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MYCOBACTERICIDAL TESTING OF CHEMICAL GERMICIDES: COMPARISON OF CONVENTIONAL CULTURE WITH A REPORTER GENE ASSAY

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

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In 1908, Leonard Williams wrote, in reference to Koch's discovery, that 'the riddle of the white plague, which had so long defied solution, had been read at last; the dreary watches of the night were over; and the dawn, with its promise of victory, peace and purity were really at hand'. Will this hope soon be translated into reality or will, in the words of Reginald Bignall, a distinguished Editor of *Tubercle*, the struggle against the mycobacteria be 'a long, long war'.

C.H. Collins and J.M. Grange.
Mycobacterial disease-old problems, new solutions. J. Appl. Bacteriol. Symposium Supplement. 1996. 81: viiS-viiiS.

ABSTRACT

Infections caused by members of the genus *Mycobacterium*, particularly *Mycobacterium tuberculosis* (TB), are responsible for significant morbidity and mortality worldwide. Cases of infections by *M. leprae*, the other well-known species of this genus, have been on the decline over the past few years. On the other hand, nontuberculous mycobacteria (NTM), which are found in the environment, are increasingly being implicated in human disease, especially in the immunocompromised.

There is concern about the escalating risk of spread of *M. tuberculosis* and NTM through the increasing use of semi-critical medical devices such as flexible endoscopes and several cases of iatrogenic infection have already been documented in recent years. This concern is further reinforced by the realization that current methods for testing the mycobactericidal activity of high level disinfectants are slow, labor-intensive, non-quantitative and use unsuitable surrogates.

Therefore, the first objective of this study was to adapt a quantitative carrier test for sporicidal activity developed in our laboratory to assess the mycobactericidal activity of chemical germicides. This method was carried out using 10 µL of a standardized test suspension of mycobacteria combined with a soil load which was inoculated and dried on flat-bottomed glass vials to simulate in-use conditions for the products tested. The dried inoculum in each test carrier was exposed to 1 mL of the germicide and the controls to the same volume of normal saline. The tests and controls were diluted with 9 mL of saline, passed separately through a membrane filter and the filters rinsed with saline to wash any residue of the germicide. The filters allowed consistent recovery of the input

inocula when placed on the recovery medium and incubated for colonies to appear. A quantitative assessment of the mycobactericidal activity of the germicide was made by comparison of colony forming units (CFU) in the tests and controls.

The quantitative carrier test was tested with seven different species of mycobacteria, including *Mycobacterium terrae*, using five types of germicides. The results expressed in $\log_{10}$ reductions in CFU, revealed the differences in the susceptibility of the tested species to the germicides. The flaws in the current methods were addressed in the quantitative carrier test and the findings demonstrate the suitability of this method in the assessment of mycobactericidal activity of chemical germicides. Although the results were found to be accurate and reproducible, it required 4 weeks or more of incubation to obtain results with the slow-growing mycobacteria.

To address this issue, we investigated approaches based on reporter genes that would generate results in a shorter time period. Recent developments in recombinant DNA technology have provided the means for introducing reporter genes into mycobacteria, thus prompting the application of such genes in assessing the activity of drugs and chemical germicides against them, while considerably reducing the turn-around time for results.

In the initial phase of this study, the firefly luciferase gene, capable of generating bioluminescence in transformed host cells, was introduced into *M. smegmatis* and *M. terrae* on the plasmid pYUB180. The transformed cells obtained, gave poor growth even after prolonged incubation and the luciferase system was also found to have other drawbacks, such as the need to add a substrate and lyse the cells prior to measuring

bioluminescence. In view of these limitations, this line of investigation was not pursued any further and the focus of our research shifted to using another reporter gene.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is a convenient reporter molecule that allows direct and repeated measurements of cell viability without the need for any substrates or cofactors. The plasmids pWES4 and pBEN containing the genes encoding, respectively, a wild type (wt) and a red-shifted, higher-intensity GFP variant, were introduced into *M. smegmatis* and *M. terrae*. GFP expression was observed in transformed mycobacteria as green fluorescence using epi-fluorescence microscopy. Mycobacteria containing the plasmid pBEN were chosen for the red-shifted, higher-intensity fluorescence with consequent ease of detection and measurement in equipment with standard FITC (fluorescein isothiocyanate) filter systems. In a comparative study using a culture method, the reporter strains of *M. smegmatis* (pBEN) and *M. terrae* (pBEN) were found to have a slightly higher and lower resistance to germicides, respectively, than the corresponding parent strains.

The second objective of this study was to develop a fluorescence assay utilizing mycobacteria expressing GFP that would reduce the turn-around time in the testing of mycobactericidal activity of germicides. A GFP fluorescence assay using *M. terrae* (pBEN) was developed for screening germicides and evaluated by comparing it with the culture method. *M. terrae* (pBEN) exposed to formulations of hydrogen peroxide, acid and alkaline glutaraldehyde, ethanol and phenolic germicides and controls exposed to saline were diluted and washed with saline and centrifuged. The pellets were resuspended in a liquid culture medium, transferred to 4 mL clear glass vials and incubated. Fluorescence was measured directly from the glass vials every 24 hours for 7

days. RFU in the controls increased progressively to a peak in 2-4 days followed by a gradual fall. In the tests, lethal concentrations of germicides did not allow any increase in fluorescence above background levels. Weaker germicides suppressed GFP expression from several hours to several days after which expression indicated by fluorescence resumed at a rate similar to that of controls. Concurrent measurements of fluorescence and growth in controls and tests revealed a good correlation.

The response of fluorescent mycobacteria to germicidal challenge and its early detection and quantification provide a useful method for reducing the turn-around time in the screening of chemical germicides for mycobactericidal activity. However, further work is required to adapt the use of the GFP-expressing mycobacteria to a proper carrier test for mycobactericidal activity.

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I would like to thank my family for their continued support and encouragement. I thank my mother, Sharifunnessa Lashker, for her love and her efforts to guide her children into intellectual pursuits. I dedicate this thesis to the memories of my late father, Nisar A. Lashker, for giving me the opportunity to fulfill my quest for knowledge.

"If I have seen farther, it is by standing on the shoulders of giants".

(Sir Isaac Newton)

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LIST OF ABBREVIATIONS

ADC	Albumin dextrose catalase
AFB	Acid fast bacilli
AFNOR	Association Francaise de Normalisation (French Association for Standardization)
AIDS	Acquired immune deficiency syndrome
AOAC	Association of Official Analytical Chemists
aph	Aminoglycoside phosphotransferase
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
ATP	Adenosine tri phosphate
BCG	Bacillus Calmette-Guerin
BGA	German Federal Center for Health Education
bp	Base pairs
BSA	Bovine serum albumin
BSI	British Standards Institution
Cat	Chloramphenicol acetyl transferase
CCD	Charged Coupled Device (camera)
CDC	Centers for Disease Control and Prevention
CEN	Comite Europeen de Normalisation (European Committee for Standardization)
CFU	Colony forming unit
CGSB	Canadian General Standards Board
CPC	Cetylpyridinium chloride
CRAs	Chlorine releasing agents
DEFT	Direct epifluorescent filtration technique
DGHM	Deutsche Gesellschaft fur Hygiene und Mikrobiologie (German Society for Hygiene and Microbiology)
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EPA	Environmental Protection Agency (US)
ept	Efficient plasmid transformation
EtBr	Ethidium bromide
Et-OH	Ethyl alcohol (Ethanol)
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
gfp	Green fluorescent protein gene
GI	Growth index
HIRL	Hospital Infection Research Laboratory (UK)
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
ISO	International Organization for Standardization
IUAT & LD	International Union Against Tuberculosis and Lung Diseases

kb	Kilobase pair
kDa	Kilodalton
LacZ	β-galactosidase gene
LB	Luria Bertoni (medium)
LJ	Lowenstein-Jensen (medium)
Log	Logarithm
LRP	Luciferase reporter phages
lux	Luciferase gene
MABA	Microplate alamar blue assay
MAC	*Mycobacterium avium* complex
M-ADC-TW	Middlebrook 7H9 broth + 10% ADC + 0.05% Tween 80
M-7H11-OADC	Mycobacteria 7H11Agar + 10 % OADC + 0.5 % Glycerol
MAI	*Mycobacterium avium-intracellulare*
MAIS	*Mycobacterium avium-intracellulare scrofulaceum*
MDR-TB	Multidrug-resistant tuberculosis
MGIT	*Mycobacterial* growth indicator tube
MIC	Minimum inhibitory concentration
MOTT	Mycobacteria other than tuberculosis
MPB	Modified Proskauer-Beck (medium)
MW	Molecular weight
NCCLS	National committee for clinical laboratory standards
NTM	Nontuberculous mycobacteria
OADC	Oleic acid-albumin dextrose complex
PCR	Polymerase chain reaction
PPD	Purified protein derivative
ppm	Parts per million
QACs	Quaternary ammonium compounds
REA	Restriction enzyme analysis
RFLP	Restriction fragment length polymorphism
RFU	Relative fluorescence unit
RLU	Relative light unit
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TB	Tuberculosis
TE	Tris ethylene diamine tetraacetic acid
TNTC	Too numerous to count
Tris	Tris(hydroxymethyl)aminomethane
TTC	Triphenyltetrazolium chloride
UV	Ultra violet
WHO	World Health Organization
wt	Wild type
Z-N	Ziehl-Neelsen

1. GENERAL INTRODUCTION

1.1 Background

1.1.1 History and Epidemiology

Tuberculosis (TB), far from being conquered by modern methods of treatment, was declared a global emergency by the World Health Organization in 1993 (WHO, 1994a). TB infects 10 million people and kills 3 million each year (Raviglione *et al.*, 1995), making it the highest cause of mortality from a single infectious agent worldwide. Globally, TB is thought to be responsible for 25% of avoidable deaths in young adults (Murray *et al.*, 1990). TB has been traced as far back as the Neolithic period and reached epidemic proportions during the major periods of urbanization in the 18th and 19th century, winning TB the appellation the "White Plague".

Leprosy, the second important mycobacterial disease seems to have made its appearance in early history. Leprosy once affected every continent on the globe and engraved a terrifying image in history and human memory of mutilation, rejection and exclusion from society. Currently, leprosy remains a public health problem in developing countries or the 'poverty belt of the globe', although there has been progress in reducing this problem through improved surveillance, treatment and control measures (WHO, 1994b). In the beginning of 1998, the number of leprosy cases in the world was estimated to be 800,000 and the number of new cases detected during 1997 was reported to be 690,000 (WHO/Lep, 1998).

The discovery of the causative agents of TB and leprosy by Robert Koch in 1882 and G.H. Armauer Hansen in 1874, respectively, initiated the battle against these two evils that continues to this day. These diseases have major sociologic components, flourishing with ignorance, poverty, overcrowding, and poor hygiene and during the social disruptions of war and economic depression. Under these conditions, the poor are the major victims, but all sectors of society are at risk. In developed countries, the incidence of TB declined steadily until 1985, when the downward trend reversed itself due to a combination of factors (Table 1.1).

Table 1.1 Causes of resurgence of TB in the developed and developing countries.

Developed World	**Developing World**
Immigration	Demographic changes
HIV Infection	HIV Infection
Socioeconomic (poverty, unemployment, overcrowding, homelessness, under nutrition, drug addiction, etc.)	Socioeconomic (poverty, unemployment, overcrowding, homelessness, under nutrition, drug addiction, etc.)
Program decline	Poor programs
Increased life-expectancy	
Multidrug-resistant TB (MDR-TB)	Multidrug-resistant TB (MDR-TB)

Modified from Parry and Davis, 1996.

The resurgence of TB was first observed in the United States where the Centers for Disease Control and Prevention (CDC) estimated that since the mid-1980s, 39,000 cases of the disease occurred in excess of those expected if the pre-1985 downward trend had continued (CDC, 1992). In Canada, before the second world war, more than 14,000 new cases of TB were reported each year and over 17,000 patients were placed in TB sanatoria. The last of Canada's TB sanatoria was closed in the 1970s. Since 1987, the number of TB cases reported in Canada has remained constant. Approximately 2,000 cases are reported each year (Health Canada, 1996). In Canada, TB has, over the years, become increasingly concentrated in the foreign-born and in 1991, 50.1% of all reported cases occurred in such individuals. This population accounted for only 20% of TB cases in the early 1970s. The higher risk in foreign-born is due to two factors. First, the likelihood of having had TB previously but without adequate treatment, and second, the prevalence of past infection which confers a higher lifetime risk of developing active TB (Brancker *et al.*, 1992). TB accounts for as much as 40% of deaths in HIV-coinfected individuals in some developing countries (Abouya *et al.*, 1992). Adding to this, MDR-TB has emerged in several countries, with case fatalities ranging from 40-60% in immunocompetent individuals and >80% in the immunocompromised (Frieden *et al.*, 1993).

The risk of TB developing in individuals already exposed to the organism and exhibiting a positive tuberculin skin test (Purified protein derivative or PPD-Positive) depends on host factors which include the immunological status and the presence of any underlying disorder. Approximately 10% of PPD-positive individuals who are immunocompetent without any underlying disease will develop active TB sometime in

their lifetime. The relative risk in the same group is higher in the first two years following exposure than in subsequent years of their lifetime. Persons (PPD-positive) with compromised host defenses related to conditions defined as high risk in prior guidelines for prophylaxis (Diabetes, immunosuppressive therapy, tumors, silicosis, etc.), have about a 10-fold higher risk than the first category. At the extreme, the risk is about 100 times more in patients with HIV infection or the acquired immunodeficiency syndrome (AIDS) which has a significant role in the current resurgence of TB (Table 1.2).

Table 1.2 Relative risk of developing active TB in PPD-positive individuals with and without underlying conditions or immunosuppression.

Underlying conditions	**Relative risk of disease**
No underlying conditions (Immunocompetent)	1.0
Recent onset of TB infection (within 2 years)	15.0
Conditions defined as high risk in prior guidelines for prophylaxis (diabetes, immunosuppressive therapy, silicosis, tumors, organ transplantation, radiation etc.)	3.6 - 16
HIV infection or AIDS	13 - 117

From McGowan, 1993.

Whereas tuberculosis and leprosy have been scourges of mankind for centuries, other members of the genus *Mycobacterium*, usually referred to as nontuberculous mycobacteria (NTM) or mycobacteria other than tuberculosis (MOTT), were, until recently, considered to be harmless environmental saprophytes. Over the past few

decades, an increasing number of diseases caused by NTMs have emerged around the world, particularly in individuals with AIDS and other forms of immunosuppression (Wolinsky, 1992; Kirschner *et al.*, 1992). Nosocomial disease outbreaks caused by NTM have been recognized for more than 20 years and they continue to be a problem to this day (Wallace *et al.*, 1998). An increase in the reported number of nosocomial outbreaks and pseudo-outbreaks in recent years is attributed to several factors, including an increased awareness of the problem, better understanding of the epidemiology, reservoirs of NTM, and the mechanisms of outbreaks as well as to improved molecular methods of detection.

Before the introduction of antibiotics, disinfectants and antiseptics played an important role in the prevention and perhaps even treatment of many types of infections. The introduction and successful use of antibiotics relegated disinfectants to the background in the fight against bacterial and fungal infections. Now with the partial failure of antibiotics due to the emergence of resistant strains, antisepsis and disinfection have made a comeback. The transmission of mycobacteria from the environment and inanimate surfaces reinforces the importance of infection control practices, and in particular the proper disinfection of items in health care settings. Although scientific publications have covered techniques for testing disinfectants since the beginning of the 20th century, a general internationally accepted test scheme does not exist (Reybrouck, 1991).

Traditional methods for determining mycobactericidal activity of germicides are based on culture and require prolonged periods for results to become available, due to the slow growth of clinically relevant mycobacteria. Newer test methods excluding culture

are evolving to reduce the turn-around time. However, there is no single test suited for all germicides or organisms and the search for a better technique continues to this day.

1.1.2 Review of the Literature on Mycobacteria

The genus *Mycobacterium* comprises of at least 60 named species, classified and distinguished on the basis of nutritional and temperature requirement, growth rates, colony morphology and pigmentation, some key biochemical tests, cellular constellation of free fatty acids and range of pathogenicity in experimental animals. *Mycobacterium tuberculosis* complex includes the species – *M. tuberculosis, M. bovis, M. africanum* and *M. microti*, though they are without doubt variants of a single species (Rogall, 1990). Although *M. tuberculosis* is by far the most important human pathogen followed by *M. leprae*, NTM are assuming increasing importance as human pathogens.

1.1.2.1 Taxonomy

Mycobacteria comprise three major groups. The fast-growing mycobacteria have doubling times of 2-3 hours and will yield a colony from a single cell in 3-4 days. The slow growers double in number every 16-18 hours and thus yield colonies from a single cell in 14-28 days. There are lastly those species or strains such as *M. leprae* that have not yet been cultivated *in vitro*. In the system of Adansonian taxonomy, numerous cultural and biochemical properties were used to group most mycobacteria in distinct clusters corresponding to known species (Wayne *et al.*, 1978). The genus *Mycobacterium* was divided by immunodiffusion analysis into four antigenic groups according to the distribution of mycobacterial antigens (Stanford and Grange, 1974) and the results of this form of taxonomy correlate very closely with those of the Adansonian and genomic analysis.

Chromatographic analysis of lipid composition by thin-layer chromatographic, capillary gas chromatographic and high performance liquid chromatographic (HPLC) methods have been used for identification of mycobacteria (Nolte and Metchock, 1995). These methods provide definitive identification of an isolate in less than 2 hours, although the required time for adequate growth is extensive, and the procedure requires highly experienced technicians. HPLC analysis of species-specific mycolic acids is used by the CDC to detect and group uncharacterized mycobacteria. Uncharacterized mycobacteria are initially reported as no-common-pattern (NCP). When an NCP group contains a sufficient number of these isolates, taxonomic studies are initiated to characterize and name the mycobacteria (Tuberculosis/Mycobacteriology Branch, 1999).

Methods based on DNA technology such as DNA hybridization probes, restriction fragment length polymorphism (RFLP) and the polymerase chain reaction (PCR) are now increasingly used in mycobacterial taxonomy and identification (Marks, 1993). Restriction endonuclease analysis (REA) involves electrophoretic separation of DNA fragments following digestion of chromosomal DNA. The subtle differences between mycobacterial strains can be identified on the basis of a mobility shift in one or more fragments, or the loss or acquisition of a fragment.

A rapid method for differentiating medically relevant and other frequent laboratory isolates of mycobacteria involves PCR amplification of a 439-bp fragment encoding for the 65-kDa heat-shock protein using primers common to all mycobacteria. Restriction enzyme analysis of PCR products using *Bst*EII and *Hae*III allowed differentiation of mycobacteria to the species or subspecies level (Telenti *et al.*, 1993). RFLP involves transfer of DNA fragments from an agarose gel to a membrane by

Southern blotting, followed by hybridization with a specific probe. Since the probe will only hybridize to complementary DNA, the number of fragments detected by autoradiography is greatly reduced, simplifying the identification of differences between isolates. RFLP analysis has become the gold-standard molecular epidemiological marker for *M. tuberculosis*. RFLP typing schemes are less successful for other mycobacterial species and is an area of active investigation (Marks, 1993).

Ribotyping is a useful taxonomic approach based on the study of the genetically conserved DNA coding for the 16S ribosomal RNA, that most clearly separates mycobacteria at the species level (Rogall *et al.*, 1990).

1.1.2.2 Morphology and Structure

Mycobacteria are curved or straight rods, 0.2-0.6 × 1.0-10 μm in size. They are non-sporing, non-capsulated, non-motile bacilli. The most characteristic feature is their complex cell wall with a high lipid content (60%), which includes the large-branched mycolic acids. The cell wall makes them hydrophobic, rendering mycobacteria resistant to staining with basic aniline dyes. It also makes them resistant to breakage and relatively impermeable to chemicals including antibiotics and germicides. Mycobacterial cells tend to stick together when grown in liquid media and form macroscopic clumps. The cell membrane contains carotenoids which impart a bright yellow or orange color to bacterial colonies having sufficient quantities of these compounds. In some mycobacteria, the synthesis of carotenoid pigments depends on the presence or absence of light (Please refer to Table 1.3 and Figure 1.1 in the following pages).

Table 1.3 Distinctive properties of cultivable mycobacteria of medical importance.

This table shows some of the cultural characteristics of mycobacteria found in clinical specimens and the availability of nucleic acid probes for their identification. According to the rate of growth, mycobacteria are classified into slow (^{A}S), moderate (M), and rapid (^{A}R) growers. *M. haemophilum* requires hemin as an additional growth factor (S^{f}). Colonial morphology or appearance is grouped into rough (^{B}R), smooth (^{B}S), intermediate in roughness (SR), thin or transparent (t), and with filamentous extensions (f). According to the type of pigmentation, mycobacteria are classified into photochromogens (P) that produce nonpigmented colonies when grown in the dark and yellow to orange carotenoid pigment when exposed to light, scotochromogens (^{C}S) that produces deep yellow to orange pigmented colonies irrespective of the presence or absence of light, and nonphotochromogens (N) that do not produce pigment under any condition or have only a pale-yellow, buff, or tan pigment that does not intensify after light exposure. *M. szulgai* is an exception because it is scotochromogenic at 37°C and photochromogenic at 24°C.

Plus (+) and minus (-) signs indicate the presence or absence, respectively, of the feature.

Table 1.3 Properties of cultivable mycobacteria

SPECIES	GROWTH RATE[A] (DAYS)	OPTIMAL TEMP. (^{O}C)	COLONY MORPHOLOGY[B]	PIGMENTATION[C]	NUCLEIC ACID PROBES
M. avium complex (MAC)	S (10-21)	37	St/R	N	+
M. africanum	S	37	R	N	-
M. asiaticum	S	37	S	P	-
M. bovis	S (10-21)	37	Rt	N	+
M. celatum	S	37	S/St	N	-
M. chelonae	R (3-7)	28	S/R	N	-
M. flavescens	M	37	S	S	-
M. fortuitum	R (3-7)	28	Sf/Rf	N	-
M. gastri	S (10-21)	37	S/SR/R	N	-
M. genavense	S (5-67)	37	St	N	-
M. gordonae	S (10-25)	37	S	S	+
M. haemophilum	S[f] (14-28)	30	R	N	-
M. kansasii	S (10-21)	37	SR/S	P	+
M. malmoense	S	37	S	N	-
M. marinum	M (5-14)	30	S/SR	P	-
M. nonchromogenicum	S	37	SR	N	-
M. phlei	R	28	R	S	-
M. scrofulaceum	S (10-14)	37	S	S	-
M. shimoidei	S	37	R	N	-
M. simiae	S (7-14)	37	S	P	-
M. smegmatis	R (3-7)	28	R/S	N	-
M. szulgai	S (14-28)	37	S or R	S/P	-
M. terrae complex	S (10-21)	37	SR	N	-
M. triviale	M	37	R	N	-
M. tuberculosis	S (12-28)	37	R	N	+
M. ulcerans	S	30	R	N	-
M. vaccae	R	28	S	S	-
M. xenopi	S (28-42)	42	S	S	-

Modified from Nolte & Metchock, 1995.

