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Aminoglycoside-modifying enzymes determine the innate susceptibility to aminoglycoside antibiotics in rapidly growing mycobacteria

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Objectives: Infections caused by the rapidly growing mycobacterium (RGM) *Mycobacterium abscessus* are notoriously difficult to treat due to the innate resistance of *M. abscessus* to most clinically available antimicrobials. Aminoglycoside antibiotics (AGA) are a cornerstone of antimicrobial chemotherapy against *M. abscessus* infections, although little is known about intrinsic drug resistance mechanisms. We investigated the role of chromosomally encoded putative aminoglycoside-modifying enzymes (AME) in AGA susceptibility in *M. abscessus*.

Methods: Clinical isolates of *M. abscessus* were tested for susceptibility to a series of AGA with different substituents at positions 2', 3' and 4' of ring 1 in MIC assays. Cell-free extracts of *M. abscessus* type strain ATCC 19977 and *Mycobacterium smegmatis* strains SZ380 [*aac*(2')-Id⁺], EP10 [*aac*(2')-Id⁻] and SZ461 [*aac*(2')-Id⁺, *rrs* A1408G] were investigated for AGA acetylation activity using thin-layer chromatography (TLC). Cell-free ribosome translation assays were performed to directly study drug–target interaction.

Results: Cell-free translation assays demonstrated that ribosomes of *M. abscessus* and *M. smegmatis* show comparable susceptibility to all tested AGA. MIC assays for *M. abscessus* and *M. smegmatis*, however, consistently showed the lowest MIC values for 2'-hydroxy-AGA as compared with 2'-amino-AGA, indicating that an aminoglycoside-2'-acetyltransferase, *Aac*(2'), contributes to innate AGA susceptibility. TLC experiments confirmed enzymatic activity consistent with *Aac*(2'). Using *M. smegmatis* as a model for RGM, acetyltransferase activity was shown to be up-regulated in response to AGA-induced inhibition of protein synthesis.

Conclusions: Our findings point to AME as important determinants of AGA susceptibility in *M. abscessus*.

Keywords: *Mycobacterium abscessus*, drug resistance, amikacin, tobramycin, cystic fibrosis

Introduction

Mycobacterium abscessus is a rapidly growing mycobacterium (RGM) recognized as an important emerging human pathogen. *M. abscessus* causes serious lung infections in patients with chronic pulmonary diseases, e.g. cystic fibrosis or bronchiectasis,^{1–3} and is also associated with infections following surgical interventions⁴ or tattooing.⁵ *M. abscessus sensu lato* can be subdivided into three subtaxa, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*. Identification on a subspecies level is based on DNA sequences of *rpoB* and additional genes, including *erm*(41), which encodes a 23S rRNA methylase conferring resistance to macrolides.^{6–8} However, the taxonomy of the *M. abscessus* complex is the subject of an ongoing debate.⁹

M. abscessus sensu lato shows innate antimicrobial resistance to all major antituberculous drugs in addition to resistance to

broad-spectrum antibiotics such as β -lactams and tetracyclines.¹⁰ In consequence, infections with *M. abscessus* respond poorly to antibiotic chemotherapy.^{11,12} While no standard treatment recommendations have yet been established, current guidelines propose administration of an aminoglycoside, preferably amikacin, and a macrolide, e.g. clarithromycin or azithromycin, in combination with additional compounds, e.g. imipenem, ceftazidime, linezolid, tigecycline and/or fluoroquinolones, for *M. abscessus* pulmonary disease.¹² In addition, surgery may be required to reduce the bacterial load at the site of infection.^{11,12}

Aminoglycoside antibiotics (AGA) form a group of hydrophilic molecules, consisting of a characteristic, central aminocyclitol linked to one or more amino sugars by pseudoglycosidic bonds.¹³ Originally isolated from *Streptomyces* spp., this class of compounds has been a major therapeutic component for mycobacterial diseases since the recognition of the activity of streptomycin against

Mycobacterium tuberculosis.¹⁴ In clinically used AGA, the central 2-deoxystreptamine ring is disubstituted at positions 4 and 6 to result in the 4,6-disubstituted 2-deoxystreptamine antibiotics (Figure S1, available as Supplementary data at JAC Online). The target of AGA is the decoding A-site in the bacterial ribosome. AGA are bactericidal against most bacterial species.^{13,15} More recently, it was demonstrated that a chromosomal aminoglycoside-modifying enzyme (AME) abolishes bactericidal activity of AGA with deletion of the corresponding gene conferring bactericidal activity to AGA.¹⁶

