Comparative analysis of 16S rRNA methyltransferases conferring resistance to aminoglycoside antibiotics in producing strains

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Methyltransferases that employ S-adenosyl-L-methionine as a cofactor act on a wide variety of target molecules, including rRNAs. Nucleoside methylation is the most frequent type of rRNA modification in bacteria, and is introduced post-transcriptionally with a wide variety of site-specific enzymes. Through methylation of specific nucleosides within the tRNA acceptor aminoacyl site of 16S rRNA, which impedes binding of aminoglycosides to the 30S ribosomal subunit, S-adenosyl-L-methionine-dependant 16S rRNA methyltransferases confer a high-level resistance to aminoglycosides in antibiotic producing strains and serve as a mean of self-protection. Until recently, this mechanism of resistance was considered as a unique to the producers; however, it has been acceleratedly emerging among clinically relevant pathogens. The level of antibiotic resistance is not usually related to methyltransferases gene dosage, and expression of relatively few enzyme molecules is sufficient for complete modification of the target. A negative autoregulatory system may be involved in maintaining both constant and low concentrations of these proteins.

Keywords Aminoglycosides; Methyltransferases; Regulation; Resistance

1. Introduction

Nucleic acids, proteins, lipids, polysaccharides, and a range of small molecules are subject to methylation by highly specific methyltransferases (MTs), in both prokaryotic and eukaryotic cells. These enzymes use S-adenosyl-L-methionine (SAM), the most commonly utilized co-enzyme among methyl donor molecules, and act on various atomic targets like carbon, oxygen, nitrogen, and sulfur [1]. Methylating the substrate, such as DNA or RNA, MTs modulate its activity, function and folding; therefore, they influence cells' destiny. Bacterial SAM-dependant DNA MTs are not just involved in defense against invasion of bacteria by foreign DNA as a part of restriction modification system but also participate in the processes like chromosome replication, mismatch repair, transcriptional regulation, and regulation of transposition. Post-transcriptional methylation of the transfer RNA (tRNA) that occurs at various nucleosides and different atoms, is believed to affect the function of the tRNA through relevant alternation of "rigidity" and "flexibility" of tRNA, which impinge directly on proper base paring required for codon recognition [1]. Furthermore, post-transcriptional methylation of ribosomal RNA (rRNA) confers housekeeping roles essential for the assembly, maturation, and function of the cellular protein synthesis machinery. The sites of housekeeping methylations have been most accurately mapped in *Escherichia coli.* Its ribosome contains 24 methylated nucleosides - 10 in the 16S rRNA and 14 in the 23S rRNA [2].

Being of fundamental importance for cell viability it is not unexpected that many antibiotics target the ribosomes [3]. The main contact sites for the antibiotics are on the rRNAs, rather than on the ribosomal protein components, which is consistent with the view that the rRNAs carry out the primary functions of the ribosome while the ribosomal proteins have the supporting roles [4-6]. Several classes of antibiotics target the rRNA-rich surfaces on the 30S and 50S ribosomal subunits, interfering with their functions in protein synthesis [3]. Not surprisingly therefore, changes that confer antibiotic resistance to these chemotherapeutics mainly consist of base substitutions or nucleoside methylations [7]. A distinct class of SAM-dependant MTs that post-transcriptionally modify, i.e., methylate rRNA molecules is responsible for resistance to antibiotics produced by different bacterial species. Modifying the antibiotic-binding sites on the ribosome antibiotic-producing bacteria avoid suicide [3 and references within].

2. Antibiotics interfering with protein synthesis

Antibiotics targeting the 50S ribosomal subunit bind to its three main regions and interfere with GTP hydrolysis (e.g., thiostrepton), the formation of peptide bonds (e.g., chloramphenicol), and obstruct synthesized peptide passage through the 50S subunit tunnel (e.g., macrolides, lincosamides and streptogramin B). Among many different classes of antibiotics targeting the 50S ribosomal subunit macrolides are perhaps the clinically most important. Binding of the macrolide antibiotics in the peptide exit tunnel impedes progression of the nascent peptide and results in a general inhibition of translation. Residues A2058 and A2059 in 23S rRNA are crucial for efficient binding of macrolides to their target and mutations at these residues or methylation of A2058 negatively affects interactions with the drug [8-11]. Since 23S rRNA MTs conferring resistance to macrolides are out of scope of this paper, the reader is referred to related reviews [12, 13].