Figure 1.1 Colonies of mycobacteria grown on M-7H11-OADC agar plates.

Diluted suspensions of mycobacteria were filtered through a black-gridded, 0.45 μm membrane and plated onto M-7H11-OADC agar plates. The plates were incubated for the appropriate time and temperature in the dark. After appearance of colonies the plates were exposed to light for several hours.

A. Yellow pigmented colonies of *M. kansasii*. This picture depicts the photochromogenic characteristics of *M. kansasii*. Buff-colored colonies grown in the dark turned yellow upon exposure to light.

B. *M. kansasii* grown in the dark remained buff-colored.

C. Whitish non-pigmented colonies of *M. terrae*. No change in color occurred after exposure to light (nonphotochromogenic).

D. Colonies of *M. terrae* that grew to a large size after incubation for several weeks.

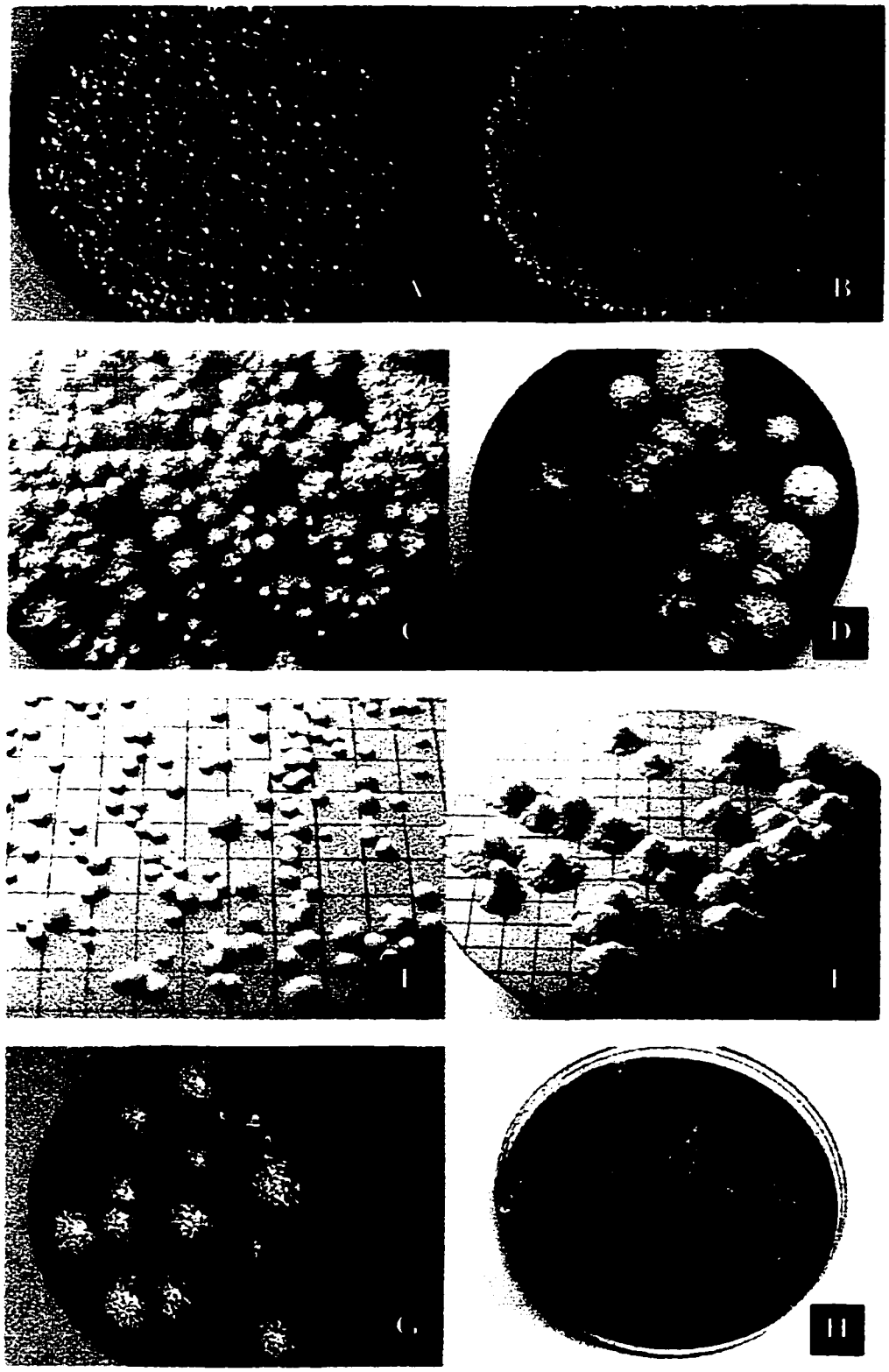
E. Smooth and spherical colonies of *M. xenopi*. The colonies were incubated at 42°C and developed a yellow pigment in the dark (scotochromogenic).

F. Yellow-orange raised colonies of *M. gordonae* (scotochromogenic).

G. Colonies of *M. chelonae* grown at 28°C (nonphotochromogenic).

H. Colonies of *M. smegmatis* grown by streaking on M-7H11-OADC agar containing charcoal (nonphotochromogenic). Colonies grew in 3-4 days of incubation at 37°C (rapid grower).

Figure 1.1



The genome consists of a circular chromosome and some strains contain one or more plasmids (Crawford *et al.*, 1981). The genome of *M. tuberculosis* H37Rv comprises 4,411,529 base pairs (bp), contains 4,000 genes and has a very high (62-70%) guanine + cytosine (GC) content. A very large portion of its coding capacity is devoted to the production of enzymes involved in lipogenesis and lipolysis (Cole *et al.*, 1998).

1.1.2.3 Growth Requirements and Dynamics

Mycobacteria are strict aerobes and some species, particularly *M. tuberculosis*, show enhanced growth in 10% CO_2 and at a pH of about 6.5 to 6.8. Nutritional requirements vary among species and range from the ability of some to multiply on the washers of water faucets to the strict intracellular parasitism of *M. leprae*, which does not grow in artificial media or even in cell cultures. Many species of mycobacteria can grow on artificial media containing nitrogen and carbon sources and mineral salts. Glycerol and asparagine are incorporated into media as sources of carbon and nitrogen, respectively. However, *M. bovis* produces minute colonies (dysgonic growth) in culture containing glycerol and requires pyruvate to produce larger colonies (eugonic growth).

While most mycobacteria including *M. tuberculosis* grow well at 37°C, some species, including *M. xenopi*, *M. smegmatis* and *M. avium* complex (MAC), are thermophilic and are able to grow at 45°C or higher such as in hospital hot water systems. Nonthermophilic mycobacteria such as *M. kansasii*, *M. gordonae*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, and *M. mucogenicum* do not appear to tolerate temperatures of 43°C and are generally found in cold water systems. NTMs even appear to survive and

grow in distilled water. *M. abscessus* and *M. mucogenicum* were experimentally grown in distilled water where they reached concentrations of 10^6 CFU/mL and were maintained over a one-year period (Wallace *et al.*, 1998).

Many species of mycobacteria grow relatively slowly because of their hydrophobic cell surface, which causes them to clump and slows down the permeability of nutrients into the cell. Detergents such as Tween-80 added to liquid media reduce clumping and aid in the preparation of single cell suspensions. For experimental work, disaggregation of clumps may be accomplished by sonication or by passing cell suspensions through a 23-gauge needle several times (Parish and Stoker, 1998). Culture requires strict aseptic measures, since most contaminants will outgrow slow growing mycobacteria. Contamination may be further reduced by adding substances such as malachite green and cycloheximide that suppress growth of contaminating organisms.

Growth of some species may require supplements or enrichments such as egg, albumin, serum, blood, hemin, oleate, mycobactins or other iron transport compounds. Solid media used in mycobacteriology are either egg-based such as Lowenstein-Jensen (LJ), Petragnani, American Trudeau Society (ATS), Ogawa (Ogawa *et al.*, 1950) or, agar-based such as Middlebrook 7H10 and 7H11. The most useful liquid media are those of Proskauer and Beck, Sauton, Kirchner, Middlebrook 7H9 and Dubos Tween Albumin Broth. Selective media are mostly used for the isolation of *M. tuberculosis* from clinical specimens and are egg or agar-based media containing different types of anti-fungal and anti-bacterial agents that help to reduce contamination.

1.1.2.4 Habitat and Transmission

The wide range of mycobacterial species includes obligate parasites, saprophytes and opportunistic pathogens. The *M. tuberculosis* complex and *M. leprae* occur almost exclusively in humans and other warm-blooded animals. In contrast, NTM form an important part of the environmental flora and have been isolated from water and soil (Yajko *et al.*, 1995; Kamala *et al.*, 1994), dust and aerosols (Goslee & Wolinsky, 1976) as well as food, raw milk, oysters, beef, pork and eggs (Thompson, 1994) and cigarettes (Eaton *et al.*, 1995). Water appears to be the most important reservoir of NTM and is a matter of great concern for immunocompromised individuals on account of the ease of transmission and consequent development of disease. Mud and soil as well as contaminated food could also serve as potentially large reservoirs (Yajko *et al.*, 1995; Kamala *et al.*, 1994). Some species such as *M. marinum* and *M. gordonae* appear to prefer water, while some others such as *M. terrae* prefer soil (Dailloux *et al.*, 1999).

Mycobacteria occur in surface water, notably ponds, streams and estuaries but are rarely found in ground water. Municipal water supplies are now recognized as a major reservoir for NTM and are responsible for most nosocomial outbreaks and pseudo-outbreaks caused by these organisms (Wallace *et al.*, 1998). The most disturbing source of mycobacteria such as MAC, are hospital water tanks, bedside carafes, sprays from toilets and shower heads (Glover *et al.*, 1994; du Moulin *et al.*,1988; Peters *et al.*,1995). Picardeau *et al.* (1997) reported finding *M. kansasii* throughout six different hospitals in and around Paris, France. von Reyn *et al.* (1993) isolated *M. avium* from water in the United States, Finland, Zaire and Kenya.

Three main routes of infection, via aerosols, skin lesions or cuts, and ingestion of water or food, have been proposed (Nolte and Metchock, 1995; Bermudez *et al.*, 1992). Mycobacteria are hydrophobic and readily form aerosols when water containing mycobacteria is disturbed. Although TB generally spreads through air, *M. tuberculosis* can survive for several days on inanimate surfaces and they may constitute potential vehicles of infection (Kunz and Gundermann, 1982).

In contrast to *M. tuberculosis*, interpersonal transmission is not known to occur with NTM, and water and aerosols generated from the environment constitute the most important source of infection. NTM responsible for infections through inhalation include *M. kansasii*, *M. avium*, *M. intracellulare* and *M. xenopi*. Contamination through the skin following an injury is seen with *M. ulcerans*, *M. marinum*, *M. haemophilum* and rarely with *M. avium*, *M. kansasii*, *M. chelonae* and *M. fortuitum* (Dailloux *et al.*, 1999). Recently, it was proposed that *M. ulcerans* could be transmitted by the bite of aquatic insects. These water bugs presumably acquire the mycobacteria when feeding on water-filtering organisms that concentrate *M. ulcerans*. If confirmed, this would be the first implication of insects in the transmission of mycobacterial disease (Portaels *et al.*, 1999). Mycobacterial disease acquired by ingestion can be caused by members of the *M. tuberculosis* complex although they are mostly transmitted by air. NTM such as *M. avium* are considered to follow both the aerosol and ingestion route and are usually seen in HIV seropositive patients (Dailloux *et al.*, 1999). It has been proposed that *M. avium* infection in AIDS patients stems from the gastrointestinal route, and therefore from ingestion of colonized water or foods (Bermudez *et al.*, 1992).

Multiple epidemics and pseudoepidemics have been reported during the past two decades due to *M. tuberculosis* and NTM (Rutala *et al.*, 1991). The NTM outbreaks have resulted from contamination followed by inadequate disinfection of medical instruments. The source of contamination has been either patients or environmental reservoirs, especially water supplies.

Collignon and Graham (1989) reported that many hospitals were not adequately cleaning and disinfecting their endoscopes. Transmission of infection has resulted through the use of contaminated endoscopes (Spach *et al.*, 1993). Serious post-operative mycobacterial infections have resulted from environmental contamination in cardiac surgery units (Grange, 1992). Mycobacterial contamination of laboratory ware from water supplies has been responsible for pseudo-outbreaks of mycobacteriosis (Collins *et al.*, 1984). Sources of contamination with respect to heat-sensitive hospital equipment include defective parts of the bronchoscopes, insufficiently disinfected instruments, solutions of local anesthetics and tap water (Gubler *et al.*, 1992). Bryce *et al.* (1993) reported on the contamination of bronchoscopy specimens at Vancouver General Hospital where two of eight bronchoscope suction valves cultured positive for *M. tuberculosis*.

Mbithi *et al.* (1993) found that when 2% alkaline glutaraldehyde was used over a recommended 14-day reuse period to decontaminate flexible fibreoptic endoscopes, the concentration of the disinfectant fell steadily to a final concentration which was no longer effective before the 14-day period ended. It is obvious that these diagnostic tools have the potential to be an important source of mycobacterial infection.

1.1.2.5 Mycobacteria and Disease

Members of the *M. tuberculosis* complex as well as an increasing number of NTM species are responsible for a variety of diseases in humans and animals. Almost any organ in the human body can be the seat of mycobacterial disease. There is considerable evidence that several 'idiopathic' or autoimmune diseases such as sarcoidosis, Crohn's disease and rheumatoid arthritis may be caused by infections with very slow-growing, possibly cell-wall defective, forms of mycobacteria that are non-cultivable or very difficult to culture by standard means (McFadden and Fidler, 1996). NTM, in particular *M. avium* subsp. *paratuberculosis*, may be the etiologic agents responsible for Crohn's disease in humans and Johne's disease in livestock, and has been isolated from bowel tissues of Crohn's patients (Nolte and Metchock, 1995). However the isolation and culture procedures from different studies are inconsistent, and the mycobacterial species isolated differ (Thompson, 1994). Certain NTM infections are increasing on a rapid scale in both healthy and immunocompromised patients in the last decade. Of these, Buruli ulcer, caused by *M. ulcerans*, poses the greatest immediate public health threat and is rapidly becoming the third most prevalent mycobacterial disease, with an impact soon to surpass that of leprosy (Dobos *et al.*, 1999). Leprosy on the other hand is declining due to improved surveillance and control measures (WHO, 1994b).

Many preexisting disease conditions, localized or generalized, can increase the risk of developing mycobacterial disease. For example, patients with diseases of the respiratory tract such as, pneumoconiosis, healed chronic infections, chronic bronchitis, emphysema and bronchiectasis are at risk of infection with numerous NTM species such as *M. avium* complex, *M. kansasii*, *M. fortuitum* and *M. xenopi* (Wolinsky, 1992; Nolte

and Metchock, 1995). Patients with generalized immunodeficiency such as those being treated with high doses of corticosteroids or other immunosuppressive drugs, or those with diabetes mellitus and, in particular AIDS, are at a higher risk of developing disease. Disease in AIDS patients is presently caused mainly by *M. avium*, *M. avium-intracellulare*, replacing the agents *M. kansasii*, and *M. intracellulare* complex, as agents of disseminated mycobacterial infection (Horsburgh, 1991). MAC has currently replaced *M. scrofulaceum* as the predominant species causing lymph node disease in children in England and the United States (Wolinsky, 1992). The disseminated infection resulting from MAC, *M. avium*, *M. kansasii* and *M. fortuitum* in AIDS patients usually occurs ≥1 year after the diagnosis of AIDS is made (Wolinsky, 1992). Although NTM disease increases in the developed world, it remains relatively low in developing countries where TB is still a major concern (Kamala *et al.*, 1994; von Reyn *et al.*, 1993).

Table 1.4 Mycobacterial diseases in human and animals.

***MYCOBACTERIUM* SPP. (*RESERVOIR).**	**TYPE OF DISEASE**
Mycobacterium tuberculosis complex	
M. tuberculosis (*Human)	Pulmonary disease commonest. Less commonly lymphadenitis, pleurisy, pericarditis, osteo-articular disease, diseases of brain, meninges, eyes, skin; most abdominal and pelvic organs.
M. africanum (*Human)	Human tuberculosis in tropical Africa.
M. bovis (*Cattle, human and other primates. Dogs, cats, swine, parrots, some birds of prey. Badger in UK and Opossum in New Zealand)	Tuberculosis in cattle, humans and other primates
M. microti (*Vole, guinea pigs, rabbits, calves)	Tuberculosis in voles, guinea pigs, rabbits and calves.

M. avium-intracellulare-complex (*Water, soil, plants, house dust, and other environmental sources)	Cavitary pulmonary disease, cervical lymphadenopathy, chronic osteomyelitis, renal and skin infection. Disseminated infections particularly in AIDS and other immunocompromised states. Most common opportunistic mycobacterial infections associated with immunodeficiency states.
M. scrofulaceum (*Water and soil)	Cervical lymphadenitis. Less commonly pulmonary and disseminated disease, conjunctivitis, oteomyelitis, meningitis, and granulomatous hepatitis.
M. kansasii (*Water and soil)	Cavitary pulmonary disease. Cervical lymphadenopathy, skin and musculo-skeletal disease, pleural effusion are rare. Second most common opportunistic mycobacterial infection associated with AIDS and includes disseminated disease with CD4+ T-lymphocyte count less than 200 cells/μL.
M. xenopi (*Water, African toad, ?Birds)	Chronic pulmonary disease. Rarely extra-pulmonary and disseminated infection in immunocompromised individuals.
M. szulgai (*Not established)	An infrequent cause of human disease. Pulmonary disease, olecranon bursitis, cervical adenitis, tenosynovitis, cutaneous infections, osteomyelitis and AIDS associated infection.
M. malmoense (*Not established)	Chronic pulmonary disease (pre-existing pneumoconiosis). Extrapulmonary and disseminated infection in AIDS patients.
M. simiae (*Monkey, tap water)	Pulmonary disease, osteomyelitis and disseminated disease with renal involvement. AIDS associated infection.
M. haemophilum (*Largely unknown. ?Human. ?Environment)	Multiple skin nodules with abscesses and fistula.
M. genavense (*Not established)	Disseminated infection in AIDS patients.
M. marinum (*Fresh and salt water. ?Human)	Skin lesions, tenosynovitis, arthritis, bursitis and osteomyelitis. Rarely disseminated and visceral infections.
M. ulcerans (*Unknown. ?Human. ?Koala. ?Water. ?Soil. ?Water bugs)	Skin and soft tissue infection (Buruli ulcer).
M. leprae (*Human. ?Armadillo)	Leprosy: Lesions in skin and peripheral nerves. Gangrene, disability and deformity are late features.
M. fortuitum complex *M. fortuitum* (*Water, soil, dust); *M. chelonae* (*Water)	Osteomyelitis, cellulitis, disseminated disease with multiple nodular soft tissue abscesses, surgical and post-traumatic infection, otitis-media, corneal ulcer, and chronic pulmonary disease.
M. shimoidei (*Data not available)	Cavitary pulmonary disease.
M. celatum (*Not established)	Pulmonary disease. HIV associated infection.

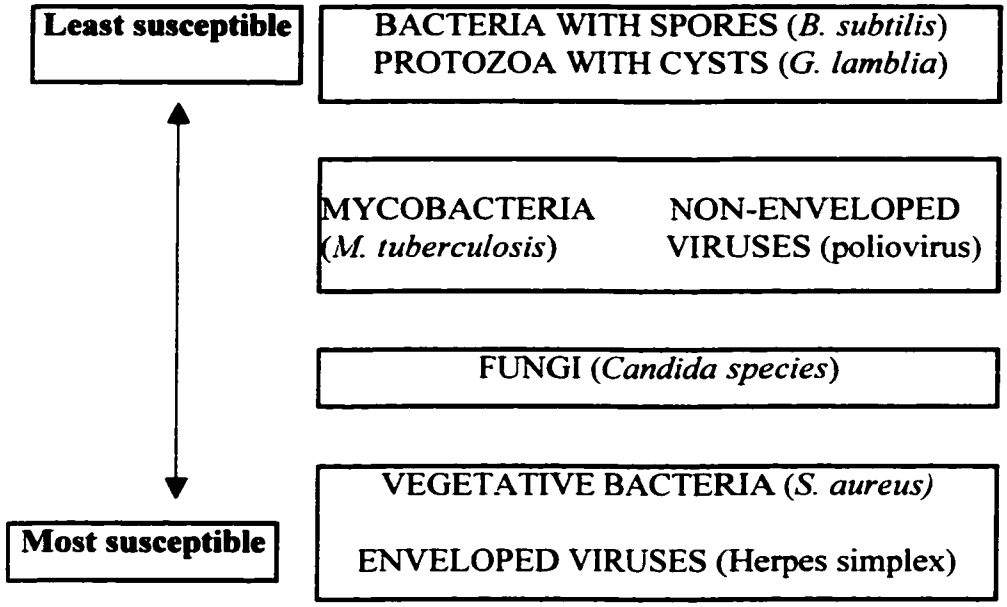
M. gastri (*Not established)	Rarely pathogenic
M. flavescens (*Water)	Rarely pathogenic.
Usually saprophytic *M. gordonae* (*Soil, water)	Rarely pathogenic. Reports of isolation from sputum and bone marrow of AIDS patient.
M. terrae complex (*Soil, water)	Infection of the lung, tendon sheath and intestine have been reported.
M. smegmatis	Most often associated with soft tissue lesions following trauma or surgery

(Nolte and Metchock, 1995; Falkinham, 1996; Dailloux *et al.*, 1999)

1.1.2.6 Viability and Inactivation

Mycobacteria are generally more resistant to germicides than other vegetative bacteria. This is most likely due to their unusually high cell wall lipid content and the resultant hydrophobicity. The susceptibility of mycobacteria to germicides is between that of other vegetative bacteria and bacterial spores (Russell *et al.*, 1986; Favero and Bond, 1991). Mycobacteria as a group are also generally considered to be more resistant to chemical germicides than non-enveloped viruses (Favero and Bond, 1991). Studies in our laboratory with a variety of formulations have shown many instances where non-enveloped viruses proved to be as resistant as, if not more resistant than, mycobacteria (Sattar *et al.*, unpublished data). This argues strongly against any generalizations with regards to the relative resistance of mycobacteria in relation to non-enveloped viruses and a modified hierarchy of microbial resistance to chemical germicides has been presented (Sattar and Springthorpe, 1998). Please refer to Figure 1.2 in the following page.

