Antibiotic therapy of infections caused by the emerging pathogen *Mycobacterium abscessus* is challenging due to the organism’s natural resistance toward most clinically available antimicrobials. We investigated the bactericidal activity of antibiotics commonly administered in *M. abscessus* infections in order to better understand the poor therapeutic outcome. Time–kill curves were generated for clinical *M. abscessus* isolates, *Mycobacterium smegmatis*, and *Escherichia coli* by using antibiotics commonly categorized as bactericidal (amikacin and moxifloxacin) or bacteriostatic (tigecycline and linezolid). In addition, the impact of aminoglycoside-modifying enzymes on the mode of action of substrate and nonsubstrate aminoglycosides was studied by using *M. smegmatis* as a model organism. While amikacin and moxifloxacin were bactericidal against *E. coli*, none of the tested compounds showed bactericidal activity against *M. abscessus*. Further mechanistic investigations of the mode of action of aminoglycosides in *M. smegmatis* revealed that the bactericidal activity of tobramycin and gentamicin was restored by disruption of the chromosomal *aac(2)* \(_\text{R}\) gene in the mycobacterial genome. The lack of bactericidal antibiotics in currently recommended treatment regimens provides a reasonable explanation for the poor therapeutic outcome in *M. abscessus* infection. Our findings suggest that chromosomally encoded drug-modifying enzymes play an important role in the lack of aminoglycoside bactericidal activity against rapidly growing mycobacteria.

*Mycobacterium abscessus* is a rapidly growing mycobacterium that has been associated with a variety of conditions, including chronic lung disease in patients with cystic fibrosis (CF) or bronchiectasis (1, 2). The major threat posed by this pathogen is not the least due to its high natural resistance to a broad range of antibiotics, which is of particular concern in public health institutions (3, 4).

Clinical studies on therapeutic outcome are rare, and yet no standardized antibiotic regimens leading to cure rates of >30 to 50% have been established (5, 6). Lengthy courses of antimicrobial chemotherapy, often complemented by surgery, are required to lower the burden of disease, especially in patients suffering from pulmonary manifestations (5). Antibiotic treatment of *M. abscessus* infection is guided by *in vitro* antimicrobial susceptibility testing (AST) and usually includes a macrolide, like clarithromycin (CLR) or azithromycin (AZM), amikacin (AMK), and various combinations of moxifloxacin (MOX), linezolid (LIN), tigecycline (TIG), imipenem (IMI), or cefoxitin (CEF) (1, 2). Determination of MICs by the broth microdilution method is the standard AST procedure, and a corresponding guideline has been released by the Clinical and Laboratory Standards Institute (CLSI) (7). However, little is known about basic time- and dose-dependent inhibition kinetics of individual antimicrobials against clinical *M. abscessus* isolates (8).

Antibacterial compounds are traditionally categorized into two classes: bacteriostatic and bactericidal antimicrobials. In order to assign a compound–pathogen combination to one of these two classes *in vitro*, two parameters are useful (9, 10). The MIC is the lowest drug concentration that prevents a bacterial suspension of \(1 \times 10^5\) to \(5 \times 10^5\) CFU/ml from becoming turbid after a defined incubation period (which depends on the generation time of the studied organism). The minimal bactericidal concentration (MBC) actively reduces the bacterial input from \(1 \times 10^5\) to \(5 \times 10^5\) CFU/ml to at least \(1 \times 10^2\) to \(5 \times 10^2\) CFU/ml (i.e., by more than 99.9%) following a defined incubation period (7, 9–11). For slow-growing mycobacteria, this threshold has been lowered to 99.0% by some authors (10). For bactericidal antibiotics, the MBC is typically \(\leq 4\) times the MIC. In contrast, the MBC/MIC ratio is usually >4 for bacteriostatic drugs (9). Macrolides like CLR and AZM, oxazolidinones (LIN), tetracyclines and the glycyclcline tigecycline (TIG) are considered to act as bacteriostatic antibiotics. In contrast, aminoglycoside (AG) antibiotics and fluoroquinolones are considered to exhibit bactericidal activity against most species (9).

