Details of Research Work

Background

Antibiotic resistance is a silent tsunami which is projected to kill over 10 million people annually by 2050. Due to extensive antibiotic abuse past few decades have witnessed a rampant upsurge in multidrug resistant strains (MDRs) [1]. Alarming increase in MDRs compounded with the scarcity of potential drug candidates is a serious concern for the healthcare sector. To combat this issue, continuous isolation of new antimicrobials, as well as purposeful synthesis of next generation of antibacterial by medicinal chemists, is an ongoing uphill task[7]. However, resistance develops within months of introduction of these variants. Emergence of superbugs like NDM1, MRSA, CRE, XDR TB etc in past few years, which have acquired resistance to the last line of antibiotics, pose an enormous health threat[1, 2]. Hence, it has become imperative to focus on strategies that are associated with the origins of resistance itself. Therapies developed via this route can be used in conjunction with existing antibiotics, thereby, re-sensitizing the pathogen to the obsolete drugs[3].

Using the structural biology skills as a versatile tool we have studied this antibiotic resistance which have immense societal implications. Our contribution in the arena of decoding antibiotic resistance has pushed the envelope of research towards providing solutions for these critical problems. Bacterial adaption to antibiotics is a result of their genetic plasticity and rapid time scale of evolution[4]. Pathogens commonly gain resistance either via mutational adaptation or via transfer of specific genetic elements by lateral gene transfer[4]. Several pathogens acquire resistance, mostly via horizontal gene transfer from *Streptomyces*, the original progenitors.[5] In 2014 our group solved the X-ray structure of the tetracycline receptor (TetR) in complex with DNA from the antibiotic producer organism, *Streptomyces coelicolor (**Nucleic Acid research 2014***)[6]. This structure was a breakthrough as it helped in providing a glimpse as to how the antibiotic producers protect themselves against the antibiotics they synthesize. This discovery was important as these regulators pass into pathogens via lateral gene transfer and result in creation of superbugs. Her work on efflux pump regulators showed that the TetRs found in pathogens such as the multi-drug resistant organism *Staphylococcus aureus*, are similar to those in these progenitors. This work paved the way towards understanding evolutionary links and helped shed light on how cellular antibiotic concentration is regulated (***NAR2014, JPC2014, BBA2015, JSB2017)***.[6,7,8,9] Subsequently, our group solved structures of unique TetRs from *Streptomyces fradiae,* another antibiotic producer, that further helped decode the various architectures and mechanisms by which drug regulation occurs (***JBC 2017)***.[9] Based on the information harnessed here, developing efflux pump blockers can be envisioned that will help reverse resistance and re-sensitize the pathogens towards existing antibiotics.

Ribosome is the central protein synthesis machinery of the cell. Several commonly occurring antibiotics bind to the ribosome and inhibit/alter protein synthesis, thereby leading to cell death (Figure 1A).[10] Structural analysis has revealed that most of the antibiotics targeting ribosome act on mechanistically active regions of this macromolecule. For example, in the 50S subunit they target mechanisms like GTP hydrolysis, peptide bond formation and polypeptide release.[11] Macrolide antibiotics, MLS, target the highly conserved peptidyl transferase region, wherein they block the exit of nascent polypeptide leading to premature termination of translation. A large number of them interact with the important base adenine A2058 which is replaced by guanine in eukaryotes (Figure 1B).[12] This difference protects eukaryotes from the action of MLS drugs and has been exploited by the drug industry to synthesize several commercial variants [11]. Crystal structures of macrolides bound to ribosome have revealed that apart from the central polyketide scaffold it is the fine play of the rare sugars mycaminose and cladinose that play an integral role in drug specificity[16]. For instance, the hydroxyl group of the mycaminose sugar forms hydrogen bond with A2058, thereby facilitating anchoring to the ribosome.[13] To outplay this interaction as a survival strategy pathogens have acquired enzymes such as Erms (Erythromycin Resistant Methyltransferase) that either mono or di-methylate at this position.[14] Presence of methyl group at A2058 position is sufficient to create a steric clash with the methyl amine groupand the O2A of the mycaminose sugar leading to dislodging of the antibiotic from the ribosome(Figure1C,D)[15]. While mono-methylation results in moderate level of resistance, di-methylation results in aggressive resistance (Figure 1D)[14]. However, since di-methylation in general slows down and leads to inefficient protein synthesis it is not generally a preferred mark[16].

