Mycobacterium abscessus Smooth And Rough Morpohotypes Form Antimicrobial-Tolerant Biofilm Phenotypes But Are Killed by Acetic Acid

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Abstract

*Mycobacterium abscessus* has emerged as an important pathogen in people with chronic inflammatory lung diseases such as cystic fibrosis, and recent reports suggest that it may be transmissible by fomites. *M. abscessus* exhibits two major colony morphology variants: a smooth morphotype (*Ma*<sup>Sm</sup>) and a rough morphotype (*Ma*<sup>Rg</sup>). Biofilm formation, prolonged intracellular survival, and colony variant diversity can each contribute to the persistence of *M. abscessus* and other bacterial pathogens in chronic pulmonary diseases. A prevailing paradigm of *M. abscessus* chronic infection is that *Ma*<sup>Sm</sup> is a noninvasive, biofilm-forming, persistent phenotype and *Ma*<sup>Rg</sup> an invasive phenotype unable to form biofilms. We show that *Ma*<sup>Rg</sup> is hyper-aggregative and forms biofilm-like aggregates, which, like *Ma*<sup>Sm</sup> biofilm aggregates, are significantly more tolerant than planktonic variants to acidic pH, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and to treatment with amikacin or azithromycin. We further show that both variants are recalcitrant to antibiotic treatment inside human macrophage-like cells and that *Ma*<sup>Rg</sup> is more refractory than *Ma*<sup>Sm</sup> to azithromycin. Our results indicate that biofilm-like aggregation and protracted intracellular survival may each contribute to the persistence of this problematic pathogen in the face of antimicrobial agents regardless of morphotype. Biofilms of each *M. abscessus* variant are rapidly killed, however, by acetic acid, which may help to prevent local fomite transmission. [209]
Introduction

*Mycobacterium abscessus* is a pathogenic nontuberculous mycobacterium (NTM) and the leading cause of infection attributed to rapidly growing mycobacteria (RGM). *M. abscessus* can cause skin and soft tissue infections in patients with healthy immune systems as well as a variety of infections on medical implants. It has recently gained attention as the most common cause of RGM infections worldwide in people with chronic inflammatory lung diseases such as cystic fibrosis (CF), non-CF bronchiectasis, and chronic obstructive pulmonary disease (COPD), resulting in both nodular and cavitary granulomas and persistent lung infection. Unlike many NTM infections, antibiotic therapy often fails to lead to lasting sputum conversion and no antibiotic regimen reliably cures *M. abscessus* infection. A recent systematic review of NTM pulmonary disease (PNTM) found that without adjunctive surgical resection, the rate of sputum conversion (SC) with medical treatment alone of *M. abscessus* infection was 34% in studies that specifically determined *M. abscessus sensu stricto*, SC rates were even lower (25-32%). Mortality due to PNTM in the US has increased by over 8% per year and now outpaces mortality due to tuberculosis, with PNTM fibrocavitary disease concomitant with increased severity of disease and mortality. Pathogenic RGM such as *M. abscessus*, *Mycobacterium chelonae* and *Mycobacterium fortuitum* are widely distributed in the environment, often in nutrient-poor, low pH environments. These NTM are also difficult to eradicate in nosocomial settings. We have previously shown that pathogenic RGM readily form aggregated structures (biofilms) by colonizing surfaces independently of other microorganisms. Biofilms may contribute to the transmission of RGM by protecting bacteria from desiccation and by harboring high numbers of
bacteria, which if aerosolized may lead to the inhalation of a condensed infective dose in aerosolized aggregates. Recent reports suggest that the increase in *M. abscessus* infections may be due to the global emergence of transmissible virulent clones that are possibly spread by aerosols or by fomites. Understanding the virulence mechanisms of *M. abscessus* is therefore clinically relevant, particularly with regard to pulmonary infections.

Biofilm formation, extended intracellular survival and colony variant diversification each can contribute to the persistence of select bacterial pathogens in CF. CF pathogens also share important traits in pathoadaptation to the CF airway, including antibiotic tolerance and evasion of innate immune effectors. For example, *Pseudomonas aeruginosa*, the most common pathogen causing lung infections in CF patients, forms biofilms that protect bacteria from antibiotic therapy and from effective host clearance during chronic lung infection.

*P. aeruginosa* colony morphology variants isolated from CF sputum include mucoid colonies and aggregative rugose small colony variants, both of which are linked to extended antibiotic treatment and correlate with the onset of persistent infection. *M. abscessus* biofilm aggregates have recently been demonstrated in the lungs of patients with CF, non-CF bronchiectasis and COPD. *M. abscessus* exhibits two colony morphology variants: a smooth colony variant (*Ma*<sup>sm</sup>) that expresses glycopeptidolipid (GPL) on its cell wall, and a rough colony variant (*Ma*<sup>rg</sup>) with diminished GPL expression on the cell surface. Both variants are found in patients with chronic lung infections, however the *Ma*<sup>rg</sup> variant is associated with more aggressive pulmonary disease and is hyper-virulent in a zebrafish infection model. Previous research using an *M. abscessus* clinical isolate showed that a smooth variant formed biofilms, but a rough variant did not. These studies led to the
proposition that GPL expression enhanced $Ma^{Sm}$ sliding motility in CF mucus and was a colonizing, biofilm-forming phenotype, whereas $Ma^Rg$ was a non-biofilm forming, invasive phenotype. According to this paradigm persistent infection with *M. abscessus* is thought to be due to the transition (switching) between $Ma^Rg$ and $Ma^{Sm}$ colony variants. More recently genomic sequencing studies showing extensive disruption of the GPL locus in $Ma^Rg$ have cast doubt on the ability of $Ma^Rg$ to transition to a $Ma^{Sm}$ variant. Furthermore, studies with many bacteria show that although biofilm phenotypes may be non-motile, aggregation is necessary for antimicrobial tolerance.

