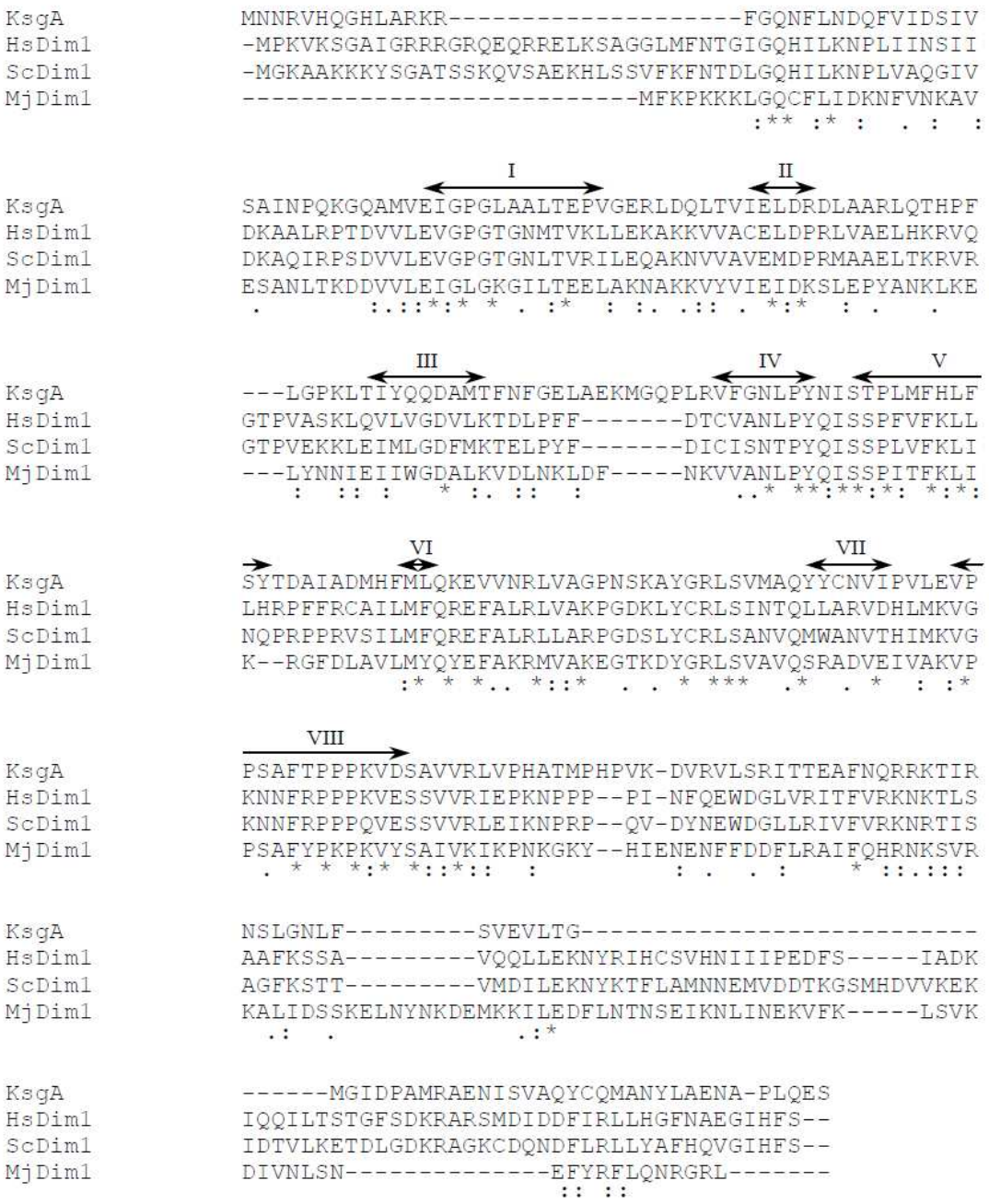

Figure 15. Structure-based sequence alignment of KsgA (P06992), putative Dim from *Homo sapiens* (HsDim1, Q9UNQ2), ScDim1 (P41819), and MjDim1 (NP_248023).
The alignment was generated using the TCoffee Web server[114]. Identical residues are denoted with a star, and strongly conserved residues with a colon; weakly conserved residues are marked with a period. Double-headed arrows indicate motifs common to SAM-dependent methyltransferases. Structures used for alignment were those of KsgA[84] (PDB ID 1QYR) and HsDim1[115] (PDB ID 1ZQ9).

In vivo analysis

ScDim1 has been shown to complement for KsgA function in *ksgA*$^{-}$ *E. coli* cells[60], demonstrating functional equivalence of the two proteins. We asked whether MjDim1 could also complement KsgA knockout. In vivo activities of both ScDim1 and MjDim1 were assessed using a modified minimal inhibitory concentration (MIC) assay, which takes advantage of the fact that loss of KsgA function renders bacteria resistant to kasugamycin (ksg)[59, 65]. Plasmids containing the two proteins were transformed into a ksg^{R} strain of *E. coli*, which lacks endogenous KsgA activity. This strain was constructed from BL-21 (DE3) cells; this allows leaky expression from the pET15b T7 promoter. Growth on ksg was compared to cells transformed with pET15b-KsgA plasmid (positive control) and cells transformed with empty vector (negative control).

Unlike in a traditional MIC, untransformed cells are naturally resistant to the antibiotic and become sensitive when transformed with a functional dimethyltransferase. Therefore, cells transformed with KsgA have a low MIC of 400 μg/ml ksg, while cells transformed with empty vector have a high MIC, greater than 3000 μg/ml ksg. As shown in Figure 16, MjDim1 is fully functional in this in vivo system, with an MIC of 400 μg/ml. ScDim1, on the other hand, shows partial activity on bacterial ribosomes *in vivo*, with an MIC of 1200 μg/ml. While ScDim1 does not restore full sensitivity to the antibiotic, it does show increased sensitivity, indicating that the enzyme is able to recognize the small subunit as a substrate. Lack of full complementation may correlate with slower and/or incomplete methylation of 30S as compared to the other two enzymes (see nucleoside analysis).

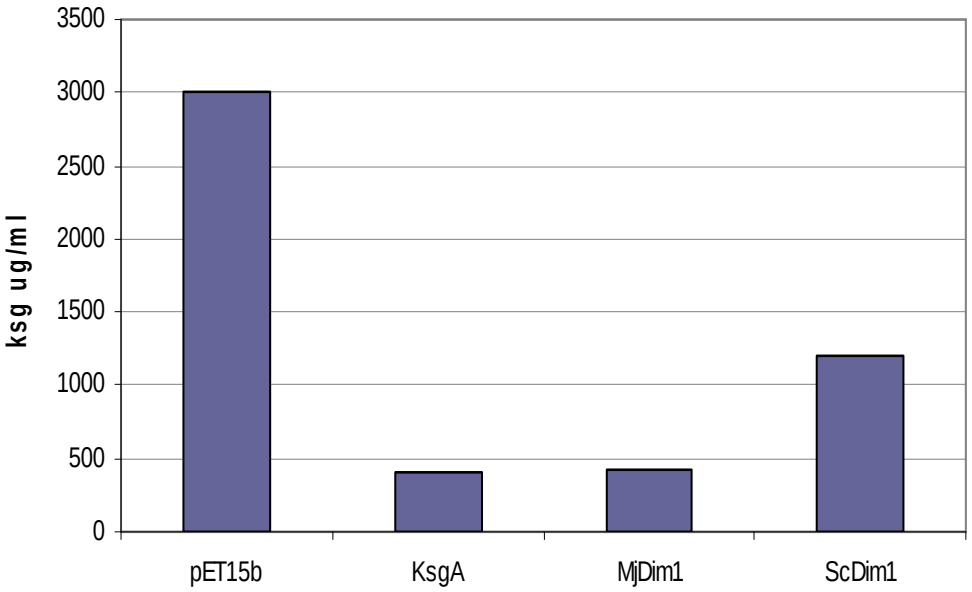

Figure 16. In vivo activity of KsgA orthologs

In vitro analysis

We next asked how efficiently ScDim1 and MjDim1 were able to methylate *E. coli* 30S in an *in vitro* assay. Unmethylated 30S subunits were prepared from the ksg^R strain described above. Incorporation of ^{3}H-methyl from labeled SAM by each enzyme was followed at discrete time points over an interval of two hours. Control experiments were performed with 30S subunits purified from wild-type *E. coli* cells, which are methylated by endogenous KsgA and thus do not serve as substrates. Initial experiments were performed with 10 pmol of 30S substrate and 1 pmol of enzyme. This amount of protein did not allow for completion of the reaction within two hours, so experiments were also performed using 10 pmol each of 30S and enzyme.

Figure 17, A-C, shows the time-course of methylation for KsgA, ScDim1, and MjDim1 respectively. Methylation of *E. coli* 30S by ScDim1 and MjDim1 closely followed the KsgA time-course, both in rate of incorporation and final level of methylation. In reactions with 1 pmol of protein, MjDim1 showed a slightly higher rate of ^{3}H incorporation than KsgA and ScDim1 at later time points. With stoichiometric amounts of protein relative to 30S, the time-course of methylation was essentially indistinguishable between the three proteins, confirming the ability of the enzymes from archaea and eukaryotes to recognize bacterial 30S subunits as substrates.

We then estimated the amount of methyl groups transferred at the 2h time point by each enzyme, with both 1 pmol and 10 pmol amounts of enzyme, by constructing a standard curve of cpm versus concentration of ^{3}H-methyl-SAM (Figure 18). With 10 pmol of 30S per reaction, and four methylation sites per 30S molecule, we would expect

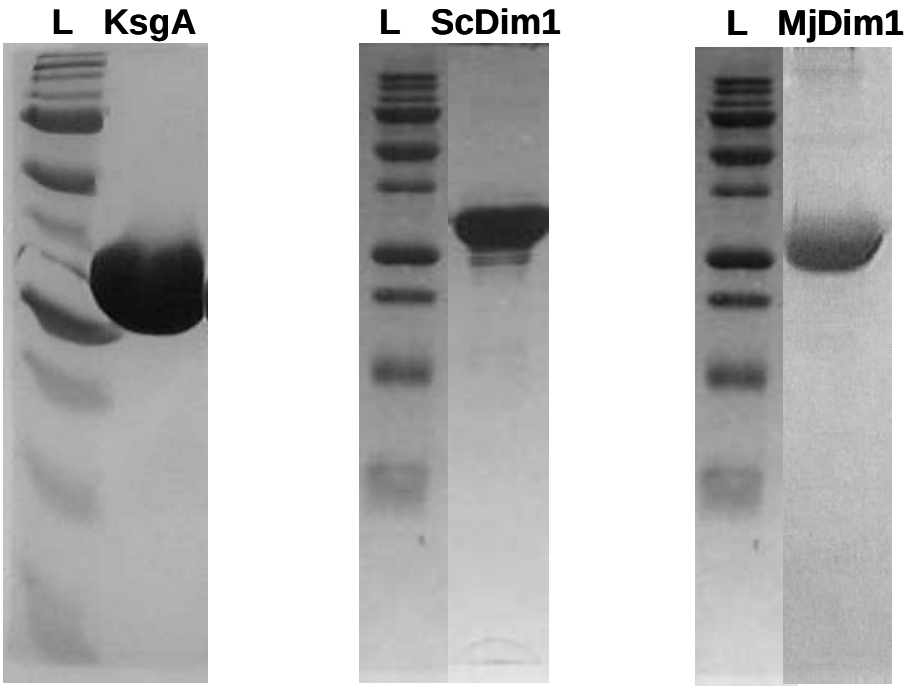

Figure 17. SDS PAGE analysis of KsgA, ScDim1 and MjDim1 proteins to assess purity. 'L' represents reference protein ladder (Precision Plus Protein Dual Color Standards, Bio-Rad).

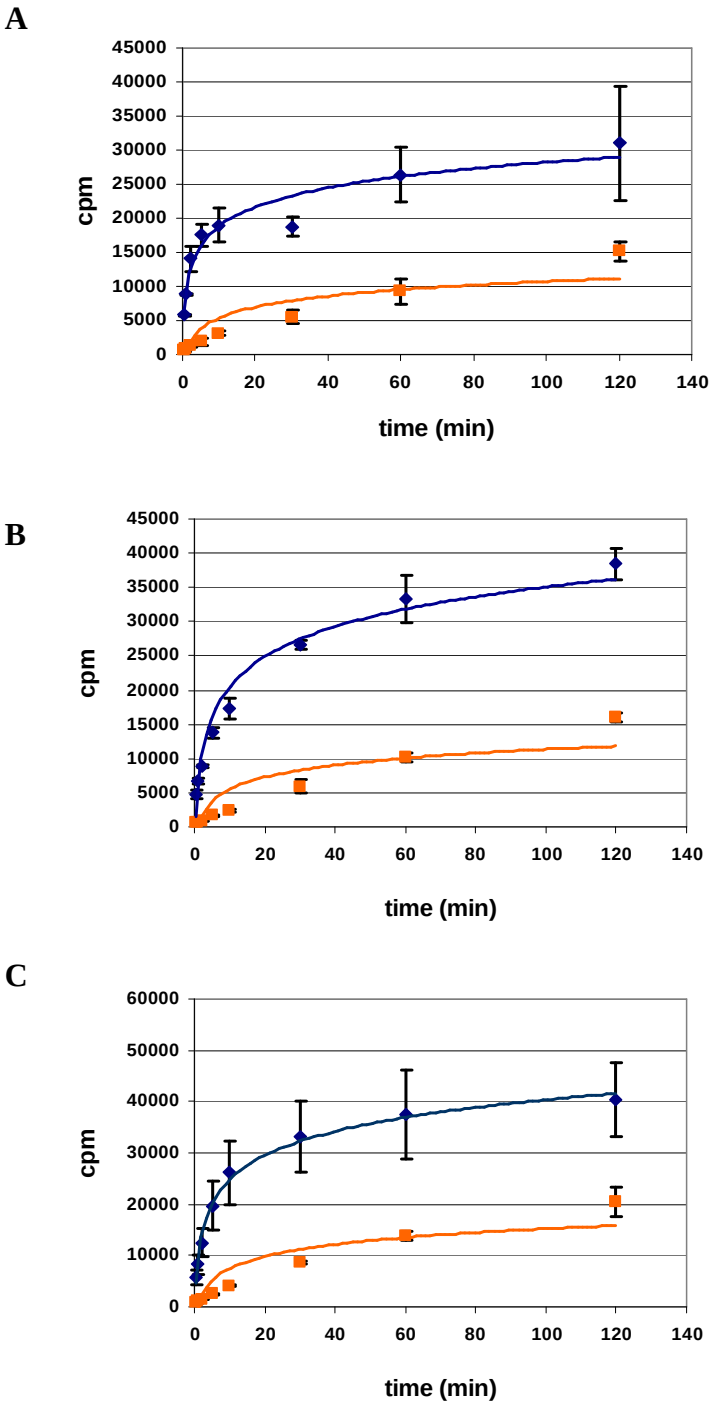

Figure 18. In vitro methylation of 30S. Time-course assays for KsgA (A), ScDim1 (B), and MjDim1 (C). Blue lines indicate assays containing 10 pmol ksgR 30S, 10 pmol enzyme and orange lines indicate assays containing 10 pmol ksg^{R} 30S, 1 pmol enzyme. Assays were performed in triplicate; error bars represent standard deviation.

