Biofilms of *Mycobacterium abscessus* Complex Can Be Sensitized to Antibiotics by Disaggregation and Oxygenation

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ABSTRACT Pulmonary infection with the multidrug-resistant *Mycobacterium abscessus* complex (MABSC) is difficult to treat in individuals with cystic fibrosis (CF). MABSC grows as biofilm aggregates in CF patient lungs, which are known to have anaerobic niches. How aggregation and anoxic conditions affect antibiotic tolerance is not well understood. We sought to determine whether disaggregation and oxygen availability sensitize MABSC isolates to recommended antibiotics. We tested the susceptibilities of 33 isolates from 22 CF patients with MABSC infection and a reference strain to the following antibiotics: amikacin, azithromycin, cefoxitin, ciprofloxacin, clarithromycin, imipenem, kanamycin, linezolid, moxifloxacin, rifampin, tigecycline, and sulfamethoxazole-trimethoprim. Isolates were grown in Mueller-Hinton broth with and without the disaggregating detergent Tween 80 (5%). Time-kill curves at days 1 and 3 were generated for oxic and anoxic amikacin treatment in 4-fold dilutions ranging from 2 to 512 mg liter⁻¹. Scanning electron microscopy was used to visualize the aggregation patterns, while confocal laser scanning microscopy and microrespirometry were used to visualize biofilm growth patterns. Disruption of MABSC aggregates increased susceptibility to amikacin, tigecycline, kanamycin, azithromycin, imipenem, cefoxitin, and clarithromycin ($P < 0.05$, $n = 29$ to 31). Oxygenation enhanced the killing of disaggregated MABSC isolates by amikacin ($P < 0.05$) by 1 to 6 log units when 2 to 512 mg liter⁻¹ of amikacin was used. This study explains why current drug susceptibility testing results correlate poorly with treatment outcomes. The conditions achieved by oxic culturing of planktonic isolates *in vitro* do not resemble the hypoxic conditions in CF patient lungs. Biofilm disruption and increased O₂ availability during antibiotic therapy may be new therapeutic strategies for chronic MABSC infection.

KEYWORDS *Mycobacterium abscessus* complex, oxygenation, cystic fibrosis, antimicrobial resistance, biofilm

The *Mycobacterium abscessus* complex (MABSC) is part of the rapidly growing pathogenic nontuberculous mycobacteria (NTMs) and is increasingly isolated from sputum samples from patients with cystic fibrosis (CF) (1, 2). The MABSC severely impacts lung function and life expectancy in patients with CF (3). It is clinically challenging to treat MABSC infections (4), and antibiotic therapy often fails (5, 6). Intensive, multidrug treatment regimens are required for months to years, but cure rates are still disappointing (7–9). It is recommended that several antibiotics be combined in an initial, intravenous phase, followed by a long maintenance phase with two or more antibiotics, administered orally. Susceptibility testing is common, but there are only weak correlations between the results of *in vitro* susceptibility studies and clinical outcomes (10).

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The genus *Mycobacterium* is comprised of more than 170 species (11), the majority of which are not implicated in human infection, while *Mycobacterium tuberculosis* is the most well-known. NTMs include species that are ubiquitous in soil and aquatic environments, as well as human pathogens (12, 13). NTMs may exist as free-living, planktonic cells or embedded in large, biofilm aggregates. Aggregation was clearly demonstrated in the early days of mycobacteriology (14, 15), with the subsequent focus being on growth patterns, cord formation, and other manifestations of complex bacterial communities (16–19). Bacteria in a biofilm differ from planktonic cells, are more tolerant to antimicrobial treatments, and can resist challenges from inflammatory cells (20). Recently, it was demonstrated that cells of the MABSC cluster together in aggregates in mucus and in the endothelial wall of patients with CF and that the resulting infection induces a humoral immune response, which is correlated with disease severity (21, 22). MABSC exhibits two distinct morphotypes when plated on agar media: the smooth morphotype (SM) and the rough morphotype (RG). The main difference between these two morphotypes is the complete lack of surface-associated glycopeptidolipids (GPL; molecules that localize to the outermost layer of the mycobacterial cell wall) in the rough morphotype (23). These morphotypes appear to differ in their virulence and pathogenicity traits, as well as in their ability to form biofilms (24).

