Structural Analysis of Biofilm Formation by Rapidly and Slowly Growing Nontuberculous Mycobacteria[∇]

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***Mycobacterium avium* complex (MAC) and rapidly growing mycobacteria (RGM) such as *M. abscessus*, *M. mucogenicum*, *M. chelonae*, and *M. fortuitum*, implicated in health care-associated infections, are often isolated from potable water supplies as part of the microbial flora. To understand factors that influence growth in their environmental source, clinical RGM and slowly growing MAC isolates were grown as biofilm in a laboratory batch system. High and low nutrient levels were compared, as well as stainless steel and polycarbonate surfaces. Biofilm growth was measured after 72 h of incubation by enumeration of bacteria from disrupted biofilms and by direct quantitative image analysis of biofilm microcolony structure. RGM biofilm development was influenced more by nutrient level than by substrate material, though both affected biofilm growth for most of the isolates tested. Microcolony structure revealed that RGM develop several different biofilm structures under high-nutrient growth conditions, including pillars of various shapes (*M. abscessus* and *M. fortuitum*) and extensive cording (*M. abscessus* and *M. chelonae*). Although it is a slowly growing species in the laboratory, a clinical isolate of *M. avium* developed more culturable biofilm in potable water in 72 h than any of the 10 RGM examined. This indicates that *M. avium* is better adapted for growth in potable water systems than in laboratory incubation conditions and suggests some advantage that MAC has over RGM in low-nutrient environments.**

Many species of nontuberculous mycobacteria (NTM) are commonly isolated from potable water (PW) supplies and have been implicated in both community-acquired and health care-associated infections (7, 13, 18, 21, 30, 31). Much attention has been paid to slowly growing mycobacteria, especially *Mycobacterium avium* (1, 10). However, several health care-related outbreaks and pseudo-outbreaks caused by rapidly growing mycobacteria (RGM; e.g., *M. abscessus*, *M. chelonae*, *M. mucogenicum*, and *M. fortuitum*) demonstrate the importance of these organisms in causing infections (9). Examples of diseases caused by RGM in PW supplies include infections in hemodialysis patients (23), postsurgical wound infections (6), furunculosis caused by *M. fortuitum* (33), and bacteremia caused by *M. mucogenicum* (20).

Although many studies have linked environmental mycobacteria to clinical isolates, NTM can be difficult to culture from the complex community found in most drinking water distribution systems (WDS) due to competition on media from many faster growing fungi and heterotrophic bacteria (7, 18, 25). In addition to linking infections to their source, quantification of environmental NTM will help to determine their ecological role in WDS biofilms, possibly leading to more-effective point-of-use treatment to prevent transmis-

sion to susceptible populations. For example, previous work has demonstrated a positive correlation between lower levels of assimilable organic carbon and the concentration of NTM in WDS biofilms (25, 30). In other work, clinical isolates of *M. avium* formed more biofilm when incubated in water than when incubated in Middlebrook 7H9 broth (4, 22). The presence of divalent cations and carbon in the water also increased biofilm production (4). Although laboratory studies help define parameters for NTM biofilm growth, little is known regarding environmental settings, such as the numbers of each species in multispecies biofilms, how often they are sloughed off into the water supply, and most importantly for human health, what their virulence is when they reach the user during bathing/showering, reprocessing of medical devices, or other exposures. Given that free-living mycobacteria are part of the water flora, it may ultimately be more relevant to determine the virulence of NTM reaching exposed individuals than to merely confirm their presence and numbers.

Some researchers have linked biofilm formation ability, glycopeptidolipid (GPL) production, and in some cases, microcolony morphology to virulence in NTM (19, 34). Yamazaki et al. (34) created mutants of *M. avium* that could not form as much biofilm as the wild type, and these mutants were also less infective than the wild type. The opposite was found for *M. abscessus*, where more-invasive strains formed less biofilm in a static laboratory model (19). The rough colony type of *M. abscessus* was associated with virulence more than the smooth colony type was, as tested in human monocyte and mice mod-

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TABLE 1. Clinical, environmental, and reference strains of NTM examined for biofilm formation ability