Resistance to AGA is conferred by several mechanisms: drug modification, target mutation or alteration, reduced uptake and/or increased efflux.¹³ Chemical synthesis efforts have focused on AGA both with a view to confer protection against AME and to reduce unwanted toxic side effects.^{17–19} In bacterial organisms that carry a single copy of the 16S rRNA (*rrs*) gene, e.g. *M. abscessus* and the closely related *Mycobacterium chelonae*, point mutations in *rrs*, particularly at 16S rRNA position A1408 (*Escherichia coli* numbering), confer high-level resistance (MICs >1000 mg/L) to all 2-deoxystreptamines, including amikacin, tobramycin, gentamicin, dibekacin, kanamycin A and kanamycin B.^{20–24} In addition, AGA resistance can be conferred by target alteration via rRNA methylases.^{25,26} Three classes of AME¹³ have been described: (i) AGA acetyltransferases catalyse the acetyl-CoA-dependent *N*-acetylation of an amino group; (ii) AGA phosphotransferases transfer the γ -phosphoryl group of ATP to a hydroxyl substituent; and (iii) AGA adenyltransferases catalyse the transfer of an adenyl group to a hydroxyl substituent. In general, expression of AME is considered to be constitutive.²⁷ However, studies in *Providencia stuartii*,^{28,29} *Serratia marcescens*,³⁰ *Salmonella enterica*³¹ and *Pseudomonas aeruginosa*³² indicate that AME expression can be regulated in response to unfavourable environmental conditions, e.g. exposure to subinhibitory AGA concentrations.

The presence of AME has been reported to show little correlation with intrinsic AGA resistance in RGM.^{33–35} To study the role of AME in the intrinsic AGA resistance of *M. abscessus* in more detail, we determined: (i) MICs for a panel of AGA with defined substituents at positions 2', 3' and 4' of ring 1; (ii) acetyltransferase activity in cell-free extracts of *M. abscessus* ATCC 19977 and *Mycobacterium smegmatis* strains SZ380 [*aac*(2')-Id⁺], EP10 [*aac*(2')-Id⁻] and SZ461 [*aac*(2')-Id⁺, *rrs* A1408G]; and (iii) susceptibility of *M. abscessus* ribosomes to various AGA in cell-free translation assays.

Materials and methods

Strains

Clinical strains of *M. abscessus* subsp. *abscessus* ($n=4$), *M. abscessus* subsp. *bolletii* ($n=4$), *M. abscessus* subsp. *massiliense* ($n=3$) and *M. chelonae* ($n=5$) were isolated from patient specimens. The term '*M. abscessus* subsp. *massiliense*' is used throughout this paper to refer to strains carrying distinct deletions within the *erm*(41) gene.^{6,7} *M. smegmatis* SZ380³⁶ is a genetically constructed, single rDNA-allelic derivative of *M. smegmatis* mc² 155.³⁷ *M. smegmatis* SZ461 is a derivative of *M. smegmatis* SZ380 with a 16S rDNA mutation A1408G, conferring high-level resistance to all 2-deoxystreptamine AGA.²³ *M. smegmatis* EP10 was derived from *M. smegmatis* mc² 155 by insertional inactivation of the *aac*(2')-Id gene (GenBank accession number U72743) using an *aph*(3') cassette.³⁸

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed based on CLSI document M24-A2.³⁹ Amikacin, tobramycin, gentamicin, kanamycin A, kanamycin B

(Sigma-Aldrich, Buchs, Switzerland) and dibekacin (Anawa, Wangen, Switzerland) were dissolved in sterile water, filter sterilized, aliquotted into stock solutions of 5 g/L and stored at -80°C . Working solutions were prepared by diluting aliquots of the respective stock solutions in cation-adjusted Mueller–Hinton broth (CAMHB; Becton Dickinson, Allschwil, Switzerland) at pH 7.4. Two-fold serial dilutions of the working solutions were prepared in CAMHB using sterile 96-well microtitre plates (Greiner Bio-One, St Gallen, Switzerland). In all tests, a growth control without antibiotic and a negative control containing only CAMHB were included. All antimicrobial solutions were thawed once and leftovers were discarded after use. For inoculum preparation, colonies were taken from fresh cultures grown on LB agar using sterile cotton swabs and suspended into glass vials containing 2 mL of sterile saline. Bacterial suspensions were adjusted to a turbidity equivalent to or greater than that of a 0.5 McFarland standard and diluted in CAMHB to generate a final inoculum suspension of $1-5 \times 10^5$ cfu/mL. Each inoculum suspension was checked for purity and correct concentration of viable cells by obtaining cfu counts of suitable dilutions plated on LB agar. After inoculation, microdilution plates were covered with adhesive seals and incubated at 37°C . Growth was assessed by visual inspection of the microtitre plates after 3, 7 and 12 days of incubation.