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Small ribosomal subunit is a target for antibiotics that prevent the association of tRNA with the ribosomal tRNA acceptor aminoacyl site, i.e., A-site (e.g., tetracyclines) [14, 15], trap the ribosome in an intermediate state of translation (cyclic peptides) [16], and induce misreading of the genetic code (e.g., aminoglycosides). The best studied are aminoglycoside antibiotics, a structurally diverse family of polycationic compounds containing central aminocyclitol ring, most frequently 2-deoxystreptamine or streptamine, connected via glycosidic bonds to amino sugars. They can be conveniently divided into three structural classes based upon the position of these bonds: 4,5-disubstituted 2-deoxystreptamine (4,5-DOS), 4,6-disubstituted 2-deoxystreptamines (4,6-DOS) while the third class consists of those compounds that do not fit into either of previously described groups, such as 4-monosubstituded 2-deoxystreptamines (apramycin, neamine), streptomycin, hygromycin B, and spectinomycin. Only 4,5-DOS and 4,6-DOS classes have been presented in Fig. 1.

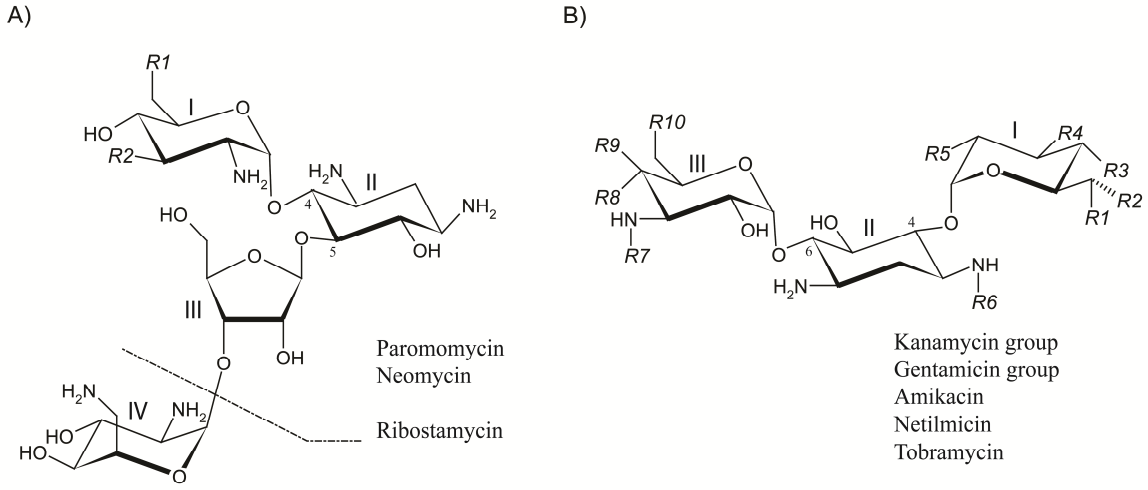

Fig. 1 Structures of 4,5- and 4,6-disubstituted aminoglycoside antibiotics. A) 4,5-disubstituted 2-deoxystreptamines and B) 4,6-disubstituted 2-deoxystreptamines. Kanamycin group includes: kanamycin A and kanamycin B, and gentamicin group includes: gentamicin B, gentamicin C1, gentamicin C1a and gentamicin C2. Substituents (R) could be: H; OH; NH_2; $NHCH_3$; CH_3; CH_2OH. Roman numbers represent rings: II – aminocyclitol ring (2-deoxystreptamine); I, III and IV – amino sugars.

Aminoglycosides inhibit the translation process by causing misreading and/or hindering the translocation step. The fidelity of translation depends on two steps, an initial recognition between the codon of the mRNA and the anticodon of an aminoacylated-tRNA, and a proofreading step that follows [17]. Kanamycin, gentamicin, neomycin, and paromomycin bind in a similar fashion to the base of 16S rRNA helix 44 (h44), that together with portions of the 530 loop and helix 34 constitute A-site. It has been shown that aforementioned antibiotics have two binding positions in common, A1408 and G1494 within h44, with which they form almost similar hydrogen bonding pattern. On the other hand, four bases, A1408, A1492, A1493, and G1494, in the 16S rRNA A-site interact with tRNA, although with different affinities [18]. Figure 2 shows aminoglycosides binding sites and MTs target nucleosides.

