

Deletion of RsmE 16S rRNA methyltransferase leads to low level increase in aminoglycoside resistance in *Mycobacterium smegmatis*

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Abstract

Owing to its central role in cellular function, ribosome is one of the most common targets of antibiotics in bacteria. Mutations in *rrs* gene, ribosomal protein genes, methyltransferases or drug influx/efflux are often found to overcome the drug response. Despite modulation of methylation pattern in the ribosome through mutations in the methyltransferases as one of key modulators of drug response, *rsmG* (*gidB*) is the only conserved methyltransferase associated with low-level drug resistance in large number of mycobacterial isolates. Here, we present the first evidence of association of methylation by mycobacterial RsmE, that methylates U1498 of 16S rRNA, with low levels of drug resistance. Deletion of the RsmE-homolog of *Mycobacterium smegmatis* leads to at least two-fold increase in the inhibitory concentration of aminoglycosides that bind in the decoding center proximal to U1498 in the 30S subunit. The change in inhibitory concentrations was highly specific and does not show any cross-resistance to drugs of other classes. Surprisingly, Rv2372c, the RsmE-homolog of *Mycobacterium tuberculosis* has the largest number of mutations among conserved ribosomal methyltransferases, after *gidB*, highlighting the role of mutations in the RsmE methyltransferase as a key emerging mechanism of drug resistance in clinical strains of *M. tuberculosis*. Our work underlies the association of methylation by the RsmE-homolog with drug resistance and lays the groundwork to tackle this emerging mechanism of drug resistance in mycobacteria.

Introduction

M. tuberculosis is one of the most prevalent pathogens and responsible for largest number of deaths by a single infectious pathogen, annually, worldwide (WHO 2018). Treatment involves multidrug therapy with the four frontline drugs for extended periods of time (Sotgiu *et al.*, 2015). However, a rapid increase in resistance towards the first line of drugs, over the last few years, has led to an increased dependence on a prolonged and complex treatment that includes the second line of drugs, namely, fluoroquinolones and injectable aminoglycosides (kanamycin, amikacin, capreomycin) (Sotgiu *et al.*, 2015; WHO 2018). Any resistance towards the second line of drugs compounds the problem towards extensively drug resistant disease (XDR-TB). Resistance to fluoroquinolones may occur due to mutations in gyrases (Nosova *et al.*, 2013; Zhang and Yew 2015), to kanamycin by overexpression of *eis* drug-modifying enzyme (Sowajassatakul *et al.*, 2014; Kambli *et al.*, 2016) or of efflux pumps (Louw *et al.*, 2009; Reeves *et al.*, 2013), and to other aminoglycosides by modification of target binding site through mutations in *rrs* (Gygli *et al.*, 2017; Hameed *et al.*, 2018), ribosomal protein genes (Finken *et al.*, 1993) or changes in rRNA methylation pattern (Buriánková *et al.*, 2004; Maus *et al.*, 2005; Okamoto *et al.*, 2007).

Methylations in ribosomal RNA are brought about by highly specific rRNA methyltransferases. The complete set of conserved ribosomal methyltransferases has been identified in *E. coli* and indicates ten conserved methyltransferase members for the 30S subunit (Golovina *et al.*, 2012). These rRNA methyltransferases are known to modulate the structure of ribosome by addition of methyl group to key nucleotides in the decoding center of ribosome. Mutation in some of the rRNA methyltransferases is associated with altered function of the ribosome as well as with drug resistance. For instance, mutations in *gidB*, the RsmG 16S rRNA methyltransferase has been reported to be associated with low level resistance towards streptomycin and leads to at least two-fold increase in MIC to streptomycin in *E. coli* and *M. tuberculosis* (Okamoto *et al.*, 2007; Wong *et al.*, 2011). Mutations in *tlyA*, that methylates C1920 in 23S rRNA and C1409 in 16S rRNA, are linked to resistance to capreomycin in *M. tuberculosis* (Maus *et al.*, 2005). Despite key roles of methylated nucleotides in the decoding center, Rv3919c (RsmG/GidB, Wong *et al.*, 2011), Rv2966c (RsmD, Kumar *et al.*, 2011) and Rv2372c (RsmE, Kumar *et al.*, 2014) are the only conserved 30S rRNA methyltransferases identified in *M. tuberculosis*. Moreover, the role of these methyltransferases in drug resistance, apart from *gidB*, remains poorly characterized.

We have earlier identified Rv2372c as a RsmE homolog of *M. tuberculosis* that methylates U1498 in helix 44 of 30S subunit of ribosome in a highly specific manner (Kumar *et al.*, 2014). U1498 is one of the conserved methylated nucleotides present in the decoding center of the ribosome that together form a compact hydrophobic cage around the anticodon stem loop structures at the P-site and monitor the codon-anticodon interactions (Korostelev *et al.*, 2006; Selmer 2006). However, deletion of *rsmE* had no significant effect on growth and survival of *E. coli* (Basturea 2006). Apart from its key roles in ribosomal function and fidelity, U1498 also likely to affect the response to antibiotics, as U1498 lies in the vicinity of binding site of various aminoglycosides as seen in the crystal structures of ribosome-antibiotic complexes (Borovinskaya *et al.*, 2007, 2008; Demirci *et al.*, 2013; Coccozaki *et al.*, 2016). A mutation of U1498C is among three mutations that confer different levels of hygromycin resistance in *M. smegmatis in vitro* (Pfister *et al.*, 2003). However, this mutation has not been reported in clinical mycobacterial strains. Moreover, the association of methylation at U1498 has not been previously investigated with altered drug response in mycobacteria.

Given the association of antibiotic response with methylated nucleotides in the decoding center, and the key role of helix 44/ U1498 in the P-site of ribosome, here, we investigate the role of methylation by mycobacterial RsmE with antibiotic response by generating a deletion mutant of the RsmE homolog in *M. smegmatis*. Growth of wild-type, knockout and complemented strain was analyzed with varying concentration of ribosomal- and non-ribosomal-targeting drugs and showed

at least two-fold increase in the inhibitory concentration of several antibiotics. Moreover, Rv2372c, the RsmE-homolog of *M. tuberculosis* was found to harbor the largest number of mutations among conserved ribosomal methyltransferases, apart from *gidB*, suggesting a possible correlation of RsmE mutations with drug response in clinical strains. In this work, we show U1498 methylation is conserved in mycobacteria and present the first evidence of association of U1498 methylation with modulation of antibiotic response in any bacteria.

Methods

Mutational analysis of Rv2372c in pathogenic *M. tuberculosis* strains

Whole genome sequencing data for available 9745 strains of *M. tuberculosis* were extracted from PATRIC database (www.patricbrc.org, PATRIC March 2019 Release). Rv1010, Rv1407, Rv2966c, Rv2372c, Rv3919c, Rv2165 and Rv1003 were identified as homologs of conserved 16S rRNA methyltransferases of *E. coli* on the basis of sequence similarity and selected for further analysis. Non-synonymous mutations in the mycobacterial 16S rRNA methyltransferases were identified from genome data of all the strains, with *M. tuberculosis* H37Rv as the reference strain.

Bacterial strains and growth conditions

M. smegmatis mc²155 (Ms-WT) or *RsmE*-depleted strains (Ms- Δ RsmE) were grown in Difco Middlebrook 7H9 broth or on 7H10 agar plates, supplemented with 0.05% Tween 80, 0.25% glycerol and 0.4% glucose. Growth profiles were obtained by monitoring growth at 600 nm at 37 °C with continuous shaking using the BioScreen growth curve analyzer and plots were generated. The strains, plasmids and the primers used in the study are listed in Table 1.

Construction of *M. smegmatis* knockout and complementation

Rv2372c was earlier identified as the RsmE ortholog in *M. tuberculosis* (Kumar *et al.*, 2014). A knockout of *MSMEG_4502*, the sequence homolog of Rv2372c in *M. smegmatis*, was generated by a suicidal vector strategy using pPR27, as described earlier (Pelicic *et al.*, 1996; Pelicic *et al.*, 1997). Briefly, for the targeted gene knockout, the 5' and 3' ends of *MSMEG_4502* and its flanking sequences were amplified to generate 'UP' (813 bp) and 'DOWN' (823 bp) fragments. A zeocin resistance gene (657 bp) excised from pMSG360-zeo (Barkan *et al.*, 2010) was introduced between them to generate UZD (UP-Zeocin-DOWN) deletion cassette. The UZD deletion cassette was then cloned between XbaI and NotI sites in *M. smegmatis* – *E. coli* shuttle vector pPR27 yielding plasmid pPR27-MSM_UZD. Plasmid pPR27-MSM_UZD was then

transformed into *M. smegmatis* mc²155 via electroporation, to generate the knockout by homologous recombination method.

The pPR27-MSM_UZD transformants of *M. smegmatis* were selected on 7H10 agar plates containing 10 µg/ml gentamicin and 40 µg/ml zeocin. Knockout strains were selected by growing at 42 °C to promote loss of the plasmid as it has a temperature sensitive origin of replication, Ori_Myco_TS. The knockouts were screened by a two-step selection method. Firstly, the mutants were grown at 42 °C on 7H10 plates along with 10% sucrose for positive selection of knockouts that have lost *sacB* gene. Mutants were further screened through negative selection by growing on 7H10 plates containing 10 µg/ml gentamicin. The gene knockout of *MSMEG_4502* was finally confirmed by PCR for incorporation of UZD disruption cassette and the knockout strain designated as Ms-ΔRsmE.

For complementation of the knockout strain, *MSMEG_4502* was cloned in pTC0X1L (Guo *et al.*, 2007; Klotzsche *et al.*, 2009) as well as in pSMT3 expression vector (Gaora *et al.*, 1997) and designated as Ms-ΔRsmE/RsmE or Ms-ΔRsmE/RsmE', respectively. Ms-ΔRsmE/RsmE' was used for complementation when the response with kanamycin and neomycin was tested. pTC-MCS (Klotzsche *et al.*, 2009) or pSMT3 were used as parent vector controls along with indicated antibiotics for both wild type or knockout strain.

***In-vivo* methylation assay**

To test the activity of *MSMEG_4502* in *M. smegmatis* cells, primer extension assay was carried out (Kumar *et al.*, 2014). Briefly, total RNA was isolated from the *M. smegmatis* strains and 16S rRNA was gel purified from the total RNA. A primer was synthesized, complementary to the sequence 1497 to 1515 of 16S rRNA of *M. smegmatis* (corresponding to 1513 to 1531 of *E. coli* 16S rRNA). The primer was labelled at 5' end with ³²P-labelled ATP, using T4 Polynucleotide kinase. Primer extension was done using Verso reverse transcriptase as per the manufacturer's protocol. The reaction was terminated by heating at 80 °C for 15 minutes and the products were analysed by running on a 15% urea-PAGE. The gel was further imaged by phosphorimaging. The expected size of the product is 33 bp long if U1482 is methylated (1483 bp to 1515 bp). U1482 of *M. smegmatis* 16S rRNA (NCBI Accession No. NC_008596.1:c5029475-5027948) corresponds to U1498 of *E. coli* 16S rRNA (NCBI Accession No. NC_000913.3:4166659-4168200). An oligonucleotide of 33 bp size was 5' end labelled with ³²P and used as an indicative size marker.