Thin-layer chromatography (TLC)

For preparation of cell extracts, bacteria were grown to log phase in CAMHB. For preincubation, liquid cultures were exposed to subinhibitory concentrations of tobramycin (1/4 MIC) for 84 h before cell pellets were harvested, washed twice in drug-free medium and disrupted using the OmniLyse HL cell lysis kit (Claremont Bio Solutions, Upland, CA, USA). Cell lysates were centrifuged at 10000 g for 30 min at 4°C . The supernatant was passed through filter paper to remove residual lipids and subsequently centrifuged at 22000 g for 40 min at 4°C to obtain cell extracts. Total protein concentration was determined using a bicinchoninic acid assay (Micro BCA Protein Assay, Thermo Fisher Scientific, Reinach, Switzerland). Phosphate-buffered acetylation reactions (pH 7.0, total volume 50 μL) containing cell extract (standardized to 30 μg of total protein), acetyl-coenzyme A (Sigma-Aldrich; final concentration 4 mM) and one AGA per reaction (final concentration 500 mg/L) were incubated for 16 h at 37°C . For each experiment, a control reaction containing water instead of cell extract was incubated in parallel. A 20 μL aliquot of the incubated reaction mixtures was transferred to a silica gel 60 TLC plate (Merck, Darmstadt, Germany). For normalization, 10 μL of the respective antibiotic stock solution (1 g/L) was spotted on every TLC plate. A 5% KH_2PO_4 solution was used as the mobile phase. Following separation, TLC plates were air-dried overnight and stained by spraying with ninhydrin solution (5 g/L acetone). After staining, TLC plates were incubated at 60°C for 20 min and digitalized; spot intensities were quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Isolation of bacterial ribosomes and cell-free luciferase translation assays

Ribosomes and ribosome-free S100 cell extracts were purified from bacterial cell pellets as described previously using sucrose gradient (10%–40%, w/v) centrifugation.¹⁷ Concentration of 70S ribosomes was determined by absorption measurements using a standard value of 23 pmol ribosomes per A260 unit. Firefly luciferase (F-luc) mRNA was produced *in vitro* using T7 RNA polymerase (Thermo Scientific Fermentas, Reinach, Switzerland) on templates of modified plasmid pGL4.14 (Promega, Mannheim, Germany).¹⁷ A typical translation reaction with a total volume of 30 μL contained 0.25 μM 70S ribosomes, 4 μg of F-luc mRNA, 40% (v/v) of ribosome-free S100 extract, 200 μM amino acid mixture, 24 U of RNase inhibitor (Ribo Lock, Thermo Scientific Fermentas), 0.4 g/L total *E. coli* tRNA and 12 μL of commercial S30 Premix without amino acids (Promega).

After addition of serially diluted AGA, the reaction mixture was incubated at 37°C for 35 min and subsequently placed on ice. F-luc activity was quantified using the Luciferase Reporter Assay System (Promega). Luminescence was measured using a luminometer (FLx800; Bio-Tek Instruments, Luzern, Switzerland). IC₅₀ values represent the AGA concentrations that inhibit F-luc activity by 50%.

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics version 20 (IBM, Armonk, USA) and Microsoft Excel (Microsoft, Redmont, USA).

Student's *t*-test was used where applicable. The level of significance was set at *P* values of <0.05.

Results

Determination of AGA MICs

MICs were determined for a range of chemically well-defined compounds (tobramycin, kanamycin B, amikacin and kanamycin A; see Figure S1 for chemical structures) using clinical strains of *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and

