

**Rapid, Quantitative Assessment of Antimycobacterial Water
Disinfection based on the Firefly Luciferase Reporter Gene**

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ABSTRACT

Mycobacterium avium causes disseminated infection in humans with immunodeficiency, pulmonary infections in individuals with predisposing lung conditions (e.g., pneumoconiosis), and cervical lymphadenitis in children. Twenty-five to fifty percent of late stage AIDS patients are infected with *M. avium*. *M. avium* has been recovered from drinking water and strains from water share identical DNA fingerprints with isolates recovered from patients exposed to the water.

Further, *M. avium* is resistant to chlorine, a disinfectant commonly used in municipal water supplies. Because of the slow growth of *M. avium*, measuring its susceptibility to disinfectants is laborious and reaction to a potential problem is delayed. Thus, there exists a need for a rapid test to measure the antimycobacterial disinfectant capability of chlorine containing water samples. The objective of this research was to develop a rapid and quantitative assay for the viability of mycobacteria using firefly luciferase as a reporter gene for disinfection survival studies.

Derivatives of *M. avium* strains MD1 and A5, *Mycobacterium smegmatis* strain VT307 and *Mycobacterium bovis* BCG strain Pasteur carrying the firefly luciferase gene (pLUC10) were constructed. In pLUC10-carrying strains of *M. avium* strain A5 and *M. smegmatis* strain VT307, a direct correlation was shown between the quantity of light produced and the number of cells recovered as colony forming units. In disinfection studies of both pLUC10-carrying derivatives of *M. avium* strain A5 and *M. smegmatis* strain VT307, survival, as measured in colony forming units, correlated with survival in relative light units. Luciferase measurements appear to offer a method for rapid enumeration of mycobactericidal disinfection capacity of chlorinated water.

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I am indebted to my Father. He has always been there to encourage me to make the right decisions in life. As a little girl learning to ski, he would tell me that if I wasn't falling down, then I wasn't trying hard enough. Thanks for reminding me that the most precious accomplishments in life are hard-won.

To my husband, Jim, I can not say how much I appreciate your love and support. You made me smile and keep my perspective, even when things did not go as I had planned. Thanks for the optimism and encouragement. I love you very much.

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BACKGROUND

Epidemiology:

Mycobacterium avium causes pulmonary disease in individuals with predisposing lung conditions such as pneumoconiosis (Contreras *et al.*, 1988), disseminated infection in immunodeficient individuals caused by HIV infection (Hoover *et al.*, 1995), and cervical lymphadenitis in children (Wolinsky, 1995). Twenty-five to fifty percent of AIDS patients in the United States are infected with nontuberculous mycobacteria, caused primarily by *M. avium* (Horsburgh, 1991). Rather than pulmonary infection, the most common form of *M. avium* infection in AIDS patients is mycobacteremia, in which *M. avium* can be isolated from blood and tissue (Ruf *et al.*, 1989). The lack of evidence linking person-to-person contact with infection, suggests that the source of the infection is environmental (Wolinsky, 1979).

Current research has focused on determining whether individuals may become exposed and infected by *M. avium* through water intended for public consumption. The use of water for bathing and drinking is more likely than natural waters (e.g. lakes and rivers) to result in exposure to *M. avium* complex bacteria (von Reyn *et al.*, 1993). The persistent colonization of a hospital hot water system by an *M. avium* isolate was found to be the only link between three individuals with AIDS who were found to have disseminated infections resulting from *M. avium* (von Reyn *et al.*, 1994). Isolates from that same hospital water system were identical to isolates from the patients as determined by pulsed-field gel electrophoresis of restriction profiles (von Reyn *et al.*, 1994).

Primates with immunodeficiency syndromes are also susceptible to *M. avium* infection. Simian Macaques were infected with SIV, tested at the beginning of the study to verify that they were free of *M. avium*, and housed in a facility in which the monkeys had no contact with each other or the outside environment. The only water to which the macaques were exposed was the piped water in the facility. The piped water yielded isolates of *M. avium* with the same banding pattern by PCR of spaces between conserved inverted repeat sequences as isolates recovered from the infected monkeys (Mansfield

and Lackner, 1997). These studies lend very strong support in favor of a piped water *M. avium* reservoir.

Presence of *M. avium* in Natural Waters

M. avium has been isolated from aquatic environments all over the world. *M. avium* has been found in ponds (Kazda and Brook, 1988), swamps (Kirshner *et al.*, 1992), sphagnum bogs (Kazda *et al.*, 1979), rivers, surface waters, oceans (von Reyn *et al.*, 1993; Falkinham *et al.*, 1984), and aerosols (Wendt *et al.*, 1980). Due to its apparent ubiquitous presence in natural waters, *M. avium* should be considered a natural inhabitant of most aqueous environments. Therefore, *M. avium* is likely to be present in reservoirs for municipal drinking supplies, and, consequently, in water treatment facilities and distribution systems.

***M. avium* in Treated Water Supplies**

M. avium has been recovered from many types of treated water systems. *M. avium* has been recovered from chlorinated municipal water systems (duMoulin and Stottmeier, 1986). *M. avium* has been recovered from hospital water systems all over the world (duMoulin, *et al.*, 1988, von Reyn *et al.*, 1993; von Reyn *et al.*, 1994). Non-tuberculous mycobacteria have been recovered from biofilms in drinking water systems (Shultze-Röbbeck and Fischeder, 1989). And finally, *M. avium* was recovered from public baths (Saito and Tsukamura, 1976).

Physiology of *M. avium*

Understanding the physiology of *M. avium* is important in that it explains the reason for the presence of *M. avium* in both natural and treated water supplies. First, *M. avium* can utilize a wide range of compounds as a nutrient source. Second, *M. avium* will grow in a variety of conditions such as temperature, pH, and salt concentration, as would be observed in an aqueous environment over time. Third, *M. avium* grows slowly due to a number of physical characteristics, and its slow growth may serve as an advantage in aqueous environments for a number of reasons. Last, The pleiomorphic nature of the

organism is likely to enhance adaptation of the organism to changes in its aqueous environment.

***M. avium* Nutrient Sources**

M. avium is an oligotroph, able to grow on very low concentrations of nutrients, and may be cultured in natural waters from a wide geographic area (George *et al.*, 1980). It has long been asserted that mycobacteria have a fatty acid requirement for growth, though mycobacteria may be able to meet this need by scavenging humic and fulvic acids from water (Kirschner *et al.*, 1992).

Range of Tolerance of *M. avium* to Environmental Conditions

M. avium is capable of adapting to different aqueous surroundings due to its ability to grow in a wide spectrum of environments. *M. avium* is able to grow at 17.8° C (George *et al.*, 1980) and as high as 45 ° C (Kirschner *et al.*, 1992; Wayne *et al.*, 1991) enabling it to grow in lakes and rivers as well as hot water systems. Fifty percent of *M. avium* are able to grow on media containing 5% (w/v) NaCl (Wayne *et al.*, 1991) though *M. avium* is not found in ocean waters as frequently as brackish or fresh waters (George *et al.*, 1980). *M. avium* grows on media with a pH as low as 4 and as high as 8 (George and Falkinham, 1985). More MAIS (*M. avium*, *intracellulare*, and *scrofulaceum*) were isolated from water samples with lower pH (Kirschner *et al.*, 1992), and a pH of 5.5 is optimum for growth of *M. avium* (George and Falkinham, 1985). Finally, the number of *M. avium* isolated is positively related to the presence of zinc in the water sample (Kirschner *et al.*, 1992), and this may also influence the ability of *M. avium* to colonize piped water systems since most pipes are galvanized.

Rate of Growth

The slow growth of *M. avium* may be important for two reasons. First, since a rapid rate of growth adversely affects the susceptibility of microorganisms to antimicrobial agents in biofilms (Gilbert *et al.*, 1990; Shigeta *et al.*, 1997), slow growth would likely serve as an advantage to survival in treated waters. Slow growth may allow *M. avium* to produce enzymes and cell wall components that are important for adaptation

to new conditions. Further, the slow rate of growth may allow *M. avium* to exist in areas of low nutrient concentration as discussed.

M. avium grows slowly. The generation times of *M. avium* in rich medium comprised of Middlebrook 7H9 broth prepared as directed, including enrichment additives glycerol and oleic-acid albumin, with 0.5% (w/v) glucose was 15 h. The generation time of *M. avium* was 91 h. in poor medium comprised of Middlebrook 7H9 broth, without the enrichment additives of glycerol and oleic-acid albumin, and only 0.5 % (w/v) Tween 80 as a carbon source (Rastogi and David, 1981). The slow growth rate of *M. avium* has been attributed to the fact that there is only one copy of the 16S-23S operon in the chromosome (Bercovier *et al.*, 1986) probably resulting in fewer ribosomes, and hence, slower protein synthesis. Another possible reason for the slow growth of *M. avium* is the metabolic expense of synthesizing the cell wall which is Gram positive with an outer layer of mycolic acids (Trias *et al.*, 1992 and Liu *et al.*, 1996), a process which uses an enormous amount of ATP.

***M. avium* Morphology**

M. avium is a pleiomorphic species with varied cellular morphologies, from short rods to filaments (McCarthy and Ashbaugh, 1981). The different cellular morphologies are growth dependent, and the different growth stages displaying these morphologies have different properties such as transformation efficiency (Beggs *et al.*, 1996) and H₂O₂ sensitivity (Laochumroonvorapong *et al.*, 1997). These properties may be representative of adaptive mechanisms.

M. avium interchangeably forms a variety of colonies which vary from transparent to opaque and rough to smooth (McCarthy, 1981) which indicates that there are different cell wall properties as well. It is well known that the transparent colony type, but not the opaque colony type, is virulent (Schaefer *et al.*, 1965) That difference may represent different adhesion properties of the cell wall. Transparent, as opposed to opaque, colonies are more fastidious in their nutrition requirements (McCarthy, 1981), possibly indicating different porin properties on the surface of the cell wall. Pigmented colonial variants, as opposed to unpigmented colonial variants, are more sensitive to antimicrobial agents (Stormer and Falkinham, 1989), possibly indicating a permeability difference in the cell

wall. Since the colony types are inter-convertible, the differing colony types are an adaptive mechanism. Since aqueous environments are always in a state of flux due to rainfall, drought, and seasonal changes, these adaptive mechanisms probably play an important role in adaptation of *M. avium* to these types of changes as well as explain its prevalence in a variety of locations.

Susceptibility of *M. avium* to Chlorine

Chlorination of water supplies is commonly used for disinfection in the United States (White, 1993). In order to infect people through drinking water, *M. avium* must survive the disinfection process and be released into the water distribution system. The organism most commonly used in measuring the disinfection potential of chlorine as a disinfectant is *Escherichia coli*. That use is based on the assumption that the susceptibility of *E. coli* is indicative of the susceptibility of pathogens in the water supply (Mir *et al.*, 1997). *E. coli* would be a good indicator for *Salmonella*, *Shigella*, and *Campylobacter* disinfection, but it is not a good indicator if disinfection of mycobacteria because mycobacteria are highly resistant to chlorine.

Engelbrecht *et al.* (1977) demonstrated that *Mycobacterium phlei* and *Mycobacterium fortuitum* were 10 to 100 fold more resistant to chlorination than enteric bacteria. *M. marinum* was isolated from swimming pools containing chlorine at concentrations ranging from 0.65 and 0.75 mg chlorine/ L, and observed that the isolates would survive for more than 30 minutes at 1.5 mg chlorine/L (Park *et al.*, 1976). Most importantly, *M. avium* is 10,000-fold more resistant to chlorination than *E. coli*, (Taylor, 1998). This resistance to disinfection by chlorine is alarming considering the possibility that drinking water may be a source of infection (vonReyn *et al.*, 1994) and the prevalence of *M. avium* in drinking water systems (du Moulin and Stottmeyer 1986).

Mechanism of Chlorine Disinfection

The mechanisms of bacterial inactivation by chlorine are not fully understood (Russel *et al.*, 1997). Hypochlorous acid, the predominant form of chlorine in water, is a strong electrophile and will oxidize biological molecules, especially those containing thiols, thioesters, and conjugated double bonds (Chesney *et al.*, 1996). Surface proteins,

like porins, likely contain SH bonds, and the mycolic acids contain double bonds which provide a potential target for oxidation. Hypochlorite biocides halogenate amino groups within proteins and inhibit bacterial DNA synthesis (Russell, *et al.*, 1997), another possible means of inactivation of the cell. There is no evidence of a single, specific method of bacterial inactivation by chlorine.

Mechanism of *M. avium* Resistance to Chlorine

There is no specific data on the mechanism of *M. avium* resistance to chlorine, however there are reports that suggest potential mechanisms. LeChevallier *et al.*, (1988) suggested that encapsulation and aggregation (as a biofilm) of *Klebsiella pneumoniae* may have contributed to chlorine resistance by shielding the bacterial cell from exposure to chlorine. The encapsulation of *K. pneumoniae* by a polysaccharide capsule may have acted to shield the cell in much the same way as the lipid outer layer of mycobacteria. Mycobacteria have been observed in large proportions within aquatic habitats as biofilms, and within these biofilms they constituted a considerable proportion of the total bacterial population (Schulze-Röbbecke and Fischeder, 1989). The aggregation of mycobacteria on particles or to one another may have resulted in enhanced chlorine resistance by shielding inner cells from chlorine exposure.

The hydrophobic cell wall may prevent chlorine from contacting the more valuable and sensitive cellular structures mentioned previously. It has been proposed that the hydrophobic, lipid cell wall of mycobacteria confers greater resistance to antibiotics and to chlorine than that of the Gram negative structure of enteric bacteria (Trias *et al.* 1992; Engelbrecht *et al.*, 1977.) The permeability of the mycobacterial cell wall, as exemplified by *M. chelonae*, to cephaloridine, cephacetrile and nitrocefin was 1,000 times lower than that of *E. coli* and 10 times lower than that of the very impermeable *Pseudomonas aeruginosa* (Jarlier and Nikaido, 1990). This suggests that the resistance of mycobacteria to biocides may be due to the impermeability of the cell wall.

Rapid Assays for Mycobacteria

There is a need for rapid assessment of antimycobacterial disinfection capacity for use in monitoring drinking water systems and in monitoring contained water systems like

hospitals. Enumeration of mycobacteria is a time consuming process in studies of water disinfection because pathogenic mycobacteria grow very slowly, and current enumeration practices involve dilution and plating of samples, which may involve 7-10 days of incubation. Great benefit can be achieved by using the luciferase gene as an indicator of surviving cells in studies of mycobacterial susceptibility to chlorine. The firefly luciferase reporter gene is time-efficient, since results to chlorination treatments can be obtained at the time of treatment. The development of a luciferase assay for water treatment facilities is also useful in its sensitivity since luciferase activity is readily measured from as few as 500 to 5000 mycobacterial cells in a given sample (Jacobs *et al.*, 1993), allowing sufficient cell concentration range in which to measure mycobacterial inactivation.

The firefly luciferase gene is the best choice for studies of the susceptibility of mycobacteria to chlorine when compared to bacterial luciferase, green fluorescent protein, and β -galactosidase. Firefly luciferase is superior to bacterial luciferase because *Vibrio harveyi* luciferase may lead to oxidative stress in mycobacteria as it has been shown to do in *Escherichia coli* (Gonzales-Flecha and Demple, 1994), thereby artificially lowering susceptibility data by stressing the host. Firefly luciferase is more useful in viability studies because it is the only enzyme of those mentioned that uses ATP as a substrate, providing the potential for ATP measurements. The luciferase gene is also more useful in measuring cell inactivation compared to β -galactosidase and green fluorescent protein because of the sensitivity of luciferase to stress. Comparing the protein stability as a factor of heat denaturation, the green fluorescent protein was stable at up to 90° C for thirty minutes (Vang *et al.*, 1996), β -galactosidase was stable at 55° C for 500 hours (Hirata *et al.*, 1985), and the luciferase enzyme had a half life of ten minutes at 50° C (Kajiyama *et al.*, 1994). Thus the firefly luciferase is the most sensitive to denaturation of all of the enzymes studied. Possibly, the firefly luciferase is less likely to contribute to light output after the inactivation of the cell because luciferase is more likely to be inactivated when exposed to the disinfection agent than the more stress-tolerant enzymes.

Luciferase has been used as a reporter gene to measure cell viability based on light emitted in studies of susceptibility of mycobacteria to antimicrobial agents. A

number of researchers used inhibition of growth, hence a lack of luminescence, as an indicator of antimycobacterial activity (Jacobs *et al.*, 1993; Cooksey *et al.*, 1993; Arain *et al.*, 1996; Shawar *et al.*, 1997). Inhibition of growth was measured weeks faster using the luciferase method than the standard dilution and plating (Arain *et al.*, 1995). The luciferase systems were also useful in screening of novel compounds for antimycobacterial activity since smaller quantities of these compounds were required (Shawar *et al.*, 1997; Arain *et al.*, 1996). Other researchers have used *Vibrio harveyi* luciferase genes, *luxA* and *luxB*, as a measure of bactericidal activity of hibitane, phenol, and gluteraldehyde by observing the decrease in light emitted (Andrew and Roberts, 1993). Firefly luciferase has also been employed *in vivo* for the evaluation of antimicrobial activity of isoniazid, ethambutol, ansamycin, rifampin, and amakacin against recombinant *M. bovis* BCG in mice with great advantages in time, labor and expense (Hickey *et al.*, 1996).

HYPOTHESES

I propose to test the following hypotheses:

- ❖ The pLUC10 derivatives of mycobacteria will have stable expression of the *luc* reporter gene.

- ❖ The light output from the lysate of the pLUC10 derivatives will be proportional to the number of cells used to create the lysate.