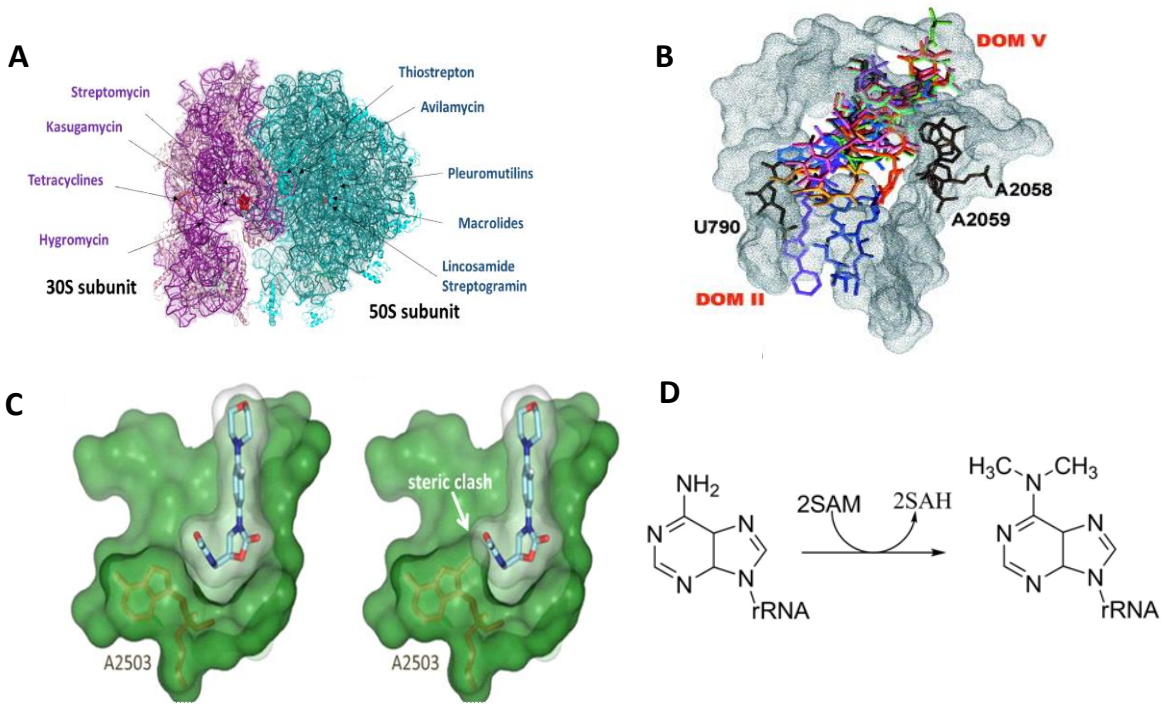


Figure 1 Mode of binding of antibiotics to the ribosome. (A) Binding sites of several antibiotics to the bacterial ribosome (PDB ID: 3J3V). The 16S rRNA is coloured Purple and 23S rRNA is coloured teal. (B) Mode of interaction of MLS antibiotics near peptidyl transferase region (adapted from Auerbach T., *et.al*. Trends biotech, 2004, 12, 570-576) (C) Model showing resistance by methyltransferase. Left: Antibiotic binds to ribosome interacting with A2503.Right: Methylation at A2503 leads to steric cash preventing antibiotic from binding thus conferring resistance (adapted from Locke J., et.al, Antimicrob Agents Chemother, 2010, 54(12):5337-43) (D) Schematic representation of general methylation reaction (SN2 reaction) at N6 exocyclic position of adenine

Structural analysis of Erm reveals that eight to nine conserved motifs identified in DNA Mtases, involved in sequence identification, catalysis and SAM binding are similar and are also present in rRNA Mtases. Motif I is highly conserved and forms the binding site that harbours the cofactor SAM. Motif IV, VI and VIII that surround Motif I constitute the active/catalysis region. Moreover, studies by our group ***(JACS 2019)*** have demonstrated that select loops, adjacent to Motif I and VII are essential for RNA recognition and specificity.[17] Switching these loops is sufficient to swap rRNA methylation. Our group was able to decipher the mechanism of targeting of these ribosomal methyltransferases and has unraveled an allosteric pocket that can be exploited to stop methylation. This knowledge can now be used to explore design of new drugs that will stop pathogens from attaining superbug stature.[17]

Delineating structural determinants which help pathogenic ribosomal methyltransferases confer resistance [17]

Erm methyltransferases selectively and specifically methylate a single base in the nascent peptide exit tunnel of the ribosome. The resulting methylation of the base leads to a steric clash dislodging the antibiotic which sits in the same place preventing the antibiotic from functioning.[18] Methylation by Erms occurs via the universal methyl donor S-adenosylmethionine (SAM).[19] SAM-dependent methylation is a ubiquitous modification that not only occurs in RNA but is also an epigenetic mark conferred on both DNA and proteins that govern gene regulation. Structural analysis showed that Erms have almost more than 50% homology with another methyltransferase known as KsgA.[20] KsgA is a biogenesis factor that acts on the 30S small subunit of the prokaryotic ribosome and demethylates A1518 and A1519 (*E.coli* numbering). Methylation by KsgA is imperative for the

smaller subunit to attain maturation and the lack of the enzymatic modifications help conferring resistance to kasugamycin, an aminoglycoside.[21] Interestingly, irrespective of the structural homology, Erms and KsgA completely act on two different substrates and recognize different structural motifs.