Phenotypic Characterisation of knockout strains

For the growth assay, Ms-WT, Ms- Δ RsmE or Ms- Δ RsmE/RsmE were grown till an OD (600 nm) of \sim 1.0 at 37 °C. It was then diluted 1:1000 with fresh media and growth was monitored every 2 hours using the Bioscreen growth analyzer.

Growth assay was also performed to investigate the effect of stress conditions on the growth of knockout. For this, *M. smegmatis* strains were grown in 7H9 media along with varying concentrations of different stress agents, as indicated. Growth under oxidizing conditions was monitored by growing cells in media containing increasing concentrations (0, 0.25, 0.5, 1.25 and 1.5 mM) of hydrogen peroxide while effect of osmotic stress was monitored with increasing concentrations (0, 0.4, 0.6, 0.8 and 1.0 M) of NaCl. Knockout and wild type cells were grown in sodium nitroprusside (0, 10, 20, 30, 40 and 50 mM) to monitor SNP-mediated stress, which is a NO donor and induces nitrosative stress. For acid stress, knockout or wild type strains were grown in 7H9 media at pH 6.0, pH 5.5, pH 5.0 or pH 4.5.

Drug sensitivity of knockout strain

Drug sensitivity of the knockout strain was tested by microbroth dilution method. Briefly, *M. smegmatis* strains, Ms-WT, Ms- Δ RsmE and Ms- Δ RsmE/RsmE (or Ms- Δ RsmE/RsmE') were first grown until OD (600nm) \sim 1.0 at 37 °C. The respective strains were then diluted 1:1000 with fresh media and grown with serial dilution of indicated antibiotics added to the culture. Untreated culture and blank media was taken as positive and negative control, respectively. The growth was monitored at 600 nm every 2 hours at 37 °C with continuous shaking in the Bioscreen growth analyzer. The growth curve was plotted using the average value of three replicates. MIC was reported as the lowest drug concentration showing no visible growth in the respective antibiotics.

Antibiotics used in the study belonged to the following three groups: 30S ribosomal-targeting drugs - kanamycin, amikacin, gentamicin, neomycin, paromomycin, tetracycline and streptomycin; 50S ribosomal-targeting drugs - chloramphenicol and erythromycin and non-ribosomal-targeting drugs - moxifloxacin and rifampicin.

Results

Generation of *rsmE* knockout of *M. smegmatis*

MSMEG_4502, was identified as the homolog of RsmE of *E. coli* (e-value: $4e^{-26}$) and Rv2372c of *M. tuberculosis* (e-value: $3e^{-107}$) on the basis of sequence similarity. Knockout of MSMEG_4502 in *M. smegmatis* was generated with the help of a suicidal vector strategy (Figure 1A) as described in the Methods section. The mutants were screened and confirmed by PCR (Figure 1B) as well as by DNA sequencing. For confirmation by PCR, primers were designed flanking the

UP and DOWN fragments (primer F1 and primer F2, Table 1, respectively). An expected amplified product size of 2535 bp in the knockout (lane 1) as compared to that of 2160 bp in Ms-WT (lane 2), confirms the successful knockout of *MSMEG_4502* in *M. smegmatis* to generate Ms- Δ RsmE.

In-vivo* methylation of 16S rRNA by MSMEG_4502 in *M. smegmatis

The methylation status at U1498 of 16S rRNA by the mycobacterial homolog was examined by primer extension assay on purified 16S ribosomal RNA extracted from the wild-type (Ms-WT), knockout (Ms- Δ RsmE) and complemented (Ms- Δ RsmE/RsmE) strains. Methylation occurs at N-3 position of U, disrupting hydrogen-bonding with a complementary A-nucleotide during reverse transcription. A primer extension by reverse transcriptase hence stops 3' to the methylated m³U. As seen in Figure 2, extension of primer (Primer A, Table 1) complementary to 1497 to 1515 by reverse transcriptase stops at U1483 if U1482 is methylated (equivalent to U1498 of *E. coli* 16S rRNA) resulting in a 33 bp product in wild type and complemented strains while reverse transcriptase reads through position 1482 to yield longer products in Ms- Δ RsmE (Figure 2B). Hence, *MSMEG_4502* is the RsmE-homolog in *M. smegmatis* that specifically methylates the equivalent m³U1498 position in 16S rRNA in *M. smegmatis*.

Effect on growth of Ms- Δ RsmE in the absence of U1498 methylation

M. smegmatis strains Ms-WT and Ms- Δ RsmE, with respective empty vector controls, along with Ms- Δ RsmE/RsmE were grown in 7H9 media at 37 °C and growth monitored at OD (600 nm) in the Bioscreen growth analyzer. Ms- Δ RsmE showed slightly slower growth as compared to Ms-WT (Figure 3) that is partially restored in the complemented strain.

The growth of Ms- Δ RsmE was also monitored under different stress conditions. Effect of oxidative stress, osmotic stress, SNP induced stress and acid stress on the growth of Ms-WT, Ms- Δ RsmE and Ms- Δ RsmE/RsmE strains were examined. For effect on growth of Ms- Δ RsmE, under oxidative stress, cells were grown in the presence of increasing concentrations of hydrogen peroxide. Deletion of the RsmE-homolog showed no significant difference on growth under oxidative stress conditions and the growth of Ms- Δ RsmE was largely similar to Ms-WT (Figure 4A). To induce osmotic stress to the mycobacterial strains, cells were grown in increasing concentrations of sodium chloride and growth was monitored by OD₆₀₀. Ms- Δ RsmE showed similar growth as Ms-WT under osmotic stress as well (Figure 4B). The growth of Ms- Δ RsmE was slow in response to sodium nitroprusside, a nitrosative stress agent which induces stress by generating NO inside the cells (Figure 4C).

The growth of Ms- Δ RsmE as a function of decreasing pH, however, was more dramatic. Ms-WT showed slower growth as pH of the media was varied from pH 6.8 to pH 4.5. However growth of Ms- Δ RsmE, at pH 5.5 or lower was severely compromised compared to the Ms-WT cells (Figure 4D). The growth was restored similar to Ms-WT in the complemented Ms- Δ RsmE/RsmE strain, suggesting that expressing the gene in the knockout can reverse the sensitivity towards acidic stress (Figure 4D, right panel).

Taken together, these results highlight a possible role of RsmE in the acidic stress that mycobacteria might encounter during infection.

Methylation at U1498 can modulate antibiotic response

U1498 is present in helix 44 of 30S subunit and is part of the functional unit, namely, P-site, of ribosome. Aminoglycosides and tetracyclines are the two major classes of drugs that bind at the functional site of 30S subunit of ribosome and were tested for drug susceptibility.

Role of m³U1498 methylation in modulating drug response in mycobacteria was monitored by drug susceptibility assays of Ms-WT, Ms- Δ RsmE and Ms- Δ RsmE/RsmE (or Ms- Δ RsmE/RsmE') (Figure 5). Growth in presence of kanamycin shows that a two fold higher concentration is required for growth inhibition of Ms- Δ RsmE as compared to Ms-WT (Figure 5A). In the MSMEG_4502 expressing complement strain, the growth inhibition was restored to similar kanamycin concentrations as Ms-WT, suggesting the difference of growth is associated with the methylation at U1498 (Figure 5A, right panel).

At least two fold higher concentration of drugs for growth inhibition is also required with amikacin (Figure 5B), gentamicin (Figure 5C) and paromomycin (Figure 5D) as compared to Ms-WT, which are restored in the Ms- Δ RsmE/RsmE complemented strain. Although a two fold higher concentration for growth inhibition is also required with neomycin (Figure 5E), it was not restored in the complemented strain.

The growth response of Ms-WT, Ms- Δ RsmE, Ms- Δ RsmE/RsmE with streptomycin (Figure 5F) and tetracycline (Figure 5G), which bind to A-site of the 30S subunit, farther from helix 44, however, showed no change in inhibitory concentrations.

Deletion of the RsmE-homolog of *M. smegmatis* hence leads to a low level of resistance to all the tested aminoglycosides that bind in the decoding center proximal to U1498 in helix 44 of 30S subunit of ribosome. The susceptibility is restored to lower concentrations of each aminoglycoside in the respective complemented (Ms- Δ RsmE/RsmE) strain in all cases (except neomycin), suggesting a correlation of methylation at U1498 with drug binding and antibiotic response for aminoglycosides that bind at this site.

Antibiotic response to non-30S targeting drugs

To confirm whether the observed low level resistance was specific to aminoglycosides in Ms- Δ RsmE or did the deleted strain exhibit cross-resistance to any other antibiotics, growth of Ms-WT, Ms- Δ RsmE and Ms- Δ RsmE/RsmE was monitored with non-30S targeting drugs, namely; non-ribosomal drugs (rifampicin and moxifloxacin) and 50S-targeting drugs (erythromycin and chloramphenicol). Growth of Ms- Δ RsmE with rifampicin (Figure 6A) that targets RNA-polymerase and with moxifloxacin (Figure 6B) that targets DNA gyrase was similar to that of Ms-WT and the inhibitory concentration for Ms-WT and Ms- Δ RsmE was same for both the drugs.

Growth of Ms- Δ RsmE as compared to Ms-WT was found to be marginally affected by other drugs. Growth was slightly slower in response to erythromycin, that targets 50S subunit at the exit tunnel (Wilson 2014) (Figure 6C) but slightly faster in response to chloramphenicol, that targets ribosomal A-site in the 50S subunit (Wilson 2014) (Figure 6D). However, no change in inhibitory concentration was observed as compared to Ms-WT for both the drugs. Growth of Ms- Δ RsmE/RsmE complemented strain was similar to that of Ms-WT for all the non-30S targeting drugs (Figures 6, right panels).

Hence, the change in inhibitory concentrations, in the knockout strain of *M. smegmatis* (Ms- Δ RsmE) was specific towards antibiotics belonging primarily to aminoglycoside class among the tested drugs and does not show any cross-resistance to drugs of other classes (Table2).