Isolate	Source ^a	Comment(s)
<i>M. abscessus</i> 23007	ATCC	Reference strain
<i>M. abscessus</i> BF6	Patient	NY type 2 strain; one of two types isolated from patients who received illegal cosmetic procedures in 2002
<i>M. abscessus</i> 4AU	Vial of adrenal cortex extract	Contaminated vials caused multistate outbreak, 1995–1996
<i>M. chelonae</i> 35752	ATCC	Reference strain
<i>M. chelonae</i> 34	Cornea of LASIK patient	Strain associated with contaminated contact lenses used during illegal LASIK procedure
<i>M. chelonae</i> 56	Cornea of LASIK patient	Strain associated with contaminated contact lenses used during illegal LASIK procedure
<i>M. chelonae</i> 99	Blood of bone marrow transplant patient	Infection associated with indwelling catheter
<i>M. fortuitum</i> 32	Blood of bone marrow transplant patient	Infection associated with indwelling catheter
<i>M. fortuitum</i> 89	Leg of nail parlor patient	Caused outbreak of furunculosis of the legs in patients using contaminated footbaths
<i>M. smegmatis</i> 19420	ATCC	Reference strain
<i>M. avium</i> 91	AIDS patient	Serotype 4 strain; most common type isolated from patients with AIDS
<i>M. avium</i> EPA 61151	Water	Strain persistent at point of use for at least 26 mo
<i>M. avium</i> EPA 88126	Patient	Respiratory isolate
<i>M. intracellulare</i> EPA 88144	Patient	Respiratory isolate

^a LASIK, laser-assisted in situ keratomileusis.

els. The rough phenotypes formed microscopic cording structures, while the smooth phenotype did not. GPL was expressed in smooth types but little in rough types. Rough types formed little biofilm compared to smooth types. The “hypervirulence” of a rough colony morphotype was also observed in another strain of *M. abscessus* (5), indicating that biofilm formation ability, biofilm structure, and virulence can be linked in at least some mycobacteria. The link between biofilm formation and structure has been examined previously in *M. chelonae*, *M. fortuitum*, and *M. marinum* (2, 14, 15, 16). *M. marinum* formed cords similar to that of the rough *M. abscessus* strains mentioned above (16).

The surfaces to which mycobacteria attach are likely determined, at least in part, by the hydrophobicity and mycolic acid composition of the organisms' cell walls. The environmental conditions and nutrients available inside pipes and other surfaces in health care environments also affect mycobacterial growth. In the health care setting, some important surface materials include the pipes comprising the WDS, shower fixtures, sink faucets, ice machines, and medical devices. Relevant materials include metals, such as stainless steel (SS) and copper, and plastics, such as polyvinyl chloride (PVC) and polycarbonate (PC). In a previous study, *M. fortuitum* developed more biofilm on SS, PVC, and PC than on copper or glass (32). Similar amounts of biomass were measured on PVC and PC. PC was chosen as a substrate for biofilm growth for this investigation, along with SS, because of their inclusion in many medical devices as materials that can be disinfected through steam autoclaving, the use of ethylene oxide, or irradiation.

Given the association between NTM infections and drinking water, clinical isolates of NTM would be expected to form biofilm in WDS. The goal of this study was to determine the effects of two substrate materials (SS and PC) and nutrient level (autoclaved municipal tap water and a microbiological culture medium) on the ability of NTM to form biofilms in a laboratory model. A second objective of this study was to evaluate biofilm structure to clarify the role that microcolony

morphologies such as cord and pillar formation may play in the survival and maintenance of mycobacteria in WDS biofilms.

MATERIALS AND METHODS

Mycobacterial isolates and culture conditions. The 14 mycobacterial isolates included in this study are listed in Table 1. All non-ATCC (American Type Culture Collection, Manassas, VA) isolates were obtained from health care-related outbreaks investigated by the CDC, except for three. *Mycobacterium avium* EPA 61151, *M. avium* EPA 88126, and *M. intracellulare* EPA 88144 were obtained during a research study (18). All isolates were cultivated on Middlebrook 7H10 agar (Becton, Dickinson and Co., Sparks, MD). RGM were incubated at 35°C; *M. avium* complex (MAC) species isolates (*M. avium* and *M. intracellulare*) were incubated at 37°C.

