

**Effect of growth in biofilms upon antibiotic- and chlorine- susceptibility of
Mycobacterium avium and *Mycobacterium intracellulare***

Keesha Ashanna Steed

Thesis submitted to the faculty of Virginia Polytechnic Institute & State University in
Fulfillment of the requirements for the degree of
Master of Science in Biology

Approved

Joseph O. Falkinham, III

Muriel L. Lederman

Aaron S. Goldstein

March 10, 2003

Blacksburg, VA

Keywords: *Mycobacterium avium*, *Mycobacterium intracellulare*, biofilms, antibiotic and chlorine susceptibility

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Keesha Ashanna Steed
Joseph O. Falkinham, Chair
Department of Microbiology

ABSTRACT

Mycobacterium avium and *Mycobacterium intracellulare* are environmental opportunistic pathogens whose source for human infection is water and soil. *M. avium* and *M. intracellulare* cause pulmonary infections (tuberculosis) in immunocompetent individuals and bacteremia in immunodeficient individuals (e.g. AIDS). One factor likely influencing the lack of success of antibiotic therapy in patients would be their ability to form biofilms. Growth in biofilms might result in antimicrobial resistance because (1) cells are protected by layers of other cells and extracellular material (2) and differences in physiologic state of cells as a consequence of growing on surfaces.

The objectives of the work were to (1) establish methods for reproducible growth of mycobacterial biofilms (2) measure the formation of biofilms on surfaces by cells of *M. avium* and *M. intracellulare* (3) measure the antibiotic- and chlorine- susceptibility of *M. avium* and *M. intracellulare* strain TMC1406^T in cell grown in suspension, cells grown in biofilms and suspended and of cells grown in biofilms (4) measure the hydrophobicity of *M. avium* and *M. intracellulare* grown in suspension and in biofilms.

Methods were developed for growing mycobacteria in biofilms in polystyrene flasks and on glass beads. Although both strains formed biofilms, *M. intracellulare* strain

TMC 1406^T more readily formed biofilms than *M. avium* strain A5 in polystyrene flasks.

The majority of *M. intracellulare* strain TMC 1406^T cells grew on the walls of the flasks rather than in suspension like *M. avium* strain A5.

The susceptibility of M7H9 medium-grown cells of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T cells grown in suspension, cells grown in biofilms and suspended and cells grown in biofilms was measured against clarithromycin, ethambutol, kanamycin, rifampicin and streptomycin. Cells grown in biofilms and exposed to antibiotics in biofilms were five-fold resistant to antibiotics than were cells grown in biofilms and exposed in suspension. Cells grown and exposed in suspension were ten-fold more sensitive to antibiotics than were cells grown in biofilms and exposed in suspension.

The chlorine susceptibility of cells grown in medium and water was also measured. Cells grown in biofilms were more resistant to chlorine than cells grown in biofilms and suspended. Cells grown in suspension were more sensitive to chlorine than cells grown in biofilms and suspended.

The hydrophobicity data (i.e., hexadecane adherence and contact angle measurements) showed that cells grown in biofilms are more hydrophobic than cells grown in biofilms and suspended and cells grown in suspension. It is clear that there are physiological changes between cells grown in suspension, cells grown in biofilms and suspended and cells in biofilms.

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Joseph Falkinham for his guidance and knowledge of mycobacteria. To my committee members, Dr. Muriel Lederman and Dr. Aaron Goldstein, thank you for your insight and support.

I would like to thank Dr. Sible for the use of the inverted compound microscope, and Dr. Saraf for the use of the goniometer. I would like to thank the Minority Academic Opportunities Program (M.A.O.P.) under Dr. Moore and Dr. Grayson for their funding and support.

I would like to thank members of my lab, past and present, for their questions and suggestions.

I would especially like to thank my family: Mr. Keith Sr., Mrs. Carol, Keith Jr. and Kyle Steed, for your immeasurable contributions in my life. Thank you for your prayers, continual support and for giving me “shoulders” upon which to stand. I love you!

LaRone Gardner, thank you for *all* that you have been to me. Thank you for bringing me to “Beautiful.” Thank you for believing in me...you inspire. I love you baby!

To my extended family, godparents and Seeds of Greatness Ministries, thank you for your prayers and support.

CHAPTER I: Introduction

Epidemiology of Environmental Opportunistic Mycobacteria

Mycobacteria are aerobic to microaerophilic bacteria, which form slightly curved or straight rods ranging from approximately 0.2-0.6 by 1.0-10 μ m in size (Gangadharam & Jenkins, 1998). Mycobacteria are acid-fast, slow-growing bacilli. Acid-fast is the property of bacteria with lipid-rich cell walls; mycobacteria cell walls contain 40% mycolic acids (Gangadharam & Jenkins, 1998). Mycolic acids are β -hydroxy acids substituted at the α -position (Gangadharam & Jenkins, 1998). Mycobacteria grow to form turbid cultures in approximately two weeks at a temperature of 30°C to 37°C. Slow growth is attributed to the impermeable lipid-rich wall (Brenan and Nikaido et. al., 1995) and fact that they have one rRNA cistron (Gangadharam & Jenkins, 1998).

Mycobacterium avium and *Mycobacterium intracellulare*, the *M. avium* complex (MAC) are environmental, opportunistic human and animal pathogens (Falkinham, 1996; Inderlied et al., 1993; Wolinsky, 1979). MAC organisms are found in natural (Keinanen et al., 2002) and drinking water (Covert et al., 1999; Falkinham et. al., 2001), and in food and soils (Brooks et al., 1984; Yajko et al, 1995). These organisms have also been recovered from biofilms on pipes (Covert et al, 1999; Falkinham et al., 2001), water droplets ejected into air (Wendt et al, 1980; Dobos et al, 2000), and can grow in amoeba (Cirillo et al, 1997; Strahl et. al, 2001).

M. avium and *M. intracellulare* pulmonary infections are found in patients with predisposing lung conditions that include silicosis and black lung (Contreras et al., 1988, Wolinsky, 1979), patients with pulmonary alveolar proteinosis (Witty et al., 1994) and in

patients with cystic fibrosis (Kilby et al., 1992). Skin and soft tissue infections have also been reported. *M. avium* complex infections in immunodeficient individuals have also been reported (Young, 1998). The onset of the AIDS epidemic has resulted in the increase of *M. avium* complex infections in the final stages of the disease (von Reyn et al., 1996; Horsburgh, R.C, Jr., 1999). These infections usually become disseminated which makes them difficult to treat with antibiotics (Horsburgh, R.C., Jr., 1999).

Over the past ten years, it has been noted that the frequency of “slight, slender” elderly women with tuberculosis cause by *M. avium*, *M. intracellulare*, or rapidly growing mycobacteria has been increasing (Prince et al., 1989; Reich and Johnson, 1991; Kennedy and Weber, 1994). These “slight, slender” elderly women have none of the predisposing factors typically associated with mycobacteria pulmonary infections. Treatment of infections caused by *M. avium* and *M. intracellulare* organisms is difficult because of their antibiotic resistance (Rastogi et al., 1982; Gangadharam et al., 1983; Brenan et al, 1995).

Biofilm Formation and Consequences

Biofilms are defined as heterogeneous environments composed of structured communities of sessile bacteria and extracellular material (e.g., polysaccharide) that adhere to themselves or to inanimate objects (Costerton et al., 1999). Biofilms provide a protected mode of growth for bacteria. Bacterial cells in biofilms are less susceptible to antibiotics due to changes in the cellular metabolism as well as slow growth rate of the bacteria (Stewart, 1996; Stewart, 2002). The slow growth of bacteria in biofilm

infections may also contribute to the slow expression of symptoms. (Costerton et. al., 1999).

M. avium and *M. intracellulare* are found in biofilms in drinking water systems (Falkinham et al., 2001) and may also form biofilms in the lung (Rao et al., 2000). As a consequence of biofilm formation, cells of *M. avium* and *M. intracellulare* would be expected to exhibit an altered pattern of susceptibility to antibiotics (Stewart, 1996). In addition, *M. avium* and *M. intracellulare* cells in biofilms would provide a continuous and sustained source of cells for dissemination leading to persistence and infection of other tissues (Costerton et al., 1999).

M. avium complex biofilms would be defined as a population of cells growing irreversibly adherent to submerged living or non-living surfaces. Though biofilms have been shown to consist of extracellular polymeric material surrounding the cell (Costerton et al., 1978; Pratt and Koiter 1998; Costerton et al., 1999), this may not be the case for mycobacteria. The composition and structure components of the mycobacteria biofilms have not been described.

Biofilm Formation by Pulmonary Bacterial Pathogens in the Lungs of Infected Patients

Recently, it has been pointed out that the pathogenesis of a number of human infections involves bacterial biofilm formation (Costerton et al., 1999). Pulmonary infection in cystic fibrosis patients caused by *Pseudomonas aeruginosa* or *Burkholderia cepacia* is due, in part, to biofilm formation in lung tissue (Lam et al., 1980; Saiman et al., 1990; Buret et al., 1991; Govan and Deretic, 1996; Costerton et al., 1999; Singh et al.,

2000). Biofilm-grown cells of *P. aeruginosa* are more resistant to antibiotics than are cells grown in medium (Anwar et al., 1989a, b; Shigeta et al., 1997; Singh et al., 2000).

Mycobacteria have also been identified in the lung (Rao, 2000). The route of transmission is most likely through aerosols (Iseman, 1996). *M. avium* and *M. intracellulare* have been linked to geriatric lung disease (Kennedy and Weber, 1994) and are found in small numbers in the lungs of cystic fibrosis patients (Kilby et al., 1992). The biofilm formation of *M. avium* and *M. intracellulare* in the lungs would add to the persistence of infection.

Biofilm colonization of water distribution systems

One source of *M. avium* and *M. intracellulare* infection in humans is water (von Reyn et al., 1994). *M. avium* and *M. intracellulare* are present in drinking water (duMoulin et al., 1988; Fischeder et al., 1991; von Reyn et al., 1993; Glover et al., 1994; Covert et al., 1999; Iivanien et al., 1999; Taylor et al., 2000; Falkinham et al., 2001). DNA fingerprints of *M. avium* AIDS patient isolates matched those of isolates recovered from water samples drunk by the patients (von Reyn et al., 1994). Mycobacteria form biofilms (Schulze-Röbbecke and Fischeder, 1989; Rao et al., 2000) and can be recovered from biofilms in drinking water distribution systems (Iivanainen et al., 1999; Falkinham et al., 2001). *M. avium* and *M. intracellulare* were recovered from biofilm samples collected from eight drinking water systems across the United States (Falkinham et al., 2001). *M. intracellulare* biofilm numbers were particularly high (600 colony-forming units per cm²). Mycobacteria have also been recovered from reverse osmosis membranes used for drinking water purification (Ridgway et al., 1984). Two characteristics that may promote

the formation of biofilms by *M. avium* and *M. intracellulare* are: high cell surface hydrophobicity (Stormer and Falkinham, 1989) and sliding mobility (Martínez et al., 1999).

Hypotheses

- (1) Cells of *M. avium* and *M. intracellulare* grown in biofilms are more resistant to antibiotics and chlorine compared to cells in suspension

Specific Experimental Objectives

- (1) Establish conditions to reproducibly grow *M. avium* and *M. intracellulare* biofilms.
- (2) Measure the formation of biofilms on surfaces by cells of *M. avium* and *M. intracellulare*.
- (3) Measure the antibiotic and chlorine susceptibility of cells grown in suspension in comparison with biofilm-grown cells of *M. avium* and *M. intracellulare* to antibiotics.
- (4) Measure the hydrophobicity of *M. avium* and *M. intracellulare*.

CHAPTER II: Materials & Methods

Experimental Method (performed in duplicate)

Strains

Bacterial strains

M. avium strain A5 is an isolate from an AIDS patient and received from Marjorie Beggs McClellan Veterans Hospital, Little Rock, AR. *M. intracellulare* strain TMC 1406^T is an isolate recovered from a human lymph node.

Antibiotics

Clarithromycin was donated by Abbott Laboratories (Chicago, IL). Kanamycin sulfate, ethambutol hydrochloride, streptomycin sulfate, and rifampicin, were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions 0.1% of the antibiotics were made in distilled water except for that of clarithromycin that was made in DMSO. All antibiotics were filter-sterilized with a 0.2 µm pore size filter and stored at 4°C.

Table 1. Antibiotics

Antibiotic	Mode of action	Target
Clarithromycin	semi synthetic antibiotic that inhibits bacterial protein synthesis	50S ribosomal subunit
Ethambutol	tuberculostatic agent that interferes with formation of arabinan and cellular metabolism	lipid and cell wall metabolism
Rifampicin	blocks bacterial RNA polymerase	β subunit of DNA-dependent RNA polymerase
Kanamycin	bactericidal agent that inhibits initiation of translation and causes misreading of mRNA	30s ribosomal subunit
Streptomycin	bactericidal agent that inhibits protein synthesis	binds to S12 protein of the 30s ribosomal subunit and causes misreading

(Scholar and Pratt, 2000).

Table 2. Definition of Cell types

Cell type	<i>Definition</i>
Cells grown in suspension	Cells of <i>M. avium</i> strain A5 or <i>M. intracellulare</i> strain TMC 1406 ^T that have been grown in suspension and exposed to antimicrobials agents in suspension
Cells grown in biofilms and suspended	Cells of <i>M. avium</i> strain A5 or <i>M. intracellulare</i> strain TMC 1406 ^T grown in biofilms and suspended and exposed to antimicrobials agents in suspension
Intact biofilms	Cells of <i>M. avium</i> strain A5 or <i>M. intracellulare</i> strain TMC 1406 ^T grown and exposed to antimicrobials agents in biofilms.

Stock cultures

Each strain was streaked on Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5 % (vol/vol) glycerol and 10 % (vol/vol) oleic acid-albumin (M7H10) and the plates were incubated for 12 days at 37°C. Two isolated colonies were used to inoculate 2 ml of M7H9 broth medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5 % (vol/vol) glycerol and 10 % (vol/vol) oleic acid-albumin or 2 ml of sterilized raw water. After 7 days of incubation at 37°C, 1 ml of that culture was used to inoculate 9 ml of M7H9 broth medium or 9 ml of raw water from the Mississippi River to make a 10ml stock culture. These stock cultures were grown in polystyrene flasks and incubated for 4 days at 37°C. See appendix for flow chart of experimental design.

Acid-fast Stain

Ziehl Neelsen stain (Manual of Methods for General Bacteriology, 1981).

Culture characterization

Cultures were streaked on Plate Count Agar (PCA, Difco Sparks, MD) plates and on M7H10 medium. The cultures were also acid-fast stained to insure purity. The positive control was (*M. avium*) and a negative control was (*E. coli*). Slides were viewed under microscope. Slides were examined for the presence of non acid-fast cells. In all experiments reported here, no acid-fast cells were seen in the culture.

Growth

Growth media

M. avium strain A5 and *M. intracellulare* strain TMC 1406^T were grown in both M7H9 and raw water. The raw water was input water that likely originated from the Mississippi River.

Growth in polystyrene flasks

One milliliter of a 10 ml stock culture of *M. avium* strain A5 or *M. intracellulare* strain TMC 1406^T was added to 9 ml of M7H9 broth media or sterile raw water in polystyrene flasks (BD Biosciences Labware, Franklin Lakes, NJ) from Mississippi River. Flasks were incubated at 30°C and gently shaken at 100 rpm to provide aeration. Growth was monitored daily by removing 1 ml samples of each culture and measuring turbidity in the spectrophotometer (Abs_{580nm}). Cells were harvested when cultures were in mid log phase.

Growth on polystyrene flasks

One milliliter of a 10 ml stock culture of *M. avium* strain A5 or *M. intracellulare* strain TMC 1406^T was added to 9 ml of M7H9 broth media or sterile raw water in polystyrene flasks from Mississippi River. Flasks were incubated at 30°C and gently shaken at 100 rpm to provide aeration. The surface of the polystyrene flask was

thoroughly scraped to remove adherent biofilms. Growth was monitored daily by removing 1ml samples of each culture and measuring turbidity in the spectrophotometer ($\text{Abs}_{580\text{nm}}$). Cells were harvested when cultures were in mid log phase. See appendix for flow chart of experimental design.

Growth on glass beads

Sterile glass beads were added to polystyrene flask that contained 1) 40 ml of M7H9, 4 ml of oleic-acid albumin, 4 ml of culture or 2) 40 ml of raw water, 4 ml of cultures. Flasks were incubated at 30°C and gently shaken at 100 rpm to provide aeration. Growth was monitored every five days by removing 1ml samples of each culture and measuring turbidity. To measure colony-forming units five 4mm glass beads were removed from the canted neck polystyrene flasks, placed in 1ml of M7H9 broth medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5 % (vol/vol) glycerol and 10 % (vol/vol) oleic acid-albumin or sterile raw water with 100 μ l of 0.5% (vol/vol) Tween 80. The tubes containing the glass beads were vortexed at maximum setting for 60 seconds. On (3) M7H10 agar plates, 100 μ l of the vortexed sample (biofilms) was plated. The plates were incubated at 37°C for 7 days. See appendix for flow chart of experimental design.

Microscopic examination of biofilms development on polystyrene flasks

In a sterile 50 ml polystyrene flask, 9 ml of sterilized raw water or M7H9 and 1 ml of the 10 ml stock culture of *M. avium* strain A5 or *M. intracellulare* strain

TMC1406^T culture was added. Flasks were incubated for 1 to 4 weeks in 30°C incubator, and slowly shaken at 100 rpm to provide aeration. The polystyrene flask was capped, however the cap is vented so that air can flow into the flask.

After cells reached mid-log phase, the suspension was removed for susceptibility tests and the polystyrene flasks were washed with distilled water and dried at 50°C in the oven. Photographs of representative areas of biofilm development were taken using an inverted compound microscope at a magnification of 400x. See flow chart of experimental design.