Biofilms are most often described as assemblages of microbial cells that are attached to a surface. However biofilms also form as suspended aggregates at air–liquid interfaces and non-adherent aggregated bacteria show a similar antibiotic tolerance phenotype to adherent aggregates. In CF lung infections the majority of aggregated P. aeruginosa are found within the inspissated mucus in larger airways, rather than adherent to pulmonary epithelium. A consensus definition of biofilm development as inherently anchored to a substratum has recently been modified to accommodate biofilm-associated infections and growing evidence that free floating biofilm aggregates can profoundly affect the interplay between nutrient resources, spatial structure, bacterial fitness, and multicellular assembly.

We hypothesized that *M. abscessus* antimicrobial recalcitrance was not restricted to one colony morphology variant and investigated each morphotype using isogenic $Ma^{Sm}$ and $Ma^Rg$ variants isolated from the sequenced *M. abscessus* ATCC# 19977 reference strain. Our results show that each *M. abscessus* colony variant formed biofilms that exhibited antimicrobial tolerance and that neither biofilm formation, nor prolonged survival inside macrophages is
morphotype-restricted. $Ma^{Rg}$, however, is more refractory than $Ma^{Sm}$ to antimicrobial treatment overall. We also show that although $M. abscessus$ variants in biofilm-like aggregates are significantly more tolerant to antimicrobial treatment, biofilms were rapidly killed with acetic acid, which may prevent the potential transmission of $M. abscessus$ in clinical settings.

**Materials and Method**

**Bacteria**

$M. abscessus$ ATCC 19977 was obtained from American Type Culture Collection (Manassas, VA), reconstituted and grown as directed for the establishment of frozen stocks. Since $M. abscessus$ contains a mixture of both smooth and rough morphotypic variants, frozen stock was streaked to isolation and individual variants cultured on 7H10 agar containing 10%OADC and glycerol. For frozen stocks each morphotype was scraped into 7H9 broth containing 10%OADC/20%glycerol and frozen at -80°C. For mCherry transformation, each morphotype was grown, rinsed, and a mCherry cassette containing kanamycin-resistance (provided by Sarah Fortune, Harvard University) electroporated into $M. abscessus$ and transformants selected on kanamycin-containing selective agar medium. Electrocompetent 19977 were prepared by growing bacterial cultures to mid-log phase (OD600 = 1.0) and harvesting the bacterial cells by centrifugation at 4°C, 2000xg for 10 min. Cells were washed 3 times in freshly prepared sterile-filtered, ice-cold 10% glycerol/H$_2$O and pellets were then resuspended in 1 mL ice-cold 10%glycerol/H$_2$O. Electroporation of mCherry plasmid construct DNA was carried out using a
Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA) set at 2500 V, 1000 Ω, 25 μF, according to manufacturer’s instructions. Briefly, 0.5 -1.0 μg of DNA was added to 200 μL of washed electrocompetent cells, mixed gently in an electroporation cuvette, incubated on ice, and inserted into electroporation chamber followed by an electrical pulse. Transformants were grown on 7H10 containing 100 μg/mL kanamycin for 3-5 days and frozen stocks prepared as above. Bacteria were grown from frozen individual colony variant stocks on 7H10 plates for 5-7 days at 37°C/5%CO₂. Morphotypes were checked using a stereoscopic microscope (Stereo Zoom Microscope, AmScope, USA) and plated separately for use in experiments.

Planktonic and biofilm growth

Single cells of each M. abscessus morphotype were obtained by direct counts as previously described⁴⁷. Briefly, for each experiment, isolated morphotypic colony variants were placed in Eppendorf tubes, each containing 1 mL of 7H9/OADC/0.5%Tween 80, pulse vortexed with glass beads and allowed to settle for 30 min, after which 600μL of supernatant was transferred to a second tube. After 10 min, 300μL was transferred from each tube to a new tube. Bacteria were transferred to Petroff-Hausser chamber and counted. For colony-forming unit (CFU) experiments 4x10⁸ bacteria in 4 mL 7H9/OADC without Tween were dispensed into 6 well tissue culture plates (Costar) and half the medium was replenished daily. For the assessment of biomass using crystal violet (CV) or for determining fluorescence intensity 100 μL (1x10⁷ bacteria) was dispensed into 96 well plates (Costar) as described⁴⁸. Cultures were incubated at 37°C/5%CO₂ and biofilm development was measured using a Spectra max Plate Reader (Molecular Devices, Sunnyvale, CA) over 7 days.
Biofilm development was also determined using mCherry transformed bacteria. Ma\textsuperscript{Sm} or Ma\textsuperscript{Rg} variants were each inoculated into 96 well black, clear bottom plates (Costar) at a density of 1\times10^7 bacteria/well. At each time point wells were rinsed to remove suspended bacteria and mCherry (red fluorescence) Ex\textw{575}/Em\textw{610} was quantified using a SpectraMax Plate Reader to determine relative fluorescence intensity (RFI). For the measurement of lipid, biofilms in separate wells were rinsed and incubated with FM 1-43 (Molecular Probes, Eugene, OR) as per manufacturer’s instructions and rinsed before measuring RFI.

**Bacterial Growth and Aggregative assays**

Growth of each variant was determined using The OmniLog\textsuperscript{®} Phenotype MicroArray\textsuperscript{™} System. (Biolog Inc., Hayward, CA). Ma\textsuperscript{Sm} or Ma\textsuperscript{Rg} was grown up to 48h in the OmniLog\textsuperscript{®} Incubator in 7H9 with OADC with or without Tween 80 and in the presence of Biolog Redox Dye A at a density of 1 \times 10^7 bacteria/well and readings taken every 15 min.

The ability of M. abscessus to aggregate was assessed using an optical density aggregative index as described with modifications\textsuperscript{49}. Briefly, Ma\textsuperscript{Sm} or Ma\textsuperscript{Rg} was grown for 48h in 7H9 with OADC with or without Tween 80. After removal from the shaking incubator cultures were gently agitated and the OD\textw{600} taken at time 1 (T\textsub{1}), and taken again after 15 min (T\textsub{2}) for each variant. The aggregative index (AI) was determined by the equation: OD\textsub{1} – OD\textsub{2}/OD\textsub{1} \times 100 = AI.