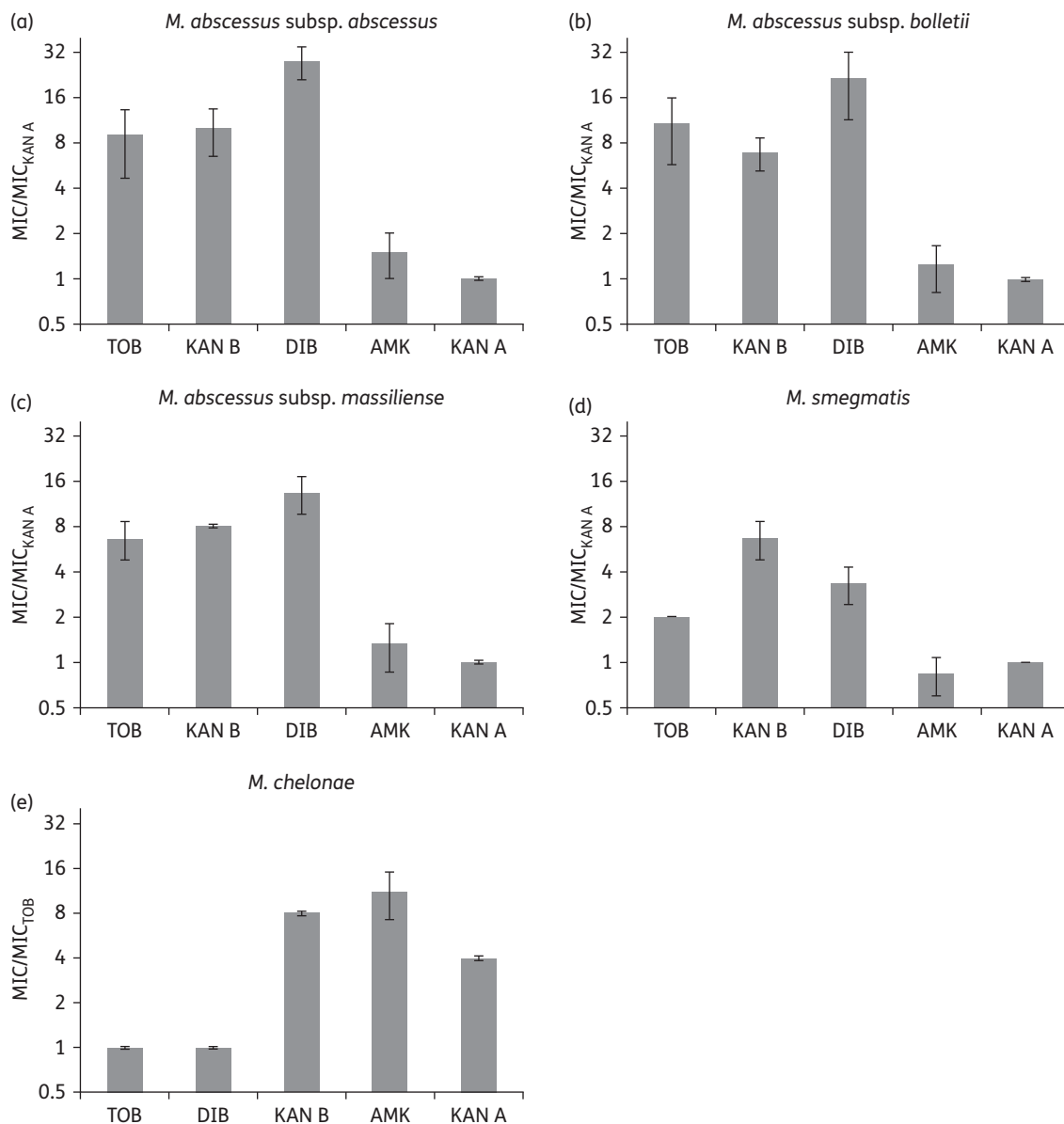
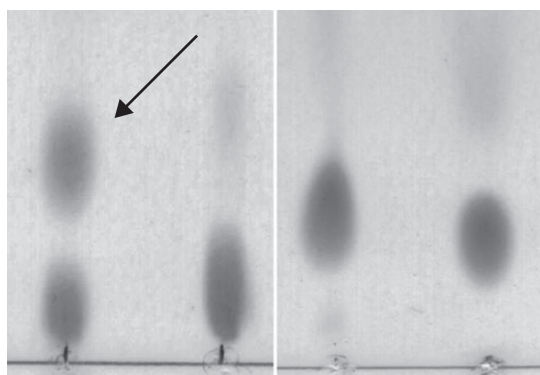


Figure 1. Average MIC ratios of selected aminoglycosides for clinical strains of RGM and *M. smegmatis* strain SZ380. (a) *M. abscessus* subsp. *abscessus* (four isolates). (b) *M. abscessus* subsp. *bolletii* (four isolates). (c) *M. abscessus* subsp. *massiliense* (three isolates). (d) *M. smegmatis* strain SZ380 (measured in triplicate). (e) *M. chelonae* (five isolates). MICs were normalized to the compound showing the lowest MIC (kanamycin A for *M. abscessus* and *M. smegmatis*, and tobramycin for *M. chelonae*). Absolute MICs of kanamycin A were: *M. abscessus* subsp. *abscessus*, one isolate 1 mg/L, two isolates 8 mg/L and one isolate 16 mg/L; *M. abscessus* subsp. *bolletii*, all isolates 8 mg/L; *M. abscessus* subsp. *massiliense*, one isolate 4 mg/L and two isolates 8 mg/L; and *M. smegmatis*, 0.5 mg/L. The absolute MIC of tobramycin for *M. chelonae* was 16 mg/L for all isolates. Error bars: ±1 SD. TOB, tobramycin; KAN B, kanamycin B; DIB, dibekacin; AMK, amikacin; KAN A, kanamycin A.