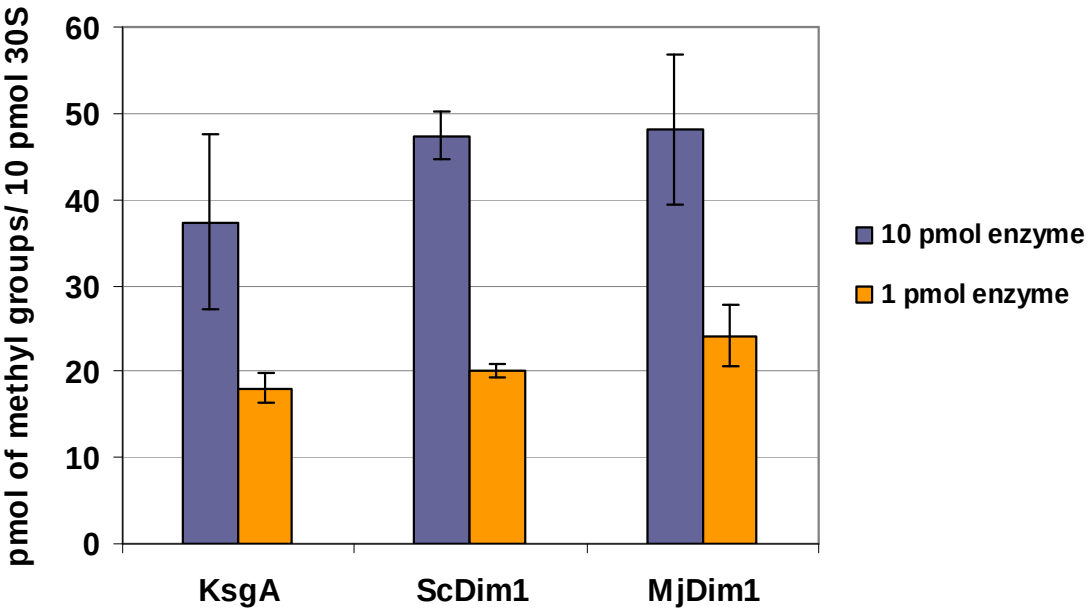

Figure 19. Quantitation of methyl groups transferred after two hours. Blue bars represent assays performed with 10 pmol enzyme; orange bars represent assays using 1 pmol enzyme. Assays were performed in triplicate; error bars represent standard deviation.

to see transfer of 40 pmol methyl groups per 10 pmol of 30S if the reactions have gone to completion. As shown in Figure 19, our calculations lead to slight overestimation of methyl group transfer, probably due to error in ^{3}H counting. Nevertheless, the reactions performed with 10 pmol enzyme appear to be more or less complete after 2 hours. Reactions performed with only 1 pmol enzyme are approximately halfway completed after 2 hours.

Nucleoside analysis

In vitro assays of the three proteins, performed as described above for the time course, were incubated for two hours and analyzed to determine relative amounts of m^6A and $m^6{}_2A$ (Figure 20; Table 1). 16S rRNA isolated from 30S subunits methylated by 10 pmol of either KsgA or MjDim1 contained no detectable labeled m^6A; radioactive incorporation was seen only in the dimethyladenosine peak. This agrees with the in vitro data suggesting that these reactions have gone to completion (Figure 19). ScDim1, on the other hand, produced a mixture of m^6A and $m^6{}_2A$; approximately 28% of the incorporated radiolabel was found on monomethylated adenosine. This indicates that at most 80% of the potential sites were methylated after two hours.

Partially methylated 30S from reactions using 1 pmol of enzyme showed radioactive peaks at both m^6A and $m^6{}_2A$. Surprisingly, although the total level of methylation was similar for all three enzymes (Figure 19), rRNA methylated by the different enzymes showed different ratios of m^6A to $m^6{}_2A$. ScDim1 produced approximately 1.4 times as much m^6A as $m^6{}_2A$. MjDim1, conversely, produced almost no m^6A; only about 1% of the incorporated methyl groups were found on m^6A. KsgA fell

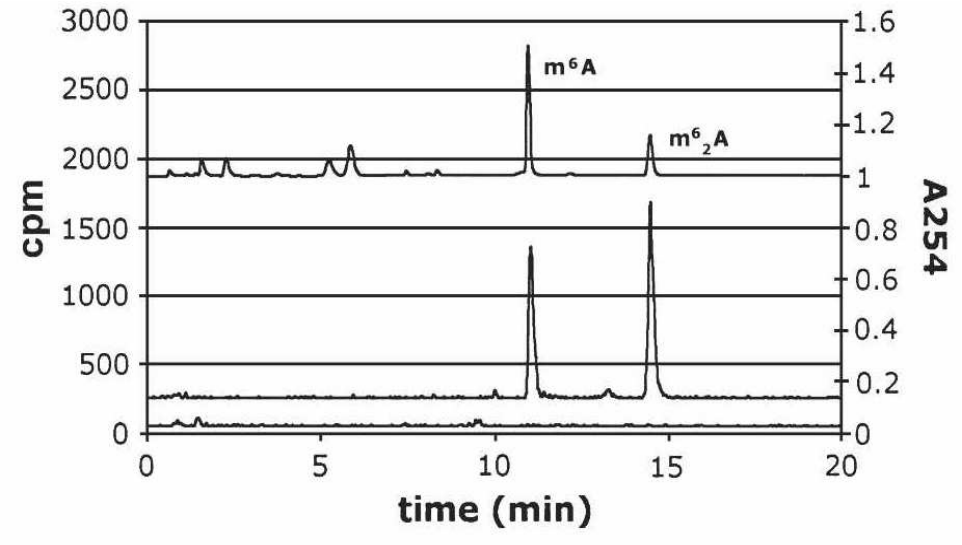

Figure 20. Representative HPLC trace. The bottom trace, plotted as cpm *vs.* time (shifted upward by 50 cpm), represents scintillation data from a control assay using wild-type 30S The middle trace (shifted upward by 250 cpm) represents scintillation data obtained from an assay using 1 pmol ScDim1 and 10pmol ksg^R 30S. At the top is the UV trace, plotted as A254 *vs.* time (shifted upward by 1 OD unit), representing the reference nucleotides, with m^6A and $m^6{}_2A$ peaks labeled.

Table 1. Quantitation of methylated adenosine species

	Total CH_3 (pmol)[a]	Ratio m^6A:m^6_2A	m^6A (pmol)	m^6_2A (pmol)[b]
KsgA				
1 pmol	18.1 ± 1.7	0.8:1	5.2	6.5
10pmol	37.4 ± 10.1	–	ND[c]	18.7
ScDim1				
1 pmol	20.1 ± 0.7	1.4:1	8.3	5.9
10pmol	47.4 ± 2.7	0.8:1	13.5	16.9
MjDim1				
1 pmol	24.1 ± 3.6	0.02:1	0.2	11.9
10pmol	48.1 ± 8.8	–	ND[c]	48.1

[a]Data from Figure 19

[b]1 pmol m^6_2A corresponds to 2 pmol methyl groups

[c]ND = none detected

somewhere between the other two, producing both m^6A and $m^6{}_2A$, with only 0.8 times as much m^6A as $m^6{}_2A$. These results could be an outcome of assaying enzymes from different species on the bacterial substrate, or they could reflect a difference in reaction mechanism.

The KsgA/Dim1 enzymes transfer a total of four methyl groups from four SAM molecules to two adenosines. The exact mechanism of transfer has not yet been established; questions remain as to order of addition, if any, and the number of binding events required for the four methylations. The above data begin to address the question of the multiple methyl group transfers. Partially methylated 30S were produced in reactions containing 10 pmol 30S subunits and 1 pmol enzyme. Therefore, m^6A produced in excess of 2 pmol (corresponding to two adenosines available for methylation per subunit) will only be seen if the enzyme releases the substrate after monomethylation and rebinds to a new substrate. With 20.1 pmol of methyl groups incorporated by 1 pmol ScDim1, the 1.4:1 m^6A: $m^6{}_2A$ ratio represents approximately 8.3 pmol labeled m^6A and 5.9 pmol labeled $m^6{}_2A$ (1 pmol $m^6{}_2A$ represents 2 pmol incorporated methyl groups). Therefore, under our assay conditions, ScDim1 clearly releases the m^6A intermediate, which is subsequently converted to the $m^6{}_2A$ product after an additional binding event.

In contrast, there is no indication that MjDim1 produces m^6A as anything but a transient intermediate. Of the 24.1 pmol methyl groups transferred, only 0.3 pmol were found on m^6A. These results suggest that the archaeal enzyme preferentially forms dimethyladenosine, without release of the monomethyl intermediate. While release of a monomethyl intermediate and subsequent re-binding and addition of the second methyl

cannot be ruled out, such a model requires that MjDim1 prefer the monomethylated substrate to the unmethylated substrate to a large degree.

In terms of m^6A versus $m^6{}_2A$ production, KsgA falls somewhere in between ScDim1 and MjDim1. Unlike ScDim1, KsgA produces less m^6A than $m^6{}_2A$; however, of the 18.1 pmol of methyl groups transferred, 5.2 pmol are found on m^6A, which is still indicative of a released intermediate. Although it is possible that these differences are a result of suboptimal assay conditions, these results also allow the possibility of distinct mechanisms for the three enzymes, thus demonstrating a need for future analysis to dissect the exact scheme of methyl transfer.

Conclusions

Substrate recognition by the KsgA/Dim1 methyltransferases is complex. KsgA is able to methylate 30S subunits under conditions of low Mg^{2+}, but it can also methylate a pre-ribosomal particle containing 16S and a partial complement of ribosomal proteins[96]. Dim1 is essential for early processing of the pre-18S rRNA, but does not methylate 18S until very late in the 40S maturation process[70]. Despite evolutionary divergence of ribosomal assembly and processing pathways, eukaryotic and archaeal KsgA orthologs are able to methylate *E. coli* 30S both in vivo and in vitro. This requires the conservation of similar structural cues in small ribosomal subunits across evolution. Also complex is the mechanism of the modification performed by these enzymes. A total of four methyl groups are transferred, from four SAM molecules, to two separate adenosines. It is clear from the crystal structure of KsgA[84] that only one SAM molecule is bound at a time, and that the adenosines enter the active site separately. It has not been determined in what

order, if any, the methyl groups are transferred, or if all four of the transfers take place within a single or multiple binding events. The work presented here demonstrates clear differences in the reaction intermediate profiles produced in vitro by KsgA enzymes from bacteria, archaea and yeast, despite the fact that the three enzymes methylate bacterial 30S to a similar extent and at similar rates in the assay used.

However, we cannot exclude the possibility that the differences in the respective rates of m^6A and $m^6{}_2A$ production seen here are a result of suboptimal substrate or assay conditions rather than a reflection of true differences in mechanism. For example, the yeast and archaeal enzymes may show different activity if assayed on their respective small ribosomal subunits rather than on bacterial 30S. Our results demonstrate the remarkable cross-recognition of a complex substrate by evolutionarily distant members of an enzyme family and emphasize the need to further investigate the multi-step reaction mechanism.

Experimental

Cloning

KsgA gene from *E. coli* and *MjDim1* gene from *M. jannaschii* were previously cloned in our laboratory into the pET15b vector, in-frame with the vector-encoded N-terminal poly-histidine tag, and was confirmed by sequencing. The pET15b-ScDim1 construct was provided by Dr. Jean Vandenhaute, and was also confirmed by sequencing.

Protein expression and purification

pET15b-KsgA and pET15b-MjDim1 plasmids were transformed into BL-21 (DE3) cells for overexpression. Cell cultures were grown to an OD600 of 0.6 in the

presence of ampicillin and induced with 1 mM IPTG (Sigma-Aldrich). After 4 hours at 37 ºC, cells were harvested by centrifugation. Pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), broken with two passages through an Emulsiflex cell breaker (Avestin), and centrifuged to remove cell debris. Cleared lysate was loaded onto a HiTrap Chelating column (Amersham) equilibrated with 0.1M $NiSO_4$, washed twice with increasing amounts of imidazole (wash buffer 1: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0; wash buffer 2: 50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, pH 8.0), and the protein eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0).