**Antimicrobial/Antibiotic assays**
Amikacin (Pharmacy-Main OSUMC), and azithromycin (Sigma-Aldrich) were added to RPMI/10% heat inactivated fetal bovine serum (HI-FBS) and diluted to obtain a range of final concentrations between 2 \(\mu\)g/mL and 256 \(\mu\)g/mL. Each antibiotic was added to planktonic suspensions containing 1x10^7 bacteria/well each of mCherry Ma^{sm} or Ma^{Rg} in 96 well black, clear bottom plates (Costar) and incubated at 37°C/5%CO_2 for 24-48h. Inhibitory activity was assessed by measuring RFI using a modification of the 96 well assay. The minimal inhibitory concentration (MIC) or lowest concentration exhibiting reduced RFI was determined based on statistically significant reduction relative to untreated control growth. For biofilm assays, 1x10^7 bacteria/well were dispensed in RPMI/10%HI-FBS and incubated at 37°C/5%CO_2 for 24h after which antibiotic was added to each well at the appropriate concentration and compared with untreated controls.

For hydrogen peroxide (H_2O_2) assays, H_2O_2 (30%) (Fisher Scientific) was diluted in RPMI/10%HI-FBS fresh for each assay and added to 1x10^7 bacteria/well of Ma^{sm} or Ma^{Rg} planktonic or 24h biofilms for a final range of concentrations from 0.1 to 100 mM. RFI was quantified as described above. For pH assays, acidic pH (5.5, 4.5 and 3.5) was obtained by diluting 7H9/OADC with HCl acid. Acetic acid was diluted to 5, 2.5 or 1% in 7H9/OADC.

Macrophage culture and infection

THP-1 cells (ATCC) were cultured as recommended by ATCC in RPMI 1640 medium with 2 mM L-glutamine/10%HI-FBS and 100 U/mL penicillin/100 \(\mu\)g/mL streptomycin at 37°C/5%CO_2. For differentiation to macrophage-like cells, THP-1 cells were added to 24 well tissue culture plates (Costar) in antibiotic free media and treated with 10 ng/mL of phorbol 12-myristate 13-acetate
(PMA) (Sigma) for 48h to promote attachment. Monolayers were checked before each experiment and cells were infected using a single cell suspension of \( M\alpha^{sm} \) or \( M\alpha^{rg} \) with a multiplicity of infection (MOI) of 2.5 bacteria to 1 macrophage (2.5) in RPMI/20 mM Hepes (RH)/1 mg/mL human serum albumin (RHH) for 2h at 37°C/5%CO\(_2\). Each variant was opsonized with human serum for 30 min before incubating with THP-1 macrophages on a rotating platform for 30 min to ensure even infection, followed by a stationary incubation for 90 min. After infection, macrophages were washed 3 times to remove extracellular or non-associated bacilli before the addition of fresh RPMI/10%HI-FBS and either left untreated, or treated with 100 \( \mu \)L/mL amikacin or 32 \( \mu \)L/mL azithromycin for 24 or 48h.

For CFU counts, plates were removed after 2, 24 and 48h, washed 3 times and lysed with 0.25%SDS in PBS for 10 min in the presence of 50 \( \mu \)g/mL of DNase (Sigma) as described\(^{46}\). The lysate was then placed into 7H9/OADC/0.5%Tween 80 with glass beads, pulse vortexed, serially diluted and plated onto 7H10 agar. Agar plates were incubated 37°C/5%CO\(_2\) for a minimum of 3 days before enumerating CFUs.

For microscopy, PMA-treated THP-1 cells (4x10\(^5\)) were plated on 35mm, glass bottomed (#1.5) MatTek dishes (MatTek Corp, Ashland, MA) for 48h prior to infection with opsonized mCherry \( M\alpha^{sm} \) or \( M\alpha^{rg} \) at an MOI of 2.5:1. After each time point the cells were imaged with an Olympus FluoView FV10i confocal laser scanning microscope system (Olympus, Center Valley, PA). The mean number of infected macrophages on each MatTek plate was determined by counting ≥300 consecutive THP-1 cells per duplicate plate using phase-contrast and red fluorescence channels\(^{50}\).
For scanning electron microscopy, 72h biofilms were rinsed using PBS and fixed as described in glutaraldehyde, post-fixed with osmium tetroxide and dehydrated with ethanol. The samples were coated with gold–palladium using a sputter coater and imaged using a Nova NanoSEM 400 (FEI Co., Hillsboro, OR).

Confocal microscopy was performed using a Nikon A1R confocal system equipped with 60x (N.A. 1.4) and 100x oil-immersion objective lenses (N.A. 1.45) on mCherry MaSm or MaRg 48h biofilms alone or stained using FM 1-43 (Molecular Probes, Eugene, OR). Images were processed for presentation with Nikon Elements software (ver. 4.30.02).

Statistical analysis was performed using GraphPad Prism (v 5.0 for Windows, GraphPad Software, San Diego California USA) using unpaired t-tests or 2-way ANOVA between data sets.

Results

MaRg is more aggregative than MaSm

M. abscessus smooth and rough variants have been shown to differ in their sliding motility and ability to colonize the surface of pegs in MBEC plates. We found that MaRg settled rapidly when not shaken and hypothesized that this might be due to its ability to aggregate. MaSm and MaRg isolates were readily distinguishable by colony morphology on 7H10 agar (Fig. 1a,d). MaRg colonies were also distinguished using a low magnification stereomicroscope by cording at the
edge of the colonies (Fig. 1b,e). Colony variant cell structures were not globally distinguishable by SEM (Fig. 1c,f), although inter-connecting threadlike structures are visible on rough bacilli (Fig. f). Growth was similar for both variants in 7H9 broth with and without Tween 80 (OmniLog) (Fig. 1g). The OD$_{600}$ of isolated $Ma^{Rg}$ or $Ma^{Sm}$ grown with shaking for 48h with and without Tween showed that $Ma^{Rg}$ settled within 15 min in the absence of Tween, whereas $Ma^{Sm}$ remained suspended during this time indicating that $Ma^{Rg}$ was significantly more aggregative compared to $Ma^{Sm}$ (Fig. 1h). $Ma^{Rg}$ but not $Ma^{Sm}$ aggregates were visible on the side and bottom of the test tube and aggregation was significantly inhibited in the presence of Tween (Fig. 1i,j).