Method for growth and quantification of biofilms. (i) Preparation of materials. PC and SS grade 316L disks, each measuring 13 mm in diameter and 4 mm in thickness (BioSurface Technologies, Bozeman, MT), were washed in dilute laboratory soap (Versa-Clean; Fisher Scientific, Pittsburgh, PA), rinsed at least five times in reverse osmosis-purified water, rinsed once in 70% ethanol, air dried, and autoclaved before use.

(ii) Method for growth of biofilms. Biofilms were developed on autoclaved SS or PC disks incubated in a 24-well tissue culture plate (Corning Incorporated, Corning, NY), with one disk per well. Each disk was covered with 1.5 ml of either sterile R2A broth (R2A medium without the agar) (26) or autoclaved PW. Suspensions of each isolate, collected from the surface of a Middlebrook 7H10 agar plate, were made in Middlebrook 7H9 broth; concentration was determined by measuring absorbance in a MicroScan turbidity meter (Dade Behring, Deerfield, IL). Suspended cells were diluted in 0.00425% monopotassium phosphate (Butterfield's buffer; Becton, Dickinson and Co.), and approximately 10⁴ CFU were inoculated per well. Inoculation concentration was confirmed by enumerating CFU from a subsample on Middlebrook 7H10 agar. The well plates were incubated for 72 h at 35°C with gentle shaking on a rocker platform (setting 9, 15 rotations/min; Cole-Parmer, Vernon Hills, IL). At the end of the incubation time, disks were removed with sterile forceps, dipped three times with gentle up and down motions in a beaker containing phosphate-buffered saline (PBS) to remove loosely attached bacteria, and processed for either plate count enumeration or microscopy analysis. Disks destined for microscopic structural analysis were fixed in 4% formaldehyde and stored at 4°C until examined.

(iii) Biofilm quantification by viable plate counting. After disks were rinsed in PBS, mycobacteria were enumerated by culturing on Middlebrook 7H10 agar. Disks were placed in 50-ml polypropylene centrifuge tubes containing 10 ml of PBS plus 0.1% Tween 80. Bacteria were removed from the disk surface with three cycles of sonication in a water bath sonicator (frequency of 42 kHz [±6%]; Branson Ultrasonics Corp., Danbury, CT) for 1 min, followed by vortexing

(maximum setting) for 30 s. This bacterial suspension was diluted in 0.00425% monopotassium phosphate and spread on 7H10 agar. Plates were incubated at 35°C or 37°C for rapid growers or slow growers, respectively, until colonies were observed (3 to 5 days for rapid growers and 10 to 14 days for slow growers, typically). Removal efficiency was estimated by microscopic examination of 12 isolates grown in triplicate on each substrate under each nutrient condition. Percent coverage at the substrate level was calculated as described in "Microscopy and image analysis" below.

(iv) **Biofilm measurement by microscopic methods.** Bacterial DNA within biofilms was stained with Sybr green I (Molecular Probes, Inc., Carlsbad, CA) for visualization of all bacteria attached to the SS or PC surface. Disks were removed from formaldehyde, dipped in PBS, and placed in a 5× solution of Sybr green I in room temperature Tris-EDTA buffer at pH 8 (10 mM Tris-1 mM EDTA; Mediatech, Inc., Manassas, VA). Staining was carried out for 10 min in the dark, followed by a 1-min rinse in sterile distilled water. Disks were air dried in the dark and mounted onto glass microscope slides with double-sided tape. Coverslips (22 mm², with 1-mm thickness) were mounted onto the disks with ProLong Gold antifade mounting fluid (Molecular Probes, Inc.); the coverslips were taped down on either side of the disk to maintain stability.