In this study, we investigated the inhibition kinetics of AMK, MOX, LIN, and TIG against *M. abscessus* to better understand the poor correlation between AST results and treatment outcome. As *M. abscessus* is poorly, if at all, amenable to genetic manipulation (12, 13), we used *Mycobacterium smegmatis* as a model organism to investigate the mode of action of aminoglycosides in rapidly growing mycobacteria.

**MATERIALS AND METHODS**

**Strains.** *Escherichia coli* laboratory strain AS19 (14), *M. smegmatis* strain mc\(^c\) 155 (15), *M. smegmatis* strain EP10 (16) [a derivative of *M. smegmatis* mc\(^c\) 155 with a disrupted chromosomal aac(2)\(_\text{R}\)-Id gene]. *M. abscessus* subsp. *abscessus* (clinical strains 179363/08, 500043/08, and 500042/08), *M. abscessus* subsp. *bolletii* (clinical strains 179709/08, 183177/08, and 181739/08), and *M. abscessus* subsp. *massiliense* (clinical strains 177217/10, 186139/07, and 500044/09) were used in this study. The clinical strains were isolated from mostly respiratory specimens from CF patients. While it has recently been proposed to combine the two subspecies *bolletii* and *massiliense* into *M. abscessus* subsp. *bolletii* comb. nov. on the basis of low
sequence divergence (17), the previous nomenclature will be used throughout this article (18). Assignment of the clinical isolates to subspecies level was based on 16S rRNA gene (rrs), rpoB, and erm(41) sequences (19–23).

AST. Antibiotic susceptibility testing (AST) was performed based on CLSI document M24-A2 (7). In brief, AMK, tobramycin (TOB), gentamicin (GEN), MOX, LIN, and TIG (Sigma-Aldrich, Buchs, Switzerland) were dissolved according to the manufacturer’s recommendations, filter sterilized, aliquoted into stock solutions of 5 g/liter, and stored at −80°C. Working solutions of all compounds were prepared by diluting thawed aliquots of the respective stock solutions in cation-adjusted Müller-Hinton broth (CAMHB) (Becton, Dickinson, Allschwil, Switzerland) at pH 7.4 to a final concentration of 256 mg/liter. Two-fold serial dilutions of the working solutions were prepared using CAMHB in sterile 96-well microtiter plates (Greiner Bio-One, St. Gallen, Switzerland). For all antibiotics, a growth control lacking antibiotic and a negative control containing only CAMHB were included. For inoculum preparation, colonies were transferred from fresh pure cultures grown on LB agar into glass vials containing 2 ml of saline using sterile cotton swabs. Bacterial suspensions were adjusted to a McFarland standard of 0.5 or higher and diluted in CAMHB to generate a final inoculum suspension of 1 × 10^5 to 5 × 10^5 CFU/ml. Each solution was checked for purity and the correct concentration by obtaining CFU counts of suitable dilutions plated on LB agar. Inoculated microdilution plates were covered with adhesive seals and incubated at 37°C for 3 days before growth was assessed by visual inspection.

Determination of concentration- and time-dependent bactericidal activity. Bacterial suspensions from strains grown on LB agar plates were prepared in LB broth, adjusted to a McFarland standard of 0.5, and diluted 100-fold. Twenty-milliliter aliquots of these suspensions were incubated with shaking (120 rpm) at 37°C. Each aliquot contained AMK, TOB, GEN, MOX, TIG, or LIN at a final concentration of 1, 2, 4, 8, 16, or 32 times the respective MIC. As a control, aliquots containing no antimicrobials were incubated. Following defined time periods, a volume of 400 µl was removed from each liquid culture using a sterile pipette, and 10-fold serial dilutions were prepared in LB broth. One hundred microliters of these dilutions were plated on LB agar plates and incubated at 37°C to determine CFU counts. Mean CFU counts are expressed as relative percentages compared to the CFU counts observed after 0 h of incubation and plotted against time.