ScDim1 protein was expressed in BL21-CodonPlus (DE3)-RIL cells (Stratagene). The cells were grown at 37 ºC to an OD600 of 1.2 in the presence of ampicillin. Then the protein was induced under mild conditions with 0.1 mM IPTG and transferred to 25 ºC for four hours. Cells were harvested and broken as for KsgA and MjDim1. Purification was carried out by affinity chromatography using a Ni^{2+} column; buffers included 15% glycerol and 3 mM β-mercaptoethanol along with the above concentrations of NaH_2PO_4, NaCl and imidazole. To increase the stability of the protein, glycerol and β-mercaptoethanol were added to the purified protein to final concentrations of 25% and 6 mM, respectively.

Proteins were estimated to be >95% pure by SDS-PAGE analysis. Protein concentration was measured using the Bradford method (Bio-Rad Protein Assay).

30S purification

An *E. coli* strain lacking functional KsgA was constructed by growing BL-21 (DE3) cells on kasugamycin (ksg) to select for loss of the dimethylations. 30S ribosomes from this ksg^R strain were used in an in vitro assay to confirm that the adenosines were able to be methylated and therefore that the resistance to ksg was due to lack of KsgA activity. 30S subunits from both the ksg^R strain and the wild-type strain were prepared as previously described[116], except that cells were broken as described above. Purified subunits were dialyzed into reaction buffer (40 mM Tris, pH 7.4; 40 mM NH_4Cl; 4 mM MgOAc; 6 mM β-mercaptoethanol) and stored at –80 ºC in single-use aliquots. 30S concentration was estimated by measuring the absorbance at 260 nm and using a relationship of 67 pmol 30S per 1 unit of optical density.

In vivo assay

ksg^R cells were transformed with the pET15b constructs and selected on LB plates containing ampicillin. Transformed colonies were picked into liquid media and grown in overnight culture. These cultures were diluted 1:25 in fresh LB (Luria-Bertani media) containing 50 μg/ml ampicillin and grown to OD600 of 0.7-0.8, diluted 1:100 in fresh LB, and plated onto LB/ampicillin containing increasing amounts of ksg, from 0 to 3000 μg/ml. Plates were incubated at 37 ºC overnight and visually inspected for colony formation.

In vitro assay

The in vitro assay was adapted from Poldermans et al.[58]. Time-course reactions were performed in 500 μl volumes containing 40 mM Tris, pH 7.4, 40 mM NH_4Cl, 4 mM

MgOAc, 6 mM β-mercaptoethanol, 0.02 mM ^{3}H-methyl-SAM (780 cpm/pmol; MP Biomedicals), 100 pmol 30S subunits, and 10 or 100 pmol enzyme; volume and components were sufficient for 10 reactions. Buffer and reagents were pre-warmed to 37 ºC and added into pre-warmed tubes to minimize any lag in the reaction start. At each of eight designated time points 50 µl was removed and added to a pre-chilled tube containing 10 µl of 100 mM unlabeled SAM (Sigma-Aldrich) to quench the reaction; the remaining 100 µl was stored at –20 ºC and used for HPLC analysis (see below). The quenched reactions were deposited onto DE81 filter paper (Whatman), washed twice with ice-cold 5% TCA, and rinsed briefly with ethanol. Filters were air-dried for one hour, placed into scintillation fluid, and counted.

HPLC analysis

Labeled 16S rRNA was extracted from 30S subunits with phenol/chloroform/isoamyl alcohol. 16S was digested and dephosphorylated as described by Gehrke and Kuo[117], and subjected to nucleoside analysis by reversed-phase HPLC. Nuclease P1 was obtained from USBiological, shrimp alkaline phosphatase was from MBI Fermentas. HPLC analysis was performed on a Polaris C-18 column (Varian). The HPLC system used consisted of a Waters 600 Controller, a Waters 2487 Dual λ Absorbance Detector, and Waters Empower software. Radioactivity was monitored with a Packard 150TR Flow Scintillation Analyzer. Buffer A was 20 mM NaH_2PO_4, pH 5.1. Buffer B was 20 mM NaH_2PO_4 (pH 5.1): acetonitrile, 70:30. Separation was performed at room temperature using a linear gradient from 100%A-100%B over 20 min, at a flow rate of 1.0 ml/min. Nucleoside standards used were N^6-methyladenosine (Sigma-Aldrich) and

N^6 N^6-dimethyladenosine, synthesized as in Rife et al.[102] Peak integration was calculated by the Empower software and used to determine ratios of m^6A:$m^6{}_2A$.

CHAPTER 4: A preliminary examination of the 16S rRNA sites implicated in KsgA binding

Ribosome biogenesis is a fundamental cellular process yet it follows strikingly different pathways in prokaryotic, eukaryotic and archaeal cells[24, 104].Similarities are extremely rare. Within such divergence, the conservation of KsgA/Dim1 family of methyltransferases is unique, since it represents the only rRNA post-transcriptional modification enzymes that appear to be conserved in all three domains of life[59-61]. The conservation also extends into mitochondria and chloroplasts[63, 64].

The enzyme was first described in *E. coli*, where it catalyzes the conversion of two adjacent adenosines A1518 and A1519, also conserved, in the 30S subunit rRNA to N6,N6-dimethyladenosines[57, 58]. The methyltransferase has since been described in eubacteria[59], archaeabacteria[62], eukaryotes[60, 61] and in cellular organelles[63, 64].

Connolly et al. recently offered a mechanistic explanation for the extreme conservation of the KsgA/Dim1 family of enzymes[69]. They suggest that KsgA functions as a late stage ribosome biogenesis factor and that the methylation triggers release of KsgA from the assembling subunit, allowing it to finally mature and enter the translation cycle with the speculation that it functions as a gatekeeper. On the other hand, Dim1 had the additional role of being an essential member of the processome. Connolly et al. also reported that a Δ*ksgA* genotype in *E. coli* is linked to cold sensitivity and altered

ribosomal profiles, and that a methyltransferase-inactive form of KsgA is profoundly deleterious to cell growth and ribosome biogenesis[69].

The binding site of KsgA on the 30S ribosomal subunit is thought to overlap with that of IF3 binding site and 50S subunit association sites[91]. Xu et al. observed that addition of IF3 lead to a loss of directed cleavage of 16S rRNA by KsgA in their hydroxyl radical probing experiments[91]. Hence, one of the roles of KsgA could be to occlude these translational components from binding until an 'appropriate' assembly of structurally and functionally competent 30S subunit has occurred[91]. These recent additions to the roles and importance of KsgA in prokaryotes prompt the consideration of KsgA as a potential antimicrobial drug target.

In order to gain complete insight into the role of KsgA in ribosome biogenesis and how it interacts with other ribosome biogenesis factors or chaperons, it is extremely important to determine the binding site of KsgA on the 30S subunit. However, there seems to be a great deal of incongruity in the literature regarding this information. The model of KsgA-16S rRNA complex, proposed by Xu et al.[91] based on hydroxyl radical probing and footprinting data, positions KsgA such that its N-terminal domain interacts with the 790 loop of 16S rRNA and almost the entire protein interacts with the top portion of helix 44 in a linear fashion. This arrangement puts the target adenosines of helix 45 within close proximity to the active site of KsgA (motif VIII)[84], but KsgA never binds helix 45.

On the other hand, Tu et al. recently reported the crystal structure of *Aquifex aeolicus* KsgA bound to double stranded RNA via the cleft between its N- and C-terminal

domains[99]. The RNA used in their study represents the helix 45 of *E. coli* 16S rRNA. Helix 45, according to the structure of *E. coli* 30S subunit[18], forms a hairpin structure capped by a GGAA tetraloop that contains the target adenosines methylated by KsgA. However, the KsgA-RNA crystal structure reported by this group shows a duplex formed by the association of two RNA molecules instead of a hairpin formed by a single RNA molecule. This unnatural form of RNA leaves room for skepticism on whether KsgA truly binds helix 45 in vivo. Moreover, the mode by which KsgA interacts with 16S rRNA is also different as per the above two reports. The different binding modes require dramatically different mechanistic details of KsgA.

We examined the 16S rRNA sites implicated by the above models in binding to KsgA using site directed mutagenesis and in vitro methods. Our results indicate that KsgA does not bind at the site of methylation and support the proximity model, whereby KsgA remains anchored to helix 44/790 loop and awaits the presentation of the target adenosines in the loop of helix 45.

Sites of mutation

To interrogate, we made mutations in the helix 24, helix 44 and helix 45 of the *E. coli* 16S rRNA (Figure 21).

Helix 24 mutations

The hydroxyl radical probing and footprinting data reported by Xu et al. indicates that the 790 loop, G785-C797, of helix 24 makes direct interactions with KsgA[91]. However, their model of KsgA-16S rRNA complex puts this region of 16S rRNA into a steric clash with KsgA. There is a possibility that the conflict is an adverse effect of

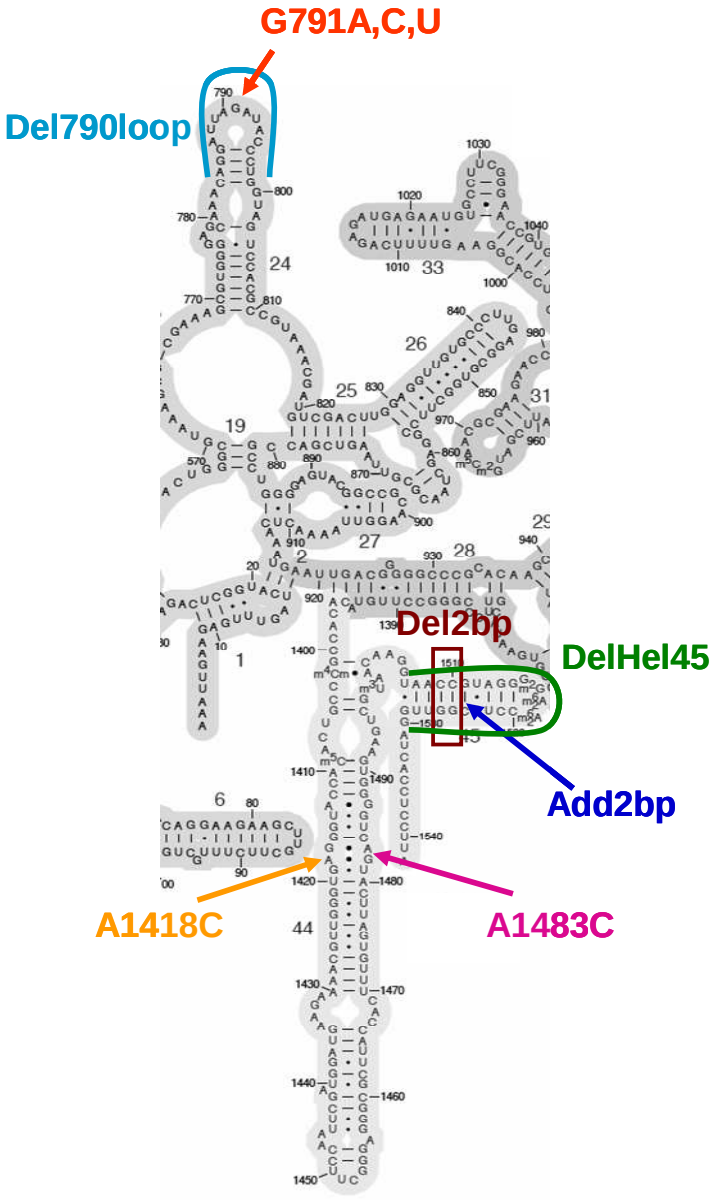

Figure 21. Location of the mutated nucleotides in the sequence and secondary structure of *E. coli* 16S rRNA. rRNA image adapted from Noller et al.[118].

creating the model using 16S rRNA of 30S subunit in active conformation, which is not the true substrate for KsgA[108]. Therefore, we wanted to study how KsgA interacts with the 790 loop of 30S subunit that is in the inactive conformation.

G791 of the 790 loop is a highly conserved nucleotide. Cunningham et al. has mutated this nucleotide to analyze its role in protein synthesis[119]. From their work, we know that G791 mutations retain at least intermediate activity and therefore such mutant 30S can be assembled properly to a significant proportion. Thus, we chose G791 for our study and independently mutated it to A, C and U. Also, we created the Del790loop mutant by removing nucleotides G785-C797 and replacing them with a GAAA tetraloop to retain the overall topology of the region.

