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Intrinsic rifamycin resistance of *Mycobacterium abscessus* is mediated by ADP-ribosyltransferase MAB_0591

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Objectives: Rifampicin, a potent first-line TB drug of the rifamycin group, shows only little activity against the emerging pathogen *Mycobacterium abscessus*. Reportedly, bacterial resistance to rifampicin is associated with polymorphisms in the target gene *rpoB* or the presence of enzymes that modify and thereby inactivate rifampicin. The aim of this study was to investigate the role of the *MAB_0591* (arr_{Mab})-encoded rifampicin ADP-ribosyltransferase (Arr_*Mab*) in innate high-level rifampicin resistance in *M. abscessus*.

Methods: Recombinant *Escherichia coli* and *Mycobacterium tuberculosis* strains expressing *MAB_0591* were generated, as was an *M. abscessus* deletion mutant deficient for *MAB_0591*. MIC assays were used to study susceptibility to rifampicin and C25 carbamate-modified rifamycin derivatives.

Results: Heterologous expression of *MAB_0591* conferred rifampicin resistance to *E. coli* and *M. tuberculosis*. Rifamycin MIC values were consistently lower for the *M. abscessus* Δarr_{Mab} mutant as compared with the *M. abscessus* ATCC 19977 parental type strain. The rifamycin WT phenotype was restored after complementation of the *M. abscessus* Δarr_{Mab} mutant with arr_{Mab}. Further MIC data demonstrated that a C25 modification increases rifamycin activity in WT *M. abscessus*. However, MIC studies in the *M. abscessus* Δarr_{Mab} mutant suggest that C25 modified rifamycins are still subject to modification by Arr_*Mab*.

Conclusions: Our findings identify Arr_*Mab* as the major innate rifamycin resistance determinant of *M. abscessus*. Our data also indicate that Arr_*Mab*-mediated rifamycin resistance in *M. abscessus* can only in part be overcome by C25 carbamate modification.

Introduction

Mycobacterium abscessus, an environmental saprophyte, is one of the most pathogenic and drug-resistant organisms among rapidly growing mycobacteria (RGM).[1,2] It accounts for ∼80% of all lung infections due to RGM, particularly exacerbations of chronic lung disease in patients with cystic fibrosis or bronchiectasis. *M. abscessus* is also responsible for skin or soft tissue infections, usually following trauma, plastic surgery or aesthetic procedures (tattooing and body piercing).[3–11] Currently, there is no reliable antibiotic regimen for the treatment of infections with *M. abscessus*, as this bacterium demonstrates a high level of intrinsic and acquired resistance to commonly administered antibiotics.[2,3,7,11]

Rifampicin is a major chemotherapeutic agent of the rifamycin group.[12,13] The rifampicin mechanism of action is based on its ability to inhibit transcription by binding with high affinity to the *rpoB*-encoded β-subunit (RpoB) of the DNA-dependent RNA polymerase of prokaryotes.[14,15] Bacterial rifampicin resistance is mostly attributed to alterations in *rpoB*, responsible for decreased affinity of the RpoB for rifampicin.[15–18] Less frequent mechanisms of rifampicin resistance include limited membrane permeability to rifampicin and enzymatic inactivation of rifampicin through modification processes.[19–24]

Rifampicin resistance mutations were originally identified and extensively studied in *Escherichia coli* and are mapped in four distinct sequence clusters within the *rpoB* gene, known as the N-terminal cluster (N) and clusters I, II and III.[25–30] It has subsequently been shown that the majority of rifampicin resistance mutations occur within an 81 bp region of cluster I, denoted as the rifampicin resistance-determining region, across all bacterial species.[28,31–33] In *Mycobacterium tuberculosis*, high-level clinically acquired rifampicin resistance is almost always conferred by mutations in *rpoB*.[34] In contrast, *Mycobacterium smegmatis*, which is naturally resistant to rifampicin, has rifampicin ADP-ribosyltransferase activity conferring innate rifampicin resistance.[22,35,36] Combrink *et al.*[37]

delineated that the mechanism of Arr_*Msm*-mediated rifampicin inactivation can be overcome by a series of 3-morpholino rifamycins in which the C25 acetate group of the rifamycin core was replaced by a bulky carbamate group. The C25 carbamate prevents ribosylation of the adjacent C23 alcohol catalysed by Arr_*Msm* and thus C25 rifamycins exhibit improved antimicrobial activity compared with rifampicin against *M. smegmatis*.[37]

WGS revealed that *M. abscessus* carries a putative ADP-ribosyltransferase (Arr_*Mab*), encoded by *MAB_0591* (arr_{Mab}).[11,38] To date, no experimental data have defined the exact function of *MAB_0591* nor confirmed its association with *M. abscessus* innate high-level rifampicin resistance.

We here used heterologous expression of *MAB_0591* and targeted deletion of *MAB_0591* in *M. abscessus* to identify Arr_*Mab* as the major relevant rifampicin resistance determinant in the type strain *M. abscessus* ATCC 19977. In addition, our finding that Arr_*Mab*-mediated rifampicin resistance in *M. abscessus* can hardly be overcome by rifamycin C25 carbamate modification has important consequences for the development of new rifamycin derivatives active against *M. abscessus*.

Materials and methods

RpoB amino acid sequence alignment

The RpoB sequences of *E. coli* K-12, *M. tuberculosis* H37Rv, *M. smegmatis* mc^2155 and *M. abscessus* ATCC 19977 were collected from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/protein/). Multiple sequence alignment was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Bacterial strains and growing conditions

(i) *E. coli* strains were cultivated in LB medium at 37 °C overnight. Ampicillin was added to the medium at a final concentration of 120 mg/L when necessary. For all cloning steps, *E. coli* XL1-Blue or *E. coli* MC1061 were used, whereas the *E. coli-gfp* control strain and *E. coli-*arr_{Mab} testing strain were used for MIC assays. (ii) *M. abscessus* strains were grown in Middlebrook 7H9 liquid medium or on LB agar plates at 37 °C for 5 days. When needed, apramycin was added at a final concentration of 50 mg/L. The *M. abscessus* ATCC 19977 type strain, *M. abscessus* Δarr_{Mab} mutant strain, *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant strain and *M. abscessus* Δarr_{Mab} pMV361-*aac(3)IV* vector backbone control strain were used throughout this study. (iii) The *M. smegmatis* mc^2155 type strain was grown on LB agar plates at 37 °C for 3 days. (iv) *M. tuberculosis* strains were cultivated in Middlebrook 7H9 liquid medium or on Middlebrook 7H10 agar plates at 37 °C for 2–4 weeks and apramycin was added at a final concentration of 50 mg/L when necessary. The *M. tuberculosis* H37Rv type strain, Mtb-*aac(3)IV* control strain and Mtb-*aac(3)IV-*arr_{Mab} testing strain were used in this study.

Antibiotics

Ampicillin, apramycin, amikacin, isoniazid, rifampicin, rifapentine and rifaximin were bought from Sigma–Aldrich, Switzerland. The following C25 modified rifamycin derivatives were synthesized according to the literature protocol:[37] (i) 5f, 25-*O*-desacetyl-(4-methoxybenzylaminocarbonyl) 3-morpholino rifamycin S; (ii) 5k, 25-*O*-desacetyl-{C-[3-(2-methoxy-phenyl)-isoxazol-5-yl]-methylaminocarbonyl} 3-morpholino rifamycin S; and (iii) 5l, 25-*O*-desacetyl-[C-(3-pyridin-2-yl-isoxazol-5-yl)-methylaminocarbonyl] 3-morpholino rifamycin S. The identity of the compounds was confirmed by NMR and MS analysis. Purity of each compound was >97% as analysed by HPLC. Compounds were dissolved in H_2O or DMSO according to the manufacturer's recommendations, were filter sterilized, aliquotted into stock solutions of 5–50 g/L and finally stored at −20 °C.

Expression of MAB_0591 in E. coli

MAB_0591 was amplified from *M. abscessus* ATCC 19977 genomic DNA by Phusion High-Fidelity DNA Polymerase PCR (5′-ATATATGCTCTTCTA GTACGATGCCCAACTTTTTGA-3′ and 5′-TATATAGCTCTTCATGCGTCA TAGATGACCGCGTTTCC-3′). Following initial cloning into the pINIT vector, the sequence-verified *MAB_0591* amplicon was inserted downstream of the arabinose-inducible pBAD promoter into the multicopy expression vector pBXNH3 via a fragment exchange cloning system.[39] The resulting *E. coli* MC1061 pBXNH3-arr_{Mab} testing strain is referred to as *E. coli-*arr_{Mab}. As a control, *gfp* was amplified from the pOLYG-*gfp-hyg* vector (5′-ATATATGCTCTTCTAGTATCTCGAAGGGCGAGGAGCT-3′ and 5′-TATATA GCTCTTCATGCCTTGTACAGCTCGTCCATGCCG-3′) and cloned directly into the pBXNH3 expression vector. The resulting *E. coli* MC1061 pBXNH3-*gfp* control strain expressing *gfp* is referred to as *E. coli-gfp*. Gene expression of *MAB_0591* and *gfp* was conducted by induction of 30 mL bacterial cultures (OD_{600} = 0.85–1) with L-arabinose at a final concentration of 0.2% v/v, at 37 °C for 4.5 h.

Expression of MAB_0591 in M. tuberculosis

MAB_0591 (including its native promoter) was PCR amplified from *M. abscessus* ATCC 19977 genomic DNA using KpnI-linker modified primers 5′-AGGGTACCCGGATATGTGCAGCGGCATG-3′ and 5′-GAGGTACCCACCGAAG CACTGAAGGTGC-3′ and cloned into the KpnI site of the pMV361-*aac(3)IV* vector to result in the pMV361-*aac(3)IV-MAB_0591* complementing vector. The control backbone vector [pMV361-*aac(3)IV*] and the complementing vector [pMV361-*aac(3)IV-MAB_0591*] were transformed into the electrocompetent *M. tuberculosis* H37Rv type strain, as previously described for *Mycobacterium bovis* BCG.[40] Briefly, 400 μL of *M. tuberculosis* H37Rv competent cells were mixed with 1 μg of supercoiled plasmid DNA and electroporated in a Bio-Rad Gene Pulser II (settings: 2.5 kV, 1000 Ohms and 25 μF). Following electroporation, cells were resuspended in 4 mL of 7H9-OADC-Tween 80 and incubated for 20 h at 37 °C. Appropriate dilutions were plated on selective agar and after 3 weeks of incubation, single colonies were picked, restreaked and grown in liquid broth when necessary. The Mtb-*aac(3)IV* control strain and the Mtb-*aac(3)IV-*arr_{Mab} testing strain were obtained by positive selection on 7H10 plates containing apramycin. The presence of the *aac(3)IV* and/or the *MAB_0591* gene(s) in these strains was confirmed by colony PCR.

Deletion of MAB_0591 in M. abscessus

An 1.3 kbp PscI/NotI fragment from position 591427 to 592720 (5′arr_{Mab} flanking sequence) and an 1.3 kbp NotI/XbaI fragment from position 593015 to 594322 (3′arr_{Mab} flanking sequence) were PCR amplified using genomic DNA from *M. abscessus* ATCC 19977 [(5′-GAAATTACATGT GTCACGATCTCCTGGACTGCCTC-3′, 5′-GAAAGCGGCCGCCATGGAAGTACGCA CCCGATTCG-3′) and (5′-GATAGCGGCCGCCCGAATTCATGGAAACCTTCCGGG-3′, 5′-GTCTAGAGTCCTGTGTGAACAGGTCGGTG-3′), respectively)] and stepwise cloned into the pSE-*katG-aac(3)IV* suicide vector resulting in the knockout vector pSE-*katG-aac(3)IV-*Δ*MAB_0591*. Details on the construction of the *M. abscessus* deletion mutant will be described elsewhere (A. Rominski, P. Selchow and P. Sander, unpublished results). Briefly, pSE-*katG-aac(3)IV-*Δ*MAB_0591* was transformed into electrocompetent *M. abscessus* ATCC 19977. For electroporation, 100 μL of competent cells were mixed with 1–2 μg of supercoiled plasmid DNA and electroporated in a Bio-Rad Gene Pulser II (settings: 2.5 kV, 1000 Ohms and 25 μF). After electroporation, cells were resuspended in 0.9 mL of 7H9 medium and incubated for 5 h with constant shaking (1000 rpm) at 37 °C. Appropriate dilutions were subsequently plated on selective agar and after 5 days of incubation, single colonies were picked, restreaked and grown in liquid broth when necessary.

Transformants were selected on LB agar plates containing apramycin and identified by *aac(3)IV* PCR. Single crossover transformants were identified by Southern blot analysis with a 0.2 kbp EcoRI 5′arr_{Mab} DNA probe (the same probe was also used for all subsequent Southern blot analyses) and subjected to counterselection on LB agar plates containing isoniazid (32 mg/L). Single colonies were screened for deletion of *MAB_0591* by PCR and the genotype was finally confirmed by Southern blot analysis. In this way, a 0.3 kbp region of the *MAB_0591* was deleted. For complementation of the *M. abscessus* Δarr_{Mab} mutant with *MAB_0591*, the previously described complementation vector pMV361-*aac(3)IV-MAB_0591* was transformed into the Δarr_{Mab} mutant strain. Transformation was verified by Southern blot analysis.

Susceptibility testing of M. tuberculosis strains

Drug susceptibility testing (DST) was performed using the MGIT 960 system as recommended by the manufacturer[41] and the results were interpreted as described previously by Springer *et al.*[42] Briefly, 0.5 mL dilutions of positive MGIT vials of the Mtb-*aac(3)IV* control strain and the Mtb-*aac(3)IV-*arr_{Mab} testing strain were inoculated into fresh vials containing constant levels of apramycin for plasmid maintenance and different concentrations of the test drugs.[43] Rifampicin was tested at concentrations of 1, 4 and 20 mg/L, while the control drug isoniazid was tested at concentrations of 0.1, 1, 3 and 10 mg/L. For the drug-free growth control, the bacterial working suspension was diluted 1:100 with sterile NaCl and 0.5 mL of the diluted working suspension was inoculated into the tube (proportion testing).[44] All MGIT tubes were incubated in the MGIT 960 instrument and monitored using EpiCenter (version 5.53) software equipped with the TB eXiST module (Becton Dickinson).

Rifampicin Etest

Bacterial suspensions of *M. abscessus* strains ATCC 19977, Δ$arr_{Mab}$and Δarr_{Mab}-arr_{Mab} were adjusted to a turbidity equivalent to that of a 0.50 McFarland standard and subsequently spread on LB agar plates using a sterile cotton swab. Then, a rifampicin Etest strip (bioMérieux, Switzerland) was placed on each plate and plates were incubated for 5 days at 37 °C. The point of intersection between bacterial growth and the Etest device was read as the MIC value.

MIC assays

MIC assays were performed according to CLSI guidelines.[45] Working solutions were prepared by diluting the antibiotic stock solutions in CAMHB (pH 7.4) (Becton Dickinson, Switzerland) to a concentration corresponding to twice the desired final concentration [working solutions of 128, 512 and 1024 mg/L were prepared when the highest concentrations tested in the MIC assay were 64, 256 and 512 mg/L, respectively (Table 1 and Table 3)]. By using CAMHB in sterile 96-well microtitre plates (Greiner Bio-One, Switzerland), 2-fold serial dilutions of the working solutions were prepared. A positive growth control lacking antibiotic and a sterile negative control containing only CAMHB were included in each 96-well microtitre plate. For the preparation of the inoculum, three to four colonies from each bacterial strain grown on LB agar were transferred into a glass tube containing 2 mL of NaCl using a sterile cotton swab. In order to achieve a final inoculum titre of $1–5 \times 10^5$ cfu/mL, all bacterial suspensions were adjusted to a turbidity equivalent to that of a 0.50 McFarland standard and subsequently diluted in CAMHB. The final test volume in each well of the microtitre plate was 0.1 mL. The correct titre of each inoculum was checked by obtaining cfu counts on LB agar plates. All microdilution plates were capped with adhesive sealing covers and incubated at 37 °C for (i) 16 h for *E. coli* strains and (ii) 3, 5, 7 and 12 days for *M. abscessus* and *M. smegmatis* strains, before the MIC values were assessed by visual inspection. All MIC assays were conducted in triplicate.