Figure 1.2 Classes of microorganisms ranked in descending order from least to most susceptible to chemical disinfectants (Sattar and Springthorpe, 1999).



Environmental mycobacteria are incredibly hardy, able to grow in municipal and distilled waterand thrive at temperatures of 45°C or above. They are able to resist the activity of organomercurials, chlorine (0.05-0.2 μg/mL of free chlorine) found at the tap, formaldehyde and alkaline glutaraldehyde (2%) as well as other germicides at concentrations that are lethal to most other vegetative bacteria (Wallace *et al.*, 1998).

The viability of NTM in the environment can be explained by their ability to grow over wide ranges of temperature, pH, salinity and oxygen tension (Collins *et al.*, 1984; Kirschner *et al.*, 1992). The relative resistance to heavy metals and oxyanions also explains the persistence of *M. avium-intracellulare-scrofulaceum* (MAIS) in water distribution systems which use galvanized pipes. Biofilms, the slimy layers at the solid-

water interface, have come to be recognized as a haven for NTM. In a study in Germany, 90 % of biofilms from water pipes contained mycobacteria in densities of 10^3-10^4 CFU cm^{-2} and microcolonies, indicating replication, were often seen (Schulze-Robbecke *et al.*, 1992). Species identified were *M. fortuitum*, *M. chelonae*, *M. flavescens*, *M. gordonae*, *M. kansasii*, and *M. terrae*. Biofilms appear to provide the nutritional support for the organisms and release mycobacteria and other species in the free-flowing liquid.

The presence of mycobacteria in water supplies indicates that the contaminated waters from streams and rivers retain viable organisms after entering the distribution system because of inadequate water treatment or re-establishment of the organisms upon contact with biofilms in piping (von Reyn *et al.*, 1993). Recently, many water treatment plants have started employing chloramine instead of chlorine for water disinfection. Although chloramine kills most water contaminants, it allows mycobacteria to thrive with no competition for resources. Sand filtration on the other hand removed acid-fast organisms from water supplies and the densities of these organisms decreased with increasing distance from the treatment plant (Haas *et al.*, 1983).

Inactivation of environmental mycobacteria through appropriate disinfection is an important component in the environmental control of infection. Potential target sites for germicides includes the mycobacterial cell wall, cytoplasmic membrane and the cytosol (proteins, enzymes, DNA and RNA). The germicidal property of a chemical agent may be altered by various factors. Please refer to Table 1.5 in the following page.

Table 1.5 Factors influencing antimycobacterial activity of germicides.

FACTORS	INFLUENCE ON ANTIMYCOBACTERIAL ACTIVITY
Related to disinfectant	Stability of solution: The temperature, pH, container and storage period influence the efficacy. Concentration: Activity rises as concentration increases. pH: Depends on individual agents, e.g. chlorine releasing agents (CRAs) & Phenols: more active at acid pH; chlorhexidine, quaternary ammonium compounds (QACs), glutaraldehyde: more active at alkaline pH.
Related to material to be disinfected	Nature of material: Surfaces that are smooth, nonporous and cleanable such as a scalpel blade are easily disinfected. Crevices, joints and pores constitute barriers to the penetration of liquid germicides. Delicate or sensitive materials may undergo corrosion or damage especially with prolonged contact time. Size of medical devices may limit the types of germicides and disinfection procedures that can be used. Number of contaminating microorganisms in a medical devise may influence the outcome of a procedure. Resistance of microorganisms: The more resistance an organism is, the more difficult it is to achieve appropriate disinfection. Among the mycobacterial species, some are more resistant than others. Presence of organic soiling matter (blood, serum, mucus, feces, urine, pus, milkstone) may interfere with disinfection in two ways. The organic soil may occlude microorganisms and prevent penetration of chemical germicides. The soil may inactivate certain germicides such as chlorine and iodine based compounds and QACs.
Related to procedure	Period of contact: Long periods may be necessary to get optimum anti-microbial effect. Temperature of treatment: Activity generally increases with rise in temperature. Efficiency of pre-cleaning of instruments prior to disinfection.

Modified from Russell, 1996.

1.1.2.7 Nosocomial and Iatrogenic Infections

Nosocomial disease outbreaks caused by NTM have been recognized since 1975 (Wallace *et al.*, 1998). The incidence has escalated in recent years due to the increase in medical and surgical procedures involving the use of heat-sensitive devices. HIV infection and increased awareness of NTM as human pathogens along with improved methods for their detection. Most involve wound infections following cardiac or plastic surgery and post-injection abscesses. A wide variety of other surgical procedures such as laparoscopy, surgical irrigation of chronic otitis media and spinal disk removals were occasionally followed by outbreaks of mycobacteriosis. Sporadic cases of peritonitis in patients undergoing peritoneal dialysis is well recognized (Wallace *et al.*, 1998). Six out of seven hospital outbreaks of TB with a total of 228 cases in USA were investigated by the CDC between 1990 and 1992. In each instance, evidence of nosocomial transmission of MDR-TB from person-to-person was compelling and supported by RFLP analysis and more than 80% occurred in persons infected with HIV (Ellner *et al.*, 1993). These outbreaks depict the way in which HIV can both amplify and accelerate TB outbreaks.

Iatrogenic transmission of mycobacteria via flexible endoscopes and other heat-sensitive, semi-critical medical devices is attributed to the failure to follow recommended disinfection procedure including pre-cleaning, the complexity of the mechanical configuration of the endoscopes with the resulting difficulty of decontaminating component valves or channels, and the use of chemical germicides with inadequate mycobactericidal activity (Spach *et al.*, 1993; Hanson *et al.*, 1992). Depending on the origin of contamination, transmission of infection can occur from patient to patient or

environment to patient as depicted for endoscopes (Figure 1.3). Environmental contamination typically results from flushing or cleaning endoscopes with contaminated solutions.

Figure 1.3 Mycobacterial infections transmitted by endoscopes.

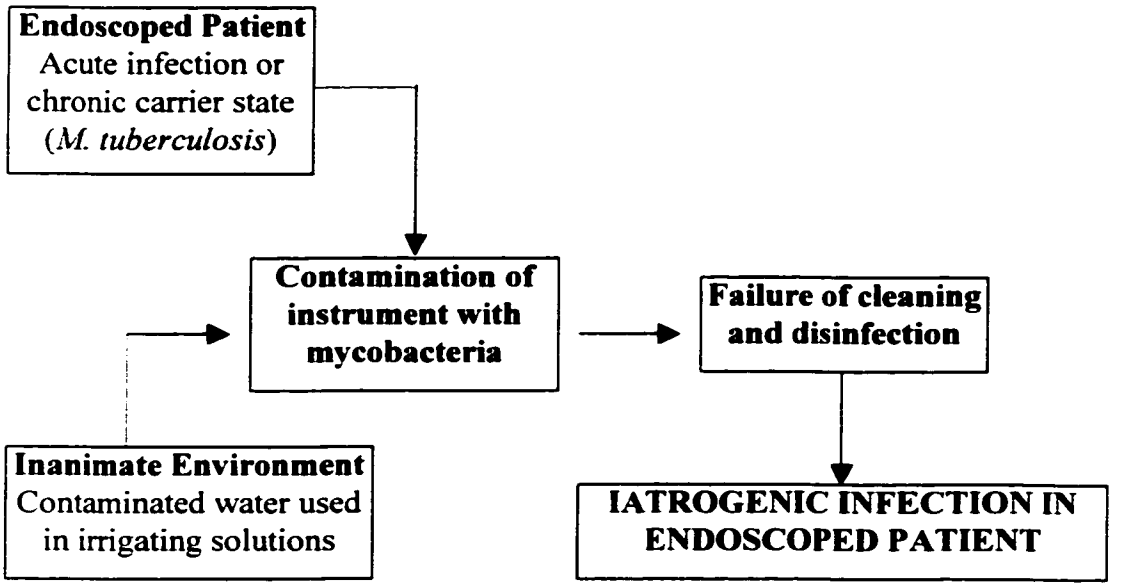


Table 1.6 Transmission of mycobacteria through flexible bronchoscopy.

REFERENCES	Nelson *et al.* 1983	Wheeler *et al.* 1989	Pappas *et al.* 1983	Wheeler *et al.* 1989
Organism	*M. tuberculosis*	*M. tuberculosis*	*M. chelonae*	*M. avium*
Isolates	2	3	72	2
Cases of disease	1	1	2	0
Deaths	0	0	1	0
Source of contamination	Iodophor disinfection	Suction valve	Punctured channel	Suction valve

Adapted from Spach *et al.*, 1993.

1.1.3 Disinfection Assay Strategies

Although a general internationally accepted test scheme for disinfectants does not exist, standard methods have been developed in different countries. In the United States, standard methods are published by the Association of Official Analytical Chemists (AOAC International, 1995) and the American Society for Testing and Materials (ASTM); the U.S. Environmental Protection Agency publishes guidelines in this regard (U.S. EPA, 1988). Standard methods have been laid down by the German Society for Hygiene and Microbiology (DGHM, 1996), and the French Association for Standardization (AFNOR, 1989). The British Standards Institution has also published some test methods (BSI, 1960 and 1987). The Technical Committee (TC) 216 Chemical Disinfectants and Antiseptics, founded in 1990 by the European Committee for Standardization (CEN) with 18 member countries, concentrates on the development of new European standards for disinfectant testing (CEN, 1996). There is close technical cooperation between the CEN and the International Organization for Standardization (ISO).

According to the test structure, disinfectant testing is classified into three broad categories (Reybrouck, 1999): *in vitro*, practical and in-use tests. *In vitro* tests are further classified as suspension, capacity and carrier tests. Suspension tests determine specific activity where the disinfectant solution is mixed with the microbial suspension and finally sub-cultured in liquid or semi-solid media to obtain a qualitative or quantitative assessment of effectiveness. In capacity tests, the test dilution of the disinfectant is loaded with several sequential additions of a microbial suspension, and after each addition, survivors are assessed by sub-culture to determine the germicidal

capacity of the disinfectant. Carrier test methods attempt to simulate surface disinfection and employ inanimate articles or germ carriers such as rubber tubing or glass vials, on which the organisms are dried. Data based on carrier tests are generally preferred by regulatory agencies even though certain 'official' carrier methods are known to be flawed (US General Accounting Office, 1990). The practical tests are used to assess the disinfection of instruments such as endoscopes (Hanson *et al.*, 1992; Cutler and Wilson, 1993), surfaces, cubicles and rooms, hands, skin, cloths, etc., experimentally contaminated with micro-organisms, under conditions of use. Tests on actual endoscopes are likely to provide more meaningful data about disinfection processes, including biocide concentration and periods of contact (Russell, 1996). Although these tests can be arranged in the laboratory without any difficulty, most of them have a limited application due to poor reproducibility (Reybrouck, 1999). Finally, the in-use tests are aimed at testing product performance under actual conditions of use. In-use tests are very helpful in some situations such as monitoring of disinfection practices in hospitals.

Disinfection tests conducted by different methods have their inherent advantages and drawbacks and require culture of the test organism. However, many clinically relevant species of mycobacteria are slow growers (Grange, 1996). This means that results of disinfection tests employing conventional culture of slow-growing mycobacteria would require several weeks to months to obtain. In addition, these methods are usually expensive, labor intensive and unsafe for laboratory personnel.

Most of the newer methods represent significant savings in time but are mostly designed for testing drugs rather than germicides for antimycobacterial activity. Radiometric culture methods for determining susceptibility of mycobacteria to

antimicrobial agents include the BACTEC TB system, where the release of $^{14}CO_2$ from a radiolabelled [^{14}C] amino acid substrate (palmitic acid) in the growth medium can be detected and linked with the growth of mycobacteria in the medium (Broadley *et al.*, 1995; Siddiqi *et al.*, 1993). Other available radiometric culture methods utilize radiolabelled [^{35}S] methionine (Holton *et al.*, 1994), [^{14}C] acetate (Ashtekar *et al.*, 1987) and [^{3}H] uracil (Chung *et al.*, 1995). Owing to the strict regulation of radio-chemicals in Japan, a modified BACTEC system has been developed where the radioactive substance [^{14}C] was replaced with alpha-antigen, a widely distributed secretory protein in mycobacteria, detectable by reverse passive latex agglutination (Shigeto and Taska, 1996). Methods employing flow cytometry (Norden *et al.*, 1995) and measurements of differential light scattering (Conville *et al.*, 1994) that utilize laser technology have been reported for rapid assessment of drug susceptibility of mycobacteria. The Mycobacterial Growth Indicator Tube (MGIT) allows drug susceptibility determination of organisms directly from clinical (respiratory) specimens in 5 days (Palaci *et al.*, 1996). The inoculated MGITs are examined daily for fluorescence under a 365 nm UV-light and the results correlated with those obtained by the indirect proportion method. Failure of the test bacteria to fluoresce indicated sensitivity to the anti-mycobacterial agents. Mycolic acid analysis through HPLC (Garza-Gonzalez *et al.*, 1997), the microplate alamar blue assay or MABA (Collins and Franzblau, 1997) and the gel micro-drop encapsulation technique (Ryan *et al.*, 1995) are other innovations in the rapid assessment of drug susceptibility of mycobacteria. Several methods have evolved for the rapid assessment of germicides, including the direct epifluorescent filtration technique (DEFT), triphenyltetrazolium (TTC) reduction and microcalorimetry (Hugo and Russell, 1999).

The DEFT is based on the principle that viable microorganisms fluoresce (orange-red) when stained with acridine orange, whereas non-viable cells do not. In practice, viability of the bacteria after exposure to the germicide is determined by filtering through a membrane filter, which is then stained with acridine orange and examined microscopically. Similarly, TTC reduction by viable bacteria has also been utilized for rapid evaluation of germicides. Microcalorimetry is a physical method based on the measurement of the small amount of heat produced by metabolizing bacteria.

More recently, reporter genes have been used for assessing anti-mycobacterial agents. The reporter genes, luciferase (*lux*) and the green fluorescent protein (*gfp*) confer bioluminescence and fluorescence, respectively, that allow convenient detection of cells harboring them as well as ascertain their physiological status or viability. Assays based on reporter genes have been shown to be sensitive, cheap and generate results in a much shorter time than with conventional culture (Andrew and Roberts, 1993; Collins *et al.*, 1998). The application of reporter genes in the evaluation of germicides for mycobactericidal activity would be an obvious advantage in terms of saving time, labor and cost.

1.2 Objectives and Hypotheses

The current methods to assess the mycobactericidal activity of chemical germicides are slow, labor-intensive, non-quantitative and rely on inappropriate surrogate organisms. Therefore, the **first objective** of this study was to adapt a quantitative carrier test for sporicidal activity developed in our laboratory to assess the mycobactericidal activity of chemical germicides. We hypothesized that the level of mycobactericidal activity of a germicide would be indicated by the extent of reduction in the number of CFU of the test organism.

The above-mentioned procedure was still slow in the generation of data because it relies on the detection and quantitation of the colony forming units of the surrogate organism. To address this issue, we investigated approaches that would generate results in a shorter time period. Therefore, the **second objective** of this study was to assess the viability of mycobacteria using recombinant cells expressing luciferase and green fluorescent protein (GFP) and to apply this understanding in developing a method for mycobactericidal testing of germicides. The luciferase system was based on the hypothesis that light emission from mycobacteria expressing the luciferase enzyme would be altered by chemical inhibition of the test organism.

The difficulties faced during development of the luciferase system prompted the application of the green fluorescent protein gene (*gfp*) in the assessment of the viability of mycobacteria. Therefore, the main component of the second objective was to develop a rapid screening method for the determination of mycobactericidal activity of chemical germicides using mycobacteria expressing the green fluorescent protein (GFP). We hypothesized that germicidal inactivation of mycobacteria expressing GFP would alter

the intensity of fluorescence. We also proposed that the measurement of the altered fluorescence would allow assessment of viability, which would be a measure of the mycobactericidal activity of the formulation under test.

2. MYCOBACTERICIDAL TESTING OF CHEMICAL GERMICIDES USING A CULTURE-BASED QUANTITATIVE CARRIER METHOD

2.1 Introduction

Conventional *in vitro* methods for testing mycobactericidal activity of chemical germicides are usually suspension or carrier tests based on culture of mycobacteria. Currently used protocols suffer from several drawbacks including: (a) a lack of proper quantitation; (b) inappropriate type of carriers; (c) absence of a suitable soil load; (d) no or ineffective neutralizers; (e) unsuitable surrogates for *M. tuberculosis*; and (f) improper recovery media (Sattar *et al.*, 1995).

Mycobacterial cells in suspension are more susceptible to germicides than when dried on carriers. To evaluate germicides used on contaminated surfaces and simulate the in-use practices of surface and equipment disinfection, data based on carrier tests are generally preferred by regulatory agencies when considering the registration of germicides. Quantitative assessment of germicidal activity is preferable to qualitative or semi-quantitative methods for establishment of a criterion for product potency. A quantitative test provides information on the relative efficacy of a germicide compared to another with respect to specified microorganisms. However, many of the tests currently used do not allow proper quantitation due to the use of inappropriate carriers. The wide range of carriers used includes stainless steel and polypropylene disks and glass cover slips (Best *et al.*, 1990), glass cups (Best *et al.*, 1994; Mbithi *et al.*, 1993), glass slides (Lind *et al.*, 1986), porcelain penicylinders (Rutala *et al.*, 1991; Cole *et al.*, 1990), Millipore membranes (Collins, 1987), fabric pieces (Gundermann, 1987), rubber tubing (Anon, 1991), and glass vials with inserts (Springthorpe *et al.*, 1994). Some of the

methods using carriers such as porcelain, metal or glass penicylinders and rubber tubing have variations in quantitating the input inocula. Inaccuracies also arise during recovery of organisms from porous materials (Lloyd-Evans *et al.*, 1986) and from rinsing carriers that leads to wash-off of cells, such as in the AOAC method (Cole *et al.*, 1990).

An appropriate carrier should provide a small non-porous flat surface and allow for complete recovery of organisms without any wash-off. Aggregation of mycobacteria in nature as well as in laboratory systems produces further confusion as to the reliability of results, and methods for disaggregating mycobacterial clumps, such as the addition of surfactants, vortexing with or without glass beads and coarse filtration are among many of the approaches taken to address this issue (Sattar *et al.*, 1995).

Organic substances used to prepare the test inoculum are generally preferred to simulate in-use conditions for germicide use. The addition of sputum (Best *et al.*, 1990) and guinea-pig spleen homogenates (Lind *et al.*, 1986), though attractive, are difficult to obtain and cannot be standardized. Various other materials including 0.5% yeast extract (Holton *et al.*, 1994), human serum, horse blood (Lind *et al.*, 1986), 20% defibrinated blood on carrier and 0.5% albumin in the disinfectant (Anon, 1991), 5% fetal bovine serum (Best *et al.*, 1994) and tryptone (Best, 1993) have been used in different situations.

In germicidal tests, adequate elimination of inhibitory residual disinfectant activity at the time of culture is a fundamental issue. Three procedures used individually or in combination are possible to attain this objective: neutralization, dilution and washing. Neutralizers which inactivate germicides include glycine for aldehydes, catalase for hydrogen peroxide, letheen broth for QACs and chlorhexidine, Tween 80 for phenol and Tego compounds (ampholytic surface active agents) and sodium thiosulfate

for hypochlorites and iodines (Russell, 1991). Sodium bisulphite and sodium thiosulfate have been used as neutralizers for *Ortho*-phthalaldehyde and peracetic acid, respectively (Walsh *et al.*, 1999). An ideal neutralizer that is harmless for the test organism(s), fast acting, readily available, relatively inexpensive and effective against most germicides is yet to be found. Dilution of the organism-disinfectant mixture may not be very efficient especially for some germicides such as QAC or chlorhexidine (Leers *et al.*, 1974). It is however preferred (Best *et al.*, 1994; Cole *et al.*, 1990) particularly when combined with washing with the diluent followed by mechanical separation of the microbial cells and the disinfectant, using membrane filtration. This also facilitates colony counting on the membrane itself (Collins, 1987).

A surrogate for testing mycobactericidal activity of germicides should represent not only *M. tuberculosis* but also the growing number of NTMs. Mycobacterial species used in disinfectant assays include the fast growing *M. smegmatis* (Collins, 1986; van Klingeren and Pullen, 1987; Best *et al.*, 1988 and 1990), *M. chelonae* (Walsh *et al.*, 1999; HIRL, 1993) and the slow growing *M. bovis* (Rutala *et al.*, 1991; Cole *et al.*, 1990), *M. bovis* BCG (Collins, 1987; Best *et al.*, 1994), *M. terrae* (Walsh *et al.*, 1999; Sattar *et al.*, 1998; Gundermann, 1987; DGHM, 1991), and *M. tuberculosis* (Rutala *et al.*, 1991; Best *et al.*, 1990; Cole *et al.*, 1990; Collins, 1987; Gundermann, 1987). As detailed below, each one has its own advantages and drawbacks including turn-around time for results, susceptibility to germicides, and biosafety issues.

Although *M. smegmatis* is non-pathogenic and grows in 48-72 hours, it is more sensitive to germicides than other mycobacteria (Best *et al.*, 1990; Collins, 1986). *M. terrae* was reported to be a good choice for having similar responses or slightly higher

resistance to germicides than *M. tuberculosis*, its ease of handling and its non-pathogenic nature (van Klingeren and Pullen, 1987; Griffiths *et al.*, 1998). *M. terrae* ATCC 15755 was used in suspension and carrier tests (Hingst *et al.*, 1990; Sattar *et al.*, 1998), the quantitative suspension test of the DGHM (1996) and the tests for surface and instrument disinfection of the German Federal Health Office (BGA, 1994; Robert Koch Institute, 1995).

A germicidal test must also take into account other factors such as the diluent for the germicide, temperature and humidity, growth and recovery media and the number of repeats to validate the test protocol.