$Ma^{Rg}$ and $Ma^{Sm}$ form biofilms with distinct phenotypes

Since $Ma^{Rg}$ was more aggregative than $Ma^{Sm}$ we hypothesized that this would affect its biofilm forming capacity. Due to the lack of a consensus definition of biofilms, particularly with respect to mycobacteria, we used several assays to measure biofilm formation with isolated $Ma^{Rg}$ or $Ma^{Sm}$ variants over 7 days. Crystal violet (CV), a determination of biomass that includes the nonspecific measurement of extracellular matrix material, was 2-fold greater with $Ma^{Rg}$ after day 3 ($p<0.001$) (Fig. 2a). CFU/cm$^2$ enumeration however indicated that the number of bacteria in biofilms was not statistically different between variants ($p>0.05$) (Fig. 2b). We also quantified biofilm formation by measuring relative fluorescence intensity (RFI) of mCherry-expressing $Ma^{Sm}$ or $Ma^{Rg}$ over time. In agreement with CFU/cm$^2$ data, mCherry RFI showed no significant differences between the two variants (Fig. 2c,e,h). Each morphotype also exhibited a greater
RFI when probed with lipophilic FM 1-43 over time. However \( Ma^{Rg} \) showed significantly more lipophilic material associated with biofilm aggregates compared with \( Ma^{Sm} \) at days 3 and 7 (Fig. 2d,f,i). After 3 days biofilms were visible on the surface as a pellicle and on the bottom of wells; at day 7 the \( Ma^{Sm} \) pellicle appeared oleaginous and the \( Ma^{Rg} \) pellicle waxy (Fig. 2g,j). Neither variant exhibited robust attachment to surfaces, even though large structurally complex bacterial aggregates were visible in wells with both variants (Fig. 2k,m). \( Ma^{Sm} \) or \( Ma^{Rg} \) aggregates exhibited lipophilic-rich structures (Fig. 2l,n) generally co-localized with mCherry with some evidence of extracellular lipid in aggregates. Thus, \( Ma^{Sm} \) and \( Ma^{Rg} \) biofilms each demonstrated 3-D biofilm-like aggregates that were structurally contiguous.

276 **\( Ma^{Sm} \) and \( Ma^{Rg} \) biofilm formation confers tolerance to antimicrobial treatment**

277 Antimicrobial tolerance is a characteristic criterion for microbial biofilms\(^ {26,41} \). To further interrogate whether \( Ma^{Rg} \) exhibited biofilm-like behavior, we tested the ability of each variant to withstand antimicrobial treatment when grown planktonically or as biofilms. Virulent mycobacteria can resist host defense strategies and therefore we examined the susceptibility of \( Ma^{Sm} \) or \( Ma^{Rg} \) variants to hydrogen peroxide (\( H_2O_2 \)) or low pH\(^ {52,53} \). Planktonic cells showed a reduction in survival – indicated by the reduced RFI of mCherry transformed cells – for \( H_2O_2 \) concentrations of 1mM and above (Fig. 3a), however \( Ma^{Sm} \) and \( Ma^{Rg} \) biofilm-like aggregates were each more resistant to \( H_2O_2 \) between 1 and 10mM (Fig. 3b,c) (\( P<0.001 \), unpaired t-test).

Planktonic \( Ma^{Sm} \) was unaffected at pH 5.5 with no significant reduction in RFI after 2h compared with untreated controls (\( P>0.05 \)), but was significantly reduced at pH 4.5 (\( P<0.01 \))
MaRg, however, tolerated pH 4.5 (P>0.05). Both variants were susceptible to pH 3.5 (P<0.001). Biofilm MaSm showed no difference in RFI at pH 4.5 after 2h compared to untreated or 5.5 pH (P>0.05). However there was a significant difference between variants at pH 3.5 (P<0.001; t-test) at 24h evidenced also by a 2-3 log reduction in MaSm, but only ~1 log reduction of MaRg, indicating that biofilm aggregates were significantly more tolerant to low pH and MaRg more tolerant than MaSm (Fig. 4c,d and Table 1).

Biofilm formation also results in increased antibiotic tolerance compared to planktonic cells. Amikacin treatment resulted in significantly lower RFI for planktonic MaSm above 2 μg/mL, and a similar result was observed with azithromycin treatment above concentrations of 4 μg/mL (Fig. 5a,d). The MaRg RFI was also reduced with each antibiotic; however, the reduction was significantly less than for planktonic MaSm at concentrations between 2-32 μg/mL for amikacin and at concentrations between 4-8 μg/mL for azithromycin. These results are in broad agreement with the reported MIC for amikacin (2-4 μg/mL) and for azithromycin (8-16 μg/mL), but suggest that MaRg is more recalcitrant to antibiotic treatment than MaSm and consistent with data obtained with each variant on 7H10 agar showing that MaSm was more susceptible to lower concentrations of amikacin or azithromycin than MaRg by zones of inhibition (data not shown).

In contrast to results with planktonic bacteria, the RFIs of MaSm or MaRg biofilms were unaffected after 24h when treated with high concentrations of amikacin or azithromycin compared to untreated controls, providing evidence that MaSm and MaRg biofilm aggregates exhibited tolerance to each of these antibiotics (Fig. 5b-c, 5e-f). Although planktonic MaSm or MaRg showed significant reductions in RFI with significant differences between variants, biofilm-
like aggregates of both variants were tolerant to all concentrations of antibiotic after 24h of treatment. Extended contact times of 48 and 72h showed no reductions in RFI for biofilm bacteria (data not shown). By all criteria used to distinguish biofilms, including antimicrobial tolerance, *M. abscessus* colony morphology variants were comparable.