Pure, in vivo-derived mutant 30S ribosomal subunits for our assays were obtained using the MS2 affinity purification system developed by Youngman and Green[120]. In this approach, the 16S rRNA of mutant ribosomes is expressed from a plasmid-borne operon in a background of wild-type ribosomes. The system allows inducible expression of tagged ribosomes, permitting the expression of even dominant-negative 16S rRNA mutants. The mutant ribosomes are affinity tagged with an RNA stem-loop that allows specific binding to the coat protein of the MS2 bacteriophage; the MS2 coat protein is in turn immobilized as a fusion with glutathione S-transferase (GST) to a glutathione matrix. At low Mg^{+2} concentrations (0.3 mM), the 70S subunit falls apart into its constituent subunits[111], hence buffers with low Mg^{+2} concentrations were used on the affinity column to selectively retain mutant 30S and wash away any 50S subunits bound to them. The advantage of the MS2 affinity system is that it is efficient and since the

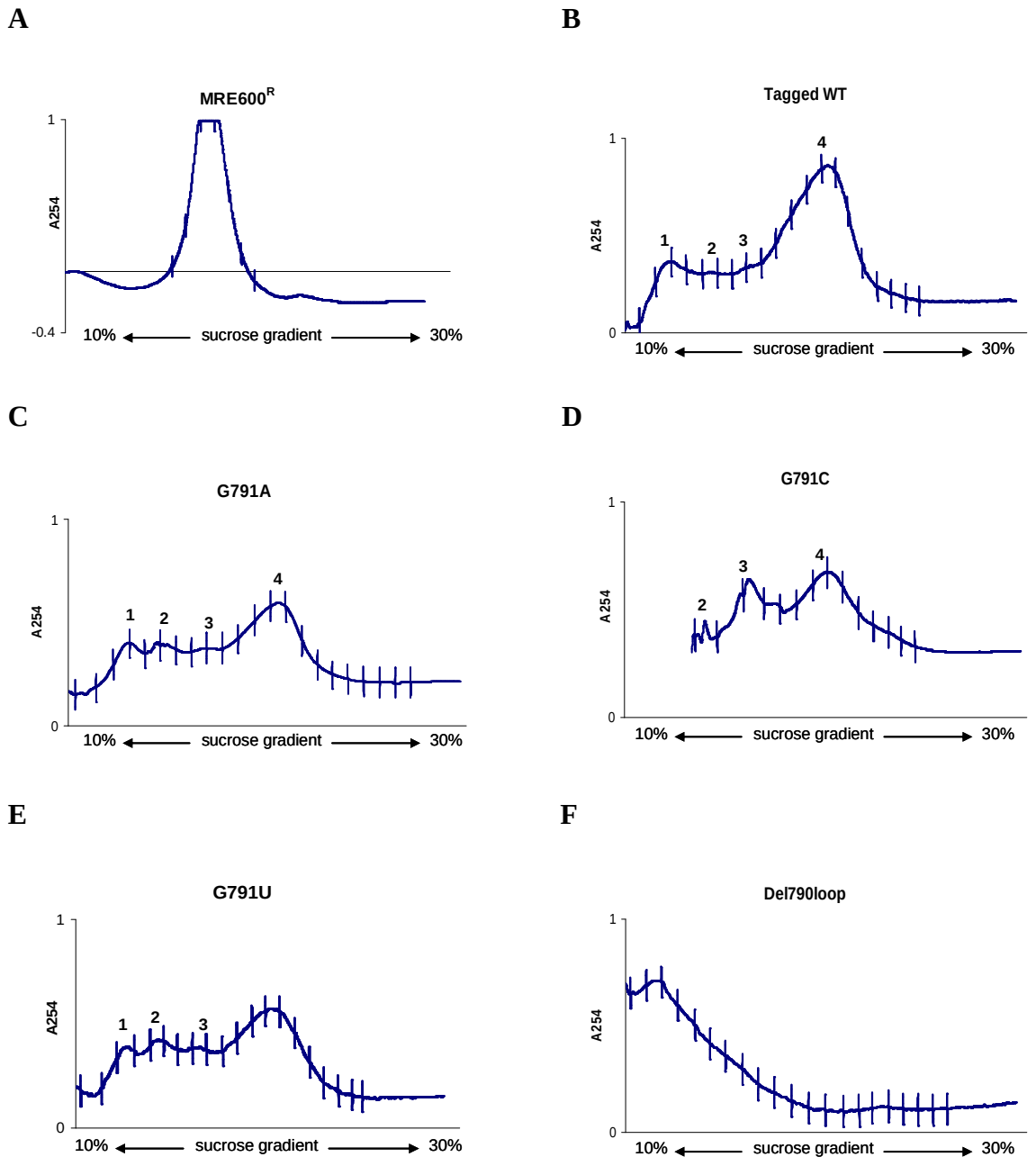

Figure 22. Sucrose gradient analysis to assess the structural integrity of 30S subunits. 250 pmols of 30S were loaded on to 10-30% sucrose density gradients. The fractions were collected from top to bottom. The 16S rRNA carrying Del790loop mutation did not form any 30S. The numbers 1-4 on the gradient profiles indicate peaks of subunit assembly products, where peak 4 contains fully formed 30S subunits whereas the others are intermediates. Peak 1 of G791C could not be collected due to a technical problem with the fraction collector.

mutant 30S subunits are assembled in vivo, they do not suffer from severe functional limitations that are encountered with in vitro reconstituted subunits.

The purified mutant 30S subunits were assessed for their structural integrity by running them on 10-30 % sucrose density gradients prepared in low magnesium buffer (Figure 22). Untagged wild-type 30S purified from MRE600^{R} strain and tagged wild-type 30S (has the MS2 tag in its 16S rRNA, but no mutation) purified from DH5α^{R}+ pcI+ pSpurMS2 strain were run as a control. The MS2-tagged 30S subunits migrate as heavier species than the untagged ones (Figure 22A, B). Youngman and Green had observed a similar phenomenon with tagged 50S and attributed it to the GST-MS2 fusion protein bound to the ribosomes[120]. The mutant Del790loop, unfortunately, did not form any detectable 30S. It was intriguing that the profiles, including that of the tagged wild-type 30S, showed similar multiple intermediate assembly products at the top of the gradient before the actual 30S peak (peak 4). This phenomenon was not observed in case of MRE 600^{R} untagged 30S. Therefore, it seems that the presence of MS2 tag causes a slight reduction in the efficiency of 30S subunits to assemble, which may then be aggravated by the presence of mutations. We collected the individual peaks for each mutant and assayed them for methylation by KsgA. For the G791C mutant, we could not collect the first peak due to a technical problem with the fraction collector.

In vitro analysis

We first compared the ability of KsgA to methylate 30S subunits bearing the MS2 tag to that of the untagged 30S (Figure 23A). Experiments were carried out with tagged wild type 30S subunits purified from Kasugamycin (ksg) resistant (Tagged-WT) and ksg

sensitive (WT Tagged-WT) DH5α cells, and untagged 30S subunits purified from ksg resistant MRE600 cells (Untagged MRE600^{R}). The incorporation of ^{3}H-methyl from S-adenosyl methionine (SAM) into the 30S was followed at discrete time points over an interval of 2 h. The concentration of Mg^{+2} in the reactions was adjusted to 4mM, a value shown to be optimal for KsgA function[58]. The Tagged-WT 30S showed fewer scintillation counts than the untagged 30S subunits, however this difference was within the range of error.

We then assayed the ability of KsgA to methylate the mutant subunits. The activity of assembly intermediates and 30S subunits collected from the sucrose gradients was compared to that of subunits not subjected to sucrose gradient, in a 2hr end point assay (Figure 23B). As control, this experiment was also performed with tagged wild-type 30S. Peaks 1, 2 and 3 did not yield enough subunits to perform a full reaction and hence, for those peaks, the reaction components and volume were reduced to half. These peaks, which represent intermediates of 30S subunit assembly, showed no activity and so were not pursued further. However, this data provided an important observation that KsgA does not methylate early intermediate assembly particles.

The G791 mutant subunits from the largest particles were approximately 50% as active as the wild type, with G791U being the most active of all. The subunits not subjected to sucrose gradients (non sucr grad, Figure 23B) are the pool of tagged subunits purified off the GST-affinity column. It was surprising that, in case of the mutants as well as the control, the non-sucrose gradient subunits showed greater activity than the pure

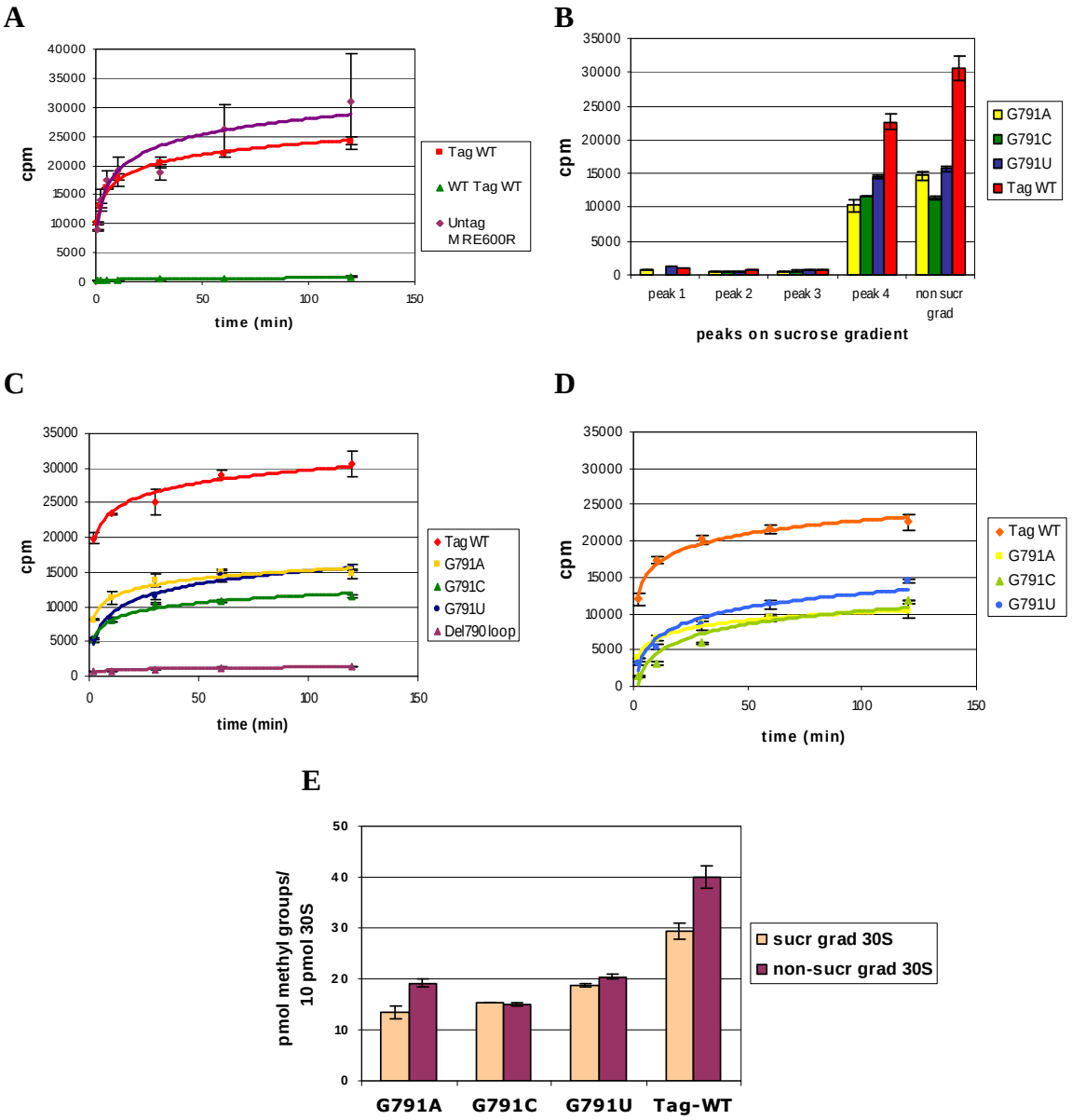

Figure 23. In vitro methylation assay of subunits. (A) Time-course assays for various control 30S subunits. (B) 2 h end point assay. The activity of assembly intermediates and 30S subunits collected from the sucrose gradients was compared to that of subunits not subjected to sucrose gradient. Time course assay for 790 loop mutants (C) and (D). The difference between (C) and (D) is that the 30S subunits used in (D) were subjected to a sucrose gradient for assessing their structural integrity. (E) Number of pmol of methyl groups per 10 pmol of 30S transferred after 2h. All the above reactions contained 200nM KsgA, 200nM subunits and 0.02 mM, 780cpm/pmol ^{3}H-methyl-SAM. However, due to insufficient yield of subunits, the reaction volumes for peak 1, 2 and 3 were reduced to half that of others. Assays were performed in triplicate; error bars represent standard deviation. CPM stands for Counts per Minute

30S subunits isolated via sucrose gradients (peak 4). To further understand this unexpected difference, we followed the methylation of both types of subunits over the course of two hours (Figure 23C, D). Although the overall activity trends in (C) and (D) did not look very different, the scintillation count of each reaction in (D) was lower than its counterpart in (C). Also notable is the slow onset of methylation in case of (D). Del790loop showed just above background activity. This was not surprising given the fact that no 30S subunits were detected for this mutant by sucrose gradient analysis (Figure 22F).

We also determined the extent of methylation at the 2 h time point for both, sucrose gradient purified 30S and non-sucrose gradient 30S subunits of each mutant (Figure 23E) using the specific activity of ^{3}H-methyl-SAM. With 10 pmol of 30S per reaction, and four methylation sites per 30S molecule, we would expect to see transfer of 40 pmol methyl groups per 10 pmol of 30S if the reactions have gone to completion. As can be seen in Figure 23E, the non-sucrose gradient Tagged-WT reaction appears to have reached completion whereas that of sucrose gradient Tagged-WT seems to be lagging behind. Also, the sucrose gradient reactions of G791 mutants show less methylation as compared to their non-sucrose gradient reactions. This may be due to partial deterioration of 30S subunits, caused by the extra handling steps involved in sucrose gradient analysis. Hence, for the subsequent mutants, only the non-sucrose gradient 30S subunits were assayed.