RsmE mutations are prevalent in the clinical strains of *M. tuberculosis*

Rv1010, Rv1407, Rv2966c, Rv2372c, Rv3919c, Rv2165 and Rv1003 were identified as homologs of conserved 16S rRNA methyltransferases RsmA, RsmB, RsmD, RsmE, RsmG, RsmH and RsmI, respectively, in 9745 clinical strains of *M. tuberculosis* in the PATRIC database (Wattam *et al.*, 2017). Non-synonymous mutations in the mycobacterial 16S rRNA methyltransferases were identified from genome data of all the strains, with *M. tuberculosis* H37Rv as the reference strain. Among all methyltransferases, RsmG, which is known to be associated with streptomycin resistance, has mutations in >40% of all strains. Surprisingly, at 19% of all strains with mutations, the RsmE homolog of *M. tuberculosis*, Rv2372c stands at second position, suggesting a key association of this methyltransferase with clinical manifestation and/or drug response in mycobacteria. Strains with mutations in RsmA, RsmH or RsmI comprised less than 3% of the strains, in each case, while mycobacterial strains carrying mutations in RsmD (Rv2966c) were negligible (Figure 7).

Discussion

U1498 is localized in the highly conserved region in the top half of helix 44 of 16S rRNA (Schuwirth *et al.*, 2005; Basturea 2006). This region is of high functional importance as it is part of the decoding centre. The decoding centre of ribosome ensures error-free selection of cognate tRNA to mRNA, promoting translation accuracy (Ogle *et al.*, 2003; Mahto and Chow 2013). Antibiotics of several classes, target the decoding centre by binding around helix 44 and inhibiting protein translation (Wilson 2014; Lin *et al.*, 2018).

Here, in this study, we have shown that methylation of U1498 of 16S rRNA is associated with altered antibiotic response of mycobacterial cells. This response is highly specific towards certain aminoglycosides targeting the 30S subunit of the ribosome. The binding site of these aminoglycosides in the ribosome was mapped from the available crystal structures in the PDB (Figure 8). Kanamycin, amikacin, neomicin, gentamicin and paromomycin belong to the class of antibiotics that bind near the top half of the helix 44, close to U1498 in 16S rRNA, whereas streptomycin and tetracycline bind at sites farther away. The fact that only the antibiotics that bind in the vicinity of U1498 exhibited altered growth response of Ms- Δ RsmE in presence of these aminoglycosides, suggests the absence of methylation in the deleted strain affects antibiotic binding at this site. A possible change in the local conformation of the binding site in the absence of m³U1498 methylation, might lead to change in the binding pattern of antibiotics, resulting in a low level increased resistance in Ms- Δ RsmE. This is the first report where methylation at U1498 of 16S rRNA is shown to be directly associated with change in the antibiotic response in mycobacteria.

RsmE is widely distributed in major bacterial groups (Baldrige and Contreras 2014; Benítez-Páez *et al.*, 2014). Despite a reductive evolution of the mycobacterial genomes, conservation of Rv2372c, the RsmE-homolog in *M. tuberculosis*, suggests m³U1498 methylation is necessary for normal cellular physiology. The m³U1498 nucleotide interacts directly with the mRNA in the ternary complex of 70S ribosome-mRNA-tRNA of *Thermus thermophilus* (Korostelev *et al.*, 2006) and *E. coli* (Selmer 2006) highlighting its role in efficient translation. Moreover, a U1498G mutation blocks the first peptide bond formation (Cunningham *et al.*, 1993) due to possible change in tertiary structure around the decoding structure (Ringquist *et al.*, 1993). The crystal structure of 70S ribosome also showed that m³U1498 is present in the intersubunit bridge of 50S and 30S and interacts with the conserved regions in 23S rRNA (Selmer 2006; Baldrige and Contreras 2014). Despite taking part in several key interactions in the assembly and function of ribosome, the RsmE knockout of *E. coli* grows normally although it fails to compete when grown together with the wild type (Basturea 2006). In case of *M. smegmatis*, although the

RsmE-homolog, *MSMEG_4502*, is not essential for its survival, knockout of *MSMEG_4502* leads to subtle changes in physiology of the mutant Ms- Δ RsmE strain.

Even a low level resistance in a microorganism *in vitro*, can serve as a stepping stone to development of high level clinically relevant resistant strains (Gomez *et al.*, 2017), as seen for streptomycin (Okamoto *et al.*, 2007). The modulation of antibiotic response by RsmE methyltransferase might lead to a potential threat and hence must be understood in greater details. Mycobacteria encounter a wide variety of stress, *viz.*, oxidative and pH stress during infection of the host. In a recent report, phagosomal acidification was shown to alter the redox physiology of *M. tuberculosis* to promote tolerance of the bacterium to antibiotics (Mishra *et al.*, 2019). Inhibition of phagosomal acidification by the antimalarial drug chloroquine eradicated drug-tolerant bacilli in *in vivo* models. The increased sensitivity of Ms- Δ RsmE, lacking methylation at 1498 position, to acid stress, however, suggests an alternate potential combinatorial way to target tubercular bacilli and prevent emergence of resistance to front line drugs.

Overall, we show that absence of methylation of U1498 of 16S rRNA leads to development of low level resistance in *M. smegmatis*. The increased resistance was specific only for some antibiotics belonging to the aminoglycoside class. Mapping of binding site in the ribosome, showed that growth of Ms- Δ RsmE was affected specifically by aminoglycosides that bind in the vicinity of U1498 of 16S rRNA. We also found that RsmE has the second highest mutation rate in the clinical strains among all conserved methyltransferases. This reinforces the possibility of association of RsmE towards development of resistance in clinical strains of *M. tuberculosis*, which must be tackled in a timely manner.

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Legends to Figures

Figure 1: Generation of *M. smegmatis* *RsmE* (*MSMEG_4502*) knockout strain.

A. A knockout of *MSMEG_4502* was generated using suicidal vector strategy with *M. smegmatis* – *E. coli* shuttle vector pPR27. The upstream fragment (UP) and downstream fragment (DOWN) of the target gene *MSMEG_4502* were cloned in the vector along with Zeocin^R, yielding pPR27-*MSM_UZD*. The knockout was generated by homologous recombination and further screening of the transformants.

B. The knockouts were confirmed via PCR using the primers flanking the UP and DOWN fragments. An amplified product of 2535 bp is obtained in *Ms-ΔRsmE* (Lane 1) and that of 2160 bp in *Ms-WT* (Lane 2).

Figure 2: Primer extension assay for m³U1498 methylation in *M. smegmatis*.

A. Overall assay design of the primer extension assay. The primer complementary to the region 1497 to 1515 of 16S rRNA of *M. smegmatis* (Primer-A, Table 1) is 5' end-labelled with ³²P. In the presence of methylation, the reverse transcription is stalled due to disruption of base pairing, giving a truncated product of 33bp, while in the absence of methylation, reverse transcription continues resulting in longer products. All indicated position numberings are with respect to *M. smegmatis* 16S rRNA. *M. smegmatis* U1482 is equivalent to U1498 of *E. coli* 16S rRNA.

B. Primer extension assay products from wild type, knockout and complemented strains of *M. smegmatis* were analysed on a 15% urea PAGE. The presence of a band indicates a truncated product of 33bp in *Ms-WT* (lane 1) and *Ms-ΔRsmE/RsmE* (lane 2) due to presence of m³U methylation. (As explained above, *M. smegmatis* U1482 is equivalent to U1498 of *E. coli* 16S rRNA). Absence of methylation in *Ms-ΔRsmE* (lane 3) leads to longer products. A ³²P end-labelled 33 base oligonucleotide was used as a marker (M) to indicate the size of the truncated product due to methylation in *Ms-WT* and *Ms-ΔRsmE/RsmE*.

Figure 3: Growth curve of *M. smegmatis* strains.

The *Ms-ΔRsmE* knockout strain (squares) shows a slightly extended lag phase compared to *Ms-WT* strain (circles), which is partially restored, similar to *Ms-WT*, in the complemented strain *Ms-ΔRsmE/RsmE* (diamonds).

Figure 4: Growth of *M. smegmatis* strains under stress conditions.

Growth curve of Ms-WT (left panels), Ms- Δ RsmE (middle panels) and Ms- Δ RsmE/RsmE (right panels) with varying levels of (A) H₂O₂, (B) NaCl, (C) Sodium nitroprusside and (D) pH are plotted.

Figure 5: Drug response towards 30S targeting antibiotics

Growth of Ms-WT (left panels), Ms- Δ RsmE (middle panels) and complemented Ms- Δ RsmE/RsmE (or Ms- Δ RsmE/RsmE'), (right panels) were monitored with increasing concentrations of (A) kanamycin, (B) amikacin, (C) gentamicin, (D) paromomycin, (E) neomycin, (F) streptomycin and (G) tetracycline.

Figure 6: Drug response towards non-30S targeting antibiotics

Growth of Ms-WT (left panels), Ms- Δ RsmE (middle panels) and Ms- Δ RsmE/RsmE (right panels) were monitored with increasing concentrations of (A) rifampicin, (B) moxifloxacin, (C) erythromycin and (D) chloramphenicol.

Figure 7: Mutations in 16S rRNA methyltransferases in clinical strains of *M. tuberculosis*

The total number of non-synonymous mutations in the coding sequence of the conserved rRNA methyltransferases in the clinical strains of *M. tuberculosis* obtained from the PATRIC database is indicated. RsmG, having the highest number of mutations, is known to be associated with streptomycin resistance. RsmE methyltransferase has the second highest mutations among conserved methyltransferases, in a total of 19% clinical strains. The conserved *E. coli* counterpart for each mapped methyltransferase is also indicated.

Figure 8: Binding site of aminoglycosides in ribosome structure

The binding of antibiotics (A) Gentamicin (green), (PDBID: 4V53), (B) paromomycin (grey), (PDBID: 4V5Y), (C) neomycin (red), (PDBID: 4V52), (D) tobramycin^a (yellow), (PDBID: 4LFC), (E) streptomycin (magenta), (PDBID: 4JI8) and (F) tetracycline (orange), (PDBID: 5J5B) was mapped using the available crystal structures in PDB and are shown as ball-and-stick structures. The distance from N-3 of U1498 to the bound drug (closest atom) is indicated in dashed lines (in Å). For clarity, only helix 44 and helix 45 of 30S ribosomal subunit are shown here (cyan). U1498 is indicated as space-filled structure (bright blue). Non-30S targeting drugs are not shown as they bind much farther away from U1498.

a: Tobramycin-bound structure is indicative of kanamycin/amikacin binding site, as a kanamycin/amikacin-bound ribosome structure could not be identified in PDB.

