Applied and Environmental Microbiology, June 2006, p. 4007–4011 Vol. 72, No. 6
0099-2240/06/$08.00+0 doi:10.1128/AEM.02573-05

Effect of Growth in Biofilms on Chlorine Susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*

Keesha A. Steed and Joseph O. Falkinham III*

Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0406

Received 1 November 2005/Accepted 29 March 2006

***Mycobacterium avium* and *Mycobacterium intracellulare* were grown in suspension and in biofilms, and their susceptibilities to chlorine were measured. *M. avium* and *M. intracellulare* readily adhered within 2 h, and numbers increased 10-fold in 30 days at room temperature in biofilms on both polystyrene flasks and glass beads. The chlorine resistance of *M. avium* and *M. intracellulare* cells grown and exposed to chlorine in biofilms was significantly higher than that of cells grown in suspension. Survival curves showed no evidence of a resistant, persisting population after 6 h of exposure to 1 μg chlorine/ml. The chlorine susceptibility of cells grown in biofilms and exposed in suspension (cells detached from bead surfaces) was also significantly higher than that of cells grown and exposed in suspension (planktonic cells), although it was lower than that of cells grown and exposed in biofilms. The higher resistance of the detached biofilm-grown cells was reversed upon their growth in suspension. There was a strong correlation between the chlorine susceptibility of cells of both *M. avium* and *M. intracellulare* and cell surface hydrophobicity measured by contact angle for both biofilm- and suspension-grown cells.**

Mycobacterium avium and *Mycobacterium intracellulare* are opportunistic environmental pathogens (17, 52). One of the sources of *M. avium* for human infection is drinking water (50). *M. avium* and *M. intracellulare* can be recovered from water (6, 11, 15, 16, 21, 50) and biofilm (19, 46) samples from drinking water distribution systems. *M. intracellulare* numbers in biofilms averaged 600 CFU per cm^2 (19), suggesting that this mycobacterium may prefer the biofilm habitat. *M. avium* has also been reported as colonizing and growing in point-of-use water filters containing silver as a disinfecting agent (37). Other mycobacteria, including *Mycobacterium kansasii* (38), *Mycobacterium flavescens* (39), *Mycobacterium chelonae* (40), *Mycobacterium fortuitum* (23), and *Mycobacterium phlei* (1), have also been shown to form biofilms.

The high cell surface hydrophobicity of *M. avium* and *M. intracellulare* is likely to contribute to the formation of biofilms (9). In fact, the adhesive properties (e.g., hydrophobicity) of mycobacteria, corynebacteria, and nocardiae are related to the chain length of their mycolic acids (3). *M. avium* and *M. intracellulare* are concentrated at air-water interfaces (51) and adhere to particulate matter in soil (5) and water (19). Strains of those species are also relatively resistant to heavy metals (20). Heavy-metal resistance (i.e., Ag) of mycobacteria undoubtedly contributed to the colonization of point-of-use water filters by *M. avium* (37). *M. avium* and *M. intracellulare* are also highly resistant to chlorine and other disinfectants used in water treatment (6, 18, 35, 45). Chlorine exposure of microbial biofilms on copper pipes in a model distribution system led to loss of almost all microorganisms except mycobacteria (34). The combination of high hydrophobicity and resistance to chlorine and heavy metals suggests that *M. avium* and *M. intracellulare* are biofilm pioneers in drinking water distribution systems, especially on copper and galvanized pipes.

Cells of *M. avium* and *M. intracellulare* in biofilms may also be more resistant to antimicrobial agents. A number of mechanisms for enhanced resistance to antimicrobials of biofilm-grown cells have been proposed (29). First, layers of cells and extracellular material comprising the biofilm (25, 33) could provide protection because penetration of chlorine is reduced in biofilms (8, 12). Second, physiological changes in cells could result from growth in the biofilm habitat (14, 24, 27, 28, 29). Cells of *M. avium* grown at low growth rates, through changes in nutrient composition, exhibited increased chlorine resistance (18). Cells in biofilms may exhibit changes, such as reduced growth rates (14), as a result of reduced oxygen (13, 47, 53) and nutrient penetration in biofilms (42). Third, the frequency of physiologically resistant cells ("persisters") could be higher in biofilm populations (26, 29). Fourth, high concentrations of organic matter in biofilms would increase chlorine demand, thereby making cells appear chlorine resistant.