Recovery of cells in biofilms

Effect of vortexing with glass beads upon mycobacteria colony-forming counts

Using a 2 ml RAF, 2 (1ml) suspensions were made in microcentrifuge tubes. 5 (4 mm) glass beads were placed into the suspensions using a sterile loop. 10µl of 0.5% (vol/vol) Tween 80 was added to the suspension and vortexed for 60 seconds. 100µl samples were plated in triplicate onto M7H10 agar plates.

Bead to bead variation of colony count

Two 4 mm glass beads were removed from the polystyrene flask using a sterile loop and rinsed in 10ml of M7H9 medium or chlorine demand free phosphate buffer (CDFPB) by swirling on shaker for 30 seconds. One bead was removed and placed in 1 ml of media. 100µl of 0.5% (vol/vol) Tween 80 was added to the microcentrifuge tube

and vortexed for 60 seconds. On (3) M7H10 agar plates, 100 µl samples were plated. Experiment was repeated with another bead.

Phosphate buffered

In 1 liter of distilled water, 5.305 g of KH₂PO₄ and 8.29 g K₂HPO₄ was added. The mixture was autoclaved for 50 minutes.

Reduced aggregate fraction (RAF)

In order to obtain individual cells, cultures of cells grown in suspension and cells grown in biofilms grown in M7H9 or sterile raw water (AOC=500mg/l) were placed in a sterile 25ml Cortex tube and centrifuged for 10 minutes at 4000 x g at 4°C (Taylor et al., 2000). The supernatant was discarded and the pellet was washed three times in 10 ml of phosphate buffered (PB) for antibiotic susceptibility or 10ml of chlorine demand free phosphate buffer (CDFPB, Cleceri et al., 1998) for chlorine susceptibility experiments. The final pellet was suspended in 10ml of M7H9 or raw water and the suspension was centrifuged for 10 minutes at 1200 x g at 4°C. The supernatant retained contained the individual cells that were used for antibiotic and chlorine susceptibility experiments. See appendix for flow chart of experimental design.

Measure of Antibiotic Susceptibility

Minimum Inhibitory Concentration (MIC) of antibiotics

M7H10 agar plates (BBL Microbiology Systems, Cockeysville, MD) containing 0.5 % (vol/vol) glycerol and 10 % (vol/vol) oleic acid-albumin and antibiotic concentrations of 1 µg/ml, 10 µg/ml, 100 µg/ml were prepared 3 days prior to use. *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T were streaked onto M7H10 agar plates in triplicate and were incubated for 12 days at 37°C. Once the range of concentrations was identified, the experiments were repeated with narrower range of antibiotic concentrations.

*Antibiotic Susceptibility of *M. avium* and *M. intracellulare* cells in suspensions*

Log phase cells grown in either suspension or in biofilms and suspended were diluted to yield 10⁴ colony-forming units (CFU/ml). *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T were suspended in M7H9 medium containing 145µg of each antibiotic/ml and incubated at 37°C. Samples were withdrawn immediately and at 1,2 and 3 hours. Samples (10µl) were removed and spread on M7H10 agar medium in triplicate. Plates were incubated for 12 days at 37°C. M7H10 agar medium was prepared 3 days prior to usage to insure proper drying. See appendix for flow chart of experimental design.

Antibiotic Susceptibility of cells in biofilms

To obtain biofilms, 5 (4 mm) glass beads were removed from the polystyrene flask with a sterile loop and washed in M7H9 medium by shaking for 30 seconds on the shaker. The five beads were added to each tube with 4.5ml of M7H9 medium and cultures were suspended in 145 μ g of each antibiotic/ml at 37°C on a dry heating block. Immediately and at 1, 2 and 3 hours beads were removed and placed in microcentrifuge tubes. To each tube, 100 μ l of 0.5% (vol/vol) Tween 80 was added and vortexed for 60 seconds. Suspensions (10 μ l) were spread in triplicate onto M7H10 agar plates. Plates were incubated for 12 days at 37°C.

Measurement of Chlorine Susceptibility

Measurement of chlorine

Chlorine concentration was measured before and during exposure using the N,N-diethyl-p-phenylenediamine (DPD) colometric method following the manufacturer's directions (Hach Co. Loveland, CO). Chlorine standards (Hach Co. Loveland, CO) were diluted in CDFPB and used to create standard curve relating free chlorine residuals with absorbance at 515nm.

Chlorine demand free flasks and chlorine demand free phosphate buffer

Chlorine demand free flasks were prepared by UV irradiating flasks containing distilled water for 3 days in the dark (Cleceri et al., 1998). Chlorine demand free phosphate buffer was prepared as described (Cleceri et al., 1998).

Chlorine susceptibility of *M. avium* and *M. intracellulare* cells in suspension

Chlorine demand free (CDF) flask and chlorine working solution bottle were covered in aluminum foil because of the light sensitivity of chlorine. In a CDF flask, 200 ml of chlorine demand free phosphate buffer (CDFPB) and 15 μ l of RAF culture was added to yield a final cfu/ml of 3.0×10^3 . A working solution of chlorine (sodium hypochlorite) was added to the flask sufficient to reach 1ppm. The suspension was stirred on low, to evenly distribute the chlorine.

A 1.8 ml sample was taken from the cell-chlorine suspension and mixed with 0.2 ml of 0.1% sodium thiosulfate was used to inactivate the chlorine. On (3) M7H10 agar plates, 0.1ml samples were plated from the 2 ml sample. From the 2 ml sample, 0.5 ml inoculated 4.5 ml of CDFPB and 0.1ml samples were plated in triplicate on M7H10 agar medium. Sampling was repeated immediately and at 10, 20, 40, 60 minutes. Plates were incubated for 7 days at 37°C. See appendix for flow chart of experimental design.

Chlorine susceptibility of cells in biofilms

Glass beads from polystyrene flasks were removed using sterile loops, washed in M7H9 medium or CDFPB by swirling on shaker for 30 seconds, and transferred to microcentrifuge tubes. Each microcentrifuge tube contained, 5 (4 mm) glass beads, 1ml of CDFPB and 10 μ l of 0.5% (vol/vol) Tween 80. Tubes were vortexed for 60 seconds.

On (3) M7H10 agar plates, 100 μ l samples 10^{-1} , 10^{-2} dilution were plated. At time 0, 5 μ l of chlorine was added to each microcentrifuge tube sufficient to reach 1 ppm. The tube contained 5 beads, 1 ml of CDFPB, and 10 μ l of 0.5% Tween 80. To inactivate the chlorine, 100 μ l of 0.1% sodium thiosulfate was added at each time point.

Microcentrifuge tubes were vortexed for 60 seconds. On (3) M7H10 agar plates, 100 μ l samples 10^{-1} , 10^{-2} were plated.

Hydrophobicity

Hexadecane adherence

The initial turbidity, Abs_{580nm} , of a log phase culture was measured. In a microcentrifuge tube, 1ml of the culture was added and centrifuged for 5 minutes at 15,000 x g. The pellet was suspended in 2 ml of Phosphate Buffered Saline (PBS) then vortexed for 60 seconds. In a 13 x 100nm tube, 2 ml were than placed. This centrifugation process was repeated two more times for a total volume of 6 ml. The initial Abs_{580nm} (x) was measured and 3 ml were placed into (2) 13 x 100nm tubes. To each 13 x 100nm tube, 0.1 ml of hexadecane was added and vortexed for 60 seconds. The cultures were left for 30 minutes and the final Abs_{580nm} (x_1) was measured the turbidity of the hexadecane layer. The addition of hexadecane was repeated until 0.3ml of hexadecane had been added. The percent adherence can be determined by completing the following equation $(x-x_1)/(x)$. See appendix for flow chart of experimental design.

Measurement of contact angle

Cells grown in suspension on filters to log phase or in biofilms and then suspended and washed twice in PBS buffer and collected on a 0.45 μm pore size 25 mm cellulose acetate filter that had been soaked in 50:50 glycerol: water (vol/vol). The glycerol solution was added to every filter prior the filtration of the culture. The microbial film covered the entire surface of filter. Each filter was mounted on a glass slide by gluing each quadrant edge to the glass. The cultures were dried to prevent the absorption of moisture.

For biofilms, the procedure was repeated using the solution of biofilms vortexed off the glass beads. Filters were allowed to dry for up to 24 hours. To measure contact angles a 20 μl drop of water, *n,n* dimethylformamide, or hexadecane was placed on one part of the bacterial film. The interfacial tension from interfacial tension from interfacial tension of three liquids and the measures average contact angles was also calculated (Table 4) (van Oss et al., 1975). See appendix for flow chart of experimental design.

Table 3. Contact angle data for liquids

Liquid	Y_{LV}
Water	72.8
Formamide	58.2
Hexadecane	27.6

(van Oss et al., 1975)

Statistical analysis

Values for different treatments were compared for statistical significance using the unpaired T-test (Graph Pad Software, Inc.). Correlation between survival values and contact angle was calculated using the correlation and linear regression program (Graph Pad Software, Inc).

CHAPTER III: Results

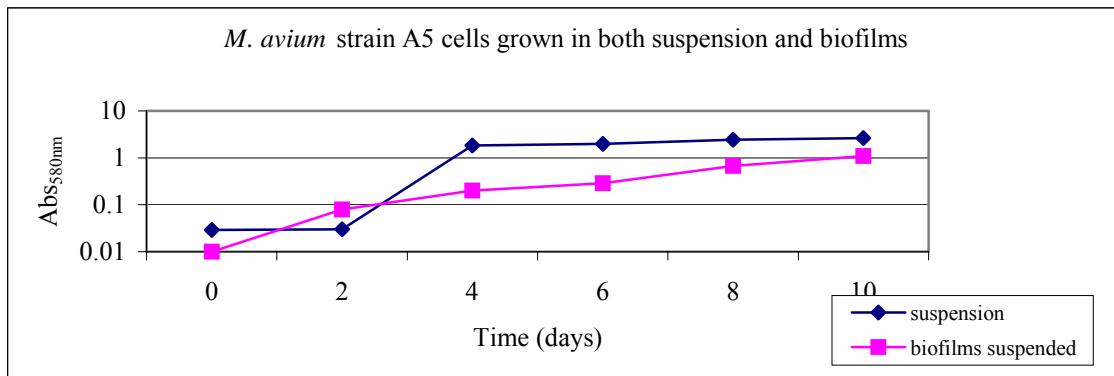
Growth of suspension and biofilms in polystyrene flasks

Polystyrene flasks were used to determine whether the mycobacteria strains formed biofilms. The growth rate of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T were measured in both M7H9 medium and raw water in polystyrene flasks.

Figure 1 illustrates the growth, as turbidity, of *M. avium* strain A5 cells grown in suspension and biofilms in M7H9 medium on the surface of polystyrene flasks. The growth rates of *M. avium* strain A5 of cells suspension and cells grown in biofilms and suspended are approximately 24 hours per doubling. Figure 2 shows the growth, as turbidity, of *M. intracellulare* strain TMC 1406^T cells grown in biofilms and suspended is approximately 24 hours per doubling. A mid-log phase culture was reached within 3 days. Logarithmic (log) phase is defined as a phase of exponential cell growth.

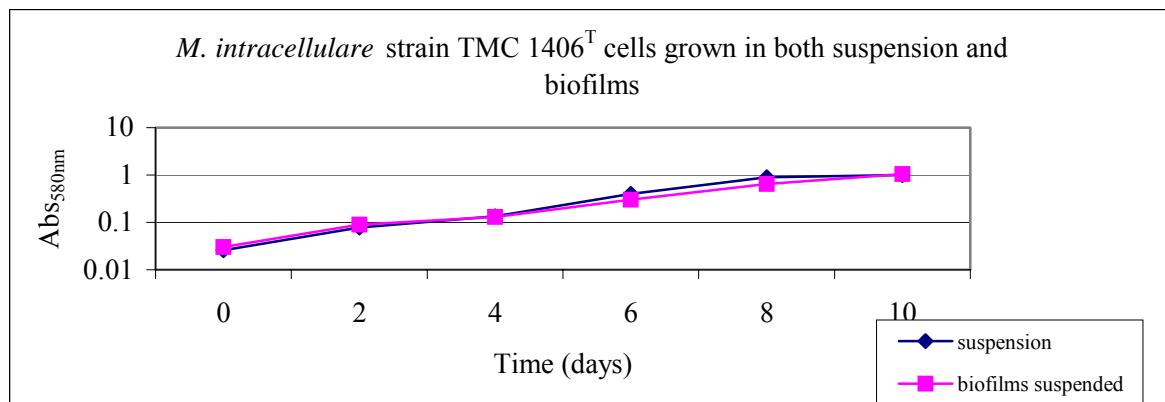
Figure 3 shows the growth, as turbidity, *M. avium* strain A5 cells in raw water both in suspension and biofilms suspended in polystyrene flasks. The growth rate of raw water grown *M. avium* strain A5 cells in suspension and cells grown in biofilms and suspended are approximately 8 hours per doubling. Figure 4 illustrates the growth, as turbidity, *M. intracellulare* strain TMC 1406^T cells in raw water in both suspension and biofilms suspended in polystyrene flasks. The growth rate of raw water grown *M. intracellulare* strain TMC 1406^T cells in suspension is approximately 36 hours per doubling and cells grown in biofilms and suspended is 12 hours per doubling.

Figure 1. Growth curves *M. avium* strain A5 in M7H9 medium in suspension and biofilms on polystyrene



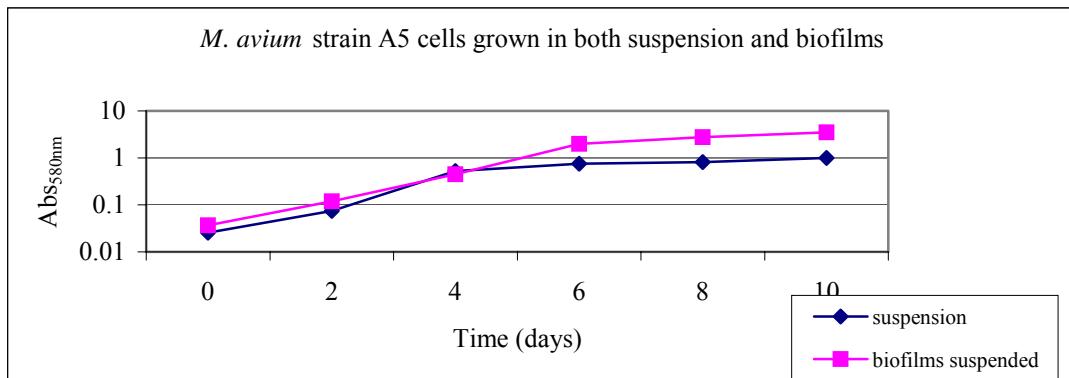
The growth of *M. avium* strain A5 cells grown in suspension and cells grown in biofilms and suspended is seen by the increase of turbidity ($\text{Abs}_{580\text{nm}}$) over time in days. Approximately 24 hours per doubling for cells grown in suspension and cells grown in biofilms and suspended. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Figure 2. Growth of *M. intracellulare* strain TMC 1406^T in M7H9 medium in suspensions and biofilms in polystyrene



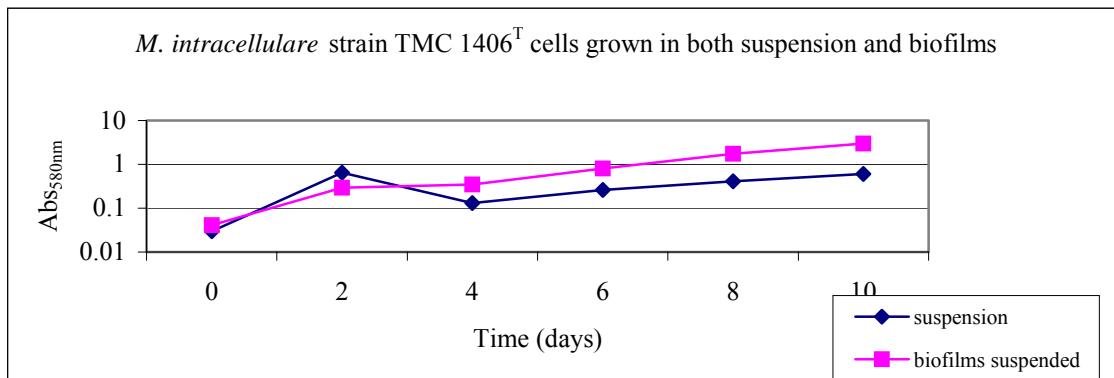
The growth of *M. intracellulare* strain TMC 1406^T cells grown in suspension and cells grown in biofilms and suspended is seen by the increase of turbidity ($\text{Abs}_{580\text{nm}}$) over time in days. Approximately 24 hours per doubling for cells grown in suspension and cells grown in biofilms and suspended. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Figure 3. Growth of *M. avium* strain A5 in raw water in suspension and biofilms in polystyrene



The growth of *M. avium* strain A5 cells grown in suspension and cells grown in biofilms and suspended in seen by the increase of turbidity ($\text{Abs}_{580\text{nm}}$) over time in days. Approximately 8 hours per doubling for cells grown in suspension and cells grown in biofilms and suspended. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Figure 4. Growth of *M. intracellularare* strain TMC 1406^T in raw water in suspension and biofilms in polystyrene



The growth of *M. intracellularare* strain TC 1406^T cells grown in suspension and cells grown in biofilms and suspended is seen by the increase of turbidity (Abs_{580nm}) over time in days. Approximately 36 hours per doubling for cells grown in suspension and 24 hours per doubling for cells grown in biofilms and suspended. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Growth on glass beads

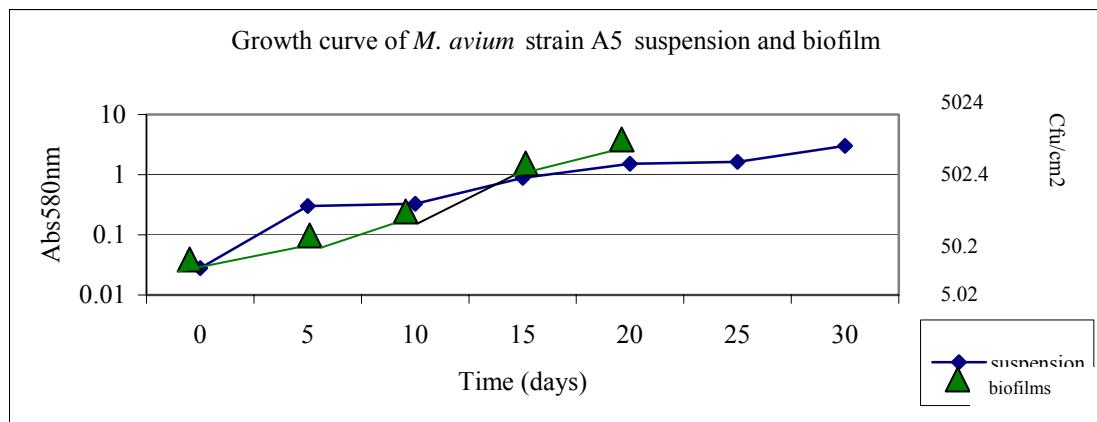
Measurement of turbidity was used to monitor growth of cells grown in suspension and the measure of colony forming unit/ cm² was used for intact biofilms. The growth rate as colony forming units of *M. avium* strain A5 and *M. intracellulare* TMC 1406^T was measured in both medium and water in polystyrene flasks containing glass beads and plated onto M7H10 agar medium. Cells grown in biofilms on glass beads permitted measurement of antimicrobial susceptibility of the biofilm.