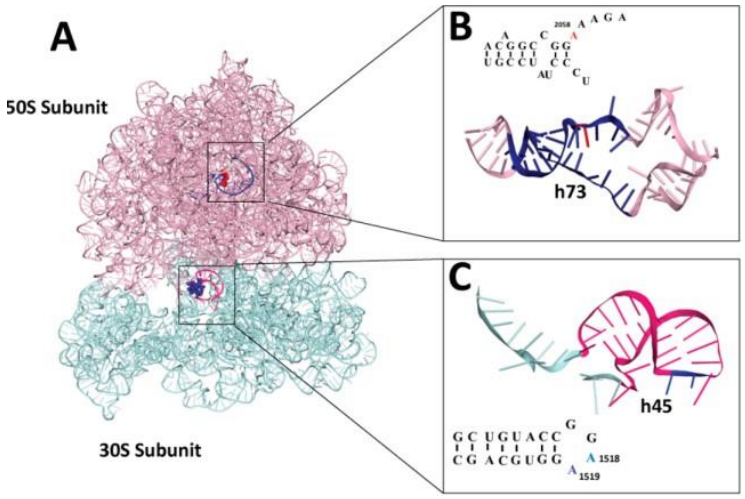
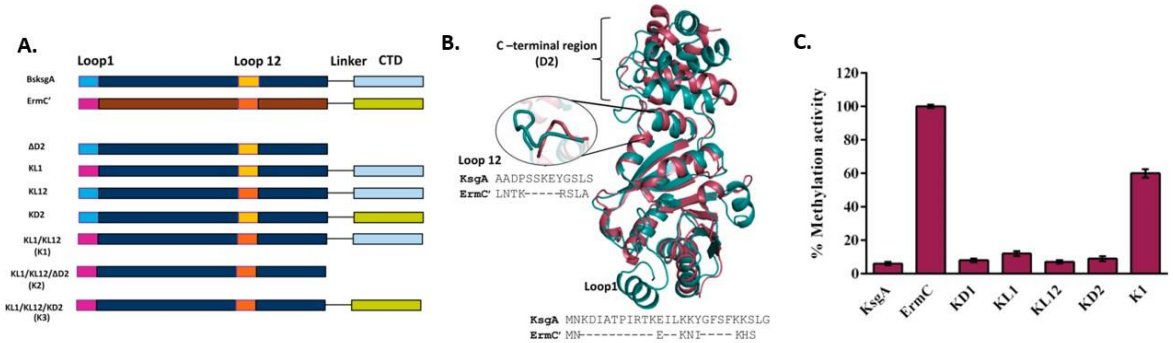


Fig 2. A. The 70S prokaryotic ribosome B. Region in the 50S which is recognized and methylated by Erms C. Region in the 30S which is recognized by KsgA

We decided to undertake and understand this problem and help elucidate what makes one protein an antibiotic resistance perpetuator and the other a house keeping biogenesis factor. In order to do so, we created a series of chimeric proteins in which loop1 or loop12 or both were exchanged onto a KsgA template. Both ErmC′ and KsgA exhibit very low sequence conservation in the C-terminal head domain, and structural comparison reveals that rmsd in this domain is quite high (~5.5 Å). Therefore, we generated variants in which the head was deleted and related chimeras that additionally had the loops switched. Among these chimeras the most important ones involved changing loop 12 and 1 of KsgA to those of ErmC (Fig 3A,B). Scintillation assays (transfer of tritium-labeled methyl group) showed that chimeras with the individual substitution of loops (chimeras KL1, KL12) do not exhibit methylation. Chimera (KD2), in which the head domain of ErmC′ was stitched onto the KsgA scaffold, also showed no methylation. However, a chimera in which the dual loop1 and loop12 regions were switched exhibited significant methylation (Figure 3C) and had a catalytic efficiency that was 50% lower than that of the native Erm enzymes (ErmC′, Erm42; Table S2). These results were confirmed via MALDI TOF assay. The results indicate that loop1 and loop12 collectively recognize the target RNA for methylation and are sufficient for the gain of Erm activity. It was further noted that K1 can still recognize 30S ribosomal subunit, but



with reduced efficiency.

Fig 3. A. Design of chimeras to switch targeting propensity. B. Structural alignment of BsKsgA (teal) and ErmC′ (pink; PDB ID: 1QAM) used as a guide for creation of the chimeras. Structural and sequence differences are highlighted in the inset C. Activity assay of chimeras toward the Erm mini-RNA substrate (scintillation assay).

The *in-vitro* results were corroborated by testing the erythromycin resistance of the chimeras in vivo. Cells transformed with a plasmid containing the competent chimeras were grown at various concentrations of erythromycin and their viability was tested. It was observed that the construct with both loops as well as the head domain-swapped was the best candidate (K3, minimum inhibitory concentration (MIC). The loop1 and loop12 swapped chimera (K1) was marginally resistant, whereas K2, the headless variant of K1, was moderately resistant to erythromycin (Fig 4 A, B). The fact that the loop1 and loop12 switched chimera efficiently methylated mini-RNA substrates in vitro shows that these structural elements suffice for local orientation of the rRNA. *In-vivo*, however, the head domain plays a more critical role in leading the enzyme to the select ribosomal region and serves as a sensor of the global environment.