- ❖ The light output from the lysate of pLUC10 derivatives in a disinfection assay (i.e., under stress) will be proportional to the number of cells used to create the lysate.

- ❖ Mycobacterial strains containing the firefly luciferase gene, *luc*, can be used to rapidly measure mycobacterial disinfection capacity.

OBJECTIVES

1. Construct pLUC10-carrying derivatives of *M. smegmatis* strain VT307, *M. avium* strains MD1 and A5, and *M. bovis* BCG strain Pasteur expressing the *luc* reporter gene using a previously constructed *E.coli*-mycobacteria shuttle-plasmid.
2. Prepare single-cell suspensions from *M. smegmatis* strain VT307 and *M. avium* strain A5.
3. Develop a method for the release of luciferase by mycobacterial cells that promotes both lysis of the cell and maximum enzyme function.
4. Develop a method for measuring the luciferase activity of mycobacterial lysates
5. Measure the effect of different concentrations of luminescence reagents on the light-producing reaction.
6. Determine the optimum growth stage for lysis of *M. avium* strain A5/pLUC10
7. Determine the range of cell numbers that may be measured using the luminometric assay in a manner such that light output is proportional to colony-forming units.
8. Measure the concentration of chlorine over time in suspensions of mycobacterial cells to determine optimum cell concentration for chlorine disinfection assays.
9. Rapidly measure antimycobacterial disinfection capacity of chlorine using the firefly luciferase reporter gene in *M. smegmatis* strain VT307/pLUC10 and *M. avium* strain A5/pLUC10 and examine the relationship between light produced and colony-forming units.
10. Investigate potential induction of chlorine resistance using both traditional enumeration and luminometric estimation of cell numbers.

MATERIALS AND METHODS

Bacterial Strains:

Mycobacterium smegmatis strain VT307 is an electrocompetent derivative of *M. smegmatis* strain mc²155, which was isolated following the procedure described by (Jacobs *et al.*, 1991).

Mycobacterium avium strain A5 is a plasmid-free, electrocompetent isolate from an AIDS patient (Hellyer *et al.*, 1991) generously provided by Dr. M. Beggs of the Medical Research Service, McClellan Veterans Hospital, Little Rock, Arkansas.

Mycobacterium avium strain MD1 is an isolate recovered from an immunocompetent patient with a pulmonary infection (Fry *et al.*, 1986). It was found to be electrocompetent by standard laboratory procedures (Wards and Collins, 1996).

Mycobacterium bovis BCG strain Pasteur was obtained from Dr. Laura E. Via.

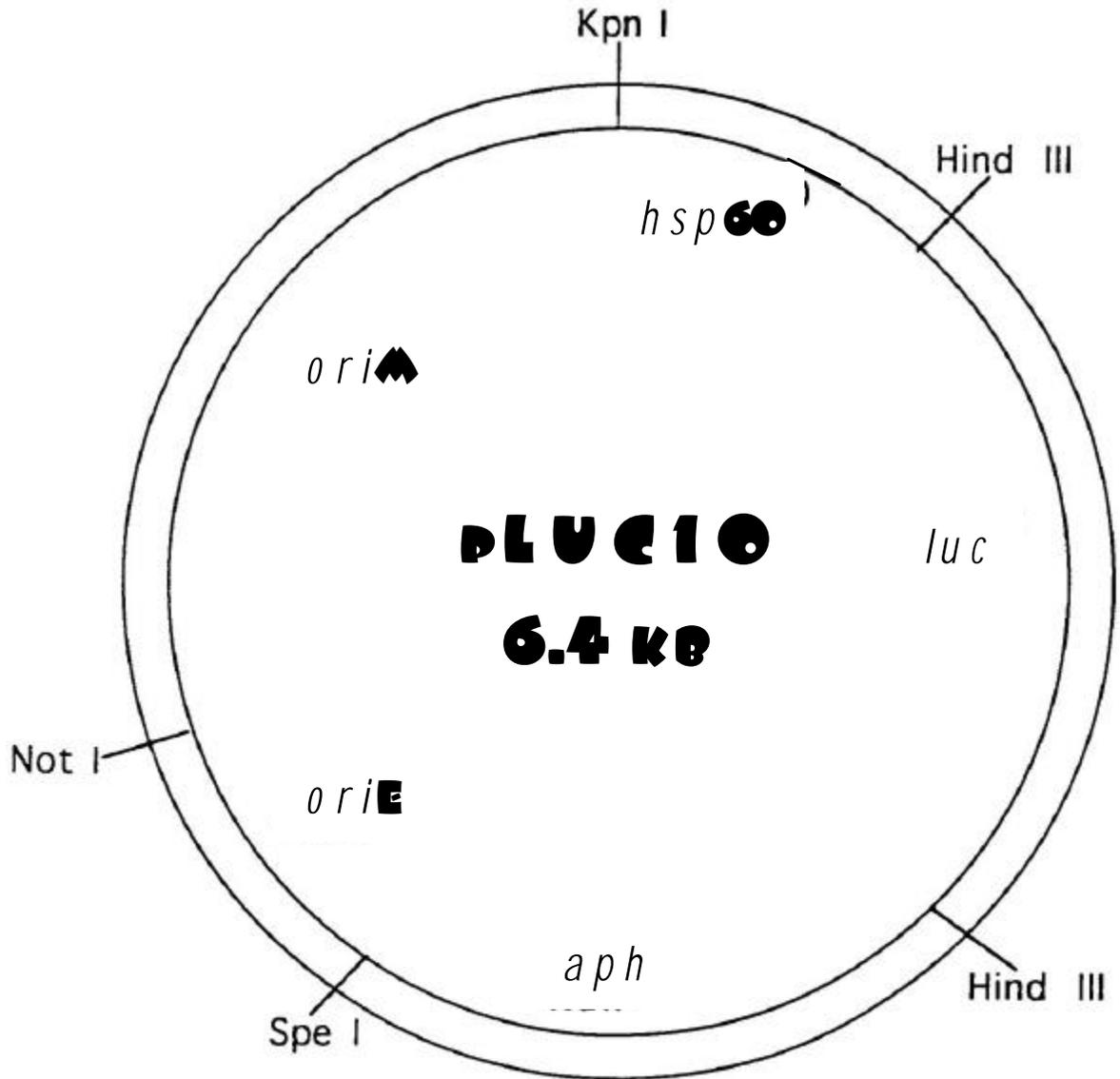
Plasmid:

Plasmid pLUC10 contains the *luc* firefly luciferase gene under the control of the mycobacterial *hsp60* promoter, the mycobacterial *oriM* pAL5000 origin of replication, the *oriE* ColE1 origin of replication for *Escherichia coli*, and the *aph* gene for aminoglycoside phosphotransferase that confers kanamycin resistance (Cooksey *et al.*, 1993). The cloned *hsp60* promoter is expressed constitutively (Stover *et al.*, 1991). The plasmid was generously supplied by Dr. R. C. Cooksey of the Centers for Disease Control, Atlanta, Georgia in *Escherichia coli* DH5 α . Please see the following page for the Map of pLUC10.

Plasmid DNA was isolated from *E. coli* strain DH5 α by an alkaline-lysis method. (Ausbel *et al.*, 1995).

Bacterial Growth and Maintenance:

Mycobacterium smegmatis strain VT307 was grown in 50 ml Nutrient Broth (Difco, Detroit, MI) containing 0.5% (w/v) NaCl and 0.5% (v/v) Tween 80 (designated NBT broth) with aeration in a Reciprocal Shaking Bath Model 25 (Precision Scientific, Chicago, IL) at 80 reciprocations per minute in a 500 ml Erlenmeyer flask at 37° C. Colonies were isolated or enumerated on agar medium containing Nutrient Broth (Difco,



Map of pLUC10: Mycobacterial vector pLUC10 carrying the *photinus pyralis* luciferase gene (*luc*) under control of the mycobacterial *hsp60* promoter. Expression of *luc* in mycobacteria is constitutive. The *aph* gene encodes aminoglycoside phosphotransferase conferring kanamycin resistance. *OriM* and *oriE* are the plasmid origins of replication recognized by *Mycobacterium spp* and *Escherichia coli*, respectively.

Detroit, MI) containing 0.5% (w/v) NaCl, 0.5% (v/v) Tween 80, and 1.5% (w/v) agar (designated NBT agar) and incubated at 37° C for 48 hours. Kanamycin was added to a final concentration of 25µg/ml as needed for selection and growth of recombinant strains carrying pLUC10.

Mycobacterium avium strains were grown in 50 ml Middlebrook M7H9 (Difco, Detroit, MI) broth medium containing 0.5% (v/v) glycerol, and 10% (v/v) oleic acid albumin (OAA) enrichment (Pethel and Falkinham, 1988) (designated MGO broth) with aeration (shaking at 80 reciprocations per minute) in a 500ml Erlenmeyer flask at 37° C. Colonies were isolated or enumerated on Middlebrook M7H10 (Difco, Detroit, MI) agar medium containing 0.5% (v/v) glycerol and 10% (v/v) OAA enrichment (designated MGO agar) and incubated at 37° C for 7-10 days. Kanamycin was added to a final concentration of 50 µg/ml as needed for selection and growth of recombinant strains carrying pLUC10.

Mycobacterium bovis BCG strain Pasteur was grown in Dubos Broth Base containing 0.5% (v/v) glycerol and 10% (v/v) OAA (Pethel and Falkinham, 1980) (designated DBB broth) in 15mm x 15 cm screw-capped tubes on a TC-6 Rollerdrum (New Brunswick Scientific Co., Edison, NJ) at 37° C. Cultures were transferred by increasing the volume of broth media three-fold every five days. Larger cultures were grown in a volume of 50 ml in a 500 ml Erlenmeyer flask in a Reciprocal Shaking Bath Model 25 (Precision Scientific, Chicago, Illinois) at 80 reciprocations per minute. *M. bovis* BCG colonies were isolated or enumerated on Dubos Broth Base (Difco, Detroit MI) agar medium consisting of Dubos Broth Base containing 0.5% (v/v) glycerol, 10% (v/v) OAA (Pethel and Falkinham, 1980), and 1.5% wt/vol agar (designated DBB agar). Fifteen µg kanamycin/ml was added for selection and growth of recombinant strains carrying pLUC10.

Construction of *M. smegmatis* strain VT307/ pLUC10

M. smegmatis strain VT307 was grown in 50 ml NBT broth as described to OD₆₀₀=0.500. The culture in the flask was placed on ice for 30 minutes, cells were then pelleted by centrifugation (14,470 x g for 10 min. at 4°C) and washed with an equal

volume of ice-cold 10 % (v/v) glycerol three times. The pellet was suspended in 0.5 ml 10% (v/v) glycerol. Fifty μ l of cell pellet were mixed with 1 μ g plasmid DNA, transferred to an ice-cold 2 mm electroporation cuvette (Bio-Rad, Hercules, CA.), and subjected to electroporation at 2.5 kV and 25 μ F at 200 Ω on the Gene PulserTM (Bio-Rad, Hercules, CA). Cells were incubated under non-selection conditions to allow for phenotypic lag by suspension in 950 μ l NBT broth medium lacking kanamycin for 2 hours at 37°C. After incubation, 10 and 100 μ l samples were spread on the surface of NBT agar medium containing 25 μ g kanamycin/ml and incubated at 37° C for 48 hours.

Transformant colonies were streaked on NBT plates containing 25 μ g kanamycin/ml. Isolated colonies were selected and grown in 5 ml NBT broth containing 25 μ g kanamycin/ml on a TC-6 Rollerdrum (New Brunswick Scientific Co., Edison, NJ) at 37° for 48 hours. After incubation, cultures were diluted to an absorbance₅₈₀ of 0.10. One hundred μ l of the culture was combined with 100 μ l Luc-LiteTM substrate/lysis solution (Packard Instruments, Meriden, CT), sonicated 5 min in a Bransonic 12 (Branson Co., Sheldon, CT.) sonic cleaner, and read in a PAL-Luminometer (Gen Probe, San Diego, CA.). The strain selected had the greatest light output as measured on the luminometer. A stock culture was grown as described in NBT broth with 25 μ g kanamycin, aliquoted into 2 ml cryotubes, and stored at -70° C.

Construction of *M. avium* strains A5/pLUC10 and MD1/pLUC10

Cultures were grown as described to OD₆₀₀= 0.200. The culture in the flask was placed on ice for 1 hour, cells were then pelleted by centrifugation (14,470 x g for 10 min. at 4°C) and washed in ice-cold 10 % (v/v) glycerol three times. The pellet was suspended in 0.5 ml ice-cold 10% (v/v) glycerol. Fifty μ l of cell pellet were mixed with 1 μ g plasmid DNA, transferred to a 2 mm-electroporation cuvette (Bio-Rad, Hercules, CA.) and incubated at 37° for 10 minutes. Cells were then subjected to electroporation at 2.5 kV and 25 μ F at 200 Ω on the Gene PulserTM (Bio-Rad, Hercules, CA.) Cells were incubated under non-selection conditions by adding in 950 μ l MGO broth medium lacking kanamycin for 2 hours at 37° to allow for phenotypic lag. After incubation, 10

and 100 μ l samples were plated on MGO plates containing 50 μ g kanamycin/ml incubated at 37° C for 7-10 days.

Strain A5 Km^R colonies and strain MD1 Km^R colonies were streaked. Isolated colonies were inoculated into 1 ml MGO broth culture containing 50 μ g kanamycin/ml and the suspensions were placed on the rollerdrum at 37° C for seven days. All cultures were then diluted ten-fold by the addition of fresh MGO medium and allowed to grow for an additional 4 days. Cells in 1 ml of culture were pelleted at 14,000 x g for 10 min. at room temperature and suspended in 100 μ l 0.1 M potassium phosphate buffer (pH 7). The suspension was mixed with 100 μ l Luc-Lite™ substrate/lysing solution (Packard Inst., Meriden, CT), frozen at -70°C, and thawed to room temperature in a water bath. Light output was then measured on a luminometer (Analytical Bioluminescence Laboratories, San Diego, CA.) One of the luminescent cultures was selected for experimentation based on the greatest amount of light exhibited. A stock culture was grown as described, aliquoted into 2 ml cryotubes and stored at -70°C.

Construction of *M. bovis* strain BCG /pLUC10

A 50 ml culture was grown as described to OD₆₀₀= 0.600. Cells were pelleted by centrifugation (14,470 x g for 10 min. at 4°C) and washed in ice-cold 10 % (v/v) glycerol three times. The pellet was resuspended in 1.0 ml ice-cold 10% (v/v) glycerol. Sixty μ g of cell pellet were mixed with 1 μ g plasmid DNA, transferred to a 2mm cuvette (Bio-Rad, Hercules, CA.) and incubated at 37° for 10 minutes. Cells were then subjected to electroporation at 2.5 kV and 25 μ F at 200 Ω on the Gene Pulser™ (Bio-Rad, Hercules, CA.) Cells were incubated under non-selection conditions to allow for phenotypic lag by adding 950 μ l DBB broth medium lacking kanamycin for 16 hours at 37° C. After incubation, 10 and 100 μ l samples were spread on the surface of DBB agar medium containing 15 μ g kanamycin/ml and incubated 32 days.

Km^R colonies were streaked on DBB plates containing 50 μ g kanamycin/ml and incubated for 32 days. One colony was selected from each plate and placed in a luminescent tube with 100 μ l Chlorine Demand-Free Phosphate Buffer (CDFPB) (Celsceri *et al.*, 1989), capped, and sonicated 5 min to break up the colonies. 100 μ l of

substrate/lysing solution was added and tubes were recapped, placed at -70° , and then thawed at room temperature. Isolated colonies were picked and inoculated into 1 ml DBB broth containing $50\mu\text{g}$ kanamycin/ml and incubated on the rollerdrum at 37° for seven days. Cultures were diluted 1:10 with fresh media and incubated for 34 days. Eight ml of culture were diluted into 32 ml fresh DBB broth media and grown in a sidearm flask as described. These were tested in the same fashion as the *M. avium*, a stock culture was grown from one culture as described, aliquoted into 2 ml cryotubes and stored at -70° .

Preparation of a Reduced-Aggregate Fraction (RAF) from Cultures of *M. smegmatis* Strain VT307/pLUC10:

In order to measure the disinfection of an organism that grows normally in aggregates, a baseline should be established for the disinfection of single cells. To create a suspension of predominantly single cells, *Mycobacterium smegmatis* strain VT307/pLUC10 was grown in broth as described. After washing twice, cells were resuspended in CDFPB. The suspension was vortexed 1 minute at full power. *M. smegmatis* strain VT307/pLUC10 was then passed through a 25 mm diameter, $5\mu\text{m}$ pore-size filter (to produce a reduced-aggregate fraction of approximately 10^7 to 10^8 CFUs/ml. The washed culture and the RAF were examined microscopically as described below.

Characterization of Reduced-Aggregate Fractions from cultures of *M. smegmatis* strain VT307/pLUC10 in a Petroff Counter.