The most severe complication in CF patients is chronic bacterial lung infection, characterized by antibiotic-tolerant biofilms in the endobronchial mucus. These biofilms are dominated by the pathogen *Pseudomonas aeruginosa* and are present in zones of oxygen (O₂) depletion (25), a consequence of O₂ consumption by polymorphonuclear leukocytes during their respiratory burst (26, 27). Slow metabolism is favored in these environments, and under the right conditions, the mycobacteria can enter a dormant state (28) with almost no metabolic activity. Aggregation in metabolically dormant biofilms offers protection from the host immune response and antibiotic challenges (29, 30). The mechanisms of the antibiotic tolerance of biofilms remain unclear, but it has been shown that the efficiency of several bactericidal antibiotics is enhanced by stimulating aerobic respiration. A lack of O₂, on the other hand, increases bacterial antibiotic tolerance (31–35). The activity of antibiotics depends on bacterial metabolic activity. Bacterial metabolic activity may lead to the increased uptake of antibiotics and expression to antibiotic targets. In addition, active aerobic metabolism enables the formation of reactive O₂ radicals (reactive oxygen species [ROS]), which may in part contribute to the bactericidal effect of antibiotics (30, 34–36). In bacteria surviving treatment with antibiotics, the formation of mutagenic ROS may lead to the development of antibiotic resistance (36). Mycobacterial growth has been shown to be O₂ dependent, and therefore, ROS formation may provide an explanation for why MABSC quickly develops resistance to bactericidal drugs, such as aminoglycosides, during chronic lung infection in patients with CF (37).

In *Pseudomonas aeruginosa*, zone formation during agar disk susceptibility testing is due to the transformation of the bacteria from single cells to a biofilm, in which the resistance to antibiotics increases when the growth of the inoculum reaches aggregates of approximately 64 cells (38, 39). Since *M. abscessus* grows as aggregates, we hypothesized that this is the reason for the high level of antimicrobial resistance of this species and why the results of susceptibility testing have a low predictive value for determination of the clinical effect of antibiotic treatment. An additional reason could be the very slow growth of the bacteria (40) because of the dormancy of the bacteria due to a lack of O₂.

The objective of this investigation was therefore to determine whether disaggregation and O₂ availability sensitize MABSC to the recommended antibiotics.

RESULTS

Aggregation of MABSC. Ziehl-Neelsen staining demonstrated the presence of MABSC aggregation and the more abundant cording in a sputum sample from a CF patient (Fig. 1A). However, scanning electron microscopy (SEM) shows that MABSC readily aggregates in shaken cultures if no detergent is added. This indicates biofilm

FIG 1 Aggregation and cording formation by MABSC with rough morphotypes. (A) Cord formation of MABSC in Ziehl-Neelsen-stained CF sputum sample. (B) MABSC aggregate with cells of the rough morphotype isolated from a CF patient sputum sample visualized by scanning electron microscopy (SEM). (C and D) Enlarged images of organized cord formation in the MABSC aggregate with cells of the rough morphotype (C) and smooth morphotype (D) isolated from a CF patient sputum sample visualized by SEM.

formation by MABSC (Fig. 1B). From the SEM images, it was possible to see the cording formation of the rough morphotype (Fig. 1C) and a chaotically scattered distribution of the cells with a smooth morphotype (Fig. 1D).

The aggregation of MABSC isolates is influenced by Tween 80. The aggregate size and shape of MABSC isolates as well as reference strain ATCC 19977 in the presence of increasing concentrations of Tween 80 were compared by confocal microscopy (Fig. 2). Variations in aggregate size were seen when cells were incubated in medium without Tween 80. Visually, the cells formed pellicles in the liquid medium (Fig. 2). Increasing the concentration of Tween 80 in the liquid medium caused cells to disperse as Tween 80 altered the structural integrity of the membrane, lipids, and proteins (41). The fraction of aggregates of $<10\ \mu\text{m}$ was the highest for cells incubated in 5% Tween 80, indicating more disaggregated cells (Fig. 3). Furthermore, 5% Tween 80 had no detectable effect on the viability of MABSC (see Fig. S1A in the supplemental material), nor did Tween 80 in combination with amikacin affect bacterial killing (Fig. S1B).