Table 1. DST results of *E. coli* expressing *MAB_0591*[a]

Strain	Rifampicin MIC (mg/L)
E.coli-gfp	4
E.coli-arr_{Mab}	>512

[a]Broth microdilution method

Results and discussion

Analysis of the RpoB rifampicin resistance sequence clusters from M. abscessus

To investigate whether innate rifampicin resistance in *M. abscessus* (MIC: 128 mg/L) is associated with polymorphisms in the RNA polymerase β-subunit, we aligned the RpoB amino acid sequence of *M. abscessus* ATCC 19977 with those of *E. coli* K-12 and *M. tuberculosis* H37Rv, known to be naturally susceptible to rifampicin,[13,16] and *M. smegmatis* mc^2155 that has no polymorphism in its *rpoB* gene corresponding to any known rifampicin resistance genotype,[35] but is naturally resistant to rifampicin through ADP-ribosylation of rifampicin.[22,36] Our analysis revealed no polymorphism known to confer rifampicin resistance within all four (N, I, II and III) rifampicin resistance sequence clusters of *M. abscessus* RpoB (Figure 1), suggesting that other mechanisms are involved in the high intrinsic rifampicin resistance of *M. abscessus*. Possible mechanisms include efflux of rifampicin, diminished uptake of rifampicin and enzymatic degradation or modification of rifampicin.[19–23] The latter resistance mechanism has been studied in *M. smegmatis* and *Legionella pneumophila*.[22,23] A recent genome analysis suggested that *M. abscessus* carries a putative rifampicin ADP-ribosyltransferase (Arr_*Mab*),[38] encoded by *MAB_0591* (arr_{Mab}). MAB_0591 has 66% amino acid sequence identity with the *M. smegmatis* ADP-ribosyltransferase that modifies rifampicin. However, Table S1 (available as Supplementary data at *JAC* Online), which lists the MIC values for selected mycobacterial standard strains[42,46,47] as well as information about the presence or absence of ADP-ribosyltransferase-like protein in these species, shows that a direct correlation between rifampicin MIC and the presence or absence of ADP-ribosyltransferase-like proteins does not exist. *M. tuberculosis* and *Mycobacterium leprae* were predicted to have no ADP-ribosyltransferase-like proteins and are susceptible to rifampicin (MIC: <1 mg/L).[42,47] ADP-ribosyltransferases were predicted to be present in *M. abscessus* (*M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*) and *Mycobacterium fortuitum* and these species are resistant (MIC: ≥64 mg/L).[46] In contrast, the presence of putative ADP-ribosyltransferases in e.g. *Mycobacteriumphlei*, *Mycobacterium gilvum* and *Mycobacterium marinum* does not correlate with high rifampicin MIC levels (MIC: <0.5 mg/L).[46] Therefore, a functional role of ADP-ribosyltransferases in rifampicin resistance has to be addressed experimentally. By using ADP-ribosyltransferase of *M. smegmatis* mc^2155 in a BLASTP search, we identified also other, non-mycobacterial species predicted to possess ADP-ribosyltransferase-like proteins (Table S2). Interestingly, mycobacterial ADP-ribosyltransferases show homology to the catalytic domain of exotoxin A from *Pseudomonas aeruginosa*,[48,49]

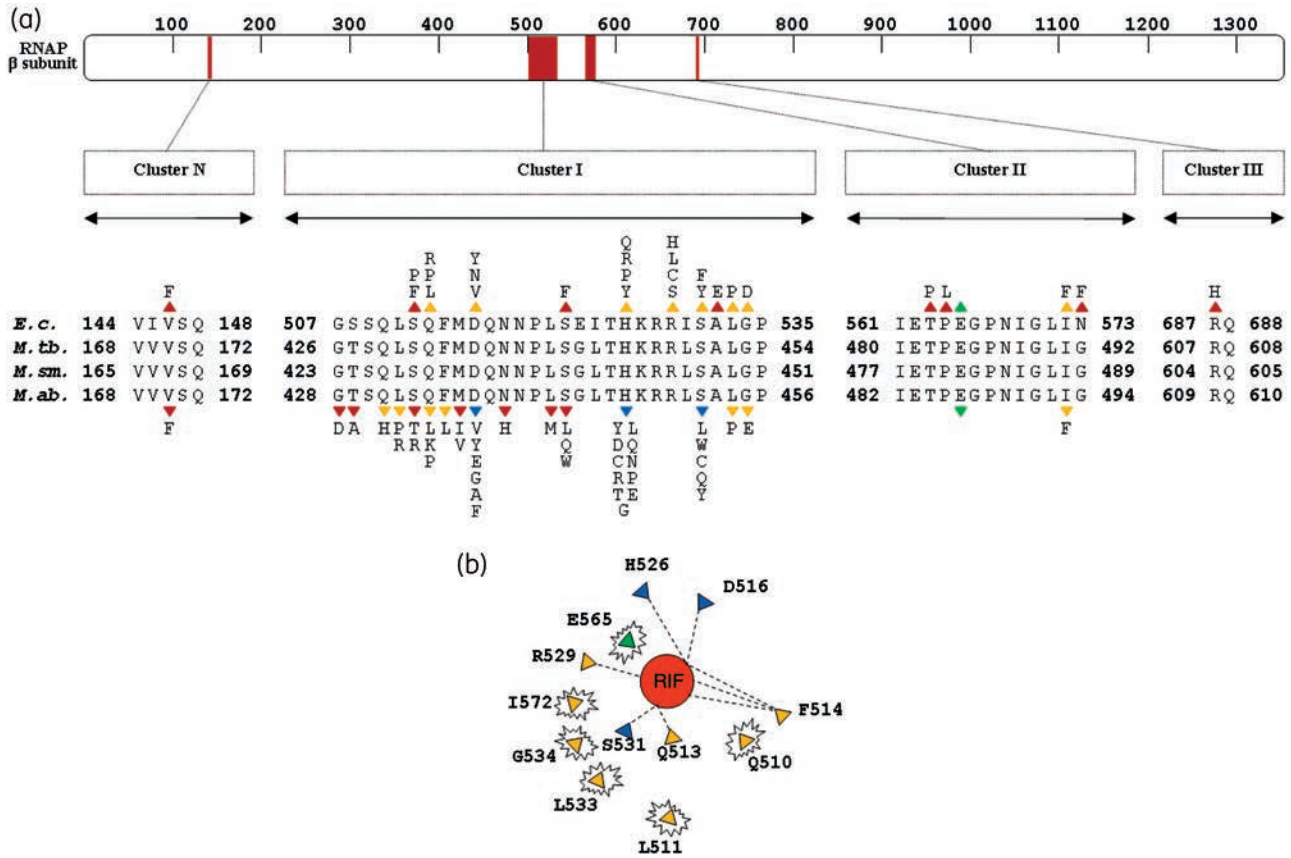

Figure 1. Rifampicin resistance regions of RpoB and their association with the interactions between rifampicin and the prokaryotic RNA polymerase (RNAP). (a) The bar at the top illustrates the RNAP β-subunit from *E. coli*. Directly above, its amino acid numbering is indicated. Dark red lines within the bar indicate the positions of the four clusters [N-terminal (N) and clusters I, II and III (I, II and III)] where the rifampicin resistance-conferring alterations are identified across all bacterial species.[24–30] Directly below follows the amino acid sequence alignment spanning the rifampicin resistance regions of *E. coli* K-12, *M. tuberculosis* H37Rv, *M. smegmatis* mc^{2}155 and *M. abscessus* ATCC 19977 RpoB. Amino acid substitutions that confer rifampicin resistance are shown as coloured triangles above and below the alignment, respectively. The possible substitutions for each position are mentioned in single amino acid code in columns directly above (for *E. coli*) or below the coloured triangles (for *M. tuberculosis*). The yellow triangles represent the residues that interact directly with rifampicin binding. In contrast, the red triangles indicate residues that are positioned too far away to have direct interaction with rifampicin. The blue triangles show the positions that are substituted with the highest frequency among *M. tuberculosis* rifampicin-resistant isolates.[31] The green triangles show a residue that is directly interacting with rifampicin, but no substitutions have been reported at this position, probably because they would be fatal for the bacterium.[15] (b) Using the same colour code for the amino acid positions as above, the residues of direct interaction with rifampicin are shown (yellow, blue and green triangles). Hydrogen bonds formed between an RpoB residue and rifampicin are depicted as broken lines and residues that form van der Waals interactions are indicated in zigzag circles. Numbering of the residues is according to *E. coli* positions.[15] RIF, rifampicin.

pointing to a putative role of mycobacterial ADP-ribosyltransferases as virulence factors. However, within this study we exclusively focus on the role of *MAB_0591* in rifamycin resistance.

Heterologous expression of MAB_0591 in E. coli and M. tuberculosis

To study if rifampicin resistance in *M. abscessus* is due to arr_{Mab}, we cloned *MAB_0591* and expressed the recombinant protein in two rifampicin-susceptible hosts, *E. coli*[16] and *M. tuberculosis*.[50] First, we addressed the question whether induced expression of *MAB_0591* would confer rifampicin resistance in a non-mycobacterial heterologous host. Arr_*Mab* was recombinantly expressed by the multicopy pBXNH3-arr_{Mab}vector in *E. coli* MC1061 under the control of the L-arabinose-inducible pBAD promoter. The *E. coli-gfp* control strain and the *E. coli*-arr_{Mab} testing strain were generated following transformation with the pBXNH3-*gfp* and pBXNH3-arr_{Mab} vectors, respectively. The recombinant strains were subsequently tested

Table 2. DST results of *M. tuberculosis* expressing *MAB_0591*[a]

Strain	Rifampicin 1.0 mg/L	Rifampicin 4.0 mg/L	Rifampicin 20.0 mg/L	Isoniazid 0.1 mg/L
Mtb-*aac(3)IV*	S	S	S	S
Mtb-*aac(3)IV*-arr_{Mab}	R	R	R	S

S, susceptible; R, resistant.
[a]Proportion method using the MGIT 960 system.

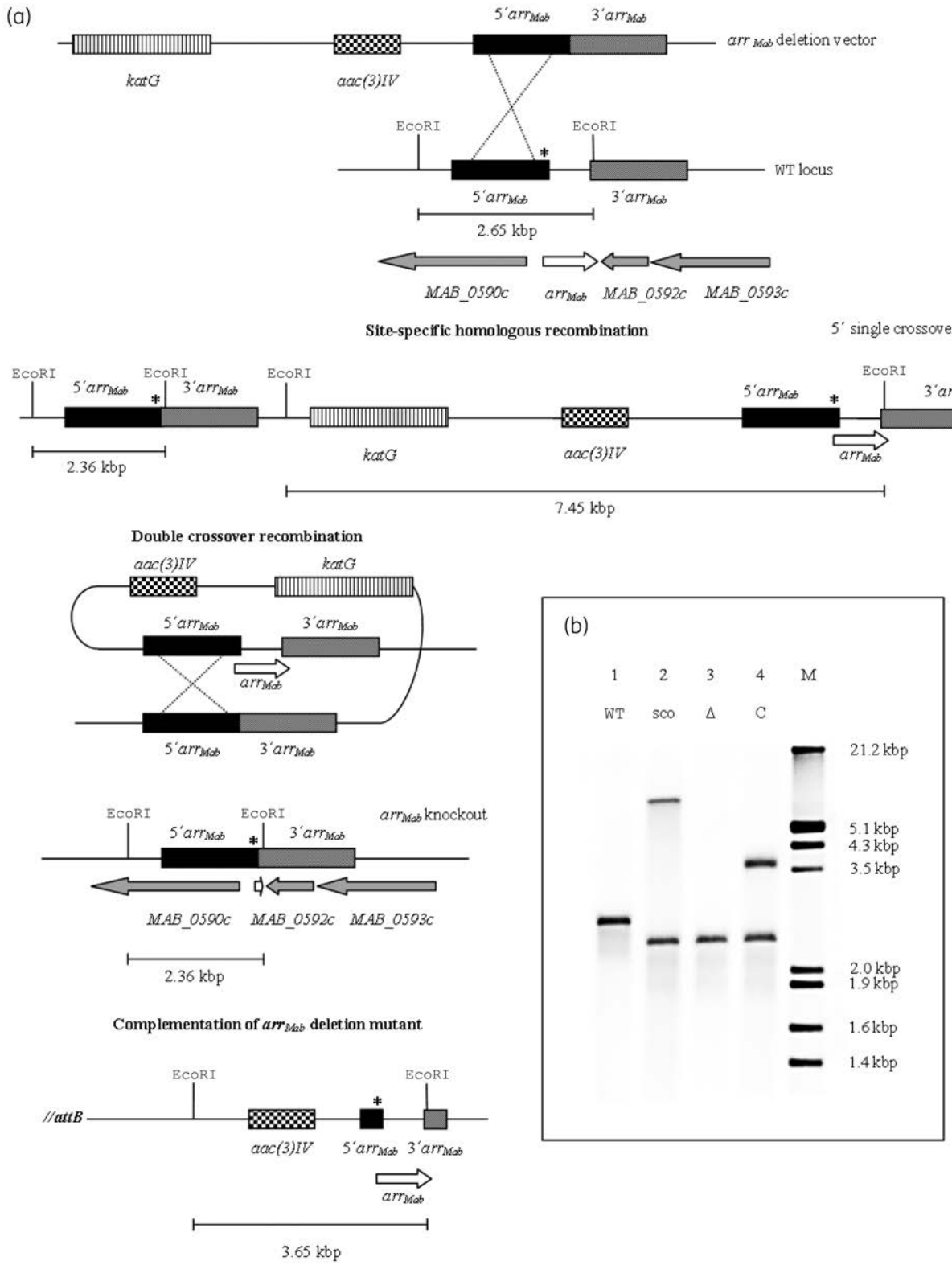

Figure 2. Genotypic analyses of the *M. abscessus* arr_{Mab} locus. (a) Schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirms the deletion of *MAB_0591* from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1), *M. abscessus* transformant with arr_{Mab} targeting vector [pSE-*katG-aac(3)IV-*Δ*MAB_0591*] prior to (2) and after KatG-dependent isoniazid counterselection (3) and after transformation of counterselected mutant with arr_{Mab} complementation vector (4) was digested with EcoRI and probed with a fragment from the 5′arr_{Mab} flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 2.65 kbp fragment of the WT parental strain, to the 2.36 and 7.45 kbp fragments after site-specific homologous recombination [single crossover (sco)], to a 2.36 kbp fragment of the Δarr_{Mab} mutant (Δ) and to the 2.36 and 3.65 kbp fragments of the *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant strain (C). M, molecular marker.

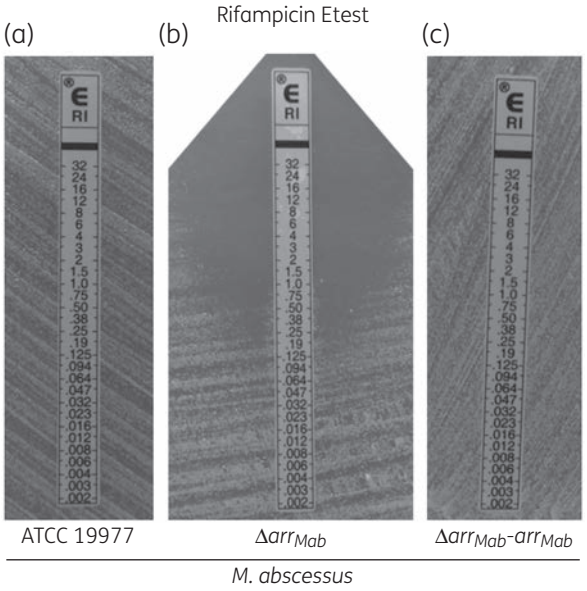

Figure 3. Contribution of *MAB_0591* to rifampicin resistance in *M. abscessus*. Rifampicin susceptibility of the (a) *M. abscessus* ATCC 19977 WT strain, (b) *M. abscessus* Δ*arr*$_{Mab}$ mutant and (c) *M. abscessus* Δ*arr*$_{Mab}$-*arr*$_{Mab}$ complemented mutant strain was examined by Etest. The picture of the rifampicin Etest results was taken after 5 days of incubation at 37 °C.

against rifampicin in MIC assays. The *E. coli-arr*$_{Mab}$ testing strain was highly resistant to rifampicin with MIC values >512 mg/L, while the *E. coli-gfp* control strain remained susceptible to rifampicin (MIC: 4 mg/L) (Table 1). These results confirm that Arr_*Mab* plays a critical role as a rifampicin resistance determinant when expressed in the rifampicin-susceptible *E. coli* host. We hypothesize that Arr_*Mab* modifies rifampicin by ADP-ribosylation at the hydroxyl group of C23, as previously shown for Arr_*Msm*,[48] and that this modification interferes with target binding.