A quantitative carrier test developed for testing sporicidal activity of germicides addresses some of the flaws inherent in culture-based methods (Springthorpe *et al.*, 1994). The objective of this chapter was to investigate this method for its usefulness, accuracy and reproducibility in the testing of germicides for mycobactericidal activity. Furthermore, the suitability of *M. terrae* as a surrogate organism and a soil load prepared in our laboratory were also assessed using the quantitative carrier test.

2.2 Materials and Methods

2.2.1 Materials

A list of solutions, chemicals and suppliers is found in Appendix I.

2.2.2 Cold Storage of Mycobacterial Cells

Mycobacterium terrae: As per instructions of the American Type Culture Collection (ATCC: Rockville, MD), a freeze-dried preparation of *M. terrae* ATCC 15755 was suspended in 400 µL of Middlebrook 7H9 broth + 10% albumin dextrose catalase supplement + 0.05% Tween-80 (M-ADC-TW). The suspension was inoculated into three cell culture flasks (25 cm^2, canted neck, 0.2 µm vented plug cap; Sarstedt, Inc., Newton, NC), each containing 10 mL of M-ADC-TW broth. In addition, two plates containing 7H11 agar + 10% oleic acid albumin-dextrose complex (M-7H11-OADC) and two Lowenstein-Jensen (LJ) slants were streaked with a 10 µL loop. After 21 days of incubation at 37°C, the agar plates and LJ slants showing growth were stored at 4°C. The liquid culture was further inoculated into large cell culture flasks (75^2 cm, canted neck, 0.2 µm vented plug cap; Sarstedt Inc.) each containing 100 mL of M-ADC-TW broth and incubated for another 21 days. The cultures were centrifuged at 2,000 ×*g* for 15 minutes. The supernatant was discarded and the pellet resuspended in a medium for frozen storage of the cells (Appendix I). The cells in the freezing medium were aliquoted in volumes of 1 mL in cryo-vials and stored at -80°C.

Cultures of *Mycobacterium gordonae* NT 1101, *Mycobacterium xenopi* NT 1901, *Mycobacterium fortuitum* NT 2707, *Mycobacterium chelonae* NT 2901 and

Mycobacterium kansasii NT 711 were propagated in M-ADC-TW broth and their frozen stocks were prepared as above. *Mycobacterium smegmatis* mc^2155 (Snapper *et al.*, 1990), an efficient plasmid transformation (*ept*) mutant, was obtained as a gift from Dr. Lucy Mutharia, University of Guelph, Guelph, Ontario. It was grown in M-ADC-TW broth and also maintained as a frozen stock.

2.2.3 Preparation of Mycobacterial Stock Culture

A frozen vial of the test mycobacterium was thawed quickly in a 37°C water bath or warm tap water and inoculated into a vented cell culture flask containing 100 mL of M-ADC-TW broth and incubated. Temperature and time requirements for individual species of mycobacteria are listed in Table 1.3.

2.2.4 Preparation of Mycobacterial Suspension

A liquid culture of mycobacteria grown to log phase was centrifuged at 2,000 ×g for 15 minutes and washed twice with sterile water. The final pellet was transferred to a sterile bijoux bottle with 10 glass beads, mixed with a sufficient volume of water and vortexed to homogenize the pellet. The optical density or turbidity of the resulting suspension was visually adjusted to match that of No. 6 McFarland standard to give approximately 1.8×10^9 cells/mL.

2.2.5 Enumeration of Mycobacteria Using Spread Plate Method

One hundred µL of a mycobacterial suspension corresponding to McFarlands No. 6 standard, was serially diluted in 900 µL of 0.85% saline. The 10-fold serial dilutions were continued up to 10^{-7}. A 100 µL volume of the last three dilutions i.e., 10^{-7}, 10^{-6} and 10^{-5} were separately spread over M-7H11-OADC agar plates in triplicate and the plates

were incubated for the required time and temperature (Table 1.3). The average CFU of triplicate plates was used to calculate the CFU/mL of the suspension using the following formula.

CFU/mL = Average CFU of 3 determinations × 10^n × 10

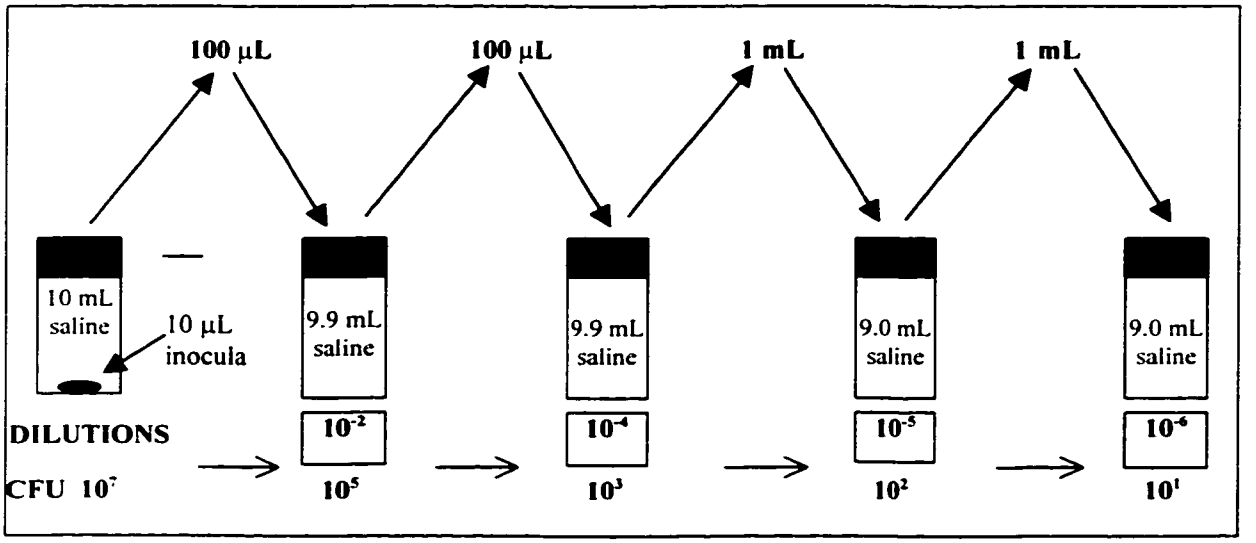
where, n = the degree of dilution.

The extra factor of 10 was used to arrive at the titer for each mL of the suspension.

2.2.6 Enumeration of Mycobacteria using Membrane Filtration Method

Glass vials (28 mm × 58 mm; Galaxy Environmental Products, Newfield, NJ) containing 10 µL of a suspension of mycobacteria or the same after overnight drying were eluted with 10 mL of saline using Teflon-coated magnetic stir bars (15 mm × 4 mm; Rehabilitation Centre Engineering Workshop, Smyth Rd., Ottawa, ON) followed by vortexing to break up any clumps. The eluates were serially diluted as depicted below.

Figure 2.1: Elution and serial dilutions for enumeration of mycobacteria using membrane filtration.



The last three dilutions that would give countable colonies were filtered through a 0.45 μm, black-gridded, mixed cellulose ester Millipore membrane filter (Millipore Corp., Bedford, MA), starting from the most dilute (10^{-6}) to the least dilute (10^{-4}) using the same filtration units (47 mm, Millipore, Bedford, MA). Each vial was rinsed three times with normal saline and the rinse passed through the same filter. Each filter membrane was washed with at least 100 mL of normal saline, aseptically removed from the unit using flamed forceps and placed on M-7H11-OADC agar in 100 mm diameter disposable plastic plates (Fisher). The plates were sealed using Shrink Seal (92 mm × 25 mm; Scientific Device Laboratory, Inc., Des Plaines, IL), placed in plastic bags and incubated for the time and temperature recommended for the species (Table 1.3). The procedure was done in triplicates. Plates were checked weekly for the slow growing species, and daily for the faster growing species, and CFU were counted. The average CFU of three determinations was used to calculate the CFU/mL of the suspension using the following formula (Figure 2.3 A-E).

CFU/mL = Average CFU of 3 determinations × 10^n × 100

n = the dilution factor in the vials

The extra factor of 100 was used to arrive at the titer for each mL of the suspension.

2.2.7 Antibiotic Susceptibility Testing of Mycobacteria

The agar proportion method was used for susceptibility testing of mycobacteria (Inderlied and Salfinger, 1995). One hundred μL of a mycobacterial suspension was serially diluted as described in Section 2.2.5. The last 3 dilutions that would give countable colonies i.e., 10^{-5}, 10^{-6} and 10^{-7} were spread over M-7H11-OADC agar plates

without and with antibiotics in different concentrations and the plates were incubated. The extent of inhibition of the organism was determined by comparing the number of CFU in the test and control plates. Once the drug-free (control) plates showed at least 50 to 150 colonies, the extent of inhibition in the antibiotic (test) plates were determined. If the percentage of colonies in the test plates is ≥1% of that of the controls, the isolate should be reported as resistant to that drug at the concentration tested.

2.2.8 Preparation of Mycobacterial Inoculum for Quantitative Carrier Test

A 342.8 µL volume of homogenized mycobacterial suspension (~10^9 CFU/mL) was mixed with 22.1 µL of 5% bovine serum albumin (BSA) + 35.1 µL of 5% tryptone + 100 µL of 0.4% mucin.

2.2.9 Inoculation of Carriers

The carriers were round (28 mm wide × 58 mm long) screw-capped, flat-bottomed clear-glass vials (catalog #5260-G; Galaxy Environ. Products, Newfield, NJ) with custom-made glass inserts (that just touched the inside bottom of the vials) which were held in place with septate caps. For inoculation, sterile carriers were placed inside an operating Class II laminar flow biosafety cabinet and 10 µL of the prepared inoculum (~10^9 CFU/mL) was pipetted at the center of the inside bottom surface of each carrier without touching the insert. After holding them to dry for about 2 hours in the biosafety cabinet, the carriers were transferred to a desiccator for overnight drying at room temperature under vacuum. The next day, the septate caps and inserts were carefully removed and replaced with normal sterile caps (Figure 2.3A and Appendix IVB).

2.2.10 Quantitative Carrier Test

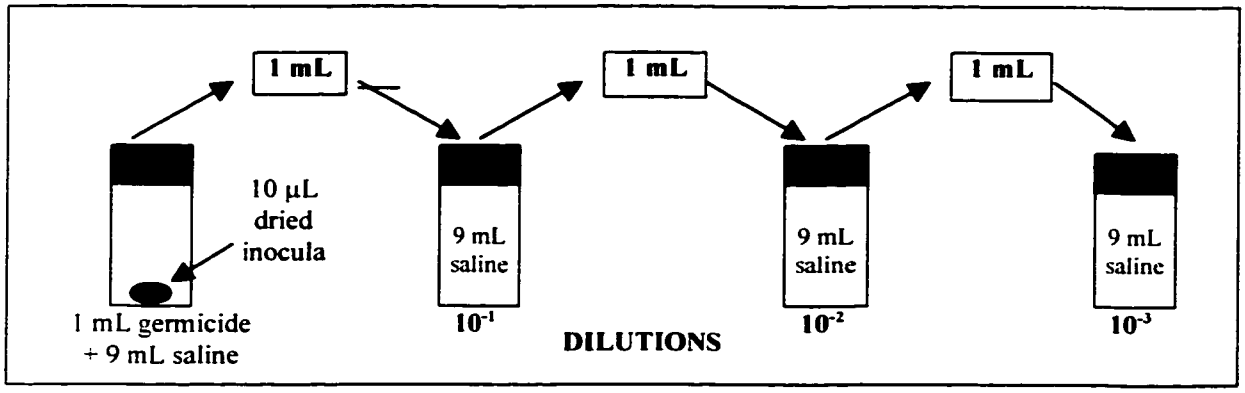
Each procedure comprised of 5 test carriers and 3 control carriers. Where indicated, dilution of the test products was done with sterile water with a standard hardness of 200 ppm as $CaCO_3$ except for QAC, where the hardness was 400 ppm.

Control carriers: The dried inoculum in each control carrier was exposed to 1 mL of sterile normal saline for a time indicated by the manufacturer for the test germicide. The contents were eluted by the addition of 9 mL of saline using Teflon-coated magnetic stir bars followed by vortexing to break up any clumps. Subsequent steps involving serial dilution, filtration, plating, incubation and CFU enumeration were performed as mentioned in Section 2.2.6.

Test carriers: One mL of the test germicide (at use dilution) was pipetted into each of the 5 test carriers at regular intervals and held at the temperature and exposure time indicated by the manufacturer. After the contact time, the germicide was diluted with 9 mL of 0.85% sterile saline and the inoculum was eluted and filtered without serial dilution. Approximately 100 mL of saline was used to wash off the carrier and the sides of the filter unit. The filter membranes were plated onto M-7H11-OADC agar and incubated. Plates were checked for growth for a maximum of 35 days and the number of CFU were recorded. Where colonies on filters were too numerous to count (TNTC), serial dilution of the test carriers were done during the next germicide test as depicted below to obtain countable colonies in the plates (Figure 2.2).

These dilutions were filtered, starting from the most dilute (10^{-3}) to the least dilute (10^{-1}) using the same filtration unit for each carrier. The filter membranes were plated

Figure 2.2: Elution and serial dilution of test carriers in a quantitative carrier test



onto M-7H11-OADC agar and incubated. The procedure was repeated for all five test carriers. Plates were checked for growth for up to 35 days and colonies if any, were counted for $\log_{10}$ reduction calculations. (Please refer to Figure 2.3 A-E).

2.2.11 Calculation of $\log_{10}$ Reduction for Assessing Mycobactericidal Activity

Mycobactericidal activity of a germicide was determined by comparing growth on the control and test plates. An average count of the number of CFU in the three controls was calculated to yield an initial value for $\log_{10}$ reduction calculations (Section 2.2.6). The number of CFU in the five test plates was counted and used separately in the following formula:

$\log_{10}$ Reduction = $\log_{10}$ (average CFU of 3 controls) – $\log_{10}$ (CFU of each test carrier)

An average $\log_{10}$ reduction of all the five tests in each experiment was calculated. Finally, three similar experiments were carried out separately to validate the results. This was reported as the mean ($\pm$SD) $\log_{10}$ reduction in the number of CFU of the three experiments for each type or dilution of germicide. Each germicide was assessed for its capacity to produce $\geq$6-$\log_{10}$ reduction in the CFU of the test bacterium.

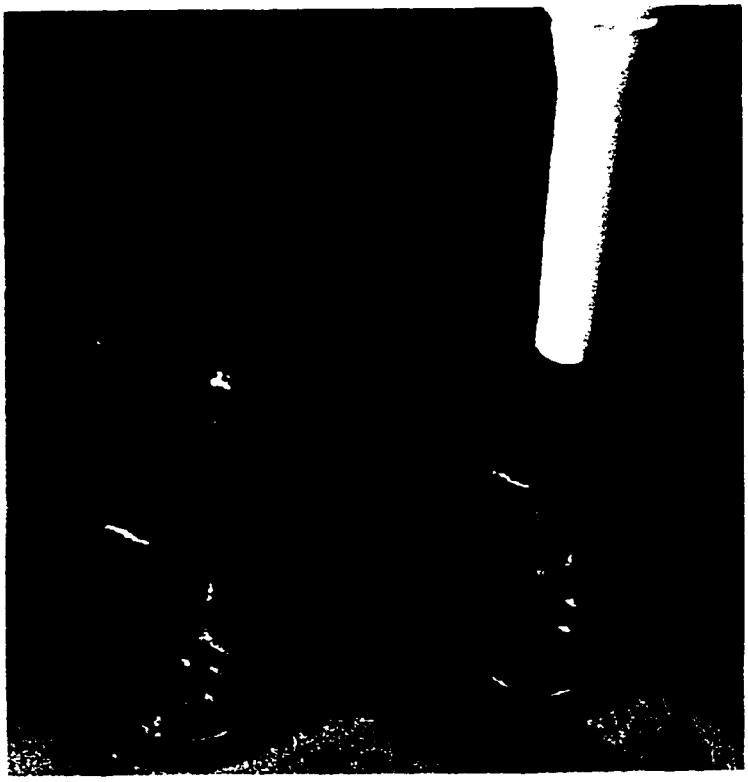
Figure 2.3 Quantitative carrier test showing (A) inoculation of carriers and (B) application of germicide on dried inoculated carrier.

(A) Ten μL of a suspension of *M. terrae* (10^8-10^9 CFU/mL) combined with a soil load was pipetted onto the bottom of glass vials with inserts and septate caps. The carriers were dried overnight in a desiccator. The next day, the septate caps and inserts were carefully removed and replaced with normal sterile caps.

(B) One mL of the test germicide was pipetted onto the bottom of the test carriers containing the dried inoculum. After the desired contact time the germicide was neutralized by dilution with saline, the inocula eluted by using Teflon-coated magnetic stir bars and vortexed to break up any clumps. Controls were exposed to saline instead of germicides and eluted in a similar manner with serial dilution.

Figure 2.3 (A-B)

A. INOCULATION OF CARRIERS



B. APPLICATION OF GERMICIDE

Figure 2.3 Quantitative carrier test showing (C) filtration of eluate (D) removal of the filter membrane and (E) plating on agar medium.

(C) The eluate from the test and control carriers were filtered separately through a 0.45 μm black-gridded, mixed cellulose ester Millipore membrane. Approximately 100 mL of saline was used to wash off the carriers and the sides of the filtration units.

(D) The filter membranes were removed aseptically from the filtration units using flame sterilized forceps.

(E) The membranes were placed onto M-7H11-OADC plates and incubated. Plates were checked weekly for growth for a maximum of 35 days and CFU recorded.

Figure 2.3 (C-E)

2.3 Results

2.3.1 Comparison of a Standard Spread Plate Method with Membrane Filtration for Enumeration of Mycobacteria

The spread plate method was performed to determine the CFU/mL in a suspension of mycobacteria used to inoculate the carriers, and membrane filtration was used to enumerate the organisms recovered from the carriers during the actual quantitative carrier test. A comparison of the counts was made to identify any differences between the two methods (Table 2.1). Four suspensions of *M. terrae* ATCC 15755 were enumerated in duplicates by a standard spread plate method (Section 2.2.5) using glass and plastic spreaders and a membrane filtration method (Section 2.2.6).

Table 2.1 Comparison of spread plate and membrane filtration methods for enumeration of mycobacteria.

Mycobacterial suspension	Log_{10} CFU/mL		
	Spread plate with glass spreader	Spread plate with plastic spreader	Membrane filtration
M. terrae (1)	8.99 ± 0.06	9.05 ± 0.01	9.31 ± 0.05
M. terrae (2)	9.61 ± 0.01	9.96 ± 0.20	10.03 ± 0.05
M. terrae (3)	8.35 ± 0.15	8.39 ± 0.08	8.85 ± 0.07
M. terrae (4)	9.38 ± 0.03	9.36 ± 0.06	9.72 ± 0.03

Each result is the mean of two determinations ± the standard deviation (SD).

The log_{10} CFU/mL for all the four tests were a little higher for the membrane filtration than those obtained with the spread plate method. In the spread plate method, results obtained using glass spreaders were similar to those of plastic spreaders. In view of this, all subsequent titrations were based on membrane filtration.

2.3.2 Effect of Combining an Organic Load with Mycobacterial Suspensions

An organic load was combined with the mycobacterial suspension for inoculating carriers. The titer of *M. terrae* with the organic load was determined so that the reduction of the titer in the suspension due to dilution, drying of the suspension and after exposure of dried carriers to germicides could be compared to those obtained with the same suspension of mycobacteria but without the organic load. Please refer to Figure 2.4.

2.3.2.1 Effect of Dilution on the Titer of *M. terrae*

A suspension of *M. terrae* in water and the same combined with an organic load (Section 2.2.8) was enumerated using the membrane filtration method (Section 2.2.6). The mean $\log_{10}$ CFU/10 µL of the suspension, with and without the organic load was 6.99 and 7.48, respectively. The titer of the suspension with the organic load was reduced by 0.49 most likely due to dilution and not because of any inhibitory effects of the components of the soil load. (Figure 2.4 A)

2.3.2.2 Effect of Drying on the Titer of *M. terrae*

Suspensions of *M. terrae* with and without an organic load were inoculated onto glass vial carriers and dried overnight (Section 2.2.9). The mean titer ($\log_{10}$) in the dried inocula with and without the organic load was 6.50 in both cases, whereas the titers before drying were 6.99 and 7.48, respectively. The titer was therefore reduced by 0.49 and 0.98, respectively, in the inocula with and without the organic load. This indicated a lesser degree of reduction of CFU in the inocula with the organic load during drying, most probably due a protective effect on the viability of the mycobacteria. (Figure 2.4 B)

2.3.2.3 Effect on Susceptibility of *M. terrae* to Germicides

To determine whether and to what extent an organic load protects mycobacteria from germicides, a quantitative carrier test was performed using carriers contaminated with *M. terrae* with and without an organic load and following overnight drying. Contaminated carriers were exposed to diluted alkaline glutaraldehyde (0.8%) and a phenolic-based (0.33%) germicide for 10 minutes in a quantitative carrier test (Section 2.2.10).