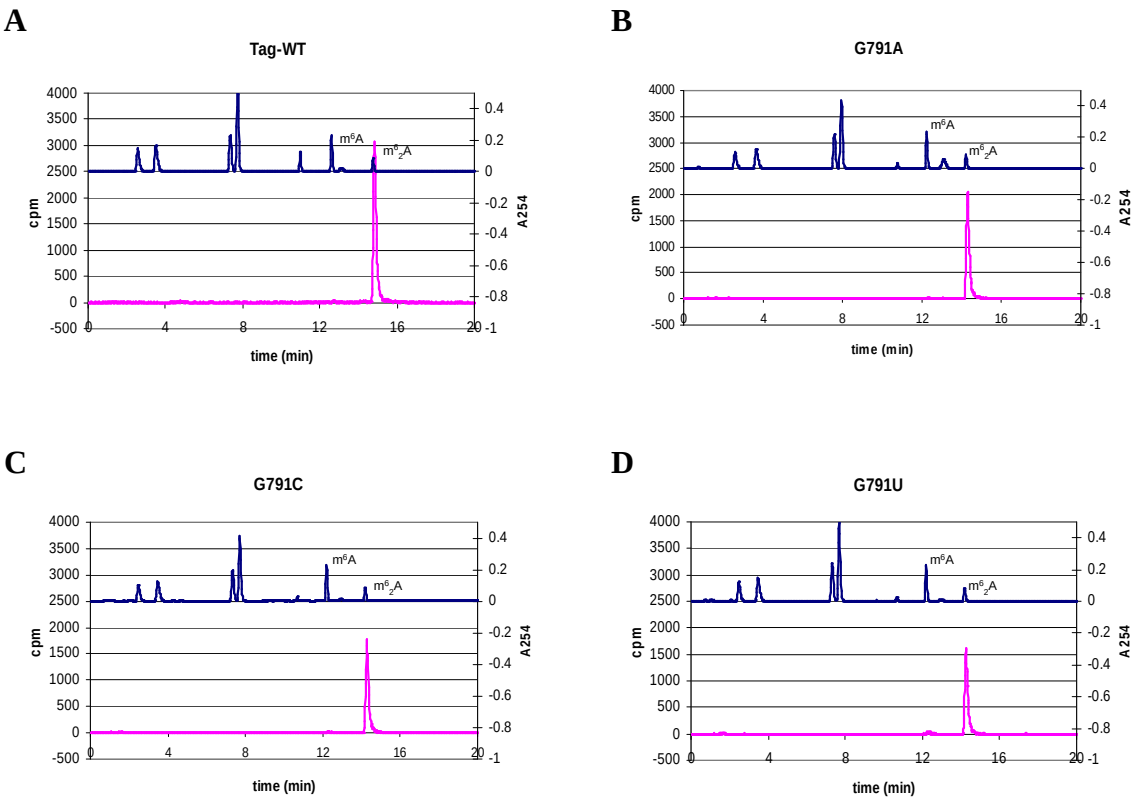

Figure 24. HPLC traces. (A), (B), (C) and (D) respectively represent the traces of Tag-WT, G791A, G791C and G791U, at the end of a 2 h reaction. At the top is the UV trace, plotted as A254 (arbitary units) *vs.* time, representing the reference nucleotides, with m^6A and $m^6{}_2A$ peaks labeled. At the bottom is the scintillation trace, plotted as cpm *vs.* time, showing the type and extent of methylation.

Mutation	Conc. of sample (nmol/80 μl)	Area under the peak*	Area under peak/nmol	Normalized percent (%)
Tag-WT	15.36	3253763	211833.52	100
G791A	19.68	2264810	115081.80	54.32
G791C	13.28	1872381	140992.54	66.55
G791U	15.2	1714992	112828.42	53.26

Table 2. Extent of methylation of wild type and G791 mutant 30S by KsgA. Normalized percent is the ratio of mutant to wild type area under peak/nmol x 100.
* Area under the dimethyladenosine scintillation peak.

Nucleoside Analysis

As seen in Figure 23E, for both sets, the G791 mutants showed approximately 50% methylation as compared to Tagged-WT. We asked if the reduced activity of the mutants came from poor binding of KsgA to such 30S or was due to reduced catalysis resulting from the disruption of important interactions between KsgA and G791. The KsgA/Dim1 enzymes transfer a total of four methyl groups from four SAM molecules to two adenosines. Therefore each adenosine should be dimethylated. However, we have previously established that the KsgA/Dim1 enzymes produce a mixture of monomethyladenosines (m^6A) and dimethyladenosines ($m^6{}_2A$) when they cannot bind their substrates tightly[62, 121]. Therefore, to determine whether the observed reduction in the methylation was the result of reduced KsgA binding, we performed nucleoside analysis using HPLC to measure the ratio of m^6A and $m^6{}_2A$.

For this analysis, only the non-sucrose gradient 30S subunits were used. In vitro assays for all three mutants and the control were incubated for two hours. At the end point, the 16S rRNA was extracted from the 30S subunits and subsequently digested and dephosphorylated. Component nucleosides were separated by reversed-phase HPLC. Peaks corresponding to m^6A and $m^6{}_2A$ were assigned by comparison to reference nucleosides (Figure 24). Extent of methylation was determined by calculating the area under the $m^6{}_2A$ scintillation peak per nmol for each mutant and then normalizing the values to that of Tag-WT.

16S rRNA isolated from none of the mutant 30S subunits contained measurable amounts of labeled m^6A. Although only about 50% of the potential sites were methylated

in case of G791 mutants, almost 100% of the incorporated radiolabel was seen in the dimethyladenosine peak. Such predominance of $m^6{}_2A$ clearly indicates that the mutations at G791 do not diminish the ability of 30S to be involved in a tight complex with KsgA. This, together with the fact that G791 lies in close proximity to the target adenosines and catalytic core of KsgA in Xu's model[91], indicates that the reduced activity of the mutants must be due to reduced catalysis resulting from the disruption of important interactions between KsgA and G791.

Helix 44 Mutations

Another region of 16S rRNA that defines the binding site of KsgA, as per the KsgA-16SrRNA model proposed by Xu et al., is helix 44[91]. To test this premise, we took advantage of the well conserved tandem sheared A•G base pairs, G1417•A14183 and A1418•G1482, of helix 44 (Figure 25). According to Xu's model, the adenosines of these base pairs make primary interactions with the highly conserved residues R221, R222 and K223 of the C-terminal domain of KsgA[83, 91].

The characteristic of two consecutive sheared A•G base-pairs is that, although each A•G base pair can distort the helix individually, when a G•A base pair is followed by another A•G base pair, they are arranged such that the distortion caused by the first base pair is compensated by that caused by the other, thus maintaining the overall regularity of the helix[122, 123]. We created the A1418C and A1483C mutants, in which the corresponding sheared A•G base pair was replaced by a C:G base pair. The helix 44 of these mutants, carrying only one of the two consecutive sheared A•G base pairs, should be fairly distorted. Therefore, if the interaction between KsgA is governed to an

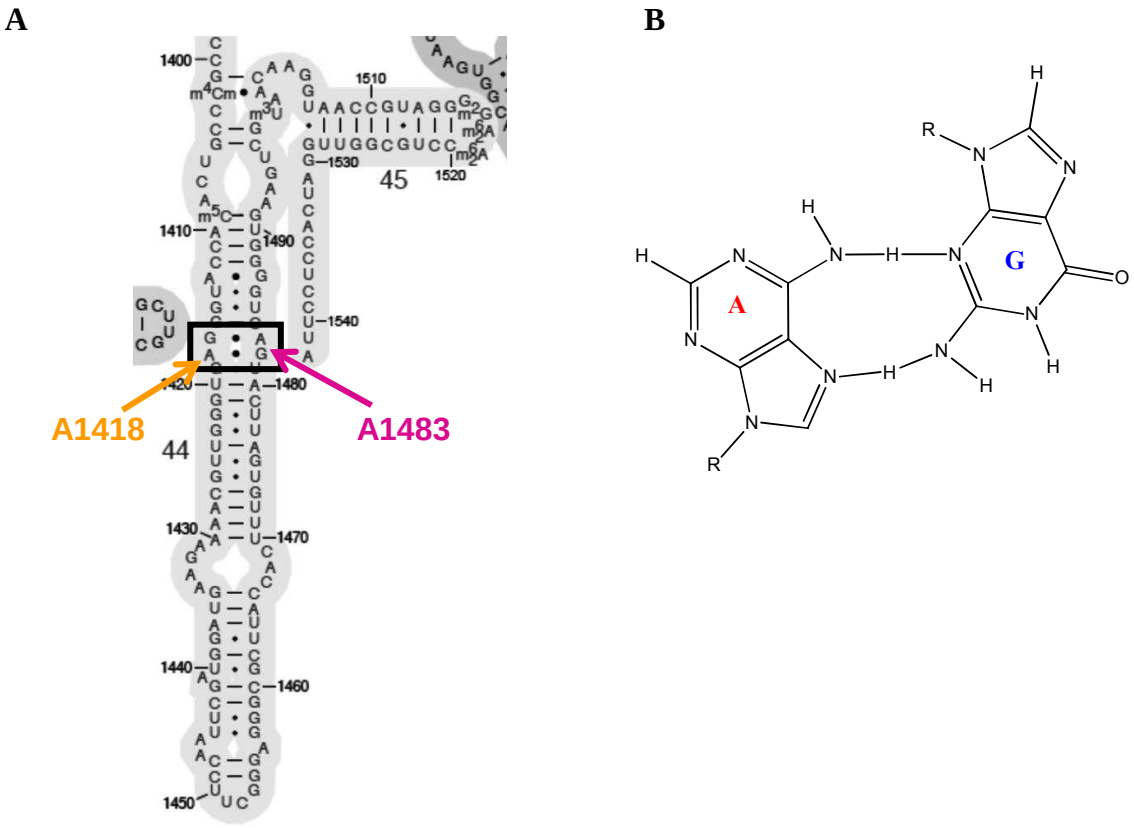

Figure 25. (A) Secondary structure of Helix 44 and 45 showing the location of the tandem sheared A•G base pairs. A1418 and A1483 were mutated to a C, one at a time. rRNA image adapted from Noller et al.[118] (B) Chemical structure of sheared A•G base pair, as per Gutell et al.[122]

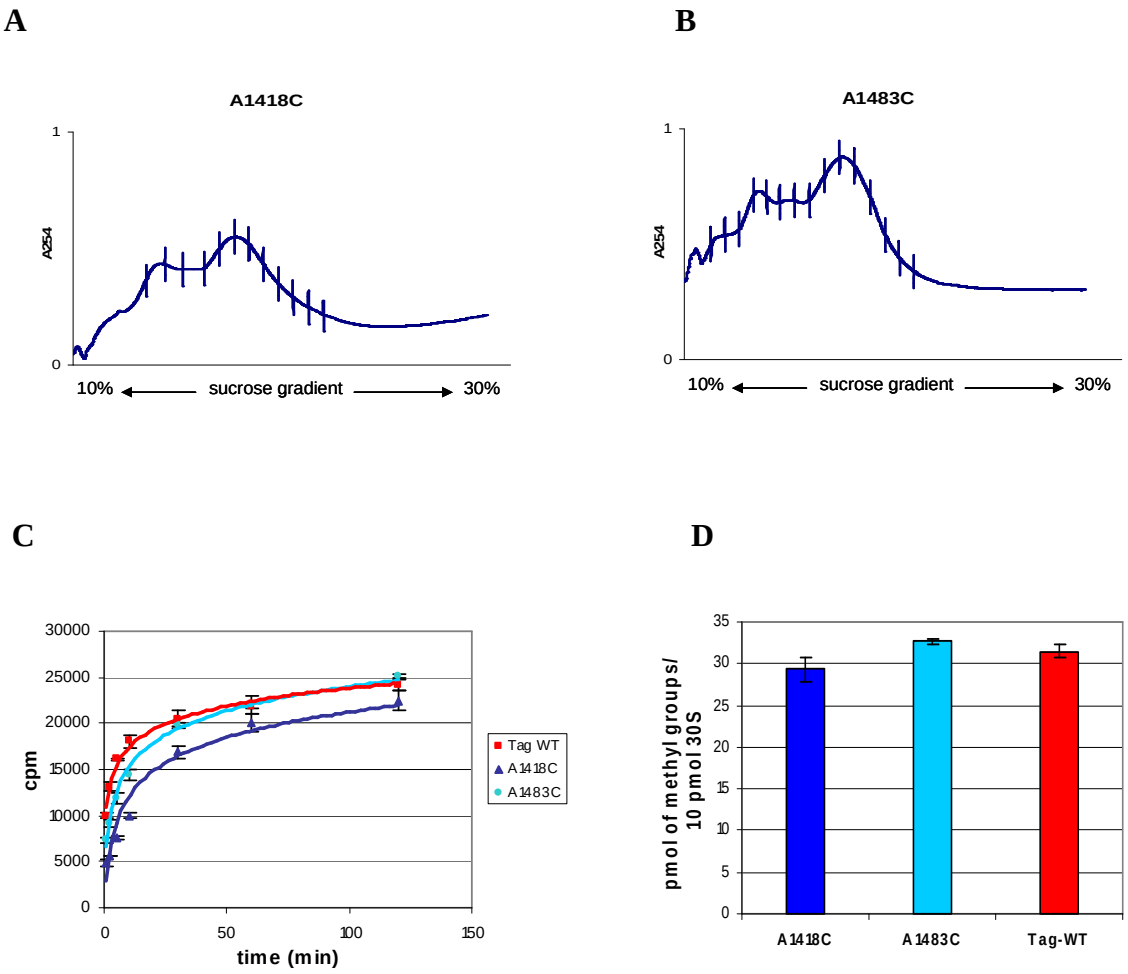

Figure 26. Sucrose gradient analysis of A1418C (A) and A1483C (B) mutants. (C) Time course activity assay of affinity purified, non-sucrose gradient, mutant 30S subunits. (D) Number of pmol of methyl groups transferred after 2h. The reactions in (C) contained 200nM KsgA, 200nM subunits and 0.02 mM, 780cpm/pmol ^{3}H-methyl-SAM. Assays were performed in triplicate; error bars represent standard deviation. CPM stands for Counts per Minute.

important degree by the local structure around A1418 and A1483, then mutation of these residues should affect KsgA's ability to bind and modify 30S subunits.