Because of the potential significance of biofilm formation to persistence in drinking water distribution systems in the presence of residual disinfectant (e.g., chlorine), we initiated a study of the characteristics of *M. avium* and *M. intracellulare* in biofilms. Here we report the chlorine susceptibility of cells in suspension, of cells in biofilms, and of cells detached from biofilms. In addition, we report the relationship between the chlorine susceptibility and hydrophobicity of those different cell types.

MATERIALS AND METHODS

Mycobacterial strains. *M. avium* strain A5 (2) and *M. intracellulare* strain TMC 1406^T (31) were used.

Growth of strains. Single isolated colonies on Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin (36) were used to inoculate 2 ml of Middlebrook 7H9 broth medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin

* Corresponding author. Mailing address: Biological Sciences, Virginia Tech, Blacksburg, VA 24061-0406. Phone: (540) 231-5931. Fax: (540) 231-7126. E-mail: jofiii@vt.edu.

(36). After 7 days of incubation at 30°C, 1 ml was used to inoculate 9 ml of M7H9 or sterilized potable water obtained from the New River Valley Municipal Water System (assimilable organic carbon range, 500 to 750 μg/liter). Those stock cultures were incubated for 7 days at 30°C and used to inoculate 40 ml M7H9 in polystyrene flasks (Falcon, 50 ml, canted neck, 0.2-μm vented plug seal cap; Becton Dickinson, Franklin Lakes, NJ). To grow mycobacterial strains in biofilms on the surfaces of polystyrene flasks or on 4.0-mm-diameter glass beads (Fisher Scientific, Pittsburgh, PA), cells from stock cultures were inoculated into 40 ml M7H9 in polystyrene flasks containing glass beads. Cultures were incubated at room temperature and slowly rotated at 100 rpm. Unless otherwise noted, cells in biofilms were tested after 30 days of growth. For some experiments, the potable water was not sterilized and was used as the growth medium (with 10% M7H9) to measure the effect of the presence of the water's normal microbial flora on mycobacterial biofilm formation.

Measurement of biofilm accumulation. To measure the number of CFU from glass bead biofilms (unexposed or chlorine exposed), a specified number of beads was transferred from a flask using a sterile loop to a petri dish containing 25 ml chlorine demand-free water (CDFW) (10). The beads were washed by swirling at 30 rpm for 30 s. The beads were transferred to a tube containing 0.8 ml CDFW, 0.1 ml 0.1% (wt/vol) sodium thiosulfate, and 0.1 ml of 0.05% (vol/vol) Tween 80. The cells were separated from the beads by vortexing for 1 min at the highest speed. The turbidity of the suspension was measured and 0.1 ml of the suspension and dilutions in CDFW broth spread on M7H10. Colonies were counted after 7 days at 30°C.

Measurement of chlorine susceptibility. To measure the chlorine susceptibility of biofilm-grown cells that were detached from biofilms on bead surfaces, beads were transferred to 25 ml CDFW in a petri dish and gently washed as described above. Five washed beads were transferred into 1 ml of CDFW, and cells were detached by vortexing at the highest speed for 1 min. The beads were allowed to settle, and the supernatant suspension was used (biofilm-grown, liberated cells). Suspensions of mycobacterial cells freed of aggregates of cells (i.e., reduced aggregate fractions) (45) were prepared from cultures grown in suspension and from suspensions of cells liberated from biofilms before chlorine exposure to reduce the possible protective effect of cells in aggregates. Suspensions were diluted to 10^4 CFU/ml in 25 ml CDFW in a foil-covered flask, and the colony count was measured by spreading 0.1 ml of the suspension and a 10-fold dilution on M7H10 agar. Chlorine was then added to a final concentration of 1 μg/ml (1 ppm), and 1.8-ml samples were removed immediately and at 1, 2, and 3 h of incubation at room temperature and then mixed with 0.2 ml 0.1% (wt/vol) sodium thiosulfate to inactivate the chlorine. Undiluted and 10-fold-diluted samples in CDFW (0.1 ml) were spread on M7H10 agar, and surviving colonies were counted after 7 to 10 days of incubation at 37°C.

The chlorine susceptibility of cells in intact biofilms was measured on cells harvested from glass beads at different times following addition of chlorine. Beads were transferred from polystyrene flasks with a sterile loop and washed in 25 ml of CDFW in a sterile petri dish by gentle rotation as described above. The washed beads were then transferred to 25 ml CDFW in a foil-covered flask. Immediately five beads were removed and placed in CDFW containing sodium thiosulfate and Tween 80, and the CFU was measured as described above. Chlorine was added to a final concentration of 1 μg/ml, and at 10, 20, 40, and 60 min, five beads were removed and the surviving CFU of cells on the beads was measured as described above for determination of growth rate. The presence of either sodium thiosulfate or Tween 80 at the concentrations used did not influence survival.