*Ma*<sup>Rg</sup> survives significantly better than *Ma*<sup>Sm</sup> in untreated or in azithromycin-treated differentiated THP-1 macrophages despite similar uptake, but variants survive equally well in macrophages treated with amikacin.

Since *M. abscessus* variants have been shown to differ in their invasiveness in cells<sup>32</sup>, we studied *Ma*<sup>Sm</sup> and *Ma*<sup>Rg</sup> uptake in PMA-differentiated human THP-1 cells and tested whether antibiotic treatment differentially affects intracellular survival. At an MOI of 2.5 there was no difference in intracellular *Ma*<sup>Sm</sup> or *Ma*<sup>Rg</sup> after 2h of infection by CFU or by confocal microscopy indicative of similar uptake kinetics by THP-1 cells of each variant (Fig. 6a,b). However, after 24h without antibiotic treatment there was significantly more *Ma*<sup>Rg</sup> than *Ma*<sup>Sm</sup> in THP-1 cells by CFU counts (*P*<0.05) (Fig. 6c). This difference was not seen at 48h by CFU (Fig. 6c), but was seen at this time point by microscopy (*P*<0.01) (Fig. 6d). Amikacin treatment inhibited intracellular growth of *Ma*<sup>Rg</sup> or *Ma*<sup>Sm</sup> compared with THP-1 cells without antibiotic and showed no significant difference between variants over 48h of treatment commensurate with the percentage of infected cells observed using confocal microscopy (Fig. 6e,f). Azithromycin effectively reduced intracellular *Ma*<sup>Sm</sup> or *Ma*<sup>Rg</sup> compared with no antibiotic treatment, however, by 48h there was significantly more intracellular *Ma*<sup>Rg</sup> compared to *Ma*<sup>Sm</sup> (*P*<0.001).
(Fig. 6g). When macrophages were examined microscopically, approximately 10-20% of cells were infected at 2h and remained consistent over the infection period for both variants (Fig. 6b,d,f,h, and Supplemental Fig. 1). These data demonstrate that whereas both variants can survive in macrophages, $Ma^{Rg}$ may have a survival advantage.

Acetic acid rapidly kills $Ma^{Rg}$ and $Ma^{Sm}$ biofilms

Acetic acid is an effective anti-tuberculocidal disinfectant that is also effective against $M. abscessus$, although distinct morphotypes or biofilms were not previously evaluated\(^5\). We therefore tested acetic acid against highly tolerant $M. abscessus$ variant biofilms. Planktonic $Ma^{Sm}$ or $Ma^{Rg}$ treated with acetic acid were significantly different from untreated ($P<0.001$) at all concentrations and time points, indicating susceptibility (Fig. 7a,b). Treatment with 1% resulted in a reduction of nearly 5 logs for $Ma^{Sm}$ and 4 logs for planktonic $Ma^{Rg}$ at 2h (Table 1). The RFIs of $Ma^{Sm}$ or $Ma^{Rg}$ biofilms were also significantly reduced after only 30 min in a dose dependent manner ($P<0.001$), although 1% resulted in only 1 log reduction for biofilms of each variant (Table 1). Notably planktonic and biofilm $Ma^{Sm}$ and $Ma^{Rg}$ were both reduced to the detection limits by RFI and CFU with exposure to 2.5% for 2h (Fig. 7a-d; Table 1). Remarkably, after only 30 min exposure to 5% acetic acid, the RFIs of both planktonic variants were reduced to the detection limits (Fig. 7a-d) with a >7 log reduction in CFUs (Table 1).

Discussion
Despite the association of Ma<sup>Rg</sup> with increased pathogenicity<sup>10,36</sup>, few studies have compared M. abscessus colony variants for pathogenic properties. Such studies have suggested that biofilm formation is restricted to the Ma<sup>Sm</sup> variant<sup>32,33,40</sup>. In contrast, using isolated isogenic colony morphology variants from the sequenced M. abscessus 19977<sup>T</sup> reference strain, we found that Ma<sup>Rg</sup> is more aggregative than Ma<sup>Sm</sup> and that each variant forms biofilms with distinct phenotypes over 7 days. Ma<sup>Sm</sup> and Ma<sup>Rg</sup> have similar numbers of bacteria (CFU/cm<sup>2</sup>) in biofilms, consistent with mCherry Ma<sup>Sm</sup> or Ma<sup>Rg</sup> biofilm RFI<sub>6</sub> over time. However, Ma<sup>Rg</sup> showed significantly more biofilm biomass by CV OD<sub>600</sub> than Ma<sup>Sm</sup> as well as increased RFI with the lipophilic probe, FM 1-43, possibly due to more extracellular lipid.

Ma<sup>Sm</sup> and Ma<sup>Rg</sup> variants, including 19977, differ in the expression of GPLs<sup>32,33,35,37,55,56</sup>. GPLs are associated with sliding motility and play a role in the development of biofilms in both non-pathogenic and pathogenic mycobacteria<sup>32,34,57,58</sup>. Our results indicate that despite low GPL expression, Ma<sup>Rg</sup> still forms pellicles and exhibits a phenotype that results in the accumulation of multicellular biofilm structures and biofilm-like aggregates with small foci of extracellular lipid, not previously shown for a rough M. abscessus variant. It is unclear how GPL expression affects aggregation by each variant. The deletion of MmpL4b, a gene in the GPL biosynthetic pathway, was found to abrogate the ability of M. abscessus to colonize surfaces, and enhance its ability to replicate in human macrophages<sup>34</sup>. The MmpL family of proteins plays a role in the biosynthesis of the cell envelope and the mutation resulted in defective GPL production and/or transport in the rough variant and the capacity to produce cords <i>in vitro</i><sup>55</sup>. Rough variants of M. <i>abscessus</i> and M. <i>bolletii</i> exhibit cording morphology in a zebrafish model of infection and genetic analysis of a spontaneous rough variant of M. <i>bolletii</i> recently identified isogenic.
smooth and rough variants that differed by a single mutation in MmpL4a\textsuperscript{37,55}. We saw no evidence of cording in Ma\textsuperscript{Sm} aggregates; however we observed variable cording morphology in Ma\textsuperscript{Rg} aggregates, suggesting that cording occurs under specific growth conditions.