(v) **Microscopy and image analysis.** Biofilms were examined with a Zeiss Axioplan epifluorescence microscope equipped with a fluorescein filter set (480/40 nm excitation, 505-nm long-pass dichroic mirror, 535/50 nm emission) and an ApoTome, an attachment that reduces light scatter in fluorescing samples (Carl Zeiss, Inc., Thornwood, NY). Images were obtained through a 40× oil objective, a Zeiss AxioCam HRm digital camera, and AxioVision imaging software. For each microscope field (x-y plane) imaged, optical sections were obtained through the thickness of the biofilm (z), from the disk substratum to the top of the biofilm, in a Z-stack. At least five Z-stacks were sampled per disk, and three disks per isolate per condition were examined. To view a representative section of each disk, at least one image was obtained from the center, and the others were obtained from different quadrants of the disk. For measuring percent biofilm removal, 10 substratum images were obtained from each of three disks for 30 images total per nutrient level and surface material. All images were exported in gray-scale tagged image file format for analysis using COMSTAT software (17).

Image analysis was performed on each image stack with manual thresholding. Variables such as maximum thickness, biomass estimate, and percent coverage were calculated using COMSTAT. For percent coverage, the maximum percent coverage of each image stack was included, rather than that of the layer closest to the substratum. The exception to this was in calculating the efficiency of biofilm removal from disks. Percent coverage was measured at the substratum on disks after sonication/vortexing and compared to those of disks containing biofilm by subtracting percent coverage after biofilm removal from percent coverage of intact biofilm, dividing by percent coverage of intact biofilm, and multiplying by 100.

(vi) **Statistical analysis.** Multiple comparisons of nutrient level and substrate material within each isolate were performed on image analysis data using the general linear model with the Bonferroni adjustment in SigmaStat (version 3.5; Systat Software Inc). Statistical comparisons of mycobacterial isolate biofilm growth detected by culturing were performed with SAS (version 9.1; SAS Institute Inc.). Significance was indicated if *P* values were <0.05.

RESULTS

Enumeration of mycobacterial biofilm by plate counting. Culturable biofilm was formed by all isolates of *Mycobacterium* spp. incubated at each nutrient level and in substratum material, except for *M. smegmatis* grown in low-nutrient conditions (PW), as measured by plate counting (Fig. 1). The lower detection limit for the plate count method was 100 CFU per disk or approximately 24 CFU/cm². In general, nutrient level had more of an effect on biofilm plate counts than did substrate material. For all RGM, biofilm plate counts on PC were significantly higher under high-nutrient conditions (R2A) than those in PW (*P* < 0.05). Biofilm plate counts were also significantly higher (*P* < 0.05) on steel (SS) in R2A than those in PW for several of the strains (*M. abscessus* strains ATCC 23007, BF6, and 4AU; *M. chelonae* 99; *M. fortuitum* strains 32 and 89; and *M. smegmatis* ATCC 19420). PC also yielded

significantly higher (*P* < 0.01) biofilm plate counts than SS for several of the isolates (*M. abscessus* ATCC 23007, *M. chelonae* strains 56 and 99, and *M. smegmatis* ATCC 19420) in R2A. In PW, the type of material did not affect RGM biofilm formation significantly.

Neither nutrient nor substratum had a significant effect on viable biofilm counts for the three *M. avium* strains (*M. avium* strains 91, EPA 61151, and EPA 88126), though these organisms tended to develop higher biofilm plate counts than the RGM in PW. However, *M. intracellulare* EPA 88144, a MAC organism, formed significantly more viable biofilm in R2A than in PW when grown on PC (*P* < 0.01).

Description of microcolonies observed by epifluorescence microscopy. The largest biofilm structures were observed in samples grown in R2A on PC, displaying several microcolony morphologies (Fig. 2). For instance, *M. abscessus* ATCC 23007 formed large, diffuse microcolonies (Fig. 2A). In contrast, *M. abscessus* strains BF6 and 4AU and *M. chelonae* ATCC 35752 formed cord structures in R2A on PC and, to a lesser extent, on SS. *M. abscessus* BF6 also formed cords occasionally in PW on SS. Examples of cording structure are shown in Fig. 2B and C. *M. fortuitum* strains 89 and 32 formed tall, narrower microcolonies, but *M. fortuitum* 32 also had curved, fingerlike projections that extended along the surface at the base of many structures (Fig. 2E). Other isolates formed moderate to very sparse biofilms, as represented by *M. smegmatis* ATCC 19420 and *M. avium* 91 in Fig. 2D and F, respectively. Although culturable *M. smegmatis* ATCC 19420 was not recovered from disks incubated in PW, small amounts of attached bacteria were observed directly (data not shown). For EPA strains *M. avium* EPA 88126 and *M. intracellulare* EPA 88144, it was necessary to scan much of each disk to find any evidence of cells or microcolonies. Image stacks of these two mycobacteria were not analyzed quantitatively.