FIG 1 Concentration- and time-dependent activity of 4 antimicrobials against E. coli strain AS19. Liquid cultures (2.0 × 10^5 to 3.6 × 10^5 CFU/ml) were exposed to linezolid (A), tigecycline (B), amikacin (C), and moxifloxacin (D) at 2-fold increasing drug concentrations (from 1× to 32× the MIC). Cell suspensions were plated on drug-free media for CFU determination after 0, 30, 60, and 120 min of incubation at 37°C.
RESULTS AND DISCUSSION

Assessment of antibiotic time-kill kinetics in E. coli. LIN and TIG showed little or no time- and dose-dependent killing in quantitative cultures of E. coli strain AS19 obtained by plating suitable dilutions of standardized inocula exposed to various drug concentrations (Fig. 1). While the highest TIG concentration tested (32 mg/liter [32× the MIC]) reduced the number of viable bacteria by 99.0% after 120 min, none of the TIG and LIN concentrations tested was sufficient to kill 99.9% of the bacteria at any time point (30, 60 and 120 min of incubation) (Table 1). In contrast, AMK and MOX showed a dose- and time-dependent reduction of CFU (Fig. 1). Concentrations of 16× to 32× the respective MICs were required to kill ≥99.9% of cells after 30 min. After 120 min of incubation, i.e., after roughly 4 replication circles (E. coli generation time of 20 to 30 min [24]), drug concentrations of 4× to 8× the respective MICs were sufficient to kill ≥99.9% of the bacterial

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/liter)</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
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<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>1</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Amikacin</td>
<td>4</td>
<td>64</td>
<td>32</td>
<td>8</td>
<td>64</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.04</td>
<td>0.64</td>
<td>0.32</td>
<td>0.08</td>
<td>1.28</td>
<td>0.64</td>
<td>0.32</td>
</tr>
</tbody>
</table>

FIG 2 Concentration- and time-dependent activity of 4 antimicrobials against M. abscessus subsp. abscessus clinical isolate 500042/08 (rrs and rrl wild type). Liquid cultures (10.4 × 10^5 to 18.2 × 10^5 CFU/ml) were exposed to linezolid (A), tigecycline (B), amikacin (C), and moxifloxacin (D) at 2-fold increasing drug concentrations (from 1× to 32× the MIC). Cell suspensions were plated on drug-free media for CFU determination after 0, 6, 12, and 24 h of incubation at 37°C.
### TABLE 2
Concentration-dependent bactericidal effect over time in *M. abscessus* and *M. smegmatis*

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Antibiotic</th>
<th>MIC (mg/liter)</th>
<th>Lowest drug concn (mg/liter) resulting in:</th>
<th>Concentration (mg/liter)</th>
<th>Time-Kill Curves of <em>M. abscessus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. abscessus</em> subsp. <em>abscessus</em></td>
<td>5000 42/08</td>
<td>Amikacin</td>
<td>8</td>
<td>0.5</td>
<td>0.25</td>
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<tr>
<td></td>
<td></td>
<td>Moxifloxacin</td>
<td>16</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Linezolid</td>
<td>8</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tigecycline</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>M. abscessus</em> subsp. <em>bolletii</em></td>
<td>1797 09/08</td>
<td>Amikacin</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Moxifloxacin</td>
<td>16</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Linezolid</td>
<td>8</td>
<td>0.5</td>
<td>2</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Tigecycline</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
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</tr>
<tr>
<td><em>M. abscessus</em> subsp. <em>massiliense</em></td>
<td>1772 17/10</td>
<td>Amikacin</td>
<td>8</td>
<td>0.5</td>
<td>2</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Moxifloxacin</td>
<td>32</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Linezolid</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tigecycline</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em> mc2155</td>
<td></td>
<td>Amikacin</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moxifloxacin</td>
<td>0.125</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Linezolid</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tigecycline</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td></td>
<td>Gentamicin</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobramycin</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Note: MIC (minimal inhibitory concentration) is the lowest concentration at which growth is inhibited. Time-Kill Curves reflect the percentage of killing over time.
cells (Table 1). Thus, in accordance with the prevailing paradigm, exposure to LIN and TIG mainly inhibited proliferation, indicating bacteriostatic activity, while exposure to AMK and MOX readily killed \textit{E. coli}, indicating bactericidal activity.