The mean $\log_{10}$ reductions in CFU with glutaraldehyde were 4.72 and 5.02, respectively, with and without the organic load. With the phenolic germicide, the mean $\log_{10}$ reductions in CFU with and without the organic load were 2.64 and 5.66, respectively. The mean $\log_{10}$ reductions in CFU were lower in carriers containing the organic load by 0.30 (5.02 minus 4.72) and 3.02 (5.66 minus 2.64) in the test with glutaraldehyde and the phenolic, respectively. This clearly indicated a higher bacterial survival in the inocula with the organic load, further reinforcing the protective effect of, and the need to include, a soil load in germicidal tests. These data also show that the activity of weaker germicides is reduced to a higher degree in the presence of a soil load. (Figure 2.4 C)

Figure 2.4 The effect of combining an organic load on the titer of *M. terrae* in suspension, after drying and on susceptibility to germicides

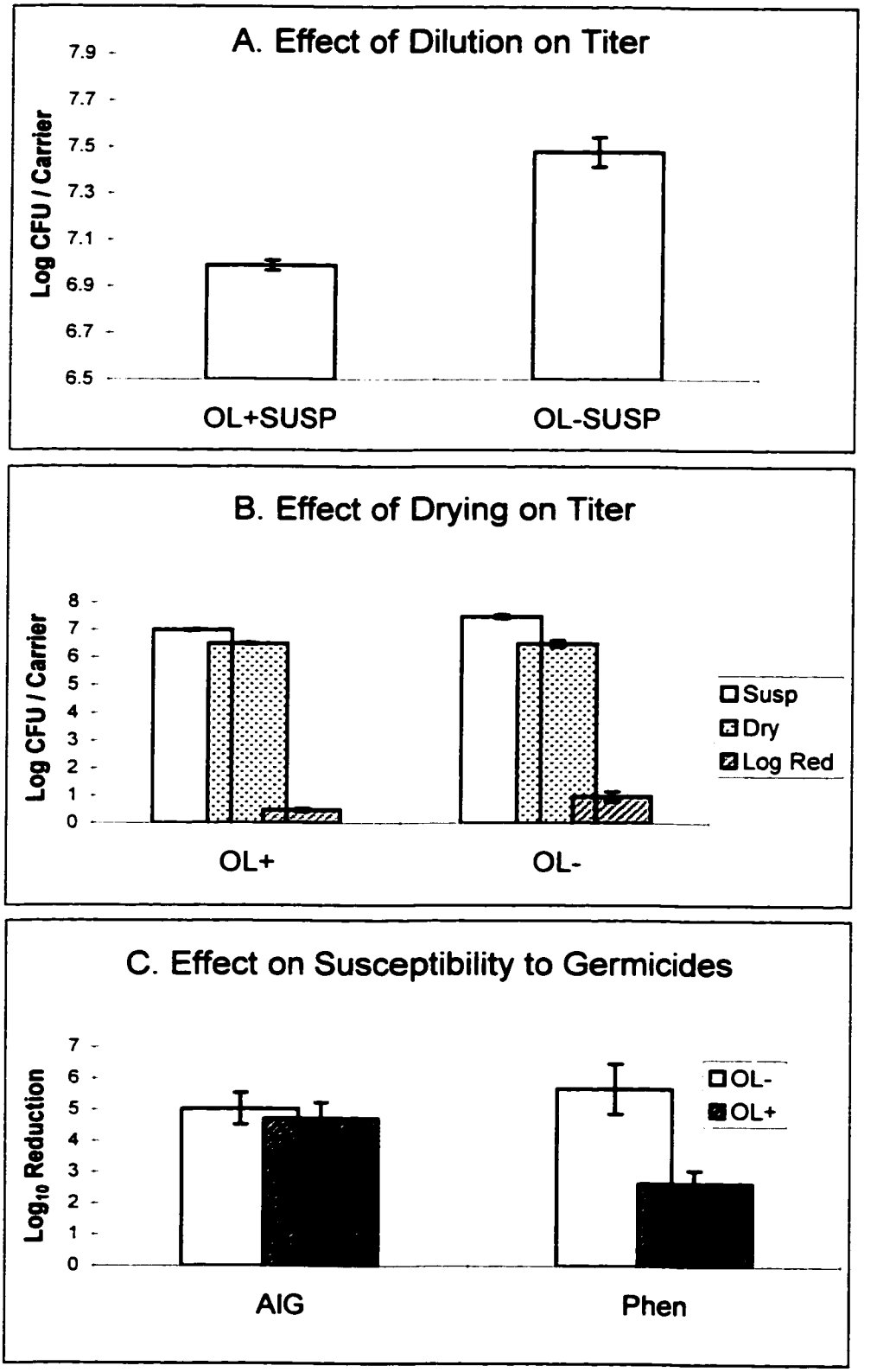
A suspension of *M. terrae* in water was mixed with a combination of organic load (*M. terrae* 342.8 µL + 22.1 µL of 5 % BSA + 35.1 µL of 5 % tryptone + 100 µL of 0.4 % mucin).

(A) Suspensions (Susp) with (OL+) and without (OL-) the organic load were enumerated using the membrane filtration method (Section 2.2.6). The $\log_{10}$ reduction due to dilution was observed in the suspension with the organic load.

(B) The suspensions (Susp) with and without the organic load were inoculated into glass vials, dried overnight and enumerated in triplicates (Dry controls). The effect of drying was observed as a $\log_{10}$ reduction in the CFU which was different in the two inocula.

(C) Dried inoculated carriers were exposed to 0.80% alkaline glutaraldehyde (AlG) and 0.33% phenolic (Phen) germicides for 10 minutes and CFU of survivors enumerated using a quantitative carrier test. The $\log_{10}$ reductions in the CFU achieved by the two germicides were lower in the inocula with the organic load than without the organic load.

Figure 2.4


A. Effect of Dilution on Titer
Log CFU / Carrier
7.9
7.7
7.5
7.3
7.1
6.9
6.7
6.5
OL+SUSP
OL-SUSP
B. Effect of Drying on Titer
Log CFU / Carrier
8
7
6
5
4
3
2
1
0
Susp
Dry
Log Red
OL+
OL-
C. Effect on Susceptibility to Germicides
Log10 Reduction
7
6
5
4
3
2
1
0
OL-
OL+
AIG
Phen

2.3.3 Mycobactericidal Activity of Germicides

Seven species of mycobacteria including *M. terrae* ATCC 15755, *M. xenopi* NT 1901, *M. gordonae* NT 1101, *M. kansasii* NT 711, *M. fortuitum* NT 2707, *M. chelonae* NT 2901 and *M. smegmatis* mc^2155 were tested in a quantitative carrier test (Sections 2.2.8, 2.2.9 and 2.2.10) to determine and compare their susceptibilities to germicides. Formulations of hydrogen peroxide, alkaline glutaraldehyde, QACs and phenolic-based germicides were used at the manufacturers recommended, as well as other, contact periods and dilutions. Each germicide was assessed for its ability to produce a $\geq$6-$\log_{10}$ reduction in CFU. The results are summarized in Table 2.2, Figure 2.5 and 2.6.

A 7.5% hydrogen peroxide-based product required 10 minutes of contact to achieve a $\geq$6-$\log_{10}$ reduction in the viability titer of *M. terrae* and *M. fortuitum*. The same product required 20 minutes for *M. gordonae* and *M. chelonae*, whereas with *M. xenopi* and *M. kansasii* a 6-$\log_{10}$ reduction was not achieved even with 30 minutes of contact. A different product based on 7.0% hydrogen peroxide used for 20 minutes achieved a $\geq$6-$\log_{10}$ reduction for *M. terrae*. The product was effective against *M. smegmatis* even when used at 2.33% for 10 minutes. Activated alkaline glutaraldehyde (2.4%) used for 20 minutes achieved a $\geq$6-$\log_{10}$ reduction in all of the species tested. It was effective against *M. smegmatis* after 10 minutes of contact. A product based on QACs used in three different concentrations (600, 800 and 1200 ppm) for 10 minutes was not effective against *M. terrae*. When used for 30 minutes at 800 and 1,200 ppm the product produced a 5.84 $\log_{10}$ reduction. The phenolic used for 10 minutes at concentrations of 0.5%, 1.0% and 2.0% achieved a >6-$\log_{10}$ reduction when tested with *M. terrae*.

Table 2.2 Log_{10} Reduction in CFU of seven species of Mycobacteria after exposure to germicides at different contact times and dilutions.

Mycobacteria	Germicide (%)	Contact Time (minutes)	Mean Log_{10} Reduction
M. terrae	Hydrogen Peroxide 7.5% (a)	10	7.23 ± 0.43
M. terrae	Hydrogen Peroxide 7.5% (a)	20 and 30	7.58 ± 0.15
M. terrae	Hydrogen Peroxide 3.75%(a)	10	2.48 ± 0.25
M. terrae	Alk. Glutaraldehyde 2.4%	20	7.22 ± 0.24
M. terrae	Hydrogen Peroxide 7.0% (b)	10	4.22 ± 0.19
M. terrae	Hydrogen Peroxide 7.0% (b)	20	6.51 ± 0.27
M. terrae	Hydrogen Peroxide 7.0% (b)	30	7.53 ± 0.06
M. terrae	Phenolic (0.5, 1 & 2 %)	10	7.58 ± 0.15
M. terrae	QAC (600 ppm)	30	5.50 ± 0.17
M. terrae	QAC (800 and 1200 ppm)	30	5.84 ± 0.16
M. fortuitum	Hydrogen Peroxide 7.5% (a)	10	6.05 ± 0.19
M. fortuitum	Alk. Glutaraldehyde 2.4%	20	6.05 ± 0.19
M. smegmatis[1]	Hydrogen Peroxide 7.0% (b)	10	6.10
M. smegmatis[1]	Hydrogen Peroxide 2.3% (b)	10	6.10
M. smegmatis[1]	Alk. Glutaraldehyde 2.4%	10	6.10
M. kansasii	Hydrogen Peroxide 7.5% (a)	10	TNTC[2]
M. kansasii	Hydrogen Peroxide 7.5% (a)	20	4.74 ± 0.38
M. kansasii	Hydrogen Peroxide 7.5% (a)	30	5.42 ± 0.54
M. kansasii	Alk. Glutaraldehyde 2.4%	20	7.11 ± 0.12
M. gordonae	Hydrogen Peroxide 7.5% (a)	10	TNTC[2]
M. gordonae	Hydrogen Peroxide 7.5% (a)	20	7.24 ± 0.48
M. gordonae	Hydrogen Peroxide 7.5% (a)	30	7.50 ± 0.11
M. gordonae	Alk. Glutaraldehyde 2.4%	20	7.60 ± 0.06
M. xenopi	Hydrogen Peroxide 7.5% (a)	10	5.23 ± 0.21
M. xenopi	Hydrogen Peroxide 7.5% (a)	30	5.73 ± 0.11
M. xenopi	Alk. Glutaraldehyde 2.4%	20	6.33 ± 0.03
M. chelonae	Hydrogen Peroxide 7.5% (a)	10	5.05 ± 0.64
M. chelonae	Hydrogen Peroxide 7.5% (a)	20	6.63 ± 0.15
M. chelonae	Hydrogen Peroxide 7.5% (a)	30	6.75 ± 0.34
M. chelonae	Alk. Glutaraldehyde 2.4%	20	6.90 ± 0.25

Each log_{10} reduction is the mean of three experiments with five test and three control carriers in each experiment (i.e. 3 x 5 tests). Hydrogen peroxide (a) and (b) are two different test products. [1]Results for *M. smegmatis* are those of one experiment (5 tests) and a soil load was not included. Where CFU was TNTC[2] (too numerous to count) log_{10} reduction could not be determined.

Figure 2.5 Log_{10} reductions in the CFU of five species of mycobacteria after exposure to germicides at different contact periods and dilutions.

Five species of mycobacteria (*M. gordonae, M. kansasii, M. chelonae, M. xenopi*, and *M. fortuitum*) were tested for their susceptibility to germicides in a quantitative carrier test (Section 2.2.10). The procedure consisted of inoculating and drying suspensions of mycobacteria on glass vials (10^6-10^7 CFU/10 μL), exposure of dried carriers to the test germicide, neutralization of the germicide by dilution, elution, membrane filtration and plating of membranes onto M-7H11-OADC agar. A comparison of growth in the test plates with controls not exposed to the germicides in a log_{10} reduction calculation allowed quantitative assessment of the mycobactericidal activity of the test germicide. Each result (represented by a column in the chart) is the mean of three experiments with five test and three control carriers in each experiment.

Figure 2.5

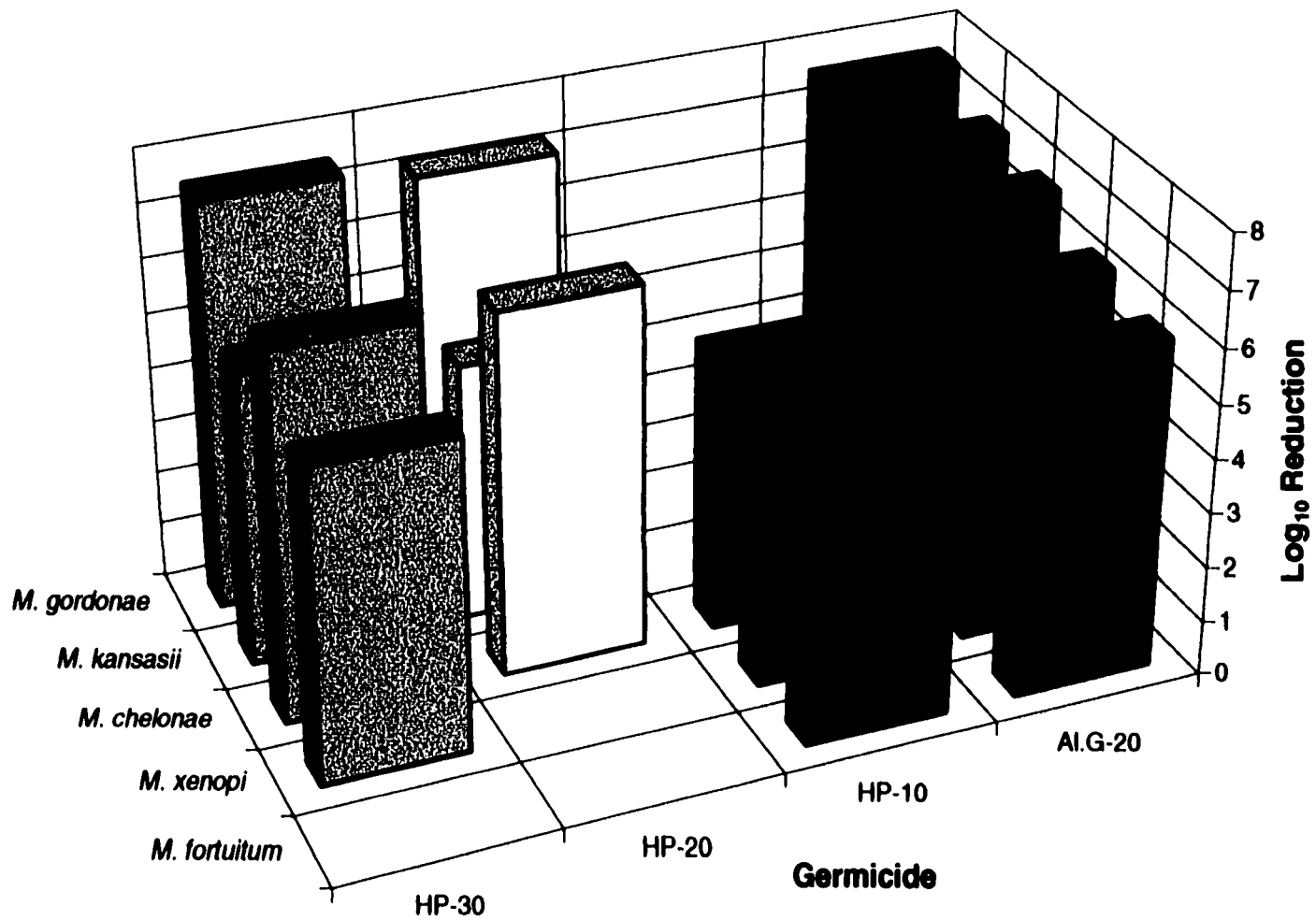

M. gordonae
M. kansasii
M. chelonae
M. xenopi
M. fortuitum
HP-30
HP-20
HP-10
Al.G-20
Germicide
Log10 Reduction
0
1
2
3
4
5
6
7
8

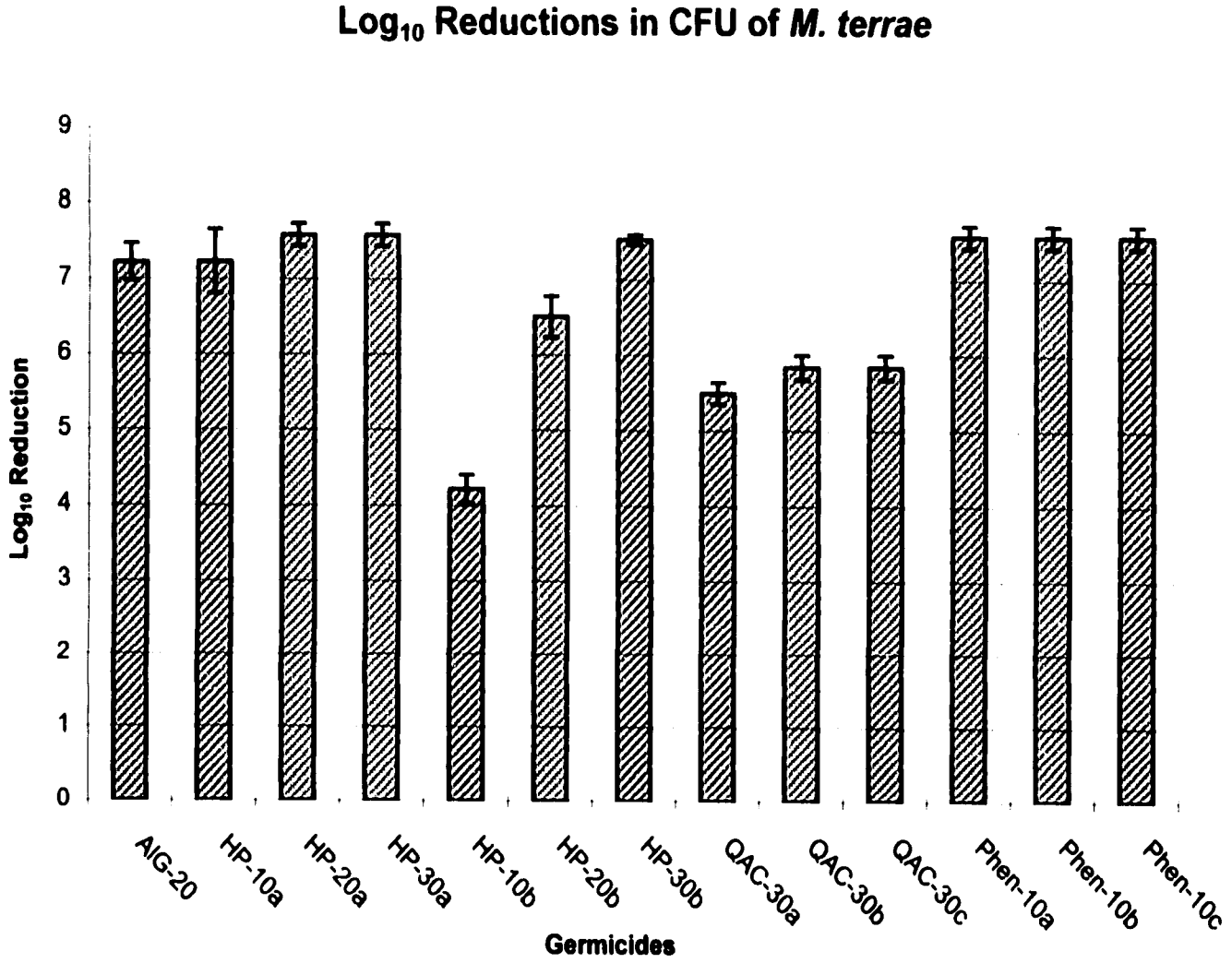
Figure 2.6 Log_{10} reduction in the CFU of *M. terrae* after exposure to germicides at different contact periods and dilutions.

M. terrae was tested for its susceptibility to several germicides in a quantitative carrier test (Section 2.2.10). A 7.5% Hydrogen peroxide was used at contact periods of 10 (HP-10a), 20 (HP-20a), and 30 (HP-30a) minutes and 2.4% alkaline glutaraldehyde for 20 minutes (AlG-20). A second product based on hydrogen peroxide (7.0%) was used for 10 (HP-10b), 20 (HP-20b), and 30 (HP-30b) minutes. The QAC-based product was tested for 30 minutes at concentrations of 600 (QAC-30a), 800 (QAC-30b), and 1,200 (QAC-30c) ppm and a phenolic germicide was used for 10 minutes at concentrations of 0.5% (Phen-10a), 1.0% (Phen-10b), and 2.0% (Phen-10c).

Dried inoculated carriers of *M. terrae* were exposed to the germicides followed by 100-fold dilution in saline, elution, membrane filtration and plating on M-7H11-OADC agar. After incubation, a comparison of growth in the test plates with controls (not exposed to the germicides) in a log_{10} reduction calculation allowed quantitative assessment of the mycobactericidal activity of the test germicide.

Each result (represented by a column in the chart) is the mean of three experiments ± SD, with five test and three control carriers in each experiment.

Figure 2.6


Log10 Reductions in CFU of M. terrae
Log10 Reduction
9
8
7
6
5
4
3
2
1
0
AIG-20
HP-10a
HP-20a
HP-30a
HP-10b
HP-20b
HP-30b
QAC-30a
QAC-30b
QAC-30c
Phen-10a
Phen-10b
Phen-10c
Germicides

2.4 Discussion

A quantitative carrier test method has been developed in our laboratory for testing the sporicidal activity of chemical germicides (Springthorpe *et al.*, 1994). Several advantages inherent in this method made it attractive for use in testing germicides for their mycobactericidal activity as well. The method was based on membrane filtration and colony counting procedures, which were described in previous studies as being reliable, sensitive and quantitative (Collins, 1987; Robison *et al.*, 1996). As described in this part of the thesis, the carrier method, with virtually no major changes, proved to be suitable for working with several species of mycobacteria and a variety of chemical germicides

The search for a suitable surrogate(s) for use in mycobactericidal tests continues because of the well-recognized limitations of the species currently under use (Sattar *et al.*, 1995; Griffiths *et al.*, 1998). *M. smegmatis* is non-pathogenic, fast-growing and yields results in 3-5 days even without the OADC supplement. However, because of its proven sensitivity to many germicides it may be used in screening tests only (Best *et al.*, 1988; van klingeren and Pullen, 1987). *M. bovis* BCG may be more sensitive to many germicides than clinically relevant species of mycobacteria (Best *et al.*, 1994). It also grows much more slowly than *M. terrae*. This, along with a higher tendency to clump, adsorption onto stir bars, and poor and inconsistent recovery are among the drawbacks associated with its use.

A suitable surrogate for testing anti-mycobacterial agents should be (a) non-pathogenic, (b) faster-growing and (c) have a susceptibility profile to germicides similar to that of most pathogenic strains of mycobacteria and in particular *M. tuberculosis*.

Although an ideal surrogate does not exist, *M. terrae* used in this study fulfills the criteria of being relatively non-pathogenic and for having susceptibility to germicides that is somewhat similar or slightly higher than that of *M. tuberculosis* (van Klingeren and Pullen, 1987; Griffiths *et al.*, 1998). Therefore, in germicidal testing, effectiveness against *M. terrae* should indicate effectiveness against *M. tuberculosis.*

The use of M-ADC-TW broth for making stock cultures of mycobacteria was found to be reliable and consistently high titer pools (~10^9 CFU/mL) could be produced. As a recovery medium for stressed and injured mycobacteria, M-7H11 agar supplemented with OADC was also found to be suitable in this study.