To follow the disappearance of the adaptive resistance of biofilm-grown cells of *M. avium* strain A5, biofilm cultures grown 30 days at room temperature on glass beads were washed in 25 ml of CDFW in a petri dish. Five beads were transferred to 1 ml of CDFW, and cells were liberated by vortexing at the highest setting for 1 min. The beads were allowed to settle, and 0.9 ml of liberated cells was added to 21.6 ml of CDFW and 2.5 ml of M7H9 broth in a 100-ml flask and incubated at room temperature. Immediately, and at daily intervals up to 1 week, reduced aggregate fractions of the cells were prepared and chlorine susceptibility was measured.

The data represent the average of a minimum of three replicates. CFU counts were used to calculate the logarithm of the percent survival with time (in minutes) for each strain and condition of growth. $CT_{99.9}$ values were calculated by multiplying the concentration of chlorine (in parts per million) by the time (duration of exposure, in minutes) for a 99.9% reduction in the CFU count.

Measurement of chlorine concentration. Chlorine concentration was measured before and during exposure using the *N*,*N*-diethyl-*p*-phenylenediamine Free Chlorine reagent (catalog no. 21055-28; Hach Chemical Co., Loveland, CO). The presence of 10% M7H9 did not alter the standard curve of chlorine.

TABLE 1. Adherence and accumulation of cells of *M. avium* strain A5 and *M. intracellulare* TMC 1406^T to glass beads in water[a]

Incubation period[b]	Mean CFU/cm^2 of bead (±SD)[c]	
	M. avium A5	*M. intracellulare* TMC 1406^T
2 h (adherence)	2.0×10^3 (±0.31)	1.8×10^3 (±0.12)
1 wk	1.6×10^3 (±0.10)	1.6×10^3 (±0.50)
2 wk	3.9×10^3 (±0.20)	1.0×10^3 (±0.32)
3 wk	5.2×10^3 (±0.09)	1.9×10^3 (±0.35)
4 wk	2.9×10^4 (±0.68)	2.5×10^4 (±0.34)

[a] Water cultures contained 10% M7H9 broth.
[b] Time after inoculation of glass bead-containing water medium incubated at room temperature.
[c] $n = 3$.

Contact angle measurement. To measure the number of CFU and harvest cells from biofilms on polystyrene, cells were scraped from the entire bottom surface using a Teflon scraper and suspended in 10 ml CDFW. The suspension was vortexed at maximum speed for 60 s to prepare a uniform suspension for measurement of turbidity and CFU per milliliter as described above. Cells grown in suspension or detached from biofilms were collected on 0.45-μm-pore-size, 25-mm-diameter cellulose acetate filters (Millipore, Bedford, MA) that had been soaked in a glycerol-water (50:50) solution. The four quadrants of the filters were glued to the surface of a glass slide, and the contact angles of 20-μl droplets of water, *n*,*n*-dimethylformamide, and hexadecane were measured with a goniometer (49). The contact angles of cells in intact biofilms were measured on polystyrene following incubation of cells. The culture was removed from the flasks, the flask contents were dried, and the wall of the flask with the biofilm was cut away. Droplets were placed on the biofilm, and the contact angles were measured with a goniometer.

Statistical analysis. Statistical analysis (e.g., *t* test and correlation) was performed using InStat, version 3.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Rates of adherence and growth in biofilms. Cells of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T readily adhered to both polystyrene and glass surfaces whether grown in M7H9 medium or water. Although the inoculated water-grown cultures contained only 5×10^6 CFU/ml, glass beads in water collected 2 h after inoculation at room temperature had approximately 2,000 CFU/cm^2 of either *M. avium* strain A5 or *M. intracellulare* strain TMC 1406^T (Table 1). Those values provide a reliable estimate of the extent of adherence, because growth of the slowly growing mycobacteria should not have affected the results.