Aggregation increases antibiotic tolerance. In total, 31 CF patient isolates of MABSC were tested for antimicrobial susceptibility using the disk diffusion assay. Isolates were crudely classified into two groups, based on zone diameters: the susceptible (zone diameter $>0\ \text{mm}$) and resistant (zone diameter $=0\ \text{mm}$) groups. It was decided to use these divisions as breakpoints due to a lack of breakpoints approved by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) for the antibiotics used for the treatment of infections caused by MABSC. The susceptibility results for the MABSC isolates were based on data obtained from samples being aggregated and disaggregated in 5% Tween 80 using plate spreading. The following 12 antibiotics were tested: amikacin, azithromycin, cefoxitin, ciprofloxacin, clarithromycin, imipenem, kanamycin, linezolid, moxifloxacin, rifampin, tigecycline, and the combination of sulfamethoxazole and trimethoprim. Disaggregation of MABSC CF patient isolates with 5% Tween 80 resulted in significantly increased zone diameters for tigecycline, amikacin, azithromy-

FIG 2 The growth of MABSC is influenced by disaggregation. The images show the effect of Tween 80, which breaks up MABSC isolates from liquid cultures. Bacterial aggregation at four different Tween 80 concentrations is shown. Samples were stained with Syto 9, and the images were obtained using a 63 $\times$ (numerical aperture [NA], 1.4) Zeiss objective on a Zeiss 880 CLSM. Bars, 20 μ m at a magnification of $\times 630$. Isolates I to III were isolated from three CF patients +10, +3, and +5 years after collection of the first sample positive for *M. abscessus*.

cin, clarithromycin, kanamycin, imipenem, and cefoxitin (Fig. 4). No significant effect of disaggregation on the zone diameter was observed for ciprofloxacin, linezolid, moxifloxacin, rifampin, and sulfamethoxazole-trimethoprim (Fig. 4).

All aggregated isolates were resistant to 4 or more antibiotics. In fact, 29/31 aggregated isolates were multidrug resistant to between 8 and 12 antibiotics. Nevertheless, when the isolates were disaggregated, the isolates become susceptible to 2.97 more drugs, on average (95% confidence interval, 2.37, 3.56; $P < 0.0001$; $n = 30$) (Fig. 5). These results indicate that the effect of Tween 80 on susceptibility depends on the type of antibiotic used. This decrease in the susceptibility of aggregating MABSC is in line

FIG 3 The size of MABSC aggregates from liquid cultures decreases as the concentration of Tween 80 increases. The fractions of large (>10 μm³; white) versus small (1 to 10 μm³; black) aggregates of the MABSC ATCC 19977 smooth reference strain are shown.

with that seen in other bacteria that have formed biofilms (42). However, some bias may have been introduced by the lack of a consistent methodology for applying the bacteria onto the plates.

To examine if the greater drug sensitivity in response to the high concentration of Tween 80 could result from an increased permeability of the cell envelope, the effect of Tween 80 on the penetration of the membrane-impermeable dye, propidium iodide (PI), was examined by flow cytometry (Fig. S1C). No difference in the PI fluorescence was observed between MABSC bacteria cultured without or with 5% Tween 80, indicating the absence of an effect of 5% Tween 80 on the permeability of the cell envelope.

Low O₂ consumption reflects dormant subpopulations. Smooth and rough MABSC isolates from 3 CF patients were dissolved in oxygenated, liquid media with various concentrations of Tween 80 and were incubated for 120 min, while O₂ consumption was recorded. It was found that all smooth isolates consumed all the O₂ present within 120 min when dissolved with 5% Tween 80 (Fig. 6A, C, E, and G) ($P < 0.0001$), while O₂ consumption by the rough isolates varied between the isolates (Fig. 6B, D, and F). On the contrary, when cells were allowed to aggregate without Tween 80, O₂ consumption was inhibited, which indicates that subpopulations of bacteria were slow growing or dormant. Furthermore, MABSC isolates grown in increasing concentrations of Tween 80 demonstrated increased bacterial growth after 120 min of incubation, as measured by plate counting ($P < 0.05$, $n = 3$) (Fig. 6H to N). Compared to the findings for the aggregated isolates, disruption resulted in increased aerobic respiration and increased bacterial growth (Fig. 6). Control experiments demonstrated that Tween 80 alone had no effect on O₂ consumption in Mueller-Hinton II bouillon medium (MH) with increasing concentrations of Tween 80 or in the supernatant of an MABSC culture grown in increasing concentrations of Tween 80 (Fig. S1).