When compared with other slow growing mycobacteria, *M. terrae* grows relatively fast in an enriched agar medium such as supplemented M-7H11 agar. In our experiments, it required 8-10 days for countable colonies to appear in the control plates and 10-28 days for cells exposed to germicides to grow. The longer lag period in the tests appears to be the result of slow recovery of stressed or injured cells before the onset of replication. The lag period was a few days longer when *M. terrae* was exposed to alkaline glutaraldehyde than to the hydrogen peroxide-based product.

It was observed that the growth of *M. terrae* was retarded when it was grown in M-7H11 agar without OADC supplement. Visible colonies appeared later and could be counted after 14 days in the controls. In a preliminary study, it was shown that despite a delay in the appearance of *M. terrae* colonies in OADC-deprived medium, there was no difference in colony count when compared with corresponding growth in OADC supplemented plates (data not shown). However, stressed or injured mycobacteria such

as those exposed to germicides may require OADC as an essential component for recovery and growth.

In order to curtail the high cost and unpredictable supply of this supplement, OADC was prepared and tested in-house as mentioned previously by other investigators (Belisle and Sonnenberg, 1998). When used for growing *M. terrae*, OADC prepared in our laboratory was found to be equivalent to that from our main commercial supplier, Difco Laboratories. The cost was about 10-times less, and it required a few hours to prepare a volume of 1-2 liters.

Other species of mycobacteria evaluated in this study did not offer any major advantages over *M. terrae*. *M. gordonae*, *M. xenopi*, *M. kansasii* and *M. chelonae* were more resistant than *M. terrae* and in addition, *M. xenopi* and *M. chelonae* required incubation temperatures higher and lower than 37°C, respectively.

In addition to having a lipid-containing cell wall, mycobacteria in general exist within a protective film of organic substances in clinical and environmental materials that allows them to better resist the effects of environmental adversities as well as germicides. An organic or soil load is often incorporated in the bacterial test inocula to simulate such conditions and to present the test germicide with a more realistic challenge. Whereas many organic substances at varying levels have been tested over the years, there is as yet no general agreement as to which one of these should be considered as a 'universal' soil load. The difficulty in identifying a universally acceptable soil load arises mainly due to a long list of criteria such a substance or a combination of substances should meet. The following is a list of such criteria:

A suitable soil or organic load in a germicidal test should: (a) be readily available commercially and be relatively inexpensive, (b) be amenable to standardization, (c) confer the desired degree of resistance to the test inoculum, (d) be non-inhibitory to the test organism(s), (e) not require the sacrifice of animals to obtain fresh cells or tissues such as would be needed in the use of guinea-pig spleen homogenates (Lind *et al.*, 1986).

Animal serum, particularly that from bovines, is often used at a final concentration of 5-10% as soil load in evaluating the germicidal activity of chemicals. In spite of its popularity, the use of serum suffers from several drawbacks and these include its lack of ready availability, expensive nature, batch-to-batch variations, presence of specific or non-specific inhibitors, etc. Therefore, one of the sub-objectives of this study was to find a soil load, which would be suitable for testing mycobactericides and other classes of chemical germicides.

The soil load evaluated in this study was a mixture of a high molecular weight (BSA), a low molecular weight (tryptone) and mucoid (bovine mucin) protein. The quantity of BSA, tryptone and mucin used to make the inoculum provided a total protein content of 4.0 mg/mL and is approximately equivalent to the level of protein in 5% fetal bovine serum (Best, 1993). The three types of protein were selected because they resemble major components of body fluids that may interact with germicides and neutralize their activity, at least in part. Mucin in the dried form may also hinder penetration of the germicide. The organic substances used in this study are readily available commercially, reasonable in cost and standardizable. A combination of BSA, tryptone and mucin, as used in this study, appeared to meet all the desirable criteria for a soil in testing for mycobactericidal activity.

The incorporation of this organic load diluted and therefore reduced the titer of 10 μL of a suspension of *M. terrae* by approximately $\log_{10}$ 0.49. In practice, this is not a problem as the initial count of cells in the suspension before the addition of the organic load is maintained at a higher level (~10^7 in 10 μL) to compensate for this loss. So, despite the decrease in cell counts resulting from dilution with the organic load, the inoculated carriers contained at least 10^6 viable cells after overnight drying. The quantitative carrier test requires that a dried carrier must have at least 10^6 viable cells in the dried state for a germicide to be able to achieve a minimum 6-$\log_{10}$ reduction. Overnight drying of 10 μL of mycobacterial suspension resulted in a reduction in titer, which was significantly less in the inocula with the soil load indicating a protective effect of the organic load against drying. This resulted in the dried carriers with and without the organic load having the same $\log_{10}$ recovery of 6.50, although carriers with the organic load started off with 0.49 $\log_{10}$ fewer organisms before drying.

The fact that the presence of the soil load was exerting a higher demand on certain germicides tested is clearly indicated by the lower levels of $\log_{10}$ reductions in its presence. For example, with the phenolic tested, more than 3 $\log_{10}$ of the test bacterium survived exposure to the germicide in the inocula with the soil load when compared to those without. Further work is necessary to establish the potential of our protein mixture as a universally acceptable soil load.

To evaluate germicides used on contaminated surfaces and simulate the in-use practices of surface and equipment disinfection, carrier tests are generally preferred to suspension tests. The glass vials used in this method allowed convenient inoculation,

desiccation and recovery of a fixed volume of standardized inocula owing to the smooth and non-porous flat-bottomed surface (Sattar *et al.*, 1995). The use of inserts and septate caps in the vials eliminated any false-positive results that may arise due to the generation of microaerosols during inoculation and drying. Once the inoculum was dried and the inserts and septate caps were replaced with normal caps, the carriers could be stored for at least one month without any further loss of viable cells or risk of contamination. The flat surface of the carriers allowed a ratio of 1:100 between the volume of the test microbial inocula and the volume of the product being tested thus providing a greater germicidal challenge. These carriers were used in the evaluation of *Ortho*-phthalaldehyde as a possible alternative to glutaraldehyde for high level disinfection using a range of organisms including glutaraldehyde-resistant mycobacteria, *Bacillus subtilis* spores and coat-defective spores (Walsh *et al.*, 1999).

The combined use of Teflon-coated magnetic stir bars and vortexing allowed nearly complete removal and disaggregation of the inocula thereby making glass vials a suitable carrier for use in this study. Other tests have used carriers such as disks, penicylinders and objects of different dimension and material that were designed to resemble components of a medical device or an environmental surface. However, some of these carriers are associated with variations in quantitating the input inocula or during recovery of the organisms (Sattar *et al.*, 1995). In our laboratory, stainless steel disks (Best *et al.*, 1990) have been used in carrier tests. Compared to glass vials, there is a smaller ratio of 1:5 between the volume of the inocula and the test product. Recovery of the inoculum may be less consistent because a Teflon-coated magnetic stirrer is not used to dislodge and remove cells from the disks. Grooves and pitting in stainless steel and

porcelain surfaces may confer protection to mycobacteria against germicide and also prevent their physical removal. Glass surfaces, on the contrary, have been found to be very smooth when examined under a scanning electron microscope (Cole *et al.*, 1987). Glass penicylinders are substituted for stainless steel ones in the current AOAC hard-surface carrier test (Rubino *et al.*, 1992). In the current study, glass vials with inserts and septate caps were shown to be suitable carriers for easy, accurate and reproducible testing of germicides for mycobactericidal activity.

The chemical composition, amounts of disinfectants such as chlorine and the level of hardness of tap water vary in different locations and at different times at the same location. Therefore, water of a standard hardness of 200 ppm calcium carbonate was used to make all dilutions of the germicides as recommended in several standard test methods (CGSB, 1997). The QACs were diluted in water with a hardness of 400 ppm as recommended by the manufacturer. The use of standard hard water in these tests, therefore, eliminated any variables that could have been introduced by the incorporation of tap water as a diluent. Moreover, it presented the test germicides with a greater challenge due to the presence of an inorganic load higher than that encountered under most field conditions.

As stated earlier, a fully quantitative carrier test, such as the one used in this study, is preferable to any qualitative or semi-quantitative methods to determine the potential of a germicidal formulation to perform well in actual in-use conditions. Our method also eliminated the variability seen in the AOAC test protocols (Beloian, 1993). In the quantitative carrier test, glass vials were inoculated with a fixed volume of standardized inocula using a calibrated micropipette. The whole procedure of germicidal

challenge, dilution and elution occurred in the same glass vial followed by complete recovery on membrane filters without any wash-off of cells, thereby permitting accurate quantitation.

A ≥6-$\log_{10}$ reduction was set as an arbitrary criterion for mycobactericidal activity of a test product. The germicides tested included those that are commonly used in hospitals, nursing homes and other institutional facilities to provide low level (QAC) and intermediate level (phenolic and 7.0% H_2O_2) disinfection of environmental surfaces and non-critical items and those that are used to provide high level disinfection (2.4% glutaraldehyde and 7.5% H_2O_2) of semicritical patient care items such as flexible endoscopes.

Activated alkaline glutaraldehyde (2.4%) used for 20 minutes was consistently effective against all *Mycobacterium spp.* although *M. smegmatis* was tested and inactivated at 10 minutes owing to its proven sensitivity (Best *et al.*, 1988; van Klingeren and Pullen, 1987). This supports previous studies that demonstrated glutaraldehyde as a good high-level disinfectant (Russell, 1994; Best *et al.*, 1990; Cole, 1990; Rutala and Cole, 1987). However, despite its effectiveness, as well as being non-corrosive to metals, rubber and lenses, glutaraldehyde is not uniformly popular owing to adverse effects on the eyes, skin and the respiratory system (Gannon *et al.*, 1995; Russell, 1994). There have also been reports of potential mutagenic and carcinogenic effects associated with the use of glutaraldehyde (Hugo and Russell, 1999).

A product based on 7.5% hydrogen peroxide achieved a ≥6 $\log_{10}$ reduction when tested against *M. terrae* and *M. fortuitum* for 10 minutes or one-third of the recommended

contact time. When tested against *M. gordonae* and *M. chelonae* it required 20 minutes to be effective. *M. xenopi* and *M. kansasii* proved to be the most resistant and could not be inactivated to the ≥6 $\log_{10}$ even at 30 minutes at room temperature. A different product based on 7.0% hydrogen peroxide required 20 minutes to achieve a similar ≥6-$\log_{10}$ reduction for *M. terrae*. When tested against *M. smegmatis*, the same undiluted product (7.0%) as well as a 1:2 dilution (2.33%) required only 10 minutes to achieve the same degree of effectiveness. This indicates a much higher sensitivity of *M. smegmatis* to the germicide than any of the other NTM as was observed in other studies (Best *et al.*, 1990; van Klingeren and Pullen, 1987). Although hydrogen peroxide-based products are not as effective as aldehydes, the safety associated with their use has increased their application in many situations including the reprocessing of heat-sensitive medical devices.

A phenolic-based germicide used at dilutions (1:200 or 0.5%) and contact period (10 minutes) recommended for tuberculocidal activity was also effective against *M. terrae*. Despite their effectiveness as mycobactericidal agents even in the presence of organic substances, phenolics are sometimes avoided in hospitals and laboratories and are unsuitable for use in food preparation areas and in places housing infants due to the strong odor and toxicity (Best *et al.*, 1990).

The product based on QACs (which had no mycobactericidal claim on the label) used at 600, 800 and 1,200 ppm for 10 minutes was found to be ineffective against *M. terrae*. This is consistent with previous findings where inadequate mycobactericidal activity was demonstrated by QAC-based products, especially in the presence of organic

matter (Rutala *et al.*, 1991; Best *et al.*, 1990). When used for 30 minutes at 800 and 1200 ppm, the product was found to be moderately effective (log_{10} reduction 5.84), although residual inhibitory activity should be properly ruled out when using QACs (Leers *et al.*, 1974).

Elimination of inhibitory residual germicidal activity at the time of culture is of paramount importance for accurate estimation of the potency of a test product, failing which an overestimation of mycobactericidal activity leading to false claims of efficacy may be made. An ideal chemical neutralizer effective against most germicides is yet to be found and those that are available have their own drawbacks, such as toxicity for the test organism, slow action, expense or lack of ready availability. We found quenching by a 1:9 dilution with sterile normal saline in combination with membrane filtration followed by rinsing with 100 mL of the diluent to be convenient for all the products tested except for the QACs. Earlier studies have also demonstrated the bacteriostatic activity of QACs in subculture media with consequent false claims of mycobactericidal activity (Leers *et al.*, 1974). When a new germicide is tested, there is a need to verify the effectiveness of quenching by dilution or chemical neutralization. If chemical neutralizers are to be used, they should also be evaluated for potential toxic effects to the test microorganisms (Walsh *et al.*, 1999; Cole *et al.*, 1990).

Vacuum filtration using Millipore membranes allowed complete capture of the cells in the eluate from the vials. It also permitted appropriate washing of the cells while flushing away any trace of germicide in the flow-through fluid. The cells were trapped on the surface of the black gridded membranes without any wash off and were able to grow when placed on the recovery medium. This allowed complete recovery of the

organisms facilitating accurate colony counts for quantitative assessment of germicidal activity as demonstrated in previous studies (Collins, 1987; Robison *et al.*, 1996). In a comparative assessment of membrane filtration and spread plate methods, higher numbers of CFU were obtained using the membrane filtration method, indicating it to be a more precise means for enumerating mycobacteria (Table 2.1).

Robert Koch (1881) introduced the first carrier tests by impregnating silk threads with the spores of *Bacillus anthracis*. To date, various methods have evolved for determining the mycobactericidal activity of liquid chemical germicides. This points to the fact that results generated from different test protocols tend to vary with a consequent variation of mycobactericidal claims on products with similar formulations. Even with the same method, significant inter-laboratory variations have been observed that are often related to the culture and composition of the test suspension (Robison *et al.*, 1996). Although suspension tests do not take into consideration the environmental aspects of disinfection, they provide a convenient means to test specific activity of the product. Carrier tests, which are preferred by manufacturers and regulators are inherently more variable than suspension tests. This reinforces the need for appropriate selection of the types of carriers. The contact time, temperature, changes in pH and soil loading as well as testing germicides under reuse should also be taken into consideration when determining the activity of a product.

The quantitative carrier test described in this study provides a convenient, accurate and reproducible method for testing germicides while taking into consideration in-use practices of disinfection, an appropriate surrogate for *M. tuberculosis*, avoidance of potentially toxic neutralizers and a fully quantitative test protocol. It is also an

appropriate method for comparing with or evaluating a new method. This method, however, continues to be an obstacle for the rapid determination of mycobactericidal activity owing to the prolonged periods of incubation necessary for the growth of most of the clinically relevant species of mycobacteria. In addition, the cost, labor and biosafety issues when considering the use of a pathogen such as *M. tuberculosis* may outweigh its benefits.

3. RAPID ASSESSMENT OF VIABILITY IN MYCOBACTERIA EXPRESSING LUCIFERASE AND GREEN FLUORESCENT PROTEIN

3.1 Introduction

3.1.1 Background

The study of mycobacteria has in recent years benefited enormously from the use of reporter genes. The reporter genes of interest include the *Escherichia coli LacZ* gene (Timm *et al.*, 1994), the chloramphenicol acetyl transferase (*cat*) gene (Das Gupta *et al.*, 1993), the *E. coli phoA* gene (Kremer *et al.*, 1995a; Lim *et al.*, 1995), the *xylE* gene from *Pseudomonas putida* coding for a catechol 2,3-dioxygenase (Curcic *et al.*, 1994), the firefly luciferase (*Lux*) gene (Jacobs *et al.*, 1993) and the green fluorescent protein gene (*gfp*) (Kremer *et al.*, 1995b). The introduction of luciferase and *gfp* into mycobacteria and their expression as bioluminescent and fluorescent phenotypes, respectively, has provided convenient tools for understanding gene regulation in response to environmental changes. Rapid detection of host cells and assessment of their physiological status or viability in real time are the obvious advantages of reporter gene techniques over traditional culture methods.

3.1.2 Luciferase Reporter Gene System

The enzyme luciferase, most commonly observed in fireflies, confers bioluminescence on living cells that harbor it. It catalyzes the oxidation of luciferin in the presence of ATP, Mg^{2+}, and O_2, to generate oxyluciferin and light. The quantum yield of the reaction is the highest known for bioluminescent reactions. At room temperature and pH 7-8, the light emitted has a wavelength of 562 nm (yellow-green). The light

emissions are proportional to the amount of luciferase in a sample and can be measured with luminescence spectrophotometers (Cooksey *et al.*, 1993; Gould and Subramani, 1988). Alternative detection methods include scintillation counters (de Wet *et al.*, 1987) or exposure of assay solution to x-ray or photographic films (Wood and DeLuca, 1987).

The enzyme is composed of single polypeptide 550 amino acids in length (62 kDa) and is active in the monomeric form. Detection of luciferase can be accomplished *in vitro*, usually requiring lysis or destruction of cells or *in vivo* (Gould and Subramani, 1988): the *in vitro* assay was 100-1000 times more sensitive than that for bacterial chloramphenicol acetyl transferase (*Cat*) and was considerably more rapid and less expensive. *In vivo* assays for detection of luciferase activity in bacteria, yeasts (Wood and DeLuca, 1987) and plants (Ow *et al.*, 1986) have also been reported. The major limitation of *in vivo* methods is the delivery of the substrate into cells, which must be permeabilized.

Jacobs and colleagues reported a rapid and sensitive drug susceptibility assay by transfecting mycobacteria with luciferase reporter phages (LRPs) and avoiding lysis of host cells (Jacobs *et al.*, 1993). This assay was tested in strains of BCG and *M. tuberculosis* to distinguish drug-resistant from drug-susceptible organisms. This study revealed that as few as 500 to 5,000 *M. smegmatis* cells expressing luciferase could be detected by luminometry. Andrew and Roberts transformed *M. smegmatis* mc^2155 with luciferase from *Vibrio harveyi*, and used it to test the activity of antibiotics and biocides (Andrew and Roberts, 1993). Rapid methods to screen the efficacy of antimicrobial agents against *M. tuberculosis* (Cooksey *et al.*, 1993; Arain *et al.*, 1996), *M. avium*

(Cooksey *et al.*, 1995; Arain *et al.*, 1996), *M. bovis* BCG and *M. intracellulare* (Arain *et al.*, 1996) have also been reported.

In the initial phases of this study, *M. smegmatis* and *M. terrae* were transformed with the plasmid pYUB180 (Jacobs *et al.*, 1993). This plasmid contains the mycobacterial plasmid pAL5000 origin of replication (oriM), the *E. coli* (ColE1) origin of replication (oriE), the promoter of the BCG heat shock protein 60 (P_{hsp60}) gene, the aminoglycoside phosphotransferase gene (*aph*) that confers kanamycin resistance and the firefly luciferase gene (*FFlux*) that expresses luciferase. It was proposed to use the bioluminescent mycobacterial cells resulting from this transformation for developing an assay for the rapid determination of mycobactericidal activity of chemical germicides. However, the luciferase system was soon found to have certain inherent limitations and these included (a) the need for a substrate (luciferin) for light emission, (b) difficulty in transporting the substrate across the intact cell membranes, (c) problems in sensitive measurement of light emission and (d) the appearance of scanty colonies of transformed mycobacteria. In view of this, efforts were directed to investigating the potential of the *gfp* as a reporter gene for mycobacteria.

3.1.3 GFP Reporter Gene System

The green fluorescent protein gene (*gfp*) is a more adaptable reporter molecule than luciferase. Although discovered by Shimomura and colleagues, in 1962, the green fluorescent protein (GFP) has only recently become established as one of the most widely used proteins in biology (Tsien, 1998). The *gfp* has been cloned (Prasher *et al.*, 1992) and expressed in heterologous systems such as *E. coli* (Inouye and Tsuji, 1994; Chalfie *et al.*, 1994), *M. smegmatis* and *M. bovis* BCG (Dhandayuthapani *et al.*, 1995) as well as in

Caenorhabditis elegans and *Drosophila melanogaster* (Chalfie *et al.*, 1994). Fluorescence persisted even when tissues were fixed in glutaraldehyde or formaldehyde indicating the resistance of GFP to these fixatives. However, the chemicals in nail polish used to seal cover slips, did appear to interfere with the *C. elegans* GFP fluorescence. Like other reporter genes such as luciferase, *gfp* and its expression in mycobacteria has provided a convenient tool for understanding gene regulation in response to environmental changes.

The GFP of the jellyfish *Aequorea victoria* is a unique protein (30-kDa monomer) of 238 amino acids (Prasher *et al.*, 1992) that absorbs light with an excitation maximum of 395 nm (with a minor peak at 470 nm) and emits green light with an emission maximum of 509 nm (with a shoulder at 540 nm) (Morise *et al.*, 1974; Ward *et al.*, 1980). The fluorescence is relatively stable at a wide range of pH between 6 to 12 and at temperatures up to 65°C and there is virtually no photobleaching (Tsien, 1998). This allows investigations to be performed repeatedly and for prolonged periods such as imaging and quantitation in a variety of ways including epifluorescence microscopy, confocal laser scanning microscopy, flow cytometry and fluorometry (Dhandayuthapani *et al.*, 1995; Kremer *et al.*, 1995b). Moreover, GFP does not require any exogenous substrates or co-factors for fluorescence and the harboring cells do not need to be lysed or permeabilized, allowing direct and repeated measurements of cell viability.

The utility of GFP-expressing *M. bovis* BCG was assessed in a quantitative fluorescence assay for rapid screening of anti-mycobacterial drugs (Kremer *et al.*, 1995b). In another study, a recombinant plasmid (pWES4) containing the gene encoding GFP was transformed into *M. avium*. Transformed *M. avium* were clearly visible inside human

macrophages and epithelial cells using fluorescence microscopy thus providing a useful tool for further study of intracellular behavior of this pathogen (Parker and Bermudez, 1997). In a more recent study, a strain of rapidly growing *M. aurum* expressing GFP was investigated in rapid screening of anti-tuberculosis drugs *in vitro* and in infected macrophages (Srivastava *et al.*, 1998).