Numbers of both *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T on the surfaces of the glass beads increased approximately 10-fold by 4 weeks of incubation at room temperature in water (Table 1). Microscopic examination revealed that the extent of the surface covered by microbial cells had increased by 4 weeks of incubation to the point where the entire glass or polystyrene surface appeared to be covered with mycobacterial cells (41). There was a considerable lag before colony numbers increased in the glass bead-water cultures. Using the data from 3 to 4 weeks, the doubling time for *M. avium* strain A5 on the glass beads was 2.8 days and that for *M. intracellulare* strain TMC 1406^T was 2.1 days. It should be pointed out that the data in Table 1 for CFU per square centimeter and the calculated rates of growth shown above represent the rates of accumulation of cells on the surfaces of glass beads, not true growth rates. Because the cells in the biofilms were enumerated from beads in flasks containing cells

TABLE 2. Chlorine susceptibility of cells of *M. avium* and *M. intracellulare* grown in M7H9 medium or water in suspension or in biofilms

Species and growth medium	$CT_{99.9}$ (mean ± SD)[a] for the following cell population[b]:		
	Planktonic	Detached	Sessile
M. avium strain A5			
M7H9	150 ± 75[c]	358 ± 173[c]	417 ± 249[c]
Water	209 ± 71	356 ± 162[c]	835 ± 236[c]
M. intracellulare strain TMC 1406^T			
M7H9	380 ± 183	458 ± 248	607 ± 322[c]
Water	330 ± 166	435 ± 228	593 ± 477[c]

[a] For four independent triplicate counts. $CT_{99.9}$ is calculated as the product of the chlorine concentration (in parts per million) and the duration of exposure (in minutes) for a 99.9% reduction in the colony count.

[b] Planktonic, cells grown in suspension and exposed in suspension; detached, cells grown in biofilms and exposed in suspension; sessile, cells grown in biofilms and exposed in biofilms.

[c] Significantly different ($P < 0.05$ by Student's t test) from other values in the same row.

growing in suspension (planktonic) as well as in the biofilms, the term "accumulation" is a more accurate representation of the measurement. The increase in the number of cells in the biofilms was likely due to growth of adherent cells plus the binding of new cells to the biofilm minus the cells spontaneously detached from the biofilms. The increases in numbers of mycobacteria on beads in the water cultures were expected because the water had an assimilable organic carbon level of approximately 500 μg/liter and contained 1/10-strength M7H9 medium from the inoculum. Further, both *M. avium* and *M. intracellulare* can grow in natural and drinking waters (19, 22) and above an assimilable organic carbon level of ≥50 μg/liter (34). The numbers of CFU per square centimeter in the experiments here are comparable to the numbers in biofilms in drinking water systems (19).

Separate experiments were performed with biofilms produced after inoculation of *M. avium* strain A5 into nonsterile water containing glass beads and incubation for 30 days at room temperature. The presence of the water's normal microbial flora in biofilms reduced the number of *M. avium* bacteria by a factor of 3, from 3.0×10^4/cm^2 (*M. avium* strain A5 alone) to 1.0×10^4/cm^2 (*M. avium* strain A5 plus normal flora).

Vortexing the glass beads removed almost all the cells from the beads as judged by acid-fast microscopy of beads stained after vortexing. There was little bead-to-bead variation in the number of cells recovered from beads. In two separate experiments, glass bead biofilms contained 384 ± 96 and 342 ± 111 *M. avium* strain A5 CFU/cm^2, and the differences were not significant. Exposure of cells to 0.005% Tween 80 or sodium thiosulfate and vortexing, as was performed for recovery of cells from glass bead biofilms, did not change the number of CFU compared to vortexing in water alone (data not shown), in agreement with the findings of Naik et al. (32).

Increased chlorine resistance of cells in biofilms and cells harvested from biofilms. The chlorine susceptibilities of the three cell populations (sessile, detached, and planktonic) of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T are displayed in Table 2. Chlorine susceptibility is expressed as $CT_{99.9}$, the product of chlorine concentration in micrograms per milliliter (parts per million) and duration of chlorine exposure (in minutes) required to kill 99.9% of cells (as CFU). Cells were grown on glass beads in water plus 10% M7H9 for the experiments reported, but similar results were obtained for cells grown on polystyrene (41). Cells of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T grown in suspension (planktonic) and exposed in suspension were the most susceptible. Cells grown in biofilms (sessile) and exposed in biofilms were the most resistant. Cells of either strain grown in biofilms but detached from beads and exposed to chlorine in suspension displayed intermediate susceptibility (Table 2). Chlorine concentrations remained within the range of 1 ± 0.1 ppm throughout the duration of the experiments.

Separate experiments employed the water without sterilization to permit the formation of a mixed biofilm (i.e., *M. avium* and the normal flora). Following establishment of the biofilm (30 days at room temperature), the biofilms were exposed to chlorine, and survival was measured as described above. The presence of the water's normal microbial biofilm increased the susceptibility of biofilm-grown cells of *M. avium* in the biofilm. The survival (expressed as a percentage of initial CFU) of *M. avium* cells grown alone in biofilms was 1.6 times higher than that of *M. avium* cells grown in the presence of the normal biofilm flora.