Compound	KAN B	KAN B	KAN A	KAN A
Cell extract	+	-	+	-
Water control	-	+	-	+

Figure 2. Aminoglycoside modification by *M. abscessus* cell extracts. Aminoglycoside acetylation was studied using TLC with ninhydrin staining. Shown is a representative example using cell-free extracts prepared from a liquid culture of *M. abscessus* ATCC 19977 incubated with acetyl-CoA and kanamycin B (2'-amino) or kanamycin A (2'-hydroxy). Acetylation reactions containing cell extract, acetyl-CoA and antibiotic were incubated for 16 h before they were transferred to TLC membranes. Control reactions including sterile water instead of cell extract are also shown. The spot indicative of acetylated kanamycin B is indicated by an arrow. Baseline is indicated by the horizontal line.

M. abscessus subsp. *massiliense* (Figure 1a–c). *M. smegmatis* strain SZ380 and *M. chelonae* were used as comparators (Figure 1d and e). In all subspecies of the *M. abscessus* complex, MICs of amikacin and kanamycin A were significantly lower than those of tobramycin, kanamycin B and dibekacin ($P \leq 0.05$). A single structural feature distinguishes both amikacin and kanamycin A from tobramycin, kanamycin B and dibekacin, i.e. a hydroxyl group instead of an amino group at the 2'-position (Figure S1). The MIC results of *M. abscessus* complex suggest the presence of a functional Aac(2'). In contrast to *M. abscessus*, *M. chelonae* showed the lowest MICs for compounds with a 3'-deoxy configuration (tobramycin and dibekacin) ($P \leq 0.01$), consistent with the presence of an Aph(3') in this species.²⁷

Analysis of aminoglycoside acetylation activity in *M. abscessus* and *M. smegmatis*

AGA acetylation at the 2' position was first investigated in cell extracts of *M. abscessus* ATCC 19977 using TLC. Control reactions, in which cell extracts were replaced by sterile water, were performed in parallel (see Figure 2). The 2'-amino-AGA kanamycin B and the 2'-hydroxy-AGA kanamycin A were studied for modifications. Two well-separated spots were observed for kanamycin B when incubated with cell extract of *M. abscessus* ATCC 19977. One spot corresponds to the unmodified compound while the other represents the acetylated compound. The control reaction only showed the unmodified compound. For kanamycin A, incubation with *M. abscessus* cell extract only showed a single spot, which corresponded to the unmodified compound in the control reaction. These data corroborate the MIC results and demonstrate the presence of a functionally active 2'-acetyltransferase, Aac(2'), in cell extracts of *M. abscessus* ATCC 19977.

Cell extracts of *M. smegmatis* SZ380 and *M. smegmatis* EP10, with a disrupted *aac(2')*-Id gene, were used in combination with different 2'-amino-AGA (kanamycin B and tobramycin) and 2'-hydroxy-AGA (kanamycin A and amikacin). Similar to *M. abscessus*, the 2'-amino-AGA showed spots representing acetylation in *M. smegmatis* SZ380, but not in the *aac(2')* knockout strain *M. smegmatis* EP10. In contrast, the 2'-hydroxy-AGA showed only a single spot corresponding to the unmodified compound in both *M. smegmatis* SZ380 and *M. smegmatis* EP10 (Figure 3a).

Regulation of Aac(2') activity

M. abscessus is poorly, if at all, amenable to genetic manipulation.^{40,41} We thus used the closely related rapidly growing *M. smegmatis* (for which genetic manipulation protocols are well established)²⁰ as a model to further investigate the role of Aac(2') enzymes in the activity of substrate and non-substrate AGA. In these experiments we wanted to study the regulation of Aac(2') expression. In particular, we wanted to address the question of whether these enzymes are induced by treatment with AGA. To investigate whether acetylation activity is up-regulated in response to AGA exposure, cell extracts of *M. smegmatis* SZ380 [*aac(2')*-Id⁺] and *M. smegmatis* EP10 [*aac(2')*-Id⁻], which had been preincubated with subinhibitory concentrations of tobramycin for 0 and 84 h prior to cell extract preparation, were used in TLC experiments (Figure 3b). For *M. smegmatis* SZ380, which had been preincubated with tobramycin, spots indicative of acetylated AGA were found to be increased in cell extracts by 1.7-fold when using tobramycin as a substrate and by 2.8-fold when using gentamicin as a substrate for modification. As expected, no acetylation activity was observed using cell extracts of the *aac(2')* knockout *M. smegmatis* EP10 irrespective of preincubation.