Aerobic conditions increase bacterial killing by amikacin. We investigated the impact of normoxic and anoxic conditions on the efficacy of amikacin on clinical MABSC isolates. To obtain a uniform suspension of cells, all MABSC isolates were cultured in medium with 5% Tween 80. We tested both smooth and rough morphotypes isolated from 3 CF patients 10, 5, 3, and 2 years after their first sample was positive for MABSC. After 3 days of incubation with 5% Tween 80, increased killing was observed for cells treated with amikacin under oxic conditions compared to that observed for cells treated with amikacin under anoxic conditions ($P < 0.05$) (Fig. 7). Two exceptions were

FIG 4 Disaggregation with Tween 80 sensitizes MABSC to antimycobacterial drugs. The effect of 5% Tween 80 on MABSC isolates ($n = 31$) was tested by disk diffusion susceptibility testing using amikacin, azithromycin, cefoxitin, ciprofloxacin, clarithromycin, imipenem, kanamycin, linezolid, moxifloxacin, rifampin, tigecycline, and sulfamethoxazole-trimethoprim. Aggregated MABSC isolates were grown and incubated in MH and plated by normal plate spreading on blood agar plates. Disaggregated MABSC isolates were suspended in MH plus 5% Tween 80, incubated for 2 to 5 days, and subsequently added onto blood agar plates and uniformly distributed by gently tipping the plates. Excess liquid was discharged. All isolates that were completely resistant regardless of Tween 80 are represented by the flat red line on the x axis. Statistical significance was determined using a paired t test ($P \leq 0.05$). NS, not significant.

seen, for smooth morphotype isolates $I^{SM}+10$ and $III^{SM}+5$, indicating variations between morphotypes (Fig. S2A and C). Oxidic conditions increased the killing of both smooth and rough isolates, with maximum bacterial killing exceeding a 2- to 4- $\log_{10}$ reduction when the bacteria were treated with $8 \mu\text{g ml}^{-1}$ amikacin compared to the level of killing seen under anoxic conditions.

FIG 5 Multidrug resistance to antimycobacterial drugs in MABSC is lowered by disaggregation of the isolates with Tween 80. MABSC isolates ($n = 31$) were tested by disk diffusion susceptibility testing, using (per disk) 30 μ g amikacin, 15 μ g azithromycin, 30 μ g ceftiofur, 5 μ g ciprofloxacin, 15 μ g clarithromycin, 10 μ g imipenem, 30 μ g kanamycin, 10/30 μ g linezolid, 5 μ g moxifloxacin, 5 μ g rifampin, 15 μ g tigecycline, and 1.25 μ g/23.75 μ g sulfamethoxazole-trimethoprim. Isolates with zone diameters of 0 mm were considered resistant. Aggregated MABSC isolates were grown and incubated in MH and plated by normal plate spreading on blood agar plates. Disaggregated MABSC isolates were suspended in MH plus 5% Tween 80, incubated for 2 to 5 days, and subsequently added onto blood agar plates and uniformly distributed by gently tipping the plates. Excess liquid was discharged. Statistical significance was determined using a paired t test ($P \leq 0.05$).

DISCUSSION

MABSC is recognized as a severe pulmonary pathogen in people with CF, in whom it leads to chronic infection and an accelerated loss of lung function. Clearing of the infection is difficult, but not impossible (3, 43). Conventional antibiotic resistance mechanisms are insufficient to explain the ability of susceptible MABSC strains to survive in the presence of antibiotic concentrations above the MIC, and this study proposes that other mechanisms are in play, namely, antibiotic tolerance through aggregation and metabolic dormancy (44, 45).