Table 1: Summary of strains, plasmids and primers used in the study

A. List of Strains		
Strain name	Description	Source
Ms-WT	<i>M. smegmatis</i> mc ² 155 (Wild type)	Laboratory stock
Ms-ΔRsmE	<i>MSMEG_4502</i> knockout strain of <i>M. smegmatis</i>	This study
Ms-ΔRsmE/RsmE	<i>MSMEG_4502</i> -expressing complement strain of Ms-ΔRsmE in pTC0X1L	This study
Ms-ΔRsmE/RsmE'	<i>MSMEG_4502</i> -expressing complement strain of Ms-ΔRsmE in pSMT3	This study

B. List of Plasmids		
Plasmid name	Description	Source
pPR27	<i>M. smegmatis</i> – <i>E. coli</i> shuttle vector used for knockout generation in <i>M. smegmatis</i> , (Genta ^R)	(Pelicic <i>et al.</i> , 1997)
pMSG360-zeo	Plasmid containing zeocin resistance gene, used for excising Zeo ^R	(Barkan <i>et al.</i> , 2010)
pPR27-MSM_UZD	pPR27 with UZD cassette, (Genta ^R , Zeo ^R)	This study
pTC0X1L	Vector for expressing <i>MSMEG_4502</i> in Ms-ΔRsmE/RsmE, (Kan ^R)	(Guo <i>et al.</i> , 2007)
pTC-MCS	Vector control for pTC0X1L	(Klotzsche <i>et al.</i> , 2009)
pSMT3	Vector for expressing <i>MSMEG_4502</i> in Ms-ΔRsmE/RsmE, (Hyg ^R)	(Gaora <i>et al.</i> , 1997)

C. List of Primers		
Primer name	Description	Sequence
A	Primer for Primer extension assay	TCCAGCCGCACCTTCCGGT
B1	Forward primer for UP fragment	TATATCTAGAGTCGGCGAGGTGATCCCCGATC
B2	Reverse primer for UP fragment	TATAGGATCCAGCGTCACCTGTTCGGTGATG
C1	Forward primer for DOWN fragment	TATAGGATCCGTCCACCGATTCTGTGGTGCT

C2	Reverse primer for DOWN fragment	TATAGCGGCCGCCACCACCGAACTCGGGATGA
D1	Forward primer for complementation in pTC0X1L	ATACATATGAGCGCGGGCGCTGTTCTACGTC
D2	Reverse primer for complementation in pTC0X1L	ATATTATAACTAGATGTTCCA GCGCGACGT
E1	Forward primer for complementation in pSMT3	ATACTGCAGCATGAGCGCGGGCGCTGTTCTAC
E2	Reverse primer for complementation in pSMT3	ATAGATATCCTAGATGTTCCAGCGCGACGT
F1	Forward primer for confirmation of knockout by PCR amplification	CAACGGCAACTCGTCGCCT
F2	Reverse primer for confirmation of knockout by PCR amplification	AGATGTGGCTGAGGTGGTCG

Table 2: Observed MICs for *M. smegmatis* strains

Antibiotic	Class of antibiotic	Target of antibiotic	Ms-WT MIC	Ms- Δ RsmE MIC	Ms- Δ RsmE/RsmE MIC
A. 30S targeting drugs					
Kanamycin	Aminoglycosides	30S ribosome	2.0 μ g/ml	4.0 μg/ml	2.0 μ g/ml
Amikacin	Aminoglycosides	30S ribosome	0.25 μ g/ml	0.5 μg/ml	0.25 μ g/ml
Gentamicin	Aminoglycosides	30S ribosome	2.0 μ g/ml	4.0 μg/ml	2.0 μ g/ml
Neomycin	Aminoglycosides	30S ribosome	4.0 μ g/ml	8.0 μg/ml	8.0 μg/ml
Paromomycin	Aminoglycosides	30S ribosome	2.0 μ g/ml	>4.0 μg/ml	2.0 μ g/ml
Streptomycin	Aminoglycosides	30S ribosome	0.25 μ g/ml	0.25 μ g/ml	0.25 μ g/ml
Tetracycline	Tetracyclines	30S ribosome	0.5 μ g/ml	0.5 μ g/ml	0.5 μ g/ml
B. 50S targeting drugs					
Erythromycin	Macrolides	50S ribosome	64.0 μ g/ml	64.0 μ g/ml	64.0 μ g/ml
Chloramphenicol	Chloramphenicols	50S ribosome	16.0 μ g/ml	16.0 μ g/ml	16.0 μ g/ml
C. Non-ribosomal targeting drugs					

Moxifloxacin	Quinolones	DNA gyrase	0.06 µg/ml	0.06 µg/ml	0.06 µg/ml
Rifampicin	Rifamycins	DNA dependent RNA polymerase	1.0 µg/ml	1.0 µg/ml	1.0 µg/ml

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Fig. 1A

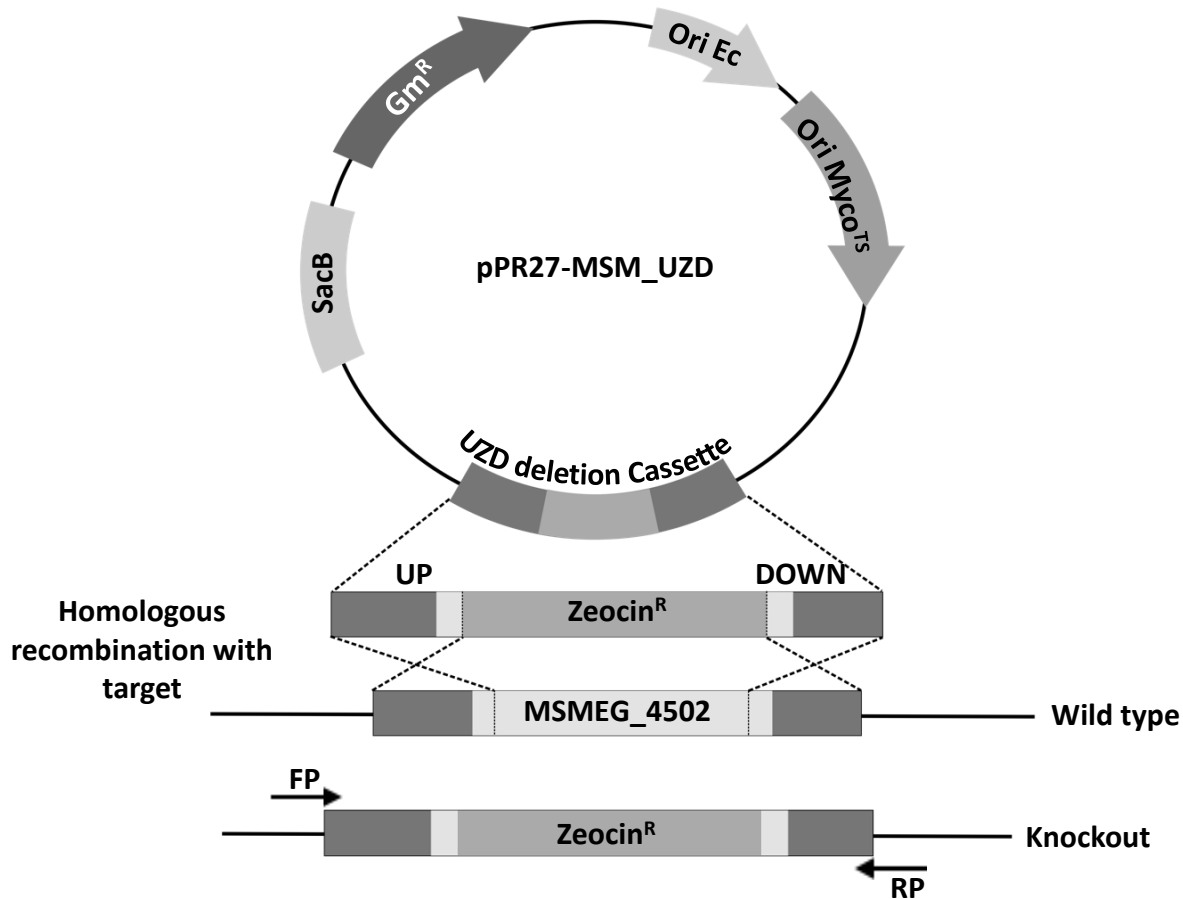


Fig. 1B

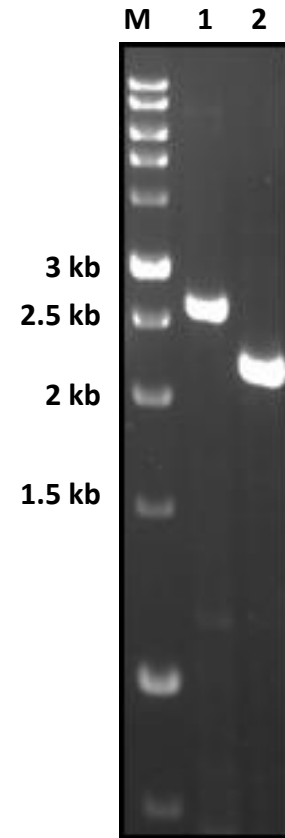
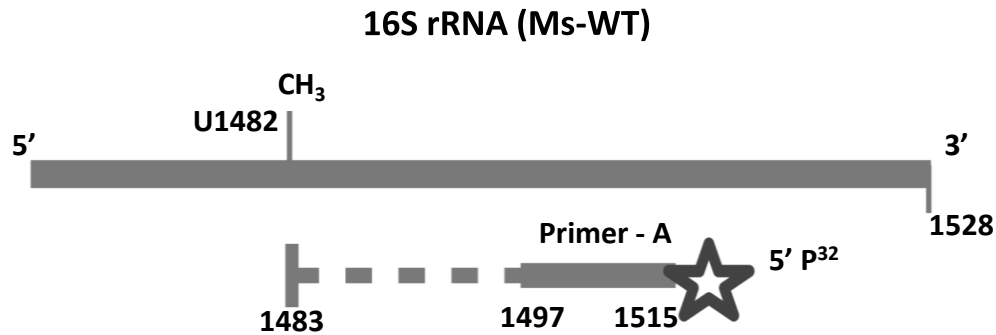
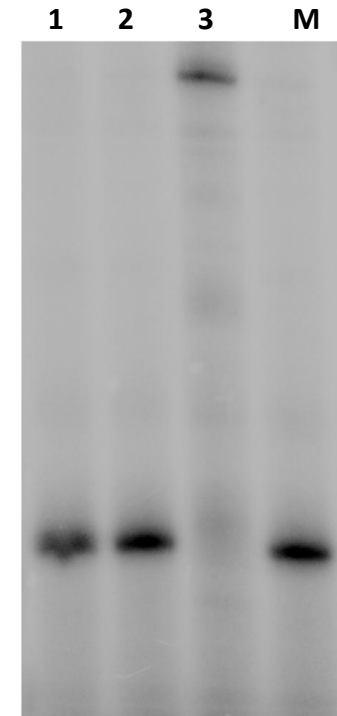


Figure 1: Generation of *M. smegmatis* *RsmE* (*MSMEG_4502*) knockout strain.