The search for GFP with higher intensity fluorescence led to the construction and isolation of high-intensity mutants. Delagrave *et al.* (1995) isolated mutants of the cloned *A. victoria* GFP that show red-shifted excitation spectra similar to that of *Renilla reniformis* GFP. Cormack *et al.* (1996) isolated three GFP mutants, which when expressed in *E. coli*, fluoresce with approximately 100-fold higher intensity than bacteria expressing wt GFP. The excitation maxima of the three mutants show a severe spectral shift from 395 for parental GFP to between 480 nm and 501 nm for the three mutants, although emission wavelengths remain the same as the parent type (507-511 nm). Changes in the excitation spectra and an increase in soluble protein in the mutant GFP were considered to be responsible for the increased fluorescence.

The mycobacterial expression vector pMV261 (Stover *et al.*, 1991) has been used to express mutant GFP in *M. marinum* to study host-pathogen interactions (Valdivia *et al.*, 1996). The fluorescence of transformed mycobacteria was enhanced at least 20-fold and the transformed mycobacteria could be easily identified within live mammalian cells or in frozen thin sections from the spleen and liver of infected frogs, allowing real-time imaging of interactions between mycobacteria and host cells. Furthermore, GFP expression did not adversely affect bacterial survival, nor did it compromise entry into mammalian cells. Collins *et al.* (1998) reported on the improved expression of the

mutant GFP in *M. tuberculosis* and assessed the potential of a microplate-based fluorometric assay for screening of anti-tuberculosis agents.

In the current study the plasmids pWES4 and pBEN containing the gene encoding, respectively, a wt and a red-shifted, high-intensity mutant GFP were used to develop a GFP-based fluorescence assay. The plasmid pWES4 was obtained as a gift from San Francisco State University and was created by ligation of the *gfp* gene of the jellyfish *A. victoria* into the hsp60 gene in an *E. coli/mycobacteria* shuttle vector, pMV261 (Parker and Bermudez, 1997). Also present in pMV261 are the origins of replication for both *E. coli* and mycobacteria (Stover *et al.*, 1991). The wt GFP expressed by pWES4 is excited at 395 nm and fluoresces with an emission maximum at 510 nm (Morise *et al.*, 1974; Ward *et al.*, 1980). The plasmid pBEN was constructed by Ben Chang and Lalita Ramakrishnan and was obtained as a gift from Stanford University. A PCR fragment of *gfp* mutant 3 was inserted at the *Bam*H1-*Hind*III site of pMV262 to obtain the plasmid. In addition to *gfp*, pBEN contains the origin of replication for mycobacteria and *E. coli*, the mycobacterial hsp60 promoter and a kanamycin resistance gene (personal communication with R.H. Valdivia, Stanford University. The mutant GFP (GFPmut3) expressed by pBEN is excited at 501 nm and fluoresces with an emission maximum at 511 nm (Cormack *et al.*, 1996). Please refer to Figure 3.1 for plasmid maps.

The plasmids pWES4 and pBEN were introduced by electroporation into *M. smegmatis* mc^2155 (Snapper *et al.*, 1990) and *M. terrae* ATCC 15755 (ATCC, Rockville, MD). Expression of the wt and mutant GFP were observed in transformed mycobacteria using epifluorescence microscopy. We investigated approaches based on the measurement of GFP expression in *M. terrae* that would allow the assessment of viability

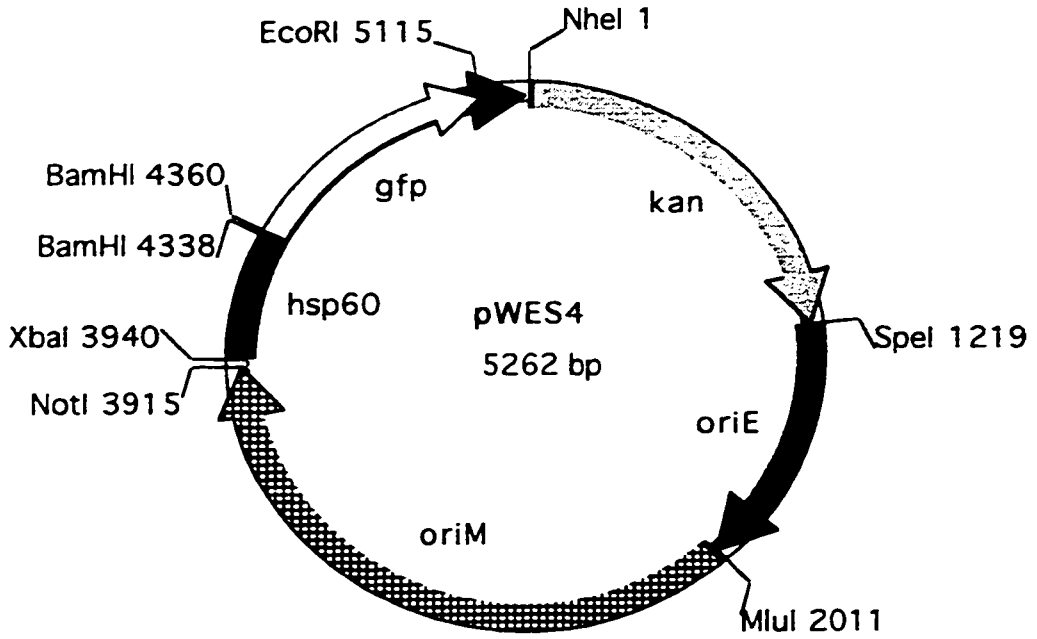
in mycobacteria and monitor any altered levels of expression in response to germicidal challenge. The objective of this study was to use the GFP expressing *M. terrae* for developing an assay for the rapid screening of chemical germicides for mycobactericidal activity.

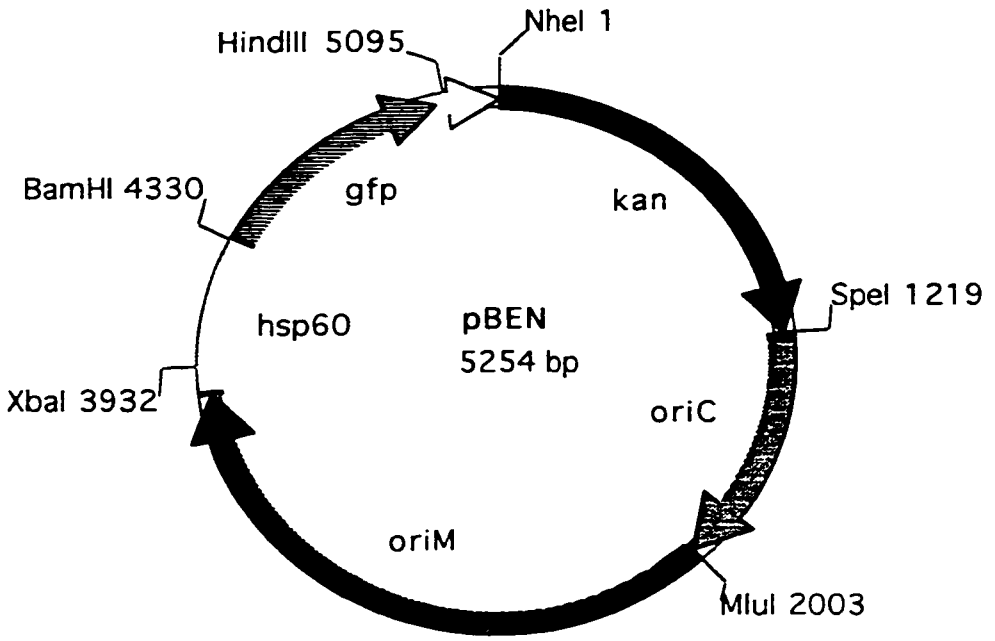
Figure 3.1 Map of the plasmids, pWES4 and pBEN containing the GFP gene.

(A) The plasmid pWES4 was created by ligation of the *gfp* gene from pGFP into the *hsp*60 multi-cloning site of pMV261 (Parker and Bermudez, 1997). The *hsp*60 gene drives the transcription of the *hsp*60/*gfp* fusion gene. The plasmid acts as a shuttle vector containing the origins of replication for both *Escherichia coli* (*oriE*) and mycobacterium (*oriM*). The kanamycin resistance gene (*kan*) and some of the restriction sites are shown.

(B) The plasmid pBEN was constructed by inserting a PCR fragment of *gfp* mutant 3 at the *Bam*H1-*Hind*III site of pMV262 (Ben Chang and Lalita Ramakrishnan, Stanford University). In addition to *gfp*, pBEN contains the origin of replication for mycobacteria (*oriM*) and *Escherichia coli* (*oriE*), the mycobacterial *hsp*60 promoter and a kanamycin resistance gene (*kan*).

Figure 3.1


EcoRI 5115
NheI 1
BamHI 4360
BamHI 4338
gfp
kan
hsp60
pWES4
5262 bp
XbaI 3940
NotI 3915
SpeI 1219
oriE
oriM
MluI 2011


HindIII 5095
NheI 1
BamHI 4330
gfp
kan
SpeI 1219
hsp60
pBEN
5254 bp
XbaI 3932
oriC
oriM
MluI 2003

3.2 Materials and Methods

3.2.1 Materials

A list of solutions, chemicals and suppliers is found in Appendix I

3.2.2 Culture of *Escherichia coli* Cells

A loopful of cells from a frozen stock (-20°C) or a single freshly grown colony from a plate was inoculated into a sterile conical flask or a centrifuge tube depending on the volume of liquid medium used. For strains containing plasmids, appropriate antibiotics were added to the medium for antibiotic resistance selection. The flask or tube was incubated at 37°C with overnight shaking (200 cycles/minute). Wherever indicated, growth was monitored by UV spectrophotometry (OD_{600}=0.5-1.0). For medium requirements of different cells and procedures, please refer to Table 3.1.

Table 3.1. *Escherichia coli* strains used for plasmid preparations and transformations

E. COLI STRAIN	PROCEDURE	CULTURE MEDIUM	ANTIBIOTIC
DH5α	Plasmid prep	Luria Broth (LB) / Terrific broth + Antibiotic	kanamycin: 15μg for pYUB180. 30μg for pBEN / pWES4.
JM109	Plasmid prep	As above	As above
DH5α	Transformation	LB / SOB / SOC / Terrific broth (No antibiotics in broth)	As above (added to agar plates for selection of transformants)
JM109	Transformation	As above	As above (added to agar plates for selection of transformants)

Adapted from Sambrook *et al.*, 1989.

3.2.3 Cold storage of *Escherichia coli* Cells

A 5 mL overnight culture was centrifuged at 2,000 ×*g* for 15 minutes and the pellet resuspended in 1 mL of 10 mM $MgSO_4$. The cell suspension was added to 1 mL of 80% glycerol in a sterile bottle and stored at -20°C. Alternatively, 0.85 mL of the liquified pellet was mixed with 0.15 mL of pure sterile glycerol (99.5%) and stored in cryovials at -80°C.

3.2.4 Storage and Culture of Mycobacterial Cells

Please see Section 2.2.2 and 2.2.3.

3.2.5 Small Scale Preparation of Plasmid by Alkali Lysis Method (Sambrook *et al.*, 1989)

A sterile loop of glycerol stock of *E. coli* DH5α cells containing the plasmid (pYUB180, pBEN or pWES4) was inoculated into 5 mL of LB broth or Terrific broth with the appropriate antibiotics and incubated overnight with shaking (200 cycles/minute) (Section 3.2.2). The cells were harvested at 2,000 ×g for 15 minutes in a centrifuge (IEC, Int., Needham Hts., MA) with a horizontal rotor and the supernatant was decanted. The cells were resuspended in 180 µL of plasmid preparation solution I (pH 8.0) and transferred to an Eppendorf tube to which was added 10 µL of lysozyme solution (10 mg/mL made in Solution I or, 10 mM Tris-Cl, pH 8.0) followed by brief vortexing. After incubation at RT for 5 minute, 400 µL of fresh plasmid preparation solution II was added and mixed by inverting the tube a few times. After incubating the tube on ice for 5 minutes, 300 µL of pre-cooled solution III (pH 4.8) was added and vortexed briefly. The tube was spun for 1 minute in a microcentrifuge (IEC Micromax, Needham Hts., MA) at 13,400 ×g and the supernatant transferred to a fresh tube. DNA was precipitated with 0.6

volumes of isopropanol (540 µL) followed by centrifugation for 1 minute. The supernatant was discarded and the pellet dried for 10 minutes in a vacuum desiccator. The pellet was resuspended in 200 µL of TE (pH 8.0) containing DNAse-free pancreatic RNAse (20 µg/mL) and incubated for 30 minutes at 37°C. 80 µL of 5 M ammonium acetate was added and the sample was mixed with twice the volume of isopropanol (560 µL). The tube was allowed to stand at RT for 10 minutes and then centrifuged at 13,400 ×g for 10 minutes. The supernatant was removed and the pellet was briefly dried in a vacuum desiccator. The pellet was resuspended in 100 µL TE (pH 8.0), to which was added 20 µL solution III (pH 5.6) and the tube was inverted to mix the contents. 240 µL of ice cold 95% ethanol was added to the tube and left for 20 minutes at -80°C or overnight at -20°C. The tube was spun for 12 minutes at 13,400 ×g, the supernatant removed and the pellet washed in 70% ethanol. The pellet was dried for 10 minutes in a vacuum desiccator and then resuspended in 15 µL TE (pH 8.0). The plasmid was stored at 4°C. (Please refer to Appendix I for plasmid preparation solutions I, II and III).

3.2.6 Large Scale Preparation of Plasmid by Sodium Dodecyl Sulphate (SDS) Lysis Method (Sambrook *et al.*, 1989)

This is the method of choice for isolating large plasmids (>15 kb) such as pYUB180 (35 kb). A 5 mL overnight culture of *E.coli* DH5α (pYUB180) was inoculated into 500 mL of LB broth or Terrific broth containing 15 µg of kanamycin per mL in a 2 L flask and incubated with vigorous shaking (200-300 cycles/minute). The growth was monitored by spectrophotometry, until the absorbance reached 0.5 at 600 nm. The cells were harvested by centrifugation at 2,800 ×g for 15 minutes at 4°C (4,000 rpm in a Beckman JA-10 rotor). The pellet was resuspended in 100 mL of ice-cold STE and

recentrifuged. After discarding the supernatant, the pellet was resuspended in 10 mL of an ice-cold solution of 10% sucrose in 50 mM Tris-Cl (pH 8.0) and transferred to a 50 mL centrifuge tube (Oak Ridge style). Two mL of a freshly prepared solution of lysozyme (10 mg/mL in 10mM Tris-Cl [pH 8.0]) was added followed by 8 mL of 0.25 M EDTA (pH 8.0). The suspension was mixed by inverting the tube several times and then placed on ice for 10 minutes. Four mL of 10% SDS was added and was immediately but gently mixed by inverting the tube a few times. Six mL of 5 M NaCl was added and mixed gently with a glass rod. The tube was placed on ice for at least 1 hour and then centrifuged at 48,000 ×g for 30 minutes at 4°C (20,000 rpm in a Beckman JA-20 rotor). The supernatant was carefully transferred to a 50-mL Teflon-coated centrifuge tube (Oak Ridge Style) which is resistant to phenol and chloroform and extracted once with phenol:chloroform and once with chloroform. The aqueous phase was transferred to a 250-mL centrifuge bottle and DNA precipitated by adding 2 volumes (~60mL) of 99% ethanol at RT. The tube was mixed well and allowed to stand at RT for 1-2 hours. The nucleic acids were recovered by centrifugation at 5,000 ×g for 20 minutes at 4°C (5,000 rpm in a Beckman J-10 rotor). The supernatant was discarded and the pellet washed with 70% ethanol at RT and then re-centrifuged at 5,000 ×g for 20 minutes. Ethanol was discarded by inverting the tube on a pad of paper towels and the pellet dried in a vacuum desiccator. The DNA pellet was dissolved in 3 mL TE (pH 8.0) and stored at 4°C.

3.2.7 Small Scale Plasmid Preparation by SDS Lysis Method (Sambrook *et al.*, 1989)

A small scale preparation by SDS lysis method is similar to the large scale procedure described in section 3.2.6, except that the culture and all other corresponding

volumes were 100 times less. After initial harvesting of the cells at 2,000 ×g for 15 minutes at 4°C, all subsequent centrifugations were carried out in a micro-centrifuge (IEC Micromax) at 13,400 ×g for 10 minutes in Eppendorf tubes. The final DNA pellet was resuspended in 15-30 μL of TE (pH 8.0) and stored at 4°C.

3.2.8 Plasmid Extraction From Agarose Gel

A 1% electrophoresis gel stained with ethidium bromide was used to separate the DNA fragments in the sample (Section 3.2.11). The gel was seen under UV light and the fragment containing the desired plasmid DNA was excised with a clean, sharp scalpel. Plasmid DNA was extracted and purified from agarose gels according to the manufacturers protocol (QIAquick gel extraction kit, Qiagen, Inc., Canada).

3.2.9 Plasmid Preparation and Purification Using an Extraction Column

E. coli DH5α containing the plasmids pWES4 and pBEN were streaked onto LB agar containing 30 μg of kanamycin per mL and grown overnight. A single colony from the plates were inoculated into a starter culture of 5 mL LB medium containing kanamycin (30 μg/mL) and incubated for 8 hours with vigorous shaking (200-300 cycles/minute). One mL of this culture was inoculated in 500 mL of LB containing kanamycin (30 μg/mL) in a 2 L flask and incubated at 37°C for 12-16 hours with vigorous shaking (200-300 cycles/minute). The cells were harvested by centrifugation at 6,000 ×g for 15 minutes at 4°C (6,000 rpm in a Beckman JA-10 rotor) and the supernatant discarded.

Plasmid DNA was extracted and purified using an extraction column according to the manufacturers protocol (Qiagen-tip 500, QIAGEN, Inc., Canada). The final pellet consisting of pure plasmid DNA was dissolved in 1-2 mL TE (pH 8.0).

3.2.10 Quantitation of DNA by Spectrophotometry (Sambrook *et al.*, 1989)

Quantitation of DNA was done by spectrophotometry performed at wavelengths of 260 and 280 nm. The readings at 260 nm allowed calculation of the concentration of nucleic acid. An optical density (OD) of 1 corresponds to approximately 50 μg/mL for double-stranded DNA. The ratio of OD_{260}/OD_{280} provides an estimate of the purity of the nucleic acid. For pure preparations of DNA the value of this ratio is 1.8.

Two μL of plasmid DNA in TE was diluted 500 times by adding to 998 μL of pure water in a quartz cuvette. The OD of the sample was determined by UV spectrophotometry (DMS 200, Varian Techtron Pty. Ltd., Victoria, Australia) against a blank containing 998 μL of pure water and 2 μL of TE at 260 nm. The quantity of DNA was calculated by using the following formula.

Quantity of DNA (μg/mL) = OD at 260 nm × Absorption Coefficient (50) × dilution factor (500).

When larger volumes of sample are available, the dilution of the DNA can be reduced for more accurate measurements.

3.2.11 Electrophoresis

DNA samples were separated on 1% agarose gels in 1 × Tris-acetate EDTA buffer at a constant voltage of 65 Volts. The gel was stained with 1 mg/L of ethidium bromide

and DNA was visualized under a UV transilluminator (Fotodyne Inc., WI, USA). Images were saved in computer diskettes and printed (Mitsubishi video copy processor, P40U).

3.2.12 Preparation of Competent *Escherichia coli* Using $CaCl_2$ (Sambrook *et al.*, 1989)

One hundred mL of LB broth or SOB medium (in a 1-Liter flask) was inoculated with a single colony (2-3 mm in diameter) of *E. coli* DH5α, from a plate freshly grown for 16-20 hours at 37°C. The culture was incubated for ~3 hours at 37°C with shaking (200-300 cycles/minute). The growth was monitored by spectrophotometry until the OD_{600} reached 0.5.

The culture was cooled to 0°C before centrifuging in 50 mL ice-cold tubes (Oak Ridge style) at 1,900 ×g for 10 minutes at 4°C (4,000 rpm in a Beckman JA-20 rotor). The supernatant was completely removed and the pellet was resuspended in 10 mL of ice-cold 0.1 M $CaCl_2$ and stored on ice for 15 minutes. The suspension was centrifuged as before and the supernatant discarded. The final pellet was resuspended in 4 mL of ice-cold 0.1 M $CaCl_2$, aliquoted into Eppendorf tubes in volumes of 200 µL each and stored at 4°C for 12-24 hours to increase transformation efficiency.

3.2.13 Transformation of Competent *Escherichia coli* Using $CaCl_2$ (Sambrook *et al.*, 1989)

Plasmid DNA (25-50 ng in a volume of 10 µL) was added to 200 µL of chilled competent *E. coli* DH5α in Eppendorf tubes. The contents were mixed by swirling gently and stored on ice for 30 minutes. As a positive control, a known amount of a standard preparation of plasmid DNA was added to competent cells. Negative controls consisted of competent bacteria without any plasmid DNA. The tubes including the controls were transferred to a water bath preheated to 42°C for exactly 90 seconds. The

tubes were rapidly transferred to an ice bath to chill for 2 minutes. Eight hundred μL of liquid medium (Terrific broth or SOC) was added and the tubes were incubated for 1 hour in a water bath at 37°C to allow the cells to recover and to express the antibiotic resistance marker encoded by the plasmid. The cells were plated onto LB or SOB agar plates (up to 200 μL cells per 90 mm plate) containing the appropriate antibiotic (Table 3.1), and the plates were left at RT for excess liquid to dry and then incubated overnight at 37°C.

3.2.14 Preparation of Competent Mycobacteria Using $CaCl_2$

Mycobacterial cells from a frozen stock were inoculated into 100 mL of M-ADC-TW broth and grown to log phase (Section 2.2.3). *M. smegmatis* mc^2155 was incubated for 48 hours at 37°C in a 1-liter conical flask with shaking (200 cycles/minute). *M. terrae* ATCC 15755 was grown in a cell culture flask for 21 days.

Subsequent steps of the procedure were the same as those used for preparing competent *E. coli* using $CaCl_2$, in the second paragraph of Section 3.2.12.

3.2.15 Transformation of Competent Mycobacteria Using $CaCl_2$

The procedure was essentially the same as those described in Section 3.2.13. During the recovery period, 800 μL of M-ADC-TW broth was added to the tubes to resuspend the cells. *M. smegmatis* mc^2155 cells were incubated for 3 hours and *M. terrae* for 12 to 16 hours at 37°C to allow the cells to recover and to express the antibiotic resistance marker encoded by the plasmid. The cells were plated onto M-7H11-OADC agar plates containing the appropriate antibiotic and incubated at 37°C (Table 3.2).