Next, we studied whether *arr*$_{Mab}$ confers rifampicin resistance in a mycobacterial host, *M. tuberculosis*. In order to simulate veritable levels of Arr_*Mab* expression, we expressed MAB_0591 under control of its native promoter. The single-copy integrating plasmids pMV361-*aac(3)IV* and pMV361-*aac(3)IV-MAB_0591* were engineered and transformed into the rifampicin-susceptible *M. tuberculosis* H37Rv strain (MIC: <1 mg/L).[42] The *aac(3)IV* gene that confers resistance to apramycin was used as a positive selection marker. The resulting strains referred to as Mtb-*aac(3)IV* control strain and Mtb-*aac(3)IV-arr*$_{Mab}$ testing strain, respectively, were subjected to DST using the MGIT 960 system equipped with EpiCenter TB eXiST software. Susceptibility to different concentrations of rifampicin (1, 4 and 20 mg/L) and the non-substrate control drug isoniazid (0.1, 1, 3 and 10 mg/L) was determined. The Mtb-*aac(3)IV* control strain was, as expected, susceptible to both rifampicin and isoniazid at all concentrations tested (MIC: rifampicin <1.0 mg/L; isoniazid <0.1 mg/L). In contrast, the Mtb-*aac(3)IV-arr*$_{Mab}$ testing strain was specifically resistant to rifampicin, even at the highest concentration tested (MIC: >20 mg/L), but remained susceptible to the unrelated control drug isoniazid (MIC: <0.1 mg/L) (Table 2). These findings demonstrate that *MAB_0591* confers high-level resistance

Table 3. DST results of *M. abscessus* and *M. smegmatis* strains[a]

	Strain and day, MIC (mg/L)																			
	M. abscessus ATCC 19977				*M. abscessus* Δ*arr*$_{Mab}$				*M. abscessus* Δ*arr*$_{Mab}$-*arr*$_{Mab}$				*M. abscessus* Δ*arr*$_{Mab}$ pMV361-*aac(3)IV*				*M. smegmatis* mc^{2}155			
Antibiotic	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12
Rifampicin	128	256	256	>256	0.25	0.5	1	1	128	256	256	>256	0.25	0.5	1	1	128	128	128	128
Rifapentine	256	>256	>256	>256	1	2	2	4	128	256	>256	>256	1	2	2	4	64	128	128	128
Rifaximin	64	128	256	256	1	2	4	8	64	128	256	>256	1	2	4	4	256	256	>256	>256
5f	8	>64	>64	>64	0.016	0.063	0.063	0.25	8	64	>64	>64	0.016	0.125	0.125	0.25	0.125	0.25	0.25	0.5
5k	4	32	>64	>64	0.063	0.125	0.25	0.25	4	16	>64	>64	0.031	0.125	0.25	0.25	0.25	0.25	1	1
5l	2	4	4	8	0.0078	0.125	0.25	0.25	2	4	4	4	0.016	0.125	0.25	0.25	0.25	0.5	1	1
Amikacin	1	2	4	4	1	2	4	4	1	2	4	4	1	2	4	4	<0.5	<0.5	<0.5	<0.5

[a]Broth microdilution method.

to rifampicin, but not to other drugs, when expressed in a rifampicin-susceptible mycobacterial host.

Generation of M. abscessus MAB_0591 deletion mutant

Heterologous expression of *MAB_0591* indicated that arr_{Mab} is able to confer rifampicin resistance to a susceptible host; however, its role in innate rifampicin resistance in *M. abscessus* remained to be determined. We recently developed tools for genetic manipulation of *M. abscessus* (A. Rominski, P. Selchow and P. Sander, unpublished results) and wished to exploit this technique to generate an *M. abscessus MAB_0591* deletion mutant. This mutant would allow us to directly address the role of MAB_0591 in innate rifampicin resistance. The arr_{Mab} deletion mutant was constructed by transformation of *M. abscessus* ATCC 19977 with suicide plasmid pSE-*katG-aac(3)IV-ΔMAB_0591* applying apramycin positive selection[51] and a *katG*-dependent isoniazid counterselection strategy that we previously established (A. Rominski, P. Selchow and P. Sander, unpublished results) (Figure 2a). Deletion of *MAB_0591* was confirmed by Southern blot analysis (Figure 2b). A complemented mutant strain was constructed by transformation of the *M. abscessus* Δarr_{Mab} mutant with the complementation vector pMV361-*aac(3)IV-MAB_0591* expressing Arr_*Mab*. The complemented mutant strain is referred to as *M. abscessus* Δarr_{Mab}-arr_{Mab}. Genetic complementation was confirmed by Southern blot analysis (Figure 2b).

DST of M. abscessus ΔMAB_0591

For determination of the Δarr_{Mab} mutant's phenotype, a rifampicin Etest was carried out with *M. abscessus* ATCC 19977, *M. abscessus* Δarr_{Mab} mutant and *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented strain. Etest results after 5 days of incubation at 37 °C revealed high-level rifampicin resistance in *M. abscessus* ATCC 19977 (MIC: >32 mg/L). In contrast, the Δarr_{Mab} mutant showed susceptibility to low rifampicin concentrations (MIC: ∼0.5 mg/L). Rifampicin resistance was restored upon complementation of the Δarr_{Mab} mutant strain with arr_{Mab} (MIC: >32 mg/L; Figure 3). MIC values were subsequently determined in detail for rifampicin, rifapentine and rifaximin. *M. abscessus* ATCC 19977, *M. abscessus* Δarr_{Mab}, *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant, *M. abscessus* Δarr_{Mab} pMV361-*aac(3)IV* vector backbone control strain and *M. smegmatis* mc^2155 were subjected to DST. For *M. abscessus* Δarr_{Mab}, the MICs of all rifamycins were consistently and significantly lower than for the *M. abscessus* ATCC 19977 WT strain, indicating that all tested rifamycins are modified by

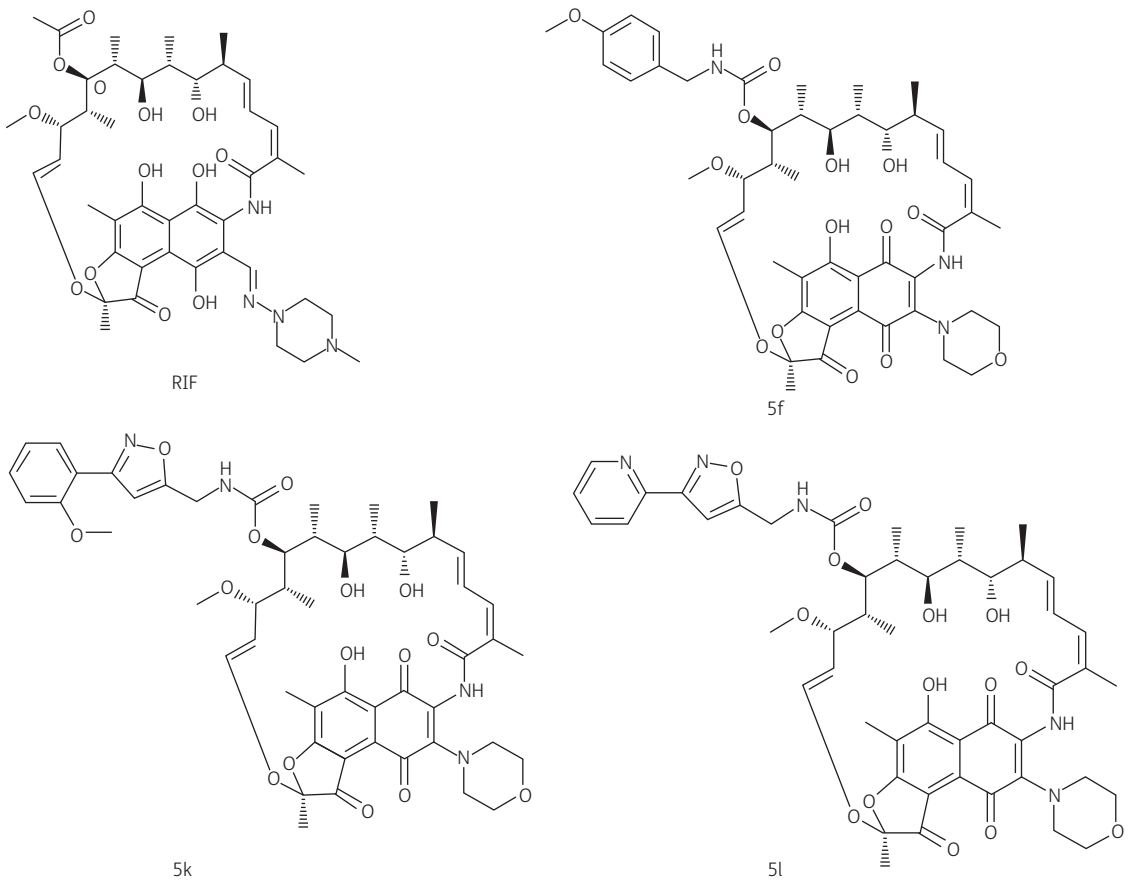

Figure 4. Rifampicin and the 5f: 25-*O*-desacetyl-(4-methoxybenzylaminocarbonyl) 3-morpholino rifamycin S, 5k: 25-*O*-desacetyl-{C-[3-(2-methoxyphenyl)-isoxazol-5-yl]-methylaminocarbonyl} 3-morpholino rifamycin S and 5l: 25-*O*-desacetyl-[C-(3-pyridin-2-yl-isoxazol-5-yl)-methylaminocarbonyl] 3-morpholino rifamycin S, C25 carbamate rifamycin derivatives, which are numbered per the original work[37] for ease of comparison. RIF, rifampicin.

Arr_*Mab* (Table 3). Transformation of the *M. abscessus* Δarr_{Mab} mutant with arr_{Mab} restored WT levels of rifamycin resistance while transformation with the empty vector backbone did not. Expectedly, amikacin MICs were independent of the *arr* genotype. These findings identify Arr_*Mab* as the major rifamycin resistance determinant in *M. abscessus*.

DST of C25 modified rifamycin derivatives

Carbamate modification at the C25 position of the rifamycin core has been shown to improve antimicrobial activity against *M. smegmatis* mc^{2}155.[37] We wanted to test whether these compounds also overcome rifamycin resistance in *M. abscessus*. C25 modified rifamycin derivatives 5f, 5k and 5l (Figure 4) were custom synthesized and tested for antimicrobial activity. The compounds showed potent activity against *M. smegmatis*—on average these compounds were 100–200-fold more active than rifampicin. These results confirm former findings that C25 modified rifamycins apparently are resilient to modification by Arr_*Msm*. The C25 modified rifamycins also showed increased activity in *M. abscessus* as compared with rifampicin, rifapentine and rifaximin (Table 3). Of note, C25 modification not only increases rifamycin activity against the *M. abscessus* WT, but also against the *M. abscessus* Δarr_{Mab} mutant, although to a lesser extent. These data indicate that the increased activity of C25 rifamycin derivatives is only partially due to resilience to Arr_*Mab* modification. Compared with compounds 5f and 5k, we observed little time-dependent increase in the MIC values of compound 5l for WT *M. abscessus* and the *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant, indicating that 5l is probably least modified by Arr_*Mab*, but still WT MIC values are high (4 mg/L; 16-fold higher than against the Δarr_{Mab} mutant).

Conclusions

Taken together, our study identified Arr_*Mab* as the major determinant of innate rifamycin resistance in *M. abscessus*. Our data indicate significant species-specific differences in rifamycin C25-mediated resilience for Arr_*Msm* and Arr_*Mab*, since Arr_*Mab*-mediated rifamycin resistance can only partly be overcome by C25 modification. These findings testify to the need to develop novel compounds that are able to escape Arr_*Mab*-mediated rifamycin resistance in *M. abscessus*. Structural similarity between Arr_*Msm* and protein ADP-ribosyltransferases, prominently domain III of *P. aeruginosa* exotoxin A, have been described.[48,49] These structural features are also conserved in Arr_*Mab*. Therefore, it is tempting to speculate on a dual role of Arr_*Mab* in drug resistance and virulence.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Elucidation of *Mycobacterium abscessus* aminoglycoside and capreomycin resistance by targeted deletion of three putative resistance genes

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Objectives: *Mycobacterium abscessus* is innately resistant to a variety of drugs thereby limiting therapeutic options. Bacterial resistance to aminoglycosides (AGs) is conferred mainly by AG-modifying enzymes, which often have overlapping activities. Several putative AG-modifying enzymes are encoded in the genome of *M. abscessus*. The aim of this study was to investigate the molecular basis underlying AG resistance in *M. abscessus*.

Methods: *M. abscessus* deletion mutants deficient in one of three genes potentially involved in AG resistance, *aac(2′)*, *eis1* and *eis2*, were generated by targeted gene inactivation, as were combinatorial double and triple deletion mutants. MICs were determined to study susceptibility to a variety of AG drugs and to capreomycin.

Results: Deletion of *aac(2′)* increased susceptibility of *M. abscessus* to kanamycin B, tobramycin, dibekacin and gentamicin C. Deletion of *eis2* increased susceptibility to capreomycin, hygromycin B, amikacin and kanamycin B. Deletion of *eis1* did not affect drug susceptibility. Equally low MICs of apramycin, arbekacin, isepamicin and kanamycin A for WT and mutant strains indicate that these drugs are not inactivated by either AAC(2′) or Eis enzymes.

Conclusions: *M. abscessus* expresses two distinct AG resistance determinants, AAC(2′) and Eis2, which confer clinically relevant drug resistance.

Introduction

Mycobacterium abscessus is a rapid growing mycobacterium of increasing medical importance. This emerging pathogen causes bronchopulmonary infections in individuals with cystic fibrosis[1] and chronic pulmonary disease, such as pneumoconiosis and bronchiectasis.[2] It also causes severe infections following surgery, transplantation, tattooing and mesotherapy.[3–8] Treatment of an *M. abscessus* infection is difficult due to the bacteria's high degree of intrinsic resistance to chemotherapeutic agents.[9] The pathogen is naturally resistant to many major classes of antibiotics used for the treatment of Gram-positive and Gram-negative bacterial infections, such as β-lactams, aminoglycosides (AGs) (kanamycin B, gentamicin C) and macrolides (erythromycin). In addition, it is also resistant to first-line TB drugs, for instance isoniazid and rifampicin. *M. abscessus* has been called an antibiotic nightmare since treatment options against *M. abscessus* infection are more limited than for *Mycobacterium tuberculosis* infection.[10] While no standard treatment recommendations for pulmonary *M. abscessus* infections have yet been established, current guidelines propose administration of an oral macrolide (clarithromycin or azithromycin) for clinical isolates susceptible to macrolides and the intravenous AG amikacin in combination with a parenteral β-lactam antibiotic, cefoxitin or imipenem.[11] AGs and macrolides inhibit protein biosynthesis by binding to the small and large ribosomal subunit, respectively. Clinically acquired pan-AG and pan-macrolide resistance has been attributed to mutations in ribosomal RNA genes *rrs* and *rrl*, coding for 16S and 23S rRNA, respectively. Owing to the presence of a single ribosomal RNA (*rrn*) operon, corresponding resistance mutations have readily been observed in *M. abscessus*.[12–14] However, distinct mechanisms have been proposed to be responsible for innate AG resistance in *M. abscessus*.[9,15]

AG antibiotics form a group of hydrophilic molecules, consisting of a characteristic, central aminocyclitol linked to one or more amino sugars by pseudoglycosidic bond(s). They inhibit prokaryotic protein biosynthesis by binding to the A-site of the 16S rRNA. AGs are active against a wide range of aerobic Gram-negative bacilli, staphylococci and mycobacteria. Resistance to AGs is due to low uptake, increased efflux, target modification

or enzyme-mediated drug modification. Originally isolated from microorganisms (mainly *Streptomyces* spp.), semi-synthetic derivatives of AGs have been generated to improve the pharmacological properties of the drugs and to overcome bacterial resistance mechanisms.[16] The semi-synthetic drug amikacin is a cornerstone of *M. abscessus* infection therapy, whereas other AGs are not frequently used. *M. abscessus* genome annotation suggests the presence of various AG-modifying enzymes such as AG phosphotransferases, AG nucleotidyltransferases and AG acetyltransferases (AACs).[17] The individual contribution of these genes to *M. abscessus* AG susceptibility cannot be predicted reliably due to overlapping specificities.