A. A knockout of *MSMEG_4502* was generated using suicidal vector strategy with *Mycobacterium smegmatis* – *E. coli* shuttle vector pPR27. The upstream fragment (UP) and downstream fragment (DOWN) of the target gene *MSMEG_4502* were cloned in the vector along with *Zeocin^R*, yielding pPR27-MSM_UZD. The knockout was generated by homologous recombination and further screening of the transformants.

B. The knockouts were confirmed via PCR using the primers flanking the UP and DOWN fragments. An amplified product of 2535 bp is obtained in Ms- Δ RsmE (Lane 1) and that of 2160 bp in Ms-WT (Lane 2).

Fig. 2A**Fig. 2B****Figure 2: Primer extension assay for m³U1498 methylation in *M. smegmatis*.**

A. Overall assay design of the primer extension assay. The primer complementary to the region 1497 to 1515 of 16S rRNA of *M. smegmatis* (Primer-A, Table 1) is 5' end-labelled with ³²P. In the presence of methylation, the reverse transcription is stalled due to disruption of base pairing, giving a truncated product of 33bp, while in the absence of methylation, reverse transcription continues resulting in longer products. All indicated position numberings are with respect to *M. smegmatis* 16S rRNA. *M. smegmatis* U1482 is equivalent to U1498 of *E. coli* 16S rRNA.

B. Primer extension assay products from wild type, knockout and complemented strains of *M. smegmatis* were analysed on a 15% urea PAGE. The presence of a band indicates a truncated product of 33bp in Ms-WT (lane 1) and Ms-ΔRsmE/RsmE (lane 2) due to presence of m³U methylation. (As explained above, *M. smegmatis* U1482 is equivalent to U1498 of *E. coli* 16S rRNA). Absence of methylation in Ms-ΔRsmE (lane 3) leads to longer products. A ³²P end-labelled 33 base oligonucleotide was used as a marker (M) to indicate the size of the truncated product due to methylation in Ms-WT and Ms-ΔRsmE/RsmE.

Fig. 3

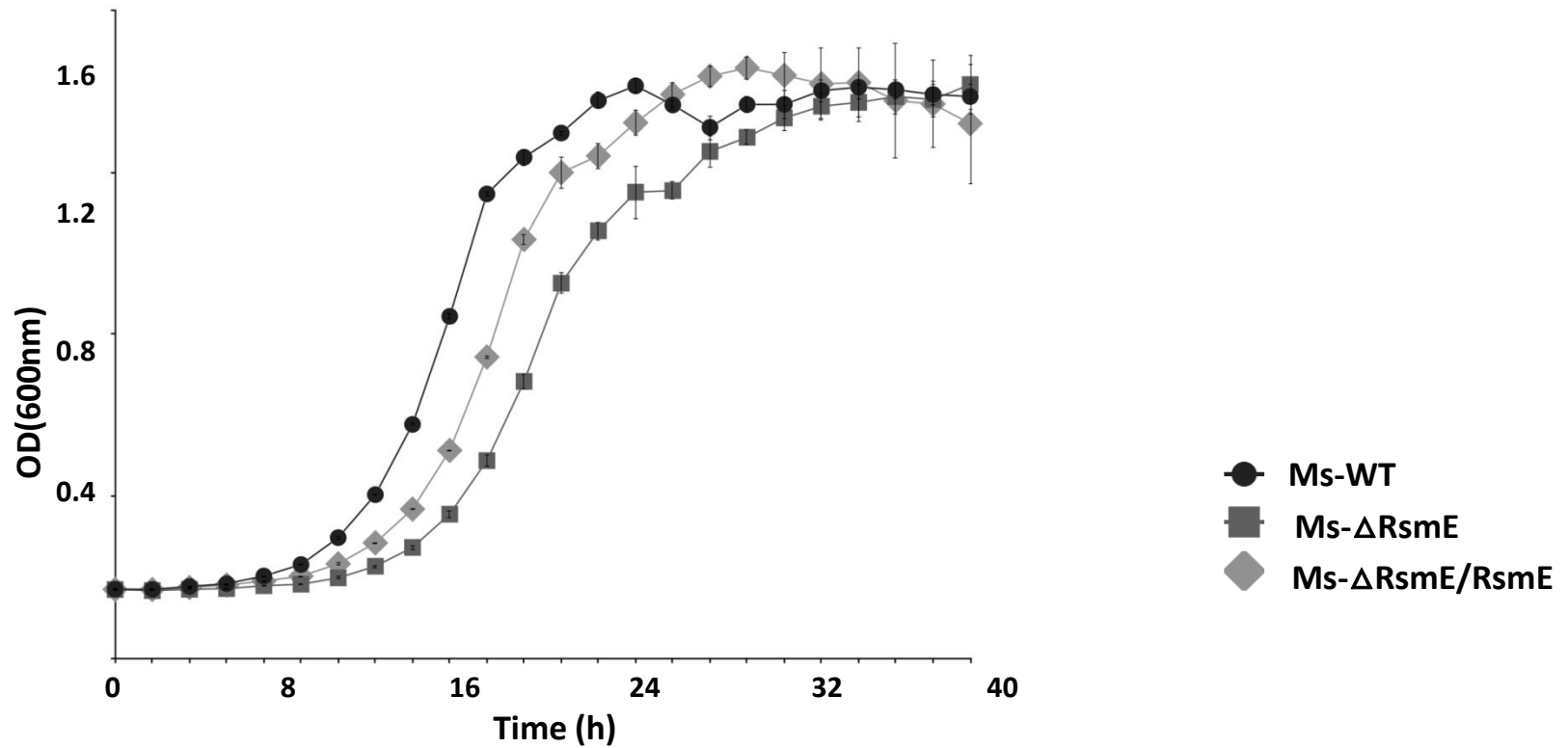


Figure 3: Growth curve of *Mycobacterium smegmatis* strains.

The Ms-ΔRsmE knockout strain (squares) shows a slightly extended lag phase compared to Ms-WT strain (circles), which is partially restored, similar to Ms-WT, in the complemented strain Ms-ΔRsmE/RsmE (diamonds).