Because the chlorine resistance of all three populations of cells was so high (at least 300-fold higher than that of *Escherichia coli* [45]), we sought to determine whether there existed a resistant, persisting population (26, 29). Survival measurements were carried out with higher numbers of beads and up to 6 h exposure to 1 μg chlorine/ml. Chlorine concentrations remained at 1 μg chlorine/ml for the full 6-h exposure. There was no evidence of a resistant or "persister" (26, 29) population. The survival curves for cells of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T were linear from 0 to 6 h. The extreme chlorine resistance of these mycobacteria is illustrated by the fact that approximately 5% of cells survived after a 6-h exposure to 1 μg chlorine/ml.

Loss of chlorine resistance by detached, biofilm-grown cells. The increased chlorine resistance of cells of *M. avium* strain A5 and *M. intracellulare* TMC 1406^T grown in biofilms in water, detached from bead surfaces, and exposed to chlorine in suspension was reversed by growth of cells in suspension. The increased chlorine resistance of the detached cells was lost following a 1-day incubation in suspension for both *M. avium* strain A5 ($CT_{99.9}$, 330 ± 125 to 106 ± 40) and *M. intracellulare* strain TMC 1406^T ($CT_{99.9}$, 440 ± 64 to 122 ± 46).

Relationship between hydrophobicity and antimicrobial susceptibility. Cell surface hydrophobicity, measured by the contact angle of cells grown on polystyrene (Table 3), was correlated with survival following exposure to chlorine for both *M. avium* and *M. intracellulare* whether grown in M7H9 medium or water (Table 4). Cells grown on polystyrene, detached from that surface, and exposed to chlorine in suspension exhibited the same $CT_{99.9}$ values as did cells grown on glass beads (41) (data not shown). The correlation coefficients ranged from a low of 0.781 to 0.997, demonstrating that cell surface hydrophobicity was a major determinant of the chlorine susceptibility of both *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T.

TABLE 3. Hydrophobicity[a] of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T

Growth and medium	Cosine of contact angle (°)	
	M. avium A5	*M. intracellulare* 1406^T
Suspension		
M7H9	0.927	0.840
Water	0.883	0.826
Detached from biofilms		
M7H9	0.883	0.716
Water	0.798	0.588
Intact biofilms		
M7H9	0.669	0.569
Water	0.602	0.340

[a] Expressed as cosine of contact angle.

TABLE 4. Correlation between cosine of contact angle and chlorine $CT_{99.9}$ values

Growth medium	Correlation coefficient[a]	
	M. avium A5	*M. intracellulare* 1406^T
M7H9	0.781	0.992
Water	0.997	0.987

[a] For correlation between chlorine $CT_{99.9}$ and paired cosine of contact angle of water on cells. Three paired values were used for each data point.

DISCUSSION

M. avium and *M. intracellulare*, like *M. kansasii* (38), *M. flavescens* (39), *M. fortuitum* (23), and *M. phlei* (1), readily form biofilms. Whether adherence to the glass or polystyrene required the presence of proteins or other compounds was not determined. Biofilm formation of M7H9-grown cells of a variety of *M. avium* strains, including A5, in water and M7H9 was dependent on Ca^{2+}, Mg^{2+}, and Zn^{2+} (7). However, adherence independent of growth was not measured (7). It is possible that the hydrophobicity of mycobacterial cells is all that is needed for adherence.

The results demonstrate that the chlorine resistance of *M. avium* and *M. intracellulare* cells in biofilms is significantly increased over that of cells grown in suspension (Table 2). Those data agree with the disinfectant resistance of biofilm-grown *M. phlei* (1). Further, cells detached from glass bead biofilms and exposed to chlorine in suspension were more resistant than cells grown in suspension (Table 2). Although Tween 80 did not reduce the CFU of unexposed cells, it might have increased the chlorine susceptibility of the biofilm-grown cells (18, 32), even considering the short duration of exposure and the fact that chlorine was inactivated by sodium thiosulfate when Tween 80 was added. Thus, the chlorine survival and $CT_{99.9}$ values reported here for the biofilm-grown cells exposed to chlorine while in biofilms might be underestimates.

It is likely that growth rate differences influenced the chlorine susceptibility of biofilm-grown cells measured, as they do for cells in suspension (18). Growth, independent of adherence of cells to surfaces or liberation of cells from surfaces, was not measured. The reduced growth rate of cells in biofilms has been cited as a factor contributing to antibiotic resistance (14). Slowly growing cells of *M. avium* and *M. intracellulare* are more resistant to chlorine than rapidly growing cells (18).