Elucidation of gene functions, drug target and host–pathogen interaction heavily relies on the generation of isogenic mutants. Usually, antibiotic resistance markers are used for the primary selection of transformants. Tools for genetic manipulation of mycobacteria in general, and *M. tuberculosis* in particular, have been developed during the past two or three decades.[18] In contrast, genetic manipulation of *M. abscessus* has been reported to be very difficult and attempts have often been unsuccessful,[19–21] although some progress has been made.[22–25] We recently developed genetic tools for *M. abscessus*[26] and have now exploited them for elucidating intrinsic AG resistance mechanisms in the *M. abscessus* ATCC 19977 type strain. We particularly addressed the role of putative AACs in *M. abscessus* AG and capreomycin resistance. We generated single, double and triple mutants of *M. abscessus* by targeted deletion of three genes potentially involved in AG resistance and characterized those mutants by phenotypic drug susceptibility testing. Interestingly, a double mutant proved to be susceptible to a wide variety of AGs and to the peptide antibiotic capreomycin.

Materials and methods

Bacterial strains and media

The *Escherichia coli* laboratory strain XL1-Blue MRF′ (Stratagene, Switzerland) and ER2925 *dam*$^{-}$, *dcm*$^{-}$ were used for cloning and propagation of plasmids. The strains were grown in LB medium containing one of the antibiotics: ampicillin (120 mg/L), kanamycin A (50 mg/L), apramycin (50 mg/L) or hygromycin B (100 mg/L). Antibiotics (Sigma–Aldrich, Switzerland) were dissolved in water according to the manufacturer's recommendations and stored as a stock solution until usage.

M. abscessus ATCC 19977 was grown in Middlebrook 7H9-OADC-Tween 80 or LB containing one of the antibiotics, if required: kanamycin A (50 mg/L), apramycin (50 mg/L) or isoniazid (32 mg/L). For preparation of electrocompetent cells two cell culture flasks each containing 200 mL of 7H9-OADC-Tween 80 were inoculated 1:100, incubated at 37°C and gently shaken daily. When OD_{600} reached 0.4–0.8, cultures were set on ice for 90 min. Bacteria were harvested by centrifugation and repeatedly resuspended in ice-cold glycerol (10% v/v) while gradually reducing the volume.[27] Finally, bacteria were resuspended in 2 mL of glycerol (10% v/v) and either frozen in liquid nitrogen or directly used for electroporation, as we recently described.[26] Genomic DNA was isolated by phenol/chloroform/isoamyl alcohol extraction, as described previously.[28]

Generation of vectors

The apramycin resistance cassette from plasmid pSET152[29] was PCR amplified (primers Apr_F and Apr_R) (see Table S1, available as Supplementary data at *JAC* Online, for list of primers) and cloned as a 1 kbp SpeI fragment into SpeI-digested plasmid pMV361[30] to result in plasmid pMV361-*apr*. An *M. tuberculosis katG* fragment (2.8 kbp) was PCR amplified (primers KatG_F and KatG_R) and cloned into pGEM-T-easy to result in pGEM-T-*katG*. Subsequently, the 2.8 kbp SwaI-*katG*-HpaI fragment was cloned into HpaI-linearized pMV361-*apr* to result in pMV361-*apr-katG*. Removal of mycobacteriophage *attP/int* from plasmid pMV361-*apr-katG* by XbaI excision and self-ligation of the vector backbone resulted in the prototype suicide vector pSE-*apr-katG* into which flanking regions of the target genes were cloned.

Disruption of MAB_4395 [aac(2′)] in M. abscessus

A 1.6 kbp NdeI/NheI fragment of *M. abscessus* ATCC 19977 from position 4477184 to 4478794 comprising the 5′ flanking sequence and a 1.5 kbp NheI/MluI fragment from position 4479125 to 4480674 comprising the 3′ flanking sequence of *MAB_4395* [*aac(2′)*] were PCR amplified (primers 4395_UP_F, 4395_UP_R and 4395_DW_F, 4395_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Subsequently, fragments were ligated stepwise into saving vector pMCS5 with respective enzymes resulting in subcloning vector pMCS5-*MAB_4395*. The region comprising 5′ and 3′ flanking sequences of *aac(2′)* was cut out with StuI and cloned into HpaI-linearized pSE-*apr-katG* vector to result in knockout vector pSE-Δ*aac(2′)*. The *aac(2′)* allele was deleted from the *M. abscessus* ATCC 19977 chromosome using apramycin for positive and isoniazid for negative selection. Deletion of *aac(2′)* was confirmed by Southern blot analysis of Van91I-digested genomic DNA with a 0.3 kbp 5′ *aac(2′)* probe amplified with primers P_4395_F and P_4395_R. A 2.2 kbp fragment from *M. abscessus* ATCC 19977 from position 4477131 to 4479338 spanning the entire *aac(2′)* gene was amplified with primers C_4395_F and C_4395_R and cloned into HindIII-digested pMV361 vector to result in pMV361-*aac(2′)* complementation vector. *M. abscessus* Δ*aac(2′)* was transformed with pMV361-*aac(2′)* and control vector pMV361, respectively. Genetic complementation was confirmed by Southern blot analysis with the same 5′ *aac(2′)* probe.

Disruption of MAB_4124 (eis1) in M. abscessus

A 1.6 kbp ApaI/NdeI fragment of *M. abscessus* ATCC 19977 from position 4187416 to 4189052 comprising the 5′ flanking sequence and a 1.7 kbp NdeI/NheI fragment from position 4189857 to 4191580 comprising the 3′ flanking sequence of *MAB_4124* (*eis1*) were PCR amplified (primers 4124_UP_F, 4124_UP_R and 4124_DW_F, 4124_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Fragments were stepwise cloned with corresponding enzymes into saving vectors and subsequently into NotI/XbaI-digested vector pSE-*apr-katG* to result in knockout vector pSE-Δ*eis1*. The *eis1* allele was deleted from the *M. abscessus* ATCC 19977 chromosome using apramycin (positive) and isoniazid (negative) selection. Deletion was confirmed by Southern blot analysis using Van91I-digested genomic DNA and a 0.3 kbp 5′ *eis1* fragment amplified with primers P_4124_F and P_4124_R.

Disruption of MAB_4532c (eis2) in M. abscessus

A 1.8 kbp HpaI/Pfl23II fragment of *M. abscessus* ATCC 19977 from position 4612843 to 4614693 comprising the 5′ flanking sequence and a 1.5 kbp PacI/Pfl23II fragment from position 4615391 to 4616869 comprising the 3′ flanking sequence of *MAB_4532c* (*eis2*) were PCR amplified (primers 4532c_UP_F, 4532c_UP_R and 4532c_DW_F, 4532c_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Fragments were stepwise cloned into vector pSE-*apr-katG* resulting in knockout vector pSE-Δ*eis2*. The *eis2* allele was deleted from the *M. abscessus* ATCC 19977 chromosome using apramycin (positive) and isoniazid (negative) selection. Deletion was confirmed by Southern blot analysis with Van91I-digested genomic DNA and a 0.3 kbp 5′ *eis2* probe amplified with primers P_4532c_F and P_4532c_R. For complementation a 1.3 kbp fragment from *M. abscessus* ATCC 19977 (position 4614320–4615651) spanning the entire *eis2* gene was PCR amplified (primers C_4532c_F, C_4532c_R) and cloned via HindIII into pJB-*apr* vector to result in pJB-*apr-eis2* complementation vector. *M. abscessus* Δ*eis2* was

transformed with pJB-*apr-eis2* and control vector pJB-*apr*, respectively. Genetic complementation was confirmed by Southern blot analysis.

MIC assays

Kanamycin B, tobramycin, dibekacin, arbekacin, gentamicin C [C1 (<45%), C2 (<35%) and C1a (<30%)], isepamicin, amikacin, kanamycin A, apramycin, streptomycin, hygromycin B and capreomycin were bought from Sigma–Aldrich. Antibiotics were dissolved in water according to the manufacturer's recommendations, were filter sterilized, aliquoted into stock solutions and stored at −20°C. MIC determination for *M. abscessus* strains was done according to CLSI guidelines[31] and basically as we previously described.[26]

Results

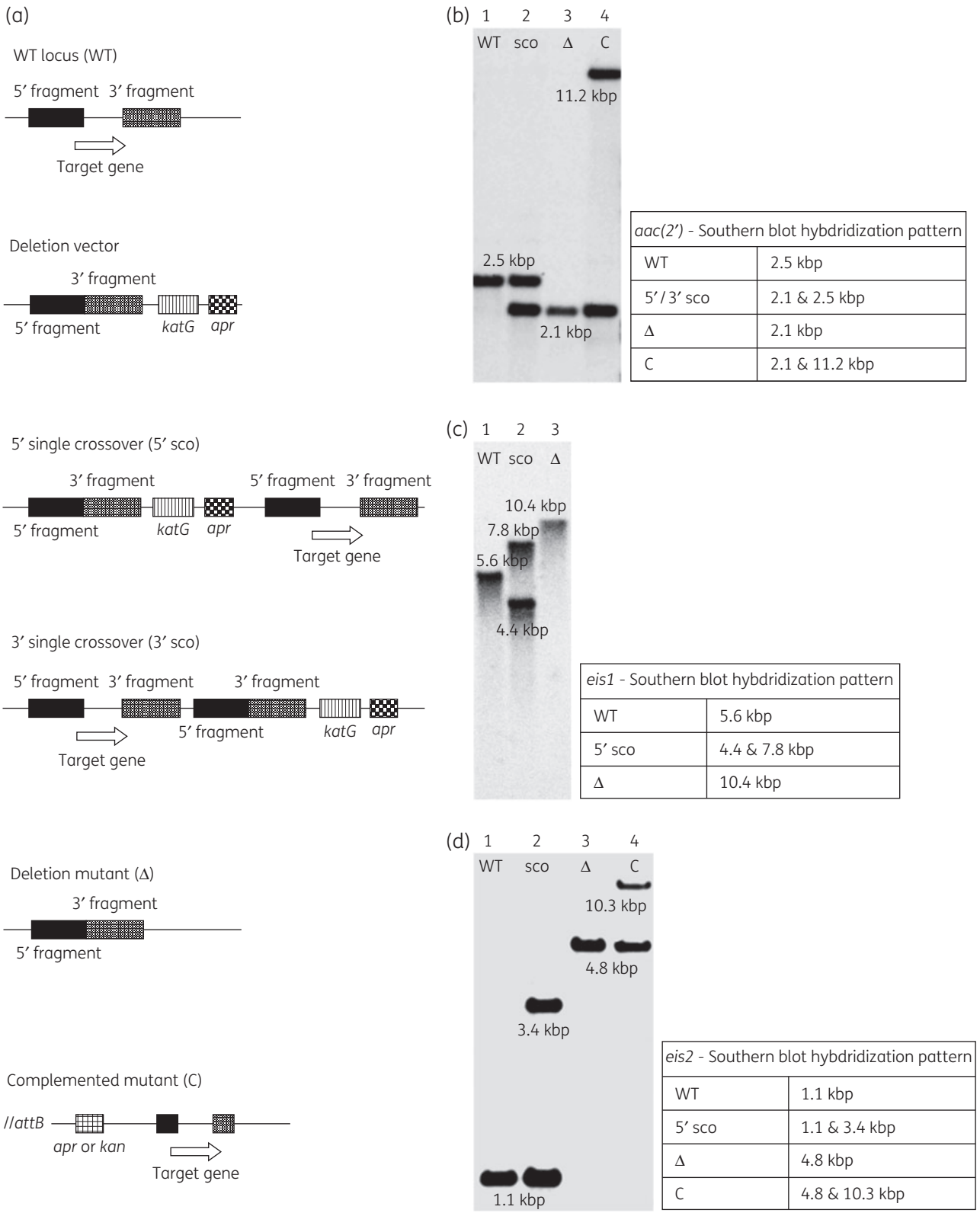
Targeted inactivation of putative AG resistance genes of M. abscessus

Genome annotation,[17] genome analysis and AG drug susceptibility testing[15] suggest the presence of several putative AACs in *M. abscessus*. ORF *MAB_4395* is annotated as a putative AG 2′-*N*-acetyltransferase [*aac(2′)*],[17] while *MAB_4124* and *MAB_4532c* show homology to *eis*. Eis (enhanced intracellular survival) proteins are found in a variety of mycobacterial and non-mycobacterial species.[32,33] Overexpression of Eis (Rv2416c) confers increased kanamycin resistance on *M. tuberculosis*.[34] *M. abscessus MAB_4124* is the closest homologue of *M. tuberculosis Rv2416c* (33% identity), which we therefore name *eis1*. Interestingly, a second Eis homologue encoded by *MAB_4532c* is present in the *M. abscessus* genome. In a phylogenetic tree constructed from 29 Eis homologues,[33] MAB_4532c (which we name Eis2) is clustered with Eis homologues from the non-mycobacterial clade instead of the mycobacterial group, e.g. *M. abscessus* Eis2 shows 23% identity to *Anabaena variabilis* Eis. We addressed the role of the three acetyltransferases in *M. abscessus* AG resistance by engineering and characterizing corresponding unmarked deletion mutants in the *M. abscessus* ATCC 19977 type strain.

Flanking fragments of the target genes *aac(2′)*, *eis1* and *eis2* were cloned into plasmid pSE-*apr-katG* to result in suicide vectors pSE-Δ*aac*, pSE-Δ*eis1* and pSE-Δ*eis2*, respectively. These flanking fragments enable homologous recombination of the suicide vector. The apramycin resistance cassette of the vector backbone facilitates selection of single crossover transformants resulting from vector integration by intermolecular homologous recombination at the target locus. Catalase–peroxidase KatG, the activator of the TB prodrug isoniazid,[35] serves as a negative selectable marker since it sensitizes *M. abscessus* to isoniazid. Expression of KatG facilitates screening for deletion mutants resulting from resolving the integration of the suicide vector by a second intramolecular homologous recombination.[26] Electrocompetent *M. abscessus* were transformed and apramycin selection and Southern blot analysis were applied to identify single crossover transformants. Subsequently, isoniazid counterselection and Southern blot analysis were applied for the identification of *M. abscessus* Δ*aac(2′)*, *M. abscessus* Δ*eis1* and *M. abscessus* Δ*eis2* mutants (Figure 1). Deletion mutants showing a phenotype [*M. abscessus* Δ*aac(2′)* and *M. abscessus* Δ*eis2*] were transformed with single copy, i.e. pMV361-based complementation and control vectors, respectively (see Table 1 for list of strains and Table 2 for list of plasmids). Furthermore, single deletion mutants served as parental strains for construction of double deletion mutants by transformation with a second targeting vector. Double deletion mutants, *M. abscessus* Δ*aac(2′)* Δ*eis1*, *M. abscessus* Δ*aac(2′)* Δ*eis2* and *M. abscessus* Δ*eis1* Δ*eis2*, were confirmed by Southern blot analyses. Finally, by transformation of pSE-Δ*aac(2′)* into *M. abscessus* Δ*eis1* Δ*eis2*, the triple mutant *M. abscessus* Δ*aac(2′)* Δ*eis1* Δ*eis2* was constructed (Figure 2).