**Bactericidal and bacteriostatic activities in \textit{M. abscessus}.**

Time-kill curves were generated for \textit{M. abscessus} subsp. \textit{abscessus} clinical isolate 500042/08 (Fig. 2). As per the generation time of \textit{M. abscessus} (4 to 5 h for \textit{M. abscessus} [25] versus 20 to 30 min for \textit{E. coli} [24]), the time points at which aliquots of the liquid cultures were plated for CFU determination were adjusted to 6, 12, and 24 h. As expected, LIN and TIG showed no bactericidal activity (Fig. 2). None of the concentrations tested (up to 32\texttimes \text{MIC}) led to a significant reduction in CFU at any time point (Table 2).

Surprisingly, AMK and MOX showed no time- and dose-dependent killing. Similar to LIN and TIG, none of the concentrations tested achieved a significant reduction in CFU (Fig. 2 and Table 2).

Time-kill curves for AMK were generated against additional clinical strains of \textit{M. abscessus} subsp. \textit{abscessus}, \textit{M. abscessus} subsp. \textit{bolletii}, and \textit{M. abscessus} subsp. \textit{massiliense} to further substantiate the observation of a lack of aminoglycoside bactericidal activity (Fig. 3). DNA sequencing was used to confirm that the strains carried wild-type \textit{rrs} and \textit{rrl} genes. Mutations in these genes are associated with resistance to aminoglycosides and macrolides (26–28). For none of the \textit{M. abscessus} subspecies did exposure to AMK at concentrations of up to 32\texttimes \text{MIC} result in a reduction of viable bacteria of \( \geq 99.0\% \) at any of the time points studied (Table 2).

The chromosome of \textit{M. abscessus} subsp. \textit{abscessus} (29) contains a large number of open reading frames (ORFs), which code for homologs of aminoglycoside (AG)-modifying enzymes (AME), among them at least 12 AG phosphotransferases, a single representative of the 2\textsuperscript{\textsuperscript{-}}-N-acetyltransferase family, and a putative 6\textsuperscript{\textsuperscript{-}}-
N-acetyltransferase, which may be capable of modifying AMK (30). Of note, a 6'-N-acetyltransferase has also been related to modification of and resistance to fluoroquinolones (31, 32). Similar to what has previously been observed for nonmycobacterial pathogens (e.g., Gram-positive cocci containing a 3'-phospho-transferase or a 2'/H11033-phosphotransferase-6'-acetyltransferase [33]), we hypothesized that the presence of AME may not only contribute to the relatively high AG MICs but may also abolish the bactericidal activity of these compounds.

**Disruption of aac(2') restores bactericidal activity in M. smegmatis**. M. abscessus is poorly, if at all, amenable to genetic manipulation (12, 13). Therefore, we used the related rapidly growing species M. smegmatis as a model to investigate the role of AME in the bactericidal activity of substrate and nonsubstrate aminoglycosides. M. smegmatis expresses the chromosomally encoded 2'-N-acetyltransferase AAC(2')-Id, and a knockout mutant (strain EP10) was constructed previously (16). AAC(2') acetylates the amino group that is present at the 2' position of some AGs, including gentamicin (GEN) and tobramycin (TOB). AMK is not a substrate of AAC(2') due to the presence of a hydroxyl group at the 2' position (16).