The following figures show growth in suspension and growth in biofilms on the same graph to indicate that both cell types are growing at the same rate. Figure 5 illustrates the growth of *M. avium* strain A5 in medium in both biofilms on the glass beads and suspension. The growth rate of medium grown *M. avium* strain A5 cells in suspension was approximately 48 hours per doubling, cells grown in biofilms and suspended approximately 86 hours per doubling. Figure 6 shows the growth of *M. intracellulare* strain TMC 1406^T in medium in both suspension and biofilms while measured in the presence of glass beads in raw water. The growth rate of medium grown *M. intracellulare* strain TMC 1406^T cells in suspension approximately 24 hours. The growth rate of *M. intracellulare* strain TMC 1406^T cells grown in biofilms and suspended approximately 36 hours per doubling.

The growth of raw water grown *M. avium* strain A5 in suspension and in biofilms is displayed in figure 7. The growth rate of *M. avium* strain A5 cells in suspension is approximately 120 hours, while cells grown in biofilms and suspended is approximately 72 hours. Figure 8 displays the growth raw water-grown *M. intracellulare* strain TMC 1406^T suspension and biofilms measured in the presence of glass beads. *M.*

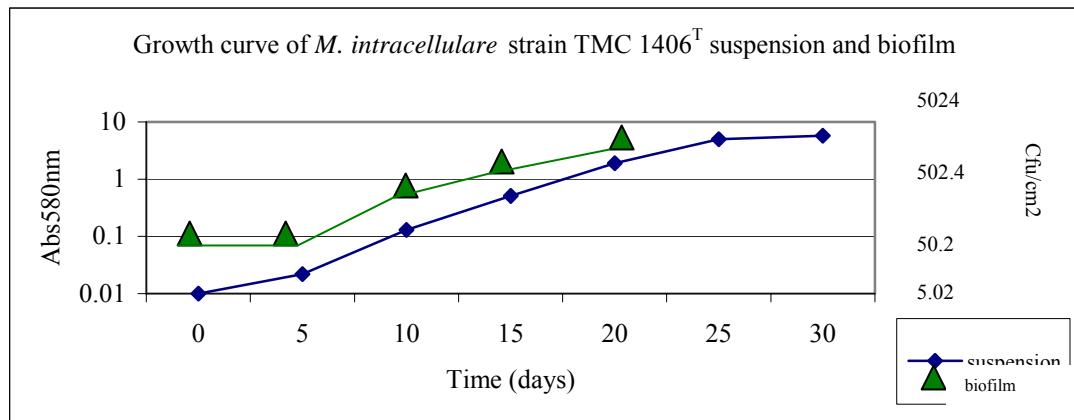
intracellulare strain TMC 1406^T suspension growth plateaus in approximately 15 to 20 days. The growth rate of *M. intracellulare* strain TMC 1406^T cells in suspension is approximately 24 hours per doubling while cells grown in biofilms and suspended is 48 hours.

Figure 5. Growth of *M. avium* strain A5 in M7H9 medium in suspension and biofilms on glass beads



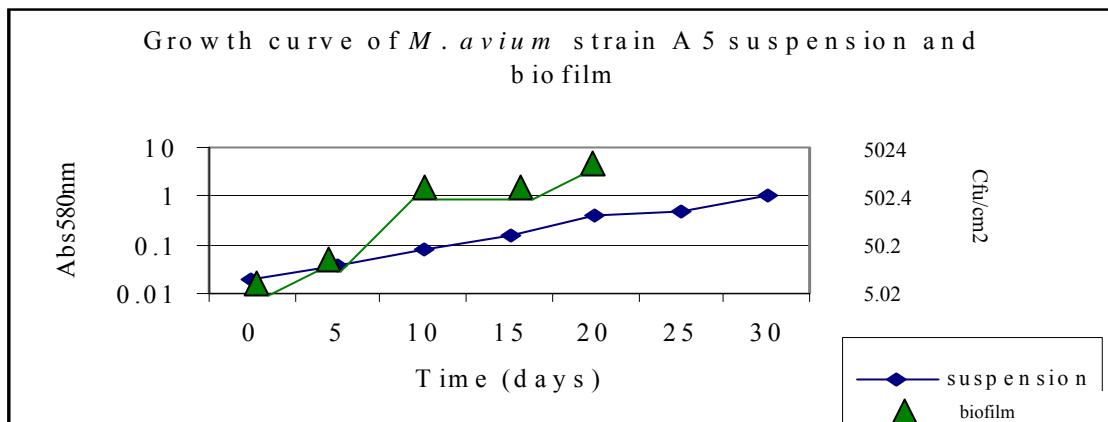
The growth of *M. avium* strain A5 cells grown in suspension and cells grown in biofilms is measured by the increase of turbidity (Abs_{580nm}) over time in days. The growth of *M. avium* strain A5 cells grown in biofilms is measured by cfu/cm² over time in days. Approximately 24 hours per doubling for cells grown in suspension and 60 hours per doubling for cells grown in biofilms. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Figure 6. Growth of *M. intracellulare* strain TMC 1406^T in M7H9 medium in suspension and biofilms on glass beads



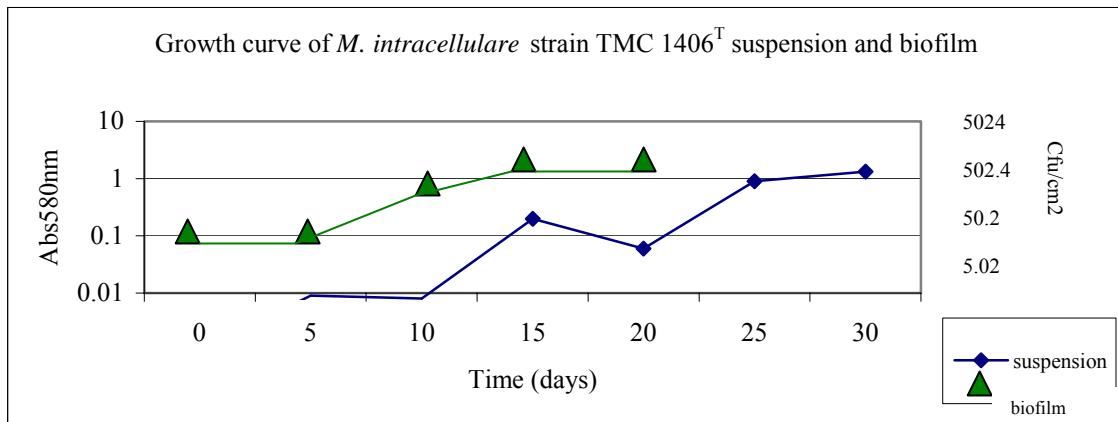
The growth of *M. intracellulare* strain TMC 1406^T cells grown in suspension and cells grown in biofilms is measured by the increase of turbidity (Abs_{580nm}) over time in days. The growth of *M. avium* strain A5 cells grown in biofilms is measured by cfu/cm² over time in days. Approximately 36 hours per doubling for cells grown in suspension and 12 hours per doubling for cells grown in biofilms. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Figure 7. Growth curve of *M. avium* strain A5 in raw water in suspension and biofilms on glass beads



The growth of *M. avium* strain A5 cells grown in suspension and cells grown in biofilms is measured by the increase of turbidity ($\text{Abs}_{580\text{nm}}$) over time in days. The growth of *M. avium* strain A5 cells grown in biofilms is measured by cfu/cm^2 over time in days. Approximately 24 hours per doubling for cells grown in suspension and cells grown in biofilms. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Figure 8. Growth of *M. intracellularare* strain TMC 1406^T in raw water in suspension and biofilms on glass beads



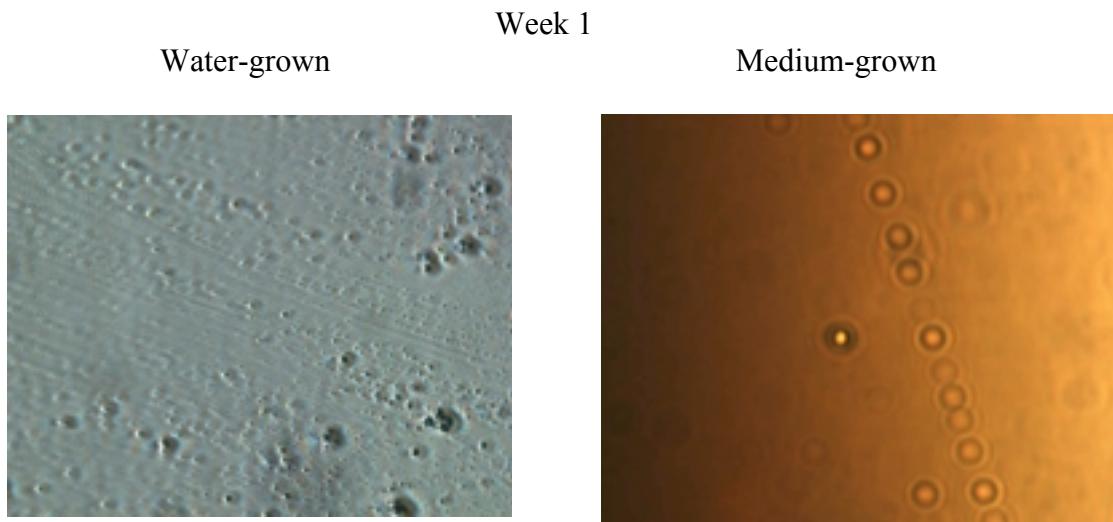
The growth of *M. intracellularare* strain TMC 1406^T cells grown in suspension and cells grown in suspension in measured by the increase of turbidity (Abs_{580nm}) over time in days. The growth of *M. intracellularare* strain TMC 1406^T cells grown in biofilms is measured by cfu/cm² over time in days. Approximately 120 hours per doubling for cells grown in suspension and 36 hours per doubling for cells grown in biofilms. Curves were plotted on the same graph to show that cultures were grown at the same rate.

Microscopic assessment of biofilm development

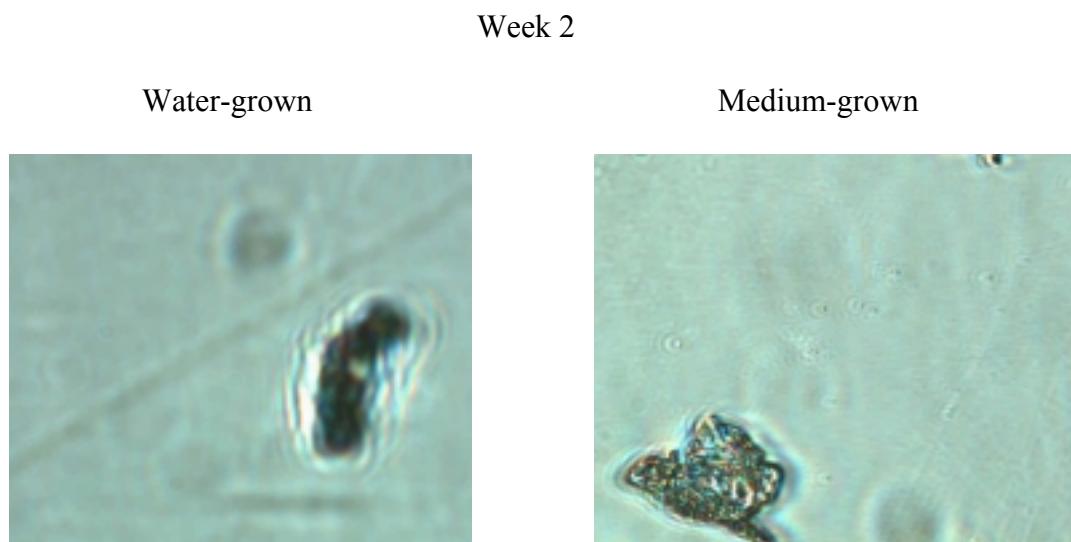
Microscopic observations show that *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T formed biofilms within 3 weeks of growth in M7H9 medium or sterile raw water. The weekly development of *M. avium* and *M. intracellulare* biofilms were observed. Biofilms were observed under an inverted compound microscope at 400x magnification. In Figure 9 the development of *M. avium* strain A5 biofilms grown in sterilized raw water and M7H9 medium was observed after 1, 2, 3, 4 weeks. Figure 10, the development of *M. intracellulare* strain TMC 1406^T grown in sterilized raw water and M7H9 was observed. No biofilms formed in one week in both media for *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T. However, after one week an initial monolayer of cells appeared on the surface of the polystyrene flask. After two weeks micocolonies appeared on the surface. By weeks 3 and 4 biofilms had developed.

M. intracellulare strain TMC 1406^T biofilms completely covered the surface of the polystyrene by week 2, whereas *M. avium* strain A5 were limited to 50% of the surface. Water-grown biofilms tended to cover a larger proportion of the surface compared to medium-grown biofilms, suggesting an effect of the medium on biofilms formation. A clear outlining layer can be seen in the week 4 biofilms of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T.

Figure 9. Microscopic appearance of *M. avium* strain A5 biofilms on polystyrene



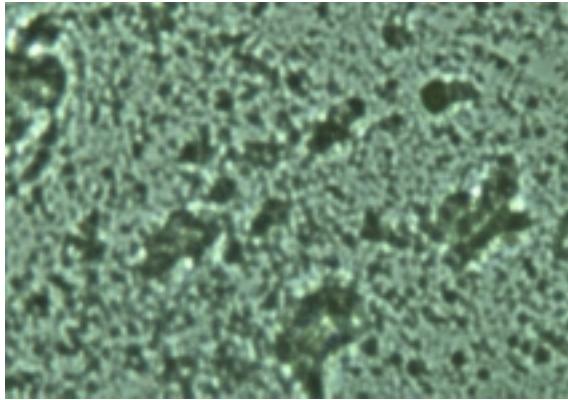
Inverted compound microscope pictures of biofilms development of *M. avium* strain A5 at week 1 grown in raw water (left) and M7H9 medium (right). Week 1 shows planktonic cells. Pictures are taken at 400x magnification.



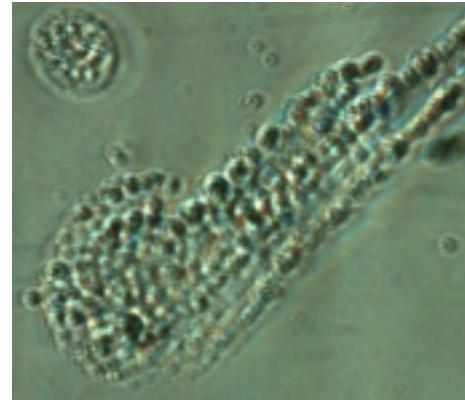
Inverted compound microscope pictures of biofilms development of *M. avium* strain A5 at week 2 grown in raw water (left) and M7H9 medium (right). Week 2 shows the attachment of the monolayer to the surface of the polystyrene flasks. Pictures are taken at 400x magnification.

Week 3

Water-grown



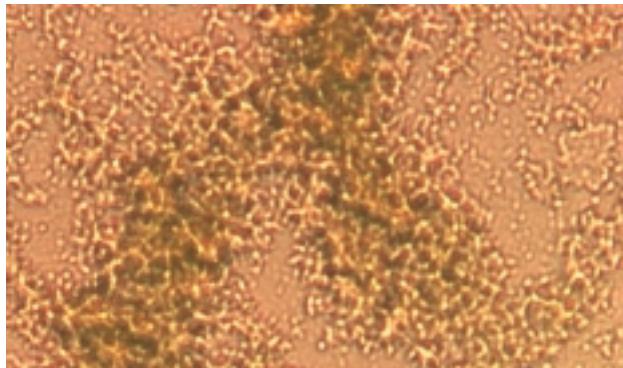
Medium-grown



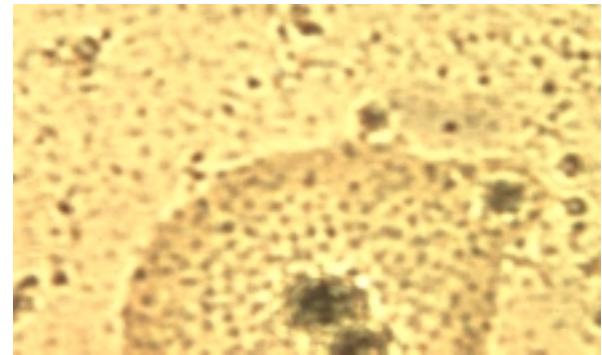
Inverted compound microscope pictures of biofilms development of *M. avium* strain A5 at week 3 grown in raw water (left) and M7H9 medium (right). Week 3 shows the development of the microcolony on the surface of the polystyrene flasks. Pictures are taken at 400x magnification.