Drug susceptibility testing of M. abscessus mutant strains

Drug susceptibility of *M. abscessus* strains towards a variety of structurally similar 4,6-disubstituted AGs (Figure 3) was tested in CAMHB. In addition, drug susceptibility to structurally atypical AGs apramycin, hygromycin B and streptomycin and to the peptide antibiotic capreomycin (also inhibiting protein biosynthesis by interaction with the 16S rRNA A-site) was determined. Growth was judged after 3, 5, 7 and 12 days of incubation and the MICs were determined. The median MIC values on day 5 are shown in Table 3. The MIC values read at all timepoints are shown in Table S2. A variety of mutant phenotypes was observed. Deletion of *aac(2′)* increased susceptibility of *M. abscessus* to kanamycin B (64-fold), tobramycin (32-fold), dibekacin (16-fold) and gentamicin C (4-fold). Deletion of *aac(2′)* did not affect MICs of arbekacin, isepamicin, amikacin, kanamycin A, apramycin, streptomycin, hygromycin B and capreomycin (Table 3). The tobramycin, dibekacin and gentamicin C WT phenotype was restored upon transformation with the complementation vector pMV361-*aac(2′)*, but not by transformation with the backbone control vector pMV361 (Table S2). The presence of a kanamycin resistance cassette [*aph(3′)*] in the backbone of the complementation vector interfered with susceptibility testing for kanamycin A and B and MICs of these compounds are therefore not reported. Deletion of *eis1* did not result in a detectable phenotype; MICs of none of the tested antibiotics were altered. Owing to the absence of a phenotype, no complementation vector was constructed. Deletion of *eis2* increased susceptibility of *M. abscessus* to several AGs. However, the susceptibility pattern of *M. abscessus* Δ*eis2* clearly differed from that of *M. abscessus* Δ*aac(2′)*. Deletion of *eis2* increased susceptibility to kanamycin B (4-fold), amikacin (8-fold), hygromycin B (16-fold) and capreomycin (32-fold). Deletion of *eis2* did not affect susceptibility to tobramycin, dibekacin, arbekacin, gentamicin C, isepamicin, kanamycin A, apramycin and streptomycin. Transformation of the Δ*eis2* mutant with the complementation vector pJB-*apr-eis2*, but not with the vector backbone pJB-*apr*, restored WT MICs of amikacin, hygromycin B and capreomycin (Table S2). The presence of the apramycin resistance cassette in the backbone of the complementation vector interfered with susceptibility testing for kanamycin (A and B), tobramycin, dibekacin, gentamicin C and apramycin, and therefore MIC values of these antibiotics are not reported. Double mutant *M. abscessus* Δ*aac(2′)* Δ*eis1* had the same phenotype as the Δ*aac(2′)* single mutant. Double mutant *M. abscessus* Δ*eis1* Δ*eis2* had the same phenotype as the Δ*eis2* single mutant. The *M. abscessus* Δ*aac(2′)* Δ*eis2* double mutant showed increased susceptibility to kanamycin B, tobramycin, dibekacin, gentamicin C,



aac(2′) - Southern blot hybridization pattern

WT	2.5 kbp
5′ / 3′ sco	2.1 & 2.5 kbp
Δ	2.1 kbp
C	2.1 & 11.2 kbp

eis1 - Southern blot hybridization pattern

WT	5.6 kbp
5′ sco	4.4 & 7.8 kbp
Δ	10.4 kbp

eis2 - Southern blot hybridization pattern

WT	1.1 kbp
5′ sco	1.1 & 3.4 kbp
Δ	4.8 kbp
C	4.8 & 10.3 kbp

Figure 1. Genotypic analyses of *M. abscessus* deletion mutants. (a) General schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirms the deletion of *aac(2′)* (*MAB_4395*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (lane 1), *M. abscessus* transformant with pSE-Δ*aac(2′)* targeting vector prior to (lane 2) and after KatG-dependent isoniazid counterselection (lane 3) and after transformation of counterselected mutant with pMV361-*aac(2′)* complementation vector (lane 4) was digested with Van91I and probed with a

amikacin, hygromycin B and capreomycin, whereas MICs of arbekacin, isepamicin, kanamycin A, apramycin and streptomycin were not altered. Thus, the phenotype of the Δ*aac(2′)* Δ*eis2* double mutant is a combination of the phenotype of the single mutants. The phenotype of the triple Δ*aac(2′)* Δ*eis1* Δ*eis2* mutant was similar to the phenotype of the Δ*aac(2′)* Δ*eis2* double mutant. Together, these data indicate a distinct role of *aac(2′)* and of *eis2* in intrinsic AG and capreomycin resistance in *M. abscessus*. Notably, AAC(2′) and Eis2 prevent antibacterial effects of different subsets of AGs. The only exception is kanamycin B, which is modified by both AAC(2′) and Eis2. However, the deletion of *aac(2′)* had a stronger effect on kanamycin B susceptibility than the deletion of *eis2* (64-fold versus 4-fold). AAC(2′) is more influential than Eis2, since the MIC for the double deletion mutant is not further decreased as compared with the *aac(2′)* deletion mutant. Deletion of *eis1* from the genome of the Δ*aac(2′)* Δ*eis2* mutant did not further increase AG susceptibility, indicating a negligible role of Eis1 in *M. abscessus* intrinsic AG resistance, even in an *aac(2′) eis2* double deletion mutant.

Discussion

M. abscessus is a pathogen of increasing medical importance, particularly in individuals with chronic pulmonary disease. The high level of intrinsic resistance to many classes of antibiotics restricts antibiotic therapy.[9] Genome annotation,[17] susceptibility testing and biochemical assays predicted a role for 2′-*N*-acetyltransferase in *M. abscessus* AG resistance.[15] A candidate gene eventually responsible for the increased MIC levels of 2′-amino-AGs, *MAB_4395* [*aac(2′)*] was inactivated by targeted gene deletion. Comparison of WT and mutant strains by phenotypic susceptibility testing demonstrated a decreased MIC for the mutant of a variety of drugs characterized by a 2′-amino group (kanamycin B, tobramycin, dibekacin, gentamicin C). Kanamycin A, which differs from kanamycin B by a single structural feature (2′-OH group instead of a 2′-amino group), has a lower MIC for the WT strain than kanamycin B and its MIC is not further decreased by *aac(2′)* deletion. These data corroborate the hypothesis that *MAB_4395* encodes a functional AG 2′-*N*-acetyltransferase, which renders *M. abscessus*

Table 1. Strains used in this study

Strain	Description	Source
E. coli XL1-Blue MRF'	cloning and propagation of plasmids	Stratagene
E. coli ER2925 *dam*$^{-}$, *dcm*$^{-}$	cloning and propagation of plasmids with methylase susceptible restriction enzymes	New England BioLabs
M. abscessus ATCC 19977	*M. abscessus* type strain	Ripoll *et al.* 2009[17]
M. abscessus Δ*aac(2′)*	*M. abscessus aac(2′)* deletion mutant; derivative of *M. abscessus* ATCC 19977	this study
M. abscessus Δ*eis1*	*M. abscessus eis1* deletion mutant; derivative of *M. abscessus* ATCC 19977	this study
M. abscessus Δ*eis2*	*M. abscessus eis2* deletion mutant; derivative of *M. abscessus* ATCC 19977	this study
M. abscessus Δ*aac(2′)* Δ*eis1*	*M. abscessus aac(2′) eis1* double deletion mutant; derivative of *M. abscessus* Δ*eis1*	this study
M. abscessus Δ*aac(2′)* Δ*eis2*	*M. abscessus aac(2′) eis2* double deletion mutant; derivative of *M. abscessus* Δ*aac(2′)*	this study
M. abscessus Δ*eis1* Δ*eis2*	*M. abscessus eis1 eis2* double deletion mutant; derivative of *M. abscessus* Δ*eis2*	this study
M. abscessus Δ*aac(2′)* Δ*eis1* Δ*eis2*	*M. abscessus aac(2′) eis1 eis2* triple deletion mutant; derivative of *M. abscessus* Δ*eis1* Δ*eis2*	this study
M. abscessus Δ*aac(2′)* pMV361-*aac(2′)*	*M. abscessus aac(2′)* mutant transformed with complementation vector pMV361-*aac(2′)*	this study
M. abscessus Δ*aac(2′)* pMV361	*M. abscessus aac(2′)* mutant transformed with pMV361 control vector	this study
M. abscessus Δ*eis2* pJB-*apr-eis2*	*M. abscessus* Δ*eis2* mutant transformed with complementation vector pJB-*apr-eis2*	this study
M. abscessus Δ*eis2* pJB-*apr*	*M. abscessus* Δ*eis2* mutant transformed with control vector pJB-*apr*	this study

Figure 1. *Continued*

fragment from the 5′ *aac(2′)* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 2.5 kbp fragment of the WT parental strain, to the 2.1 and 2.5 kbp fragments after site-specific homologous recombination (single crossover; sco), to a 2.1 kbp fragment of the *M. abscessus* Δ*aac(2′)* mutant (Δ) and to the 2.1 and 11.2 kbp fragments of the *M. abscessus* Δ*aac(2′)* pMV361-*aac(2′)* complemented mutant strain (C). (c) Southern blot analysis confirms the deletion of *eis1* (*MAB_4124*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (lane 1), *M. abscessus* transformant with pSE-Δ*eis1* targeting vector prior to (lane 2) and after KatG-dependent isoniazid counterselection (lane 3) was digested with Van91I and probed with a fragment from the 5′ *eis1* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 5.6 kbp fragment of the WT parental strain, to the 4.4 and 7.8 kbp fragments after site-specific homologous recombination (sco) and to a 10.4 kbp fragment of the *M. abscessus* Δ*eis1* mutant (Δ). (d) Southern blot analysis confirms the deletion of *eis2* (*MAB_4532c*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (lane 1), *M. abscessus* transformant with pSE-Δ*eis2* targeting vector prior to (lane 2) and after KatG-dependent isoniazid counterselection (lane 3) and after transformation of counterselected mutant with pJB-*apr-eis2* complementation vector (lane 4) was digested with Van91I and probed with a fragment from the 5′ *eis2* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 1.1 kbp fragment of the WT parental strain, to the 1.1 and 3.4 kbp fragments after site-specific homologous recombination (sco), to a 4.8 kbp fragment of the *M. abscessus* Δ*eis2* mutant (Δ) and to the 4.8 and 10.3 kbp fragments of the *M. abscessus* Δ*eis2* pJB-*apr-eis2* complemented mutant strain (C).

Table 2. Plasmids used in this study

Plasmid	Description; selectable marker	Source
pMV361	integrative *E. coli*/mycobacterial shuttle vector; Kan^R	Stover *et al.* 1991[30]
pSET152	*Streptomyces* vector with apramycin resistance gene (*aac(3)IV*), template for *apr* amplification; Apr^R	Wilkinson *et al.* 2002[29]
pGEM-T-easy	PCR saving vector; Amp^R	Promega
pMCS5	general cloning vector; Amp^R	MBio
pGEM-T-katG	intermediate vector for *katG* subcloning; Amp^R	this study
pMV361-*apr*	integrative *E. coli*/mycobacterial shuttle vector; Kan^R, Apr^R	this study
pMV361-*apr-katG*	derivative of pMV361-*apr* containing *M. tuberculosis katG*; Kan^R, Apr^R, INH^S	this study
pSE-*apr-katG*	derivative of pMV361-*apr-katG* deleted for mycobacteriophage integrase *int* and *attP*; intermediate vector used for cloning of Δ*aac(2′)*, Δ*eis1* and Δ*eis2* alleles; Kan^R, Apr^R, INH^S	this study
pMCS5-*MAB_4395*	intermediate vector containing *aac(2′)* flanking regions; Amp^R	
pSE-Δ*aac(2′)*	suicide vector; derivative of pSE-*apr-katG* carrying Δ*aac(2′)* allele; Apr^R, Kan^R, INH^S	this study
pSE-Δ*eis1*	suicide vector; derivative of pSE-*apr-katG* carrying Δ*eis1* allele; Apr^R, Kan^R, INH^S	this study
pSE-Δ*eis2*	suicide vector; derivative of pSE-*apr-katG* carrying Δ*eis2* allele; Apr^R, Kan^R, INH^S	this study
pMV361-*aac(2′)*	integrative complementation vector for *aac(2′)*, Kan^R	this study
pJB-*apr*	derivative of pMV361 in which the kanamycin resistance cassette was substituted by the apramycin resistance cassette; Apr^R	this study
pJB-*apr-eis2*	integrative complementation vector for *eis2*; derivative of pJB-*apr*; Apr^R	this study

Kan^R, kanamycin resistance cassette; Apr^R, apramycin resistance cassette; Amp^R, ampicillin resistance cassette; INH^S, isoniazid susceptibility cassette.

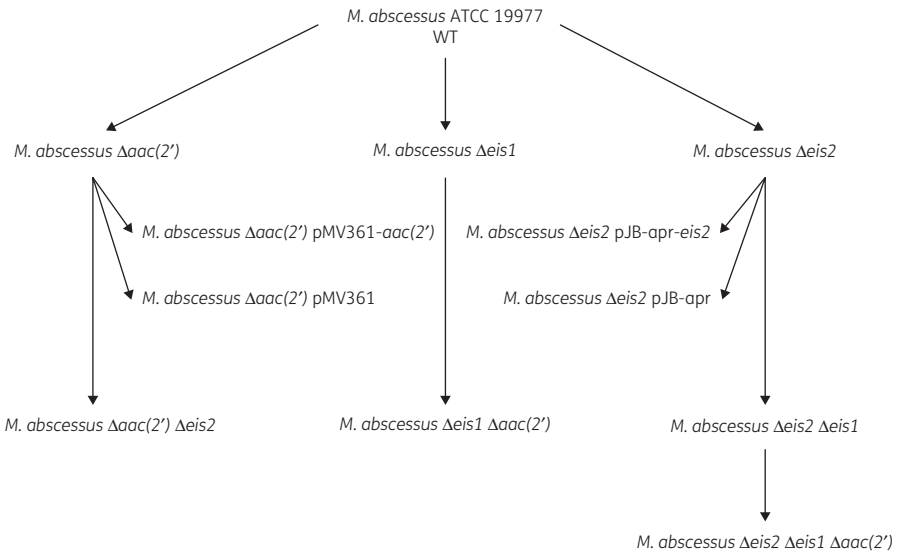


Figure 2. Genealogy of *M. abscessus* strains used in this study for the construction of single, double and triple deletion mutants.

relatively resistant to several AGs with a 2′-amino group. Amikacin is protected from AAC(2′) modification due to the presence of an OH group at position 2′. Actually, amikacin is a derivative of kanamycin A, but with an L-hydroxyaminobutyryl amide (L-HABA) side chain at position 1 of the 2-deoxystreptamine core. Tobramycin (3′-deoxy-kanamycin B) is inert to modification by 3′-phosphotransferases, but susceptible to modification by AAC(2′). This is indicated by the observation that the kanamycin B and tobramycin MICs are similarly decreased for the Δ*aac(2′)* mutant strain as compared with the parental strain. Since tobramycin is not a substrate for the 3′-AG-phosphotransferase [which is encoded on the backbone of the complementation vector pMV361-*aac(2′)*], restoration of the tobramycin phenotype could be addressed by transformation with the complementation vector. Phenotypic complementation was observed, indicating that deletion of *aac(2′)* is responsible for increased tobramycin susceptibility of the mutant. The MIC of arbekacin is similarly low for the WT strain and the Δ*aac(2′)* mutant and similar to the MIC of dibekacin for the Δ*aac(2′)* mutant, while the dibekacin MIC for the WT strain is much higher. Dibekacin (3′,4′-dideoxy-kanamycin B) differs from arbekacin at position 1 of