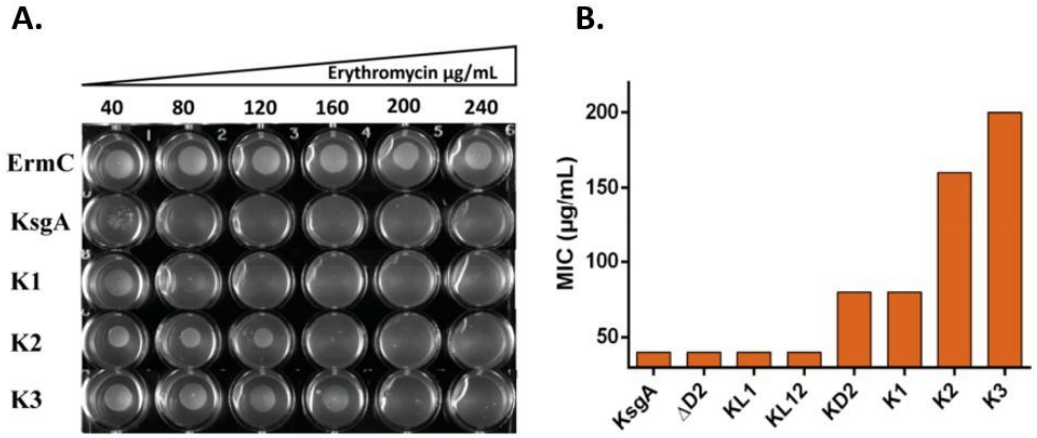


Figure 4. Sensitivity of chimeras toward the macrolide antibiotic erythromycin in vivo. (A) Spot assay for chimeras with increasing erythromycin concentration. (B) MIC studies with various chimeras.

This work highlights the mechanism through which nature achieves specific targeting in methyltransferases via either addition or deletion of the flexible loop elements that govern substrate recognition. These loop variations influence the positions they target. In KsgA, these loops are longer because substrate specificity is attained via extensive interaction with both the target site and the surrounding region. On the contrary, in the case of Erms, specificity is achieved owing to the fact that Erms target only precursor ribosomal subunits and the Erm site is inaccessible in mature ribosome. Hence, less complex loop architectures suffice for Erms. Because these loop regions are the divergent elements, specific inhibitors that target only the pathogenic Erms can now be developed. The power of this approach is that this line of design does not target the common SAM binding motif and instead focus on unique RNA selection elements. In the immediate context, inhibitor searches using this strategy can pave the way toward the development of combination therapy aimed at reversing antibiotic resistance.

Although, mini-RNA studies warrant a lot of information, a closer biological setting would be to study rMTase, in action, in a ribosomal setting. Hence, our group embarked on working with the true substrate of theses ribosomal MTases, that is the ribosome, so as to determine a holistic picture of the interaction and dynamics of these enzymes.

Decoding the Mechanism of Specific RNA Targeting by Ribosomal Methyltransferases [23]

Erms are able to methylate short RNA stretches but unable to act on the assembled 50S subunit. In contrast, KsgA does not methylate short RNAs but acts only on the 30S subunit.[22] The ability of rMTases to recognize their cognate substrates is encoded within the enzyme and how they achieve this is an interesting question that remains to be addressed. In order to further understand the mechanism of substrate specificity and targeting and the molecular details of N6 adenosine methylation, KsgA was chosen as a target as the absence of KsgA-mediated methylation at position 1518 and 1519 in 16S rRNA entails aminoglycoside kasugamycin resistance in *E. coli* and other bacteria due to conformational rearrangement in the binding site of the antibiotic. We went on to solve the cryo electron microscope structure of KsgA in complex with the 30S ribosomal subunit at a global resolution of 3.5 Å. The map of the KsgA−30S complex from the full data set showed heterogeneity, and subsequent 3D classification yielded five main classes (K1, K2, K4, K5, and K6) with KsgA bound to the ribosomes and the overall resolutions of the cryo-EM maps range between 3.17 and 3.6 Å. (Fig 5)

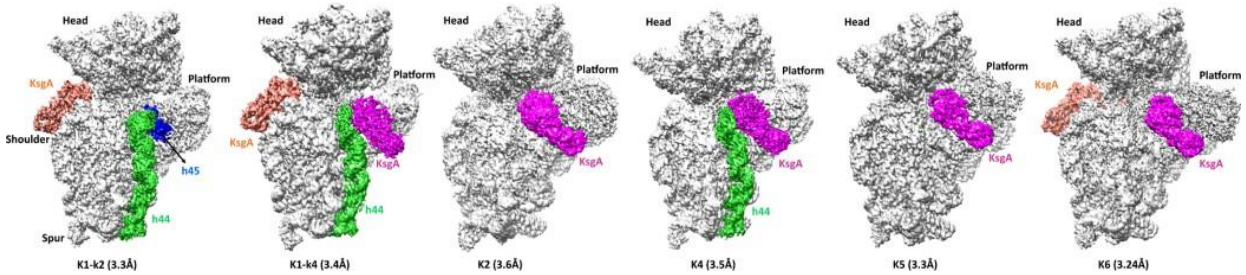


Fig 5. Cryo-EM structure of the KsgA−30S complex. The maps of cryo-EM classes reported in this study