Motility per se is not required for biofilm development in many bacteria, although aggregation is necessary\textsuperscript{26,42,44,45}. The aggregative phenotype exhibited by Ma\textsuperscript{Rg} may result in an antibiotic-tolerant phenotype similar to rugose colony variants of P. aeruginosa from CF patients\textsuperscript{25}. Notably the M. abscessus genome possesses non-mycobacterial virulence genes, including some from P. aeruginosa\textsuperscript{46}. Our results suggest that neither biofilm development, nor survival in macrophages is morphotype-restricted and that patients may be colonized with either or both M. abscessus variants. The expression of different lipids on the cell wall, however, may lead to variant-specific host cell responses that are important in delineating mechanisms that contribute to persistent infection and to M. abscessus virulence\textsuperscript{41}.

Our studies differ from previous studies that showed Ma\textsuperscript{Rg} failed to form biofilms. First, these studies assessed biofilm formation using a method based on bacterial attachment to pegs in MBEC microtiter plates. This method, however, has limitations in measuring bacterial aggregates (such as observed with Ma\textsuperscript{Rg}), which would be more likely to settle on the well bottom rather than attach to the pegs\textsuperscript{59}. Second, previous studies measured biofilm formation up to 72h, whereas Ma\textsuperscript{Rg} pellicle formation took longer in our study, although the antimicrobial tolerant phenotype was present after 24h. Third, previous studies used a clinical isolate, characterized by a spontaneous rough to smooth reversion, and this strain may exhibit a strain-specific non-biofilm phenotype compared to M. abscessus 19977.
Antimicrobial tolerance is a hallmark trait of biofilm formation\textsuperscript{26,43}. Our data show that 

\textit{M. abscessus} biofilm-like aggregates of each variant are significantly more tolerant to acidic pH. Biofilm aggregates also conferred significantly better survival compared to planktonic cells when treated with H\textsubscript{2}O\textsubscript{2}, but biofilms were significantly inhibited with 100 mM H\textsubscript{2}O\textsubscript{2}. \textit{M. abscessus} biofilms were also significantly more tolerant to two antibiotics commonly used to treat mycobacterial infection: amikacin and azithromycin. Planktonic \textit{Ma}\textsuperscript{Sm} or \textit{Ma}\textsuperscript{Rg} was significantly decreased compared to untreated controls when treated with amikacin or azithromycin in a dose dependent manner. In this case, \textit{Ma}\textsuperscript{Rg} was more recalcitrant to antibiotic treatment between 2-32 \textmu g/mL of amikacin and 4-8 \textmu g/mL of azithromycin. When \textit{Mo}\textsuperscript{Sm} and \textit{Ma}\textsuperscript{Rg} formed biofilm aggregates, however, each tolerated antibiotic concentrations of 256 \textmu g/mL with no significant reduction in RFI, a concentration 10-100 times the MIC or greater than the MBC/MIC ratio (>4) generally accepted for bactericidal or bacteriostatic antibiotics\textsuperscript{12}. Thus by all criteria used for biofilms, including antimicrobial tolerance, \textit{M. abscessus} colony morphology variants were comparable. Overall our data suggest that conditions \textit{in vivo} where \textit{M. abscessus} may aggregate can contribute to the recalcitrance to antibiotic treatment. Furthermore, these results may help to explain the high rates of ototoxicity associated with the high concentration of antibiotic required to achieve a maximum killing in 70% of patients with amikacin\textsuperscript{60}. The ability to survive intracellularly in macrophages can also contribute to mycobacterial persistence and it has been suggested that the \textit{Ma}\textsuperscript{Sm} variant is less invasive\textsuperscript{32-34}. Our results show that the uptake of opsonized \textit{Ma}\textsuperscript{Sm} or \textit{Ma}\textsuperscript{Rg} by THP-1 macrophages is similar. However, THP-1 macrophages infected with \textit{Ma}\textsuperscript{Sm} or \textit{Ma}\textsuperscript{Rg} without antibiotic treatment harbored
significantly more $Ma^{Rg}$ than $Ma^{Sm}$ at 24h, suggesting that $Ma^{Rg}$ may not be more invasive, but rather it may survive better intracellularly. Alternatively, $Ma^{Rg}$ may be able to replicate better than $Ma^{Sm}$ in human macrophages\textsuperscript{34}. We are currently investigating colony variant survival in human monocyte-derived macrophages to better assess these possibilities. In contrast to the results obtained without antibiotics, variant survival in macrophages treated with amikacin did not differ at 48h, suggesting a bacteriostatic effect on both variants or diminished antibiotic levels in THP-1 macrophages. Amikacin is widely used to treat $M$. abscessus infections and a recent study showed that $M$. abscessus clinical strains exhibited bacteriostatic responses to amikacin, suggesting that the discrepancy between antibiotic susceptibility in vitro and in vivo responses may be compounded by variable drug concentrations during in vivo conditions\textsuperscript{12}.

More $Ma^{Rg}$ than $Ma^{Sm}$ was present after 48h of treatment with azithromycin suggesting that although each variant can survive intracellularly, $Ma^{Rg}$ may be more recalcitrant to this antibiotic than $Ma^{Sm}$, consistent with our data showing that this variant is more refractory than $Ma^{Sm}$ to other antimicrobial treatments. Azithromycin penetrates cells more effectively, aligned with the reduced intracellular burden of $Ma^{Sm}$ or $Ma^{Rg}$ in THP-1 macrophages and in agreement with a study showing that $M$. abscessus survived intracellularly in human macrophages treated with clarithromycin\textsuperscript{40}. Although our studies with planktonic mCherry $M$. abscessus variants showed a range of antibiotic concentrations similar to published results, microscopic data with both mCherry variants and CFU data with non-transformed variants indicate that intracellular $Ma^{Sm}$ and $Ma^{Rg}$ both persisted over 48h. Azithromycin has multiple effects on host cell responses, including increasing ion efflux, reducing TNF-α production and interfering with
autophagy, and it is possible that these contributed to the survival of *M. abscessus* despite the ability of azithromycin to concentrate inside cells\(^{61}\).