Ten μl of sample were added to a Petroff-Hausser chamber and described(Hausser Scientific, Blue Bell, PA.) Aggregates were counted as 1-4 cells, 5-25 cells, and greater than 25 cells per clump. The total number of cells was calculated as the sum of the number of clumps within an aggregate type times their clump size. The percentage of total cells was calculated as the number of clumps within an aggregate type times their clump size and divided by the total number of cells.). The number of cells (i.e., <5 cells/unit,) small aggregates (i.e., 5-25 cells/unit,) and large aggregates (i.e., >25

cells/unit) were counted in 4 of the 25 40 x 40 μm squares, averaged, and the percent of total cells for each fraction calculated by the following equation:

$$\frac{\text{Percent of Total} = (\text{Number of Aggregates})(\text{Size of Aggregates})(100)}{\text{Total Number of Cells}}$$

***Mycobacterium smegmatis* strain VT307/pLUC10: Determination of Lysis by Colony Forming Units**

Before measuring the activity of an intracellular enzyme, the efficiency of the lysis procedure was measured. A sample RAF of *Mycobacterium smegmatis* strain VT307/pLUC10 RAF was diluted and plated to determine original colony-forming units (CFU). Another sample of the same fraction was treated with the Luc Lite® lysing solution (Packard Instruments Co. Meriden, CT) and then diluted and plated to determine the surviving colony forming units. Results are expressed as the percent lysis calculated as follows:

$$(\text{CFUs of the RAF}) - (\text{Surviving CFUs})(100\%) / \text{CFUs of the RAF}.$$

***Mycobacterium smegmatis* strain VT307/pLUC10: Measurement of the Release of Intracellular Protein**

In order to assess the amount of protein released by the cell lysis procedure, a commercial protein assay was adapted for use in cell lysis buffer. Cells from 10 ml of a 48 hour cell culture were collected by centrifugation (14,470 x g for 10 min. at room temperature). The cell pellet was suspended in 1ml of CDFPB. Rather than using a reduced-aggregate fraction, the cells were passed through a 22 gauge needle five times to break up clumps in the suspension. This was required to yield a suspension of cells where the concentration was high enough to be above the lower limit of the protein assay. The resulting suspension was enumerated.

Samples were set up as follows: (a) 100 μl cells and 100 μl Luc Lite™ lysing solution, (b) 100 μl cells and 100 μl CDFPB, (c) 100 μl cells and 100 μl Luc Lite™ lysing solution, incubated 10 min at room temperature and filtered through a 0.42μm

Spin-X® filter (Corning, Cambridge, MA), and (d) 100 µl cells with 100 µl CDFPB filtered through a Spin-X® filter. Samples were treated as follows: 50ul sample were combined with 50 ul Luc Lite™ lysing solution and 1 ml bicinchoninic acid working reagent added, [50 vol of bicinchoninic acid solution (Sigma, St Louis, MO) and 1 vol of 4% copper (II) sulfate pentahydrate]. Each sample was incubated at 37° C for 1 hour, and absorbance was measured at 562nm. The Luc Lite™ lysing solution was added because the absorbance is affected by the presence of the detergent. The total protein (i.e., sample a) should be equal to the sum of the leaked and surface reactive protein (i.e., sample b) and the intracellular and leaked protein (i.e., sample c) minus the leaked protein (i.e., sample d).

Identification of Concentration of Reagents for Maximal Light Output

The Luc Lite™ luciferase assay system (Packard Instruments, Meriden, CT) standard reaction involves three steps. The washed cells were placed in an equal volume of Luc Lite™ substrate suspended in the Luc Lite™ lysing solution, incubated 10 minutes at room temperature, and read in a Lumicount™ 96 well microplate luminometer (Packard Instruments, Meriden, CT) on the raw data setting. Because the Packard substrate formulas are unpublished, the Promega Luciferase Assay System (Promega, Madison, WI) components were used as a guideline since it is the most similar assay available. The components of the Promega Assay substrate are 500 µM Dithiothreitol, 270 µM Coenzyme A, 470 µM Luciferin, and 530 µM ATP.

Preparation of *M. smegmatis* strain VT307 Lysate

Cultures were grown as described. Cells were washed twice and suspended in CDFPB equal to the original volume. One ml of suspension was transferred to a 1.8 ml screw-capped polystyrene tube with 0.15 g of 0.1mm glass beads. This was placed in a Mini Bead-Beater (Biospec Products, Bartlesville, OK) on high speed for 80 seconds. The lysate was suitable for use as a substitute for *M. smegmatis* strain VT307/pLUC10 lysate for the quantification of luciferase.

Measurement of Chlorine Susceptibility of *M. smegmatis* Strain VT307/pLUC10

Twenty-five ml of a RAF prepared as described was diluted 10-fold in 225ml of CDFPB chlorine-demand free screwcapped 500 ml bottle (Cesleri *et al.*, 1992) wrapped in aluminum foil. Chlorine was added as free available chlorine to a concentration of 0.25 mg/L by adding 280 μ l of 0.22 g HOCl/L. Three samples were immediately withdrawn and treated as follows: (1) an 1.8ml sample was placed in 0.2ml 0.1 % w/v $\text{Na}_2\text{S}_2\text{SO}_3 \cdot 5(\text{H}_2\text{O})$ for dilution and plating, (2) a 10ml sample was withdrawn for a chlorine concentration reading, and (3) a 10ml sample was placed in 0.3ml 0.1 % w/v $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5(\text{H}_2\text{O})$ for a luminescence reading. $\text{Na}_2\text{S}_2\text{SO}_3 \cdot 5(\text{H}_2\text{O})$ was used to neutralize the remaining reactive chlorine. Samples were removed before the addition of chlorine and at thirty-five and sixty-two seconds after the addition of chlorine.

Luminescence Assay for *Mycobacterium smegmatis* strain VT307/pLUC10

Cells from a 10 ml suspension in CDFPB from the susceptibility study were concentrated by centrifugation (14,470 x g for 10 min. at room temperature) and resuspended in 1ml CDFPB. To 100 μ l were added 100 μ l of Luc Lite™ substrate reconstituted in Luc Lite™ substrate lysing solution containing 1mM additional luciferin in a luminescence cuvette in triplicate. Each sample was incubated at room temperature for 10 minutes. Luminescence was measured on a luminometer (Analytical Luminescence Laboratories, San Diego, CA) on the raw data setting.

Measurement of Chlorine Concentrations

To measure the chlorine concentration, a 10 ml sample from the chlorine susceptibility study was immediately placed into an optically matched 16 X 125 mm borosilicate tube containing N,N-Diethyl-p-Phenylenediamine (DPD) Reagent (Hach Permachem Reagents, Loveland, CO). Absorbance was immediately measured at 515 nm. All samples were processed in that manner to avoid reduction in chlorine concentration that was observed upon standing. Values for chlorine concentration in samples were obtained by comparison with a standard curve. The curve was generated by

measuring dilutions of a 68.2 mg free chlorine/L standard (Hach Permachem Reagents, Loveland, CO) with the above method.

Comparison of Light Output to Concentration of Luciferase

To determine the amount of luciferase present in cell lysates, the relationship between light output and luciferase concentration was measured. One hundred μ l samples of recombinant luciferase (Sigma Chemical Company, St. Louis, MO) in CDFPB contained the following concentrations; 1 μ M, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM and 1 pM . In addition, these same luciferase concentrations were added to a cell lysate of untransformed *M. smegmatis* strain VT307. Samples were combined with 100 μ l Luc Lite™ substrate/lysing solution (Packard Instruments, Meriden, CT) and light-output measured in a luminometer (Analytical Luminescence, San Diego, CA).

Preparation of the Reduced-Aggregate Fractions (RAF) of *Mycobacterium avium* strain A5/pLUC10

In order to measure the disinfection of an organism that naturally grows in aggregates, a baseline should be established of the disinfection of single cells. *M. avium* strain A5/pLUC10 was grown as described to early log phase (OD_{600} = 0.15 to 0.2 .) After washing twice, cells were suspended in CDFPB. The suspension was vortexed 1 min at full power. *M. avium* strain A5/pLUC10 was then centrifuged 1,300 X g for 5 min and the supernatant transferred to a fresh tube to produce a reduced-aggregate fraction of between 10^6 and 10^7 CFUs/ml The washed culture and RAF were examined microscopically as described below.

Characterization of Reduced-Aggregate Fractions from Cultures of *Mycobacterium avium* Strain A5/pLUC10 in a Petroff Counter.

Ten μ l of sample was added to the Petroff-Hausser chamber per protocol (Hausser Scientific, Blue Bell, PA.) Aggregates were counted as 1-4 cells, 5-25 cells, and greater than 25 cells per clump. The total number of cells was calculated as the sum of the number of clumps within an aggregate type times their clump size. The percent of total

cells was calculated as the number of clumps within an aggregate type times their clump size and divided by the total number of cells.

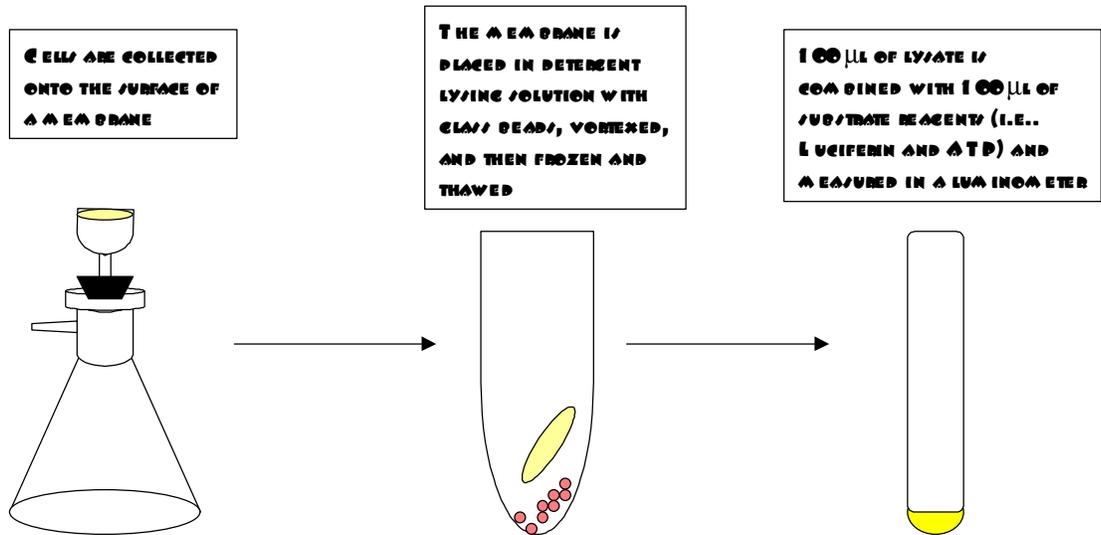
Measurement of Chlorine Susceptibility of *M. avium* strain A5/pLUC10

The appropriate volume of CDFPB was placed in a chlorine demand free (Celsceri, 1989) glass reaction vessel. Cells were placed in solution at 1:10 dilution and a before treatment sample was removed. One ml of 380 mg chlorine/L was added to produce a chlorine residual of approximately 1mg chlorine/L. At 30 seconds, 10 minutes, 20 minutes, 40 minutes, and 60 minutes the following samples were removed: one 10 ml chlorine residual sample, three 10ml luminescence samples in 0.3 ml 0.1% w/vol sodium thiosulfate, and one 1.9ml plating sample in 0.1 ml 0.1% w/vol sodium thiosulfate. Thiosulfate inactivates chlorine (Celsceri *et al.*, 1989).

Recovery and Luminescence of Reduced-Aggregate Fractions of *M. avium* strain A5/pLUC10

In order to increase the cell concentration from a diluted reduced-aggregate suspension to a concentration useful for luminescent measurement, a cell concentration and retrieval system was developed. The 10 ml sample was collected on a 25 mm diameter 0.2 um pore-size filter. The filter was transferred to a 30ml Corex glass centrifuge tube containing 1ml Luc Lite™ detergent lysing agent and 0.15g 0.1mm glass beads and vortexed for 1 min at high power. This treatment differs from bead-beating in its severity and serves only to separate the cells from the membrane without inactivating the enzyme. It was then placed at -20° C for up to 24 hours. It was thawed in water at room temperature for 10 minutes. One hundred microliter samples were transferred in triplicate into luminometer cuvettes (Analytical Luminescence Laboratories, San Diego, CA.) These were combined with 100 µl Luc Lite™ substrate (Packard Instruments, Meriden, CT) reconstituted in CDFPB with 1mM additional luciferin. Luminescence was measured on a luminometer (Analytical Luminescence Laboratories, San Diego, CA) for 10 seconds on the Raw Data setting. Please see Diagram 1.

LUMINO METRIC ASSAY FOR *M. avium*



Potential Induction of Chlorine Resistance of *M. avium* strain A5/pLUC10

The possibility that *M. avium* resistance to chlorine was due to the induction of resistance mechanisms was examined. Two 35ml samples from the same reduced-aggregate suspension were added to two separate chlorine-demand free vessels (Cesleri, *et al.*, 1992) containing 315 ml CDFPB, to one of the two was added 92 µl of 380 mg chlorine/L to produce a 0.1 mg/L final concentration and both were further incubated at 37° for 15 hours. Disinfection experiments were performed on the two by adding chlorine to 1.4 mg HOCl/L and proceeding with the susceptibility protocol described earlier. An additional 120-minute sample was taken for each of the two vessels. This experiment was repeated with the incubation time shortened to two hours and the disinfection chlorine concentration reduced to 1.0 mg HOCl/L.

RESULTS AND DISCUSSION

Strain Construction

Mycobacterium smegmatis strain VT307/pLUC10 was constructed as described in Materials and Methods. The number of Km^R transformants were 8.5×10^3 , 8.1×10^3 , and 1.2×10^4 per μg of plasmid DNA in three separate experiments. Twelve Km^R transformants were streaked on NBT agar medium and from each streak a single isolated colony was recovered and grown in NBT broth. Following growth each culture was diluted to absorbance₅₈₀ = 0.1 and light output was measured on the PAL-luminometer (Gen Probe, San Diego, CA). Using recombinant luciferase as a positive control and untransformed *M. smegmatis* strain Vt307 as a negative control, all of the Km^R transformants were found to be luminescent. The range of values was 841 to 537 relative light units for the Km^R transformants. The values for light units will vary by machine since the photomultipliers vary in sensitivity and relative gain. Isolate twelve, the strain with the highest light-output, was selected for further studies (VT307/pLUC10).

Mycobacterium avium strain MD1/pLUC10 was constructed as described in Materials and Methods. Transformation efficiency was 2.9×10^3 Km^R transformants per μg of pLUC10 plasmid DNA. Ten Km^R transformants were streaked on MGO agar medium for isolated colonies, all grew under selective pressure of 50 μg kanamycin/ ml agar medium. Four isolated colonies from each of 4 Km^R transformants were picked and grown in MGO broth with 50 μg kanamycin/ ml. Each of the Km^R transformants were tested in triplicate and demonstrated a luminescent phenotype on the Monolight luminometer (Analytical Luminescence Laboratories, San Diego, Ca.) with a range of values from 173,505 to 153,272 relative light units. Isolate 2 was selected for further studies because it had the greatest light output.

Mycobacterium avium strain A5/pLUC10 was constructed by electroporation as described in Materials and Methods. The transformation efficiency was 5.5×10^2 Km^R transformants per μg of pLUC10 plasmid DNA. Seven Km^R transformant colonies were streaked on MGO agar medium containing 50 μg kanamycin/ml and from each streak a single isolated colony was picked and was grown in MGO broth containing 50 μg

kanamycin/ml. Both cultures and colonies were tested for *luc* expression. Of the seven isolates demonstrating Km^R phenotypes, 5 (71%) demonstrated a luminescent phenotype. Of these five, values ranged between 22,630 to 51,463 relative light units in the Monolight luminometer (Analytical Luminescence Laboratories, San Diego, CA). Isolate 1 had the most typical growth curve for *M. avium* and was selected for further examination due to its apparent stability. Isolate 1 was selected for further studies as A5/pLUC10.

Mycobacterium bovis BCG strain Pasteur/pLUC10 was constructed by electroporation as described in Materials and Methods. Transformation efficiency was 1.6×10^2 Km^R transformants per μg of plasmid DNA. Six Km^R transformants colonies were streaked on DBB agar medium containing 50 μg kanamycin/ ml and from each streak a single isolated colony was picked for further study. Each colony was suspended in 100 μl CDFPB, capped, sonicated as described, and combined with 100 μl of Luc Lite™ lysing / substrate solution. The suspension was frozen at - 70 ° C, thawed to room temperature, and light output was measured on a Monolight luminometer (Analytical Luminescence Laboratories, San Diego, CA). Of the six Km^R colonies, 5 (83%) were luminescent. Of the five, values ranged between 369,976 to 4,585,085 relative light units. Three colonies from the five luminescent isolates were used to inoculate 1 ml DBB broths containing 15 μg kanamycin/ ml as described. After 7 days, 10 ml of DBB broth were added to each 1 ml culture and grown for an additional 35 days. Only isolate 4-2 grew turbid (absorbance₆₀₀= 0.1). That culture was also luminescent. It was stored for further use as described in Materials and Methods.

With the exception of *M. bovis* BCG strain Pasteur/pLUC10, the luminescent phenotypes were stable when the strain was maintained with selective pressure as indicated for each strain in Materials and Methods, and the strain demonstrated no other notable differences from the parent strain (e.g. growth rate, colony morphology, aggregation, or pigmentation). It is important to note that the growth of *M. bovis* BCG strain Pasteur was less than predictable prior to transformation, and that the nature of the transformant was not different from the parent strain in colony morphology, pigmentation, cell morphology, or characteristic cording-like aggregation. The sporadic

growth of the transformant may become more stable as experience with the organism is acquired.

Characterization of the *M. smegmatis* strain VT307/pLUC10 Reduced-Aggregate Fractions

Reduced-aggregate fractions were needed in order to eliminate the variability caused by the clumping of cells. Chlorine requires contact for disinfection. Therefore, cells in clumps that are not exposed to chlorine would be expected to survive. For example, disinfection resistance of mycobacteria could be due to survival of cells within aggregates. Inner cells would be protected from exposure to chlorine by the outer cells thereby preventing contact with the disinfectant. Alternatively, the inner environment of a large aggregate may be substantially different and lead to the expression of genes resulting in higher or lower survival. Therefore, aggregated cells should not be used as indicators of susceptibility of individual cells to disinfectants.