Based on these results, it appears that two factors influence the chlorine susceptibility of *M. avium* and *M. intracellulare* cells in biofilms. One factor is likely the limited diffusion and penetration of chlorine (8, 12) resulting from the presence of layers of cells and extracellular material (25, 33). That is illustrated by the fact that the highest $CT_{99.9}$ values were measured in cells grown and exposed to chlorine in biofilms. Unfortunately, there has been no description of the composition of mycobacterial biofilms to date. A second factor is necessary to explain the adaptive, increased resistance of biofilm-grown cells exposed to chlorine in suspension. For those liberated cells, there was no extracellular matrix protecting the cells, because of the washing steps involved in their preparation and dilution before exposure to chlorine. Possibly, physiological changes in cells as a consequence of the biofilm habitat altered their susceptibility to chlorine.

Because the chlorine susceptibilities of all three types of cells were measured here, it was possible to identify the increased resistance of cells grown in biofilms but liberated from them. Although Schulze-Röbbecke and Fischeder (39) measured the chlorine susceptibilities of cells of *M. kansasii* and *M. flavescens* grown and harvested from biofilms (i.e., liberated), they compared the susceptibilities only to those of cells grown and exposed in biofilms. The chlorine susceptibility of suspension-grown cells was not reported (39). Thus, the data did not permit identification of the increased and adaptive chlorine resistance of biofilm-grown cells. Physiological changes of other microorganisms as a consequence of growth in biofilms have been postulated to result in increased antimicrobial resistance of biofilm-grown cells (24, 27, 28, 29). Although oxygen availability is reduced for cells in biofilms (13, 47, 53), it is not likely a factor here, because growth of *M. avium* cells in suspension at 6% oxygen resulted in increased chlorine susceptibility (18).

One physiological characteristic influencing the chlorine susceptibility of *M. avium* and *M. intracellulare* was cell surface hydrophobicity. It has been well established that mycobacteria are among the most hydrophobic of microorganisms (49), and hydrophobicity has been shown to promote surface adherence (48). Chlorine susceptibility, expressed as $CT_{99.9}$ values, correlated with hydrophobicity, as reflected by the cosine of contact angle (Tables 3 and 4). It is possible that *M. avium* and *M. intracellulare* change the cell surface lipid and fatty acid composition in response to whether cells are in suspension or on surfaces. Mycobacteria have been shown to alter fatty acid composition in response to temperature differences (43, 44). Because the mycolic acid structure is a determinant of membrane fluidity in mycobacteria (4, 30), alterations in mycolic acids would be expected to change membrane permeability to chlorine.

The discovery that *M. avium* and *M. intracellulare* cells liberated from biofilms are transiently more resistant to chlorine has important ramifications for water treatment. First, the $CT_{99.9}$ values of suspension-grown and -exposed *M. avium* and *M. intracellulare* are significantly lower than those of cells in biofilms or released from biofilms. Thus, such $CT_{99.9}$ values are misleading. Not only are cells in biofilms more chlorine resistant, but cells freed from biofilms can survive and adhere to other pipe locations to initiate biofilm formation downstream.

ACKNOWLEDGMENTS

K.A.S. was supported by a Minority Academic Opportunities Program scholarship. This work was supported by funds provided by Applied Microbiology and Genetics.

We acknowledge the technical assistance of Myra Williams and the guidance of Aaron Goldstein (Virginia Tech, Department of Chemical Engineering) for measurement of contact angles.