When reproducing the O_2 depletion known to be created by the host response to invading pathogens within the endobronchial mucus, we were able to establish anaerobic MABSC cultures with slow growth and tolerance to amikacin, which is a common anti-NTM antibiotic inhibiting translation (Fig. 7). This is in line with the effect of anaerobiosis on the tolerance to antibiotics in other CF patient pathogens (30). Under similar circumstances, MABSC has found a successful niche in the endobronchial mucus in CF lungs to aggregate in megaclusters (21), offering essential biofilm protection by means of slow growth and low metabolic activity (46). Our results show that bacterial aggregation leads to diminished O_2 consumption, while disaggregated cells deplete O_2 within 30 min (Fig. 6). This low O_2 consumption in MABSC aggregates indicates slow-growing or metabolically inactive subpopulations with low levels of aerobic respiration. The aminoglycoside amikacin belongs to a class of antibiotics targeting 16S rRNA and is an essential drug for the treatment of the multidrug-resistant NTM infections (47). We show that treatment with amikacin induces higher levels of killing under oxic conditions than under anoxic conditions, which can be explained by the increased bacterial growth typically associated with increased ribosomal activity (48, 49). Bacterial aggregation and the availability of O_2 are thus essential to consider when searching for novel treatment targets.

In this study, we confirmed that both smooth and rough CF isolates aggregate into large clusters (Fig. 2), a phenomenon also recently demonstrated in the MABSC ATCC 19977 laboratory strain (45). Most noticeably, rough isolates have pronounced cord formation, whereas smooth aggregates appear as chaotically scattered clusters. Cord formation is required for mycobacterial persistence in the host (50), whereas GPL expression in the smooth morphotype has been suggested to play a role in environ-

FIG 6 The oxygen consumption of MABSC is influenced by the degree of bacterial aggregation. The graphs show the microrespirometry of O_2 in aerobic cultures with smooth and rough CF MABSC isolates and the ATCC 19977 strain during different stages of bacterial disaggregation, as determined by the Tween 80 concentrations: 0% (black), 1.25% (red), 2.5% (green), 5% (blue). Isolates I to III were isolated from three CF patients +10, +3, and +5 years after the first sample was positive for MABSC. (A to G) The oxygen consumption with 5% Tween 80 was significantly higher than the O_2 consumption with 0% Tween 80 for all isolates ($P < 0.0001$). Error bars indicate the mean $\pm$ standard error of the mean ($n = 3$). Statistical significance was assessed using two-way ANOVA, followed by Bonferroni multiple-comparison tests. (H to N) Effect of the Tween 80 dilution on the numbers of CFU per milliliter. Error bars indicate the mean $\pm$ standard error of the mean ($n = 3$). Statistical significance was determined using one-way ANOVA, followed by Dunnett's multiple-comparison test (*, $P \leq 0.05$).

mental colonization, as seen by the sliding ability and biofilm formation (51). However, one study reported that the infecting MABSC strain was of the smooth morphotype (21), indicating the controversies in the detection and characterization of MABSC morphotypes. More studies are needed, though, to disentangle the virulence of the smooth and rough morphotypes of MABCS.

However, MABSC strains are intrinsically resistant to most antibiotics that are currently available, and it is proposed that MABSC develops resistance to common antibiotics (52, 53). In addition to these well-accepted mechanisms of antimicrobial resistance, our results indicate that *in vivo*-like conditions, such as aggregation and O_2 depletion, contribute significantly to the resistance of MABSC against amikacin. Our finding of reduced susceptibility to amikacin in MABSC when O_2 is missing is predictable due to a lack of respiration (54). We have, however, no knowledge of previous reports on the effect of the absence of O_2 on the susceptibility of MABSC to amikacin. However, other studies have demonstrated that the minimal bactericidal concentra-

FIG 7 Aerobic conditions increase the bacterial killing of *M. abscessus* complex isolates during amikacin treatment. Isolates from three CF patients were cultured for 0, 1, or 3 days with amikacin, as indicated. On each day, the number of CFU per milliliter was recorded for each amikacin treatment for triplicate individual experiments. Killing was calculated as the log reduction relative to the number of CFU per milliliter of untreated samples on each day. Isolates I to III were from three CF patients +10, +3, and +5 years after first sample was positive for *M. abscessus*. (A) Effect of increasing amikacin concentrations ranging from 0 to 512 $\mu\text{g ml}^{-1}$ on smooth CF isolates ISM+10, IISM+3, and IIISM+5 incubated for 0, 1, and 3 days under oxic (red) and anoxic (black) conditions. (B) Effect of increasing amikacin concentrations ranging from 0 to 512 $\mu\text{g ml}^{-1}$ on rough CF isolates I^{RG}+10, II^{RG}+3, and III^{RG}+5 incubated for 0, 1, and 3 days under oxic (red) and anoxic (black) conditions. Error bars indicate the mean $\pm$ standard error of the mean ($n = 3$). The statistical significance of the difference between oxic and anoxic treatments was assessed using a two-way ANOVA, followed by Bonferroni multiple-comparison tests ($P \leq 0.05$).

tions of amikacin are higher for mature biofilms of MABSC than for planktonic cells (55). In this respect, the effect of O_2 on susceptibility to other antibiotics may add even more information for the implications of low metabolic activity due to a lack of O_2 in antimicrobial resistance in MABSC.