Week 4

Water-grown

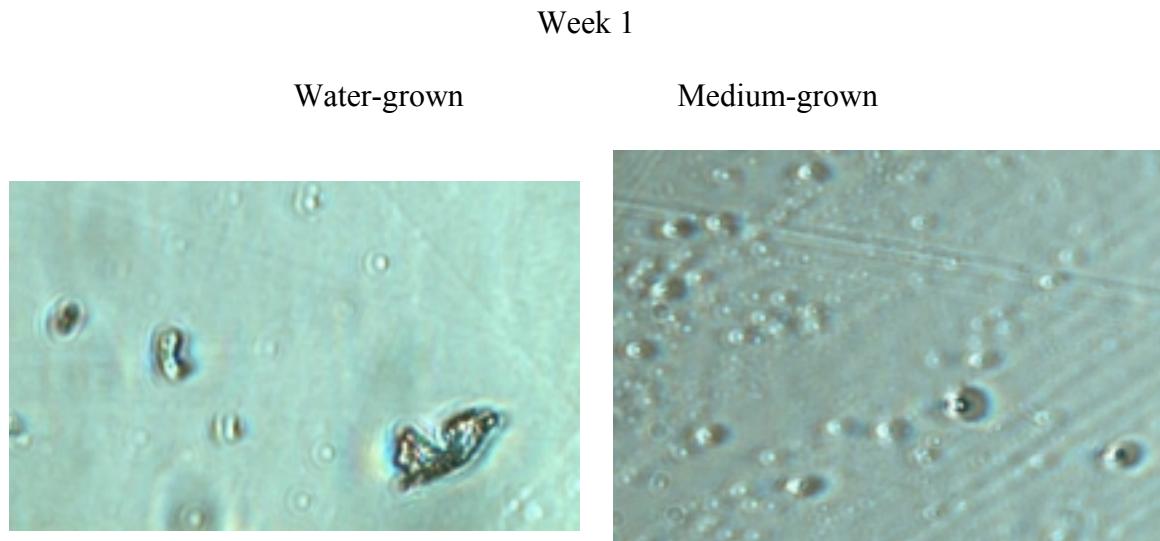


Medium-grown

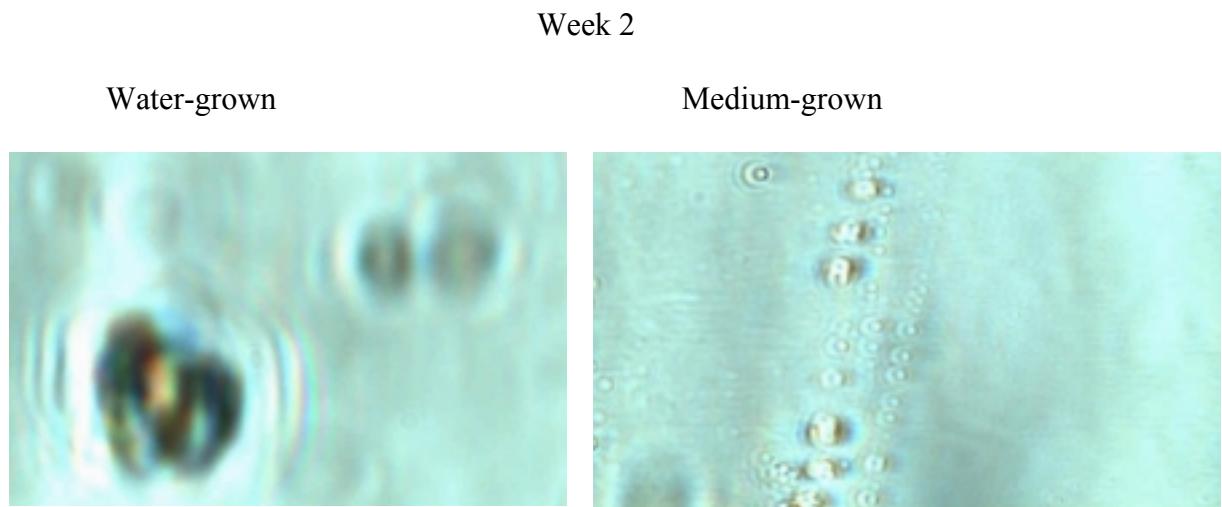


Inverted compound microscope pictures of biofilms development of *M. avium* strain A5 at week 4 grown in raw water (left) and M7H9 medium (right). Week 4 shows the formation of the mature biofilms. Pictures are taken at 400x magnification.

Figure 10. Microscopic appearance of *M. intracellulare* strain TMC 1406^T biofilms on polystyrene



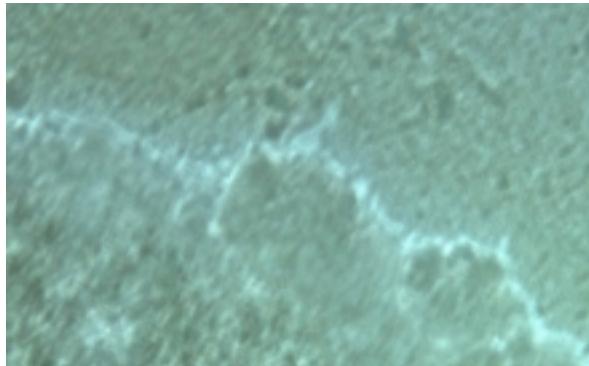
Inverted compound microscope pictures of biofilms development of *M. intracellulare* strain TMC 1406^T at week 1 grown in raw water (left) and M7H9 medium (right). Week 1 shows planktonic cells. Pictures are taken at 400x magnification.



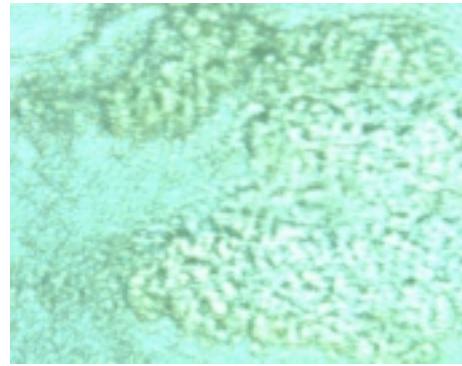
Inverted compound microscope pictures of biofilms development of *M. intracellulare* strain TMC 1406^T at week 2 grown in raw water (left) and M7H9 medium (right). Week 2 shows the attachment of the monolayer to the surface of the polystyrene flasks. Pictures are taken at 400x magnification.

Week 3

Water-grown

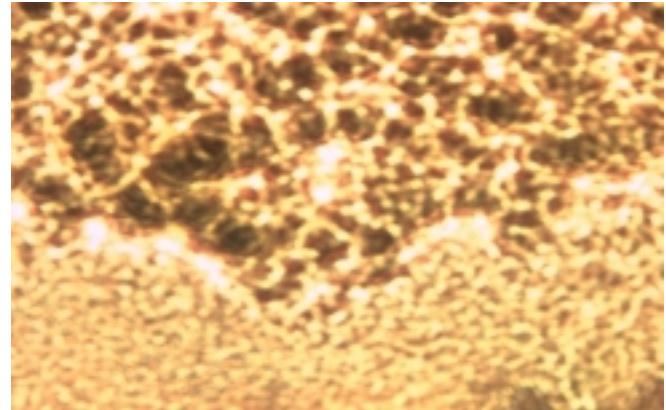
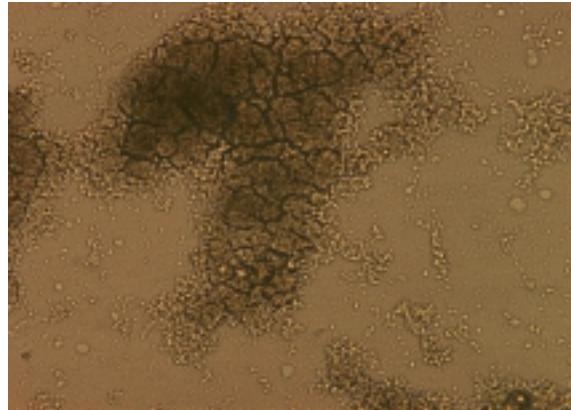


Medium-grown



Inverted compound microscope pictures of biofilms development of *M. intracellulare* strain TMC 1406^T at week 3 grown in raw water (left) and M7H9 medium (right). Week 3 shows the development of the microcolony on the surface of the polystyrene flask. Pictures are taken at 400x magnification.

Week 4



Inverted compound microscope pictures of biofilms development of *M. intracellulare* strain TMC 1406^T at week 4 grown in raw water (left) and M7H9 medium (right). Week 4 shows the formation of the mature biofilm. Pictures are taken at 400x magnification.

Effect of vortexing on recovery of colony forming units

Vortexing glass beads enabled the isolation of cells from biofilms, biofilms and the measurement of the susceptibility of cells grown on glass beads in M7H9 medium. Table 4 shows the effect of vortexing in 0.5% (vol/vol) Tween 80 with glass beads on colony forming ability of *M. avium* strain A5 (Harris and Angal, 1990). Colony forming units were counted before and after vortexing in the presence of 4 mm glass beads for 60 seconds. The difference between experiment 1 pre vortexing and post vortexing had a two-tailed P value of 0.8527 considered not significant. The difference between experiment 2 pre vortexing and post vortexing had a two-tailed P value of 0.4884 considered not significant. This shows that there was no effect of vortexing on the mycobacterial cells.

Bead-to-Bead variation in biofilm colony counts

Biofilms were grown on 4 mm glass beads. Measuring bead-to-bead variation was necessary in order to determine whether the variation would be greater than the variation due to exposure to antibiotics or chlorine. *M. avium* strain A5 was grown in M7H9 medium, in a polystyrene flask in the presence of 4 mm glass beads. Incubation of the culture containing beads, led to biofilm development and growth on the beads. Growing bacteria on glass beads and then suspending them by vortexing, yielded a cell suspension from biofilms for comparison with cells in intact biofilms. In Table 5 the variation of colony-forming units/cm² between 4 mm glass beads is shown. The difference between experiment 1 and 2 had a two-tailed P value of 0.6736 not considered

significant using a T test. Large bead-to-bead variations would result in inconsistent cell densities in susceptibility experiments, which could bias the results.

Table 4. Effect of vortexing with glass beads on colony forming ability of
M. avium strain A5^a

Experiment	Number of replicates	Colony forming units/ cm ²	
		Pre vortexing ^b	Post vortexing ^c
	3	225 ± 20	228 ± 17
	3	369 ± 49	343 ± 33

^a Vortex at maximum setting for 60 seconds with 4 mm diameter glass beads

^b Mean ± standard deviation of suspension before vortexing with beads

^c Mean ± standard deviation of suspension after vortexing with beads

Table 5. Bead-to-Bead variation in adherent *M. avium* strain A5 colony counts/cm²

Experiment	Number of replicates	Colony forming units/cm ² ^a
1	2	384 ± 96
2	2	342 ± 111

^a Mean ± standard deviation

Antibiotic susceptibility of cells grown in polystyrene flasks

The antibiotic susceptibility was measured on log phase, medium-grown *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T cells grown in suspension and cells grown in biofilms and exposed to antibiotics in suspension in polystyrene flasks. Log phase cultures were used to reduce effects of differences of growth phase on susceptibility.

Mycobacteria suspensions of approximately 10⁴ cfu/ml were used because these are reflective of the highest numbers in the blood of infected AIDS patients (Inderlied et al., 1993). The relevance of the 3 hour incubation period reflects the half-life of the antibiotics of 2 to 5 hours (Scholar and Pratt, 2000). The desired result of the antibiotics is 99% effectiveness.

Clarithromycin, ethambutol, kanamycin, rifampicin, and streptomycin were chosen because they are currently used to treat *M. avium* complex infections (Scholar and Pratt, 2000). Table 6 summarizes the antibiotic minimal inhibitory concentration (MIC) on agar media after 14 day incubation. These values served to guide the selection of concentrations to expose cells in suspension or biofilms. Higher concentrations were used on suspensions reach significant killing in the short period of exposure (0-3 hours)

Figure 11 shows the percent survival of *M. avium* strain A5 cells grown in suspension and cells grown in biofilms and exposed in suspension at a concentration of 145µg of rifampicin/ml of M7H9 for 3 hours in polystyrene flasks. Rapid killing was seen between 0 and 1 hour while 1 to 3 hours the rate decreased. This pattern of rapid followed by slow killing was seen for both strains in all antibiotics. Table 8 summarizes

the antibiotic susceptibility of cells in suspension and biofilms suspended at 1 hour for *M. avium* strain A5.

Figure 12 shows percent survival of *M. intracellulare* strain TMC 1406^T cells grown in suspension and cells grown in biofilms and exposed in suspension at a concentration of 145µg of rifampicin/ml of M7H9 for 3 hours on polystyrene flasks. Table 8 is a summary of antibiotic susceptibility of cells grown in suspension and cells grown in biofilms and tested in suspension at 1 hour for *M. intracellulare* strain TMC 1406^T. The difference between cells in suspension and cells grown in biofilms and tested in suspension of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T had a two-tailed P value of < 0.0001, considered extremely significant using a T test. This demonstrates that cells grown in biofilms are significantly more resistant to antibiotics compared to cells grown in suspension. This difference is independent of protective layers of cells because the biofilms-grown cells were exposed to antibiotics in suspension.

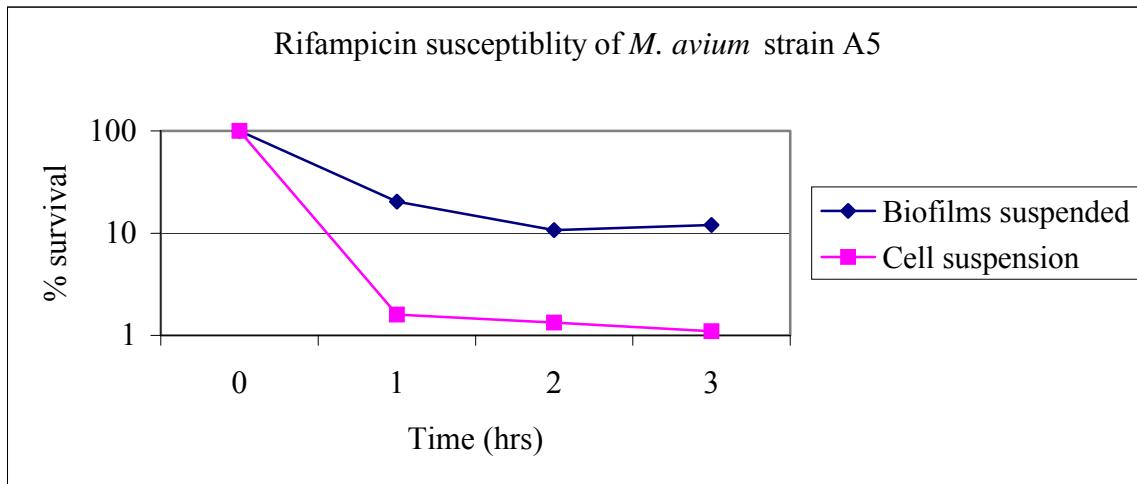
To determine whether cells grown in biofilms would regain sensitivity to antibiotics after growth in media, cells of *M. avium* strain A5 that had been grown in were suspended in M7H9 and the culture was incubated for one week at 37°C. Figure 13 shows the percent survival of cells grown in biofilms and suspended for one week at 37°C and exposed to rifampicin. The data demonstrates that the cells regained sensitivity by comparison to cells grown in biofilms (Figure 12). Table 9 summarizes the percent survival of *M. avium* strain A5 exposed to different antibiotics after growth in M7H9 medium for one week at 37°C. Cells did not regain sensitivity to Clarithromycin (Table 9). This suggests that the reversible resistance of biofilms grown cells to antibiotics does

not include Clarithromycin. Perhaps, a longer incubation period of cells would result in regaining clarithromycin sensitivity.

Table 6. Antibiotic Minimal Inhibitory Concentration (MIC) µg/ml

Antibiotic	<i>M. avium</i> strain A5	<i>M. intracellulare</i> strain TMC 1406 ^T
Clarithromycin	1-10	1-10
Ethambutol	1-10	1-10
Rifampicin	<1	<1
Kanamycin	1-10	1-10
Streptomycin	1-10	1-10

Figure 11. Rifampicin susceptibility of *M. avium* strain A5 cells grown suspension and on polystyrene and exposed to rifampicin in suspension



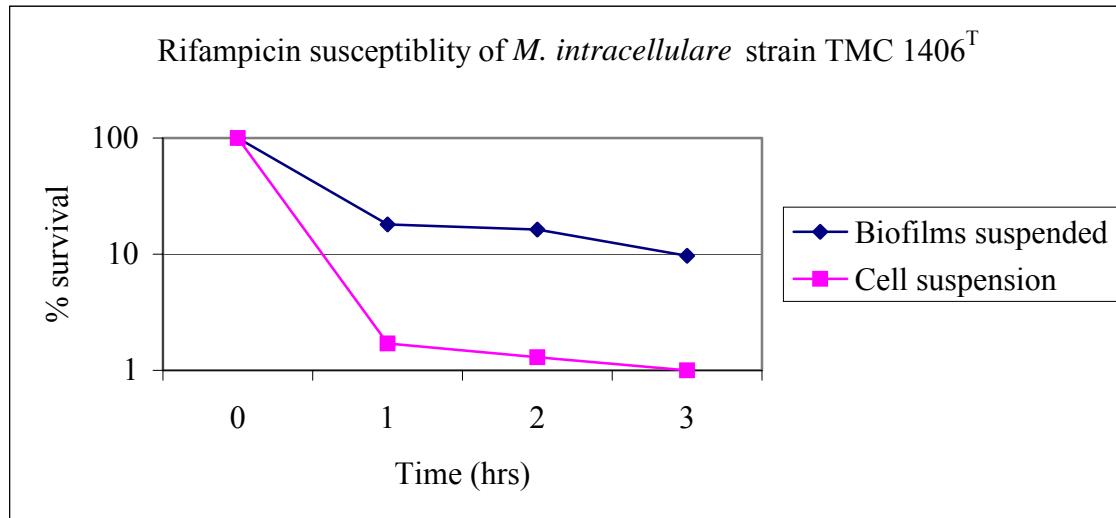
Representative graph of the percent survival of *M. avium* strain A5 cells grown in suspension and cells grown in biofilms and suspended against time at 0,1,2,3 hours. Rapid killing is seen between time 0 and 1 hour in both cell types. A ten-fold increase in percent survival is seen in cells grown in biofilms and suspended in comparison with cells grown in suspension.