For our assays, pure mutant 30S subunits were obtained using the MS2 affinity purification system. Structural integrity of the mutant 30S subunits was assessed by sucrose density gradients. The sucrose gradient profiles were comparable to that of the G791 mutants (Figure 26A and B). Time course assays were performed in triplicate for the affinity purified, non-sucrose gradient, mutant 30S subunits (Figure 26C). As a control, Tagged-WT 30S were also assayed. The amount of methyl groups transferred at the end of 2 h was determined using specific activity of ^{3}H-methyl-SAM (Figure 26D).

A1418C showed 10% less activity as compared to Tagged-WT, whereas A1483C mutant showed wild type activity in terms of the total number of methyl groups added. It was remarkable that although their reactions reached near completion at the end of 2 h, both mutants showed slower onset of activity as compared to wild type. It is possible that the distortion in mutant helix disrupts KsgA binding, causing it to fall off more frequently, thereby reducing the initial rate of reaction. If this is true, KsgA must produce a mixture of m^6A and $m^6{}_2A$. To follow the type of methylation produced by KsgA along the course of two hours, we performed nucleoside analysis of the time course assays.

For both mutants, even at the 2 minute time point, almost all incorporated radiolabel was in the form of $m^6{}_2A$ (Figure 27). No detectable m^6A was found to indicate that the slow onset of activity is due to poor binding of KsgA to mutant 30S subunits. It therefore appears that the slow onset and the marginally reduced activity at the end of 2 h are the result of helical distortion affecting catalysis. However, mutation of R221-K223

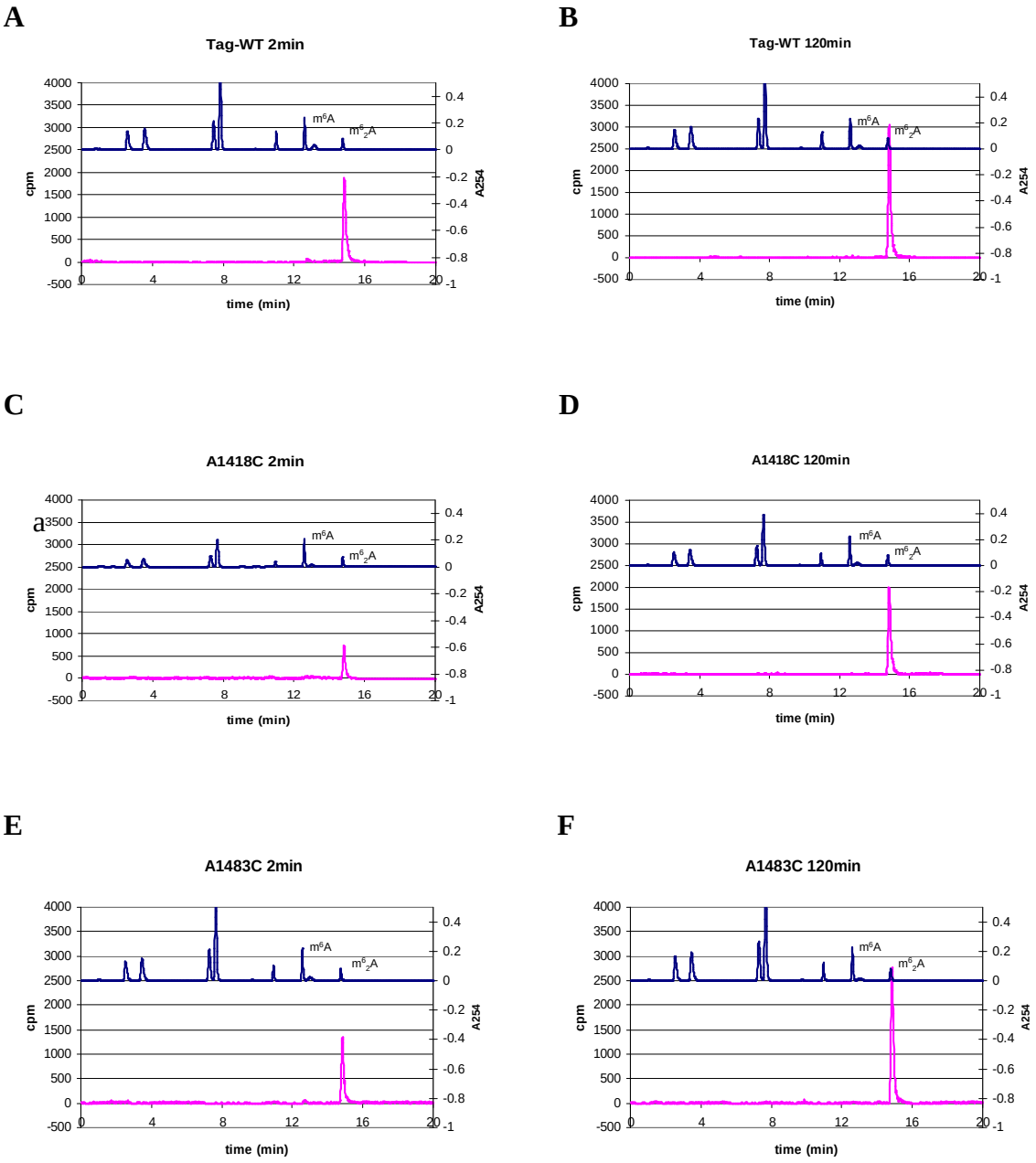

Figure 27. HPLC traces. (A), (C) and (E), respectively, represent the traces of Tag-WT, A1418C and A1483C reactions at the 2 min time point, whereas (B), (D) and (F) represent the respective traces at the end of 2 h. The top and bottom traces are as described in Figure 24.

Mutation	Time (min)	Conc. of sample (nmol/80 μl)	Area under the peak*	Area under peak/nmol	Normalized percent (%)
Tag-WT	2	14.88	2094513	140760.28	66.44
	5	14.56	2335944	160435.71	75.73
	10	15.2	2657800	174855.26	82.54
	30	14.88	2807826	188697.98	89.07
	60	15.68	3044237	194147.76	91.65
	120	15.36	3253763	211833.52	100
A1418C	2	10.08	716950	71125.99	33.57
	5	13.28	1536793	115722.36	54.62
	10	12.64	1792748	141831.32	66.95
	30	10.4	1602480	154084.61	72.73
	60	9.28	1509784	162692.13	76.80
	120	12.16	2258209	185707.97	87.66
A1483C	2	13.6	1452401	106794.19	50.41
	5	16.32	1968382	120611.64	56.93
	10	15.68	1969140	125582.90	59.28
	30	13.76	2176047	158142.95	74.65
	60	13.12	2498835	190459.98	89.91
	120	15.2	3147252	207056.05	97.74

Table 3. Extent of methylation of wild type and helix 44 mutant 30S by KsgA at 2, 5, 10, 30, 60 and 120 min. Normalized percent is the ratio of mutant to wild type area under peak/nmol x 100.
* Area under the dimethyladenosine scintillation peak.

in KsgA, which represent three of the six conserved C-terminal residues, leads to reduced activity by virtue of reduced binding[121](O'Farrell, H. C.; Rife, J. P., unpublished results). This suggests that either RRK residues do not directly interact with the sheared base pairs of helix 44 or the mutations and distortion of the helix are insufficient to affect binding with RRK residues, only reducing catalysis marginally. More work is required to resolve between these possibilities.

Helix 45 mutations

We wanted to determine whether KsgA binds directly on the helix 45 of 16S rRNA or if it binds the surrounding structures and helix 45 only reaches out and presents the target adenosines to the active site of KsgA.

Ofengand and group made point mutations in helix 45 and tested the reconstituted mutant 30S subunits for methylation by KsgA[93, 124]. The results, however, do not indicate whether the reduced methylation observed in case of some mutants (G1523A and C1524U) is due to the disruption of KsgA binding site on helix 45 or due to conformationally altered presentation of the target adenosines at the active site of KsgA. We wanted to make mutations which did not affect the helical conformation of helix 45.

Therefore, we made a pair of mutations, Add2bp and Del2bp, at the base of helix 45 with the intention of preserving the local helical structure while significantly altering the position of the target adenosines relative to the bulk of the 30S subunit. In the first, two G:C base pairs were inserted between C1510:G1525 and G1511:C1524, whereas in the latter, two base pairs, C1509:G1526 and C1510:G1525, were deleted from the base of helix 45. These mutations, respectively, elongate or shorten the helix. The greatest

conservation for this part of helix 45 is the fact that there are a constant number of Watson-Crick base pairs. While there is some conservation of sequence, the greatest conservation appears to be the conserved helical structure. Therefore, adding two base pairs or deleting two such base pairs should not affect the conformation of target adenosines or the overall conformation of the helix. Also, the crystal structure of KsgA-RNA reported by Tu et al. shows that this region is outside the KsgA binding site[99], which means that at least the insertion of two base pairs in this region should not affect the binding of KsgA on helix 45, should it truly bind as reported by the authors of this study. On the other hand, if KsgA binds in such a way as to include direct interactions with helix 44 and the 790 loop, then helix 45 must reach out into the active site of KsgA. Therefore, helix 45 with two additional base pairs added might be too long to properly fit into the active site of KsgA. Conversely, a helix with two base pairs removed might be too short for the target adenosines to be able to reach it. In either case, the target adenosines would not be in proper position for efficient methylation. We also made one other mutant, DelHel45, in which the entire helix 45 was deleted, hoping to determine if KsgA can bind 16S rRNA in the absence of helix 45.

Like for the previous mutants, we examined the structural integrity of affinity purified, mutant subunits by running them on 10-30 % sucrose density gradients prepared in low magnesium buffer (Figure 28A-C). Unfortunately, the Del2bp and DelHel45 mutants did not form any detectable 30S subunits. The Add2bp mutant did produce a peak at the expected position; however, a 50S subunit admixture was also observed (Figure 28B). 2D gel electrophoresis analysis of the Add2bp mutant subunits revealed

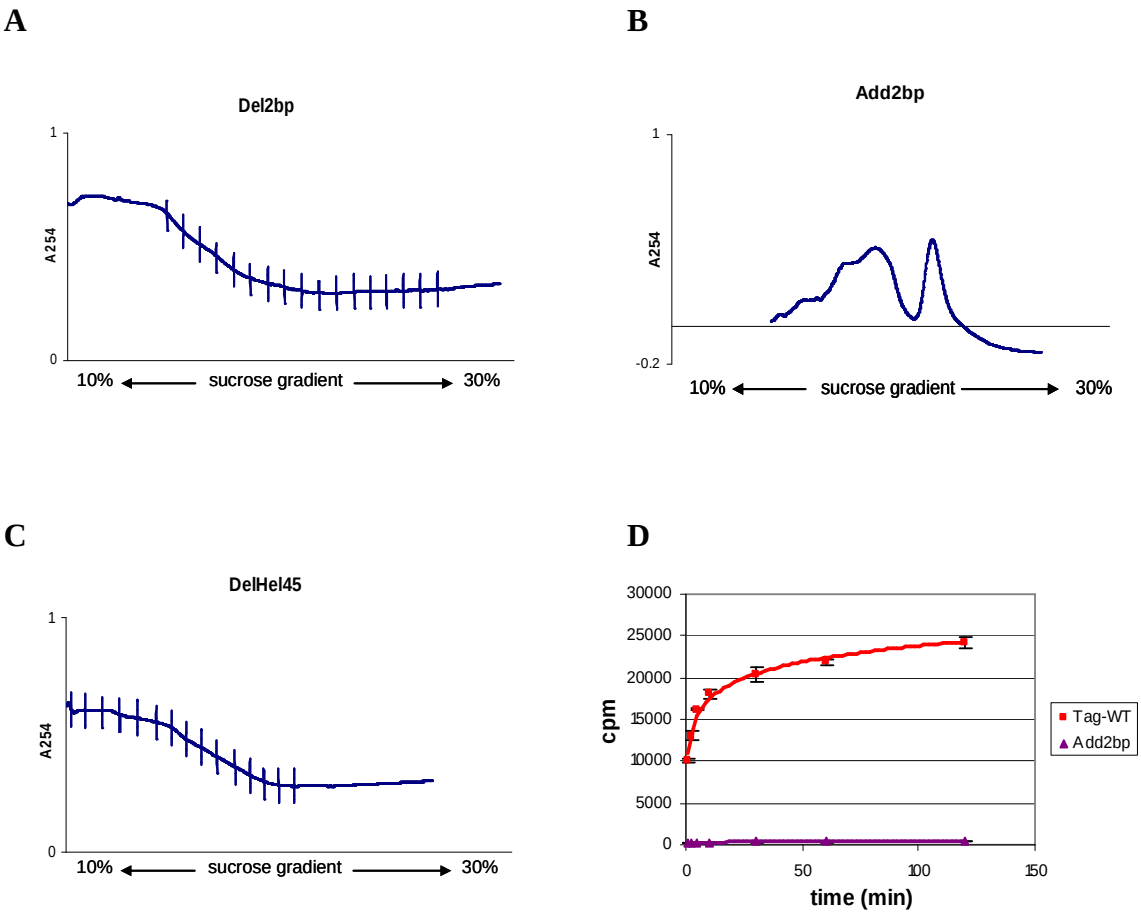

Figure 28. Sucrose gradient analysis of Del2bp (A), Add2bp (B) and DelHel45 (C) mutant 30S subunits. Del2bp and DelHel45 did not form any detectable 30S. The sharp peak on right, in case of Add2bp, was confirmed by 2D gel electrophoresis as 50S contamination (data not shown). (D) Time course activity assay of affinity purified, non-sucrose gradient, Add2bp 30S subunits. The reactions contained 200nM KsgA, 200nM subunits and 0.02 mM, 780cpm/pmol ^{3}H-methyl-SAM. Assays were performed in triplicate; error bars represent standard deviation. CPM stands for Counts per Minute.