Collectively our results indicate that the antibiotic concentrations required to kill either *M. abscessus* variant in biofilm-like aggregates or in host cells may be clinically unachievable or may result in cytotoxic adverse effects because of high, prolonged drug concentrations. Reports in immunodeficient mice show that although amikacin or azithromycin treatment resulted in lower bacterial burdens *in vivo*, antibiotic treatment did not eradicate *M. abscessus* in the lungs\(^{19,62}\). In a study of patients with pulmonary infection, combination therapy with amikacin, cefoxitin, and clarithromycin failed to successfully treat pulmonary *M. abscessus*\(^{13}\). Our study further highlights the limited efficacy of antibiotic therapy in treating *M. abscessus* infections and emphasizes the need for new therapeutic approaches.

Historically, acquisition of *M. abscessus* has been attributed to the exposure of individuals to environmental sources. Recent studies, however, report that in some outbreaks *M. abscessus* strains in CF patients have greater genomic identity than would be predicted by acquisition from environmental sources, suggesting that some clones may be transmitted from nosocomial sources\(^{24}\). *M. abscessus* aggregates harboring viable bacteria that were refractory to antimicrobial treatment were recently demonstrated in a resected lung cavity in a patient with COPD\(^{31}\). Biofilm *M. abscessus* was also found embedded in alveolar walls and in mucus in the conductive zone of the airways of a patient with CF in an end-stage transplanted lung\(^{28}\). In both cases, infectious aerosols containing aggregated bacilli may facilitate *M. abscessus* transmission. *M. abscessus* CF isolates survived in aerosolized droplet nuclei particles\(^{63}\) and particulates enhanced fomite survival\(^{64}\). Biofilm formation by *Streptococcus pneumoniae*, a
pathogen thought to die quickly outside of the human host, resulted in prolonged survival on fomites\(^6\). Together these studies suggest that biofilm-like aggregates may survive outside the host and contribute to the non-random global transmission of \(M.\ abscessus\) clones in CF patients\(^2\). We propose that the ability of \(M.\ abscessus\) variants to form biofilms should be recognized as a virulence factor.

Although biofilm formation by \(Ma^Rg\) and \(Ma^Sm\) significantly increases bacterial survival to antimicrobial treatment, acetic acid was remarkably effective in killing \(M.\ abscessus\). Acetic acid can kill several species of planktonic mycobacteria with short contact times\(^5\). Our data show that 5% acetic acid kills planktonic as well as biofilm \(Ma^Rg\) and \(Ma^Sm\) within 30 min and extend the existing evidence that acetic acid effectively eradicates \(M.\ abscessus\) to include biofilms. Thus acetic acid may help to prevent transmission in clinical settings where aggregates of \(M.\ abscessus\) may be present on fomites.

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**Supplementary Methods**

THP-1 cells were plated and infected as described for confocal microscopy. Once infected, cells were treated with Amikacin (100µg/ml) or azithromycin (32µg/ml). Untreated cells for each morphotype served as controls. The infection was stopped at desired time points by washing the monolayers with warm RPMI 3 times to remove the extracellular bacteria. The monolayers were then with fixed with 4% paraformaldehyde in RPMI (Electron Microscopy Sciences, Hartfield, PA) for 15 minutes at room temperature and images taken at 24 and 48h following treatment. Nuclei were visualized using Syto9 (Molecular Probes, Eugene, OR) and images were captured using a Nikon A1R confocal system equipped with a 60x oil-immersion objective lens (N.A. 1.4). Image processing was performed Nikon Elements software (ver. 4.30.02). Maximum intensity projections of the image stacks were created and combined with a single DIC image from that stack (Supplemental Fig. 1). Nuclear signal was pseudocolored blue for enhanced contrast with the bacterial signal (red).
Figure 1. Characterization of M. abscessus smooth (MaSm) and rough (MaRg) variant isolates.

MaSm and MaRg isolates were distinguishable by colony morphotype on 7H10 agar (a,d). MaRg colonies were also distinguishable by cording at the periphery of colonies on agar at low magnification (b,e), but not by SEM (c,f). Isolated MaRg and MaSm colonies were grown in 7H9 broth and growth was similar for both variants in 7H9 broth with and without Tween 80 (g). MaRg was more aggregative compared to MaSm. When cultures were removed from shaking after 15 min MaSm remained suspended, but MaRg rapidly settled out in the absence of Tween (h,i). Aggregation was significantly reduced with 0.5% Tween (j).

Figure 2. MaSm or MaRg variants each develop aggregated biofilm structures over time.

Biomass (CV) was greater with MaRg (black bars) compared to MaSm (white bars) (a) and similar results were obtained using the lipophilic probe FM 1-43 to label variants (d). Biofilm development was not statistically different (p>0.05) between variants measured by CFU/cm² (b) or by relative fluorescence intensity (RFI) of mCherry variants (c). Error bars = SEM (3 replicate wells and 3 biological replicates for CFU: n=9) and 6 replicate wells and 3 biological replicates for CV and RFI (n=18). Pellicle biofilms showed distinct morphology between MaRg and MaSm variants after 7 days (g,j). Confocal slices show mCherry expressing MaSm (e) and MaRg (h) to be similar. The lipophilic probe FM 1-43 showed more RFI for MaRg (i) compared to MaSm (f) (arrows indicate extracellular lipid). Finally, orthogonal confocal z-stack images, panels (k,m), and 3-D images pseudocolored to highlight the depth of bacterial biofilms, panels (l,n), show that complex aggregated biofilm structures were present after 48h for both variants.
Figure 3. Biofilm MaSm or MaRg is more tolerant than planktonic variants to hydrogen peroxide. Planktonic MaSm or MaRg were susceptible to concentrations of H₂O₂ at or above 1mM and MaSm was more susceptible to 10 mM H₂O₂ than MaRg (a). MaSm or MaRg biofilms were both significantly more tolerant to concentrations of H₂O₂ 1-10 mM compared to planktonic MaSm and MaRg (b,c respectively). MaRg biofilms were more tolerant than MaSm between 5-10 mM (d). Data represent 6 wells per exp; 3 biological replicates (n=18); P<0.05*; 0.01**; 0.001***.