Fig. 4A

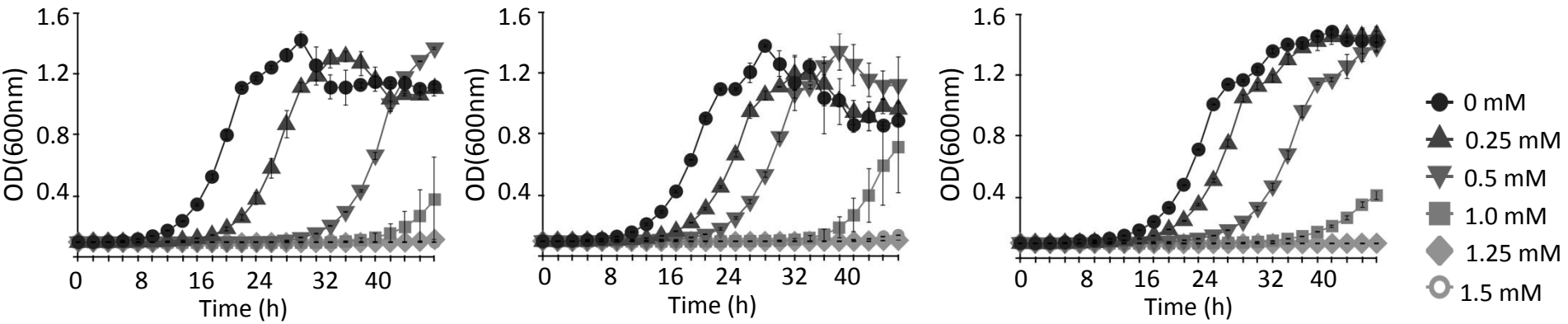


Fig. 4B

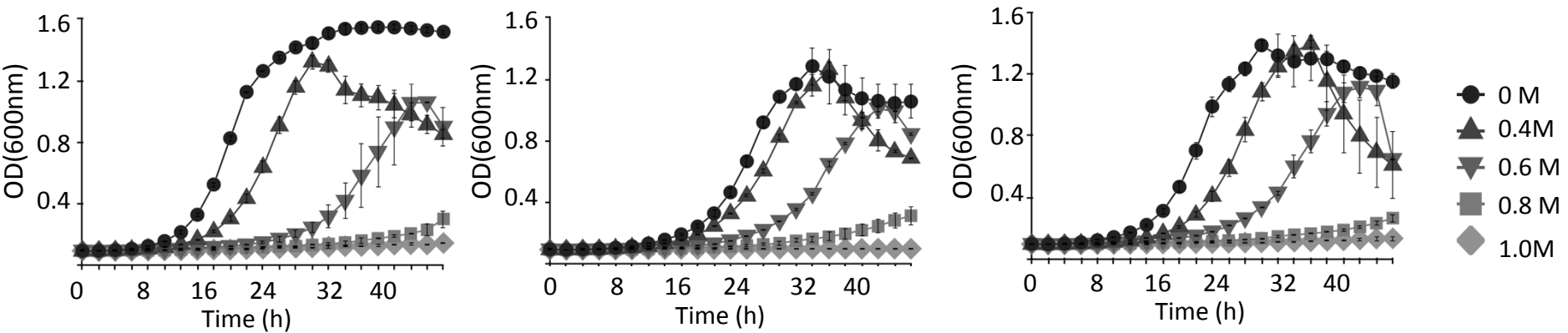


Fig. 4C

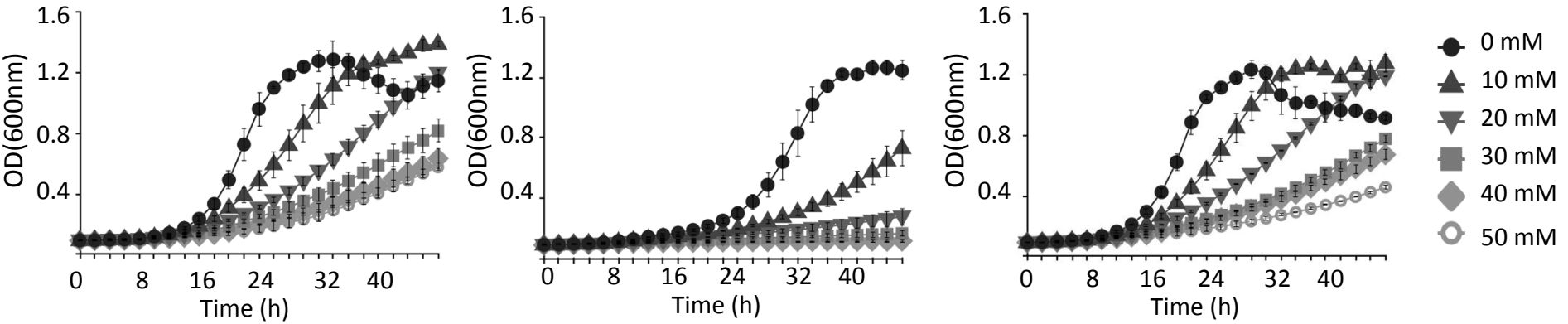


Fig. 4D

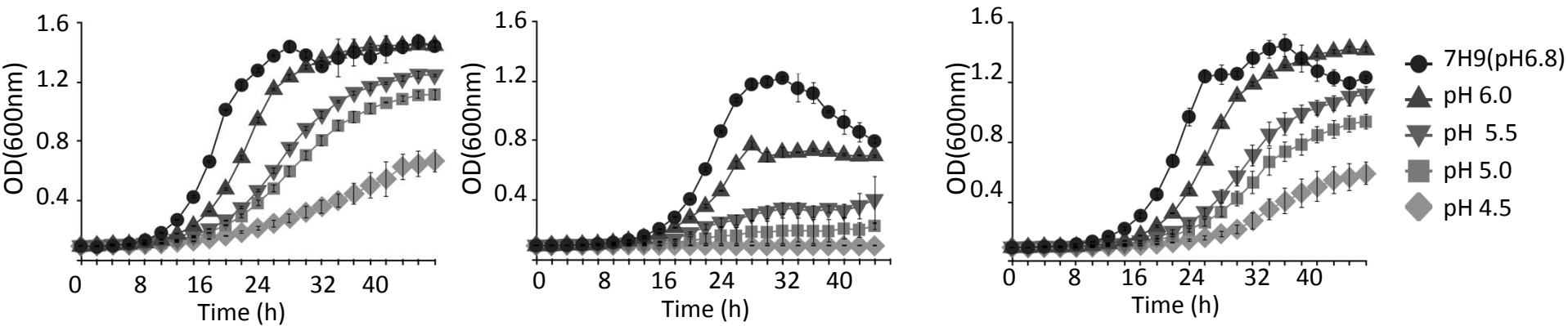
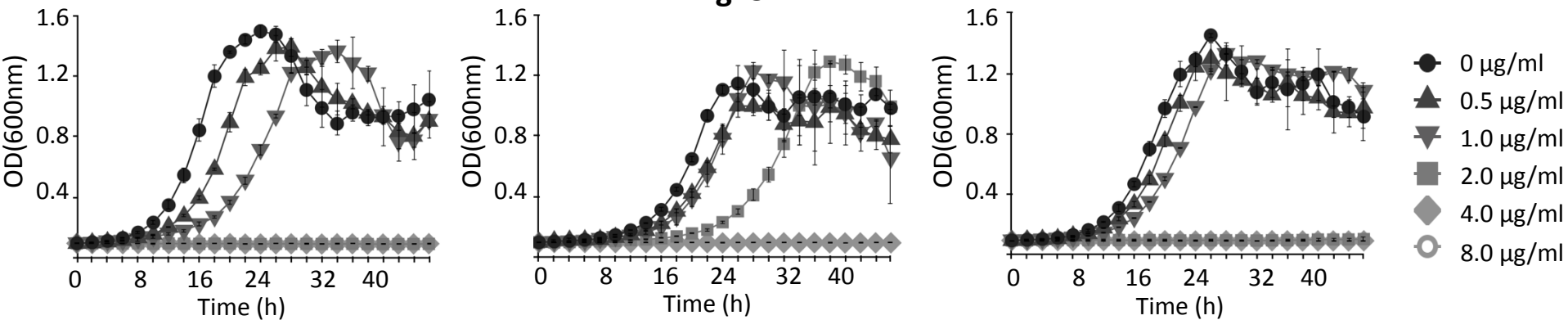
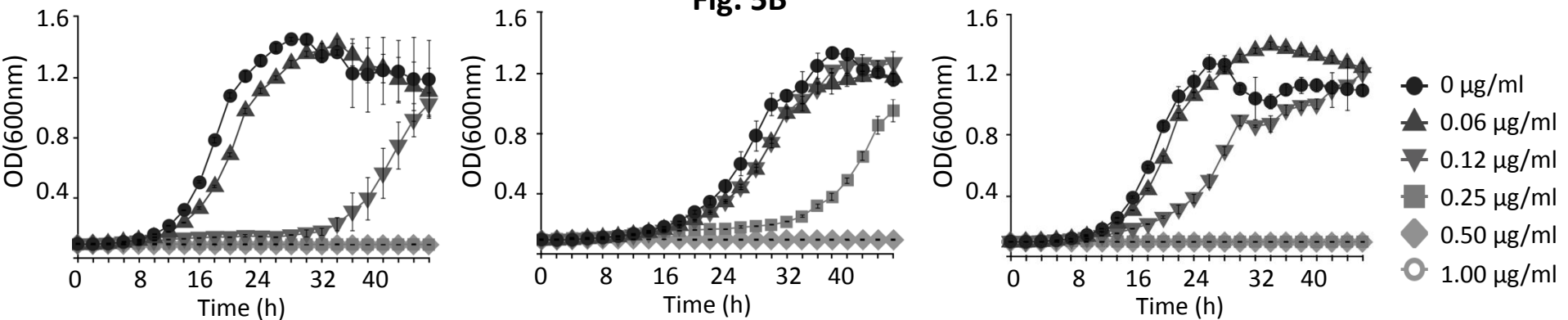
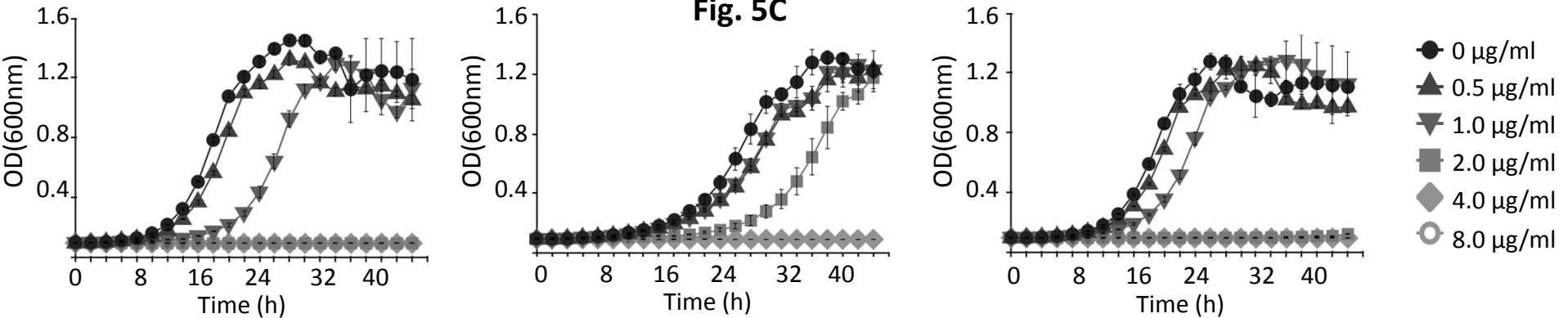


Figure 4: Growth of *Mycobacterium smegmatis* strains under stress conditions.

Growth curve of Ms-WT (left panels), Ms- Δ RsmE (middle panels) and Ms- Δ RsmE/RsmE (right panels) with varying levels of (A) H₂O₂, (B) NaCl, (C) Sodium nitroprusside and (D) pH are plotted.