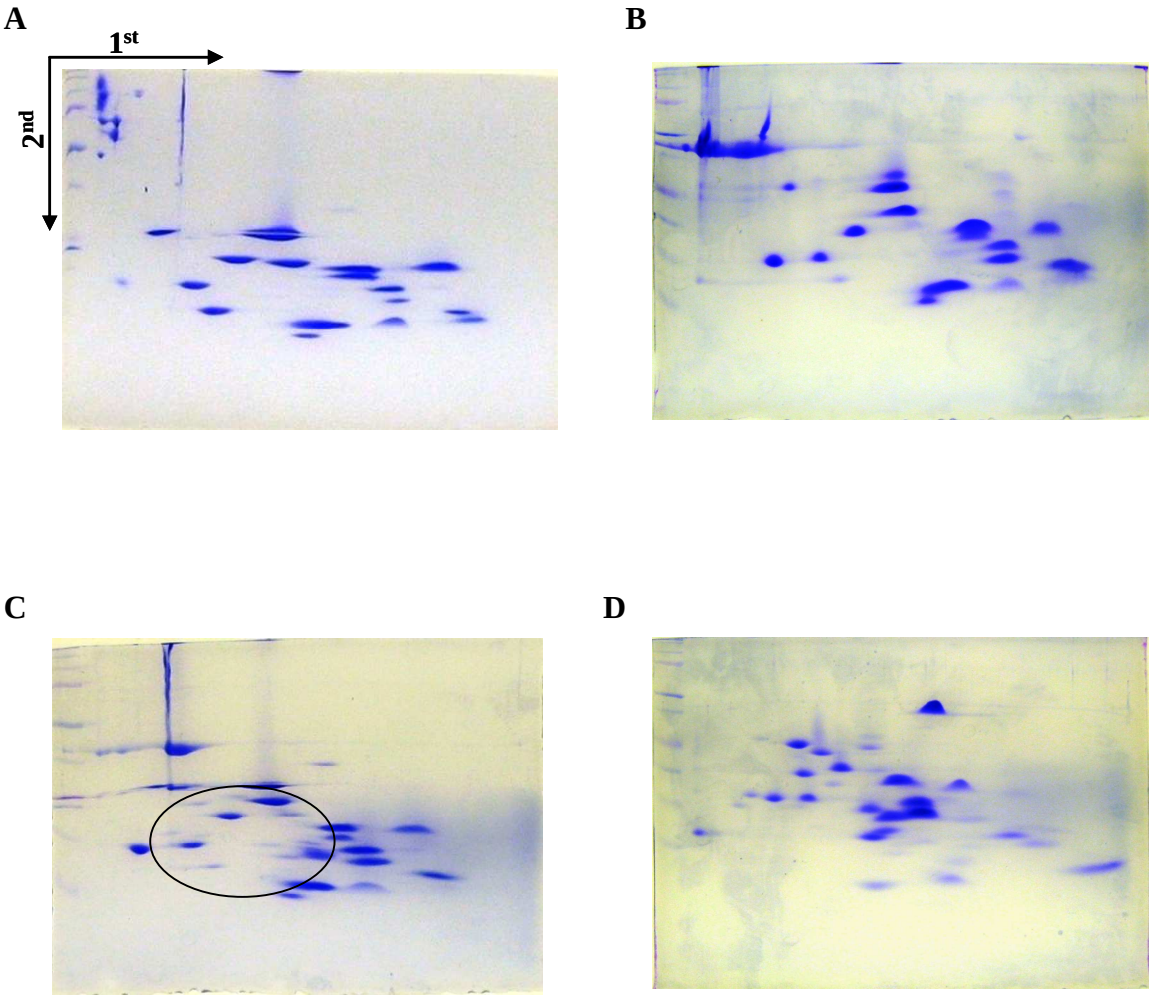

Figure 29. Two-dimensional gel electrophoresis analysis. Ribosomal proteins extracted from 30S subunits of MRE600^{R} isolated by sucrose gradient (A), affinity purified 30S subunits of Tag-WT (B), affinity purified mutant 30S subunits of Add2bp (C), 50S subunits of Tag-WT isolated by sucrose gradient (D). The indicated region of (C) shows additional protein spots comparable to those in (D), indicating that the 30S of Add2bp were contaminated with 50S subunits.

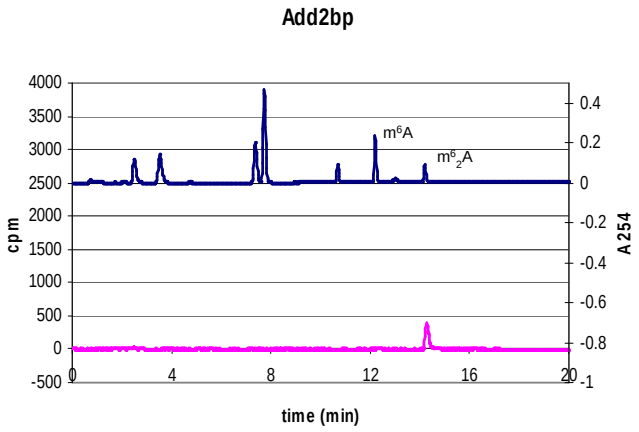

Figure 30. HPLC trace of Add2bp mutant at the end of a 2 h reaction. The top and bottom traces are as described in Figure 24.

Mutation	Conc. of sample (nmol/80 μl)	Area under the peak*	Arear under peak/nmol	Normalized percent (%)
Tag-WT	15.36	3253763	211833.52	100
Add2bp	13.28	398633	30017.54	14.17

Table 4. Extent of methylation of wild type and Add2bp mutant 30S subunits by KsgA at the end of a 2 h reaction. Normalized percent is the ratio of mutant to wild type area under peak/nmol x 100.
* Area under the dimethyladenosine scintillation peak.

that the second peak appeared due to the presence of 50S subunit impurity (Figure 29). Previous work by us confirmed that under the salt conditions used, even stoichiometric amounts of 50S have no deleterious effect on methylation. Therefore, the Add2bp mutant was used without further purification for qualitative analysis. A time course assay (Figure 28D) revealed very low levels of activity, which was confirmed by HPLC (Figure 30) and the presence of $m^6{}_2A$. No m^6A was observed, indicating that tight binding between KsgA and this mutant 30S particle remained. Certainly, a more complete picture would have emerged had the Del2bp and the DelHel45 mutations produced fully formed 30S-like particles. Nevertheless, these results with Add2bp support our proximity model, whereby KsgA remains anchored to helix 44/790 loop and awaits the presentation of the target adenosines in the loop of helix 45.

Considering the position of helix 45 in the structure of *E. coli* 30S subunit[18], if KsgA were to bind on helix 45, it would require the enzyme to be fairly embedded in the 16S rRNA and a corresponding large alteration of 16S conformation, something not seen in chemical protection experiments[91]. Although it has been indicated that A1518 and A1519 are more exposed to the solvent in the inactive state than in the active state[100], this shift cannot be so drastic as to allow KsgA to bind helix 45 without facing spatial clashes with the surrounding rRNA. Also, in this case, KsgA will have to significantly disrupt the tertiary structure of the region in an attempt to detach after completion of methylation. This seems highly improbable, especially since KsgA release has been thought to bring about the final maturation and passage of the 30S subunits into the translation cycle[69].

Additional mutations

We have made several other mutations in the 16S rRNA of *E. coli* than those discussed above. However, the purification of these mutants over the GST Trap column was problematic, and the 30S stocks were found to be severely contaminated with 50S subunits. The Rife lab will continue to work on obtaining pure 30S subunits for these mutants and analyzing them for KsgA activity. Here, I list the mutants and the rationale behind creating them.

1) AddA: KsgA transfers a total of four methyl groups from four SAM molecules to two adenosines, A1518 and A1519. However, the exact mechanism of the transfer remains to be established. Cunningham et al. reported that mutation of either adenosine does not disrupt dimethylation of the other, ruling out any obligate order of methylation[93]. We asked if KsgA simply scans its target site and adds as many methyl groups as possible, or if it is specific for transferring four methyl groups to two adenosines. To test this, we inserted an additional adenosine between the target nucleotides, A1518 and A1519.

2) G1523A and C1524U: Formenoy et al. tested the importance of the highly conserved U1512•G1523 base pair and surrounding residues as recognition element for KsgA and observed an 80% reduction in methylation when G1523 was mutated to A or when C1524 was mutated to U[124]. However, they used 30S subunits reconstituted using TP30 and in vitro transcribed 16S rRNA for their experiments. We want to determine if their results can be reproduced using in vivo derived mutant 30S subunits, and compare the efficiency of the reconstitution system with that of the in vivo derivation system.

3) G926 and A1394 mutants: The inactive state of the 30S subunit is not a mere loosening or unfolding of its active conformation. Moazed et al. described the inactive state as a structurally different state where some bases become more exposed to the solvent while some others become more sequestered[111]. Among the sites reported by the group to undergo an increase in chemical reactivity when changed from active to inactive state are the 923 to 927 and 1391 to 1401 intervals. In the tRNA binding studies carried out by Ericson et al., the delG926 mutation seemed to lock the e30S subunit in the inactive state, where as the A1394 mutations seemed to lock it in the active state[125]. We believe that these phenomena occur due to depletion of the Mg^{+2} cation that interacts with both, G926 and A1394 in the active state. According to the structure of *E. coli* 30S subunit[18], the distance between G926 or A1394 and the KsgA target site is so great that a direct interaction of KsgA with these nucleotides seems unlikely. We have created mutations of G926 and A1394 to determine how KsgA activity is dependent upon Mg^{+2} concentration and the 30S conformation.

Conclusions

The presence and function of KsgA has been conserved throughout evolution. It is anticipated that the binding site of this universally conserved enzyme on 30S subunit will also be conserved. We carried out a preliminary examination of some of the 16S rRNA sites implicated in KsgA binding. Our results indicate that G791 of the 790 loop, and to some extent, the sheared base pairs of helix 44 are important for the catalytic activity of KsgA. KsgA failed to indicate any direct interactions with helix 45 in our experiments. Therefore, it appears that helix 45 is not involved in binding to KsgA; rather it only

presents the target adenosines in its loop at the active site of KsgA. These observations support the proximity model, previously reported by Xu et al.[91]

Experimental

Bacterial strains and plasmids

The bacterial strains used in this study were *E. coli* MRE600, DH5α (Invitrogen) and BL21-DE3 (Invitrogen). Recombinant KsgA was obtained as described by O'Farrell et al.[109].

The plasmid pGSTMS2 (gift from Prof. Rachel Green, Johns Hopkins University) expresses the GST-MS2 fusion protein in large quantities. This plasmid was transformed into BL21-DE3 cells. Growth and induction were carried out according to Youngman and Green[120].

The plasmids pSpurMS2 and pCI1857 were also a generous gift from Prof. Green. pSpurMS2 had been constructed by inserting MS2 tag in the *E. coli* rrnC operon of the plasmid pLK35. The rrnC operon is under the control of the lambda operator/promoter and this promoter system allows inducible expression of tagged ribosomes[126]. Mutations Del790loop, G791A, G791C, G791U, A1418C, A1483C, Add2bp (adding two G-C base pairs in helix 45), Del2bp (deleting two G-C base pairs in helix 45) and DelHel45 were introduced into the 16S rRNA gene of the pSpurMS2 rrnC operon by sited directed mutagenesis (Quickchange XL, Stratagene and Phusion, Finnzymes). The sequences of the primers used to create the mutations are listed in Table 5 (see Appendix). Primer information is given in appendix. The sequences of all clones were confirmed by sequencing at Nucleic Acids Research Facilities, Virginia Commonwealth University.

The Dh5α cells were first transformed with pcI857, which contains the temperature-sensitive cI857 allele of the λ repressor gene. The λ cI857 repressor completely represses transcription from rrnC of pSpurMS2 at 30 °C, but is inactivated at 42 °C allowing transcription from rrnC[127]. To obtain 30S subunits unmethylated at A1518 and A 1519, a standard procedure of selecting for strains resistant (>600 ug/ml) to the antibiotic kasugamycin was followed[110]. One such resistant strain ($DH5\alpha^R$ + pcI) was then used to obtain untagged 30S or was transformed with wild type or mutant pSpurMS2 to obtain wild type or mutant MS2 tagged 30S, respectively.

Purification of His-tagged KsgA

KsgA was purified on a HiTrap Chelating column (Amersham Pharmacia) as previously described in Chapter 2. Purity was assessed by SDS-PAGE. Purified protein was dialyzed into storage buffer containing 50mM Tris, pH 7.4, 400mM NH_4Cl, 6mM BME and 10% glycerol. Concentration was estimated using Bradford method. Protein was stored at 4 °C.

Purification of GST-MS2 fusion protein

GST-MS2 fusion protein was purified as previously described[120]. Briefly, the clarified cell lysate from two liters induced culture was mixed with 10ml Glutathione–Sepharose resin (Amersham Biosciences) pre-equilibrated in 1x PBS (140mM NaCl, 2.7mM KCl, 10.2mM Na_2HPO, and 1.8mM KH_2PO_4) and stirred at room temperature for 30min. The mixture was then loaded onto an empty gravity flow column and the settled column was washed with 100ml 1x PBS. GST-MS2 protein was eluted with 40ml elution

buffer (50mM Tris–HCl pH 8.0, 10mM reduced glutathione), dialyzed against three 1-L changes of storage buffer (1x PBS, 20% glycerol) and stored at –80 °C.