A reduced-aggregate fraction was a suspension of cells in which most or all of the aggregates were eliminated. Reduced-aggregate fractions were rejected if there were any large aggregates (e.g. 25 or greater cells) present in the suspension. Such aggregates had cells that may not have been exposed to chlorine. For the studies described here, at least 75% of the suspension consisted of single cells and cells in small aggregates represented no more than 25% of the total number of cells in suspension. For a detailed discussion of the impact of aggregates, please see Appendix A. The cells were washed and a sample was examined in a Petroff Counter (Hausser Scientific, Blue Bell, PA) as described in Materials and Methods. The sample was then filtered to produce the RAF as described in Materials and Methods and counted in a Petroff Counter (Hausser Scientific, Blue Bell, PA).

The percent of total cells represented by cells, small aggregates, and large aggregates in washed culture primarily existed in large aggregates (Table 1). A predominance (70%)(Table 1) of large aggregates was not desirable because it would lead to irregular data as discussed in detail in Appendix A. In the RAF, the suspension consisted predominantly (92%) (Table 1) of single cells, with few or no aggregates of any size (8%). This type of homogeneous suspension resulted in a better sample population

for testing. The cellular concentrations of these suspensions ranged between 10^7 and 10^8 CFU/ml. Because of the relatively high number of cells in the RAFs, the concentration of cells did not limit the range of measurement of susceptibility by the decrease of light output. The threshold for measurement of light output in *M. smegmatis* was 2.4×10^4 CFU/ml, which was easily achieved with the RAF. Therefore, reductions of at least 3 orders of magnitude in cell concentration could be observed.

Table 1: The effect of filtration on washed cells to produce a Reduced-Aggregate Fraction is presented in Table 1. The aggregate sizes are based on cultures observed in a Petroff counter under phase-contrast microscopy.

Table 1 Characteristics of Washed Cultures and Reduced-Aggregate Fractions of <i>M. smegmatis</i> strain VT307/pLUC10*		
Percent of Total Cells by Particle Size		
Size of Aggregate	Washed Culture	Reduced-Aggregate Fraction
Cells <5 cells / particle	12 % ± 0.5%	92% ± 0.8%
Small Aggregates 5-25 cells / particle	18% ± 4.0%	8% ± 0.8%
Large Aggregates 5-25 cells / particle	70 % ± 4.5%	<1% ± 0.0%
* Average of two independent cultures and RAFs.		

***Mycobacterium smegmatis* Strain VT307/pLUC10: Determination of Lysis by Colony Forming Units**

If lysis of *M. smegmatis* by Luc Lite™ (Packard Instruments, Meriden, CT) lysing solution were low, then the light output would not represent a substantial proportion of cells, and would not will not accurately reflect colony forming units. To measure lysis, three separate RAFs from three separate cultures were treated with the lysing solution. Colony forming units were counted before and after exposure to the lysing solution. The dilutions of the treated cells were made in CDFPB to eliminate the effect of residual lysing solution on surviving cells. This was repeated for each RAF and the results were averaged. Lysed and whole cultures were also examined microscopically. They could not be distinguished from one another by phase-contrast microscopy. Therefore, it appears that cells are not lysed, but are being made permeable and luciferase leaks out from cells.

The average extent of lysis of RAFs of *M. smegmatis*, as determined by reduction in colony forming units, ranged from 90.6% to 98.5% under the treatment conditions described in Materials and Methods. The extent of the variation of lysis for each individual culture was less than 1% of the mean (Table 2). In light of these results, comparison of luminescence of RAF suspensions before and after disinfection were performed on the same individual RAFs. In that manner, the variation in lysis was kept to a minimum.

Table 2: The lysis of *M. smegmatis* by the detergent lysing solution provided in the Luc Lite® assay kit. Three independent cultures were examined, and the number of replicate samples is listed as *n*. The equation used to calculate the percent lysis is listed in the legend.

Table 2					
Lysis of <i>M. smegmatis</i> strain VT307/pLUC10 Measured in Colony Forming Units					
Sample	Original Colony Forming Units	Surviving Colony Forming Units	Percent Lysis*	Standard Deviation	<i>n</i>
RAF 1	2.1 x 10 ⁷	1.2 x 10 ⁶	94.2	0.5%	3
RAF 2	1.6 x 10 ⁷	9.3 x 10 ⁵	90.6	0.9%	3
RAF 3	2.4 x 10 ⁷	3.5 x 10 ⁵	98.5	0.3%	2
* Percent lysis was measured the decrease in colony forming units and calculated using the following equation: $\frac{(\text{Original CFUs minus Treated CFUs})(100)}{\text{Original CFUs}}$					

***Mycobacterium smegmatis* Strain VT307/pLUC10: Measurement of the Release of Intracellular Protein**

Protein concentrations were measured before and after addition of lysing solution to be sure that a substantial proportion of luciferase was freed from the cell to react with substrates and produce a light signal. The lysing agent for the Luc Lite™ lysing solution is Triton N-101, a detergent. Detergents interfere with both the Bradford and Lowry total protein assays, two of the most commonly employed total protein assays. The bicinchonic acid total protein assay (Smith *et al.*, 1985) is suitable in the presence of detergents and does not require any specialized equipment except a spectrophotometer. For this reason, the bicinchonic acid method was used to measure the release of intracellular protein by the Luc Lite™ lysing solution.

The protein concentration of the cells in the lysing solution represented the total protein (a) that reacted with the bicinchonic acid and are presented in Table 3, column 2. This included surface, intracellular, and 'leaked' protein. The average of three experiments for total protein was 16.5 ug/ml/10⁸ CFUs. Total protein, as measured by the Lowry method in *M. avium*, was found to be 65 µg/ml for 5 x 10⁸ CFU/ml or 13.1 µg/ per 10⁸ colony forming units (McCarthy and Ashbaugh, 1981). Therefore, the *M. smegmatis* strain VT307/pLUC10 probably contains slightly more protein/cell than the *M. avium* strain examined.

Cells suspended in CDFPB and not subjected to the lysing solution (b) had detectable protein (7.25 ug/ 10⁸ CFU) by the bicinchonic acid assay and is presented in Table 3, column 3. This may have been "leaked" protein, surface protein, or it may have been the result of lysis by the high pH of bicinchoninic acid working reagent. This protein may be distinguished from protein "leaked" prior to exposure of cells to the bicinchonic acid working reagent, (d) (<1.1 ug/ 10⁸ CFU) the filtrate of whole cells which is presented in Table 3, column 5. These were likely surface proteins or cells lysed by the high pH of the working solution because the protein concentration of the filtrate of whole cells was very low (i.e., <10 µg/ml). The measurement of protein in the filtrate of cells exposed to the lysing solution, (c) (6.3 ug/ 10⁸ CFU), indicates that protein was released from the cytoplasm and is described in Table 3, column 4. Thus, intracellular

protein was released in the presence of the Luc Lite™ lysing solution. The total protein (13.1 ug/10⁸ CFU) was approximately equal to the sum of the leaked and surface reactive protein (7.25 ug/10⁸ CFU) and the intracellular and leaked protein (6.3 ug/10⁸ CFU) minus the leaked protein (<1.1 ug/10⁸ CFU). Intracellular protein release, as determined by total protein analysis, was most accurately determined when cell numbers approached 10⁹ CFUs/ml. At lower cell concentrations, the total protein measurements were too close to the threshold of sensitivity (10-15 µg) for the protein values to be accurately determined.

Table 3: Analysis of total protein was performed by the bicinchonic acid method because of the presence of detergents in the lysing solution. High cell concentrations were used to exceed the limit of resolution of the assay, thus prohibiting the use of reduced-aggregate fractions for this study. The description of the sample type (e.g. total protein or leaked protein) represents an independent measurement. The different numbers of colony forming units represent independent cultures, not a dilution of one culture.

Table 3				
Total Protein Analysis of <i>M. smegmatis</i> strain VT307/pLUC10				
Colony Forming Units	(a) Total Protein	(b) Surface and Leaked Protein	(c) Intracellular and Leaked Protein	(d) Leaked Protein
9.0 x 10 ⁸	148 ug/ml	65 ug/ml	55 ug/ml	<10 ug/ml*
6.0 x 10 ⁸	110 ug/ml	44 ug/ml	39 ug/ml	<10 ug/ml*
3.0 x 10 ⁸	45 ug/ml	10-15 ug/ml*	32 ug/ml	10-15 ug/ml*
* The lower limit of resolution of the assay is 10 ug/ml protein, and these measurements were too close to this lower limit to determine an actual value.				

Identification of Concentration of Reagents for Maximal Light Output

In order to maximize light output, there must be no limitation of the reaction by reagents that comprise part of the light-producing reaction. The light-producing reaction catalyzed by luciferase is as follows: $\text{ATP} + \text{luciferin} + \text{O}_2 \xleftarrow{\text{CoA}} \text{Mg}^{++} \Rightarrow \text{Oxyluciferin} + \text{AMP} + \text{PP} + \text{CO}_2 + \text{Light}$ (Kricka, 1988). The reaction involves intermediate states, but the equations were combined for simplicity. To measure whether or not any of the components of the assay were limiting light output, the concentrations of the assay components directly used in the light-producing reaction (i.e., excluding cofactors) were evaluated. A commercial assay, the Luc Lite™ Luciferase Reporter Gene Assay Kit (Packard, Meriden, CT), was selected to measure luminescence, but the Luc Lite™ substrate formulas were proprietary. Promega's Luciferase Assay System (Promega, Madison, WI) components were used as a guideline because it used the same type of continuous light output technology as the Luc Lite™ assay. The concentrations of the latter assay's substrates were 270 μM Coenzyme A, 470 μM Luciferin, and 530 μM ATP.

To measure the effect of additional luciferin or ATP, the components were added to the standard Luc Lite™ assay and the light output was measured on a luminometer. Five-fold excess ATP was added to the standard reaction and compared to the commercial assay. One-fold excess luciferin was added and compared to the commercial assay. Excess of both ATP and luciferin were then added together and examined for differences in light readings against the controls. To further elucidate the effect of different concentrations of luciferase on the standard reaction, different concentrations of luciferin were added to a standard reaction (i.e., 470 μM (1X), 4mM(9X), 3.75mM, 3mM(7X), 2.75mM, 2 mM(5X), 1.75mM, 1mM(3X), and 0.7mM) and compared to the 1X reaction.

Three independent experiments were performed and the results were averaged. Light output was increased by 16% when 2.6 mM-additional ATP was added to the lysate. The light output was increased by 270% when 470 μM additional luciferin was added to the lysate. When luciferin and ATP were added in excess quantities together at the

concentrations above, the increase in light output was only 6% greater than compared to excess luciferin alone. This indicated that luciferin concentration in the Luc Lite™ assay limited light output. ATP did not limit the light-producing reaction.

The effect of different concentrations of luciferin on the standard assay was measured in detail. The relationship between the amount of luciferin added and the amount of light that was produced by lysates of RAF of *M. smegmatis* strain VT307/pLUC10 is shown in Figure 1. The percent of light produced by samples containing additional luciferin as compared to samples with no additional luciferin were plotted as a function of the excess concentration of luciferin added (Figure 1). At 1mM additional luciferin, there was a 500% increase in light production over the proprietary assay. At 2 mM additional luciferin, the light output was only 750% greater than the proprietary assay. Since the benefit achieved by adding more luciferin had to be weighed with the high cost of the reagent, 1mM was selected as the luciferin concentration added to lysates for disinfection studies.

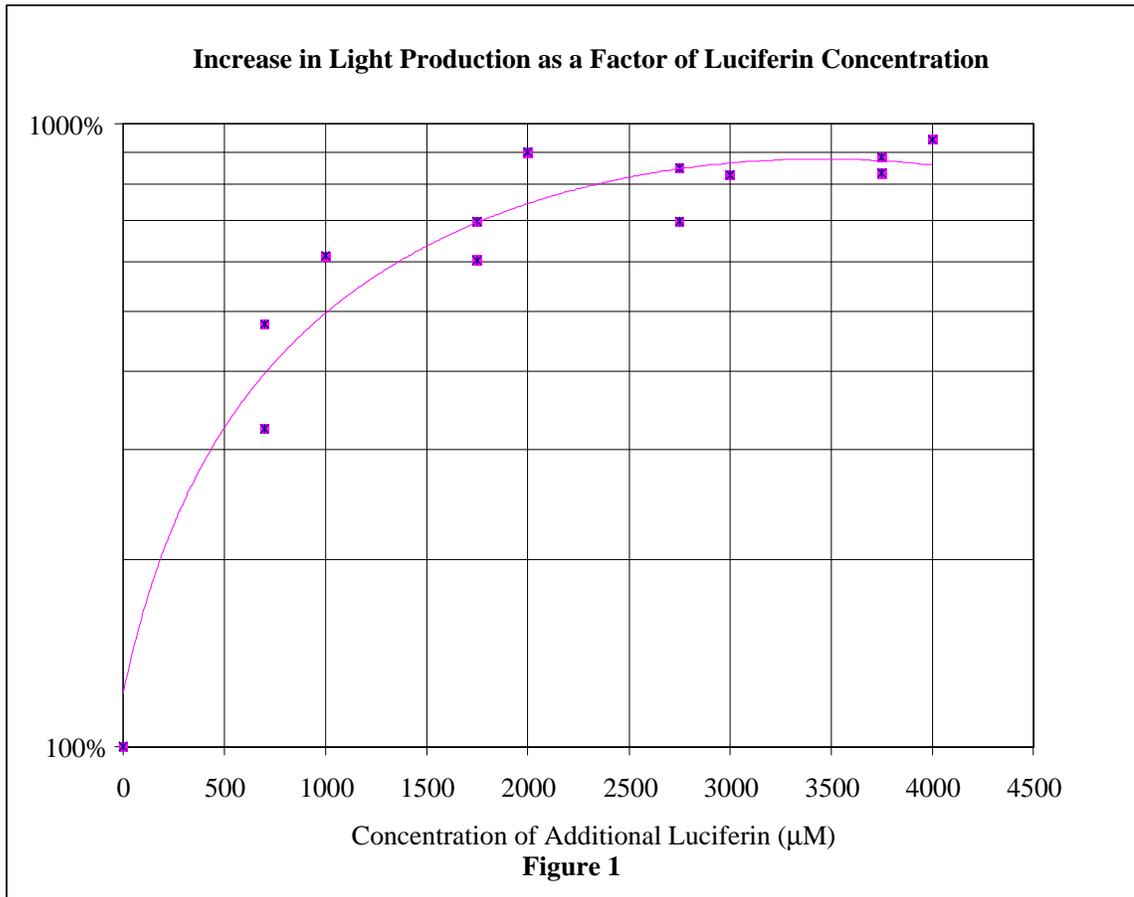


Figure 1: Light output produced is affected by the concentration of additional luciferin added to the commercial assay. Pink squares represent the percent increase in light output of samples when compared to a sample with no additional luciferin. The regression finds the best fit between all data.

Comparison of Light Output to Concentration of Luciferase

To determine the amount of luciferase present in cell lysates, the relationship between light output and luciferase concentration was measured. Different concentrations of luciferase in either CDFPB or cell lysate made from bead-beaten *M. smegmatis* strain VT307 were combined with the Luc Lite™ substrate/lysing solution and measured for light output. The relationship between light output and luciferase concentration is shown in Figure 2. Cell lysates did not have a quenching effect on the firefly luciferase. Rather, the presence of cell lysate enhanced light output of the enzyme with the light output of the enzyme in lysate being approximately double that of the enzyme in CDFPB alone. The increase in light output was likely due to the presence of additional ATP and/or cofactors in the lysate. Therefore, the concentration of luciferase released by a recombinant strain could be determined by using the light output as a standard curve.

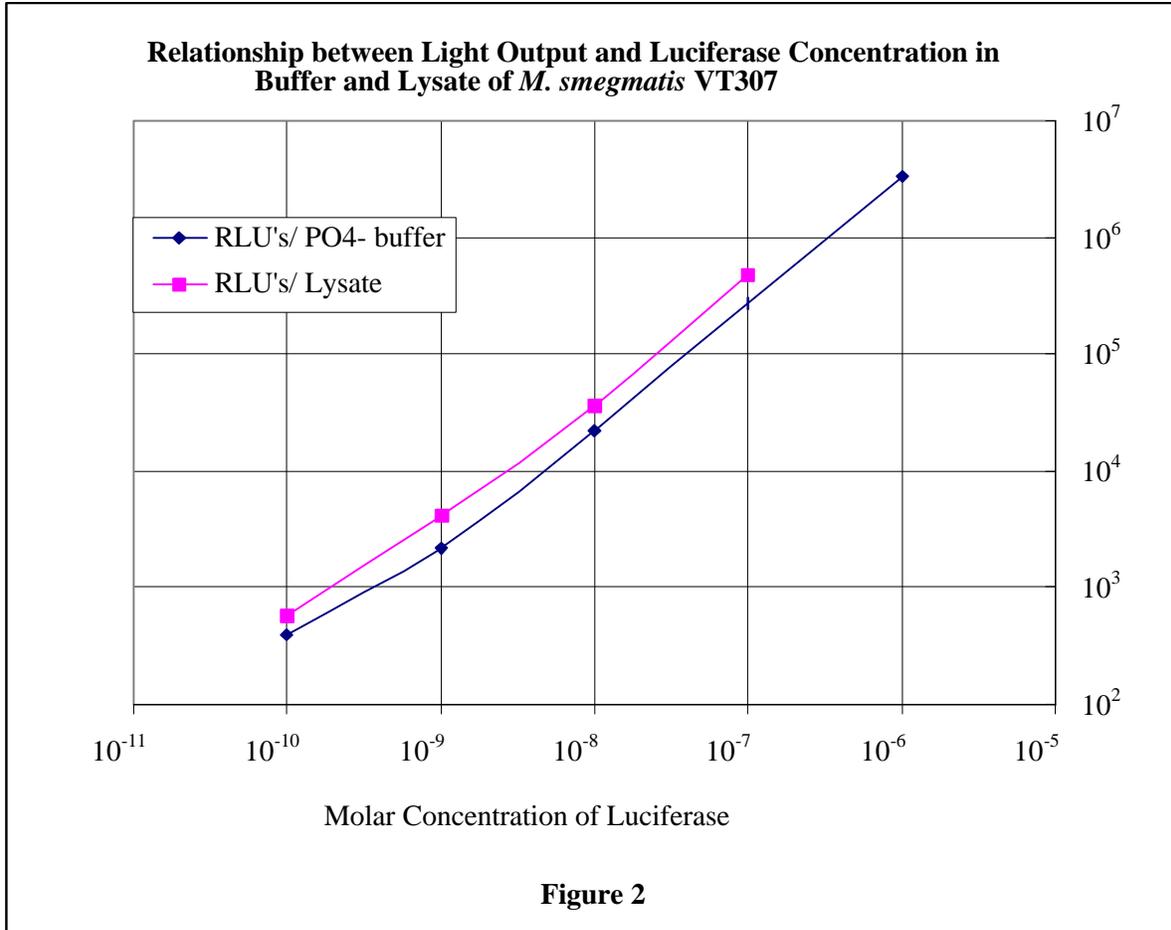


Figure 2: Relationship between relative light units and the molar concentration of luciferase. Blue diamonds indicate the values from samples in buffer. Red squares indicate values for samples in *M. smegmatis* lysate.