Delays of treatment of several weeks or even months may enable aggregate formation and O_2 restriction and thereby represent the difference between eradication and chronic progressive infection. Hence, new treatment strategies that reduce both aggregation and the metabolic inactivity of MABSC are crucial for a successful eradication strategy. We believe that early diagnosis is necessary for successful eradication, as was originally shown with *P. aeruginosa* in CF patients (56). The present study has provided *in vitro* evidence for the reasons why susceptibility testing of MABSC does not give reliable results and that the improved efficacy of antimycobacterial drugs can be obtained on disaggregated MABSC isolates from CF patients. The mechanism may be ascribed to the biofilm aggregation associated with lower metabolism, which can be reverted by disaggregation by Tween 80, leading to increased antibiotic uptake. In this respect, it may be highly relevant to determine the minimum number of bacteria providing an aggregate with tolerance. On the basis of the relation between the lower tolerance and the reduced frequency of aggregates exceeding 10 μm^3 induced by treatment with 5% Tween 80 (Fig. 3 to 5) and by assuming that the average volume of MABSC is 0.6 μm^3 , our results indicate that the aggregation of only 15 cells is sufficient to provide increased tolerance.

New therapeutic strategies in CF patients with chronic MABSC infection could therefore be early antibiotic eradication before biofilm formation combined with the disaggregation of bacterial clusters and increasing O_2 availability in the endobronchial mucus.

TABLE 1 Characteristics of 22 patients with cystic fibrosis and chronic MABSC infection^a

Characteristic	Value
Median (IQR) age (yr)	19 (12–24)
No. (%) of female patients	9 (42.9)
Median (IQR) body mass index (kg/m ²)	20 (16–23) ^b
No. (%) of patients homozygous for F508del mutation	20 (95.2)
Median (IQR) % FEV1	70 (62.5–84.1)
Median (IQR) duration of chronic MABSC infection (yr)	3 (2–6)
No. (%) of patients with chronic Gram-negative bacterial infection	10 (58.8)
No. (%) of patients receiving:	
Intravenous treatment for a minimum of 14 days	10 (62.5)
Oral treatment for a minimum of 90 days	10 (62.5)

^aAbbreviations: IQR, interquartile range; FEV1, forced expiratory volume in 1 s; MABSC, *Mycobacterium abscessus* complex.

^bData are missing for one patient.

MATERIALS AND METHODS

Patients. As defined by the Danish Act on Research Ethics Review of Health Research Projects, Section 2, the project did not constitute a health research project and was thus initiated without approval from the Committees on Health Research Ethics in the Capital Region of Denmark. The study was carried out with 36 bacterial isolates from 22 chronically infected CF patients (Table 1). Chronic infection with MABSC was defined as fulfilling the ATS/IDSA criteria (7).

Bacterial strains and media. Wild-type *M. abscessus* subsp. *abscessus* reference strain ATCC 19977, obtained from the American Type Culture Collection by Statens Serum Institute (SSI), Denmark, and 36 MABSC isolates with smooth or rough morphotypes from CF patients were used (Table 1). All experiments were initiated by streaking bacteria on chocolate blood agar plates (CBA; 36.0 g liter⁻¹ GC agar base [15 g liter⁻¹ peptone, 1.0 g liter⁻¹ corn starch, 4 g liter⁻¹ dipotassium phosphate, 1 g liter⁻¹ monopotassium phosphate, 5 g liter⁻¹ sodium chloride, 10 g liter⁻¹ agar], 10.0 g liter⁻¹ IsoVital X enrichment, 10.0 g liter⁻¹ hemoglobin; SSI), a nonselective, enriched growth medium used for the isolation of pathogenic bacteria. Bacteria were incubated until visible growth (5 to 8 days) at 37°C with 5% CO₂. MABSC was grown in Mueller-Hinton II bouillon medium (MH; 17.5 g liter⁻¹ of acid hydrolysate of casein, 3 g liter⁻¹ of beef extract, 1.5 g liter⁻¹ starch; SSI) with various concentrations of Tween 80 (Croda International PLC, United Kingdom) to reach final concentrations of 1.25%, 2.5%, and 5%. A single colony was transferred from CBA plates into 20 ml MH plus 5% Tween 80 in 50-ml culture flasks (TPP, Switzerland) and incubated for 5 days (37°C, 150 rpm) before each experiment.