REFERENCES

1. **Bardouniotis, E., W. Huddleston, H. Ceri, and M. E. Olson.** 2001. Characterization of biofilm growth and biocide susceptibility testing of *Mycobacterium phlei* using the MBEC TM assay system. FEMS Microbiol. Lett. **203:**263–267.
2. **Beggs, M. L., J. T. Crawford, and K. D. Eisenach.** 1995. Isolation and sequencing of the replication region of *Mycobacterium avium* plasmid pLR7. J. Bacteriol. **177:**4836–4840.
3. **Bendinger, B., H. H. M. Rijnaarts, K. Altendorf, and A. J. B. Zehnder.** 1993. Physiochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain length of mycolic acids. Appl. Environ. Microbiol. **59:**3973–3977.
4. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
5. **Brooks, R. W., K. L. George, B. C. Parker, J. O. Falkinham III, and H. Gruft.** 1984. Recovery and survival of nontuberculous mycobacteria under various growth and decontamination conditions. Can. J. Microbiol. **30:**1112–1117.
6. **Carson, L. A., L. A. Bland, L. B. Cusick, and M. S. Favero.** 1988. Efficacy of chemical dosing methods for isolating nontuberculous mycobacteria from water supplies of dialysis centers. Appl. Environ. Microbiol. **54:**1756–1760.
7. **Carter, G., M. Wu, D. C. Drummond, and L. E. Bermudez.** 2003. Characterization of biofilm formation by clinical isolates of *Mycobacterium avium*. J. Med. Microbiol. **52:**747–752.
8. **Chen, X., and P. S. Stewart.** 1996. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. Environ. Sci. Technol. **30:**2078–2083.
9. **Christensen, G. D., L. Baldassarri, and W. A. Simpson.** 1994. Colonization of medical devices by coagulase-negative staphylococci, p. 45–78. *In* A. L. Bisno and F. A. Waldvogel (ed.), Infections associated with indwelling medical devices, 2nd ed. American Society for Microbiology, Washington, D.C.
10. **Clesceri, L. S., A. E. Greenberg, and A. D. Eaton.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.
11. **Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma, Jr.** 1999. Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol. **65:**2492–2496.
12. **de Beer, D., R. Srinivasan, and P. S. Stewart.** 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. **60:**4339–4344.
13. **de Beer, D., P. Stoodley, F. Roe, and Z. Lewandowski.** 1994. Effects of biofilm structures on oxygen distribution and mass transport. Biotechnol. Bioeng. **43:**1131–1138.
14. **Donlon, R. M.** 2001. Biofilm formation: a clinically relevant microbiological process. Clin. Infect. Dis. **33:**1387–1392.
15. **du Moulin, G. C., and K. D. Stottmeier.** 1986. Waterborne mycobacteria: an increasing threat to health. ASM News **52:**525–529.
16. **du Moulin, G. C., K. D. Stottmeier, P. A. Pelletier, A. Y. Tsang, and J. Hedley-Whyte.** 1988. Concentration of *Mycobacterium avium* by hospital hot water systems. JAMA **260:**1599–1601.
17. **Falkinham, and J. O., III.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. **9:**177–215.
18. **Falkinham, and J. O., III.** 2003. Factors influencing the chlorine susceptibility of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*. Appl. Environ. Microbiol. **69:**5685–5689.
19. **Falkinham, J. O., III, C. D. Norton, and M. W. Le Chevallier.** 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. Appl. Environ. Microbiol. **67:**1225–1231.
20. **Falkinham, J. O., III, K. L. George, B. C. Parker, and H. Gruft.** 1984. In vitro susceptibility of human and environmental isolates of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* to heavy-metal salts and oxyanions. Antimicrob. Agents Chemother. **25:**137–139.
21. **Fischeder, R., R. Schulze-Röbbecke, and A. Weber.** 1991. Occurrence of mycobacteria in drinking water samples. Zbl. Hyg. **192:**154–158.
22. **George, K. L., B. C. Parker, H. Gruft, and J. O. Falkinham III.** 1980. Epidemiology of infection by nontuberculous mycobacteria. II. Growth and survival in natural waters. Am. Rev. Respir. Dis. **122:**89–94.
23. **Hall-Stoodley, L., and H. Lappin-Scott.** 1998. Biofilm formation by the rapidly growing mycobacterial species *Mycobacterium fortuitum*. FEMS Microbiol. Lett. **168:**77–84.
24. **Hoyle, B. D., and J. W. Costerton.** 1991. Bacterial resistance to antibiotics: the role of biofilms. Prog. Drug Res. **37:**91–105.
25. **Hoyle, B. D., J. Jass, and J. W. Costerton.** 1990. The biofilm glycocalyx as a resistance factor. J. Antimicrob. Chemother. **26:**1–5.
26. **Keren, I., N. Kaldalu, A. Spoering, Y. Wang, and K. Lewis.** 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. **230:**13–18.
27. **LeChevallier, M. W., C. D. Cawthon, and R. G. Lee.** 1988. Factors promoting survival of bacteria in chlorinated water supplies. Appl. Environ. Microbiol. **54:**649–654.