Next we addressed the mechanism that induces Aac(2') expression following treatment with AGA. In principle, two possibilities exist: induction of Aac(2') as per the chemical nature of the compound, or induction of Aac(2') expression as per the drug's effect on the bacterial ribosome. To differentiate between these two possible mechanisms we used *M. smegmatis* SZ461, a strain carrying an A1408G mutation in the 16S rRNA. This mutation confers high-level resistance to AGA with MIC levels >128 mg/L and abolishes the compounds' inhibitory activity against the bacterial ribosome.²³ The effect of the 16S rRNA A1408G resistance mutation on regulation of *aac(2')*-Id expression was studied using cell extracts of preincubated and non-preincubated *M. smegmatis* strains SZ380 [*aac(2')*-Id⁺, *rrs* WT] and SZ461 [*aac(2')*-Id⁺, *rrs* A1408G] (Figure 3c). The basal acetylation activity of Aac(2')-Id substrates using cell extracts of non-preincubated strain SZ461 was comparable to that of *M. smegmatis* SZ380 (*rrs* WT). In contrast, no increase in acetylation activity towards 2'-amino-AGA was observed for cell extracts of strain SZ461 preincubated with tobramycin. This finding indicates that up-regulation of AME expression is conferred by drug-mediated ribosomal inhibition and not triggered by the compound itself.

Susceptibility of *M. abscessus* ribosomes to aminoglycosides

Next, we studied the susceptibility to different AGA directly at the drug target level using cell-free translation assays. Purified ribosomes and ribosome-free S100 extracts were prepared from