Binding of aminoglycosides to the A-site on the 16S rRNA forces A1492 and A1493 to 'flip-out' of h44, which mimics the conformation adopted by these 16S rRNA fidelity gatewatch nucleosides in the presence of cognate mRNA-tRNA codon-anticodon association [19, 20]. Therefore, aminoglycosides shunt a molecular switch and cause a loss of translational fidelity [21]. Over time, accumulation of erroneous proteins that are truncated or incorrectly folded leads to bacterial cell death. Although apramycin binds to the decoding site, it is the only antibiotic whose primary mechanism of action is blocking the ribosomal translocation along the mRNA. This unique effect of apramycin is likely due to its unusual structure that allows interference with local conformation of ribosomal protein S12, which is involved in the translocation process [22]. Then again, streptomycin binds at the 16S rRNA in proximity of the decoding site and reduces translational fidelity by a complex mechanism that might involve interference with initial tRNA selection and proofreading [23]. Furthermore, hygromycin B binds at a site that partially overlaps with the binding site of 4,5- and 4,6-DOS aminoglycosides within the decoding site but involves only nucleosides that are conserved between bacteria and eukaryotes. Therefore, hygromycin B cannot be used as a selective antibiotic [24].

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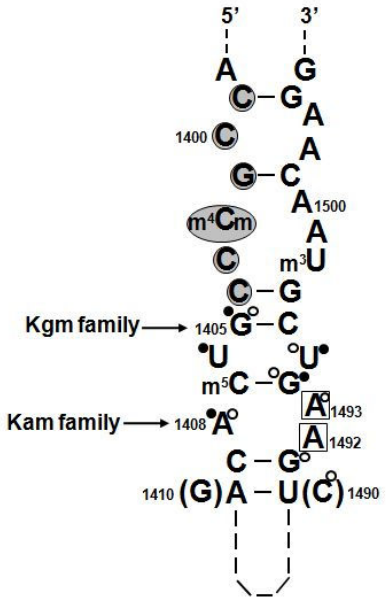

Fig. 2 Sequence and secondary structure of the 16S rRNA A-site and positions of aminoglycoside binding. *E. coli* 16S rRNA secondary structure model is presented. Experimentally determined target sites of Kgm and Kam families are indicated by arrows. A-site nucleosides (A1492 and A1493) important for tRNA anticodon–mRNA interaction are shown in squares. The sites of post-transcriptional "housekeeping" modifications in this *E. coli* rRNA region are indicated with letter m. Nucleosides involved in paromomycin binding are depicted as open dots; nucleosides involved in gentamicin C1A and kanamycin A binding are marked with black dots. Paromomycin binds to C1490; however, 16S rRNA *E. coli* has A1410:U1490 pair whereas *Actinomycetales* 16S rRNA has G1410:C1490, which is given in brackets. Motif present in *kgmB* and *sgm* 5'UTRs is indicated by shaded circles.

3. 16S rRNA methyltransferases

3.1 Housekeeping methyltransferases and resistance

KsgA is a housekeeping MT that dimethylates two nucleosides, A1518 and A1519 in the loop of helix 45, near the 3' end of the 16S rRNA, to produce N6,N6-dimethyladenosine [25]. It is the only rRNA post-transcriptional modification enzyme that appears to be conserved in all three domains of life, a conservation that extends into mitochondria and chloroplasts. KsgA methylation of helix 45 could, either directly or indirectly, act as a "mark" of a fully assembled, functionally competent 30S subunit so that KsgA functions as a gatewatch for translationally competent newly assembled small ribosomal subunits [26, 27]. There is a high degree of structural similarity between KsgA and ErmC', which methylate a single adenosine base in 23S rRNA and confer resistance to the MLS_B group of antibiotics [28]. Interestingly, inactivation of KsgA and resulting loss of the dimethylations provides a resistance to kasugamycin antibiotic with modest consequences to the overall fitness of the organism [29].

3.2 16S rRNA methyltransferases from aminoglycoside-producing bacteria

Many aminoglycoside-producing bacteria protect themselves from the toxic effects of antibiotics by methylating specific nucleosides in antibiotic-binding sites of the ribosome, thus disrupting the antibiotic binding without much interference with other functions of the ribosome. Two distinct families of 16S rRNA aminoglycoside resistance MTs, have been defined based upon their target nucleosides, G1405 or A1408, [30, 31] as Kgm (kanamycin-gentamicin methyltransferase) and Kam (kanamycin-apramycin methyltransferase) families, respectively.