Image analysis. With the exception of *M. chelonae* 56, biomass for the RGM was highest on PC in R2A and lowest on PC in PW. The high-nutrient biomass measurements are in agreement with the biofilm plate counts for PC and SS, whereas in PW, the substratum had less of an effect on plate count than on biomass (Table 2). Maximum thickness (Fig. 3) and maximum percent coverage (Table 3) for all RGM, with the exception of *M. chelonae* 56, were also highest for biofilms grown on PC in R2A (*P* < 0.05). Biomass and percent coverage for *M. avium* EPA 61151 were also significantly higher on PC in R2A. These results suggest that quantitative structural analysis methods (i.e., biomass, maximum thickness, and maximum percent coverage) can predict viable count results for these organisms, that nutrient level may predict attachment and biofilm formation, and that PC is generally most conducive to biofilm formation.

Image analysis parameters and plate counts were compared among non-cord-forming *M. abscessus* ATCC 23007 and cord-forming *M. abscessus* BF6 and 4AU biofilms incubated in R2A on PC. Plate counts for *M. abscessus* ATCC 23007 were not significantly higher than those for *M. abscessus* BF6, despite vast differences in microcolony structure. However, *M. abscessus* ATCC 23007 produced significantly more biomass and maximum percent coverage than *M. abscessus* BF6 (*P* < 0.05). Maximum thickness measurements for *M. abscessus* ATCC 23007, however, were not significantly different than those for *M. abscessus* BF6. *M. abscessus* 4AU, another cord former,

FIG. 1. Culturable biofilm of *Mycobacterium* clinical isolates and reference strains after growth for 3 days at 35°C in R2A medium (a) or PW (b) on PC or SS disks. Data were transformed by addition of 1 and converting values to log₁₀ values. The detection limit was 100 CFU/disk or approximately 24 CFU/cm² (*n* = 3). Strain identifiers are as follows: Mab23, *M. abscessus* ATCC 23007; MabBF6, *M. abscessus* BF6; Mab4AU, *M. abscessus* 4AU; Mch35, *M. chelonae* ATCC 35752; Mch34, *M. chelonae* 34; Mch56, *M. chelonae* 56; Mfo32, *M. fortuitum* 32; Mfo89, *M. fortuitum* 89; Msm19, *M. smegmatis* ATCC 19420; Mav91, *M. avium* 91; Mav61, *M. avium* EPA 61151; Mav26, *M. avium* EPA 88126; and Min44, *M. intracellulare* EPA 88144.

FIG. 2. Compiled biofilm images of six mycobacteria isolates grown in R2A medium on PC disks. Biofilm was stained with Sybr green I before image stacks were obtained. (A) *M. abscessus* ATCC 23007; (B) *M. abscessus* BF6; (C) *M. chelonae* ATCC 35752; (D) *M. smegmatis* ATCC 19420; (E) *M. fortuitum* 32; (F) *M. avium* 91.