Table 7. Percent survival of *M. avium* strain A5 cells grown in suspension and on polystyrene and exposed to antibiotics in suspension

Antibiotic	Drug Concentration	Percent survival	
		Suspension	Biofilms suspended
Clarithromycin	145 µg/ml	2.6 % ± 1.3 %	19 % ± 0.3%
Ethambutol	145 µg/ml	1.8 % ± 0.3 %	20 % ± 0.7%
Kanamycin	145 µg/ml	2.7 % ± 1.6 %	17 % ± 0.9 %
Rifampicin	145 µg/ml	2.4 % ± 1.1 %	20 % ± 1.4 %
Streptomycin	145 µg/ml	1.5 % ± 0.7 %	17 % ± 0.6 %

^a Average of duplicate experiments ± standard deviation after 1 hour exposure at 37°C

Figure 12. Rifampicin susceptibility of *M. intracellulare* strain TMC 1406^T cells grown in suspension and on polystyrene and exposed to rifampicin in suspension



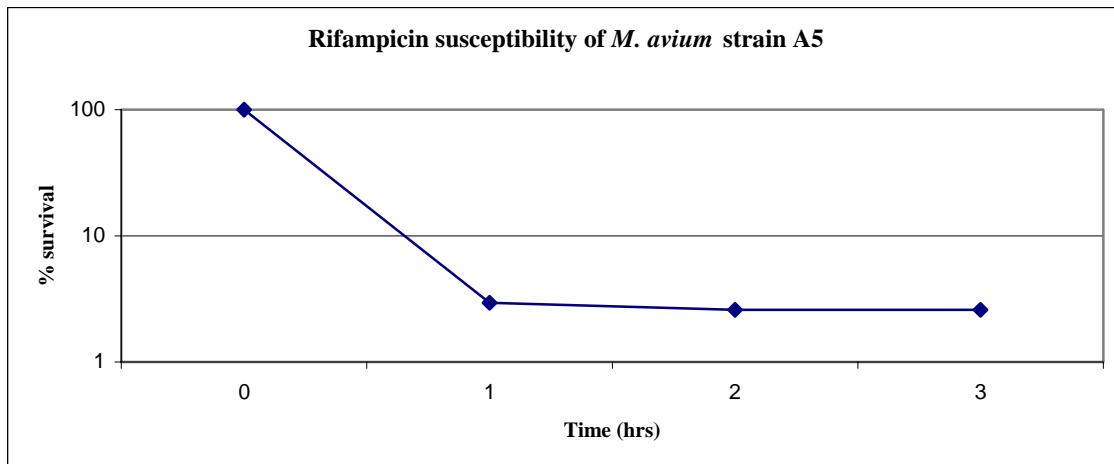
Representative graph of the percent survival of *M. intracellulare* strain TMC 1406^T cells grown in suspension and cells grown in biofilms and suspended against time at 0,1,2,3 hours. Rapid killing is seen between time 0 and 1 hour in both cell types. A ten-fold increase in percent survival is seen in cells grown in biofilms and suspended in comparison with cells grown in suspension.

Table 8. Percent survival of *M. intracellulare* strain TMC 1406^T cells grown in suspension and on polystyrene and exposed to antibiotics in suspension

Antibiotic	Drug Concentration	Percent survival	
		Suspension	Biofilms suspended
Clarithromycin	145 µg/ml	5.3 % ± 1.0 %	21 % ± 0.4 %
Ethambutol	145 µg/ml	1.8 % ± 0.1 %	18 % ± 0.6 %
Kanamycin	145 µg/ml	1.4 % ± 0.1 %	19 % ± 0.5%
Rifampicin	145 µg/ml	4.4% ± 3.4 %	19 % ± 0.3 %
Streptomycin	145 µg/ml	1.2 % ± 0.1 %	18 % ± 0.1 %

^a Average of duplicate experiments ± standard deviation, exposed for 1 hour at 37°C.

Figure 13. Rifampicin susceptibility of *M. avium* strain A5 cells grown in biofilms, suspended, and grown one week in M7H9 at 37°C



Representative graph of the percent survival of *M. avium* strain A5 cells grown in biofilms and suspended for one week in medium at 37°C against time at 0,1,2,3 hours. Rapid killing is seen between time 0 and 1 hour in both cell types. Indicates that cells grown in biofilms and suspended for one week regain antibiotic susceptibility.

Table 9. Percent survival of *M. avium* strain A5 cells grown in biofilms and growth in suspension for 1 week at 37°C

Antibiotic	Drug Concentration	Percent survival	
		Cells in suspension	No. replicates
Clarithromycin	145 µg/ml	16 % ± 7.4 %	2
Ethambutol	145 µg/ml	4.8 % ± 2.5 %	2
Kanamycin	145 µg/ml	2.9 % ± 2.7 %	2
Rifampicin	145 µg/ml	3.0 % ± 1.0 %	2
Streptomycin	145 µg/ml	2.3 % ± 1.0 %	2

^a Average of duplicate experiments ± standard deviation, exposed for 1 hour at 37°C.

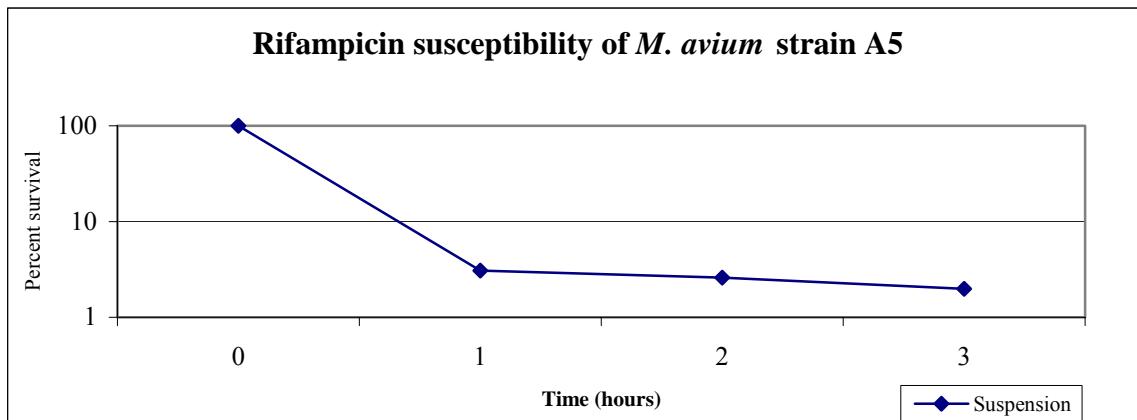
Antibiotic susceptibility of cells grown on glass beads

Cells grown in biofilms were grown on glass beads in order to accurately obtain biofilms. Cells grown in biofilms on polystyrene flasks would have to be scraped from the flask in order to be tested. Unfortunately, it is impossible to scrap exactly the same area. The glass beads allowed the biofilms to be exposed and accurately measured without changing the structure. Thus cells were grown in the presence of glass beads. The mycobacteria cells were able to grow on the glass beads, which therefore yielded biofilms for susceptibility testing. Antibiotic susceptibility was measured on log phase, medium-grown *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T cells grown in biofilms and exposed in suspension and cells grown in biofilms and exposed in biofilms grown on 4mm glass beads.

Figure 14 shows the percent survival of *M. avium* strain A5 cells grown in suspension at 145 µg rifampicin/ml of M7H9 is representative of all antibiotics. Figure 15 shows the percent survival of *M. intracellulare* strain TMC 1406^T cells grown in suspension at 145 µg rifampicin/ml of M7H9, which is representative of all other antibiotics. Table 10 is a summary of antibiotic susceptibility of cells grown in suspension and biofilms of *M. avium* strain A5. The difference between cells grown in suspension and cells grown in biofilms and tested in suspension have a two-tailed P value of < 0.0003 considered extremely significant using a T test. The difference between cells grown in suspension and cells grown in biofilms and exposed in biofilms had a two-tailed P value of < 0.0001, considered extremely significant using a T-test.

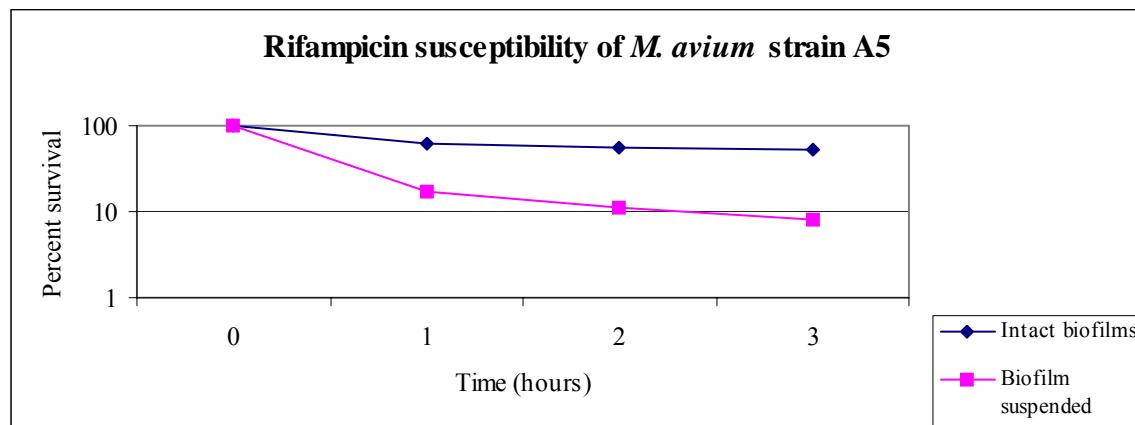
Figure 16 shows the percent survival of *M. avium* strain A5 cells grown in biofilms tested in suspension and biofilms at 145 µg of rifampicin/ml of M7H9. Figure 17 shows the percent survival of *M. intracellulare* strain TMC 1406^T cells grown in biofilms tested in suspension and cells grown in biofilms tested in biofilms at 145 µg rifampicin/ml of M7H9. Table 11 is a summary of antibiotic susceptibility of cells grown in suspension at 1 hour for *M. intracellulare* strain TMC 1406^T. The difference between *M. avium* strainA5 and *M. intracellulare* strain TMC 1406^T cells in suspension, biofilms suspended and intact biofilms had a two-tailed P value of <0.0001, considered extremely significant using a T test.

Figure 14. Rifampicin susceptibility of *M. avium* strain A5 cells grown suspension and exposed in suspension



Representative graph of the percent survival of *M. avium* strain A5 cells grown in suspension against time at 0,1,2,3 hours. Rapid killing is seen between time 0 and 1 hours in both cell types. Curve is identical to cells grown in suspension on polystyrene flask.

Figure 15. Rifampicin susceptibility of *M. avium* strain A5 cells grown in biofilms on glass beads and exposed in suspension and in biofilms



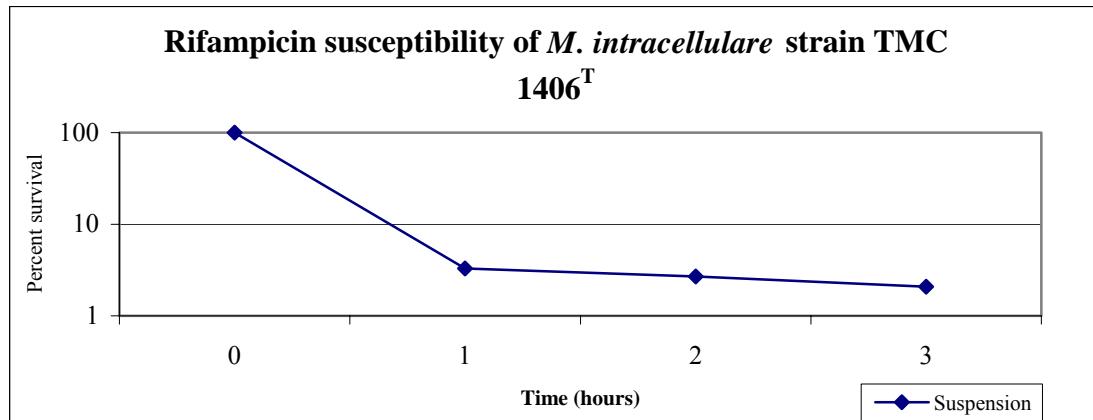
Representative graph of the percent survival of *M. avium* strain A5 cells grown in biofilms and cells grown in biofilms and suspended against time at 0,1,2,3 hours. Rapid killing is seen between time 0 and 1 hour in cells grown in biofilms and suspended. The percent survival is significantly higher in cells grown in biofilms than cells grown in biofilms and suspended.

Table 10. Percent survival of *M. avium* strain cells grown in suspension or biofilms and exposed in either suspension or biofilms

Drug	concentration	Percent survival ^a		
		Suspension	Biofilms	Biofilms suspended
Clarithromycin	145 µg/ml	3.6 % ± 0.1%	18 % ± 0.6 %	78% ± 1.2 %
Ethambutol	145 µg/ml	3.1 % ± 0.1%	18 % ± 0.4 %	66 % ± 0.3 %
Kanamycin	145 µg/ml	1.7 % ± 0.3%	16 % ± 0.9 %	64 % ± 0.2 %
Rifampicin	145 µg/ml	3.1 % ± 0.1 %	17 % ± 0.5 %	64 % ± 3.0 %
Streptomycin	145 µg/ml	3.2 % ± 0.1%	18 % ± 0.4%	65 % ± 0.1 %

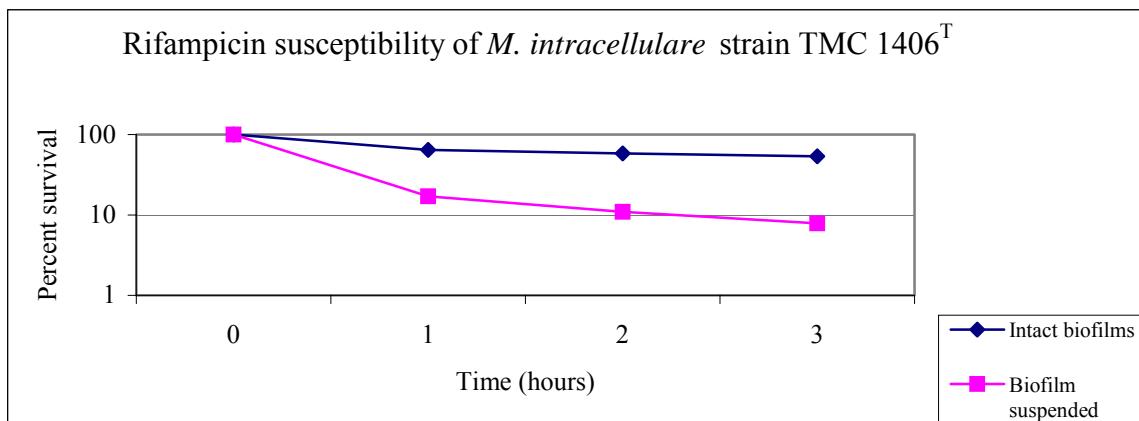
^a Cells grown on glass beads

Figure 16. Rifampicin susceptibility of *M. intracellulare* strain TMC 1406^T cells grown in suspension and exposed in suspension



Representative graph of the percent survival of *M. intracellulare* strain TMC 1406^T cells grown in suspension and cells grown in biofilms and suspended against time at 0,1,2,3 hours. Rapid killing is seen between time 0 and 1hours in both cell types.

Figure 17. Rifampicin susceptibility of *M. intracellulare* strain TMC 1406^T cells grown in biofilms on glass beads and exposed in suspension and in biofilms



Representative graph of the percent survival of *M. intracellulare* strain TMC 1406T cells grown in biofilms and cells grown in biofilms and suspended against time at 0,1,2,3 hours. Rapid killing is seen in cells grown in biofilms and suspended. The percent survival is significantly higher in cells grown in biofilms than cells grown in biofilms and suspended.

Table 11. Percent survival of *M. intracellulare* strain TMC 1406^T cells grown in suspension on biofilms and exposed in either suspension or in biofilms

	Drug concentration	Suspension	Biofilms suspended	Percent survival ^a
Clarithromycin	145 µg/ml	3.2 % ± 0.1%	17 % ± 0.5%	77 % ± 6.3 %
Ethambutol	145 µg/ml	2.7 % ± 0.1 %	18 % ± 0.4 %	69 % ± 0.1 %
Kanamycin	145 µg/ml	1.8 % ± 0.4 %	17 % ± 0.1 %	65 % ± 3.5 %
Rifampicin	145 µg/ml	3.3 % ± 0.1 %	17 % ± 0.1 %	64 % ± 1.6 %
Streptomycin	145 µg/ml	3.4 % ± 0.1 %	19 % ± 0.4 %	65 % ± 0.2 %

Chlorine susceptibility of cells grown in polystyrene flasks

Because the State of Virginia's minimum concentration of chlorine to finished (drinking water) is 1 part/million that concentration of chlorine used to measure the effect of biofilms formation on chlorine susceptibility of water-grown and medium-grown cells. The RAF method was used in order to ensure that individual cells in suspension could be tested (Taylor et al., 2000). A working solution of chlorine (sodium hypochlorite) was prepared and added to the flasks at a concentration of 1 ppm. The concentration of chlorine in the beginning and the end of the experiment was measured. Sodium thiosulfate was added to the sample tubes after exposure and before plating in order to stop the reaction of the chlorine. Comparison of percent survival between cell types and growth medium (i.e. sterile raw water or M7H9 broth medium) were made.