High-resolution structures of the 4,6-DOS aminoglycosides gentamicin C1a [32] and tobramycin [21] in complex with A-site model RNAs showed that both antibiotics make direct contacts to G1405 via their Ring III substituents. Methylation at N7 position (m7G1405) would thus directly interfere with antibiotic binding, by inducing a steric clash between the modified base and Ring III, in addition to possible electrostatic repulsion by the positive charge on modified base. In contrast, substituent at position 5 in 4,5-DOS, such as in paromomycin or neomycin, is placed at angle that directs it away from G1405; hence, methylation at this site does not interfere with their binding. Both 4,5- and 4,6- DOS antibiotics bind to 16S rRNA so that their Ring I substituents are placed in close proximity to A1408. The N1 methylated adenosine (m1A1408) is positively charged at neutral pH, and can therefore affect drug binding not only by steric hindrance but also by charge repulsion. This modification confers resistance to apramycin and the kanamycin group of 4,6-DOS aminoglycosides other than gentamicin.

KgmB from Streptoalloteichus tenebrarius (formerly Streptomyces tenebrarius), Sgm from Micromonospora zionensis, GrmA from M. echinospora (formerly M. purpurea), Krm from Frankia sp. CcI3, FmrO from M. olivasterospora, Grm from M. rosea, Srm1 from M. inyonensis, and NbrB from Streptomyces hindustanus belong to the Kgm family as well as some recently reported hypothetical proteins [33]. It has been experimentally established that KgmB, Sgm, GrmA, and Krm MTs modify N7 position of G1405 [31, 34-36]. For other mentioned members of Kgm family site of action was predicted according to the resistance profile. The m7G1405 modification by Kgm family MTs is only effective against 4,6-DOS aminoglycosides [37]. The target nucleoside G1405 is base-paired with C1496 at the

base of long h44, and its N7 atom is not accessible for methylation, hence a conformational change must occur in order for the methylation to take place.

Only three members of the Kam family: KamA from *Streptomyces tenjimariensis* [34], KamB from *Streptoalloteichus tenebrarius* [31] and KamC from *Saccharopolyspora hirsuta* [38] have experimentaly confirmed methylation at the N1 position of A1408.

Modification by Kgm MTs is effective against 4,6-DOS aminoglycosides, conferring high-level resistance to both kanamycin and gentamicin antibiotic groups, but not to apramycin, 4-monosubstituted deoxystreptamine. On the other hand, Kam family MTs confer resistance to apramycin and 4,6-DOS aminoglycosides, except to gentamicin. Comparison of antibiotic resistance patterns between Kgm and Kam family MTs unambiguously identifies functional differences correlating with modification at G1405 and A1408 in 16S rRNA.

The hygromycin B, a bactericidal aminoglycoside produced by *Streptomyces hygroscopicus* [34], has a structurally different aminocyclitol component and inhibits protein synthesis by blocking ribosomal translocation without causing significant misreading *in vivo* [39-43]. Curiously, resistance to hygromycin B is provided upon Kgm family MTs expression only in *Micromonospora* background but not in all tested streptomycetes strains. Collateral expression of resistance is attributed to some unique structural feature of the 30S ribosomal subunit in the genus of Micromonospora [44]. Otherwise, base substitutions that mediate resistance to hygromycin B exclusively involve universally conserved nucleosides within rRNA, and it is in accordance with the finding that hygromycin B is toxic for almost all organisms [24].

4. Regulation of rRNA methyltransferase gene expression

In prokaryotes, the synthesis of many RNA-binding proteins (e.g., ribosomal proteins) is regulated by a negative translational feedback mechanism involving a competition between their natural substrate, i.e., rRNA and their binding site on mRNA, which are often thought to resemble each other [45, 46]. It has been shown that *ermC* gene that codes for 23S rRNA MT employs three types of regulation [47] including translational autoregulation [48]. According to the model for *ermC* downregulation when ErmC reaches an intracellular level that exceeds the amount required to saturate the ribosome pool, free MT binds to *ermC* mRNA. More precisely, ErmC binds to the region within its mRNA which structural features resemble MT's natural target, i.e., 23S rRNA, and repress translation. Similar model for translational autoregulation of *ksgA* gene [49], supported by discovery that only a small amount of the KsgA is normally present in the cell (<1,000 molecules), has been postulated. As per the model structural similarities between region containing two neighboring adenosines within a hairpin loop close to the 3' end of the 16S rRNA (substrate site for KsgA), and *ksgA* mRNA play the essential role in autoregulation.