Helix 44 is one of the important RNA helices involved in decoding and harbors two bases at 1492 and 1493 (*E. coli* numbering) that monitors the correct interaction of codon−anticodon helix by stabilizing the first two Watson−Crick base pairs. The flexibility of h44 in the immature 30S subunit is possibly one of the reasons for the inability to trap this state in all reported structures till date. Similar flexibility in the h44−45 linker is also observed in the low-resolution cryo-EM structure of the eukaryotic pre-40S assembly factor complex where the human homolog of KsgA, Dim1, was found bound as one of the proteins. Our structure of the 30S−KsgA complex was not only able to capture this elusive helix but also showed that distortions in both h44 and h45 are introduced by the steric interaction of the N-terminal β6/7 linker of KsgA with the h44−45 linker, in order to present the target residue to the active pocket of the enzyme. In this conformation, h44 has bent away from the 30S subunit platform, and as a result, the decoding site, importantly A1492/A1493 and C1054, moved away, thereby precluding the interaction with mRNA/tRNA duplex. Comparison of the unmethylated and methylated 30S subunits reveals that dimethylation at h45 by KsgA results in the closure of the gap between h45 and h44 that finally orders h44, maturing the 30S ribosomal subunit. Thus, before methylation, h44 is largely flexible and allows the entry of KsgA (Fig. 6). KsgA inserts itself between h44 and h45, separating these helices, and

gets anchored onto the immature 30S through its C-terminal domain, which has several positively charged residues. This enables the interaction of KsgA and positions the correct substrate, h45, into the catalytic centre via flipping of G1516 into the exclusive pocket prepping the catalytic pocket for methylation by allowing the flip of A1518/A1519. In the final methylated form, both helices h44 and h45 come closer and are stabilized by both hydrogen bonding and van der Waals interactions, thereby sealing the interface that results in a structured h44 such that the biogenesis is complete and the ribosome is primed for further assembly.

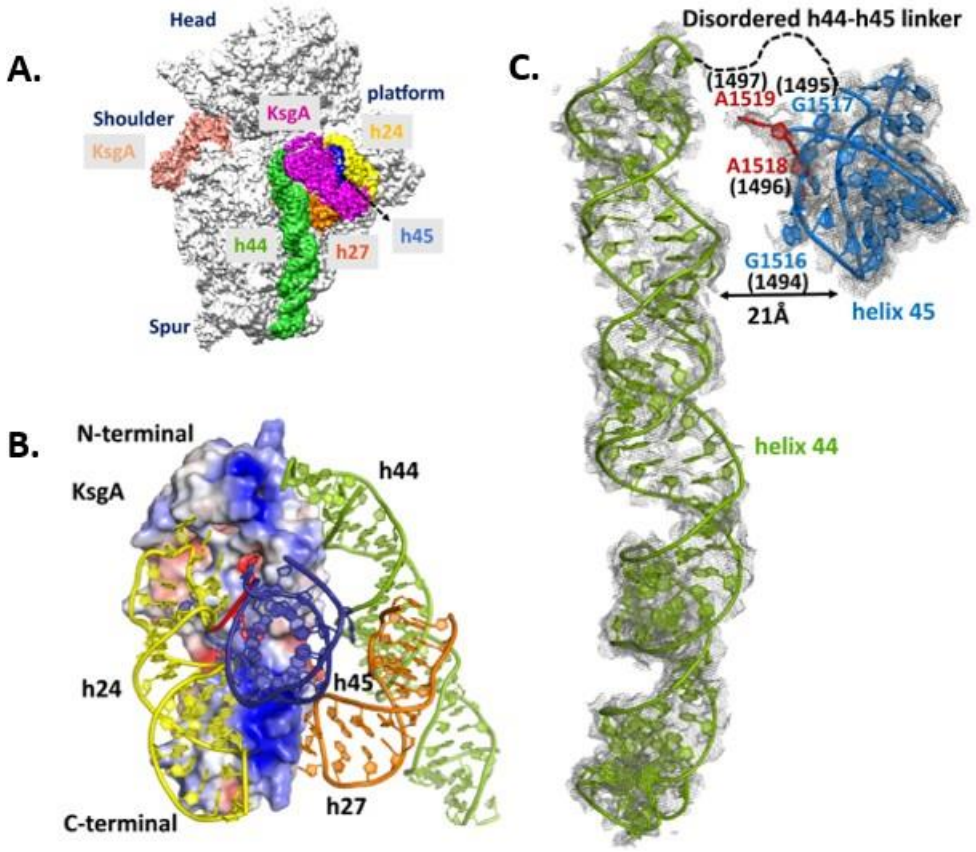


Fig 6. A. Cryo-EM reconstruction of the complex; KsgA (magenta) binds to the ribosomal platform and is surrounded by rRNA: h45 (blue), h27 (orange), h24 (yellow), and the upper region (green) of h44. B. Electrostatic surface potential map of KsgA colored by surface charge (the scale ranges from −5 kT/e (red) to 5 kT/e (blue)), in complex with h45 (dark blue), h44, h24, and h27. C. Cryo-EM densities of h44 and h45 showing the arrangement of helices in the 30S−KsgA complex.