Table 12 shows the chlorine susceptibility of medium-grown and water-grown *M. avium* strain A5 cells grown in suspension and in biofilms and exposed in either suspension or in biofilms. Table 13 shows the chlorine susceptibility of medium-grown and water-grown *M. intracellulare* strain TMC 1406^T cells grown in either suspension and in biofilms and exposed in either suspension or in biofilms. The difference between *M. avium* strain A5 cells in suspension and biofilms suspended had a two-tailed P value of 0.0002 considered extremely significant using the T-test. The difference between *M. intracellulare* strain TMC 1406^T cells in suspension and biofilms suspended had a two-tailed P value of 0.0239 considered significant using the T-test.

The difference between *M. intracellulare* strain TMC 1406^T had a two-tailed P value of 0.7084 considered not significant using the T-test. The P value reflects the high percent survival of the strain. From this value we may infer that the concentration of

chlorine was not high enough to kill *M. avium* strain A5 of *M. intracellulare* strain TMC 1406^T.

To determine whether cells grown in biofilms would regain sensitivity to chlorine after growth in sterile raw water, cells of *M. avium* strain A5 recovered from biofilms were suspended in sterile raw water and incubated for one week at 37°C. Cells harvested from biofilms and incubated one week regained the sensitivity characteristic of suspension grown cells (Table 14). The difference had a two-tailed P value of 0.569 considered not significant using the T-test.

Table 12. Chlorine susceptibility of log phase *M. avium* strain A5 cells grown in either suspension or biofilms

Growth medium	Exposure to chlorine (min)	Chlorine susceptibility ^a		
		Suspension	Biofilms	Biofilms suspended
Middlebrook 7H9	60	20 % ± 0.5 %	19 % ± 1.4 %	49 % ± 0.8 %
Sterile raw water	60	18 % ± 1.0 %	17 % ± 3.6 %	41 % ± 2.6 %
				60 % ± 1.5 %

^a Average of duplicate experiment ± standard deviation after 60 minutes of exposure to chlorine

Table 13. Chlorine susceptibility of log phase *M. intracellulare* strain TMC 1406^T cells grown in either suspension or biofilms

Growth medium	Exposure to chlorine (min)	Chlorine susceptiblity ^a		
		Suspension	Biofilms	Biofilms suspended
Middlebrook 7H9	60	50 % ± 4.2 %	50 % ± 2.5 %	57 % ± 0.9 %
Sterile raw water	60	50 % ± 3.5 %	50 % ± 1.0 %	52 % ± 11 %

^a Average of duplicate experiment ± standard deviation after 60 minutes of exposure to chlorine

Table 14. Chlorine susceptibility of *M. avium* strain A5 cells grown in biofilms and suspended one week in raw water at 37°C

Growth medium	Exposure	Chlorine susceptibility
Sterile raw water	60	13 % ± 0.6 %

^a Average of duplicate experiments ± standard deviation after 60-minute exposure to 1 ppm chlorine

Measurements of Hydrophobicity

Hexadecane Adherence

The ability of mycobacteria to form biofilms maybe due, in part to, the hydrophobicity of the cell wall. Hydrophobicity, defined as the percent of initial turbidity adhering to hexadecane, was measured by adherence to hexadecane. Table 15 shows hexadecane adherence of log phase *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T grown in suspension and cells grown in biofilms and suspended. There was very little difference between the values for hexadecane adherence of cells grown in suspension or in biofilms and suspended (Table 15). It should be noted that the cells were all relatively hydrophobic.

Contact angle

Contact angle measurements are a direct reflection of hydrophobicity of a surface based upon the liquid solution. Drops of water, *n,n*,dimethyl-formamide, and hexadecane were spotted on cells collected on polystyrene or on washed biofilms on polystyrene. Table 16 shows the contact angle measurements of *M. avium* strain A5 in log phase. Table 17 shows the contact angle measurements of *M. intracellulare* strain TMC 1406^T grown to log phase. Biofilm grown cells had the highest contact angles indicating that they were the most hydrophobic. Cells grown in suspension and collected on filters has the lowest contact angles. Cells grown in biofilms and then suspended before collection on filters had intermediate contact angles.

Contact angles of *M. intracellulare* were higher than *M. avium* and therefore *M. intracellulare* may be more hydrophobic than *M. avium*. Contact angles were also higher in raw water-grown cells compared to medium-grown cells. Therefore water-grown cells are more hydrophobic than medium grown cells. Hexadecane may be so close in surface tension to the surface of *M. avium* and *M. intracellulare* cells that it is unsuitable for discriminating between the three different cell types.

Table 15. Polystyrene flask hexadecane adherence

	Hydrophobicity ^a				
	<i>M. avium</i> strain A5		<i>M. intracellulare</i> strain TMC 1406 ^T		
Growth	Medium	Water	Medium	Water	
Cells grown in suspension	63 % ± 2.5 %	55 % ± 5.0 %	57 % ± 3.5 %	55 % ± 5.0 %	
Cells grown in biofilms and suspended	65 % ± 1.0 %	56 % ± 2.3 %	61 % ± 0.5 %	57 % ± 2.1 %	

^a Percent turbidity adhering hexadecane

^b Cultures are in exponential growth phase

^c Measurements are average of duplicate experiments ± standard deviation

Table 16. Contact Angle measurements of *M. avium* strain A5 grown to log phase^a in M7H9 medium and sterile raw water

Cell type	Water ^{oa}	<i>n,n</i>	Hexadecane ^{oa}
Dimethyl-formamide ^{oa}			
Cell suspension			
Medium	22 ± 8	25 ± 5	15 ± 1
Water	28 ± 3	24 ± 1	10 ± 0
Biofilms suspended			
Medium	28 ± 8	23 ± 3	15 ± 0
Water	37 ± 5	23 ± 3	18 ± 3
Intact Biofilms			
Medium	48 ± 3	33 ± 3	19 ± 1
Water	53 ± 3	32 ± 3	21 ± 1

^a measure of 20µl drop of water, *n,n* dimethyl-formamide, and hexadecane

^b Values are averages of duplicate measurements ± standard deviation

Table 17. Contact Angle measurements of *M. intracellulare* strain TMC1406^T grown to log phase

Cell type	Water°	n,n Dimethyl-formamide°	Hexadecane°
<hr/>			
Cell suspension			
Medium	33 ± 2	20 ± 1	19 ± 1
Water	34 ± 1	21 ± 1	20 ± 0
Biofilms suspended			
Medium	44 ± 7	16 ± 4	23 ± 6
Water	54 ± 5	29 ± 1	22 ± 3
Intact Biofilms			
Medium	55 ± 13	28 ± 3	21 ± 1
Water	70 ± 0	27 ± 4	22 ± 5

^a Measure of 20µl drop of water, n,n dimethyl-formamide, and hexadecane

^b Values are average of duplicate measurements ± standard deviation

Hydrophobicity of cells and susceptibility

Hydrophobicity was compared with susceptibility in order to determine whether a relationship existed. Table 19 shows the cosine of contact angles measured using water.

The cosine of the contact angle is a direct measure of hydrophobicity (von Oss, 1975).

Table 20 illustrates the comparison between survival of antibiotic exposure and hydrophobicity of *M. avium* strain A5 cells types grown in medium. Table 29 shows the comparison between survival of antibiotic exposure and hydrophobicity of *M. intracellulare* strain TMC 1406^T cells types grown in medium.

Table 21 illustrates the comparison between survival of chlorine exposure and hydrophobicity of *M. avium* strain A5 cell types grown in medium. Table 22 illustrates the comparison between survival of chlorine exposure and hydrophobicity of *M. avium* strain A5 cell types grown in raw water. Table 23 shows the comparison between survival of chlorine exposure and hydrophobicity of *M. intracellulare* strain TMC 1406^T cell types grown in medium. Table 24 shows the comparison between survival of chlorine exposure and hydrophobicity of *M. intracellulare* strain TMC 1406^T cell types grown in raw water. Table 25 shows the correlation between cosine contact angle and survival after Rifampicin and chlorine exposure.

Table 18. Cosine of Contact Angle of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T

Cell type ^a	Cosine Theta	
	<i>M. avium</i>	<i>M. intracellulare</i>
Cells in suspension (m)	0.927	0.840
Cells in suspension (w)	0.883	0.826
Biofilms suspended (m)	0.883	0.716
Biofilm suspended (w)	0.798	0.588
Intact biofilms (m)	0.669	0.569
Intact biofilms (w)	0.602	0.340

^a Cells grown in M7H9 medium signified by (m),

Cells grown in raw water signified by (w)

^b Contact angle data from Table 16 and 17

Table 19. Relationship between hydrophobicity and Rifampicin-susceptibility of *M. avium* strain A5

Cells	Survival of Antibiotics ^a	Hydrophobicity ^b
Suspension grown	3 %	0.927
Biofilms grown suspension	17 %	0.883
Biofilm grown	64 %	0.669

^a Antibiotic data of Rifampicin

^b Hydrophobicity is displayed as cosine of contact angle

Table 20. Relationship between hydrophobicity and Rifampicin-susceptibility of *M. intracellulare* strain TMC 1406^T

Cells	Survival of Antibiotics ^a	Hydrophobicity ^b
Suspension grown	3 %	0.840
Biofilms grown suspension	17 %	0.716
Biofilm grown	64 %	0.569

Table 21. Relationship between hydrophobicity and chlorine susceptibility of *M. avium* grown in medium

Cells	Survival of Chlorine	Hydrophobicity ^a
Suspension grown	19 %	0.927
Biofilms grown suspension	49 %	0.883
Biofilm grown	59 %	0.669

^a Hydrophobicity values are cosine of the contact angle

Table 22. Relationship between hydrophobicity and chlorine susceptibility of *M. avium* grown in raw water

Cells	Survival of Chlorine	Hydrophobicity ^a
Suspension grown	17 %	0.883
Biofilms grown suspension	41 %	0.798
Biofilm grown	60 %	0.602

^a Hydrophobicity values are cosine of the contact angle

Table 23. Relationship between hydrophobicity and chlorine susceptibility of *M. intracellulare* strain TMC 1406^T grown in medium

Cells	Survival of Chlorine	Hydrophobicity ^a
Suspension grown	50 %	0.840
Biofilms grown suspension	57 %	0.716
Biofilm grown	63 %	0.569

^a Hydrophobicity values are cosine of the contact angle

Table 24. Relationship between hydrophobicity and chlorine susceptibility of *M. intracellulare* strain TMC 1406^T grown in raw water

Cells	Survival of Chlorine	Hydrophobicity ^a
Suspension grown	50 %	0.826
Biofilms grown suspension	52 %	0.588
Biofilm grown	72 %	0.340

^a Hydrophobicity values are cosine of the contact angle

Table 25. Correlation between cosine contact angle and survival after rifampicin and chlorine exposure

Combination	Growth medium	Correlation coefficient	
		<i>M. avium</i> strain A5	<i>M. intracellulare</i> strain TMC 1406 ^T
Rifampicin-survival v Cosine	M7H9 medium	0.996	0.970
Chlorine survival v Cosine	M7H9 medium	0.801	0.996
Chlorine survival v. Cosine	Raw water	0.960	0.909

^a Fraction surviving exposure to either Rifampicin or chlorine and cosine contact angle of cells

CHAPTER IV: Discussion

Introduction

All objectives of the study were met. Conditions to reproducibly grow *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T biofilms were developed. Formation of biofilms on surfaces by cells of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T was measured and characterized. Antibiotic and chlorine susceptibility of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T cells grown in biofilms and suspension and exposed in suspension in biofilms were measured. Hydrophobicity of all three cell types of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T cells were measured. Finally, it was shows that there exists a correlation between hydrophobicity and antibiotic and chlorine susceptibility.

Reproducibility of measurements

Comparisons of cell susceptibility were made only after ensuring that cells were grown to similar growth phase because growth phase can affect susceptibility (Scholar and Pratt, 2000). Cells were grown to the same growth stage to ensure that differences in growth stages did not influence the results.

M. avium strain A5 and *M. intracellulare* strain TMC 1406^T were grown in sterile canted neck, polystyrene flasks with vented caps at 37°C. The vented cap prevented oxygen decrease during growth of cultures. Growth was monitored by measuring turbidity and colony forming ability of *M. avium* strain A5 and *M. intracellulare* strain

TMC 1406^T. Measuring growth by turbidity and colony forming units showed an increase each day of sampling (Figure 1-8).

The reproducibility of measurements can be determined by the standard deviations being relatively small. The standard deviation of the mean values for the percent survival of cells exposed to antimicrobials showed that there was no significant difference between duplicate experiments. However, there was a significant difference of percent survival amongst cell types: cells grown and tested in suspension, cells grown in biofilms and tested in suspension, and cells grown and tested in biofilms. Hydrophobicity measurements showed an increase in hydrophobicity from cells grown in suspension to cells grown in biofilms (Table 15-17).

Growth of M. avium and M. intracellulare in suspension and in biofilms

Polystyrene flasks were used to determine whether the mycobacteria strains formed biofilms. The growth rate of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T were measured in both M7H9 medium and raw water in polystyrene flasks. While both *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T formed biofilms, the *M. intracellulare* strain TMC 1406^T appeared to prefer to grow in biofilms on the walls of the flasks (Figures 9 and 10). This agreed with observations that *M. intracellulare* strain TMC 1406^T was recovered in higher numbers more frequently from biofilms in drinking water systems (Falkinham et al., 2001)

M. avium strain A5 and *M. intracellulare* strain TMC 1406^T when grown in polystyrene flask have no lag phase. This means that the cells in the inoculum were in

exponential growth and they were transferred to the same medium, and thus maintained exponential growth.

Because it was difficult to reproducibly recover cells from a specific area of polystyrene, glass beads were used to grow biofilms. Cells grown in biofilms on glass beads permitted measurement of antimicrobial susceptibility of the biofilms (Figures 5-8). To ease the removal of biofilms from glass beads, 0.5% (vol/vol) Tween 80 was used which may have slightly increased the susceptibility of mycobacteria cells to antibiotics and detergents. Removal of the biofilms by vortexing did not reduce colony-forming units (Table 4). Growth was monitored by measuring turbidity and colony-forming ability of the strains and showed similar growth rates of each cell type.

Cells grown in suspension and cells grown in biofilms were plotted on the same figure (Figures 1-4). The left hand axis showed turbidity, cells in suspension, and the right hand axis was colony-forming units, cells on glass beads. The results demonstrated that cells were growing at similar rate in both suspension and biofilms. This indicated that the growth of cells in biofilms was not limited in any way (e.g., oxygen or nutrient availability) in the experimental conditions employed.

Effect of different growth environments on antibiotic susceptibility

The effect of growth environments (suspension versus biofilms) on antibiotic susceptibility was measured on log phase, medium-grown *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T. The cells were grown and exposed in suspension. In addition, cells grown in biofilms were recovered from biofilms and finally cells were exposed in biofilms grown on glass beads. Log phase cultures were used to reduce

effects of differences of growth phase on susceptibility. Mycobacterial suspensions of approximately 10^4 cfu/ml were used because these are reflective of the highest numbers in the blood of infected AIDS patients (Inderlied et al., 1993) and CFU/ml in drinking water (Falkinham et al., 2001). There was rapid killing of cells during the first hour of exposure, but the rate of killing was reduced during the second and third hours (Figures 11-13). This could be due to a mixed population of actively growing to stationary-phase cells, susceptible and resistant (Molin et al., 2000). By extrapolating the survival curve we can see that approximately 2 % of cells grow in suspension and 20% of cells grown in biofilms and suspended can be due to mixed populations.

Cells grown in biofilms were more resistant to antibiotics than cells grown in biofilms and exposed in suspension, and cells grown and exposed in suspension. The data agrees with the idea that greater antibiotics and chlorine resistance found in biofilms (Stewart, 2002; van der Belt et al., 1998). Possibly, biofilms have protective elayers of cells and extracellular compounds prevent exposure to the antimicrobials agents (Stewart, 2002; van der Belt et al., 1998).

The data demonstrated that cells in biofilms were more resistant to antimicrobials and not simply due to the protection afforded by layers of cells. The cells grown in biofilms and tested in suspension, are more resistant to antibiotics and chlorine then cells grown in suspension, therefore biofilms resistance is not only due to the protection of layers.

To determine whether biofilms-grown cells were permanently resistant, *M. avium* strain A5 cells were grown in biofilms recovered from biofilms and grown 1 week in medium and raw water and then exposed to antibiotics or chlorine (Tables 9 and 14).

The cells grown in biofilms and then grown in medium for 1 week regained the susceptibility of cells grown and exposed in suspension. Thus there was a physiological change of cells due to growth in biofilms that could be reversed by growth in suspension. Slow growing strains should be more resistant (Costerton et al., 1999). That observation agreed with published data that of (Costerton et. al., 1999; Stewart, 2002) which showed cells that are detached from biofilms regain sensitivity to antibiotics.

Effect of different growth environments on chlorine susceptibility

As was the case for antibiotic susceptibility of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T, chlorine susceptibility was affected by growth environment. Chlorine susceptibility was highest for cells grown in suspension, lower for cells grown in biofilms and tested in suspension, and even lower for cells grown in biofilms and tested in biofilms. *M. intracellulare* strain TMC 1406^T cultures had a slightly higher resistance to chlorine, less killing of cells, than *M. avium* strain A5.

Table 12 illustrates that *M. avium* strain A5 cells grown in suspension and biofilms had different chlorine susceptibility. The highest percent survival was found in cells grown in biofilms. The differences in medium-grown and water-grown *M. avium* strain A5 were statistically significant. The differences in medium-grown and water-grown *M. intracellulare* strain TMC 1406^T were statistically significant (Table 18). This indicated that growth in biofilms does increase resistance.

The differences in medium-grown and water-grown *M. avium* strain A5 were statistically significant. The differences in medium-grown and water-grown *M. intracellulare* strain TMC 1406^T were not statistically significant.