Measurement of Chlorine-Susceptibility of *M. smegmatis* strain VT307/pLUC10

Before proceeding with measurement of chlorine-susceptibility of *M. avium* pLUC10 strains, it was necessary to demonstrate that light production decreased as colony forming units decreased in the disinfection assay with *M. smegmatis* strain VT307/pLUC10. A reduced-aggregate fraction of *M. smegmatis* strain VT307/pLUC10 was diluted tenfold in CDFPB and samples were assayed for light output, CFU, and chlorine concentration. Chlorine was added to a final concentration of 0.25 mg chlorine/L and samples were again assayed for light output, CFU, and chlorine concentration after 30 and 60 sec.

Figure 3 represents the results of the effect of 0.25 mg chlorine/L on the colony forming units and light output (expressed in relative light units) on the RAF of *M. smegmatis* strain VT307/pLUC10. Colony forming units decreased from 4×10^5 to 2.7×10^3 CFU/ ml. For the same RAF, the relative light units decreased from 6.9×10^3 to 1.0×10^2 , the threshold of the assay. The data was not linear over time because the concentration of chlorine in this assay went from a calculated 0.25 mg chlorine/L at the point chlorine was added, to 0.15 mg chlorine/L at 17 seconds, and 0.08 at 62 seconds. The data showed that chlorine exposure reduced luciferase activity at nearly the same rate as colony-forming units were reduced. Further, the decrease in CFUs is proportionately equal to the decrease in relative light units (Figure 3), encouraging the use of *M. avium* strain A5/pLUC10. This indicated that measuring luciferase activity is a good means of measuring survival.

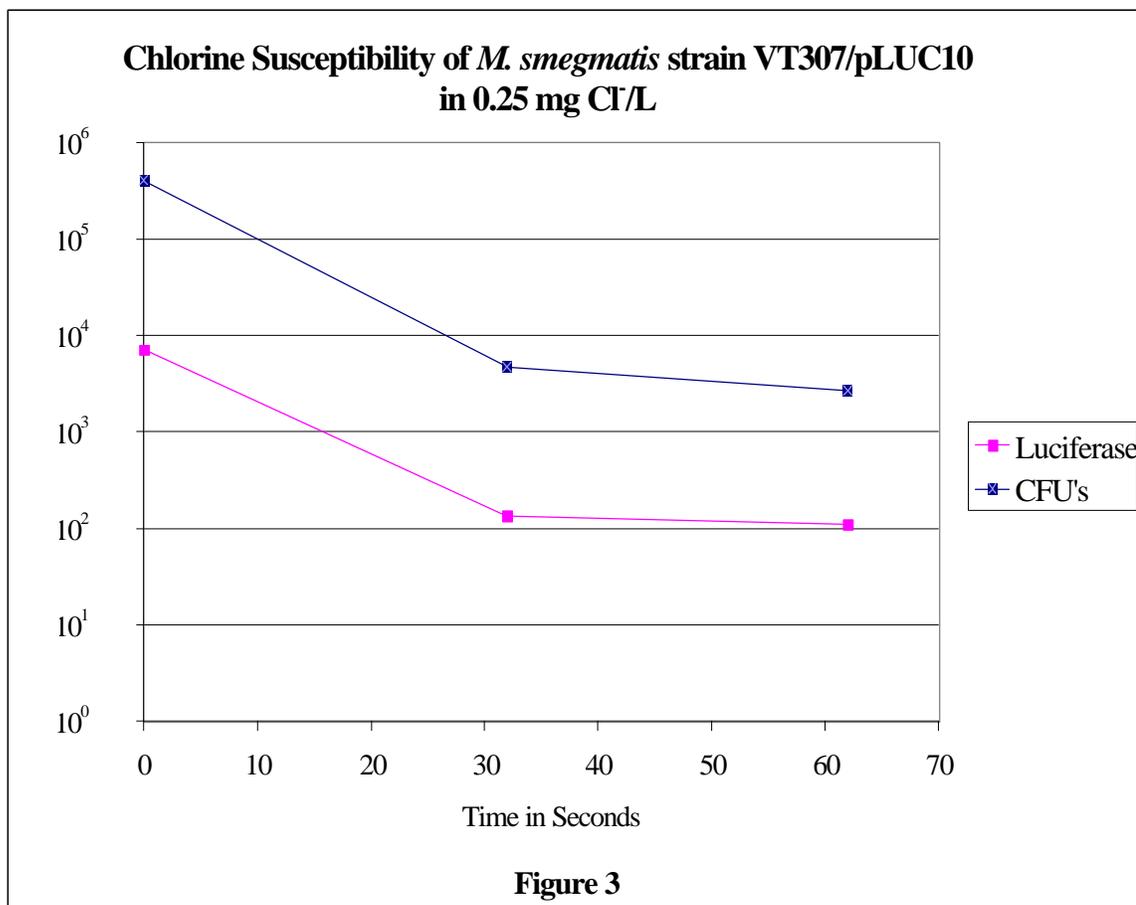


Figure 3: Susceptibility of *M. smegmatis* strain VT307/pLUC10 to 0.25 mg/L Chlorine. CFU are presented in blue and RLU are presented in red. The scale of the x-axis is in seconds. Note that the scale on the y-axis is logarithmic.

Reduced-Aggregate Fractions of *M. avium* strain A5/pLUC10

The composition of a reduced aggregate suspension was at least 75% cells and no more than 25% small aggregates. A reduced-aggregate fraction was rejected if there were any large aggregates (e.g. >25 cells) present in the suspension because these have cells which may not have been exposed to the outside environment and could have had protective value for inner cells. The percent of total cells represented by the single cells and small aggregates is outnumbered by the large aggregates (76%)(Table 4) in washed cultures of *M. avium*. The predominance of large aggregates would lead to irregular data for reasons previously discussed. This effect is discussed in detail in Appendix A. In the RAF, the suspension was principally cells (78%) (Table 4), with few aggregates of any size. The homogeneous suspension inherent in the RAF resulted in a better sample population for testing since the effect of chlorine on a suspension of single cells could be measured. The concentrations of these suspensions ranged between 10^6 and 10^7 colony forming units per ml. Since the concentration of cells required for disinfection studies was 10^5 to 10^6 CFU/ml, the RAFs were diluted 10-fold for a disinfection experiment.

Table 4: The effect of centrifugation to remove aggregates on washed cells to produce a Reduced-Aggregate Fraction is presented in Table 4. The aggregate sizes are based on cultures observed in a Petroff counter under phase-contrast microscopy.

Table 4		
Reduced Aggregate Fraction of <i>M. avium</i> strain A5/pLUC10*		
	Percent of Total Cells by Aggregate Size	
Size of Particle	Washed Culture	Reduced-Aggregate Fraction
Cells	13% ± 5%	78% ± 8%
Small Aggregates	11% ± 3%	21% ± 8%
Large Aggregates	76% ± 6%	<1% ± 0%
* Average of 4 independent cultures and RAFs		

***Mycobacterium avium* strain A5/pLUC10: Growth-Stage Dependent Lysis**

Because growth stage has been shown to influence the transformation efficiency of *M. avium* strain A5 by electroporation (Beggs *et al.*, 1996) and *M. avium* cells show dramatic differences in morphology (McCarthy and Ashbaugh, 1981), the extent of lysis of *M. avium* strain A5/pLUC10 was postulated to be growth-stage dependent as well. A culture was grown as described in Materials and Methods until the pre-determined time, the absorbance₆₀₀ was measured, it was examined by phase-contrast microscopy, and a RAF was created. This was done independently with separate cultures at 24, 36, 48 and 72 hours.

Figure 4 summarizes the growth characteristics of *M. avium* strain A5/pLUC10. Growth was measured as the increase in turbidity of cultures as measured by absorbance at 600 nm and was plotted as a function of time. Cultures were also examined under the microscope for cellular morphology characteristic of growth stages as described previously (McCarthy *et al.*, 1981) and their cell morphology was recorded. The arrows indicate times at which cultures were examined for lysis and range of light output.

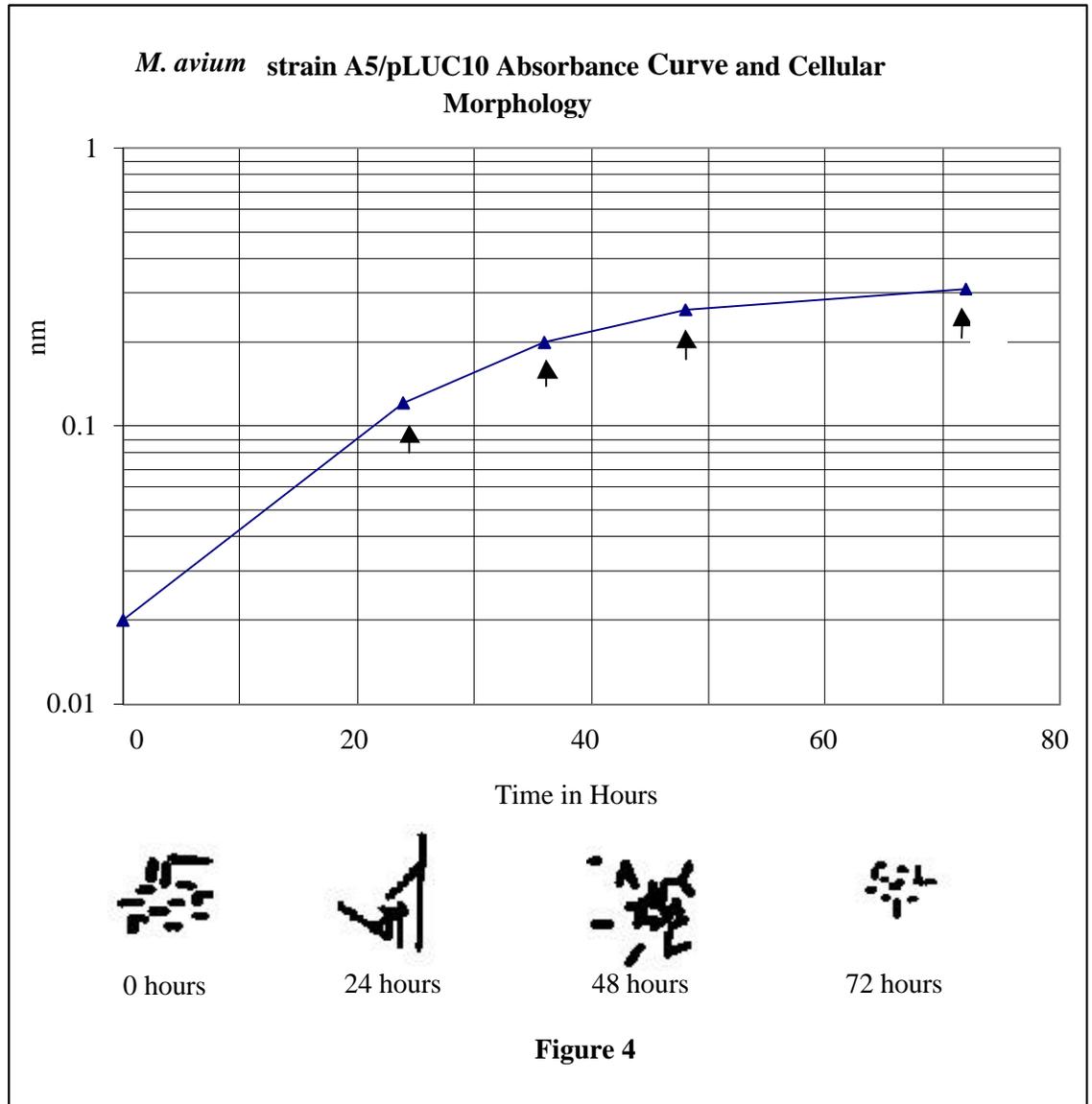


Figure 4: The absorbance curve for four cultures is shown in blue. The arrows indicate times at which RAF were created for lysis and light output studies. The cartoons below the graph represent the appearance of cells at a given absorbance.

The RAFs were diluted and plated to determine the original CFUs. The reduced-aggregate fractions were then treated with lysing solution, frozen, thawed, and plated for surviving colony-forming units. Preliminary studies indicated that sonication and bead-beating were not suitable for releasing intracellular contents because both resulted in inactivation of luciferase. There was a 99.85% reduction in recombinant luciferase activity after bead beating. A 96.5% reduction in activity was observed when the enzyme was sonicated in the presence of glass beads. A freeze-thaw with sonication resulted in 94.5% reduction in activity of the enzyme. Freeze-Thaw with the lysis buffer alone was selected as the lysis method. Percent lysis was measured as the decrease in colony forming units and calculated using the following equation:

$$\frac{(\text{Original CFUs minus Treated CFUs})(100)}{\text{Original CFUs}}$$

Examination of *M. avium* for lysis from different growth stages from different cultures showed that RAF from cultures of low absorbance (i.e., 0.12 and 0.14) contained cells that were more sensitive to the lysis conditions than other RAF as measured by reduction of colony forming units.

RAF from cultures were shown to be best for disinfection studies when isolated from cultures between $OD_{600} = 0.15$ to 0.2 based on these results. The highest transformation efficiency of *M. avium* strain A5 occurs when the cells were at low absorbance (Beggs, *et al.*, 1996). The results of this work also indicated that complete lysis of the organism was not achieved by this treatment method. In fact, because there was no microscopic evidence of lysis, it is likely that cells are being made more permeable.

Table 5: The lysis of *M. avium* strain A5/pLUC10 treated with lysing solution, frozen, and thawed are presented in Table 5. The appearance of the cells prior to treatment is indicated. The absorbance at 600 nm of each culture is indicated.

Table 5		
<i>M. avium</i> Growth Stage Dependent Lysis		
Absorbance ₆₀₀	Appearance	Percent Lysis*
0.12	Cells elongating	21.6%
0.14	Cells elongated, aggregating (early log)	30.8%
0.26	Mixed elongated and shortened cells	20.6 %
0.32	Predominantly shortened cells	9.5%
* Percent lysis is an average of three independent trials		

Relationship between CFU and Light Production for *M. avium* Strain A5/pLUC10

Since the lysis of *M. avium* strain A5/pLUC10 was incomplete, it was necessary to determine the reproducibility of the relationship between light and colony forming units. Furthermore, for the measurement of survival of *M. avium* by light output to be useful in a chlorine disinfection assay, the correlation between colony forming units and light output needed to span at least three logs to demonstrate 99.9% killing. To determine whether this was the case, a dilution series of each of six independent reduced aggregate fractions was measured for luminescence as described in Materials and Methods. Three of the six were harvested at early log phase, while the other three were from later growth stages. Colony forming units were measured by plating the dilutions in triplicate and the results averaged. The relative light units measuring light output are plotted as a function of colony forming units for each of six different reduced aggregate fractions (Figure 5).

The greatest amount of light produced per cell was found in the reduced aggregate fractions made from cultures in early log phase (i.e., absorbance₆₀₀= 0.1 to 0.2). This was most likely due to better lysis of the cells as observed earlier, or it may have been due to a greater amount of protein, specifically, luciferase, in the elongated cells. The threshold of the luminometric assay for *M. avium* strain A5/pLUC10 was 3×10^4 cells/ml. At lower cell numbers, the signal value could not be distinguished from background light levels. The dilution series demonstrated that a two-log range of 3×10^4 to 6×10^6 could be readily achieved directly from the reduced-aggregate fraction. Extending the range to three logs could be reached by concentrating the RAF suspension tenfold before measuring light output in a luminometer.

Greater light output per colony forming unit was seen in *M. smegmatis* strain VT307/pLUC10 than in *M. avium* strain A5/pLUC10 (Table 6). The light output was 78-88% less in *M. avium* than *M. smegmatis* using the same luminometer and settings. This reduced light output was most likely due to the reduced lysis seen in *M. avium*. Almost complete lysis was observed for *M. smegmatis*. It is possible, however, that the freezing and thawing of the enzyme in the lysing treatment of *M. avium* strain A5/pLUC10 may have contributed to reduced light output per cell by reducing the activity of the enzyme as was previously discussed with bead beating and sonication.