TABLE 2. Biomass estimation of 10 RGM and two *M. avium* isolates^a

Isolate	Mean biomass vol ($\mu\text{m}^3/\mu\text{m}^2$) (SD) grown with ^b :			
	R2A-PC	R2A-SS	PW-PC	PW-SS
<i>M. abscessus</i> ATCC 23007	0.73 (0.51)	0.33 (0.43)	0.0044 (0.010)	0.011 (0.0092)
<i>M. abscessus</i> BF6	0.30 (0.32)	0.026 (0.068)	0.0024 (0.0051)	0.0063 (0.0058)
<i>M. abscessus</i> 4AU	0.78 (0.77)	0.098 (0.13)	0.0016 (0.0021)	0.012 (0.019)
<i>M. chelonae</i> ATCC 35752	0.081 (0.047)	0.039 (0.050)	0.00066 (0.00093)	0.0017 (0.0022)
<i>M. chelonae</i> 34	0.0064 (0.0050)	0.0048 (0.0030)	0.00050 (0.00063)	0.0015 (0.0014)
<i>M. chelonae</i> 56	0.0041 (0.0038)	0.019 (0.021)	0.00072 (0.00051)	0.0012 (0.00079)
<i>M. chelonae</i> 99	0.48 (0.55)	0.070 (0.14)	0.0013 (0.0024)	0.0022 (0.0020)
<i>M. fortuitum</i> 32	0.87 (0.74)	0.094 (0.20)	0.00076 (0.00057)	0.0094 (0.0095)
<i>M. fortuitum</i> 89	0.23 (0.22)	0.082 (0.11)	0.0013 (0.0012)	0.0037 (0.0024)
<i>M. smegmatis</i> ATCC 19420	0.073 (0.14)	0.021 (0.029)	0.0018 (0.0030)	0.0013 (0.0014)
<i>M. avium</i> 91	0.0024 (0.0046)	0.0022 (0.0022)	0.00094 (0.00073)	0.00099 (0.00081)
<i>M. avium</i> EPA 61151	0.012 (0.033)	0.00024 (0.00011)	0.00069 (0.00056)	0.00053 (0.00030)

^a Biomass estimation was calculated for 10 RGM and two *M. avium* isolates incubated in two nutrient levels and on two substratum materials.

^b Mean value was obtained from five image stacks from each of three disks for most isolates ($n = 15$). R2A and PW were the media (nutrient levels) used; PC and SS were the surface materials. SD, standard deviation.

developed significantly more culturable bacteria than *M. abscessus* ATCC 23007 ($P < 0.01$). However, no significant differences between the biomass, maximum percent coverage, or maximum thickness measurements of *M. abscessus* ATCC 23007 and those of *M. abscessus* 4AU were observed.

Biofilm removal efficiency was estimated for 12 isolates grown in triplicate on each substrate and under each nutrient condition by comparing the percent coverage at the substrate level after sonication/vortexing to the percent coverage of intact biofilm. The majority of isolates under each nutrient level and substratum demonstrated >95% biofilm removal efficiency, and all but four calculations were above 90%. Categorized by growth condition, isolates incubated in R2A on PC produced a removal efficiency ranging from 100% (99.95%) for *M. abscessus* ATCC 23007 to 90.0% for *M. chelonae* ATCC 35752; in R2A on SS, the efficiency ranged from 99.6% for *M. abscessus* ATCC 23007 to 83.4% for *M. smegmatis* ATCC

19420; in PW on PC, the efficiency ranged from 99.9% for *M. chelonae* 99 to 90.1% for *M. abscessus* 4AU; and in PW on SS, the recovery efficiency ranged from 99.6% for *M. fortuitum* 32 to 74.6% for *M. chelonae* 56.

DISCUSSION

All clinical isolates of NTM formed detectable biofilm in PW. The highest amount of culturable biofilm was formed in PW in 3 days by the clinical isolate *M. avium* 91, a slow grower that takes more than 7 days to form colonies on laboratory medium. Ironically, the two clinical isolates *M. avium* 91 and EPA 88126 formed more culturable biofilm under each condition than the environmental isolate *M. avium* EPA 61151. A similar result was obtained by Carter et al. during a comparison of biofilm formation abilities of *M. avium* isolates from AIDS patients (4). This suggests that *M. avium* may be better adapted for growth in PW systems than some species of RGM. However, with the challenges in detecting and accurately enumerating NTM in environmental samples (7, 8, 18), more information is required to confirm this in PW supplies. Although many studies have linked MAC in PW supplies to human infection (1, 10, 12), outbreak investigations have found health care-related infections caused increasingly by waterborne RGM (9, 11, 20). In this study of single-species biofilms in a simple batch system, RGM biofilm growth was highly influenced by nutrient level, with PW restricting biofilm growth. It may be that in a multispecies PW biofilm, nutrient exchange with other organisms may enhance RGM growth. This should be determined in concert with the study of possible control measures for these organisms. Since mycobacteria demonstrate high tolerance for chlorine disinfectants typically present in PW (3, 4), especially when in biofilms (28), the best infection control intervention may be provided by measures taken at the point of use by the individual or health care professionals. Examples of infection prevention include preventing exposure of wound or catheter entry sites to PW during bathing, adequately maintaining ice machines that make ice intended for patient consumption, and performing point-of-use treatment on PW that will reach patients with compromised immune systems.