Comparison of results of hydrophobicity by two different methods

Measurement of hexadecane adherence did not demonstrate difference between the two strains and cells grown under different conditions. This could be due to the influence of acid-base character of the cell surface also influences hydrophobicity. Hydrophobicity reflected by cosine of the contact angle did distinguish between the strains and the cell types. Contact angle have been used to distinguish between cell types of other organisms (van Oss et al., 1975; van Loodsdrecht et al., 1989; van der Mei et al., 1998).

Effect of different growth environments on hydrophobicity

Cells grown in biofilms measured in suspension had a higher contact angle than cells in suspension (Table 18 and 19). Cells grown in biofilms were more hydrophobic than cells grown in suspension (Tables 18 and 19). Further, cells grown in biofilms are more hydrophobic than cells grown in biofilms and then suspended (Tables 18 and 19). Growth in biofilms evidently results in increased cell surface hydrophobicity of *M. avium* and *M. intracellulare*. The increased hydrophobicity of biofilms grown cells could be due to a change in the lipid composition of the cell wall.

The lipid content of the cell affects its hydrophobicity and thus permeability; the greater the lipid content, the greater the hydrophobicity (Brenan and Nikaido, 1995). Mycobacteria cells growing in biofilms would have a higher lipid content (more hydrophobic) and less permeable to hydrophilic compounds like antibiotics and chlorine. Slow growing strains should be more resistant (Costerton et al., 1999). The regulation of

phospholipids and glycolipids was compared (Johnston and Goldfine, 1992). The ratio of lipids was lower when cells were grown on 100% oleic acid instead of 60% oleic acid (Johnston and Goldfine, 1992). This states that lipid ratios can change due to the environment. Also, *Mycobacterium phlei* TMC 1548 when growth medium containing 2% (vol/vol) glycerol was supplement with up to 5% (wt./vol) of glucose resulted in an increase in growth yield of cells as well as total phospholipids. This article also shows that changes in substrate can affect lipid content and thus hydrophobicity.

The hydrophobicity of mycobacteria may be due to lipid composition in the outer membrane. The molecular determinants of hydrophobicity may lie within the outer membrane of the bacteria such that when the lipid content it is altered, hydrophobicity is lost. Cells growing in biofilms may alter the composition of lipids and consequently increase hydrophobicity. When the cells grown in the biofilms were grown in medium or raw water for one week, the resistance was lost. These cells were as susceptible to antibiotics and chlorine as cells that had never grown in biofilms. The resistance to antimicrobials is not permanent, suggesting that possibly lipid content or composition can be altered depending upon the growth in the environment.

Therefore growth within the biofilms provides a higher level of hydrophobicity, thus enabling the cell to remain in the biofilms (Bos et al. 2000). The hydrophobicity data agrees with the idea that water-grown cells are more hydrophobic than medium-grown cells. This indicates that water-grown cells are more hydrophobic than medium-grown cells, which is due to the availability of nutrients in medium. Due to the increased hydrophobicity, treatment regimens in patients should be changed to use more hydrophobic derivatives of current antibiotics. The elevated hydrophobicity of

mycobacteria requires the use of hydrophobic derivatives of the current antibiotics. However, the hydrophobic derivatives of the antibiotics used in this study proved to be ineffective against cells grown in biofilms, therefore a more effective antibiotic is needed.

Relationship between antimicrobial susceptibility and hydrophobicity

There was a linear relationship between hydrophobicity and susceptibility. We were able to connect hydrophobicity and susceptibility by determining whether chlorine susceptibility decreased as hydrophobicity increased (Table 19-24). This means that hydrophobicity, or another factor is a determinant of hydrophobicity is a major determinant of antibiotic susceptibility of *M. avium* strain A5 and *M. intracellulare* strain TMC 1406^T. The significance of this relationship begins to explain biofilms resistance as the effect of a hydrophobic cell type against a less hydrophobic antimicrobial.

Both measurements of hydrophobicity do show similar results in that hydrophobicity increases from cells grown in suspension to cells grown in biofilms (Table 17-18). However, a clearer representation of this change can be seen in contact angle measurements (Table 17-18).

The antibiotic and chlorine susceptibility experiments on a larger scale can begin to address some of the concerns of biofilms in patient tissues and catheters as well as biofilms in water distribution systems. The resistance of *M. avium* and *M. intracellulare* cells in biofilms is due in part to the layers of protection afforded the biofilms as well as the inherent resistance within cells of the biofilms. Treatment for diseases caused by biofilms formation will not be as effective until hydrophobic antibiotics are used in combination with the current treatments. In order to disrupt or prevent biofilms

formation in water distribution systems, hydrophobic treatments must be used as well as chlorine. Both methods, hydrophobic and hydrophilic treatments, are necessary to kill planktonic cells as well as cells within a biofilms.

Susceptibility and hydrophobicity

An interesting result from this study was the high degree of correlation between hydrophobicity, as reflected by contact angle and antibiotic and chlorine susceptibility. This suggests that a major determinant of susceptibility of both *M. avium* and *M. intracellulare* is there cell surface hydrophobicity. Further, it suggests that one way to combat infections in patients and biofilms growth in water distribution systems would be to employ hydrophobic antibiotics and hydrophobic disinfectants.

Summary

Our research allowed for a comparison of cell types in order to understand the effect of biofilm formation. This is the first report of measuring the susceptibility of cells grown in biofilms and tested in suspension. Cells grown in suspension are more susceptible to antimicrobials and less hydrophobic than cells grown in biofilms. We have shown that cells grown in biofilms and exposed in suspension are more resistant to antimicrobials agents than are cells grown in suspension. Because cells grown in biofilms and exposed to antimicrobials agents in biofilms were even more resistant, there are two factors affecting the susceptibility of cells. One factor is the layers of cells and other material in biofilms. The second is due to unknown changes in cells as a consequence of growth in biofilms. Finally, we were able to demonstrate the relationship between

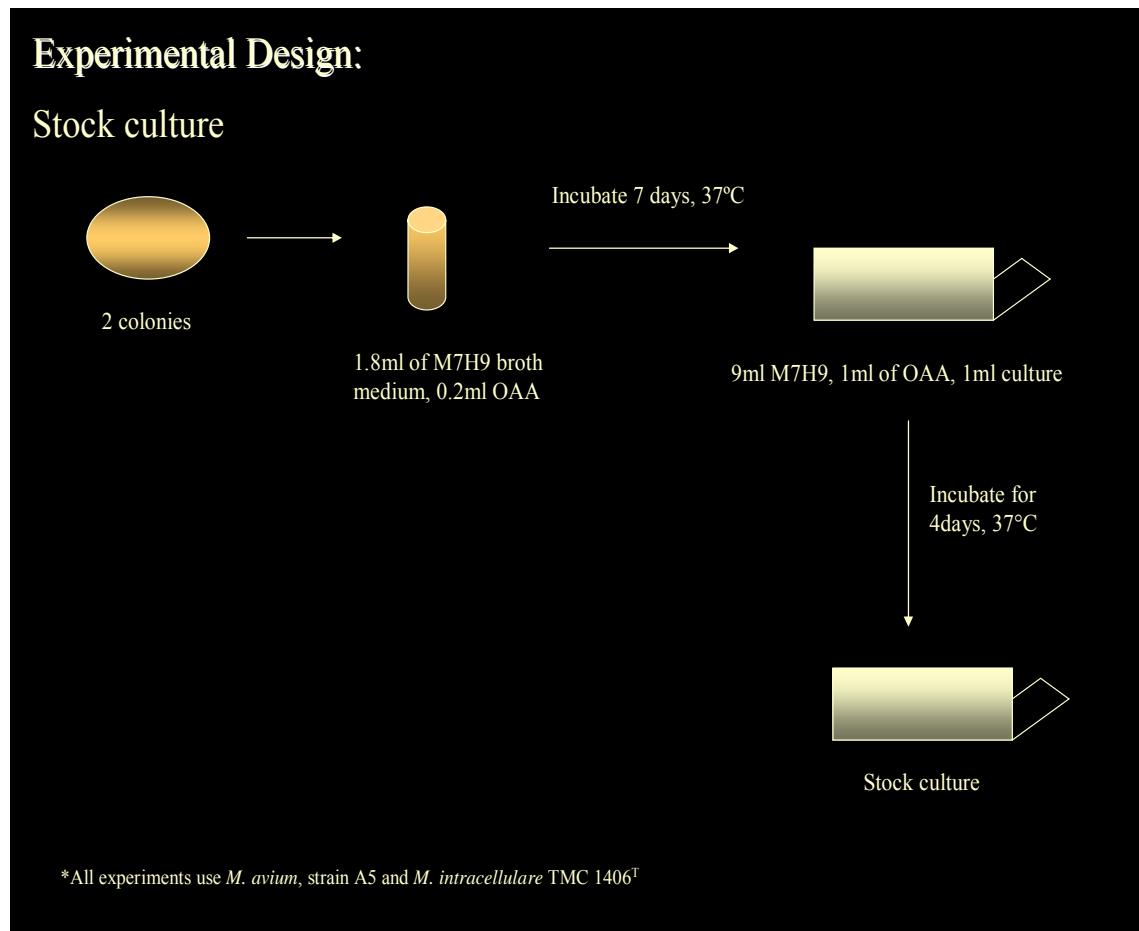
hydrophobicity and susceptibility. Because of the changes in hydrophobicity it is likely that biofilms growth induces a change in lipid composition or content.

The objective of this work was to establish the significance of *M. avium* complex biofilms in terms of antibiotic and chlorine susceptibility. Biofilms are more resistant to antimicrobials and disinfectants than biofilms suspended and cells in suspension, respectively. This resistance is due to changes in cellular metabolism and not layers of protection.

Future experiments could analyze the components and structure of *M. avium* complex biofilms and to determine the specific physiological changes necessary for biofilms formation (i.e. comparing isolated cells grown in suspension and cells grown in biofilms by comparing lipid composition). Another objective would be to identify possible subpopulations in biofilms.

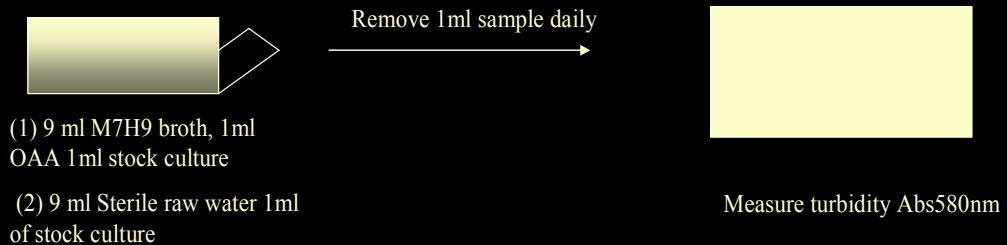
Chapter V: Appendix

Stock culture

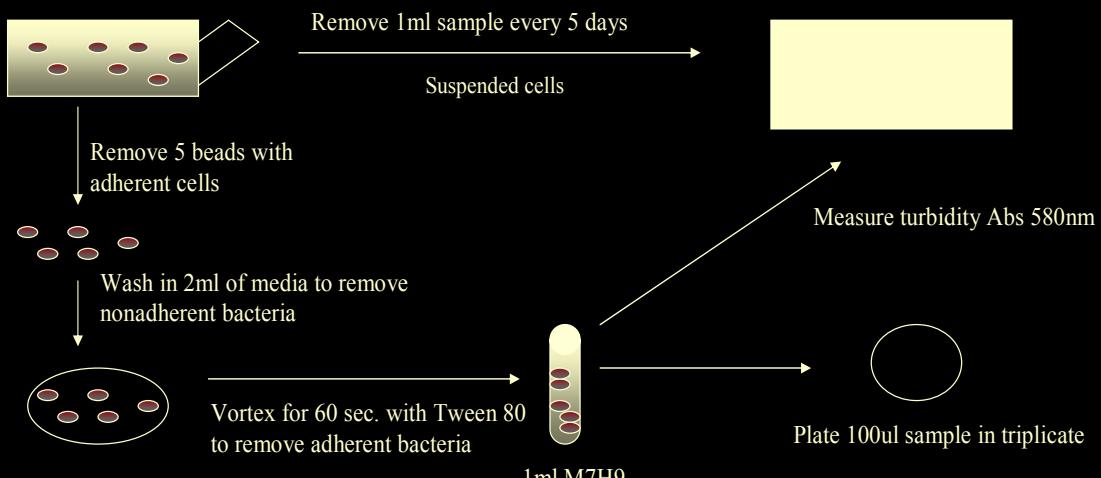


Growth

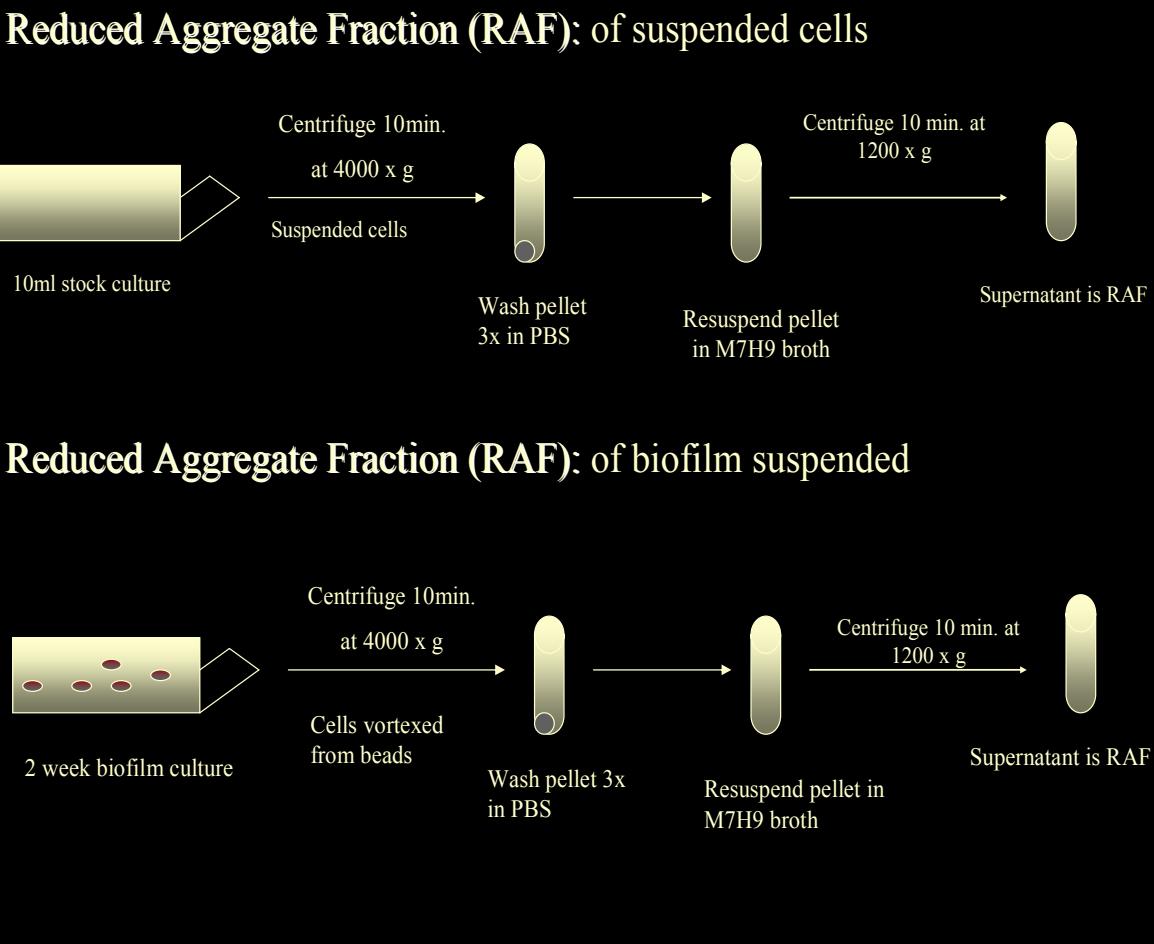
Growth curve (suspended cells):



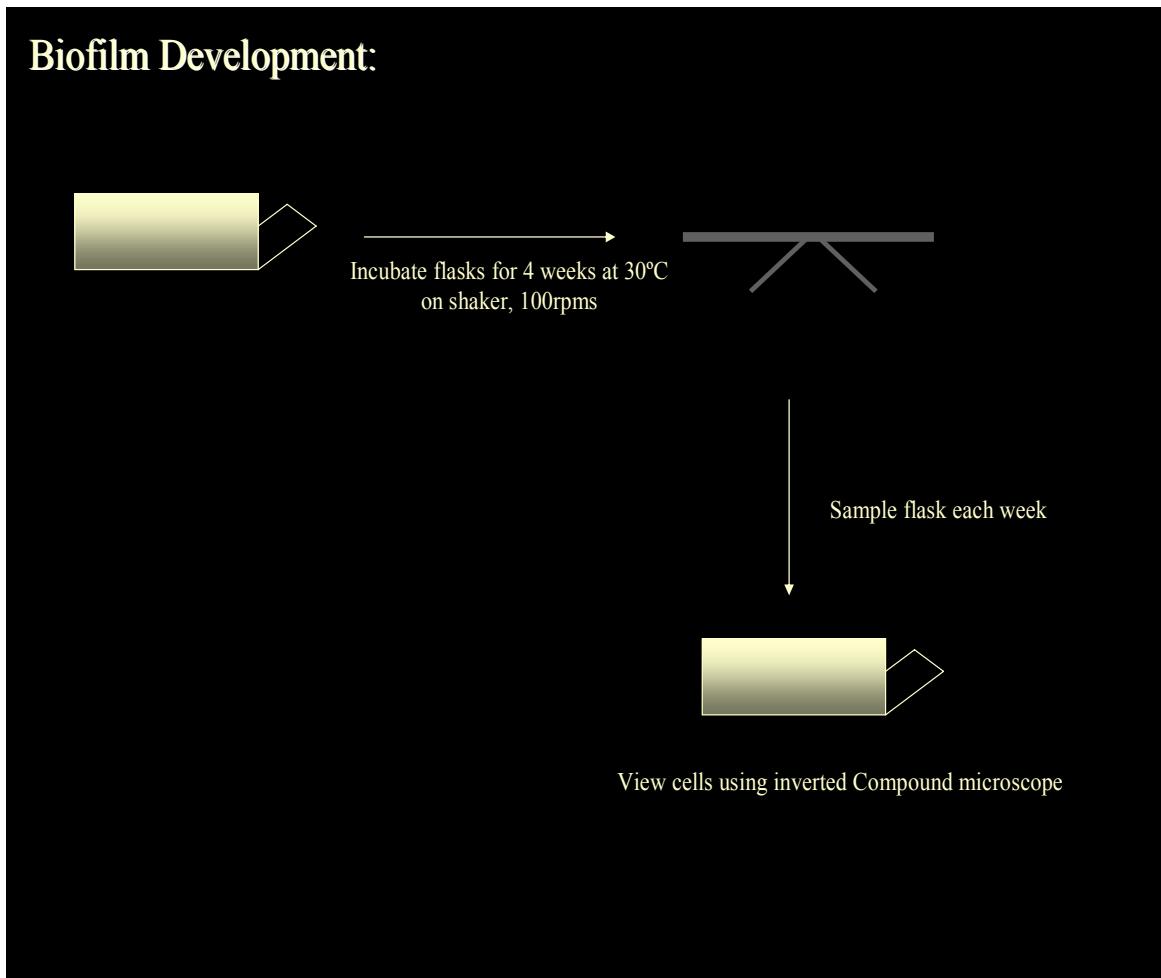
Growth curve with beads (biofilm suspended, biofilms):