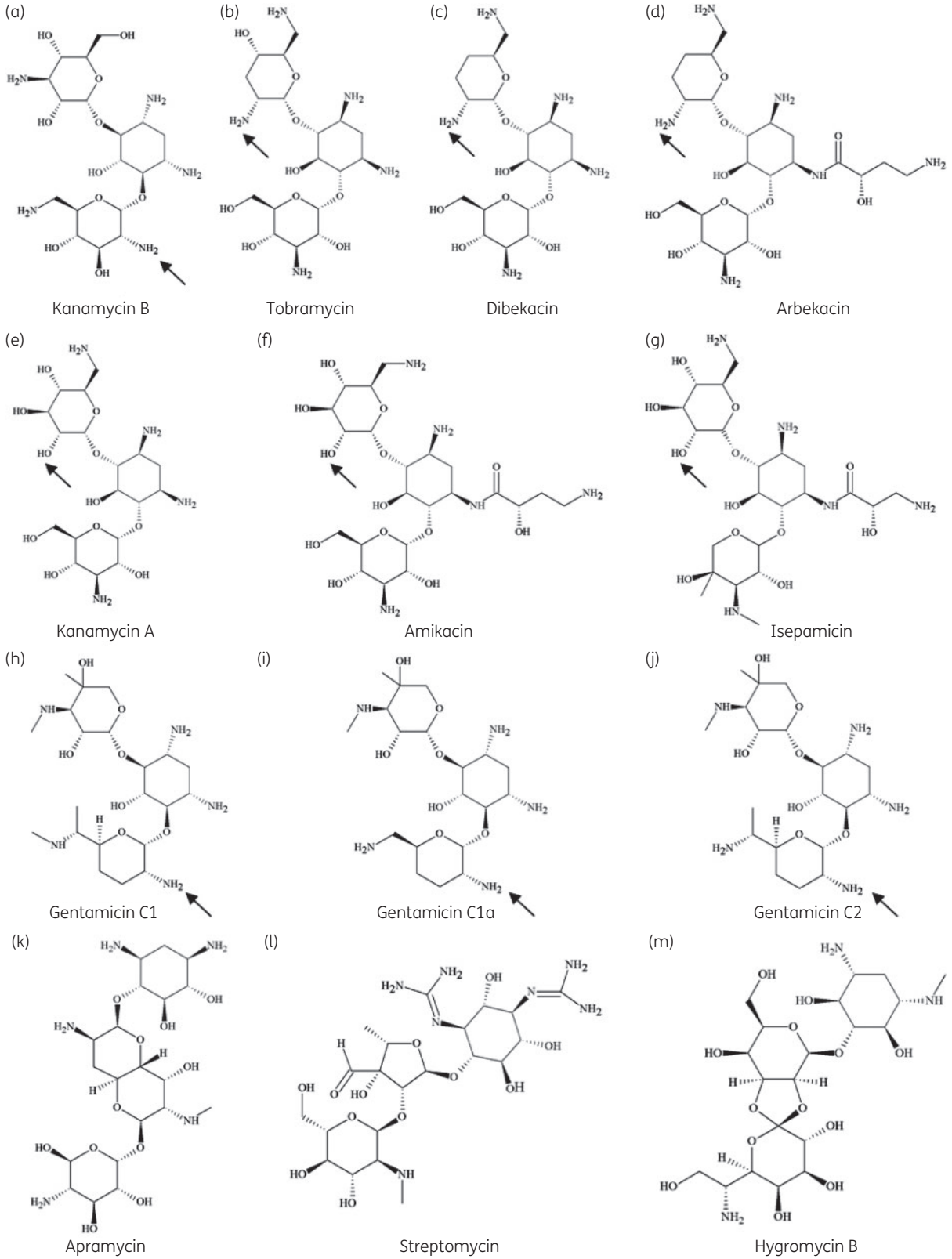


Figure 3. Structure of 4,6-disubstituted (a–j) and atypical (k–m) AGs used in this study. Black arrows show the 2′ groups of the 4,6-distubstituted AGs (a–j).

Table 3. MICs for *M. abscessus* deletion mutants (all values are medians, on day 5)[a,b]

	Strain							
Compound	WT	Δ*aac(2′)*	Δ*eis1*	Δ*eis2*	Δ*aac(2′)* Δ*eis1*	Δ*aac(2′)* Δ*eis2*	Δ*eis1* Δ*eis2*	Δ*aac(2′)* Δ*eis1* Δ*eis2*
Kanamycin B	8	0.125	8	2	0.125	0.125	1	0.125
Tobramycin	8	0.25	8	4	0.25	0.125	2	0.125
Dibekacin	16	1	16	16	2	1	16	1
Arbekacin	1	0.5	1	1	0.5	0.5	0.5	0.5
Gentamicin C	4	1	4	4	1	1	4	1
Isepamicin	1	1	1	0.5	1	0.5	0.5	0.5
Amikacin	2	2	2	0.25	2	0.25	0.25	0.25
Kanamycin A	1	1	1	0.5	1	0.5	0.5	0.25
Apramycin	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5
Streptomycin	32	32	32	32	32	32	32	32
Hygromycin B	256	256	256	16	256	16	16	16
Capreomycin	128	128	256	4	128	4	4	4

[a]For a complete overview of MIC results from three independent experiments judged at days 3, 5, 7 and 12 see Table S2.
[b]For phenotype of single deletion mutants transformed with complementation vectors and control vectors also see Table S2.

the 2-deoxystreptamine core; there arbekacin carries an L-HABA chain, which obviously protects from AAC(2′) activity.

Gentamicin C is not a pure compound, but a mixture of different subclasses (C1, C2 and C1a). C-type gentamicin subgroups carry a 2′-NH_2 group. In contrast, B-type gentamicin carries a 2′-OH group. Isepamicin is a derivative of gentamicin B with an L-HABA side chain at position 1 of the 2-deoxystreptamine core. The MIC for the WT strain of isepamicin is lower than that of gentamicin C, while both compounds have similarly (low) MICs for the Δ*aac(2′)* mutant. These findings may be explained by the enzymatic modification of gentamicin C by the AAC(2′) activity present in the WT strain. Taken together, comparison of the MICs for the WT strain and the Δ*aac(2′)* mutant indicates that AAC(2′) confers resistance to drugs carrying a 2′-NH_2 group, unless position 1 of the 2-deoxystreptamine core is modified with an L-HABA chain (compare dibekacin and arbekacin). Neither deoxygenation at 3′ nor dideoxygenation at positions 3′ and 4′ (compare kanamycin B with tobramycin and dibekacin, respectively) prevent *M. abscessus* AAC(2′)-mediated drug resistance mechanisms. An approximately 10-fold decrease in the MICs of gentamicin C and tobramycin and an even higher decrease in the MIC of dibekacin were also seen upon genetic inactivation of *Mycobacterium smegmatis aac(2′)*.[36]

The *M. abscessus* Δ*eis1* mutant did not show any resistance phenotype and therefore does not contribute to AG resistance, at least not during *in vitro* growth. Expression studies and biochemical assays would be required to address the physiological and enzymatic function of Eis1. It may be hypothesized that promoter-up mutations might increase AG resistance. This hypothesis could also be addressed by transformation of WT strains with multi-copy vectors containing *eis1*. However besides AAC(2′)-mediated AG resistance, *M. abscessus* possesses at least a second mechanism of AG resistance, which is mediated by Eis2. The Δ*eis2* mutant showed increased susceptibility to the peptide antibiotic capreomycin and to a distinct and more heterogeneous group of AGs: kanamycin B, amikacin and hygromycin B. Eis proteins of different origin have been shown to have multiple AG- and capreomycin-acetylating activities, e.g. *M. tuberculosis* and *M. smegmatis* Eis tri-acetylate neamine in a sequential manner, first at position 2′, then at position 6′ and finally at position 1. The number of acetylations depends on the AG itself, but also on the biological origin of the enzyme. Up to four acetyl residues may be transferred to tobramycin by *M. tuberculosis* Eis and *M. smegmatis* Eis, respectively.[33,37,38] Hygromycin is mono- and di-acetylated by *M. smegmatis* and *M. tuberculosis* Eis, respectively. Apramycin is diacetylated by *M. smegmatis*, but is not a substrate for *M. tuberculosis* Eis. *M. abscessus* Eis2 does not inactivate apramycin (identical MICs for the WT strain and the Δ*eis2* mutant), although this does not exclude that apramycin is an *M. abscessus* Eis2 substrate. Corresponding acetylations might just not affect antibacterial activity. A combined application of an AAC(2′) inhibitor together with specific NH_2-AGs (e.g. kanamycin B and gentamicin C) could enhance the activity of these AGs. Administration of an Eis2 inhibitor could enhance the activity of capreomycin and amikacin, the latter being a cornerstone for treatment of *M. abscessus* infections.[39] As opposed to several 2-deoxystreptamine AGs, streptomycin MICs for the mutant strains remained at the high WT MIC level, pointing to a distinct streptomycin resistance mechanism in *M. abscessus*. Our study demonstrates that apramycin, an AG with little ototoxicity,[40] arbekacin, isepamicin and kanamycin A exhibit excellent *in vitro* activities against the *M. abscessus* ATCC 19977 type strain and that the activity of these drugs is not affected by AAC(2′) and Eis proteins, respectively. Our data further support MIC determination of these AGs for a broader set of *M. abscessus* clinical isolates.[15] The results from the suggested *in vitro* studies may provide a rational basis for designing clinical trials aiming at implementation of improved treatment regimens against one of the most drug-resistant pathogens, *M. abscessus*.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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Effect of β-lactamase production and β-lactam instability on MIC testing results for *Mycobacterium abscessus*

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Objectives: Limited treatment options available for *Mycobacterium abscessus* infections include the parenteral β-lactam antibiotics cefoxitin and imipenem, which show moderate *in vitro* activity. Other β-lactam antibiotics (except meropenem) have no considerable *in vitro* activity, due to their rapid hydrolysis by a broad-spectrum β-lactamase (Bla_*Mab*). We here addressed the impact of β-lactamase production and β-lactam *in vitro* stability on *M. abscessus* MIC results and determined the epidemiological cut-off (ECOFF) values of cefoxitin, imipenem and meropenem.

Methods: By LC high-resolution MS (LC-HRMS), we assessed the *in vitro* stability of cefoxitin, imipenem and meropenem. *M. abscessus* ATCC 19977 strain and its isogenic bla_{Mab} deletion mutant were used for MIC testing. Based on MIC distributions for *M. abscessus* clinical strains, we determined ECOFFs of cefoxitin, imipenem and meropenem.

Results: A functional Bla_*Mab* increased MICs of penicillins, ceftriaxone and meropenem. LC-HRMS data showed significant degradation of cefoxitin, imipenem and meropenem during standard antibiotic susceptibility testing procedures. MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are influenced by incubation time.

Conclusions: The results of our study support administration of imipenem, meropenem and cefoxitin, for treatment of patients infected with *M. abscessus*. Our findings on *in vitro* instability of imipenem, meropenem and cefoxitin explain the problematic correlation between *in vitro* susceptibility and *in vivo* activity of these antibiotics and question the clinical utility of susceptibility testing of these chemotherapeutic agents.

Introduction

Mycobacterium abscessus is a dreadful and arduous to treat mycobacterial pathogen with a high level of innate resistance to most commercially available antibiotics, including the antituberculous agents.[1–5] Clinically relevant cases are predominantly associated with pulmonary infections in patients with cystic fibrosis or bronchiectasis and disseminated disease in immunocompromised individuals.[2–4] *M. abscessus* is also highly resistant to disinfectants and therefore it ordinarily causes skin and soft tissue infections following plastic surgery, tattooing or body piercing.[3,5–8] Several healthcare-associated outbreaks of *M. abscessus* infections, that have been reported worldwide, highlight the increasing medical importance of this MDR pathogen and the urgent need for reliable medication strategies.[3,4,9]

As *M. abscessus* clinical isolates are uniformly resistant to standard chemotherapeutic agents, so far, no reliable antibiotic regimen for *M. abscessus* pulmonary infections has been established. Antibiotic administration is empirical and heavily relies on *in vitro* antibiotic susceptibility testing (AST) by broth microdilution and definitive subspecies identification.[3,10] The clinical importance of *M. abscessus* subspecies identification (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) is attributed to the fact that *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* have a functional inducible erythromycin ribosome methyltransferase gene [*erm(41)*], which confers macrolide resistance, whereas *M. abscessus* subsp. *massiliense* has a non-functional *erm(41)*, leading to macrolide susceptibility, and thus *M. abscessus* subsp. *massiliense* infections have more favourable prognosis and treatment outcomes.[10–18] The American Thoracic Society recommends for the treatment of *M. abscessus* lung infections a combination therapy of an oral macrolide (clarithromycin or azithromycin) for clinical isolates susceptible to

macrolides and the intravenous aminoglycoside drug amikacin, administered together with a parenteral β-lactam antibiotic, cefoxitin or imipenem.[3,19–21] However, with the exception of the macrolide class, very limited data are present in the literature concerning the correlation between AST and the clinical efficacy of these recommended antimycobacterial drugs.[22] The clinical relevance of AST remains a controversy, particularly on account of technical problems associated with AST methods, reproducibility of AST results, significant discrepancies between *in vitro* susceptibility and *in vivo* activity of a given drug and solubility and stability issues of the drugs used.[23] Ideally, clinicians could take advantage of AST for *M. abscessus*, when the *in vitro* susceptibility to a drug is consistent with a clinically achievable drug exposure *in vivo*, resulting in favourable treatment outcomes. Accordingly, clinical susceptibility breakpoints must represent MIC distributions and zone diameter distributions for WT and resistant strains, resistance mechanisms, dosing regimens, drug pharmacokinetics, pharmacodynamics and epidemiological cutoff (ECOFF) values and must allow prediction of treatment outcomes.[22–25] CLSI is the only organization worldwide that has published AST guidelines for rapid growing mycobacteria[24] recommending susceptibility testing of amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline or minocycline, imipenem, linezolid, moxifloxacin, trimethoprim/sulfamethoxazole and tobramycin.[25] The breakpoint concentrations for these antibiotics have nonetheless been set on the basis of *in vitro* driven studies and their efficacy (except the new drugs linezolid and tigecycline) has been clinically evaluated by Wallace *et al.*[26] principally in patients with extrapulmonary disease. To date, EUCAST has not officially announced guiding principles for nontuberculous mycobacteria susceptibility testing.

Frequently used therapeutics for *M. abscessus* infections are the parenteral β-lactam antibiotics cefoxitin and imipenem,[24] which have moderate *in vitro* activity, with MIC values reported by Dubée *et al.*[27] for *M. abscessus* subsp. *abscessus* CIP 104536 of 32 and 8 mg/L, respectively. Meropenem, an ultra-broad-spectrum carbapenem closely related to imipenem,[28] displayed a lower MIC value (16 mg/L) than cefoxitin for *M. abscessus* subsp. *abscessus* CIP 104536.[27] Most of the other β-lactam antibiotics have no considerable *in vitro* activity due to their rapid hydrolysis by a broad-spectrum class A β-lactamase, encoded by *MAB_2875*, namely Bla_*Mab*, which was reported as the major determinant of β-lactam resistance in *M. abscessus*.[27,29] Determination of the kinetic parameters of this enzyme revealed that it can slowly hydrolyse imipenem and meropenem, while cefoxitin hydrolysis by Bla_*Mab* is immensely slow, as the methoxy group at cefoxitin's β-lactam ring was predicted to block the activity of class A β-lactamases.[27,29,30]

As imipenem, meropenem and cefoxitin are known to have limited *in vitro* stability,[31] we assessed by LC high-resolution MS (LC-HRMS) the *in vitro* stability of these β-lactams and, by exploiting a bla_{Mab} deletion mutant that we generated, we addressed the biological effect of β-lactam stability and β-lactamase production on MIC testing results after different periods of incubation. The direct impact of β-lactam stability on AST testing was further addressed by MIC determination of fresh and pre-incubated β-lactam antibiotics for the ampicillin-susceptible *Escherichia coli* XL1-Blue MRF′ strain. What is more, based on MIC distributions for *M. abscessus* clinical strains, mainly isolated from respiratory samples, we estimated ECOFFs of cefoxitin, imipenem and meropenem. Our results show that MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are severely influenced by stability issues, thus questioning the clinical utility of AST of cefoxitin, imipenem and meropenem, but not the use of these antibiotics in patients with *M. abscessus* infections.

Materials and methods

Bacterial strains and growing conditions

(i) *E. coli* strains were cultivated in LB medium or on LB agar plates, when necessary, containing either ampicillin (120 mg/L) or apramycin (50 mg/L), at 37 °C, overnight. For *E. coli* MIC testing and all cloning steps when constructing the bla_{Mab} deletion vector pSE-*katG-aac(3)IV*-Δ*MAB_2875*, *E. coli* XL1-Blue MRF′ (Stratagene, Switzerland) was used. (ii) *M. abscessus* strains were grown in Middlebrook 7H9-OADC-Tween 80 liquid medium or on LB agar plates, when needed, containing either apramycin (50 mg/L) or isoniazid (32 mg/L), at 37 °C, for 5 days. The *M. abscessus* ATCC 19977 type strain, the *M. abscessus* Δbla_{Mab} mutant strain and 62 clinical isolates of *M. abscessus* (33 *M. abscessus* subsp. *abscessus*, 17 *M. abscessus* subsp. *bolletii* and 12 *M. abscessus* subsp. *massiliense* isolates) were used throughout this study. The *M. abscessus* clinical isolates were mainly isolated from respiratory samples that were received at the Swiss National Center for Mycobacteria within the years 2007–14.