28. **LeChevallier, M. W., C. D. Cawthon, and R. G. Lee.** 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. **54:**2492–2499.
29. **Lewis, K.** 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. **45:**999–1007.
30. **Liu, J., C. E. Barry III, G. S. Besra, and H. Nikaido.** 1996. Mycolic acid structure determines the fluidity of the mycobacterial cell wall. J. Biol. Chem. **271:**29545–29551.
31. **Mayer, B. K., and J. O. Falkinham III.** 1986. Catalase activity and its heat inactivation for differentiation of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*. Int. J. Syst. Bacteriol. **36:**207–212.
32. **Naik, S. P., W. A. Samsonoff, and R. E. Ruck.** 1989. Effects of surface-active agents on drug susceptibility levels and ultrastructure of *Mycobacterium avium* complex organisms isolated from AIDS patients. Diagn. Microbiol. Infect. Dis. **11:**11–19.
33. **Nickel, J. C., I. Rueska, J. B. Wright, and J. W. Costerton.** 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. **27:**619–624.
34. **Norton, D. C., M. W. LeChevallier, and J. O. Falkinham III.** 2004. Survival of *Mycobacterium avium* in a model distribution system. Water Res. **38:**1457–1466.
35. **Pelletier, P. A., G. C. du Moulin, and K. D. Stottmeier.** 1988. Mycobacteria in public water supplies: comparative resistance to chlorine. Microbiol. Sci. **5:**147–148.
36. **Pethel, M. L., and J. O. Falkinham III.** 1989. Plasmid-influenced changes in *Mycobacterium avium* catalase activity. Infect. Immun. **57:**1714–1718.
37. **Rodgers, M. R., B. J. Blackstone, A. L. Reyes, and T. C. Covert.** 1999. Colonisation of point of use water filters by silver resistant non-tuberculous mycobacteria. J. Clin. Pathol. **52:**629.
38. **Schulze-Röbbecke, R., and R. Fischeder.** 1989. Mycobacteria in biofilms. Zbl. Hyg. Unweltmed. **188:**385–390.
39. **Schulze-Röbbecke, R., and R. Fischeder.** 1991. Growth and inactivation kinetics of mycobacteria in biofilms. Water Sci. Technol. **24:**153–156.
40. **Schulze-Röbbecke, R., C. Feldmann, R. Fischeder, B. Janning, M. Exner, and G. Wahl.** 1995. Dental units: an environmental study of sources of potentially pathogenic mycobacteria. Tuber. Lung Dis. **76:**318–323.
41. **Steed, K. A.** 2003. Effect of growth in biofilms upon antibiotic- and chlorine-susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*. M.S. thesis. Virginia Polytechnic Institute and State University, Blacksburg.
42. **Stewart, P. S.** 1994. Biofilm accumulation model that predicts antibiotic resistance of *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. **38:**1052–1058.
43. **Suutari, M., and S. Laakso.** 1993. Effect of growth temperature on the fatty acid composition of *Mycobacterium phlei*. Arch. Microbiol. **159:**119–123.
44. **Takayama, K., E. L. Armstrong, L. A. Davidson, K. A. Kuniga, and J. O. Kilburn.** 1978. Effect of low temperature on growth, viability, and synthesis of mycolic acids of *Mycobacterium tuberculosis* strain H37Ra. Am. Rev. Respir. Dis. **118:**113–117.
45. **Taylor, R. H., J. O. Falkinham III, C. D. Norton, and M. W. LeChevallier.** 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. Appl. Environ. Microbiol. **66:**1702–1705.
46. **Torvinen, E., S. Suomalainen, M. J. Lehtola, I. T. Miettinen, O. Zacheus, L. Paulin, M.-L. Katila, and P. J. Martikainen.** 2004. Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. Appl. Environ. Microbiol. **70:**1973–1981.
47. **Tresse, O., T. Jouenne, and G.-A. Junter.** 1995. The role of oxygen limitation in the resistance of agar-entrapped, sessile-like *Escherichia coli* to aminoglycoside and β-lactam antibiotics. J. Antimicrob. Chemother. **36:**521–526.
48. **Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. B. Zehnder.** 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. **53:**1893–1897.
49. **Van Oss, C. J., C. F. Gillman, and A. W. Neumann.** 1975. Phagocytic engulfment and cell adhesiveness as cellular surface phenomena. Marcel Dekker, Inc., New York, N.Y.
50. **von Reyn, C. F., J. N. Maslow, T. W. Barber, J. O. Falkinham III, and R. D. Arbeit.** 1994. Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. Lancet **343:**1137–1141.
51. **Wendt, S. L., K. L. George, B. C. Parker, H. Gruft, and J. O. Falkinham III.** 1980. Epidemiology of infection by nontuberculous mycobacteria. III. Isolation of potentially pathogenic mycobacteria from aerosols. Am. Rev. Respir. Dis. **122:**259–263.
52. **Wolinsky, E.** 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. **119:**107–159.
53. **Xu, K. D., P. S. Stewart, F. Xia, C.-T. Huang, and G. A. McFeters.** 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl. Environ. Microbiol. **64:**4035–4039.