FIG. 3. Maximum biofilm thickness of 10 RGM and two *M. avium* isolates incubated at two nutrient levels and on two substratum materials. Maximum thickness was significantly higher for all RGM grown on PC substratum ($P < 0.05$) in R2A (high-nutrient condition) than for those grown on PC in PW (low-nutrient condition). Substratum material was significant for all RGM grown in R2A, except for *M. chelonae* 56 and for *M. avium* isolates 91 and EPA 61151, at either nutrient level. Nutrient level did not significantly affect *M. avium* biofilm thickness ($n = 15$ for most isolates). Legend abbreviations for biofilm grown: R2A-PC, R2A broth on PC; R2A-SS, R2A broth on SS; PW-PC, PW on PC; PW-SS, PW on SS. Strain identifiers on the x axis are defined in the legend to Fig. 1.

TABLE 3. Maximum percent coverage of biofilm in the *x-y* plane of each image stack

Isolate	Mean % coverage (SD) grown with ^a :			
	R2A-PC	R2A-SS	PW-PC	PW-SS
<i>M. abscessus</i> ATCC 23007	9.7 (6.6)	5.9 (5.2)	0.096 (0.14)	0.54 (0.39)
<i>M. abscessus</i> BF6	4.8 (4.1)	0.69 (0.76)	0.074 (0.10)	0.30 (0.30)
<i>M. abscessus</i> 4AU	5.8 (3.1)	3.3 (3.7)	0.058 (0.083)	0.50 (0.75)
<i>M. chelonae</i> ATCC 35752	0.85 (0.37)	0.72 (0.59)	0.031 (0.028)	0.072 (0.074)
<i>M. chelonae</i> 34	0.17 (0.16)	0.16 (0.068)	0.022 (0.018)	0.085 (0.068)
<i>M. chelonae</i> 56	0.11 (0.077)	0.53 (0.44)	0.033 (0.019)	0.074 (0.036)
<i>M. chelonae</i> 99	5.8 (4.6)	1.2 (1.5)	0.048 (0.063)	0.22 (0.26)
<i>M. fortuitum</i> 32	6.7 (6.2)	1.4 (1.9)	0.036 (0.021)	0.78 (0.99)
<i>M. fortuitum</i> 89	2.8 (2.6)	0.88 (0.86)	0.046 (0.022)	0.20 (0.11)
<i>M. smegmatis</i> ATCC 19420	1.0 (1.6)	1.0 (1.2)	0.060 (0.077)	0.069 (0.073)
<i>M. avium</i> 91	0.065 (0.071)	0.13 (0.11)	0.043 (0.031)	0.057 (0.039)
<i>M. avium</i> EPA 61151	0.49 (1.3)	0.018 (0.0069)	0.034 (0.023)	0.040 (0.033)

^a Mean values were obtained from five image stacks from each of three disks for most isolates ($n = 15$). R2A and PW were the media (nutrient levels) used; PC and SS were the surface materials. SD, standard deviation.

Temperature may be an important factor in determining mycobacterial growth in PW biofilms (29). Although the recommended optimal incubation temperature for cultivation of most RGM is 28 to 30°C (13), all of the isolates included in this study are capable of growth in most of the temperature ranges found in interior plumbing, including the incubation temperature of 35°C used during this study.