The structure of the complex enabled us to design mutations in both helix 44 and 45 such that exact determinants of the interactions are revealed which has not been brought to limelight till date. Mutations have been introduced in both helix 44 and helix 45 so see how changes in specific bases affect the catalytic activity of the enzyme. Mutations designed pertain to changing base pairing in helix 44 and swapping regions between helix 44 and helix 45. All of these mutations markedly disturbed the catalytic efficiency of the enzyme (Fig. 7) thus highlighting for the first time the importance of rRNA determinants on the enzymatic activity of biogenesis factor.

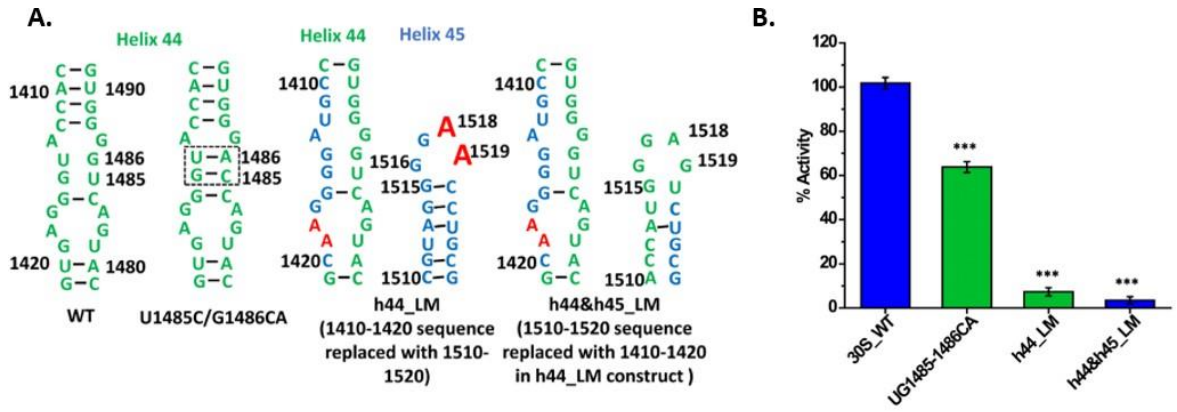


Fig 7.A. Design of h44 and h45 mutants in the 16S rRNA B. In vitro KsgA methylation assay with altered target site constructs of h44. Data for three experimental replicates are shown with the mean and standard deviation. Results show that methylation efficiency is significantly affected on perturbation of these important regions.

Our structure clearly shows that in the presence of the protein, the mix of hydrophobic and electrostatic interactions alters the conformation of the tetraloop by a rotation of approximately 90° (Figure 8), inducing significant distortions in the backbone such that it fits into the active site pocket. This helps us postulate a model of activity where, both bases G1516 and A1519 flip out and occupy tailored protein pockets. Surprisingly, the helix 45 tetraloop adopts a similar conformation in the human processome46 which indicates that this tetraloop region can exist in multiple conformations. Other studies have also shown that tetraloop structures are malleable for instance, the ribosomal sarcin tetraloop, also significantly changes conformation in the presence of the protein. Thus, this malleability allows the 3D structure of the tetraloop to vacillate between the various partially stacked states as is seen in KsgA and TFB1M complexes.[24] We propose that tandemmethylation can also be facilitated via local flipping motions concentrated around the bases which undergo methylation. Therefore, in order to facilitate consecutive methylation after A1519 gets dimethylated, we propose it flips inward, which induces A1518 to flip out and get methylated. This hypothesis is supported by the NMR structure of the tetraloop, where the analogous base position to A1519 is methylated and the state we propose is captured experimentally.[25] After dimethylation is complete, we further suggest that the methylation of A1518 triggers the release of the RNA from the protein, purporting a reorganization of the tetraloop resulting in a conformation where both the methylated bases are flipped out such that they engage closely with h44 (Figure 8). It was also observed that KsgA is tightly anchored near the methylation site via the extensive interaction of its C-terminal region with the surrounding RNA helices h24 and h27, and mutagenesis of this region also affected the methylation potential of KsgA. Thus, C-terminal along with G1516 insertion within KsgA likely serves as a pivot that allows the h45 tetraloop stretch to slightly reconfigure after each methylation, thereby enabling successive methylations without the enzyme leaving the vicinity of the rRNA.