Definitive subspecies identification of clinical isolates

Subspecies identification of *M. abscessus* isolates (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) was based on the 16S rRNA, *rpoB* and *erm(41)* gene sequences.[12–14,32,33] Subspecies attribution for *M. abscessus* complex isolates was performed according to Tortoli *et al.*[12] The obtained sequences were analysed with the use of Lasergene SeqMan software (DNASTAR, USA), the SmartGene IDNS mycobacteria and *rpoB* databases (SmartGene, Switzerland), and the BLAST algorithm (http://blast.ncbi.nlm.nih.gov).

Antibiotics

Imipenem, meropenem, cefoxitin, ceftriaxone, ceftazidime, penicillin G, ampicillin, amoxicillin, amikacin, apramycin and isoniazid were bought from Sigma–Aldrich, Switzerland. All compounds were dissolved in H_2O, according to the manufacturer's recommendations, filter sterilized, aliquoted into stock solutions of 3–50 g/L and finally stored at −20 °C.

Deletion of MAB_2875 in M. abscessus

A 1.4 kbp HpaI/Pfl23II fragment from position 2926650 to 2928011 (5′bla_{Mab} flanking sequence) and a 1.5 kbp Pfl23II/Pfl23II fragment from position 2928642 to 2930147 (3′bla_{Mab} flanking sequence) were PCR amplified using genomic DNA from *M. abscessus* ATCC 19977 (5′-GAATTAGTTAACCAGGTGTGATCCAGATGCCCG-3′, 5′-GAATTTCGTACGGGCCGCCGAAATCCTTTTCC-3′ and 5′-GAAATACGTACGCCATCGTGATGGCGGTACTCAC-3′, 5′-GAATTTCGTACGGTGTCTACCAGTCCTTGCACACC-3′, respectively) and cloned into the pSE-*katG-aac(3)IV* vector with corresponding enzymes, resulting in the bla_{Mab} deletion vector pSE-*katG-aac(3)IV*-Δ*MAB_2875*. *M. abscessus* bla_{Mab} deletion mutant was generated using a double selection strategy described previously in detail by Rominski *et al.*[34,35] In brief, pSE-*katG-aac(3)IV*-Δ*MAB_2875* was transformed into electrocompetent *M. abscessus* ATCC 19977. Competent cells (100 μL) were mixed with 1–2 μg of plasmid DNA (supercoiled) and electroporated in a Bio-Rad Gene pulser II using 4 mm gap electroporation cuvettes and the settings: 2.5 kV, 1000 Ω and 25 μF.[36] Cells were resuspended after electroporation in 0.9 mL of 7H9-OADC-Tween 80 liquid medium and incubated for 5 h at 37 °C. Proper dilutions were eventually

plated on selective agar and, after 5 days of incubation, single colonies were picked and restreaked on LB agar plates with appropriate antibiotics. Transformants were selected on LB agar plates containing apramycin and identified by *aac(3)IV* colony PCR (primers: 5′-CACCTTCTTCACGAGG CAGACCTC-3′ and 5′-GGTCTGACGCTCATGGAACTAGTAGG-3′). Isolation of genomic DNA was performed by phenol-chloroform-isoamyl alcohol extraction as described previously[37] and single crossover transformants were confirmed by Southern blot analysis with a 0.45 kbp SacI 3′bla_{Mab} DNA probe and subjected to counterselection on LB agar plates containing isoniazid. Single colonies were screened for deletion of *MAB_2875* by PCR (primers: 5′-GTACACCGTCTTCGGGACG-3′ and 5′-GAAAGTGCGAGTACGCGTCTG-3′) and their genotype was finally verified by Southern blot analysis using the same 0.45 kbp SacI 3′bla_{Mab} DNA probe. In this way, a 0.63 kbp region of the *MAB_2875* was deleted from the genome of *M. abscessus* ATCC 19977.

Nitrocefin test

For the detection of β-lactamase production in the *M. abscessus* ATCC 19977 type strain and the Δbla_{Mab} mutant strain we performed a chromogenic nitrocefin test (Becton Dickinson, USA). Tubes containing (i) pure nitrocefin solution, (ii) nitrocefin solution inoculated with five colonies of the *M. abscessus* ATCC 19977 type strain and (iii) nitrocefin solution inoculated with five colonies of the *M. abscessus* Δbla_{Mab} mutant were incubated for 1 h at room temperature before a photo was taken.

MIC assays

MIC assays were performed with the microdilution method and according to CLSI guidelines,[25] but with incubation of the 96-well microtitre plates at 37 °C, as described previously.[34,35] Directly after the preparation of the antibiotic stock solutions, working solutions were prepared by diluting the corresponding stock solutions in CAMHB (pH 7.4) (Becton Dickinson, Switzerland) to a concentration of 512 mg/L (final antibiotic concentration used for AST; 256 mg/L) and were subsequently stored at −80 °C. Shortly before the conduct of an MIC experiment (day 0), working solutions were thawed for the first time and were directly used. Alternatively, working solutions were thawed and pre-incubated for 1–7 days at 37 °C and their inhibitory effect was compared with freshly thawed antibiotics. Two-fold serial dilutions of the freshly thawed (and pre-incubated: for the pre-incubation MIC experiments) working solutions were prepared using CAMHB in sterile 96-well microtitre plates (Greiner Bio-One, Switzerland). Each 96-well microtitre plate included a positive growth control lacking antibiotic and a sterile negative control containing only CAMHB. To achieve a final inoculum titre of 1–5×10^5 cfu/mL, while final volume per well was 100 μL, bacterial suspensions of strains with smooth or rough phenotypes were adjusted to a McFarland standard of 0.5 and 3.0, respectively, and subsequently diluted in CAMHB. The proper titre of the inocula was confirmed by obtaining cfu counts on LB agar plates. MIC values were assessed by visual inspection after incubation at 37 °C for 16 h for *E. coli* strains and 3, 5, 7 and 12 days for *M. abscessus*. MIC assays for *E. coli*, *M. abscessus* ATCC 19977 and the *M. abscessus* bla_{Mab} deletion mutant were conducted in triplicate. MIC assays for *M. abscessus* complex clinical isolates were performed once.

LC-HRMS assays

In vitro antimicrobial stability of cefoxitin, imipenem and meropenem was evaluated using CAMHB and the maximum final antibiotic concentration used for AST (256 mg/L). A different test tube was prepared for each antibiotic and for every reading timepoint, i.e. 3, 5, 7 and 12 days. A day 0 control was used to define the 100% relative antibiotic concentration. The antibiotic-containing test tubes were incubated under the same temperature conditions as the microtitre plates used for AST (37 °C). Cefoxitin, imipenem and meropenem were quantified by LC-HRMS on a Q Exactive hybrid instrument (Thermo Fisher, Switzerland). Samples were precipitated using a precipitation solution consisting of methanol/acetonitrile (80/20, v/v), containing the corresponding stable-isotope labelled internal standards. After centrifugation at 11700 **g** for 10 min at 4 °C, 10 μL of the clear supernatant was injected into the turbulent flow online extraction system. As extraction column a Cyclone column (50×0.5 mm) was used and for analytical chromatography a Hypersil Gold C8 column (100×3 mm) was used. Mobile phases consisted of 10 mM ammonium acetate in water + 0.1% formic acid and 10 mM ammonium acetate in methanol/acetonitrile (50/50, v/v) + 0.1% formic acid. Samples were analysed in positive heated electrospray ionization mode and detection was done in full-scan mode with a resolution of 70000 full width at half maximum (calculated for *m/z* 200).

ECOFF determination

MIC data for 62 *M. abscessus* complex clinical isolates were collected and the resistance population analysis charts were calculated using the integrated histographical analysis tool of Microsoft Excel. Median MIC_{50} values of cefoxitin, imipenem and meropenem for the *M. abscessus* complex clinical strains were calculated using SPSS software. All ECOFF values were determined by the eyeball method.[38,39]

Results and discussion

Generation of the M. abscessus MAB_2875 deletion mutant

Similarly to our previously published techniques,[34,35] we intended to generate an *M. abscessus* Δ*MAB_2875* mutant, which would enable us to address directly the role of the β-lactamase production of *M. abscessus* on MIC testing results of β-lactam antibiotics. The bla_{Mab} deletion mutant was constructed by transformation of *M. abscessus* ATCC 19977 with plasmid pSE-*katG-aac(3)IV-*Δ*MAB_2875* applying an apramycin-positive selection[40] and a *katG*-dependent isoniazid counterselection strategy (Figure 1a).[34,35] Deletion of *MAB_2875* was confirmed by Southern blot analysis (Figure 1b). While this study was ongoing, a Δ*MAB_2875* mutant was also generated by Dubée *et al.*,[27] but with different cloning, genetic manipulation and selection strategies.

M. abscessus bla_{Mab} deletion mutant is deficient in β-lactamase production

To assess the β-lactamase production of the *M. abscessus* Δbla_{Mab} mutant, we performed a chromogenic nitrocefin test. The cephalosporin nitrocefin changes colour from yellow to red in the presence of β-lactamases that hydrolyse the amide bond in its β-lactam ring.[41] As Figure 2 shows, the tube containing yellow pure nitrocefin solution (Figure 2a) did not change colour. The tube containing nitrocefin solution inoculated with *M. abscessus* ATCC 19977 (Figure 2b) changed colour and became red, while the tube containing nitrocefin solution inoculated with the *M. abscessus* Δbla_{Mab} mutant (Figure 2c) stayed yellow, confirming that the *M. abscessus* bla_{Mab} deletion mutant is deficient in β-lactamase production. These results align with the findings from Dubée *et al.*,[27] who reported Bla_*Mab* as the single determinant of β-lactam resistance in *M. abscessus*.

AST of M. abscessus ΔMAB_2875

To explore the role of Bla_*Mab* in *M. abscessus* β-lactam resistance, MIC values for the *M. abscessus* ATCC 19977 type strain and the *M. abscessus* Δbla_{Mab} mutant were determined with the microdilution method for the β-lactam antibiotics, imipenem,

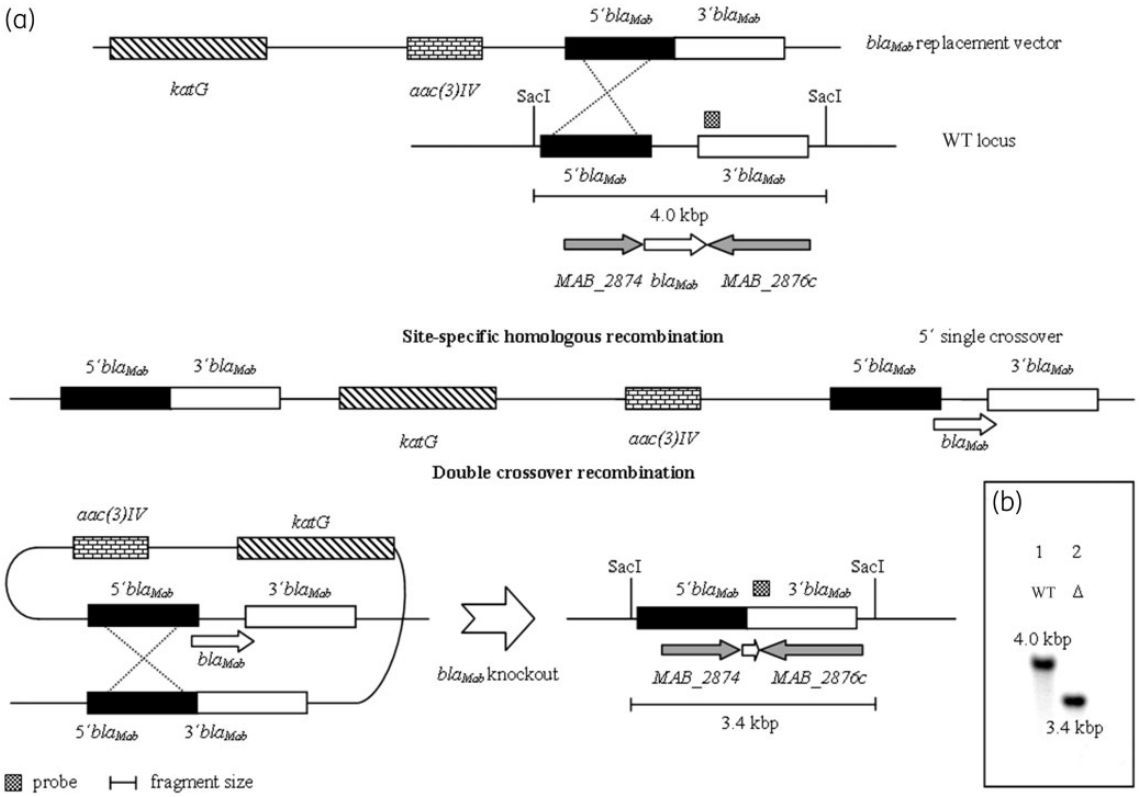

Figure 1. Genotypic analyses of the *M. abscessus bla*$_{Mab}$ locus. (a) Schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirmed the deletion of *MAB_2875* from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1) and *M. abscessus* transformant with pSE-*katG-aac(3)IV-*Δ*MAB_2875* targeting vector after KatG-dependent isoniazid counterselection (2) was digested with SacI and probed with a 0.45 kbp fragment from the 3′ *bla*$_{Mab}$ flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 4.0 kbp fragment of the WT parental strain and to a 3.4 kbp fragment of the *M. abscessus* Δ*bla*$_{Mab}$ mutant (Δ).

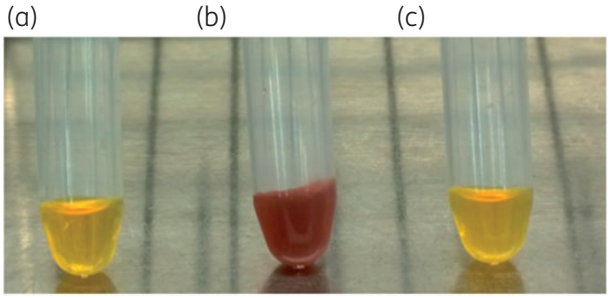

Figure 2. Chromogenic nitrocefin solution test results after incubation for 1 h at 37 °C. (a) Tube containing pure nitrocefin solution. (b) Tube containing nitrocefin solution inoculated with *M. abscessus* ATCC 19977 WT. (c) Tube containing nitrocefin solution inoculated with the *M. abscessus* Δ*bla*$_{Mab}$ mutant. NCF, nitrocefin. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

meropenem, cefoxitin, ceftriaxone, ceftazidime, penicillin G, ampicillin and amoxicillin, and a control antibiotic from the class of aminoglycosides, i.e. amikacin (Table 1). As formerly reported,[27] *M. abscessus* ATCC 19977 was highly resistant to all β-lactam antibiotics tested except imipenem, meropenem and cefoxitin. Deletion of *bla*$_{Mab}$ significantly reduced the MIC values of all penicillins tested (penicillin G, ampicillin and amoxicillin) and the cephalosporin ceftriaxone. MIC values of ≥256 mg/L of the oxyimino-cephalosporin ceftazidime for both *M. abscessus* ATCC 19977 and the *M. abscessus* Δ*bla*$_{Mab}$ mutant suggest that ceftazidime is not active against *M. abscessus*. Cefoxitin MIC values of 32 mg/L (day 3) for both *M. abscessus* ATCC 19977 and the Δ*bla*$_{Mab}$ mutant (Table 1) agree with earlier reported data,[27] corroborating previous molecular modelling and structural analyses,[42] which delineated that substitutions of the β-lactam ring by a methoxy group in cefoxitin inhibits the activity of class A β-lactamases.[30] As expected, amikacin MICs were independent of the *bla* genotype.