Time-kill curves were generated for LIN, TIG, AMK, and MOX by using the wild-type strain M. smegmatis mc² 155 (Fig. 4, Table 2). MBCs for LIN and TIG (LIN, >64 mg/liter; TIG, >16 mg/liter) (Table 2) were greater than 32× the respective MICs (LIN, 2 mg/liter; TIG, 0.5 mg/liter) after both 6 and 12 h of incubation. In contrast, AMK and MOX demonstrated bactericidal activity (MBCs, 2 mg/liter and 1 mg/liter, respectively, after 12 h of incubation, corresponding to 4× and 8× the MIC) (Table 2).

Next, we compared time-kill kinetics for three different AG antibiotics (AMK, TOB, and GEN) in M. smegmatis mc² 155 and in the aac(2')-Id knockout strain M. smegmatis EP10. After 12 h of incubation, the MBC/MIC ratios were 4 for AMK in M. smegmatis mc² 155 and 2 in M. smegmatis EP10, showing that AMK has bactericidal activity in both strains. In contrast, the MBC/MIC ratios of both GEN and TOB were 16 in the wild type but ≤4 in the

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**FIG 4** Concentration- and time-dependent activity of selected antimicrobials against M. smegmatis mc² 155. Liquid cultures (5.6 × 10⁵ to 14.2 × 10⁵ CFU/ml) were exposed to linezolid (A), tigecycline (B), amikacin (C), and moxifloxacin (D) at 2-fold increasing drug concentrations (from 1× to 32× the MIC). Bacterial suspensions were plated on drug-free media for CFU determination after 0, 6, and 12 h of incubation at 37°C.
FIG 5 Time-kill kinetics of aminoglycosides against *M. smegmatis* mc² 155 (A, C, and E) and *M. smegmatis* EP10 (B, D, and F). Liquid cultures (9.2 × 10⁵ to 16.5 × 10⁵ CFU/ml) were exposed to 2-fold increasing drug concentrations of amikacin (A and B), tobramycin (C and D), and gentamicin (E and F). Bacterial suspensions were plated on drug-free media for CFU determination after 0, 6, and 12 h of incubation at 37°C.
aac(2')-IId knockout mutant (Table 2), indicating that GEN and TOB only have bacteriostatic activity in the presence of AAC(2')-Id, which accepts both compounds as a substrate, while disruption of the aac(2')-IId gene led to a restoration of bactericidal activity. This is also reflected at CFU level as exposure to TOB or GEN led to a significantly higher reduction of CFU in the aac(2') knockout strain (i.e., roughly 100- and 1,000-fold compared to M. smegmatis mc² 155 at a drug concentration of 1 mg/liter following 12 h of incubation) (Fig. 5). We conclude that while the bactericidal activity of AMK, which is not a substrate of AAC(2')-IId, was not affected by inactivation of aac(2')-Id, the bactericidal activity of GEN and TOB is abolished in the presence of the enzyme and is restored by disruption of aac(2')-IId. Presumably, expression of AAC(2')-IId leads to an equilibrium between acetylated and non-acetylated substrate compounds, lowering the intracellular concentration of the active, nonacetylated drug. Our findings demonstrate that while the contribution of AAC(2')-Id to the AG resistance phenotype may not be immediately obvious by comparative MIC analysis of substrate (TOB and GEN) and nonsubstrate (AMK) antibiotics, it plays an important role in abolishing bactericidal activity of substrate compounds.

Taken together, our findings demonstrate a clear lack of aminoglycoside bactericidal activity in M. abscessus (Fig. 2 and 3). Based on our investigations in M. smegmatis, we hypothesize that this finding is most likely due to the presence of functional AME, as the chromosome of M. abscessus contains several genes encoding putative AG acetyl- and phosphotransferases (29). The data presented in this study demonstrate that none of the commonly administered antimicrobials exerts bactericidal activity in M. abscessus sensu lato. This effect is likely to be even more pronounced under in vivo conditions, not least due to fluctuating drug concentrations. The observation that in M. abscessus infections none of the commonly administered first line drugs exerts bactericidal activity helps to explain the high relapse rates and the poor treatment outcome for affected patients. Furthermore, it points to the need for novel therapeutic options before a significant improvement in clinical outcome can be expected.

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REFERENCES


7. Acknowledgments