Figure 4. Biofilm MaSm or MaRg is more tolerant than planktonic variants to low pH. The RFIs of planktonic MaSm and MaRg were not significantly different at pH 5.5 to untreated. MaSm, but not MaRg, was significant at pH 4.5. Both were significant at pH 3.5 (a,b). In contrast, MaSm and MaRg showed no statistical difference between untreated biofilms and those treated at pH 4.5 for 2 or 24h. MaSm and MaRg biofilms treated at pH 3.5 were significantly different compared to all other conditions by 2-way ANOVA (c,d respectively) and were significantly different at pH 3.5 between variants by t-test (P<0.001). N = 6 wells/exp; 2 experiments (P>0.05 ns; <0.01**; <0.001***).

Figure 5. Biofilm MaSm or MaRg is more refractory than planktonic variants to antibiotic treatment. Planktonic MaSm variant showed significantly lower mCherry RFI in response to amikacin concentrations between 2-32 μg/mL compared to MaRg (a). MaSm also showed significantly lower RFI than MaRg in response to azithromycin concentrations of 4-8 μg/mL (d).
However, biofilms of both *M. abscessus* variants were significantly more tolerant to antibiotic treatment than planktonic bacteria. Concentrations of amikacin that resulted in reduced RFI for planktonic cells failed to result in a significant reduction in RFI of biofilms of either variant. A similar effect was seen with azithromycin. N=12; 6 wells, 2 biological replicates. $P < 0.05^{*}; 0.01^{**}; 0.001^{***}$.

**Figure 6.** MaSm or MaRg survive inside THP-1 cells with or without antibiotic treatment. Uptake was not significantly different between variants by THP-1 cells infected with opsonized MaSm (white) or MaRg (gray) at an MOI of 2.5 for 2h by CFU (a) and by microscopy (b). Infected cell monolayers treated with antibiotics had lower intracellular burdens compared to cells without antibiotic treatment at 48h (c-h). Infected cell monolayers treated with amikacin show MaSm or MaRg both survive intracellularly in macrophage-like THP-1 cells over 48h (e,f). Azithromycin reduced intracellular bacteria, however MaRg was less susceptible to azithromycin at 48h (g,h).

For CFU experiments, n = triplicate wells/experiment; 3 biological replicates (5 replicates for no antibiotic controls). For microscopic analysis, n = duplicate plates/experiment; 2 biological replicates (3 for azithromycin). ($P<0.05^{*}; 0.01^{**}; 0.001^{***}$).

**Figure 7.** Biofilm MaSm or MaRg is susceptible to acetic acid. Planktonic MaSm or MaRg treated with concentrations of acetic acid 1% and above were statistically different compared to untreated variants by 30 min post treatment for all (a,b). Significantly, this was also observed for MaSm and MaRg biofilms with 2.5% and 5% acetic acid after only 30 min (c,d). There was no
significant difference between $Ma^{sm}$ and $Ma^{rg}$ compared by t-test ($P>0.05$). $N = 6782$ wells/experiment; 5 biological replicates; $P<0.001***$. 


Table 1. Colony forming unit (CFU) reduction

| Growth condition | Planktonic | Biofilm |
|------------------|------------|---------|
| M. abscessus variant | Ma<sub>sm</sub> | Ma<sub>Rg</sub> | Ma<sub>sm</sub> | Ma<sub>Rg</sub> |
| Exposure Time | 24h |

|          | Planktonic | Biofilm |
|----------|------------|---------|
| H<sub>2</sub>O<sub>2</sub> |          |         |
| 1        | <1         | <1      | <1         | <1         |
| 5        | <1         | <1      | <1         | <1         |
| 10       | 1.9        | 1.2     | <1         | <1         |
| 50       | >7*        | >7*     | 4.8        | 2.3        |
| 100      | >7*        | >7*     | >7*        | 6.2        |

| Exposure Time | 24h |

| pH (HCl) | Planktonic | Biofilm |
|----------|------------|---------|
| 5.5      | ND         | ND      | <1      | <1      |
| 4.5      | ND         | ND      | <1      | <1      |
| 3.5      | 4.9        | 3.4     | 2.5     | 1.1     |

| Exposure Time | 24h |

| Amikacin | Planktonic | Biofilm |
|----------|------------|---------|
| 256 µg/mL | ND         | ND      | <1      | <1      |

| Azithromycin | Planktonic | Biofilm |
|--------------|------------|---------|
| 256 µg/mL    | ND         | ND      | 0       | 0       |

| Exposure Time | 2h |

| Acetic acid | Planktonic | Biofilm |
|-------------|------------|---------|
| 1%          | 5.3        | 4       | 1.3     | 1.3     |
| 2.5%        | >7*        | >7*     | >7*     | >7*     |
| 5%          | >7*        | >7*     | >7*     | >7*     |

| Exposure Time | 30min |

|          | Planktonic | Biofilm |
|----------|------------|---------|
| 1%       | 1          | <1      | <1      | <1      |
| 2.5%     | 4          | 2       | 2.2     | 2.9     |
| 5%       | >7*        | >7*     | >7*     | >7*     |

ND (No data)

*Detection limit
Amikacin

a) Planktonic $Ma^{Sm}$ v. $Ma^{Rg}$

Percent RFI control

[b) $Ma^{Sm}$ planktonic v biofilm

[c) $Ma^{Rg}$ planktonic v biofilm

Azithromycin

d) Planktonic $Ma^{Sm}$ v. $Ma^{Rg}$

e) $Ma^{Sm}$ planktonic v biofilm

[f) $Ma^{Rg}$ planktonic v biofilm

[Amikacin mg/mL]

[Azithromycin mg/mL]