To date, none of the recent studies on β-lactam susceptibility testing of *M. abscessus* CIP 104536 and the *M. abscessus* Δ*bla*$_{Mab}$ mutant has reported MIC data obtained after 3 days of incubation.[27,29,30,43,44] Indeed, CLSI guidelines[25] for imipenem MIC testing for rapid growing mycobacteria recommends a maximum incubation period of 3 days and state that 'the reported breakpoints for imipenem are considered tentative'. Our observation that imipenem, meropenem and cefoxitin MIC values rise substantially with the incubation time for both the *M. abscessus* ATCC 19977 strain and the *M. abscessus* Δ*bla*$_{Mab}$ mutant (Table 1), in correlation with the fact that certain antibiotics undergo partial degradation

under *in vitro* testing conditions,[45] led us to investigate further the effect of the β-lactam stability on MIC testing procedures.

Effect of β-lactam stability on E. coli MIC testing

To determine experimentally whether the results of the *M. abscessus* MIC testing of β-lactams after 3, 5 and 7 days of incubation clearly reflect β-lactamase production or are affected by instability of the antibiotics tested, we performed an MIC experiment using the *E. coli* XL1-Blue model strain, which produces no β-lactamases, and fresh and pre-incubated (at 37 °C, for 1–7 days) β-lactam antibiotics. In that way, all MIC results could be obtained after 16 h of incubation of the microtitre plates and putative MIC differences between the fresh and the pre-incubated antibiotics could be directly correlated to instability of the tested antibiotics. Our results show that pre-incubation at 37 °C affects MIC results of imipenem, meropenem and to a lesser extent cefoxitin and ceftazidime (Figure 3a and b), whereas the MIC results of ceftriaxone, penicillin G, ampicillin, amoxicillin and as expected the control stable aminoglycoside antibiotic amikacin are not influenced by pre-incubation (Figure 3b, c and d). By assuming that drug degradation follows an exponential decay process and using the data displayed in Figure 3, we calculated the half-life values of imipenem as 0.75 days, of meropenem as 1.5 days and of cefoxitin as 3 days. We therefore conclude that the increased MIC values of the pre-incubated antibiotics, already before day 3, reflect instability of the antibiotics. Reading of *M. abscessus* MICs at day 3 and later gives a *de facto* misleading impression for the true extent of activity of imipenem, meropenem and cefoxitin.

Table 1. AST results of *M. abscessus* WT and Δbla_{Mab} mutant (broth microdilution method)

	MIC (mg/L)							
	M. abscessus ATCC 19977				*M. abscessus* Δbla_{Mab}			
Antibiotic	day 3	day 5	day 7	day 12	day 3	day 5	day 7	day 12
Imipenem	2	4	8	64	1	2	4	64
Meropenem	8	16	32	256	2	4	4	16
Cefoxitin	32	32	64	128	32	32	32	128
Ceftriaxone	64	64	64	64	8	16	16	16
Ceftazidime	>256	>256	>256	>256	256	>256	>256	>256
Penicillin G	>256	>256	>256	>256	2	4	4	4
Ampicillin	>256	>256	>256	>256	4	4	8	8
Amoxicillin	>256	>256	>256	>256	2	4	4	4
Amikacin	1	2	4	8	1	2	4	8

In vitro antimicrobial stability of cefoxitin, imipenem and meropenem by LC-HRMS

Based on our results suggesting that incubation of cefoxitin, imipenem and meropenem at 37 °C gives rise to misleading MIC

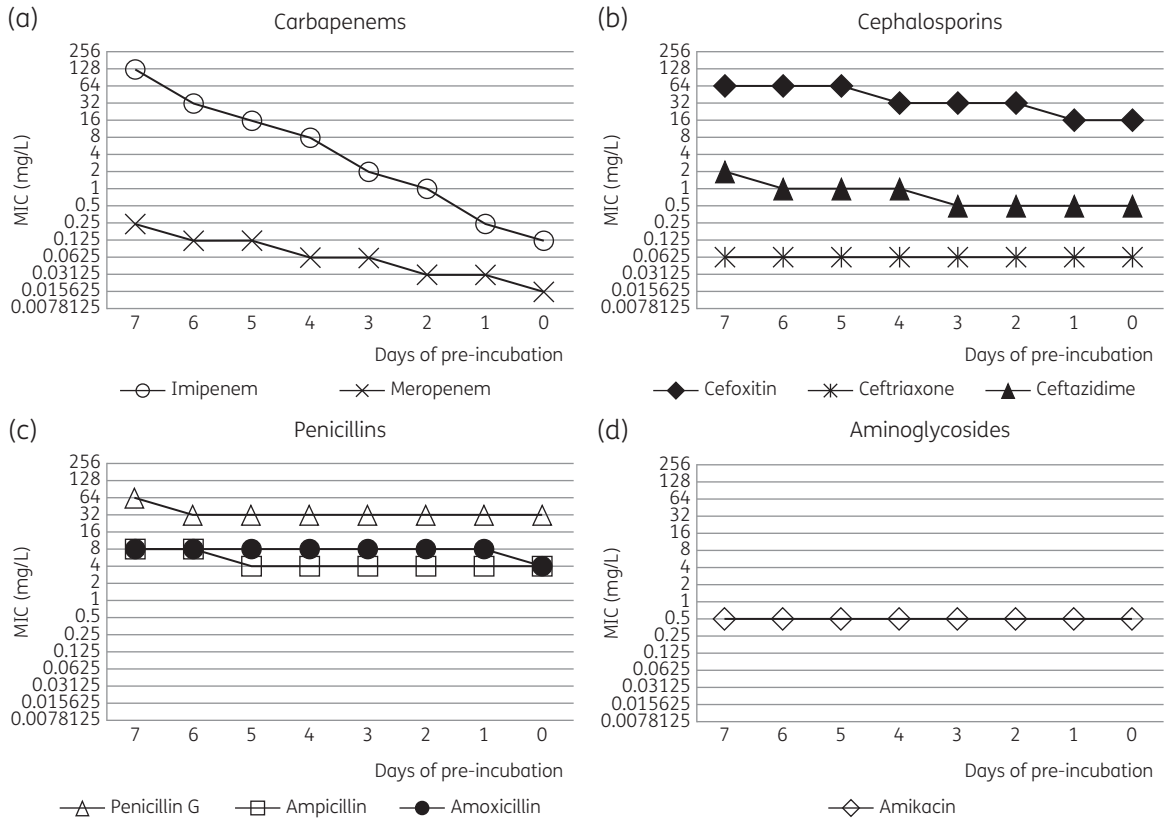

Figure 3. MIC values for *E. coli* XL1-Blue strain of fresh and pre-incubated (at 37 °C, for 1–7 days) β-lactam antibiotics. (a) Carbapenems: imipenem and meropenem. (b) Cephalosporins: cefoxitin, ceftriaxone and ceftazidime. (c) Penicillins: penicillin G, ampicillin and amoxicillin. (d) Aminoglycoside (control): amikacin.

results, we intended to address the *in vitro* stability of these β-lactams, by quantifying them using LC-HRMS. Tubes containing CAMHB and the maximum antibiotic concentration used for AST (256 mg/L) were incubated at 37 °C. The 100% relative antibiotic concentration of each antibiotic was defined by a day 0 control tube. On days 3, 5, 7 and 12 the concentrations of cefoxitin, imipenem and meropenem were quantified. Data obtained by the LC-HRMS method, clearly show degradation of cefoxitin, imipenem and meropenem. Particularly, the relative concentration on day 3 for imipenem dramatically dropped to 3.3%, whereas for cefoxitin and meropenem it dropped to 36.6% and 35.6%, respectively (Figure 4e, j and o). By using the results of this assay, the calculated half-life of imipenem was 0.6 days, that of meropenem was 2 days and that of cefoxitin was 2.1 days. These findings agree with those deducted from the *E. coli* MIC testing and confirm that the MIC values of cefoxitin, imipenem and meropenem for *M. abscessus* on day 3 and later are misleading, as concentration of the active compound is lowered. Particularly for imipenem, MIC_{50} on day 3 was 16 mg/L (Figure 4j). However, growth inhibition of half of the clinical isolates was in reality achieved by considerably lower drug amounts, as the true active relative concentration of imipenem shrank to 3.3% by day 3 due to the short half-life of this antibiotic. Consequently, due to instability of imipenem, meropenem and cefoxitin, *M. abscessus* MIC determination after 3 days of incubation does not reflect the potency of these three compounds, particularly as clinical administration[3] of these drugs is daily and in multiple doses. These data, on the one hand, question the utility of susceptibility testing of imipenem, meropenem and cefoxitin, but, on the other hand, support administration of these parenteral β-lactam antibiotics as part of combinational regimens for the treatment of *M. abscessus* infections.

ECOFF values of cefoxitin, imipenem and meropenem for M. abscessus complex

According to EUCAST, for the determination of clinical susceptibility breakpoint values and improvement of the MIC interpretation for clinical isolates, ECOFFs need to be defined to separate the WT population from any non-WT strains with acquired drug resistance mechanisms to the chemotherapeutic agents in question.[46] We therefore endeavoured to estimate ECOFF values of cefoxitin, imipenem and meropenem by visual inspection of the MIC histographic distribution analysis (eyeball method)[36,37] for 62 *M. abscessus* complex clinical strains isolated from respiratory samples, after 3, 5, 7 and 12 days of incubation at 37 °C (Figure 4). The results show that MIC distributions for our collection of *M. abscessus* complex isolates of cefoxitin, imipenem and meropenem are close to the breakpoints established by CLSI and that the eyeball-estimated ECOFF values did not show a separation of the *M. abscessus* complex isolates into two distinct subgroups, WT and resistotype. ECOFFs were set at the highest MIC value observed among the isolates tested and all of these isolates are therefore classified in the WT subgroup. According to this classification, a putative clinical isolate with an additional β-lactam resistance mechanism would have a very high MIC value (higher than the corresponding ECOFF) that would be neither detected by standard AST laboratory procedures nor relevant for clinical interpretation. Interestingly, the presence or absence of a functional or non-functional β-lactamase in *M. abscessus* could not be predicted by the MIC distribution for the clinical isolates, as the MIC values determined for *M. abscessus* Δbla_{Mab} (Table 1) fall in the MIC distribution for the 'WT' subgroup of clinical isolates of all three β-lactams (Figure 4).

Furthermore, our results show that MIC and MIC_{50} values of cefoxitin, imipenem and meropenem are greatly influenced by incubation time; the longer the incubation period, the higher the MIC for the individual strains, the MIC_{50} and the estimated ECOFF values (Figure 4). The presented MIC distribution bars, as well as the MIC_{50} and ECOFF lines, would represent valid data for MIC, MIC_{50} and ECOFF values, only if the drugs were 100% stable. However, according to the LC-HRMS results, cefoxitin, meropenem and particularly imipenem were proven as unstable compounds and thus MIC values for the *M. abscessus* complex clinical isolates of these three β-lactams on day 3 and later are certainly overestimated, as already discussed. If the concentration of the biological active form of the drug could have been kept constant during the AST procedures, we expect that the position of the MIC distribution bars and MIC_{50} and ECOFF lines would be shifted to the left in all MIC distribution analysis graphs (Figure 4). Our observations highlight the impact of cefoxitin, imipenem and meropenem stability issues on MIC, MIC_{50} and ECOFF determination for *M. abscessus* complex clinical isolates.

Conclusions

Taken together, our study confirmed Bla_*Mab* as the major determinant of innate β-lactam resistance in *M. abscessus* and addressed experimentally the biological effect of β-lactamase production and β-lactam stability on *M. abscessus* MIC testing results after different incubation periods. Our results show that MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are immensely influenced by incubation time. LC-HRMS data and MIC determination of pre-incubated drugs for the fast growing model organism (*E. coli*) proved significant degradation of cefoxitin, imipenem and meropenem during standard AST procedures, explaining the problematic correlation between *in vitro* susceptibility of these three β-lactams and their *in vivo* activity. Our findings critically question the clinical utility of cefoxitin, imipenem and meropenem susceptibility testing, but further support administration of these chemotherapeutic agents for the treatment of *M. abscessus* infections.

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Transparency declarations

None to declare.

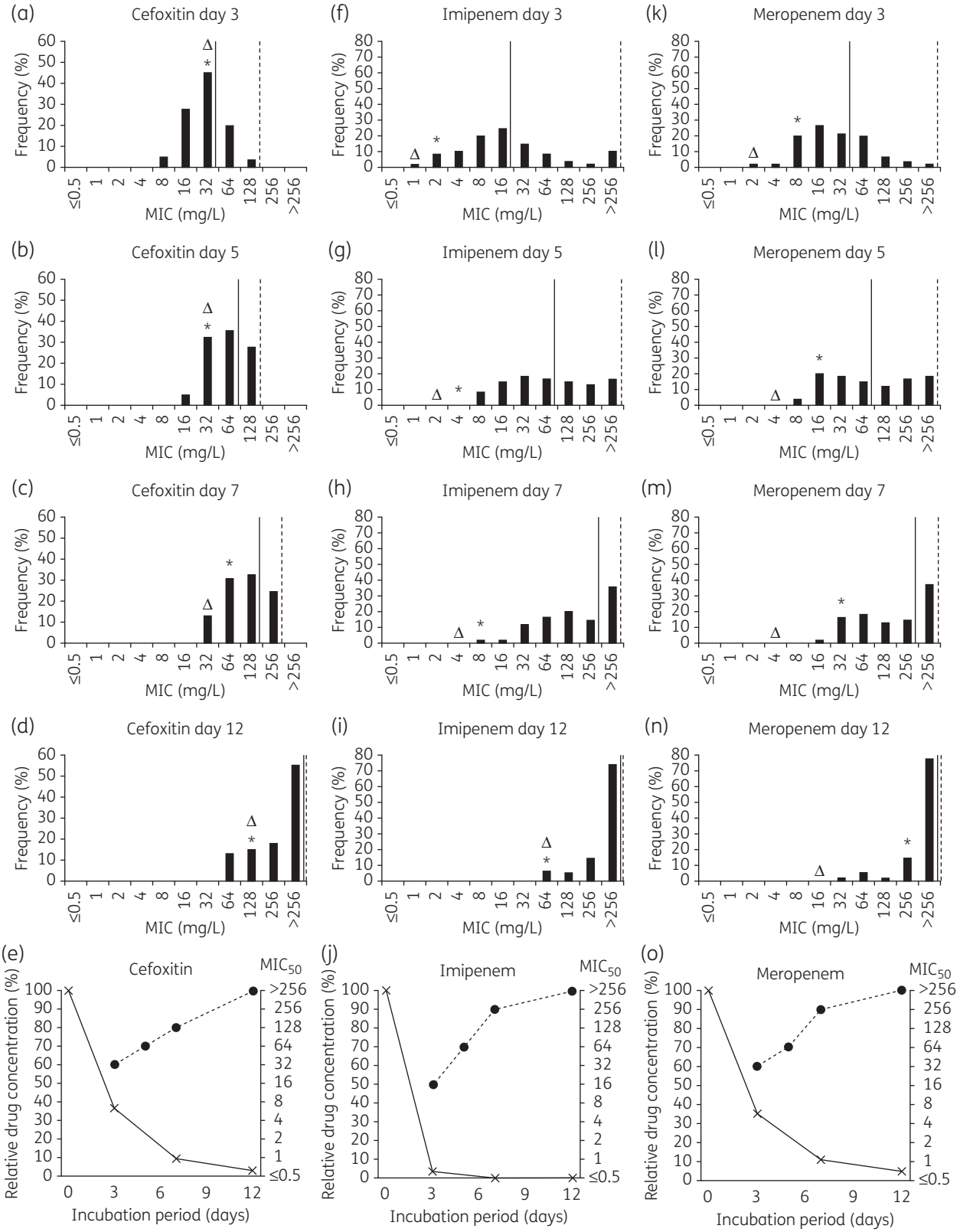

Figure 4. MIC distributions of cefoxitin (a–d), imipenem (f–i) and meropenem (k–n) for *M. abscessus* complex strains ($n = 62$). MIC_{50} values are indicated by a black vertical continuous line, whereas ECOFF values are indicated by a black vertical broken line. *, MIC values for the *M. abscessus* ATCC 19977 type strain; Δ, MIC values for the *M. abscessus* Δbla_{Mab} mutant. Drug stability of cefoxitin (e), imipenem (j) and meropenem (o) in comparison with median MIC_{50} values. (e, j and o) Crosses connected with a black continuous line show relative drug concentrations and filled circles connected with a black broken line show MIC_{50} values.

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