Bacterial susceptibility and resistance test. Aggregated MABSC isolates were grown and incubated in MH and plated by normal plate spreading, in which one MABSC colony was suspended in 2 ml 0.85% NaCl medium (bioMérieux, Ballerup, Denmark) and thereafter streaked with a sterile cotton swab onto blood agar plates (SSI) with a plate rotator (Retro C-80; bioMérieux). Disaggregated MABSC isolates were tested in MH plus 5% Tween 80, in which one MABSC colony was suspended in 12 ml MH plus 5% Tween 80 and incubated (for 2 to 5 days, depending on the isolate, at 150 rpm and 37°C in 5% CO₂). The MABSC cultures were then diluted 10-fold in MH plus 5% Tween 80 and subsequently added onto cation-adjusted horse blood agar susceptibility medium (SSI Diagnostica, Hillerød, Denmark) and uniformly distributed by gently tipping the plates. Excess liquid was discharged. First, tests for susceptibility to the following antibiotics (containing the indicated amounts per disk) were performed by the agar disk diffusion method: 30 µg amikacin, 15 µg azithromycin, 30 µg cefoxitin, 5 µg ciprofloxacin, 15 µg clarithromycin, 10 µg imipenem, 30 µg kanamycin, 10/30 µg linezolid, 5 µg moxifloxacin, 5 µg rifampin, 15 µg tigecycline, and 1.25 µg sulfamethoxazole plus 23.75 µg trimethoprim (Neo-Sensitabs; Rosco Diagnostica, Denmark). Second, Etests were performed with the following antibiotics: amikacin, azithromycin, clarithromycin, kanamycin, and tigecycline (bioMérieux). The plates were incubated for 4 to 5 days, depending on the isolate (37°C, 5% CO₂), before determining the inhibition zones and MICs by visual inspection.

Anaerobic growth. The MABSC isolates were grown and treated under anoxic conditions in an anaerobic growth chamber (Concept 400 anaerobic workstation; Ruskinn Technology Ltd., UK). The gas atmosphere consisted of N₂-H₂-CO₂ (ratio, 80:10:10). Anoxia was confirmed with an optical O₂ sensor (HQ40d multi; Hach Company, CO, US). To remove traces of O₂, all media and chemical solutions used for anaerobic work were equilibrated in the anaerobic chamber for 5 days prior to the experiment.

Killing of MABSC. Killing curves were generated to investigate the effect of O₂ on MABSC treated with amikacin for 3 days. Five-day-old cultures of reference strain ATCC 19977 and 9 MABSC isolates from CF patients were adjusted to an optical density at 600 nm (OD₆₀₀) of 0.005, corresponding to approximately 10⁵ CFU ml⁻¹. Treatment was initiated by diluting amikacin in 20 ml oxic or anoxic MH plus 5% Tween 80 in 4-fold dilutions ranging from 2 to 512 mg liter⁻¹. Cultures were aliquoted in 20-ml glass vials (Schott, Germany), after which the anoxic cultures were sealed with airtight lids, while oxic cultures were sealed with Parafilm (Sigma-Aldrich, Denmark). All O₂-depleted cultures were prepared in an anaerobic bench using equilibrated MH plus 5% Tween 80. The cultures were then incubated at 37°C and 150 rpm for up to 3 days. Samples were taken from the same cultures at days 0, 1, and 3, viable plate counts were performed by standard microbiological methods, and the plates were incubated for up to 7 days at 37°C.