Previous research has demonstrated a link between mycobacterial virulence and cord-forming ability (19). In that study, virulent *M. abscessus* strains formed cord structures, produced less biofilm, and produced less GPL than a nonvirulent strain. The three *M. abscessus* isolates examined in the current study all formed biofilm in each nutrient level on both surface materials. The numbers of culturable biofilm bacteria were equivalent or higher in the rough, cording, clinical isolates than in the smooth, noncording isolate. By contrast, quantitative analysis of biofilm structure by biomass estimation and maximum percent coverage demonstrated that the smooth *M. abscessus* 23007 produced more biofilm than the cord-forming *M. abscessus* BF6. This suggests that the cording formations are more compact, containing more cells per unit volume than the more-diffuse biofilm formed by smooth *M. abscessus*. These results suggest that in at least some cord-forming *M. abscessus* isolates, biofilm formation ability cannot be predicted by colony morphology. This indicates that the relationship among cording, biofilm formation, and virulence is not completely characterized. To understand these relationships more fully, a larger sampling of *M. abscessus* strains and other cord-forming species should be examined. Additionally, more-complete analysis of components necessary for biofilm formation in NTM is required.

Biomass and other image analysis measurement parameters may differ from culturable bacteria numbers for several reasons. Biofilm was imaged after staining with Sybr green I, a “total” stain of double-stranded DNA that does not indicate cell viability. It is possible that some attached mycobacteria did not maintain viability on PC or SS during the 3-day incubation.

Another possibility is that during removal of mycobacteria from disks for culturing, some biofilm remained clumped, even after vortexing and sonication, so that the number of culturable bacteria could have been underestimated. However, the low variance in CFU per isolate demonstrates that separation of mycobacteria during sample processing was consistent, if not 100% complete. Also, cell size may vary among species, strains, or even within a strain grown under different conditions. When measuring small microcolonies, as is the case for many of the mycobacteria grown in PW, a small difference in individual cell size and shape will greatly influence the microcolony dimensions. Although extracellular material was not visualized or quantified in this investigation, its presence could also affect biofilm structure.

Under low-nutrient conditions in PW, the low growth numbers and microcolony formation were at the lower limit of what may be considered biofilm versus attached cells. While culturable numbers of bacteria recovered from disk surfaces were high enough to indicate biofilm growth in PW for some isolates, particularly for MAC, direct observation of biofilms often demonstrated the presence of single cells or very small clusters of bacteria. This limited the use of image analysis under these conditions, since complex biofilm structure was absent. This may be due to the short incubation time for biofilm development in this experiment, so that young biofilm was observed rather than mature, complex biofilm. This was demonstrated in a comparison of percent surface coverage and maximum percent coverage to determine if structure in the *x-y* plane differed between the substratum level and elsewhere in the biofilm. The high correlation between the two measurements indicated either that the maximum percent coverage occurred at the substratum level, as seen in some samples (data not shown), or that similar percent coverage existed throughout the height of the biofilm. This corresponds to the simple microcolony structure observed for many of the mycobacteria, particularly under low-nutrient incubation.

Generally, more variability was observed in direct measurements than in plate counts, suggesting that more observations per sample are required to make direct observation consistent. This would be possible if a completely automated imaging system could be employed.

M. smegmatis, frequently chosen as a model organism for biofilm research (24, 27), may not be the ideal *Mycobacterium* species model for the study of biofilm formation in PW, since no culturable *M. smegmatis* was recovered in PW biofilms incubated under the conditions in this study.

All mycobacterial clinical isolates formed biofilm under high- and low-nutrient conditions. Nutrient level was a more important factor than the two substrate materials tested for microcolony formation by RGM in this study. Although other studies have demonstrated a difference in biofilm formation ability between cord- and non-cord-forming *M. abscessus* strains, the three *M. abscessus* isolates included in this study formed roughly equivalent amounts of culturable biofilm, despite the measurable differences in microcolony morphology. Additional study of *M. abscessus* cording ability and pathogenicity, as well as its ecology in PW supplies, may lead to a better understanding of the role played by *M. abscessus* in health care-related infections. When most measurements are considered, nutrient level did not significantly affect

MAC biofilm development. This study indicated that *M. avium* is better equipped to grow in warm PW supplies than RGM is, offering an explanation for the greater occurrence of disease caused by MAC.

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