Purification of submethylated 30S ribosomal subunits

Control untagged ribosomes were obtained from strain MRE600 and DH5α^R + pCI via ultracentrifugation across a sucrose gradient using standard techniques[111].

In order to obtain pure, in vivo-derived mutant 30S ribosomal subunits for our assays, we used the MS2 affinity purification system developed by Youngman and Green[120]. In this approach, the 16S rRNA of mutant 30S is expressed from a plasmid borne operon in the background of wild-type ribosomes. Since mutant 30S subunits are assembled in vivo, they do not suffer from severe functional limitations that are encountered with in vitro reconstituted subunits.

Briefly, saturated cultures of DH5α cells carrying the plasmids pcI857 and pSpurMS2, grown at 30 °C with amp (100ug/ml), kan (100ug/ml) and ksg (400 ug/ml), were diluted 50-fold into LB with amp (100 ug/ml) and grown to OD_{600} ~0.7-0.8 at 42 °C, to express tagged ribosomes. To obtain purified tagged 30S subunits, a 5 ml GSTrap FF FPLC column (Amersham Biosciences) was used. Per 70 mg crude ribosomes, 3mg GST-MS2 was loaded onto the column and washed with 10 ml binding buffer (20 mM Tris-HCl [pH 7.5], 100 mM NH_4Cl, 0.3 mM $MgCl_2$, 6mM BME). Crude ribosomes were then applied to the column, washed with 40 ml binding buffer and eluted with 20 ml elution buffer (50mM Tris-HCl [pH 7.5], 100mM NH_4Cl, 0.3mM $MgCl_2$, 10mM reduced glutathione, 6mM BME). Low Mg^{+2} concentration (0.3 mM) of the binding and elution buffers enable the 70S subunits to separate into constituent subunits[111], allowing selective

retention of mutant 30S on the column. Eluted 30S subunits were dialyzed into buffer K (40mM Tris-HCl [pH 7.4], 40mM NH_4Cl, 4mM MgOAc, 6mM BME) and concentrated to at least 1nmol/ml based on 1 A_{260} = 67 pmol in an Amicon Ultra centrifugal filter (Millipore, MWCO 100,000) and stored at –80 °C. Alternatively, to assess their structural integrity, eluted 30S subunits were dialyzed into a low magnesium buffer (50mM Tris–HCl, [pH 7.5], 150mM NH_4Cl, 0.3 mM $MgCl_2$, and 6mM BME) and loaded on 10–30% sucrose density gradients prepared in the same buffer. The fractions containing 30S subunits were pooled, dialyzed against buffer K and stored at –80 °C to be used later for in vitro assays.

In vitro activity assay of KsgA

The in vitro assays were carried out according to O'Farrell et al.[62]. Reactions contained 40 mMTris (pH 7.4), 40 mM NH_4Cl, 4 mM MgOAc, 6 mM BME, 0.02 mM ^{3}H-methyl-SAM (780 cpm/pmol; MP Biomedicals), 200 nM 30S subunits (10 pmol/50µl reaction), and 200 nM enzyme (10 pmol/50 µl reaction). Total reaction volume and components were adjusted based on the number of time points intended. To minimize any lag in the reaction start, buffer and reagents were prewarmed to 37 °C. At each designated time point, 50 µl was withdrawn and added to a prechilled tube containing 10 µl of 100 mM unlabeled SAM (Sigma-Aldrich) to quench the reaction. The quenched reactions were deposited onto DE81 filter paper (Whatman), washed twice with ice-cold 5% TCA, and rinsed briefly with ethanol. Filters were air-dried for 1 h, placed into scintillation fluid, and counted. Alternatively, at each designated time point, 50 µl was

removed and added to prechilled phenol/chloroform/isoamyl alcohol mixture to begin extraction of 16S rRNA for HPLC analysis (see below).

HPLC analysis

Labeled 16S rRNA was extracted from 30S subunits and separated on HPLC system as described in Chapter 3. Each sample was spiked with non-labeled N6-methyladenosine and N^6, N^6-dimethyladenosine (Sigma-Aldrich) nucleoside standard for the ease of identification in UV chromatogram. Prior to separation, concentration of total nucleosides in each sample was determined by UV spectroscopy. For each sample, 80 µl was loaded onto the column. The values of area under the dimethyladenosine scintillation peak per nmol were normalized to those of wild type.

Two dimensional gel electrophoresis analysis

Total ribosomal proteins were extracted from ribosomes using the acetic acid method previously described by Siegmann & Thomas[128]. Briefly, 200 pmol purified 30S or 50S subunits stored in Buffer K (40 mMTris (pH 7.4), 40 mM NH_4Cl, 4 mM MgOAc, 6 mM BME) were ethanol precipitated in the presence of 300mM sodium acetate. The pellet was resuspended in 50µl dH_2O and stirred together with 100µl Mg-acetate/acetic acid (1ml 1M magnesium acetate and 20 ml acetic acid) at 4°C. The mixture was centrifuged (15,000*g*, 30 min, 4 °C) to remove rRNAs. The supernatant containing the proteins was mixed with 5 volumes of acetone and allowed to precipitate overnight at −20 °C. Proteins were pelleted by centrifugation, resuspended in the sample buffer and subjected to two-dimensional gel electrophoretic analysis using the method developed by Geyl et al.[129]. The proteins were separated according to charge (pI) by isoelectric

focusing in the first dimension and according to size by SDS-PAGE in the second dimension followed by Coomassie staining of the gels for visualization.

CHAPTER 5: Future Work

Ribosome biogenesis is a fundamental cellular process that has grown more and more divergent and intricate through evolution. The structural and functional conservation of KsgA/Dim1 enzymes through all domains of life underscores the importance of this family of methyltransferase and warrants the need to study them. The work presented here contributes significantly to the characterization of KsgA and lays a foundation for future work. Several questions remain to be answered to achieve the ultimate goal of fully defining the role of KsgA/Dim1 in ribosome biogenesis and applying that knowledge in the design of novel, more selective antimicrobial agents. The three foremost questions are: Where does KsgA bind on the 30S subunit? What is the true in vivo substrate for methylation by KsgA? What is the exact mechanism of methyl transfer?

Our experiments have shown that G791 of helix 24 is important for KsgA catalysis. There were, however, no indications that KsgA interacts directly with helix 45 or the tandem sheared base pairs of helix 44. It is extremely important to determine the binding site of KsgA on the 30S subunit in order to gain complete insight into the role of KsgA in ribosome biogenesis and how it interacts with other ribosome biogenesis factors or chaperons. The attempts of crystallizing 30S subunit in complex with KsgA have turned out to be unsuccessful. However, the Rife lab is currently employing the cryo

electron microscopy technique to pursue the structure of this complex which, if successful, will tell us where KsgA binds on 30S. If not, the interaction of KsgA with helix 44 alone can be characterized by X-ray crystallography or NMR (Xu et al. showed that helix 44 is the major 16S rRNA component in binding to KsgA[91]).

We have shown that KsgA binds to naked 16S rRNA but does not methylate 21S particle in vitro, even though it has all the eight ribosomal proteins reported by Thammana and Held as absolutely essential for KsgA activity[96]. This implies that KsgA recognizes an assembly state that comes after 21S has been formed. To identify the minimal substrate, the activated counterpart 26S can be formed in vitro and tested for KsgA activity. If this particle does not prove to be a substrate, then testing of more complicated particles may be required. Such particles can be formed by batch, single omission or single addition of the 26S binding ribosomal proteins (S2, S3, S10, S14, S21) to 26S particles. Since these proteins have also been reported to be inhibitory to methylation by KsgA[96], such experiments will also allow us to learn whether the original inhibition observed with this set of proteins was accurate or was due to some impurity. In vivo methods can also be applied to study the minimal substrate of KsgA. One such approach, which will allow direct monitoring of 16S rRNA methylation events, involves pulse labeling of methylated RNA using ^{3}H-methionine. The level of methylation at A1518/A1519 can then be determined in specific populations of 16S rRNA-containing ribonucleoprotein particles (RNPs) using HPLC nucleoside analysis. Additionally, to determine whether or not the inactive conformation of 30S exists in vivo, the in vivo methylation of G926 and A1394 mutant 30S can be assessed by HPLC nucleoside

analysis. In vitro, these mutations have been shown to trap 30S subunits in the inactive or active state, respectively[125].

Another outstanding question is the exact mechanism of methyl transfer. There are no data concerning the number of binding events required to transfer four methyl groups or the order in which the methyl groups are transferred by KsgA. Kinetic studies of wild-type and mutant KsgA proteins can help probe KsgA's mechanism. Techniques such as in vitro time course analysis, HPLC nucleoside analysis and primer extension can be used in combination to determine the order of methyl group transfer. Also, analyzing the type and extent of methylation in the 30S mutant, AddA (described in Chapter 4), may shed some light on whether or not KsgA specifically transfers four methyl groups to the two adenosines.

Connolly et al. recently showed that the E66A mutant, a methyltransferase-inactive form of KsgA, profoundly impairs ribosome biogenesis and is deleterious to cell growth[69]. Their findings highlight the importance of methylation in KsgA function and ribosome biogenesis, but contrasts dramatically with the results for catalytically inactive Dim1p, where no phenotype is observed[76]. We have shown that KsgA proteins from distinct evolutionary kingdoms can function in a bacterial system, demonstrating conservation of both, the protein and the key structures of 30S subunit. However, Pulicherla et al. recently showed that neither the bacterial nor the archaeal ortholog could complement for the eukaryotic Dim1[77]. The above discussion clearly indicates that KsgA has the potential of being exploited as a selective antibacterial drug target. Future work is

required to identify those determinants which distinguish KsgA from its orthologs. Preliminary efforts for KsgA inhibitor design are already underway in the Rife lab.

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APPENDIX

Table 5. Sequence of primers used to create mutant 16S rRNA

	Primers
1 For Rev	G791A 5’- GGAGCAAACAGGATTAAATACCCTGGTAGTCC - 3’ 5’- GGACTACCAGGGTATTTAATCCTGTTTGCTCC - 3’
2 For Rev	G791C 5’- GGAGCAAACAGGATTACATACCCTGGTAGTCC - 3’ 5’ - GGACTACCAGGGTATGTAATCCTGTTTGCTCC - 3’
3 For Rev	G791U 5’ - GGAGCAAACAGGATTATATACCCTGGTAGTCC - 3’ 5’ - GGACTACCAGGGTATATAATCCTGTTTGCTCC - 3’
4 For Rev	Del790loop 5’ - CGTGGGGAGCAAACAGAAATGGTAGTCCACGCCG - 3’ 5’ - CGGCGTGGACTACCATTTCTGTTTGCTCCCCACG - 3’
5 For Rev	A1418C 5’ - CCGTCACACCATGGGCGTGGGTTGCAAAAGAAG - 3’ 5’ - CTTCTTTTGCAACCCACGCCCATGGTGTGACGG - 3’
6 For Rev	A1483C 5’ - CCACTTTGTGATTCATGCCTGGGGTGAAGTCG - 3’ 5’ - CGACTTCACCCCAGGCATGAATCACAAAGTGG - 3’

7	DelHel45
For	5’ - GCTTACCACTTTGTGATTCATGATCACCTCCTTACCTTAAA GAAGC - 3’
Rev	5’ - GCTTCTTTAAGGTAAGGAGGTGATCATGAATCACAAAGTGG TAAGC - 3’
8	Del2bp
For	5’ - CGTAACAAGGTAAGTAGGGGAACCTGCTTGGATCACCTCC - 3’
Rev	5’ - GGAGGTGATCCAAGCAGGTTCCCCTACTTACCTTGTTACG - 3’
9	Add2bp (used pSpurMS2 with DelHel45 mutation as template)
For	5’ - GCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA GGTAACCGCGTAGGGGAACCTGCGCGGTTGGATCACCTCCTTACC TTAAAGAAGC - 3’
Rev	5’ - GCTTCTTTAAGGTAAGGAGGTGATCCAACCGCGCAGGTTCCCC TACGCGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACAAA GTGGTAAGC - 3’
10	AddA (used pSpurMS2 with DelHel45 mutation as template)
For	5' - GCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA GGTAACCGTAGGGGAAACCTGCGGTTGGATCACCTCCTTACCTTAA AGAAGC - 3'
Rev	5' - GCTTCTTTAAGGTAAGGAGGTGATCCAACCGCAGGTTTCCCCT ACGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACAAAGTG GTAAGC - 3'
11	G926A
For	5' - GAATTGACGAGGGCCCGCACAAGC - 3'
Rev	5' - GCTTGTGCGGGCCCTCGTCAATTC - 3'
12	G926C
For	5' - GAATTGACGCGGGCCCGCACAAGC - 3'
Rev	5' - GCTTGTGCGGGCCCGCGTCAATTC - 3'

13 For Rev	G926U 5' - GAATTGACGTGGGCCCGCACAAGC - 3' 5' - GCTTGTGCGGGCCCACGTCAATTC - 3'
14 For Rev	A1394C 5' - CCGGGCCTTGTCCACACCGCCCG - 3' 5' - CGGGCGGTGTGGACAAGGCCCGG - 3'
15 For Rev	A1394G 5' - CCGGGCCTTGTGCACACCGCCCG - 3' 5' - CGGGCGGTGTGCACAAGGCCCGG - 3'
16 For Rev	A1394U 5' - CCGGGCCTTGTTCACACCGCCCG - 3' 5' - CGGGCGGTGTGAACAAGGCCCGG - 3'
17 For Rev	G1523A 5' - GGGGAACCTACGGTTGGATCACC - 3' 5' - GGTGATCCAACCGTAGGTTCCCC - 3'
18 For Rev	C1524U 5' - GGGGAACCTGTGGTTGGATCACC - 3' 5' - GGTGATCCAACCACAGGTTCCCC - 3'

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