Fig. 5A**Fig. 5B****Fig. 5C**

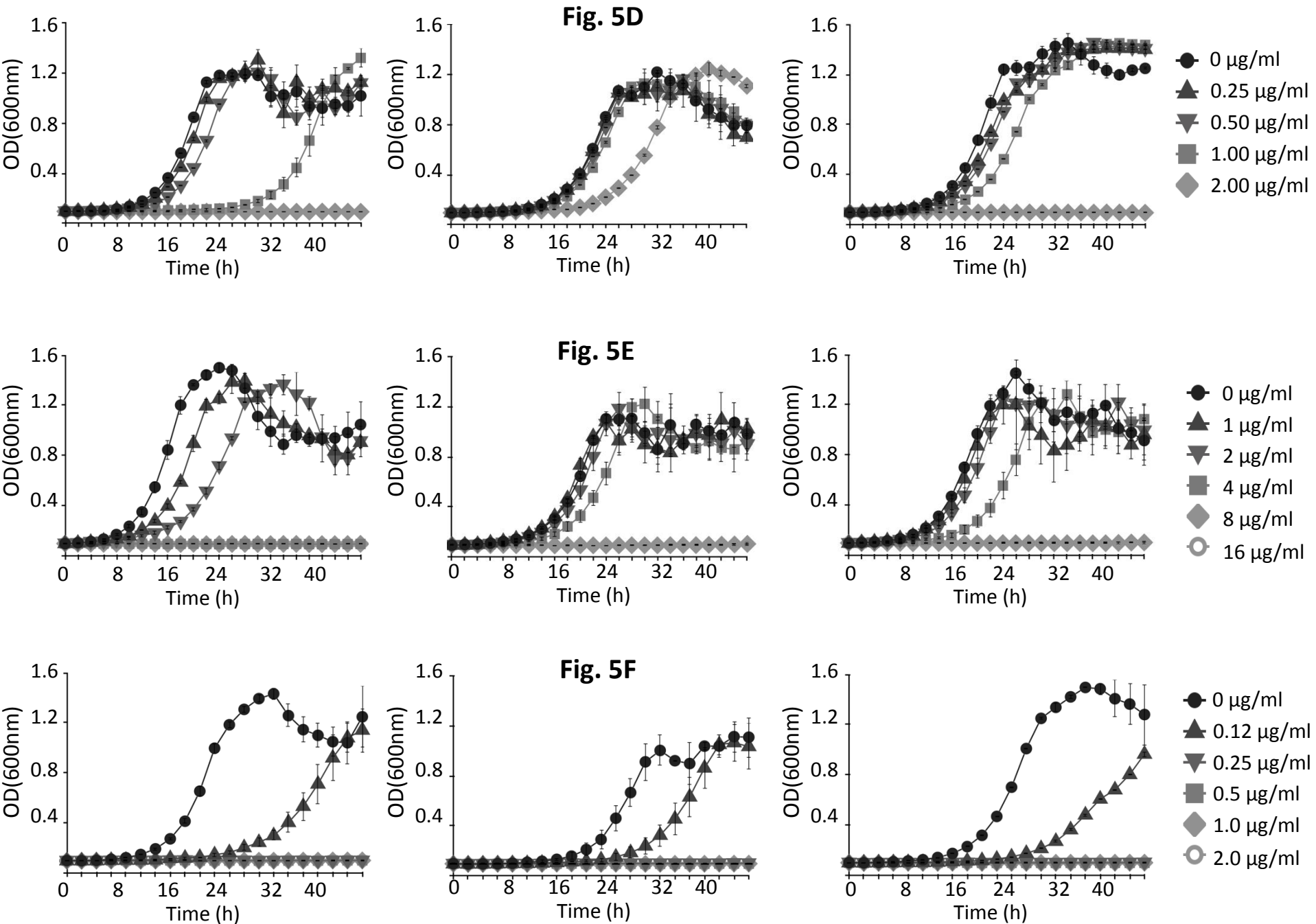


Fig. 5G

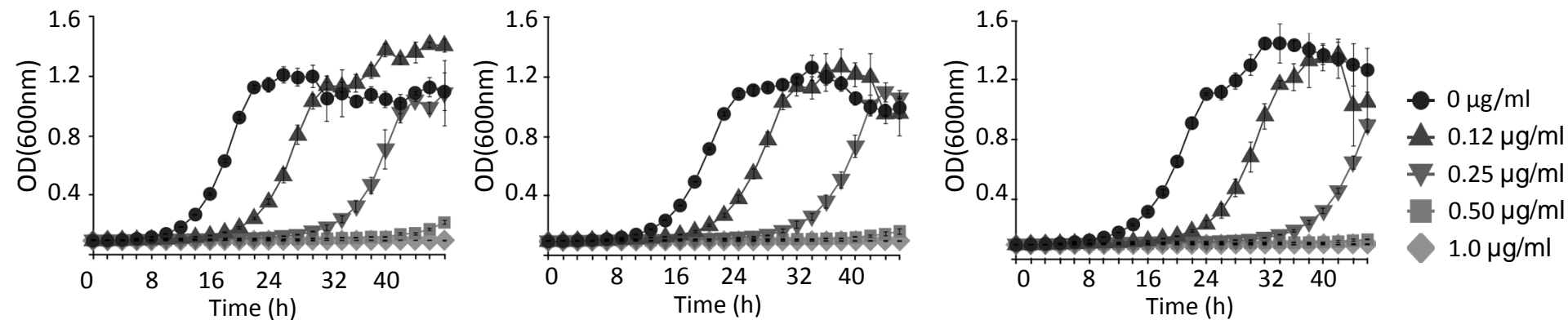
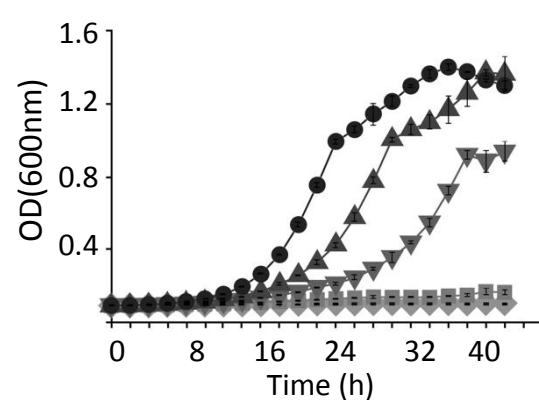
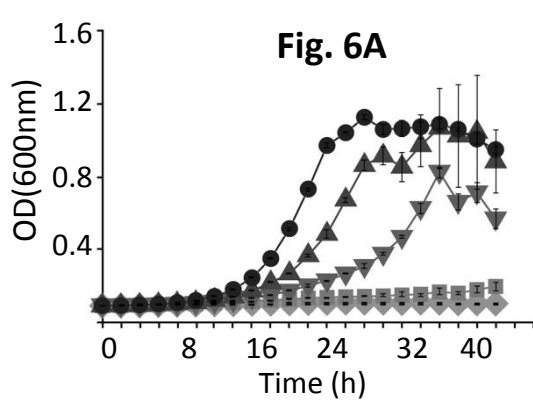
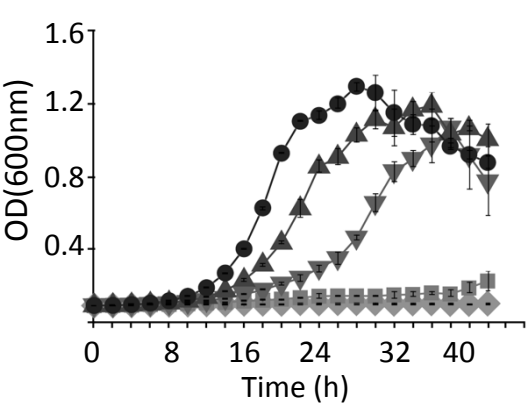
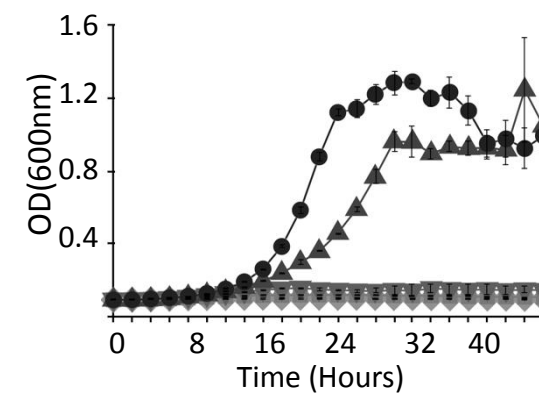
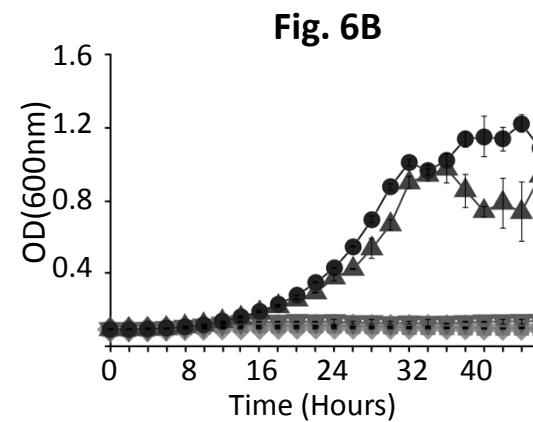
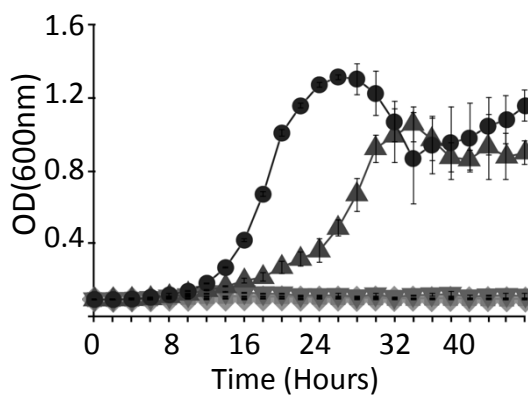


Figure 5: Drug response towards 30S targeting antibiotics

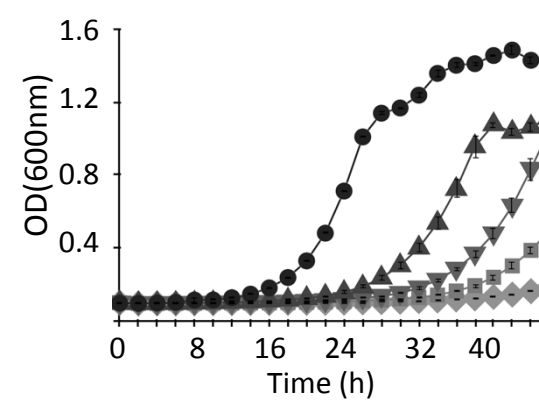
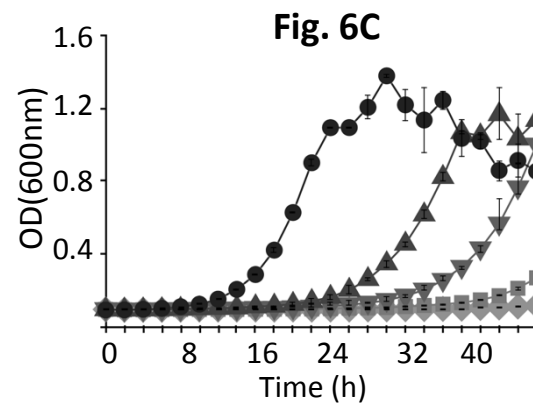
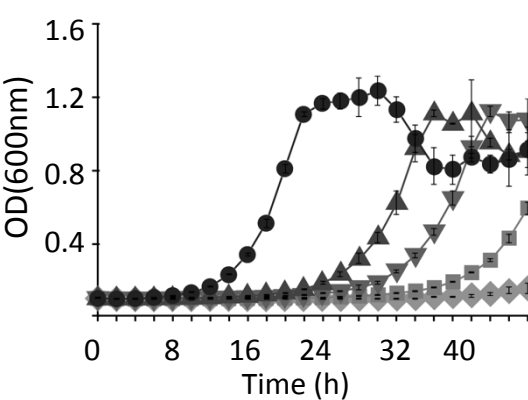
Growth of Ms-WT (left panels), Ms-ΔRsmE (middle panels) and complemented Ms-ΔRsmE/RsmE (or Ms-ΔRsmE/RsmE'), (right panels) were monitored with increasing concentrations of (A) kanamycin, (B) amikacin, (C) gentamicin, (D) paromomycin, (E) neomycin, (F) streptomycin and (G) tetracycline.