Although the importance of such mechanism for the regulation of 23S rRNA MT conferring resistance to macrolides and houskeeping MT is not completely understood, the existence of analogous type of regulation among 16S rRNA MTs from producers points out its significance in terms of survival fitness.

The nebramycin antibiotic complex producer *S. tenebrarius* and the G-52 producer *M. zionensis* accomplish resistance to their own toxic products through the activity of KgmB and Sgm MTs, respectively [50, 51]. The model proposed for translational regulation of these genes is based on *in vivo* studies and suggests that Sgm and KgmB MTs recognize the same motif(s) within 16S rRNA molecule, depicted in Fig. 2, and 5' untranslated regions (5'UTR) on their own mRNAs [52-54]. Furthermore, mutual negative translational regulation has been shown between Sgm and KgmB MTs [54], implying that they share the same *cis*-acting elements. Interestingly, although GrmA MT from *M. echinospora*, producer of gentamicin complex, does not autoregulate itself, it still downregulate Sgm and KgmB MTs (data not published). These phenomena could be attributed to the differences in their 5'UTRs. The (C)CGCCC motif within *kgmB* and *sgm* 5'UTR, hypothesized to be a regulatory sequence responsible for autoregulation of both genes, also present in the A-site of 16S rRNA, is missing in 11 base pairs long *grmA* 5'UTR. Decrease of resistance to gentamicin in both *E. coli* and *M. melanosporea* when *sgm* 5'UTR is co-overexpressed with Sgm [55] could be explained by *sgm* 5'UTR ability to titrate down Sgm MT and consequently, to reduce the number of available molecules necessary to methylate 30S subunits.

Actinomycetes, the soil microorganisms that habitate the environment where the physical and chemical conditions fluctuate constantly, as a respons to the changes switch from primary (exponential growth) to secondary metabolism (stationary growth) [56]. As antibiotics, typical products of secondary metabolism, are often toxic even to their producers, microbes first arm themselves with a defense mechanism before production commences. In other words, resistance MTs must be present in sufficiently high concentrations when antibiotic biosynthesis has begun. Antibiotic biosynthetic genes are usually clustered with corresponding resistance genes [57]. The production of antibiotics is regulated at multiple levels by a number of multifunctional signaling molecules controlling both antibiotic production and global cellular physiology. However, the signal transduction pathways still remain to be determined. Data regarding MTs gene expression while switching to secondary metabolism are very limited. Analysis of the *sgm* gene in *M. zionensis* revealed that this gene is transcribed from tandem promoters with different strengths that could enable its differential expression in the producing organism [51]. Understanding the switch to secondary metabolism is of major importance in biotechnology, where it can contribute to the optimized production of commercially relevant secondary

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metabolites, such as antibiotics. In this context, it is obvious that revealing the exact mechanism of MTs gene expression regulation is not only important from fundamental aspect but also from biotechnological point of view.

5. Emergence of 16S rRNA resistance methlytransferases among pathogenic bacteria prompts MTs structure solving

The rRNA methylation typically confers very-high-level resistance to aminoglycosides [MICs > 0.5 mg/ml] and until recently was confined solely to antibiotic producers. During the last decade, six novel plasmid-mediated 16S rRNA MTs have been worldwide found in pathogenic bacteria isolated from human and animal specimens [58-63]. According to their resistance patterns, those resistance genes can be divided into two groups based upon the nucleoside they methylate – Arm (aminoglycoside resistance methyltransferase) and Pam families (pan-aminoglycodise methyltransferase) [30]. Arm family members (ArmA, RmtA, RmtB, RmtC, and RmtD and RmtE) confer resistance solely to 4,6-DOS antibiotics, similarly to Kgm family MT from producers, modifying G1405 at N7 position. NpmA MT, the only member of the Pam MT family, confers resistance to combination of 4,6- and 4,5- DOS and apramycin by methylating A1408 at N1 position. The exact site of action has been experimentally confirmed for ArmA, RmtB, and NpmA [60, 64, 65].