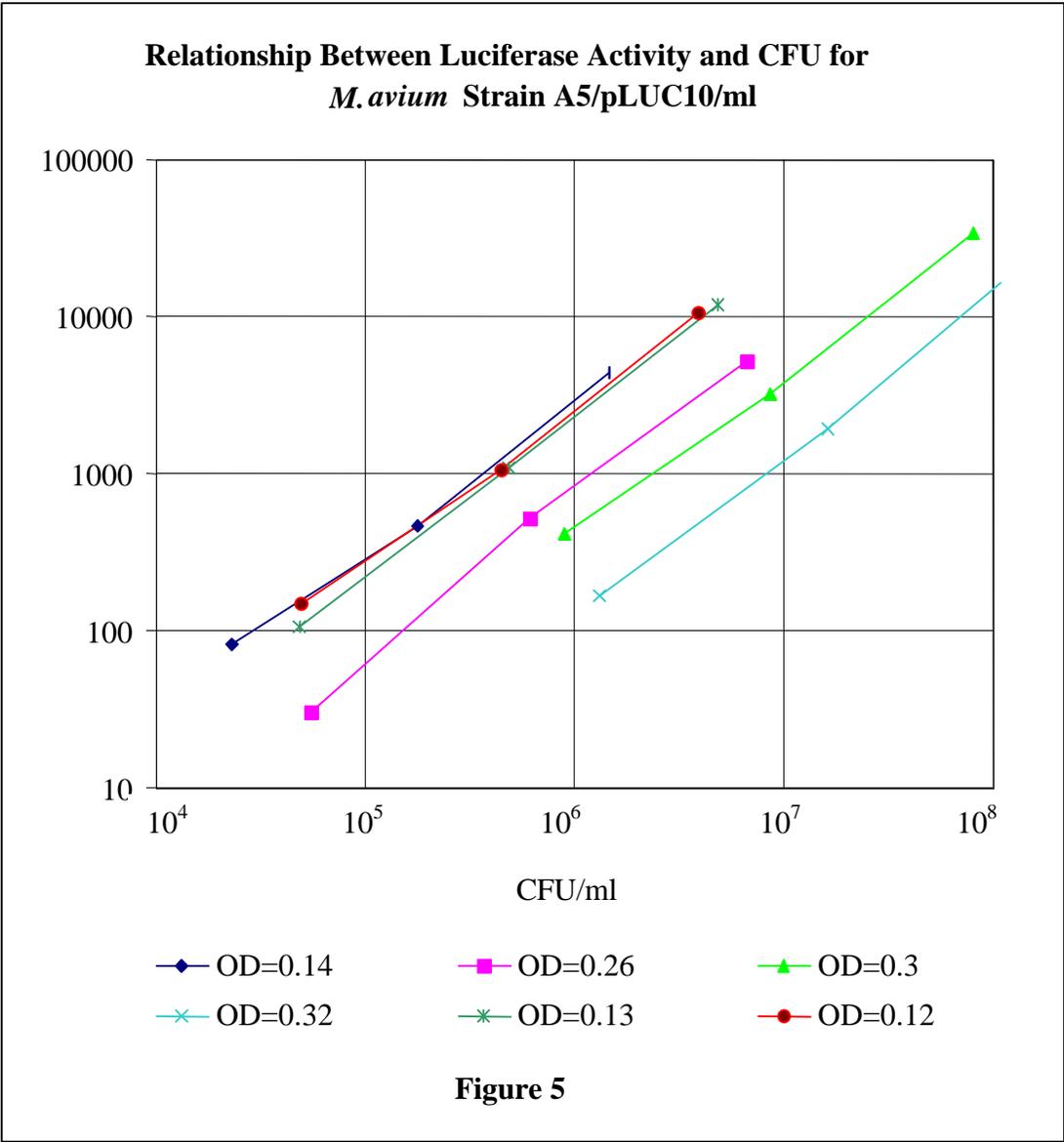


Figure 5: The relationship between relative light produced and CFU is shown. The relative light units, the y axis, and the CFU, the x axis, are both log scale. The figure legend below the graph indicates the absorbances at which RAF were made prior to dilution and examination of light output.

Table 6: A comparison of light output per colony forming unit in both *M. smegmatis* strain VT307/pLUC10 and *M. avium* strain A5/pLUC10 is shown.

Table 6		
Comparison of Relative Light Production		
Colony Forming Units	Relative light Units	
	<i>M. smegmatis</i>	<i>M. avium</i>
1.00E+05	2,137	250
1.00E+06	15,625	3,000
1.00E+07	114,677	24,526

Decline in Chlorine Concentration in *M. avium* A5/pLUC10 Reduced-Aggregate Suspensions

Chlorine is very reactive and the ability to maintain a stable chlorine concentration over time is essential to any chlorine assay. Cells may adsorb chlorine and inactivate it, thus reducing its effective concentration in suspension. Therefore, it was essential to measure the concentration of chlorine in RAF suspensions. Three RAF suspensions were prepared: (1) an undiluted RAF suspended in CDFPB, (2) a 1:10 dilution of a RAF in CDFPB, and (3) CDFPB (control). Equal amounts of chlorine were added to each suspension, and the concentration of chlorine was measured at different times as described in Materials and Methods. The concentration of chlorine in each suspension was plotted as a function of time (Figure 6).

There was a biphasic decline in chlorine concentration in reduced-aggregate fractions of *M. avium* strain A5/pLUC10. The chlorine disappeared from the suspension very rapidly at first (e.g. within 30 seconds). Then the reduction in concentration over time from 30 seconds to 60 min was equal to that of the cell-free CDFPB control. The quantity of chlorine lost by the diluted suspension (1×10^6 CFU/ml) was significantly lower than that of the undiluted suspension (1×10^7 CFU/ml), indicating that the initial chlorine reduction was cell-density dependent (Figure 6). In order to maintain chlorine concentrations for disinfection studies, the cell concentration of the RAF must not exceed 10^6 CFUs per ml.

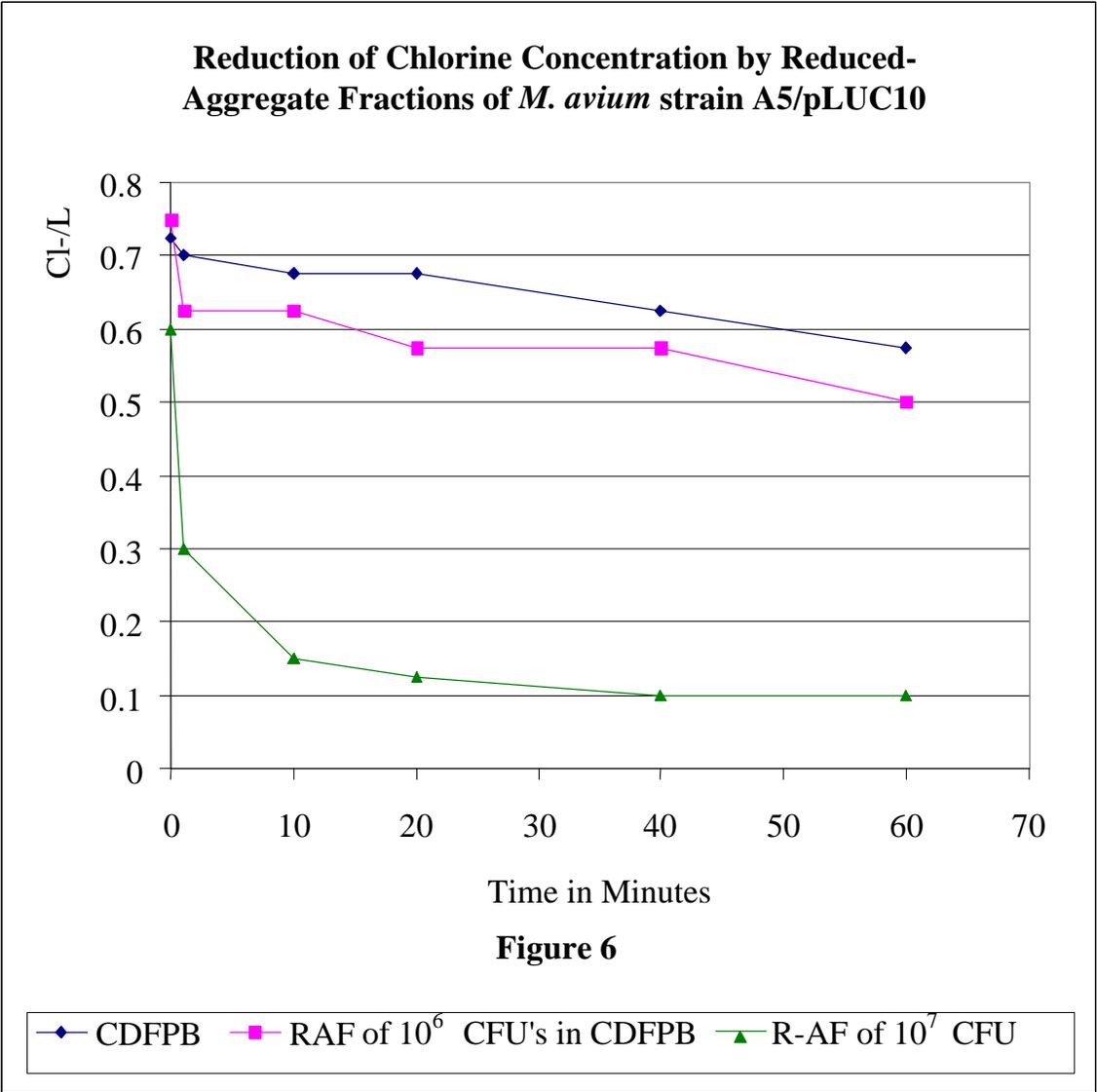


Figure 6: The effect of different concentrations of cells in CDFPB on the stability of chlorine concentration is shown. Cell concentrations of suspensions are indicated in the figure legend.

***Mycobacterium avium* strain A5/pLUC10 Disinfection**

Mycobacterium avium is indigenous to water. Conventional culture methods used to enumerate *M. avium* in water are time consuming because *M. avium* grows slowly (i.e., colonies appear on agar medium after 7-10 days incubation). To measure the susceptibility of *M. avium* strain A5/pLUC10 to chlorine, a reduced-aggregate suspension was diluted 1 to 10 in CDFPB and chlorine was added to a predetermined final concentration. Dilution was necessary to reduce the concentration of cells in a RAF (10^7 to 10^6 CFUs/ml) to the desired range of 10^6 to 10^5 for chlorine disinfection studies as described previously. Attempts at concentrating and retrieving the cells included centrifugation, centrifugation onto glass wool, and filtration. Of all of these methods, only filtration and freeze-thaw lysis of the cells on the membrane provided reproducible light output (data not shown).

Samples for the measurement of chlorine concentration, light output, and colony forming units were withdrawn at specific times following the addition of chlorine as described in Materials and Methods.

The percent survival of *M. avium* strain A5/pLUC10 at two different chlorine concentrations as reflected by decreases in CFUs is shown in Figure 7. The percent survival of *M. avium* strain A5/pLUC10 at two different chlorine concentrations as reflected by decreases in relative light units is shown in Figure 8. In the figures, the percent survival is plotted as a function of time and the different cultures at different chlorine concentrations are represented by the individual data series as indicated in the legend.

The decline in *M. avium* colony forming units (Figure 7) was similar to that represented by relative light units (Figure 8). The susceptibility of *M. avium* strain A5/pLUC10 to chlorine was the same as the susceptibility of *M. avium* strain A5 (Taylor, 1998). Thus, it appears that neither the presence of the pLUC10 plasmid, nor the growth of the bacteria in the presence of kanamycin, affected the susceptibility of the bacteria to chlorine.

The relationship between relative light units and colony forming units for 4 independent disinfection experiments including data shown in Figures 7 and 8 is presented in Figure 9. Relative light units are plotted as a function of colony forming

units, since the light output should be dependent on surviving cell numbers. A linear relationship existed between the log of colony forming units and the log of relative light units of *M. avium* strain A5/pLUC10 exposed to different concentrations of chlorine from 0.9 mg chlorine/L to 1.5 mg chlorine/L over different times from 30 seconds to 60 minutes. The range of this relationship extended from 2.5×10^3 CFU/ml to 3.5×10^5 CFU/ml. In order to increase the range to measure the point at which 99.9% killing of *M. avium* strain A5/pLUC10 was achieved, greater concentration of the cells after disinfection was necessary. Since cells are concentrated on a filter by vacuum-filtration, greater concentration could have been achieved by increasing the volume passing through the filter. Based on the correlation between CFUs and RLUs ($R^2 = 0.96$), a reasonable prediction of colony forming units could have been made from the relative light units, thereby reducing the turnaround time for results for studies involving aggregated cells as well.

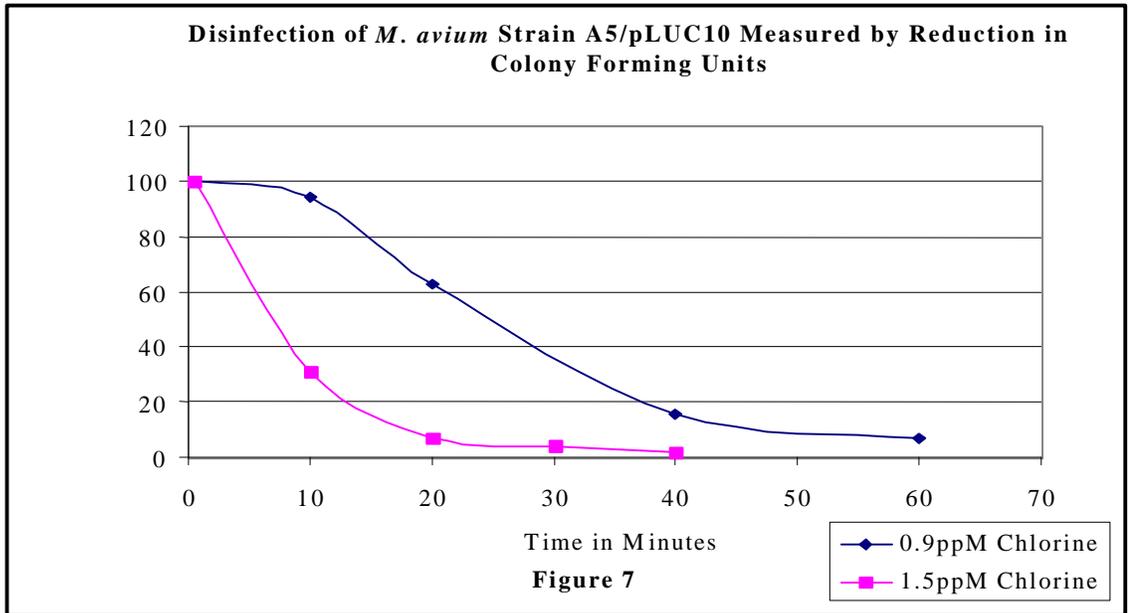


Figure 7: The survival of *M. avium* strain A5/pLUC10 at different chlorine concentrations as indicated by the figure legend is shown. The percent survival is based on the number of colony forming units remaining as compared to the original colony forming units.

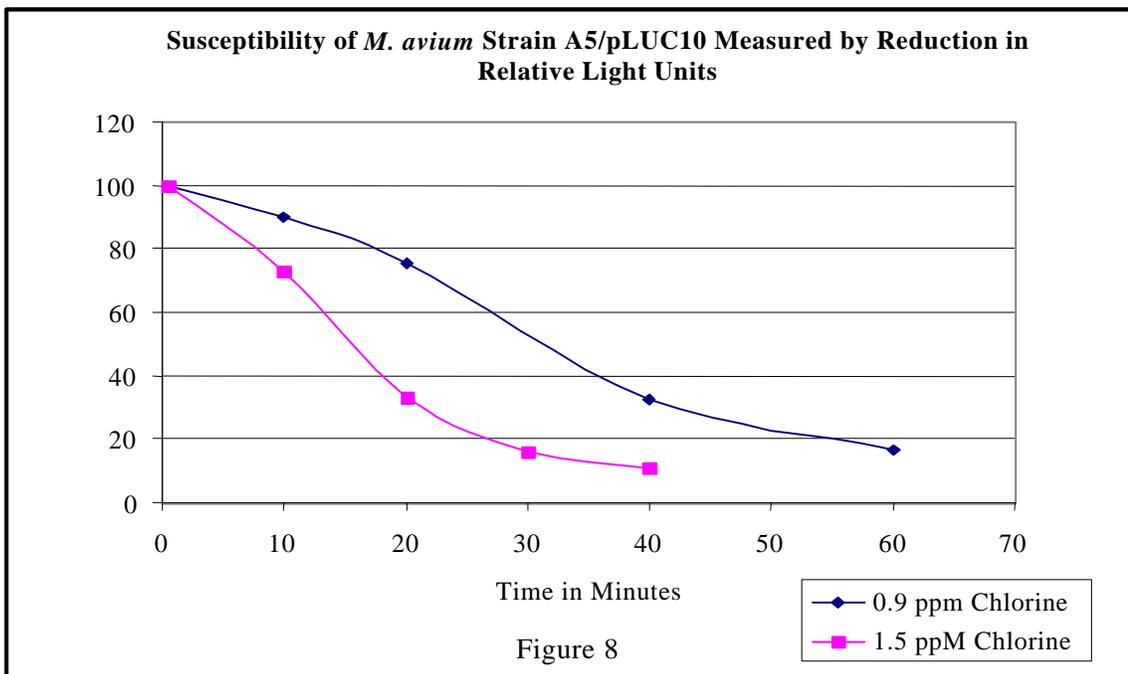


Figure 8: The survival of *M. avium* strain A5/pLUC10 at different chlorine concentrations as indicated by the figure legend is shown. The percent survival is based on the number of relative light units remaining as compared to the original colony forming units.