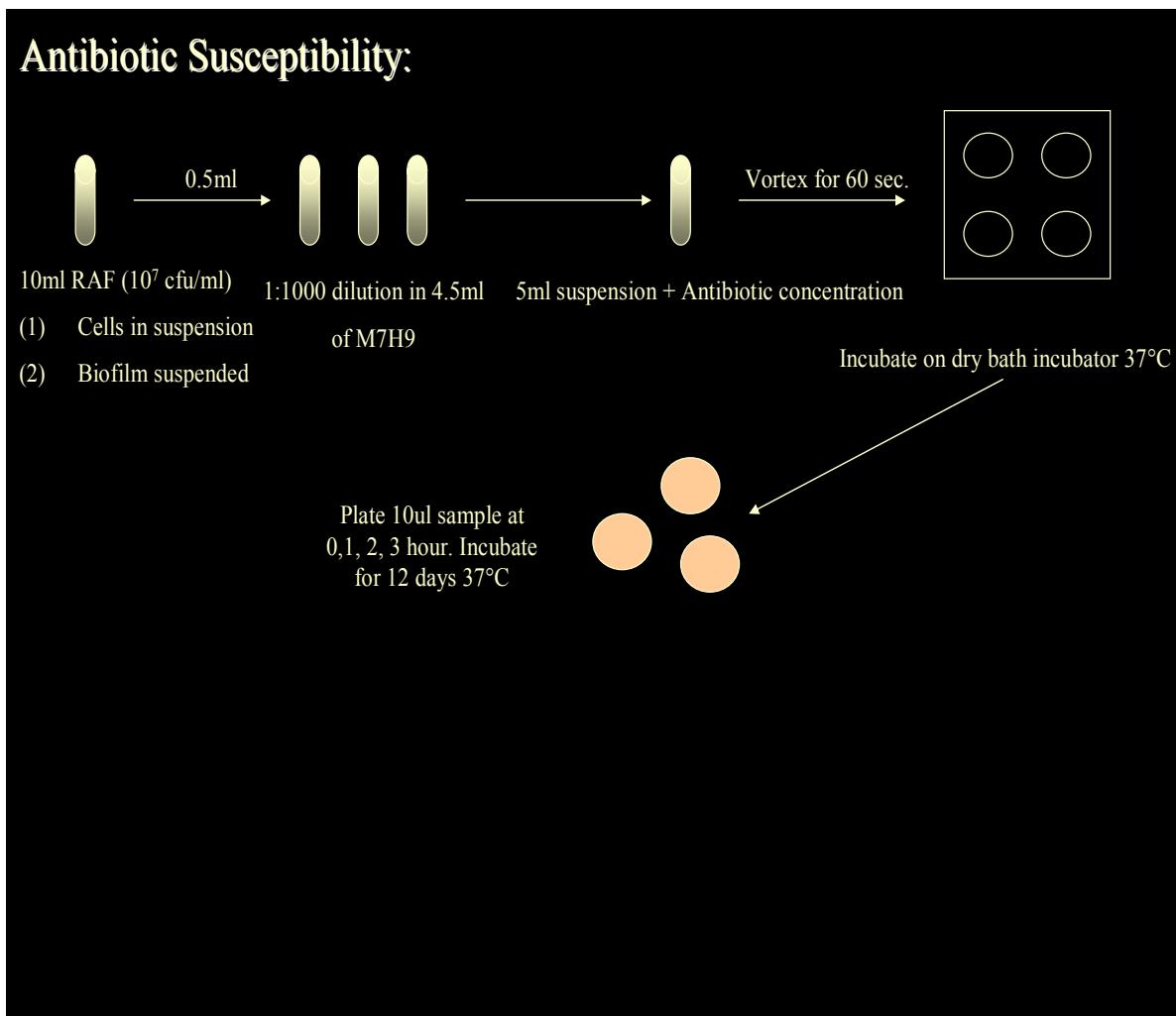
Reduced Aggregate Fraction



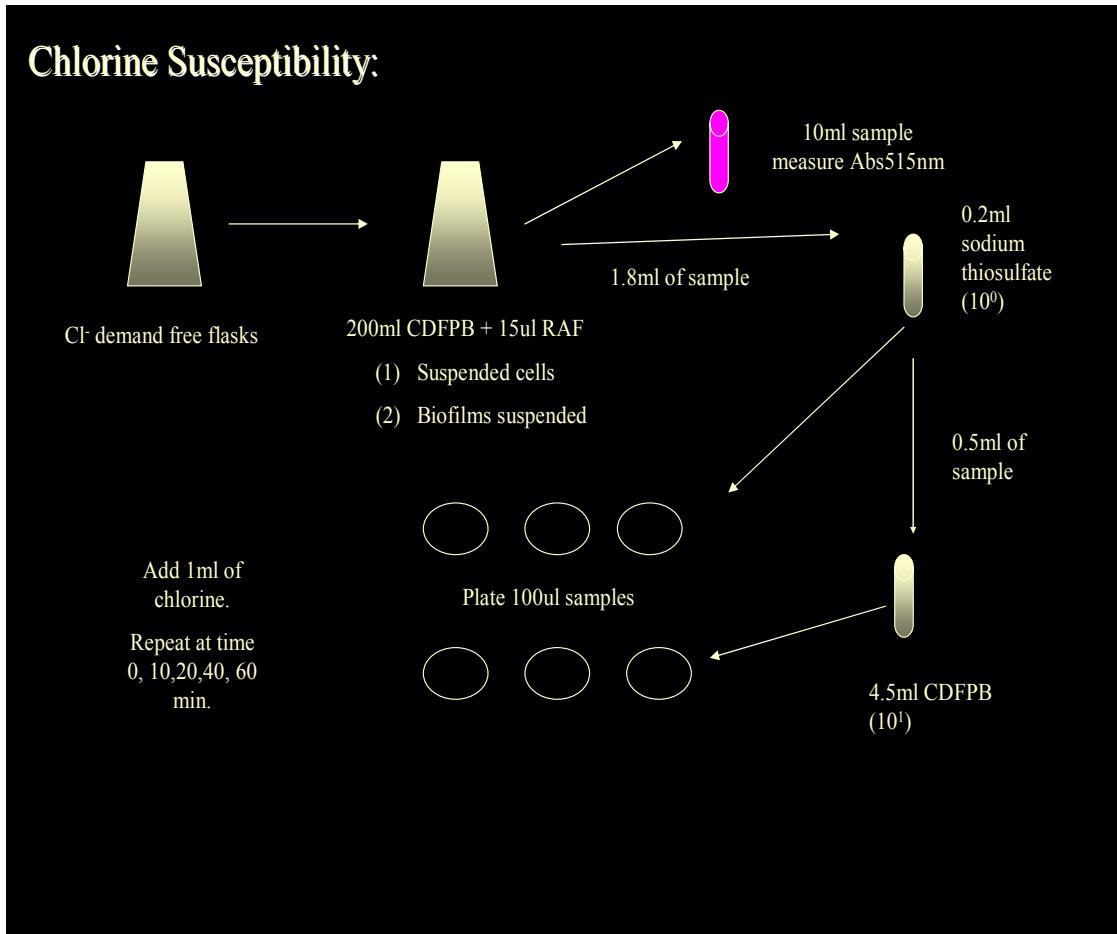
Biofilm Development



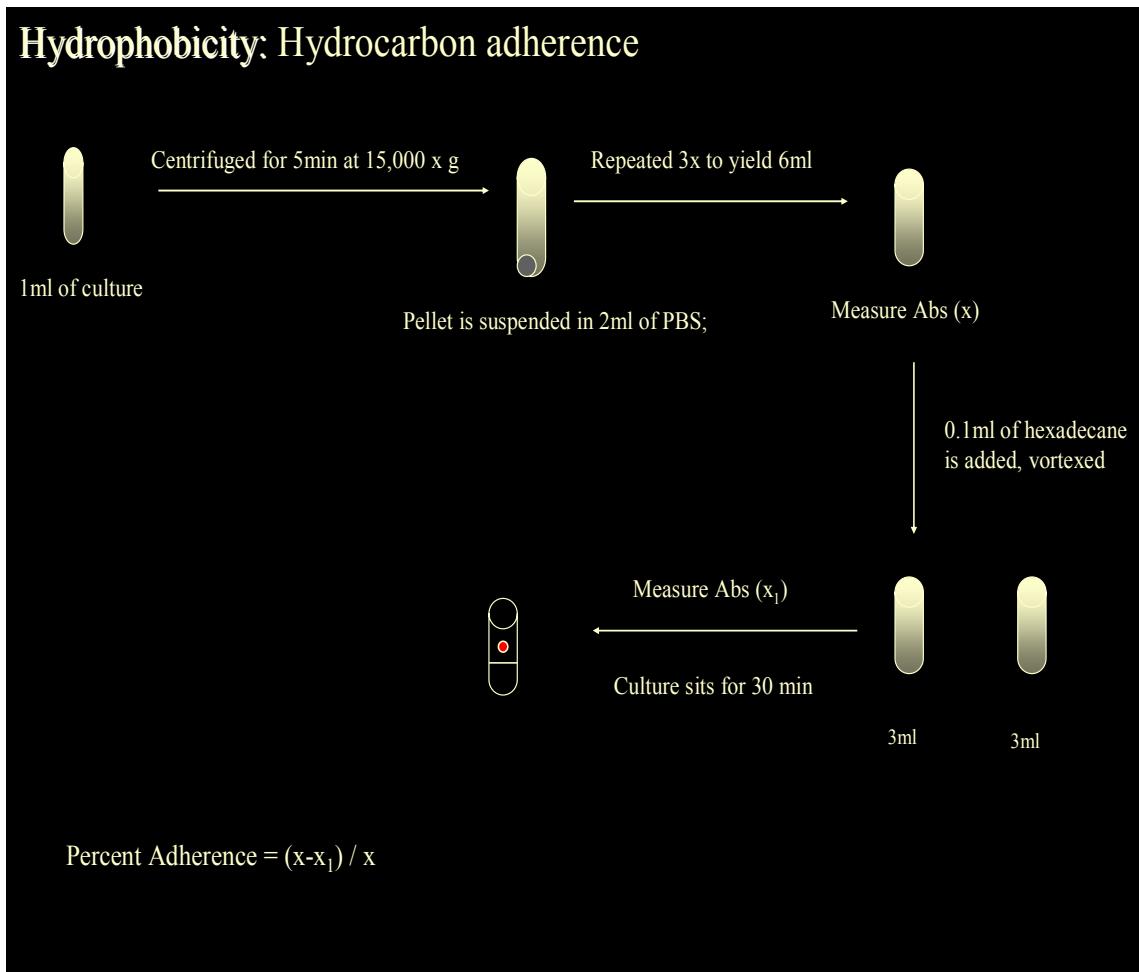
Measure of Antibiotic Susceptibility



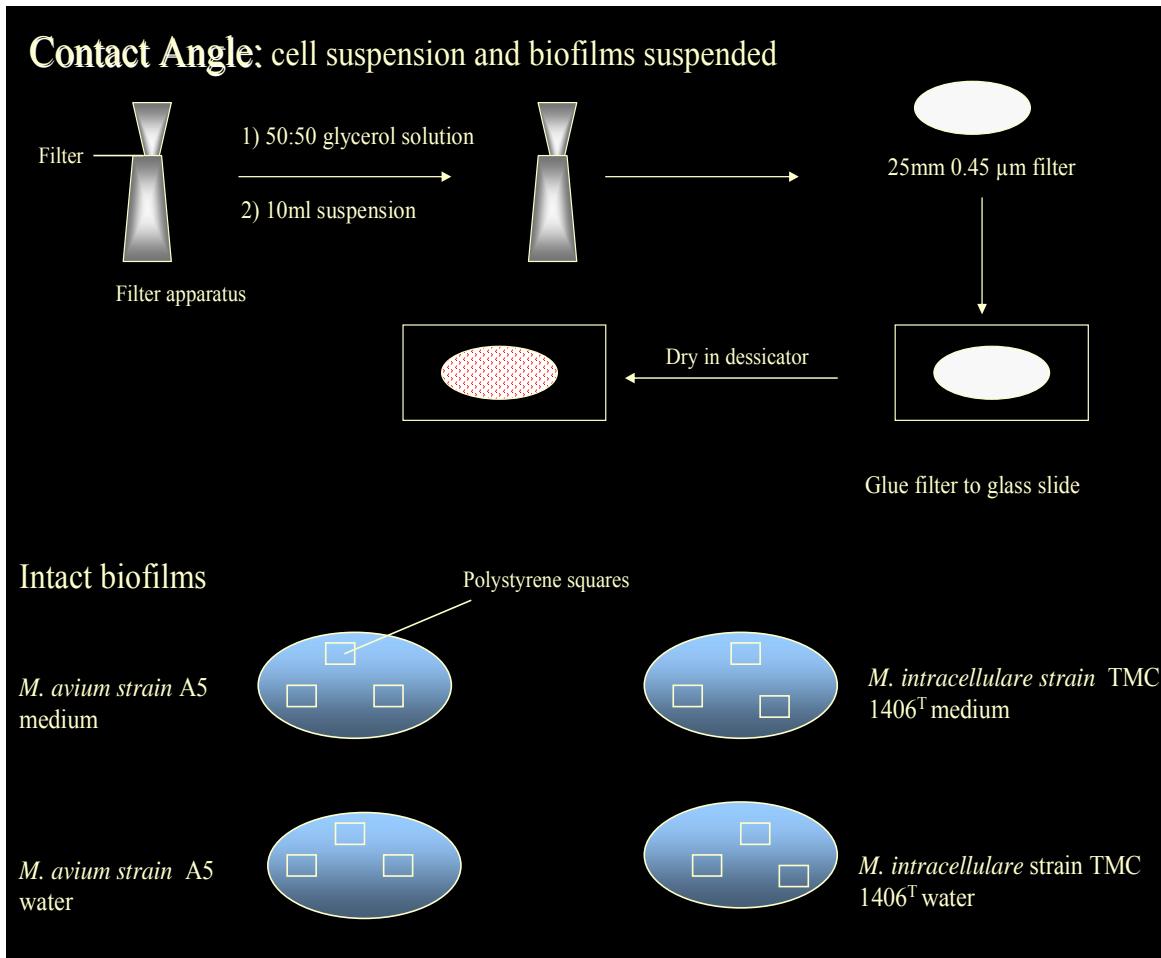
Measure of Chlorine Susceptibility



Hydrophobicity: hydrocarbon adherence

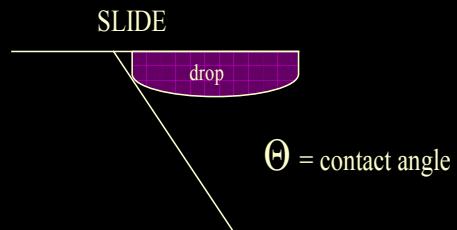


Hydrophobicity: contact angle



Hydrophobicity: goniometer measurements of contact angle

Goniometer measurement:



Thermodynamic equilibrium condition for liquid in contact with a solid surface
Young equation:

$$\gamma_{SV} - \gamma_{SL} = \gamma_{LV} \cos \theta_e$$

CHAPTER V: References

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Cirriculum Vitae
Keesha Ashanna Steed

215 Fralin Biotechnology Center ♦ Department of Microbiology ♦ **Virginia Tech** ♦ Blacksburg,
VA 24060 ♦ k_ashanna@yahoo.com

Education

Master of Science, Microbiology, Department of Microbiology, Virginia Polytechnic Institute & State University, Spring 2003.

Bachelor of Science, Biology, Department of Biology, Virginia Polytechnic Institute & State University, Spring 2000.

Teaching Experience

General Biology Lab Graduate Teaching Assistant, Spring 2002

Presentations

Minorities in Agriculture, Natural Resources, and Related Sciences (MANRRS) national conference. “Effects of growth in biofilms upon antibiotic and chlorine susceptibility of *M. avium* and *M. intracellulare*”. Portland, OR. April 4-6, 2002.

Departmental Seminar. “Bacterial Biofilm Formation.” Virginia Tech. October 31, 2001.

Grants

Minority Academic Opportunities Scholarship Program. 2001-2002.

Graduate Research Development Program (GRDP) grant. (\$300)

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