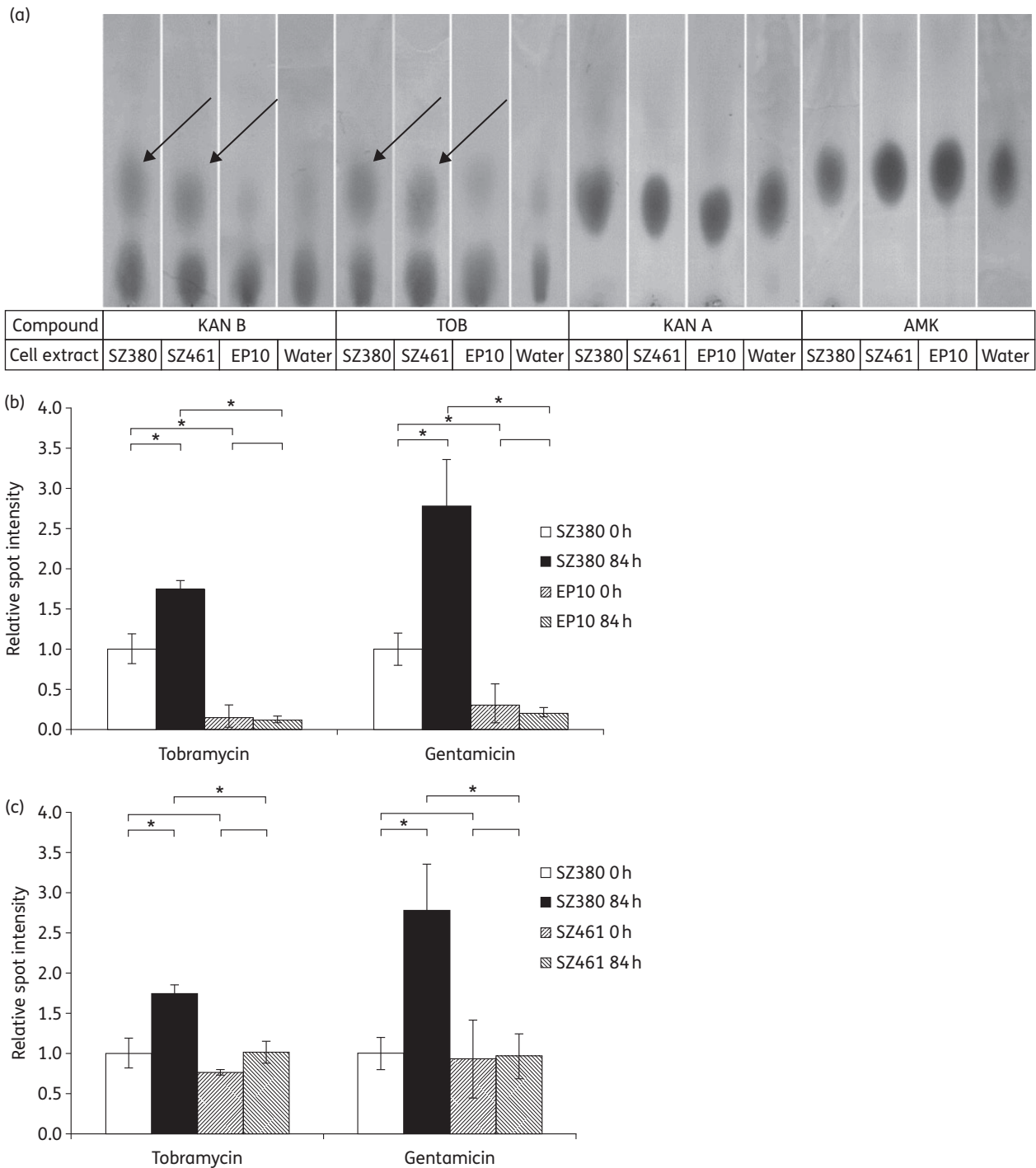


Figure 3. Aminoglycoside modification by *M. smegmatis* cell extracts. (a) Acetylation activity towards 2'-hydroxy and 2'-amino AGA was studied using TLC. Shown is a representative example using kanamycin B and tobramycin (both 2'-amino), kanamycin A and amikacin (both 2'-hydroxy) and cell extracts prepared from *M. smegmatis* strains SZ380, EP10 and SZ461. Control reactions without cell extract are also shown. Spots indicative of acetylated aminoglycosides are indicated by arrows. (b) Aminoglycoside acetyltransferase activity in cell extracts of *M. smegmatis* SZ380 (WT) and *M. smegmatis* EP10 [*aac(2)*'-Id knockout, WT *rrs*]. Cell extracts were preincubated with subinhibitory concentrations of tobramycin for 0 and 84 h prior to cell wall disruption, respectively. Shown are the relative intensities of spots indicative of acetylated aminoglycosides normalized to *M. smegmatis* SZ380 without preincubation. (c) Comparison of acetyltransferase activities between cell extracts of *M. smegmatis* SZ380 (WT) and *M. smegmatis* SZ461 [*aac(2)*'-Id WT, A1408G *rrs* mutant], which were preincubated in tobramycin for 0 and 84 h prior to cell wall disruption, respectively. Normalization was performed as in (b). Error bars: ± 1 SD. * $P < 0.05$.

Table 1. *In vitro* inhibition of protein synthesis

Purified ribosomes	S100 extract	IC ₅₀ (mg/L)				
		TOB	KAN B	DIB	AMK	KAN A
<i>M. smegmatis</i> SZ380	<i>M. smegmatis</i> SZ380	0.01	0.02	0.02	0.02	0.03
<i>M. abscessus</i> subsp. <i>abscessus</i> 500042	<i>M. abscessus</i> subsp. <i>abscessus</i> 500042	0.22	0.90	0.82	0.03	0.02
<i>M. abscessus</i> subsp. <i>massiliense</i> 186139	<i>M. abscessus</i> subsp. <i>massiliense</i> 186139	0.26	0.56	0.32	0.05	0.04
<i>M. abscessus</i> subsp. <i>abscessus</i> 500042	<i>M. smegmatis</i> SZ380	0.01	0.03	0.03	0.05	0.05
<i>M. smegmatis</i> SZ380	<i>M. abscessus</i> subsp. <i>abscessus</i> 500042	0.81	1.56	0.66	0.11	0.09

TOB, tobramycin; KAN B, kanamycin B; DIB, dibekacin; AMK, amikacin; KAN A, kanamycin A.

Translation inhibition was measured by analysis of luciferase activity following translation of F-luc template mRNA. IC₅₀ values represent the drug concentrations at which luciferase activity was reduced by 50%. Shown are the results for combinations of ribosomes and S100 preparations of two clinical *M. abscessus* complex isolates and for *M. smegmatis* strain SZ380 (all *rrs* WT).

clinical isolates of the *M. abscessus* complex and *M. smegmatis* SZ380. IC₅₀ values were determined, i.e. drug concentrations required to inhibit F-luc mRNA translation by 50%. The results of these experiments are shown in Table 1. When ribosomes and S100 extract from *M. smegmatis* SZ380 were combined, IC₅₀ values of 0.01–0.03 mg/L were determined for all AGA. When purified ribosomes and S100 extract from *M. abscessus* were combined, IC₅₀ values of 0.02–0.05 mg/L were observed for amikacin and kanamycin A. However, IC₅₀ values for tobramycin, kanamycin B and dibekacin were 0.22–0.90 mg/L.