- 0 $\mu\text{g/ml}$
- ▲ 0.25 $\mu\text{g/ml}$
- ▼ 0.50 $\mu\text{g/ml}$
- 1.00 $\mu\text{g/ml}$
- ◆ 2.00 $\mu\text{g/ml}$
- 4.00 $\mu\text{g/ml}$



- 0 $\mu\text{g/ml}$
- ▲ 0.03 $\mu\text{g/ml}$
- ▼ 0.06 $\mu\text{g/ml}$
- 0.12 $\mu\text{g/ml}$
- ◆ 0.25 $\mu\text{g/ml}$
- 0.50 $\mu\text{g/ml}$



- 0 $\mu\text{g/ml}$
- ▲ 8 $\mu\text{g/ml}$
- ▼ 16 $\mu\text{g/ml}$
- 32 $\mu\text{g/ml}$
- ◆ 64 $\mu\text{g/ml}$

Fig. 6D

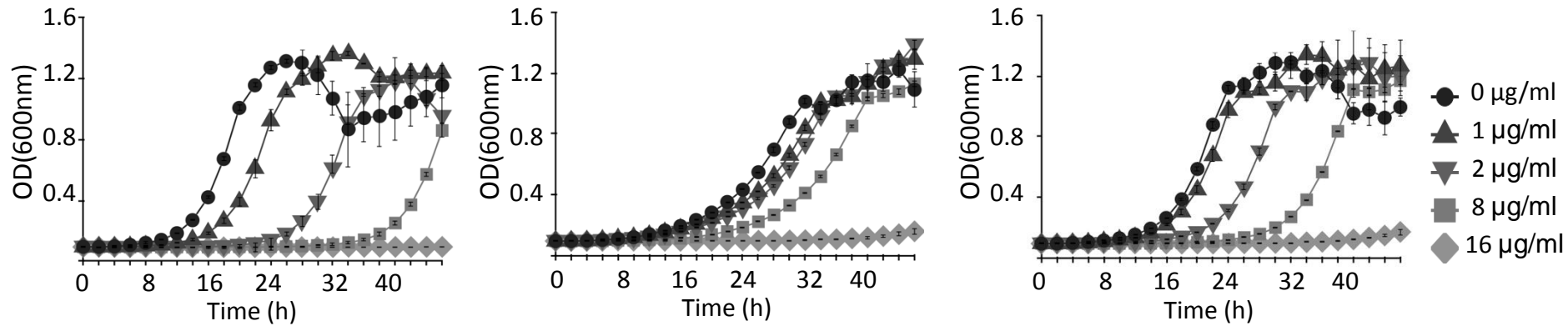


Figure 6: Drug response towards non-30S targeting antibiotics

Growth of Ms-WT (left panels), Ms- Δ RsmE (middle panels) and Ms- Δ RsmE/RsmE (right panels) were monitored with increasing concentrations of (A) rifampicin, (B) moxifloxacin, (C) erythromycin and (D) chloramphenicol.

Fig. 7

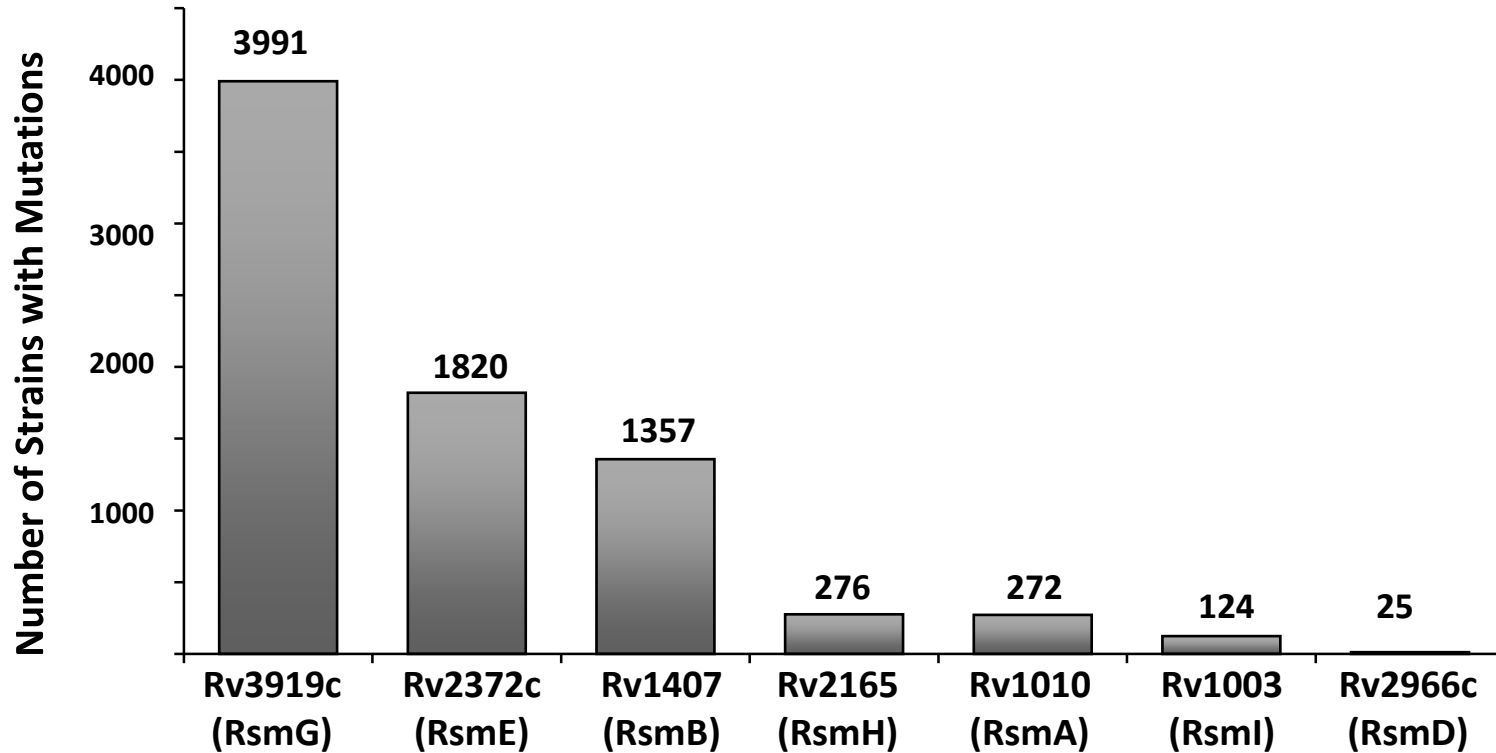


Figure 7: Mutations in 16S rRNA methyltransferases in clinical strains of *Mycobacterium tuberculosis*

The total number of non-synonymous mutations in the coding sequence of the conserved rRNA methyltransferases in the clinical strains of *Mycobacterium tuberculosis* obtained from the PATRIC database is indicated. RsmG, having the highest number of mutations, is known to be associated with streptomycin resistance. RsmE methyltransferase has the second highest mutations among conserved methyltransferases, in a total of 19% clinical strains. The conserved *E. coli* counterpart for each mapped methyltransferase is also indicated.

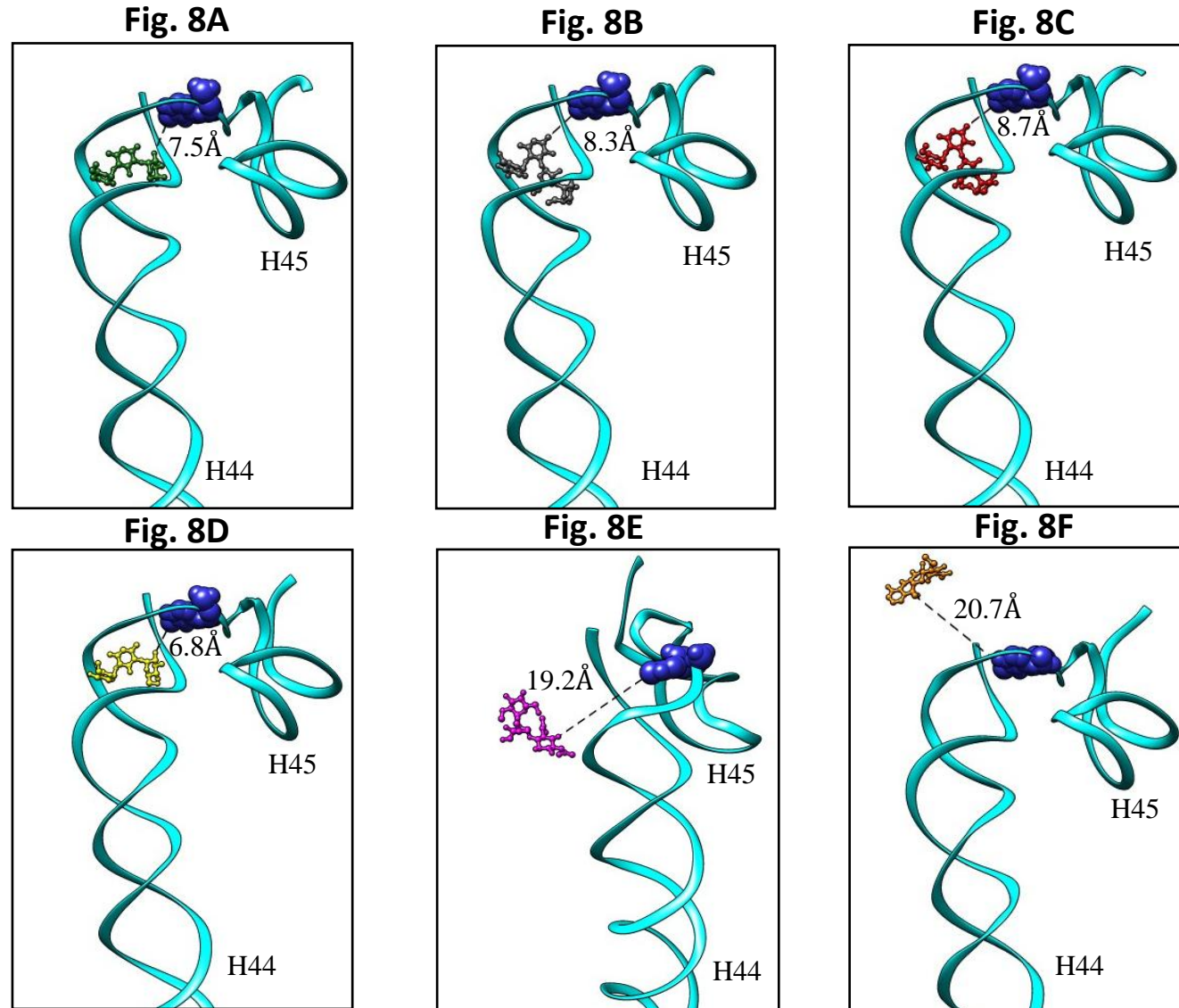


Figure 8: Binding site of aminoglycosides in ribosome structure

The binding of antibiotics (A) Gentamicin (green), (PDBID: 4V53), (B) paromomycin (gray), (PDBID: 4V5Y), (C) neomycin (red), (PDBID: 4V52), (D) tobramycin^a (yellow), (PDBID: 4LFC), (E) streptomycin (magenta), (PDBID: 4JI8) and (F) tetracycline (orange), (PDBID: 5J5B) was mapped using the available crystal structures in PDB and are shown as ball-and-stick structures. The distance from N-3 of U1498 to the bound drug (closest atom) is indicated in dashed lines (in Å). For clarity, only helix 44 and helix 45 of 30S ribosomal subunit are shown here (cyan). U1498 is indicated as space-filled structure (bright blue). Non-30S targeting drugs are not shown as they bind much farther away from U1498.

^a: Tobramycin-bound structure is indicative of kanamycin/amikacin binding site, as a kanamycin/amikacin-bound ribosome structure could not be identified in PDB.