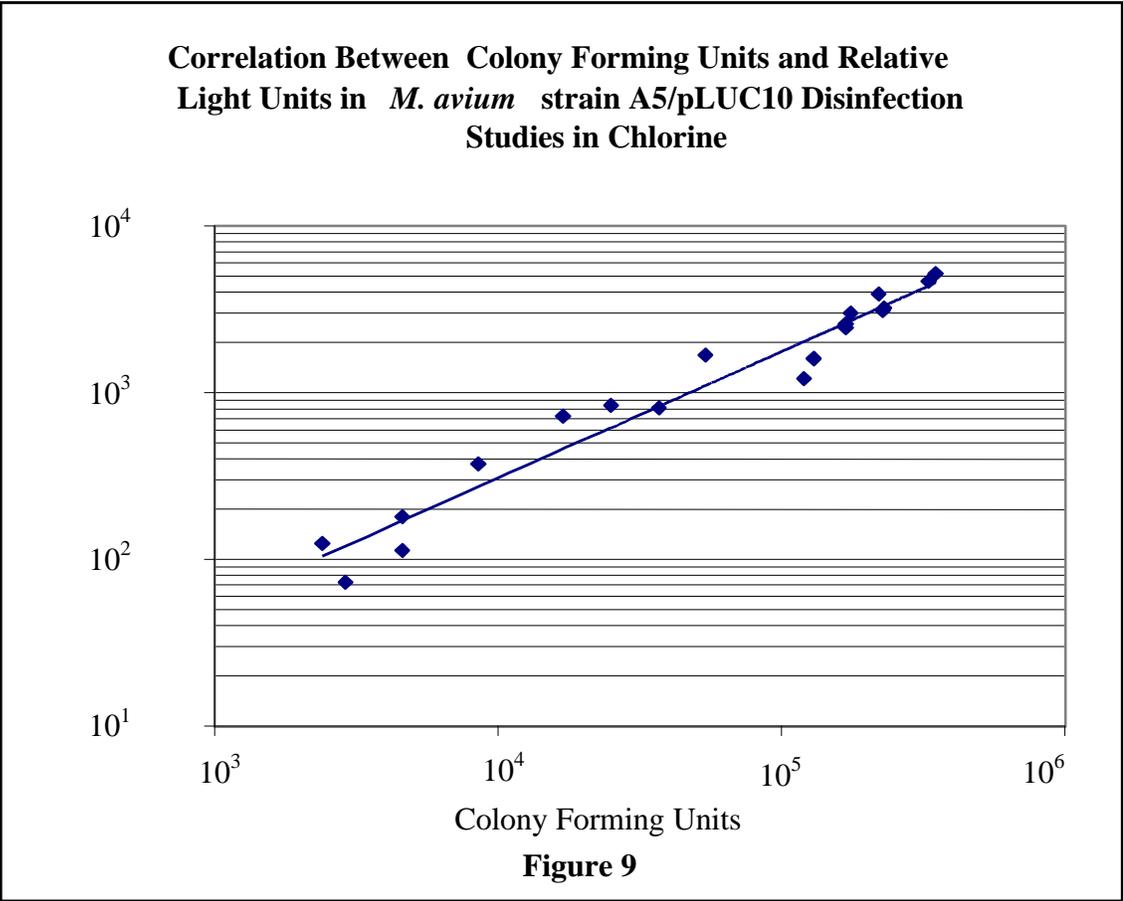


Figure 9: The linear correlation between the CFU and the RLU data from disinfection experiments is shown. Data represents four separate experiments at four different chlorine concentrations from four independently prepared RAF with samples taken at times between zero and 60 minutes. Note that the relationship is linear on a non-log scale graph, but that the data is more clearly represented on a graph where both the x and y axes are logarithmic.

Lack of Induction of *M. avium* Chlorine-Resistance

Because *M. avium* is slow growing, there would be sufficient time for induction of any possible chlorine resistance genes to be expressed before the lethal effects of chlorine. To look for induction of protective mechanisms in *M. avium* strain A5/pLUC10, diluted RAFs were incubated in the presence or absence of 0.1 mg chlorine/L for 2 or 15 hours at 37°C. The average percent survival of induced versus non-induced cultures in the presence of 0.9 mg chlorine/L is presented in Figure 10. Figure 10 contains data representative of additional experiments performed in the evaluation of whether or not resistance mechanisms could be induced by pretreatment with low levels of chlorine.

Inducible chlorine resistance was not observed based on the survival curves for both relative light units and colony forming units. Had there been induction of chlorine resistance, the curve for the induced cultures, both in CFU and RLU, would have had higher percent survival as compared to the non-induced cultures. Though the percent survival of cultures was nearly identical when examined by the same method (e.g. RLU or CFU), the comparison between methods varies greatly.

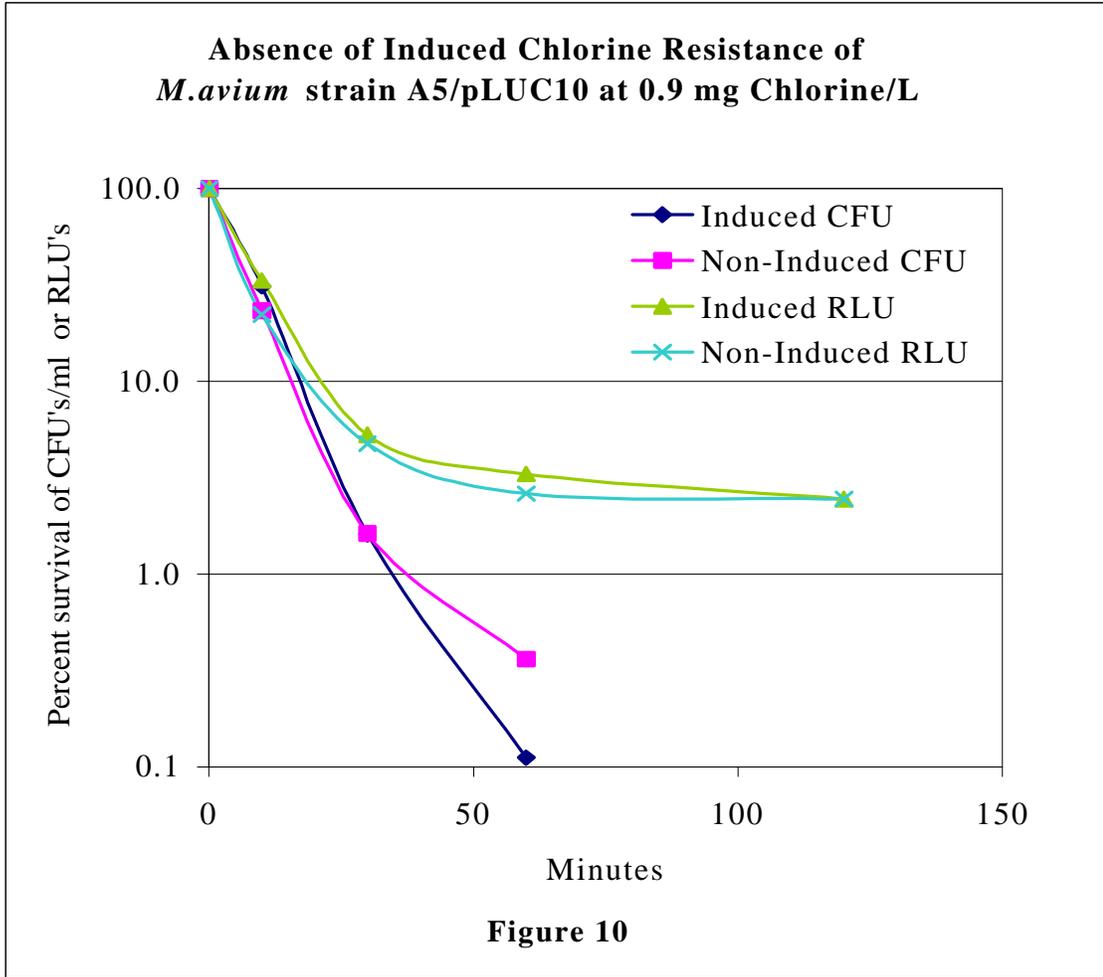


Figure 10: The percent survival of CFU and RLU for both induced and non-induced RAF of *M. avium* strain A5/pLUC10 is shown. The log of the percent survival is on the y axis, and the time is indicated on the x axis. The figure legend in the graph indicates either CFU or RLU for either an induced cell suspension or a non-induced cell suspension.

SUMMARY

Development of Recombinant Mycobacterial Strains Containing pLUC10

Recombinant strains of *Mycobacterium smegmatis* strain VT307, *M. avium* strain A5, *M. avium* strain MD1, and *M. bovis* BCG strain Pasteur expressing the *luc* gene located on pLUC10 were constructed. The phenotypic characteristics of the strains were unaltered by the presence of the plasmid based on their cellular morphology, colony morphology and growth curve, as was observed by other researchers who worked with pLUC10 (Cooksey *et al.*, 1995). Recombinant *M. smegmatis* strain VT307 and *M. avium* strain A5 were good models for the evaluation of this assay because of their stable expression of the luciferase gene when grown in the presence of selective pressure.

Improvement of a Commercial Luciferase Assay to Increase Light Output of Lysates

A commercial luciferase assay was used to measure the luciferase activity of mycobacterial lysates due to the extended half-life of luciferase activity of several hours. Because of this, the ease of measurement of this assay was vastly improved over other assay methods. The light output of the commercial assay was not at its maximum when examined for limiting factors of the light-producing reaction. Adding an additional 1mM luciferin increased the light output 5-fold, and was adopted into the protocol for all subsequent assays because of the increased sensitivity of the assay. Additional ATP was not found to improve the performance of the assay. Cell lysates had no inhibitory effect on enzyme activity, in fact, the presence of the lysate enhanced the activity of the enzyme.

The commercial assay used a detergent to lyse the cells. This was effective in *M. smegmatis*, where the detergent lysed the majority (90-99%) of all cells. Unfortunately, the detergent alone was not suitable for lysis of *M. avium*, as indicated by the 20-30% lysis of early log cells. Complete lysis of the suspension was not achieved and represents one way in which this assay might be improved for use with *M. avium*. Perhaps the addition of an enzyme or chemical that breaks down mycolic acids could improve the lysis of *M. avium* without reducing the activity of the enzyme.

Reduced Aggregation of Cell Suspensions

Cell suspensions were prepared with a significantly reduced population of aggregates and contained no aggregates large enough to interfere with cell killing in disinfection assays. The suspensions were prepared in such a way that the large aggregates were removed from suspension (<1% of the total cells were in large aggregates) and only small aggregates and single cells remained (92% single cells in *M. smegmatis* and 78% single cells in *M. avium*). This resulted in a cell population of primarily single cells for use in disinfection studies. Filtration of *M. avium* did not yield a suitable cell suspension since the single cells did not efficiently pass through a 5 µm pore size filter because the single cells were trapped by the filter (data not shown). In contrast, filtration was effective in *M. smegmatis* and resulted in a higher cell population compared to the *M. avium* suspension. The need for and rationale behind such a suspension is presented in Appendix A.

Disinfection of *M. smegmatis* strain VT307/pLUC10

The validity of the luminescence assay as a method of enumeration was confirmed with the preliminary *M. smegmatis* disinfection studies. The light signal decreased at the same rate as the decrease in colony-forming units. This indicated that as the cell was inactivated, so was the luciferase. These results provided support for the hypothesis that the light output of the recombinant strains under stress would be proportional to colony-forming units.

Conclusive data on the disinfection of *M. smegmatis* was not achieved since the cells were inactivated rapidly at the concentrations of chlorine used in the studies. Unfortunately, the threshold of sensitivity of the chlorine concentration test was such that accurate measurements at lower concentrations were impossible. Additionally, maintaining the concentration of chlorine at these low levels in the presence of cells was all but impossible because the inactivated cells formed a sink for the reactive chlorine and quickly removed it from solution.

Disinfection of *M. avium* strain A5/pLUC10

The number of cells in a suspension used for disinfection studies was limited by the stability of the chlorine concentration over time to a maximum of 10^6 colony forming units/ml. The minimum number of CFU/ml required for a light signal above background noise was 3×10^4 CFU/ml. Thus, cells in suspension would have to be concentrated in order to measure a decrease in light output.

Using the protocols developed for disinfection and subsequent light measurement, the susceptibility of *M. avium* strain A5/pLUC10 was examined. The light output decreased in proportion to the number of cells over time. Further, a linear correlation existed between the colony forming units and relative light units with a range of 2.5×10^3 CFU/ml to 3.5×10^5 CFU/ml when concentrated. The correlation was determined to be linear based on its R^2 value of 0.96. This range was not high enough to examine for 99.9% inactivation of the cells in suspension. Thus, to be used in studies of industrial significance, the range would have to be expanded either by more complete lysis of the cells or by greater concentration of the cells.

Disinfection of *M. avium* strain A5 at stationary phase had an inactivation value of 90% at 1 mg chlorine/L in 60 minutes (Taylor, 1998). This is a very similar value to the inactivation value of 90% at 0.9 mg chlorine/ L after 60 minutes observed for *M. avium* strain A5/pLUC10 in early log phase. Thus, it is likely that the presence of the plasmid does not influence the susceptibility of the bacteria to chlorine.

Future Research Possibilities

The assay described herein has some very unique advantages over traditional methods of enumeration for the study of aggregated cell suspension disinfection. An aggregated cell suspension is a suspension of cells in clumps of a specified size. The cells in the RAF were concentrated, hence, aggregated, on the membrane prior to examination for light output. Thus, the linear relationship between light and colony forming units is independent of the single-celled nature of the RAF and exists even though the cells were aggregated. One question remaining is whether or not cells inside

clumps survive disinfection based on their unique physiology, protected environment, or both.

Another possible application for the recombinant strains would be in examination of contact-type disinfectants like phenolics and quaternary ammonium compounds. To do this, a specific number of cells could be concentrated on a membrane and then the disinfectant applied. Then the membrane could be washed and treated to examine the change in light output against controls to measure inactivation of the cells. This kind of assay would not work well with conventional enumeration methods because the cells would be hard to retrieve off the membrane reproducibly for colony-forming units. It would work well with the luminescence assay since the cells could be examined from the membrane in the same way that they are examined for disinfection studies of RAFs. In this way, disinfectants used for cleaning non-autoclavable equipment such as bronchoscopes could be more effectively used to prevent the spread of disease.

Examination of biofilms could be improved with the use of the luminescent assay described herein. A culture would not need to be pure in order to enumerate the number of recombinant bacteria present. Thus, mixed biofilms, a more accurate representation of the biofilms found naturally, could be examined for growth and disinfection under a variety of conditions quickly and accurately. Further, the examination of such systems would be free of the problems of removing additional organisms through traditional decontamination methods.

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APPENDIX A:

Aggregation as a Factor in the Examination of Disinfection of Mycobacteria

I. Introduction

Aggregation of Mycobacterial Cells

Aggregation is defined as the localization and attachment of cells to one another. Aggregation or 'clumping' behavior has been observed for many microorganisms and under a variety of conditions. Some bacteria may form aggregates by producing a capsule of polysaccharides (e.g., *Klebsiella pneumoniae* or *Streptococcus mutans*) while other bacteria may form aggregates through filamentous growth (e.g., *Actinomyces* or *Mycobacteria*) (Tortora *et al.*, 1995). Aggregation has been observed in cultures of a variety of mycobacterial species including *Mycobacterium paratuberculosis* (Grant *et al.*, 1996), *Mycobacterium tuberculosis* (Ratledge and Stanford, 1982), and *Mycobacterium avium* (McCarthy and Ashbaugh, 1981). Considering the physiochemical properties of the genus *Mycobacterium*, e.g. cell wall composition and filamentous growth, it is unlikely if any of the members therein would lack aggregates.

Proposed Mechanisms of Mycobacterial Aggregation

There are intrinsic physiochemical properties of mycobacterial cells that may lead to aggregation. Mycobacteria are hydrophobic, and the interaction of these cells with other nonpolar surfaces may lead to the formation of aggregates and biofilms. Further, mycobacteria grow in filaments providing a hydrophobic center to which other mycobacteria may adhere.

Mycobacteria are hydrophobic (Stormer and Falkinham, 1994). Consequently, in aqueous solutions they are dispersed from the bulk medium toward areas of lesser polarity. These areas include other mycobacterial cells, the liquid-air interfaces in the culture or suspending medium (Parker *et al.*, 1983), and the surface of the aqueous medium (Wendt *et al.*, 1980). The concentration of hydrophobic cells in an aqueous system is caused both by hydrophobic interactions between the cells and because water bonds strongly to itself, thus creating an ordered 'cage' around the cells driving the cells together (Stryer, 1995).

Mycobacteria grow in filaments. Their growth cycle includes an elongation phase, followed by a septation phase, at which point the mother cell becomes two or more daughter cells (McCarthy and Ashbaugh, 1981; Rastogi and David, 1981). Mycobacteria often do not separate at this stage. Rather, long filaments of daughter cells can be observed, and physical shock could separate these filamentous forms, thus greatly increasing the particle count as observed microscopically (Rastogi and David, 1981). This failure of daughter cells to separate after division may lead to the formation of nonpolar nuclei within the culture. In older cultures, these nuclei will both grow and adhere to other nuclei, thereby creating large aggregates, or 'floating microcolonies'.