Microscopy and image analysis. One milliliter of untreated, 5-day-old cultures of ATCC 19977 and each of six MABSC isolates from patients with CF grown in MH supplemented with 0%, 1.25%, 2.5%, or 5% Tween 80 was mixed with 1 μ l of Syto 9 (Molecular Probes, USA). The stained samples were incubated in the dark for 15 min at room temperature. After incubation, 20 μ l of each sample was placed in the wells of a μ -slide VI channel slide (Ibidi, Germany). Samples were visualized by confocal laser scanning microscopy (CLSM) on an LSM 880 Zeiss inverted microscope running the Zen (version 2.1) program (Zeiss, Germany). Imaging was performed at a $\times 63$ magnification by use of a 488-nm laser exciting Syto 9 and an emission filter of 495 to 580 nm. Samples were imaged at 7 different randomly selected regions in images measuring 135 μ m by 135 μ m by 15 μ m per scan. The z-stacks obtained were rendered into three-dimensional projections with Imaris (version 9.2) software (Bitplane, Switzerland). Aggregate sizes were measured with the use of the Measure Pro Expansion tool for Imaris (version 9.2) software. An iso-surface was applied over the Syto 9-stained biomass. Iso-surface particles of between 1 and 10 μ m³ were distinguished from particles larger than 10 μ m³, and the fraction of the two parts was used as a measurement of aggregation versus single cells/small aggregates.

To visualize MABSC in CF patient lungs, a CF patient sputum sample was transferred to 4% formaldehyde before further preparation for microscopic investigation. The biopsy material was embedded in paraffin, cut into 4- μ m sections, and mounted on glass slides. Prior to microscopy, the sections were deparaffinized, and the tissue sections were analyzed by staining with Ziehl-Neelsen stain (an acid-fast stain). Microscopy was performed using an LSM 880 Zeiss inverted microscope. Representative MABSC bacteria isolated from a CF patient sputum sample were prepared for scanning electron microscopy (SEM) as previously described (39). Briefly, bacteria were cultured in MH without Tween 80 and harvested and fixed in 2% glutaraldehyde, postfixed in 1% OsO₄, dried to the critical point using CO₂, and sputter coated with gold according to standard procedures. Specimens for SEM were investigated with a Philips XL Feg30 SEM operated at a 2- to 5-kV accelerating tension.

Flow cytometry. The effect of 5% Tween 80 on the permeability of the cell envelope of MABSC was investigated using flow cytometry by comparison of the propidium iodide (PI) fluorescence of ATCC 19977 grown for 3 days in MH without 5% Tween 80 to the fluorescence of ATCC 19977 grown for 3 days in MH with 5% Tween 80. Before flow cytometry, PI (catalog number P4170; Sigma-Aldrich) was added to the samples to a final concentration of 20 μ M and the mixture was incubated for 15 min. Stained samples were analyzed on an Attune Next flow cytometer (Thermo Fisher Scientific) by exciting PI-stained samples at 561 nm and collecting the fluorescence through a 585/16-nm band-pass filter.

Oxygen respirometry. Respiration vials (Oxvial4) with integrated strips of O₂-sensitive, Redflash indicators glued to the inner wall (PyroScience, GmbH) were positioned on a magnetic stirring head connected to a magnetic stirrer (model 300; Rank Brothers, Cambridge, UK). Adapter rings (Advial4; Pyro-Science) were fitted around the vials to allow for the easy placement of bare optical fibers (Spfib-Bare; PyroScience) connected to an optical O₂ meter (catalog number FSO2-4; FireStingO2; Pyro Science). The oxygen concentration was measured with Pure Oxygen Logger software (version 3.206) with the optical O₂ meter (FireStingO2; Firmware, version 3.07; PyroScience) in the continuous mode with sampling every 1 s. The temperature was fixed at 37°C, and the pressure was fixed at 1.013 $\times 10^5$ Pa. The factory calibration was used to calibrate the sensors (Oxvial4) per the manufacturer's recommendation. The oxygen concentrations were measured in cultures of reference strain ATCC 19977 and 6 MABSC isolates from CF patients that had been grown in MH with final concentrations of 0%, 1.25%, 2.5%, and 5% Tween 80. All samples were homogenized with O₂ before measurements were taken. Quantitation of bacterial growth was performed.

Statistical methods. Statistical significance was evaluated by Fisher's exact test, Student's *t* test, and ordinary one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's multiple-comparison test. A *P* value of ≤ 0.05 was considered statistically significant. Data from at least 3 independent experiments were compared. Tests were performed with GraphPad Prism (version 6.1) software (GraphPad Software Inc., La Jolla, CA) and Microsoft Excel software (Microsoft Corp., Redmond, WA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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