In silico comparison, presented in Table 1, points out that although MTs from Kgm and Arm families target the same nucleoside, sequence identity at amino acid level between members of those two groups varies from 23.2-35.8%. This relatively low identity indicates that they could have a common ancestor but most likely a distant one. A huge variation of amino acid identity among members of the Kgm family, ranging from 33.2–90.5%, is also puzzling. From autoregulation point of view, it is interesting to notice that GrmA MT that lacks autoregulation but downregulate KgmB and Sgm MTs, shares with them 56.8% and 89.8% of identity, respectively, while mutually downregulated KgmB and Sgm MTs share 55.4%.

Table 1 Amino acid identities among various 16S rRNA methyltransferases.

PRODUCERS	Identity (%) of amino acid residues										
	PRODUCERS						PATHOGENS				
	Sgm	KgmB[1]	GrmA[1]	FmrO	Krm	Srm1	ArmA	RmtA	RmtB	RmtC	RmtD
Sgm							26.4	32.0	33.9	27.3	31.6
KgmB[1]	55.4						26.7	31.6	27.3	23.2	28.7
GrmA[1]	89.8	56.8					26.5	33.2	32.6	27.2	30.0
FmrO	39.2	33.2	37.1				25.5	28.5	30.5	25.4	27.2
Krm	56.5	55.3	58.0	35.4			27.5	35.8	34.4	24.6	31.4
Srm1	87.6	56.2	90.5	37.2	57.6		26.6	33.7	33.3	26.2	30.5
NbrB	60.4	85.9	61.1	34.2	57.3	59.7	27.5	32.6	29.8	25.0	29.3

Kgm family: Sgm from *M. zionensis* (accession number Q7M0R2), KgmB from *S. tenebrarius* (acc. no. AAB20100.1), GrmA from *M. echinospora* (acc. no. AAA25336.1), FmrO from *M. olivasterospora* (acc. no. BAA02451.2), Krm from *Frankia sp.* CcI3 (acc. no. YP_482456.1), Srm1 from *M. inyonensis* (acc. no. AAV28394.1), NbrB from *S. hindustanus* (acc. no. AAB95477.1); **Arm family**: ArmA from *E.coli* (acc. no. YP_724473.1), RmtA from *P. aeruginosa* (acc. no. BAC20579.1), RmtB from *S. marcescens* (acc. no. BAC81971.1), RmtC from *P. mirabilis* (acc. no. BAE48305.1) and RmtD from *P.aeruginosa* (acc. no. ABJ53409.1); [1]Anti-Sgm antibodies cross-reaction with other MTs is denoted [66-68].

Understanding of the detailed architecture of antibiotic binding sites has increased rapidly in recent years due to elucidation of high-resolution structures of antibiotic-ribosome complexes [17]. Although the first 16S rRNA resistance MT in the antibiotic-producing microorganisms has been discovered more than 20 years ago, until recently just a small number of the MTs have been partially characterized. Furthermore, knowledge on the interaction between target sites and the corresponding MTs is even more limited. Emergence and global spreading of 16S rRNA resistance MTs and their effect on usefulness of aminoglycosides gave a new significance to fundamental research related to MTs driven resistance mechanism. Very limited biochemical data on G1405 MTs were recently improved by functional probing of Sgm. These studies demonstrated existence of two structural domains [69] – smaller N-terminal domain is most likely involved in target site recognition and binding, while larger C-terminal domain is responsible for coenzyme binding and catalysis [37, 69]. Particular amino acids responsible for SAM binding, target recognition and methyl group transfer were also identified [37]. In last two years crystal structures for ArmA, RmtB and Sgm MTs have been determined [33, 70]. It has been established that MTs from pathogens are composed of two domains that structurally resemble domains in Sgm. Site-directed mutagenesis studies [37] supported by high resolution structures of ArmA and RmtB and Sgm

[33, 70] show that members of the Arm and Kgm familly MTs have identical protein fold indicating common evolutionary histories between two protein families.

Aminoglycosides are among the most commonly used broad-spectrum antibiotics in the battle against microorganisms. One of the approaches in order to circumvent raising resistance to aminoglycosides is development of MTs inhibitors. This requires in-depth understanding of MT expression and its mechanism of action, resolution of a tertiary structure of 16S rRNA MT with its cofactor, and structure of 30S-MTase complex. Taken altogether, detailed analysis of the 16S rRNA resistance MTs will be a starting point towards developing drugs that would specifically block their activity, and thus restore the therapeutic power of existing aminoglycosides.

Acknowledgements The support by Ministry of Science and Technological Development of Republic of Serbia (contract No. 143056) is gratefully acknowledged.

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