Characteristics of Mycobacterial Aggregates

Aggregation behavior differs widely within mycobacteria. Even within a species (such as *M. avium*), a large degree of variability can be seen in clumping behavior. Some *M. avium* strains produce a turbid suspension with microscopically observable aggregates, whereas others produce such large aggregates that they fall out of suspension and are visible to the naked eye (Parker *et al.*, 1983).

Aggregates may be separated into categories. Clumps of <5 cells may be considered as single cells, since they are either single cells or a group of daughter cells and will behave as single cells in culture. Large aggregates, those with >25 cells, are important in that they may provide protective value to their inner cells when under stress. Small aggregates, clumps of 6-25 cells, are not single cells because of their size, but neither do they share the protective value of large aggregates. Thus, they have their own category.

Mycobacterial aggregates tend to be pleiomorphic, generally appearing as irregular masses of filaments of variable population sizes (Rastogi and David, 1981). In washed cultures of *M. avium* strain A5 /pLUC10 from early log phase, the majority, 76%, of the total cell numbers exist in aggregates of larger than 25 cells. Only about 13% of the total cell number exist as single cells or in aggregates containing less than 5 cells.

Some proportion of cells in aggregates exist in a different microenvironment than do single celled cultures. Aggregates create a diffusion gradient, with the inner cells having the least contact with the environment. As a result, the cells within would have an

environment of reduced oxygen tension and lower concentrations of nutrients. It has been shown that physiological changes are observed for mycobacterial cells under low oxygen tension such as the induction of stationary phase (Cunningham and Spreadbury, 1998) and virulence (Bermudez *et al.*, 1997).

Aggregates may contribute to the survival of mycobacteria exposed to disinfectants. Since the effectiveness of a disinfectant is dependent on the concentration to which the cell is exposed, the diffusion barrier presented by the aggregate may create enhanced survival for cells contained within the aggregate. For highly reactive disinfectants like chlorine, chloramine, chlorine dioxide, and ozone, the cells at the surface of the aggregate may provide a 'sink' for the disinfectant, reducing its effective concentration inside the aggregate. The formation of a shield of dead cells with reactive organic matter (e.g. proteins and fatty acids with SH and double bonds) would protect the cells at the innermost layer of the aggregate. Additionally, stress may induce physiological changes, and cells within an aggregate will have a lower exposure and more time to respond to a disinfectant by inducing protective mechanisms. For example, increased levels of *aphC*, the gene encoding a subunit of alkyl hydroperoxide reductase, is inducible in the presence of peroxides in *Mycobacterium smegmatis* (Dhandayuthapani *et al.*, 1996).

Enumeration Difficulties Caused by Aggregation

Cells in aggregates present problems when attempting to enumerate individual cells. Using standard spread-plating techniques, aggregates of cells may yield a single colony-forming unit, as might a single cell. Conversely, the aggregate may be separated by shearing forces during spreading by using slightly drier plates, producing many colonies (Parker *et al.*, 1984). Thus, to obtain accurate and reproducible plate counts, it is desirable to plate a suspension with minimum aggregates. Direct observation of cell numbers is possible, but it is not feasible for disinfection studies due to the inability to microscopically distinguish live from dead cells.

Objectives

With this exercise, we intend to justify the need for using suspensions that consist of cells (<5 cells) and no more than 25% small aggregates (6-25 cells). The need to avoid problems associated with aggregates is illustrated above, and we will show mathematically that aggregates in the reduced aggregate suspension are insignificant in disinfection experiments.

II. Mathematical Exploration of the Potential Consequences of Aggregation in the Collection of Data by Colony Forming Units

Objective:

Demonstrate the improvement in data collection using a reduced-aggregate fraction compared to data collection using a washed culture.

Method:

Calculate the resulting colony forming units based on equations developed using the following assumptions and conditions:

1. Assumption #1- A clump will form one colony
2. Assumption # 2- A clump will form as many colonies as there are cells.
3. Condition # 1- A reduced-aggregate fraction which of the following proportion of cells; 75% single cells, 25% cells in small aggregates of fewer than 25 (each small aggregate will be counted as 25 for the maximum effect).
4. Condition # 2- A washed culture consists of the following proportion of cells; 20% single cells, 15% cells in clumps of fewer than 25, 65% in large aggregates of greater than 25 (We will use 100 as a baseline figure since most mycobacterial large aggregates appear to be about this size.)
5. Cells in clumps of 100 or more will survive disinfection using these assumptions and this formula:
 - A. Assumption A: 50% of cells will not be exposed to chlorine and will automatically survive disinfection.

B. Assumption B: 50% of the cells in a large aggregate will react to chlorine as do single cells.

C. With these assumptions, we equate the following for the survival of cells in large aggregates (SCLA):

$$(SCLA) = [0.5 (\# \text{ Cells in Clump}) + (\text{Percent Survival}/100)(0.5)(\text{Number of Cells in Clump})]$$

Equations for the Resulting Colony Forming Units

Equations for the combination of the assumptions and conditions above were formulated. These formulations also incorporate the effect that different amounts of survival will have on the resulting data (e.g. the percent survival impact on CFU). The population examined was limited to 10,000 for simplification of the model. For assumption #1, a clump will form only one colony, and the number for the resulting colonies cannot exceed the number of the original clumps. Thus, equations incorporating assumption #1 will have this distinction. For example, a reduced aggregate fraction of 10,000 total cells under condition 1, assumption 1, will have 7500 cells and 100 clumps of 25 cells each. If 75 % survival is the actual survival, the recorded survival in colony forming units would be 56.25 % because the small aggregates, n=100, could only form 100 colonies despite their cell number.

Equation for Assumption 1, Condition 1: For 10,000 cells

$$CFU = (\% \text{ Survival}/100)(\text{single cells}) + [(\% \text{ Survival}/100)(\text{Number of Cells in Clump}) \text{ this value cannot exceed the total number of clumps}]$$

Equation for Assumption 2, Condition 1: For 10,000 cells

$$CFU = (\text{Percent survival}/100)(\text{single cells}) + (\text{Percent Survival}/100)(\text{Number of Cells in Clump})$$

Equation for Assumption 1, Condition 2: For 10,000 cells

CFU= (Percent survival/100)(single cells)+[(Percent Survival/100)(Number of Cells in Small Aggregates) this value cannot exceed the total number of small aggregates]+[(SCLA) this value cannot exceed the total number of large aggregates]

Equation for Assumption 2, Condition 2: For 10,000 Cells

CFU=(Percent survival/100)(Number of single cells)+(Percent Survival/100)(Number of Cells in Small Aggregates)+(SCLA)(Number of Large Aggregates)

The percent survivals used in the mathematical model were: 100%, 75%, 25%, 12.5%, 4%, 2%, 1%, and 0.5%. For large aggregates, 0.1% was also used. Note that this mathematical model assumes 100% plating efficiency of viable organisms, i.e. all cells surviving are also capable of forming colonies on plates.

Results:

The results of the mathematical model are very interesting. Table A shows the number of colony forming units calculated for the percent survival of each of the possible combinations. The combination of Assumption 2 Condition 1 represents the number of colony forming units we would expect to see if all of the cells reacted individually in their plating and in their survival characteristics. Note that the effect of small aggregates will affect the culture only until the number of aggregates is equal to the number of surviving cells. For example, 1 cell makes up 4% of the size of a small aggregate. At 4% survival and lower, the resulting colony forming units will be the same as the actual cell numbers.

Table 7: The resulting colony forming units of a mycobacterial culture were calculated according to the different assumptions and conditions discussed in the Method section. The condition (e.g. a washed culture or a reduced-aggregate fraction) will determine the number of single cells present. The assumption (e.g. a clump forms one colony) pertains to the number of colonies resulting from a clump of a given size.

Table 7				
Resulting Colony Forming Units from Predetermined Percent Survival				
Percent Survival	Assumption 1, Condition1	Assumption 2, Condition 1	Assumption 1, Condition 2	Assumption 2, Condition 2
100	7600	10000	2125	10000
75	5725	7500	1625	8312
50	3850	5000	1125	6625
25	1975	2500	625	4937
12.5	1037.5	1250	375	4093
4	400	400	205	3520
2	200	200	135	3385
1	100	100	100	3317
0.5	50	50	85	3283
0.1	ND	ND	68	3256

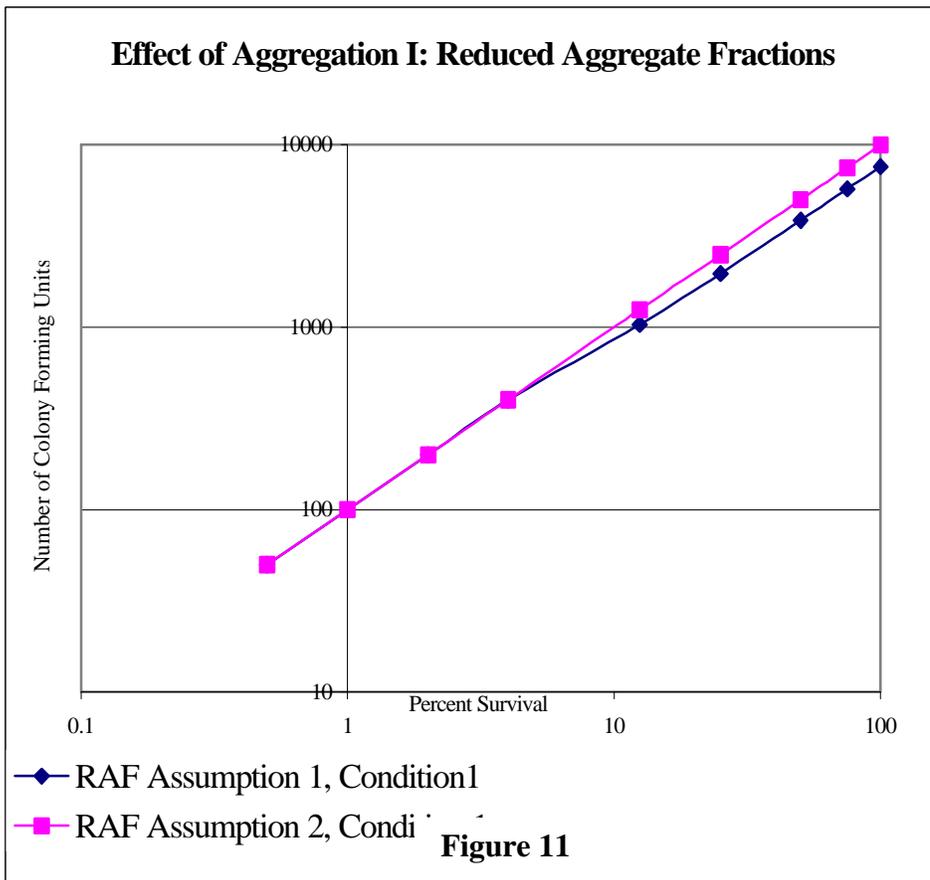


Figure 11: The graph represents the effect that a RAF would have on Colony Forming Units. The blue data series represents a culture in which each clump forms only one colony and the red data series represents a washed culture in which a clump forms as many colonies as there are cells within the clump.

The reduced aggregate fraction does not appear to have a major effect on the outcome of the colony forming units if even if the aggregates form only one colony forming unit rather than 25 colony forming units. Data below 4 % is identical to what would be observed if each cell behaved independently as exemplified by Condition 1, Assumption 2 (Figure 11), any percent survival for a reduced aggregate fraction of 4 % or below could be considered actual cell survival based on this model.

The curves of the two assumptions for the washed culture are significantly different from the curve for single cells (Figure 12). The effect of survival of cells within aggregates can be seen immediately when these surviving cells form individual colonies as they would in Assumption 2. Under this assumption, all of the cells that survive within large aggregates will form colony-forming units. This is a substantial portion of the population given that 65% of the population exists in large aggregates. Conversely, if the cells that survive in large aggregates can only form 1 colony per aggregate (Assumption #1), then there is about a 79% difference between the resulting colony-forming units (2125) and the colony-forming units that would result from a single celled culture (10000) at 100% survival. This means that the estimate of original cell population would be underestimated by 79% even before a disinfection study began. Further, as the percent survival drops below 1 %, there will always be surviving colony-forming units due to the protective nature of large aggregates.

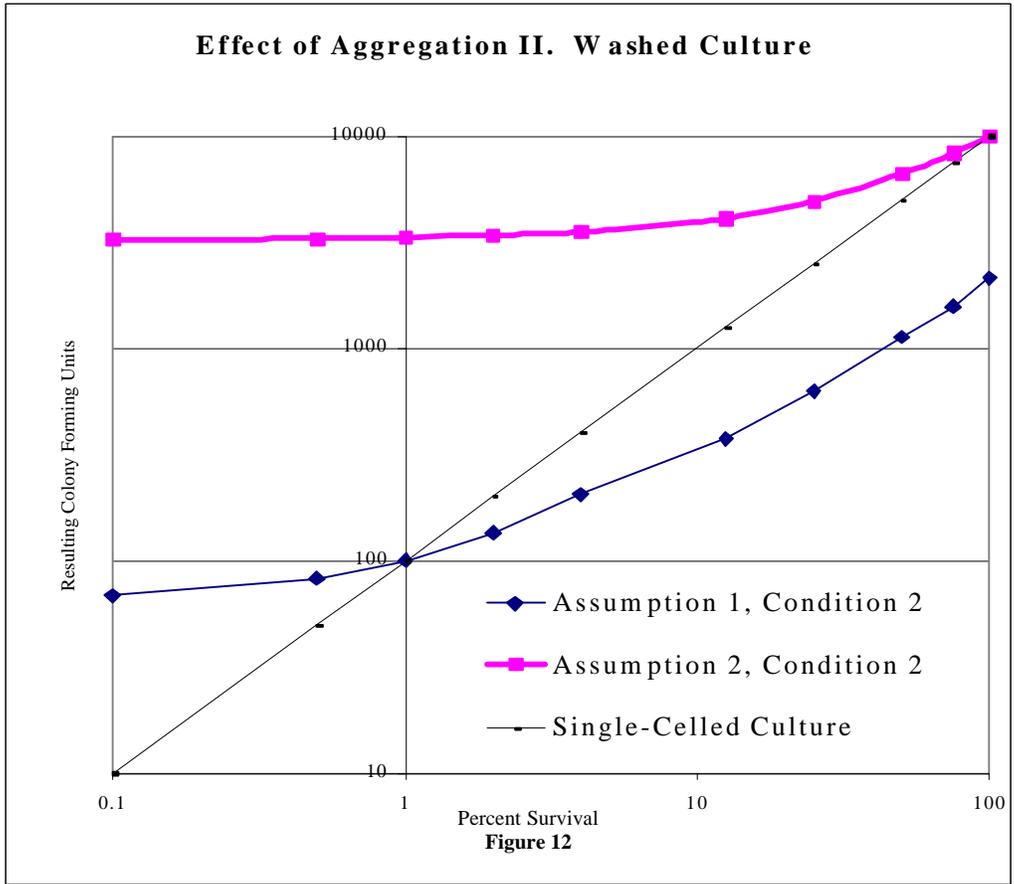


Table 12: This graph represents the effect of cell survival in clumps as observed in a washed culture. The blue data series represents a culture in which each clump forms only one colony and the red data series represents a washed culture in which a clump forms as many colonies as there are cells within the clump.

Summary

The reduced aggregate fraction composition is undoubtedly superior to the washed culture for the measurement of disinfection. Though the numbers presented here represent estimates of survival in large aggregates, the effect of these aggregates in actual experiments is likely to lead to inconsistent, inaccurate, and confusing data. Further, the effect of plating represents a best case scenario in which all cells will form individual colony-forming units, and a worst case scenario in which all cells in an aggregate will form one colony-forming unit. The actual effect of plating is likely to reside between the two, but the importance of understanding the difference between the two can not be overstated.

This work provides evidence that work, which claims to measure disinfection using aggregated cultures, should be treated with skepticism. It also provides support for the need for single-celled disinfection as a baseline for disinfection data. This work also demonstrates the value of the reduced-aggregate fraction as a tool in disinfection studies of mycobacteria.

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Grant: Graduate Research and Development Project- Funded 1997
Seminar: Rapid Detection and Identification of Mycobacterial Infection. Nov. 5, 1997
Member, American Society for Microbiology
Supervisor of Undergraduate Research
Poster Presentation at ASM General Meeting May, 1998
Project: *A Rapid, Quantitative Assessment of Anti-mycobacterial Water Disinfection based on the Firefly Luciferase Reporter Gene.*
Instructor Position, Randolph Macon Women's College, to begin July 23, 1998

Experience:

1/97 to Present Graduate Teaching Assistant - General and Principles of Biology
Instruct students as to the fundamental aspects of Biology, taking into consideration varying backgrounds in the subject. Emphasize the use of the scientific method as an approach to problem solving. Develop and implement an evaluation procedure for students in the laboratory.

Graduate Student - Department of Biology, Mycobacteriology. Skills include: Supervision of Undergraduate Research, DNA Isolation and Analysis, PCR, Cloning, Crude Extract Protein Analysis, Reporter Gene

Analysis, Culture Maintenance, General Laboratory Maintenance, and Data Collection.

8/96 to 1/97

Graduate Teaching Assistant - Microbiology

Instruct students in the essential theories and skills in a Microbiology laboratory. This includes; isolation, identification, quantification and maintenance of bacterial cultures. Relate the importance of microbiology to everyday living. Focus on industrial uses as well as clinical manifestation. Emphasize the student's critical thinking and problem-solving skills. Evaluate students based on the objectives of the department taking into consideration written communication and performance on practical and written tests.

1/96 to 8/96

Graduate Teaching Assistant - Principles of Biology

8/94 to 1/96

Director of Marketing - Diamond Healthcare

Allegheny Regional Hospital, Partial Hospitalization Program.