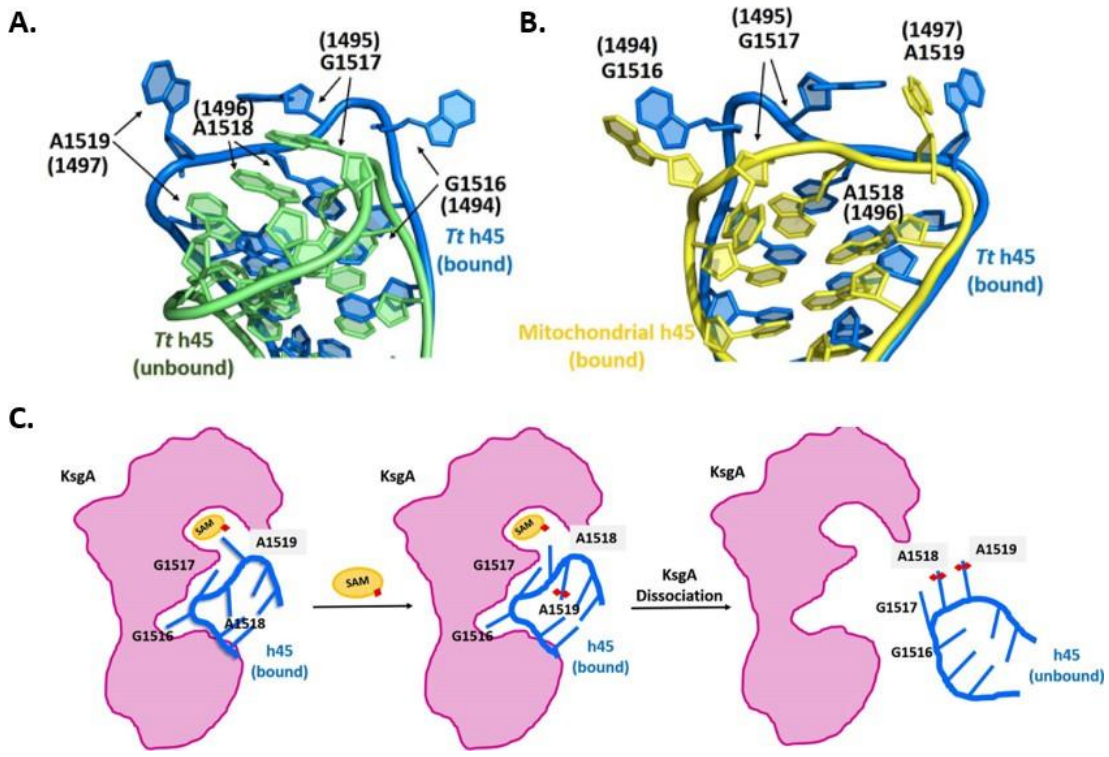


Fig 8: A. An overlay of the h45 tetraloop bound to KsgA (marine blue) and the unbound state (green) (PDB ID: 3OTO) of the Tt ribosome showing the conformational differences. B. Conformational difference of the h45 tetraloop in the bound state with KsgA (marine blue) and TFB1M (yellow) (PDB ID: 6AAX). C. Proposed mechanism of dimethylation. Tetraloop conformational change induces A1519 flips into the active pocket. Dimethylation of A1519 results in the further local flipping of the adjacent A1518 base. Finally, dimethylation of A1518 dissociates the enzyme from 30S and h45 resumes its native conformation as observed in the mature 30S ribosomal subunit

In conclusion, the cryo-EM structures of KsgA in complex with 30S ribosome along with the mutagenesis studies performed here shed light on both the mechanism of targeting and recognition adopted by N6 Rossmann fold rMTases to achieve specificity. The study asserts that the binding of KsgA onto the 30S platform is stabilized by interaction with several rRNA helices, which play an important role in tuning its methylation potential. It was established that specificity elements in KsgA such as tailored loops and the C-terminal head domain together act as a robust anchor and allow the enzyme to properly latch onto the ribosome. Furthermore, the two successive states, where the 30S−KsgA complex harbors h44 in either a disordered or in an ordered configuration, aid in capturing events that lead to the final step of biogenesis. These findings enhance our overall understanding as to how N6 A rMTases select their target substrates, a knowledge that can be further exploited for the design of selective inhibitors, tailored to inhibit a particular N6 A-rMTase.

Identifying allosteric hotspots for regulation of the binding of pathogenic methyltransferases to its cognate [26]

It is a well-established fact that Erm enzymes are extremely conserved and methylates rRNA at the conserved A2058 position and imparts resistance to macrolides such as erythromycin. As a following work to our attempts of understanding the origins of resistance and in reversing it, we show that a conserved RNA architecture, including specific bulge sites, present more than 15 Å from the reaction center, is key to methylation at the pathogenic site. Using a set of RNA sequences site-specifically labeled by fluorescent nucleotide surrogates, we show that base flipping is a pre-requisite for effective methylation and that distal bases assist in the recognition and flipping at the reaction center. The fluorophore 2-amino purine (2Ap) was introduced at nucleobase positions 2054, 2058, 2059, and 2614, and thienoguanosine (thG) was incorporated at positions 2057 and 2061 (Fig 9). All of the number are according to the *E.coli* nomenclature. All of these mutations gave negligible activity.