We next assessed whether the elevated IC₅₀ values of tobramycin, kanamycin B and dibekacin in *M. abscessus* were due to resistance of *M. abscessus* ribosomes, e.g. as per ribosomal methylation, or AME enzymatic activity present in the *M. abscessus* S100 extracts. For this purpose, cross-over experiments were performed, in which ribosomes from *M. abscessus* were combined with S100 extract from *M. smegmatis* SZ380 and ribosomes from *M. smegmatis* SZ380 were combined with S100 extract from *M. abscessus*. *M. abscessus* ribosomes in combination with S100 extract from *M. smegmatis* resulted in IC₅₀ values for tobramycin, kanamycin B and dibekacin that were similar to those of amikacin and kanamycin A (0.01–0.05 mg/L). In contrast, when S100 extract from *M. abscessus* was used in combination with *M. smegmatis* ribosomes, IC₅₀ values of 0.81, 1.56 and 0.66 mg/L were observed for tobramycin, kanamycin B and dibekacin, respectively. These findings indicate that AGA susceptibility at the drug target level (the ribosome) is similar for both *M. smegmatis* and *M. abscessus* and that the increased IC₅₀ values for tobramycin, kanamycin B and dibekacin observed in *M. abscessus* resulted from pronounced enzymatic activity [consistent with Aac(2')] in the S100 extracts of *M. abscessus*.

Discussion

The purpose of this study was to investigate the role of AME in AGA susceptibility of RGM, with special emphasis on *M. abscessus*. At the MIC level, the drug susceptibility pattern of *M. abscessus* was similar to that of *M. smegmatis* and clearly different from that of the closely related *M. chelonae*. MICs of the 2'-hydroxy AGA kanamycin A and amikacin were significantly lower than MICs of the 2'-amino-AGA tobramycin, kanamycin B and dibekacin (Figure 1). Based on these observations, we hypothesized that a homologue of Aac(2'), capable of acetylating 2'-amino-2-deoxystreptamines,

contributes to the innate AGA susceptibility of *M. abscessus*. This hypothesis is supported by the presence of an *aac*(2') homologue (GenBank accession number MAB_4395) in the *M. abscessus* chromosome.⁴² Indeed, acetylation studies indicated the presence of a functional Aac(2') acetyltransferase in *M. abscessus* (Figure 2). This finding is of clinical importance as it was recently shown that expression of AME in RGM can significantly affect both MICs and the bactericidal activity of AGA.¹⁶

Little is known regarding the mechanisms that regulate the expression of AME. In general, these enzymes are thought to be expressed constitutively.²⁷ To study the regulation of AME expression in RGM in more detail, we took advantage of *M. smegmatis*, as an *aac*(2') knockout mutant was available. Baseline acetylation activity was abolished in the *aac*(2')-Id knockout strain *M. smegmatis* EP10 as compared with *M. smegmatis* SZ380, indicating that Aac(2')-Id is the only aminoglycoside-acetylating enzyme present in *M. smegmatis* (Figure 3b). *M. smegmatis* SZ380 showed increased enzymatic activity when preincubated with subinhibitory AGA concentrations before preparation of cell extracts (Figure 3b). However, no such increase in acetylation activity was observed in *M. smegmatis* SZ461, which carries the AGA resistance mutation A1408G in the 16S rRNA gene (Figure 3c). This finding indicates that the basal expression of *aac*(2')-Id is constitutive and comparable in *rrs* WT and *rrs* A1408G mutant strains. However, basal Aac(2')-Id activity is increased upon exposure to AGA. This AGA-induced increase in AME activity was only observed in the *rrs* WT strain, indicating that drug-mediated inhibition of protein synthesis is required to up-regulate AME expression.

Our findings in *M. smegmatis* on drug-induced 'ribosomal stress' and up-regulation of genes conferring resistance to compounds that act on the bacterial ribosome are not without precedent. Nash *et al.*⁶ demonstrated that mRNA transcript levels of *erm*(41), which confers macrolide resistance by methylation of position A2058 in the 23S rRNA (*rml*) gene, increase in response to macrolide exposure. However, no such increase was observed in strains carrying a mutated position 2058, which confers resistance to MLS_B antibiotics.⁶ In addition, expression of the transcriptional regulator *whiB7* is up-regulated in response to treatment with the ribosomal inhibitors kanamycin, streptomycin, erythromycin and tetracycline.^{43,44} Among the *whiB7*-dependent transcripts are the *tap* multidrug-efflux transporter, which confers low-level resistance to AGA and tetracyclines,^{45,46} and the *erm*(37) ribosomal

methyltransferase, conferring macrolide resistance.⁴⁷ Most interestingly, in *E. coli*, formation of biofilms has been identified as a stress response, which is induced upon exposure to various translation inhibitors. This stress response is mediated via a regulatory mechanism involving the bacterial signalling molecules guanosine-bis 3',5'(diphosphate) and bis-(3'-5')-cyclic di-GMP.⁴⁸

In conclusion, this study provides evidence that the innate AGA susceptibility of *M. abscessus* is determined by a functional Aac(2') acetyltransferase. Our findings also indicate that regulation of chromosomally encoded AME in RGM involves constitutive and inducible elements. The inducible regulation appears to be mediated via the effect of AGA on the bacterial ribosome ('ribosomal stress'). It most likely involves a more general and complex signal transduction pathway consistent with an adaptive response.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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