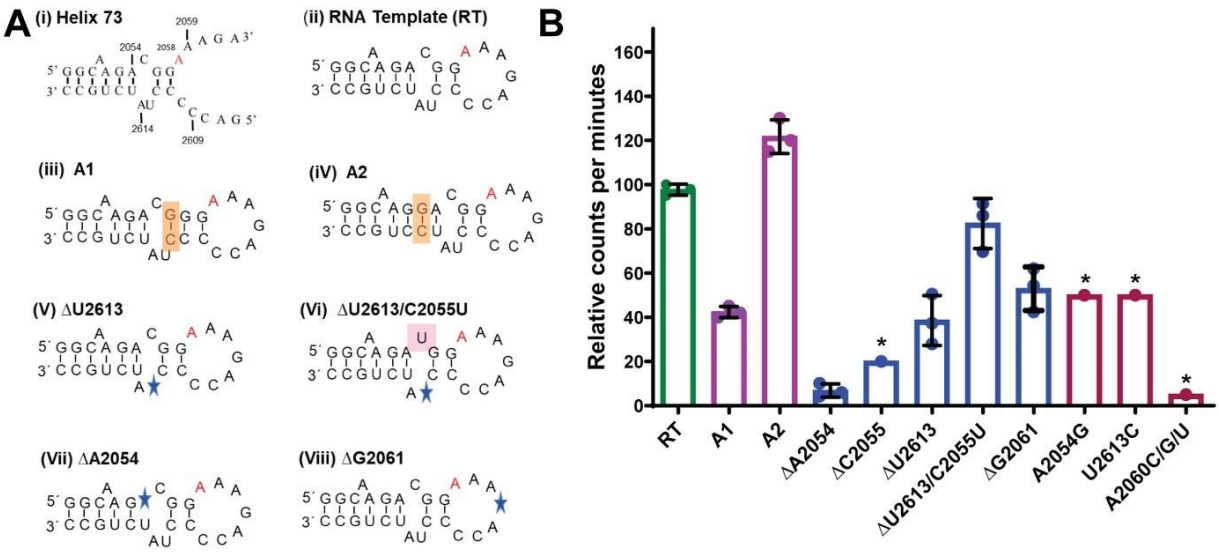


Fig. 9. A. RNA sequences tested for methylation activity. ✶ indicates deletion. The target base A2058 is highlighted in *red*. *B. in vitro* methylation assay using 3H-SAM. For comprehensive understanding, additional data are added. For A2060C/G/U construct, adenine was mutated either to C, G, or U at a time. All of these mutations gave negligible activity. SAM, S-adenosyl-L-methionine.

The Erm-RNA complex model developed by us using these fluorescent assays supplemented with molecular docking and dynamics revealed that intrinsically flipped-out bases in the RNA serve as a putative anchor point for the Erm. Further, molecular dynamic simulation studies demonstrated the RNA undergoes a substantial change in conformation to facilitate an effective protein-rRNA handshake. This study highlights the importance of unique architectural features exploited by RNA to impart fidelity to RNA methyltransferases via enabling allosteric crosstalk. In this work, we have shown that a local mechanism of RNA methylation is akin to "bind and slide" as observed for DNA Mtases is operative in Erm, wherein base flipping is a fundamental prerequisite for methylation. However, in RNA Mtases to facilitate the base flipping, reorganization of the distal extra helical region is a prerequisite. This suggests that intermittent hopping, in conjunction with the "bind and slide" approach, is used to overcome structural extrusions by RNA Mtases when tracking the target site.[27] This work throws light on how naturally programmed bulges in the rRNA sequence govern the complicated algorithm of recognition by Mtases. The allosteric sites identified here provide lucrative avenues for drug design. Instead of targeting the common conserved Mtase

catalytic site that has the potential to generate off-target effects, inhibitors can be developed for these distal bulge sites. Freezing dynamics of the specific identified out-loop region will impede protein interaction and block methylation, thereby reversing resistance.

Apart from these, we have been working to look for new drug targets by studying essential pathways that regulate the DNA and RNA pool in the cell. Our work on understanding the allosteric regulation in the purine salvage pathway has brought out key aspects of allosteric communication and molecular tunnel formation that are evolutionarily variant in human verses bacteria ***(Science Advance 2020, ACS Catalysis 2022)***.[28, 29] This aspect can be further exploited to design strategies that can target protein-protein interaction interfaces in a species-specific manner. Further, our early work on nucleobase deaminases ***(Biochemistry2013a, Biochemistry2013b)*** and recent work in ***JSB 2021*** lead us to discover a remarkable new enzyme, exclusively found in Mycobacterium, which confers innate resistance towards aza-scaffold of drugs.[30,31,32] Here, the X-ray structure of this novel enzyme and subsequent structures with substrate and their analogues aid in understanding function. It was established that this enzyme scans for mutagenic bases and eliminates them from the cell. Thus, by targeting this newly discovered pathway one can sensitize these resilient organisms towards mutagenic base harbouring drugs ***(JACS 2017)***.[33]

Our cutting-edge research has been recognized by a number of prestigious awards. I have been adorned with the ***DBT-Welcome Trust Fellowship (2019)***, ***Women's BioScientist award (2018)*** by DBT, elected as a member of the prestigious ***National Academy of Sciences in 2019*** and the ***IIT Bombay Impactful Research Award (2020).*** I have also been selected as principal investigator (5 million US dollars) from SERB, India to setup the state-of-the-art cryo-electron- microscopy centre at IIT Bombay, to take the field of structural biology further. We hope to work further towards circumventing the antibiotic resistance problem world-wide with our knowledge and expertise in this field and develop better drugs targets.

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