Table 3.2 Mycobacteria used for plasmid preparations and transformations

	***M. smegmatis* mc²155**	***M. terrae* ATCC 15755**
Liquid medium	M-ADC-TW	M-ADC-TW
Agar medium	M-7H11-OADC	M-7H11-OADC
Antibiotic for selection of plasmid pYUB180 bearing strains	Kanamycin 15 μg/mL	Kanamycin 15 μg/mL
Antibiotic for selection of pWES4 and pBEN bearing strains	Kanamycin 30 μg/mL	Kanamycin 30 μg/mL
Growth Period	48-72 hours	10-21 days

3.2.16 Preparation of *Escherichia coli* for Electroporation (Jacobs *et al.*, 1991)

Fifty mL of liquid medium (LB or SOB) was inoculated in a 500-mL flask with cells from a colony in a freshly grown plate. The culture was incubated overnight at 37°C with shaking (200 to 300 cycles/minute). One mL of this overnight culture was inoculated into 1 liter of liquid medium in a 4 L flask and incubated at 37°C for 2 to 3 hours with shaking until the OD_{550} reached 0.8. The cells were centrifuged in 250 mL bottles for 10 minutes at 2,800 ×g and 4°C (4,000 rpm in a Beckman JA-10 rotor). The supernatant was discarded and the pellets were washed twice in 250 mL of sterile 10% ice-cold glycerol followed each time by centrifugation as above. Each of the four pellets was resuspended to a final volume of 1 mL in 10% glycerol and aliquoted in Eppendorf tubes in volumes of 200 μL each tube. The tubes were kept in ice and used for electroporation the same day.

3.2.17 Electroporation of *Escherichia coli*

Plasmid DNA (pYUB180/pBEN/pWES4) (5 ng-5 μg; ≤5 μL volume) was mixed well by pipetting up and down with 50 μL of the prepared cells in microfuge tubes kept on ice. As a positive control, a known amount of a standard preparation of plasmid DNA was added to the cells. Negative controls consisted of bacteria without any plasmid

DNA. Controls were electroporated in the same way as the test sample containing the plasmid DNA of interest. Subsequent steps differed according to the type of electroporation apparatus used and described below.

(1) Cell-Porator Voltage Booster (Life Technologies, Inc., Gaithersburg, MD, USA): Twenty-five μL of cells mixed with plasmid DNA were suspended between the bosses of a chilled microelectroporation chamber (0.15 cm gap; Life Tech., Inc.) and was placed in the chamber rack set over the temperature control compartment of the chamber safe containing ice-water slurry. The chamber was closed and the cells were electroporated at 2,400 volts and 4 Kilo-Ohms as described in the Voltage Booster instructions. The electroporated cells were pipetted into 1 mL of liquid medium (Terrific broth or SOC) in Eppendorf tubes and incubated for 1 hour to allow the cells to recover and to express antibiotic resistance marker encoded by the plasmid. The cells were plated onto (LB) agar medium containing the appropriate antibiotic (Table 3.1), and the plates were left for excess liquid to dry at RT and then incubated overnight at 37°C for colonies to appear.

(2) Gene Pulsar (Bio-Rad, Richmond, CA, USA): The pulsar was set at 2,500 volts, 25 μF and 1000 ohms. 25-50 μL of prepared cells mixed with plasmid DNA were pipetted into a chilled electroporation cuvette (0.2 cm gap; Bio-Rad) and tapped to get the cells to the bottom. The cuvette was placed inside the pulsar and exposed to one pulse (time constants were between 15.0 and 25.0 milli-seconds). One mL of liquid medium (Terrific broth or SOC) was added to the cuvette to suspend the electroporated cells, which were transferred to an Eppendorf tube and incubated for 1 hour to allow the cells to

recover and to express antibiotic resistance marker encoded by the plasmid. The cells were plated onto (LB) agar medium containing the appropriate antibiotic (Table 3.1), and the plates were left for excess liquid to dry at RT and then incubated overnight at 37°C for transformed colonies to appear.

3.2.18 Preparation of Mycobacteria for Electroporation (Jacobs *et al.*, 1991)

One mL of a frozen stock or an existing culture of mycobacteria was inoculated into 100 mL of M-ADC-TW broth and grown to log phase (Section 2.2.3). *M. smegmatis* and *M. terrae* cells were incubated at 37°C for 48 hours and 21 days, respectively. The cells were centrifuged in 50 mL tubes (Oak Ridge style) for 10 minutes at 12,000 ×g at 4°C (10,000 rpm in a Beckman JA-20 rotor). The supernatant was discarded and the pellets were washed twice in sterile 10% ice-cold glycerol followed each time by centrifugation as above. Each of the two cell pellets were resuspended in 100 µL of sterile ice cold 10% glycerol and aliquoted in Eppendorf tubes in volumes of 25-50 µL each tube. The tubes were kept in ice and used for electroporation on the same day.

Rapid freezing in dry ice-ethanol bath for storage at -80°C were not undertaken as previous attempts to transform these cells by electroporation were not successful.

3.2.19 Electroporation of Mycobacteria (Jacobs *et al.*, 1991)

The procedure for electroporation of mycobacteria was essentially the same as described for *E. coli* in Section 3.2.17, except for the stage of recovery and plating.

Electroporated *M. smegmatis* and *M. terrae* were inoculated into M-ADC-TW broth and incubated for 3 hours and 12-16 hours respectively, to allow the cells to recover and to express antibiotic resistance marker encoded by the plasmid. The cells were plated

onto M-7H11-OADC agar plates containing the appropriate antibiotic, and the plates were left for excess liquid to dry at RT and then incubated at 37°C for the time required for the strain (Table 3.2).

3.2.20 Confirmation of Transformation

Agar plates containing kanamycin and streptomycin were marked with grids. Colonies of transformed cells having kanamycin but not streptomycin resistance were picked and stabbed onto corresponding squares of the grid on each of the kanamycin and streptomycin plates. The plates were incubated overnight for *E. coli* cells and for longer periods for mycobacteria (Table 3.2) at 37°C. Transformed cells would grow only on kanamycin plates and not on streptomycin plates.

3.2.21 Confirmation of Integrity of Untransformed Cells

A loop of untransformed (kanamycin sensitive) *E. coli* was streaked onto a plate of LB agar containing 15 μg of kanamycin per mL of medium and incubated overnight at 37°C. Absence of growth would indicate susceptibility to kanamycin, whereas growth would indicate a mutation of the kanamycin resistance gene, or presence of a resistance bearing plasmid. Similarly, the absence of growth of untransformed mycobacteria on kanamycin plates would indicate susceptibility to kanamycin.

3.2.22 Detection of Mycobacteria Expressing GFP with Hand-Held UV Lamp

Colonies of transformed mycobacteria expressing GFP were irradiated with a 366 nm hand-held UV Lamp (Black-Ray®, UVL-21, UV Products, Inc., San Gabriel, CA) in darkness and compared with untransformed organisms. Direct observation of fluorescent

colonies was facilitated by growing the organisms in M-7H11-OADC agar containing charcoal powder (2 grams/liter of media; BDH).

3.2.23 Detection and Imaging of Mycobacteria Expressing GFP Using Epi-fluorescence Microscopy and Photomicrography

A loop of cells from a colony of transformed mycobacteria was suspended in 5 μL of 0.85% saline in a microscope slide with a coverslip. The slide was observed under an epifluorescence (Leitz Laborlux K, Germany) microscope using a ×100 oil-immersion objective and a standard FITC excitation-emission filter system. Photomicrographs were taken in a conventional 35 mm camera with a Kodak 400 ASA film at various exposure times from 10 seconds to 2 minutes.

As a second option, the slide was viewed under an epi-fluorescence microscope (Olympus BH-2, Japan) with a charged coupled device (CCD) camera (Sony Power HAD, Japan). The CCD color video camera was used to generate images that were enhanced and integrated by computer software and visualized in a monitor. Images were saved in computer diskettes and printed in color.

3.2.24 Storage of Transformed Mycobacterial Cells

Colonies of transformed mycobacteria containing the plasmids pWES4 and pBEN were picked up from agar plates and grown in M-ADC-TW broth containing 30 μg of kanamycin per mL for the appropriate duration. The cells were frozen and stored at -80°C as described in Section 2.2.2.

3.2.25 Preparation of Inoculum for Fluorescence Assay

Transformed mycobacteria containing the plasmids, pWES4 and pBEN were grown in M-ADC-TW broth containing 30 μg of kanamycin per mL. Suspensions

containing approximately 10^9 cells per mL were prepared as described in Section 2.2.4.

3.2.26 Comparison of Susceptibility of Transformed with Untransformed Mycobacteria to Chemical Germicides By Quantitative Carrier Test

Transformed *M. smegmatis* and *M. terrae* containing the plasmid pBEN as well as untransformed mycobacteria were subjected to culture-based quantitative carrier tests as described in Sections 2.2.9 and 2.2.10. Transformed and untransformed mycobacteria were exposed to germicides and $\log_{10}$ reductions in CFU were calculated to compare their susceptibilities to germicides. The effects of drying on the survival of these bacteria were also compared.

All subsequent procedures including fluorescence measurements were performed with *M. smegmatis* (pBEN) and *M. terrae* (pBEN) expressing a red-shifted, high-intensity mutant GFP.

3.2.27 Measurement of Fluorescence Emission Using a Cuvette Type Fluorometer

A VersaFluor™ fluorometer system (Bio-Rad, Hercules, CA, USA) was used for measuring fluorescence in the FITC range. A set of excitation (490/10 nm) and emission (520/10 nm) filters were placed in the light path and the fluorometer was set to medium gain. Three mL of 0.85% saline was pipetted into a 4 mL screw-capped clear glass vial (45.5 mm long × 15 mm wide; J.G. Finneran Assoc. Inc.), placed inside the cuvette holder of the fluorometer and the instrument was set to zero. Suspensions of *M. smegmatis* (pBEN) and *M. terrae* (pBEN) were serially diluted in 0.85% saline to reach approximately 10^3 cells/mL. Three mL of each dilution were pipetted into the 4 mL clear glass vials, vortexed for 5 seconds and immediately placed inside the fluorometer for measurements. Fluorescence was recorded as relative fluorescence units (RFU) and each

sample was read twice. RFU was also measured in square-shaped disposable cuvettes recommended by the manufacturer (Bio-Rad, Hercules, CA) and the readings were compared with the 4 mL glass vials.

The instrument was set to zero using M-ADC-TW broth as the baseline fluid in all subsequent experiments for measuring RFU. Cultures of *M. terrae* (pBEN) in M-ADC-TW broth were incubated in the presence and absence of kanamycin (30 μg/mL) in 4 mL glass vials. Fluorescence was measured every 24 hours for 14 days to compare the kinetics of GFP expression during growth in the presence and absence of kanamycin.

A culture of untransformed *M. terrae* not expressing GFP was incubated in M-ADC-TW broth and fluorescence measured every 24 hours for 14 days.

Some of the commonly used germicides (hydrogen peroxide, glutaraldehyde, phenolic), neutralizers (1% glycine), culture medium (M-ADC-TW broth), and other relevant products were tested for autofluorescence. (Please refer to Appendix III and V for flow chart and equipment for fluorescence assay).

3.2.28 Fluorescence Assay for Determination of Mycobactericidal Activity of Liquid Chemical Germicides

One hundred μL of a homogenized suspension (10^8-10^9 CFU/mL) of *M. terrae* (pBEN) was pipetted into 1 mL of a test germicide in a 50 mL graduated conical tube (Falcon polypropylene tubes, Becton and Dickinson, NJ). After the required contact period, the germicide was neutralized by dilution with 50 mL of 0.85% saline followed by centrifugation at 2,000 ×g for 15 minutes. After the supernatant was removed, the dilution and centrifugation steps were repeated twice using a total of 100 mL of saline. The pellet was resuspended in 6 or 9 mL of M-ADC-TW broth containing kanamycin (30

μg/mL) and equally dispensed into 2 or 3 glass vials (45.5 mm × 15 mm; J.G. Finneran Assoc., Inc.) respectively, in volumes of 3 mL per vial. Controls were prepared by pipetting 100 μL of the suspension into 1 mL of 0.85% saline instead of a germicide. Subsequent steps were the same as performed for the tests. The fluorometer was set to zero using M-ADC-TW broth as the baseline fluid. The control and test samples suspended in M-ADC-TW broth in the 4 mL vials were vortexed for 5 seconds and immediately placed inside the fluorometer for measurement of fluorescence. Measurement of each vial was repeated after a few minutes as fluorescence was found to vary between the two readings. An average of the two readings was made for interpretation of results. Fluorescence was measured every 24 hours for at least 7 days, with incubation at 37°C and moderate shaking (100 cycles/minute) in the interim period.

When performing a germicidal assay using alkaline glutaraldehyde, a solution of 1% glycine in saline was used as a neutralizer and the fluorescence readings were compared to those where dilution was made with 0.85% saline without glycine.
(Please refer to Figure 3.2 and Appendix III and V).

3.2.29 Enumeration of CFU in Samples from Fluorescence Assay

To determine the relationship between growth (CFU) and fluorescence (RFU), the number of CFU in the samples (control and tests) were enumerated using membrane filtration every time RFU was measured (Section 3.2.28). The method consists of vortexing the sample in the 4 mL glass vials and pipetting 100 μL into a 20 mL glass vial containing 9.9 mL of 0.85% saline. Serial dilutions were made from this vial and the last three dilutions that would contain countable colonies were filtered using mixed cellulose

ester membranes (Millipore, 0.45μM, black-gridded). The membranes were plated onto M-7H11-OADC agar containing kanamycin (30 μg/mL) and incubated for the desired length of time (14-21 days for *M. terrae*). Colonies were counted and used to calculate CFU/mL of the sample as shown below:

$CFU/mL = \text{colonies in plates} \times 10^n \times 10$

n = the dilution factor in the vials

The extra factor of 10 was used to arrive at the titer for each mL of the sample.

Figure 3.2 Fluorescence assay for screening chemical germicides for mycobactericidal activity

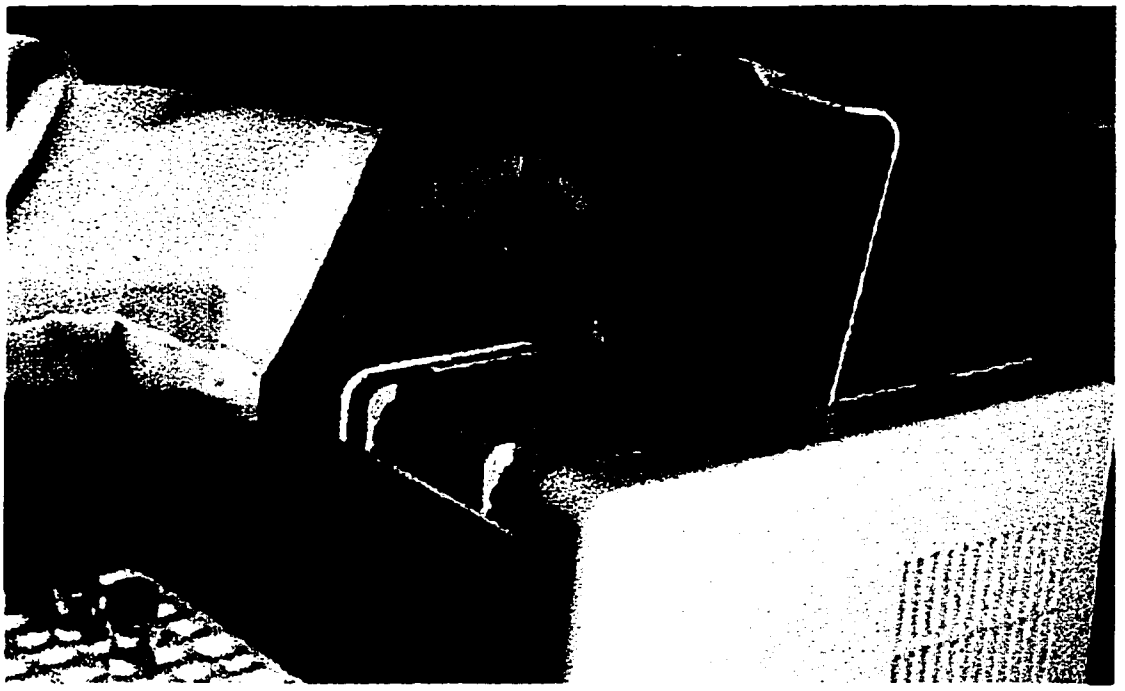
(A) Exposure of *M. terrae* suspension to germicides: One hundred μL of a suspension of *M. terrae* was pipetted into a 50 mL polypropylene tube containing 1 mL of germicide as depicted in the figure. After the required contact time, the germicide was washed with saline, centrifuged twice and the cell pellet resuspended in M-ADC-TW broth containing kanamycin. Controls were exposed to saline instead of germicides and washed, pelleted and resuspended in the same way.

(B) Measurement of Fluorescence: The resuspended cells were transferred to 4 mL clear glass vials. Each vial was vortexed for 5 seconds and placed in a VersaFluor™ fluorometer system as depicted in the figure. Fluorescence was measured every 24 hours in the test and control vials with incubation in the interim period.

Figure 3.2 (A-B)

A. EXPOSURE OF *M. terrae* (pBEN) TO GERMICIDE

B. MEASUREMENT OF FLUORESCENCE IN 4 mL GLASS VIALS



3.3 Results

3.3.1 Development of the Luciferase Reporter Gene System for Assessment of Viability in Mycobacteria

3.3.1.1 Antibiotic Susceptibility of *M. smegmatis* and *M. terrae*

M. smegmatis and *M. terrae* have an innate susceptibility to kanamycin whereas those transformed with a plasmid containing a kanamycin resistance gene could grow in the presence of kanamycin. The susceptibility of untransformed *M. smegmatis* mc^2155 and *M. terrae* ATCC 15755 to kanamycin was determined by plating them on agar medium containing kanamycin (10-50 μg/mL) and also in drug-free (control) plates (Section 2.2.7). After incubation, no growth was observed in any of the test plates indicating that untransformed *M. smegmatis* and *M. terrae* were sensitive to kanamycin.

3.3.1.2 Extraction of the Plasmid pYUB180 by Alkali Lysis and SDS Lysis Method Under Different Growth Conditions.

In the initial phases of this study, the plasmid pYUB180 was extracted from *E. coli* DH5α to develop a stock of plasmid DNA for subsequent transformation of mycobacteria. The quantity of pYUB180 obtained from 5 mL cultures of *E. coli* DH5α was found to be different using different types of growth medium and methods of extraction. The yield was very low with the alkali lysis method (Section 3.2.5) and was seen under UV light as bands of extremely low intensity in a 1% agarose gel stained with ethidium bromide. The yield was relatively higher with the SDS lysis method (Section 3.2.7) under similar growth conditions. Cells grown in Terrific broth also resulted in a

higher yield of plasmid DNA when compared to that obtained from cells grown in LB broth.

3.3.1.3 Transformation of Competent *M. smegmatis* and *M. terrae*

To obtain bioluminescent mycobacterium, the plasmid pYUB180 previously extracted from *E. coli* DH5α was used to transform *M. smegmatis* and *M. terrae* by the $CaCl_2$ method (Section 3.2.15) and electroporation (Section 3.2.19) using a cell-porator voltage booster (Life Technologies, Inc.). A few colonies appeared in the kanamycin containing agar medium after approximately 7 and 30 days, respectively for *M. smegmatis* and *M. terrae*. However, the doubtful nature of *M. terrae* transformants, an extremely slow growth rate compared to untransformed cells and other potential drawbacks of the luciferase system did not allow further development of the system.

3.3.2 Development of the GFP Reporter Gene System for Assessment of Viability in Mycobacteria

3.3.2.1 Expression of GFP Following Transformation

In the first phase of the development of the GFP reporter gene system, *E. coli* DH5α made competent using $CaCl_2$ were transformed with the plasmids pWES4 and pBEN (Section 3.2.13). Transformed cells grew abundantly after overnight culture in LB agar containing 30 μg/mL kanamycin. However, the cells did not express GFP as indicated by their failure to fluoresce under an epifluorescence microscope. Positive controls transformed with pEGFP (CLONTECH Laboratories, Inc., Palo Alto, CA, USA) grew in the presence of ampicillin but not in kanamycin and fluoresced bright green. Negative controls without plasmid DNA did not grow in the presence of either antibiotic.

Small scale preparations of the plasmids pWES4 and pBEN were made from 5 mL cultures of the transformed but non-fluorescent *E. coli* DH5α by the alkali lysis method. An extraction column (Qiagen-tip 500) allowed a large quantity of pure plasmid to be extracted from the cells (Figure 3.3). This provided a stock of plasmid DNA for subsequent transformation of mycobacteria.

M. smegmatis and *M. terrae* electroporated with pWES4 and pBEN in a gene pulsar (Bio-Rad, Richmond, CA.) required 4-5 days and 12-14 days, respectively, to appear in M-7H11-OADC agar containing 30 μg/mL of kanamycin.

Transformed *M. smegmatis* and *M. terrae* containing the plasmid pWES4 appeared as bright green fluorescent colonies in agar plates when seen under a hand-held 366 nm UV lamp (Blak-Ray UVL-21, UVP Inc., CA) (Figure 3.4). Untransformed mycobacteria as well as mycobacteria with the plasmid pBEN did not fluoresce under the UV light. When seen under an epifluorescence microscope (Leitz, Germany) with a standard FITC excitation-emission filter system, *M. terrae* and *M. smegmatis* with either plasmids were seen to fluoresce, although cells with pBEN appeared brighter than those with pWES4. GFP expression was also observed when the cells were seen under an epifluorescence microscope (Olympus, Japan) with a CCD video camera (Sony, Japan) attached to a computer imaging system. This system provided convenient microscopy and allowed images to be stored in computer disks and printed whenever necessary (Figure 3.5).

Figure 3.3 The plasmids pWES4 and pBEN obtained by using an extraction column seen in an agarose gel.

The plasmids pWES4 and pBEN were obtained by using an extraction column (Qiagen-tip 500) from *E. coli* DH5α grown in LB broth containing 30 μg/mL of kanamycin. A 1% electrophoresis gel comparing the fractions at each stage of the QIAGEN purification procedure is shown.

Lane 1: Cleared lysate containing supercoiled and open circular plasmid DNA and degraded RNA.

Lane 2: Flow-through fraction containing only degraded RNA. Plasmid DNA is bound to the QIAGEN Resin and therefore not present in this fraction.

Lane 3: First wash fraction showing remaining traces of RNA.

Lane 4: Second wash fraction showing complete removal and therefore absence of RNA and other contaminants. Pure plasmid DNA is still bound to the column.

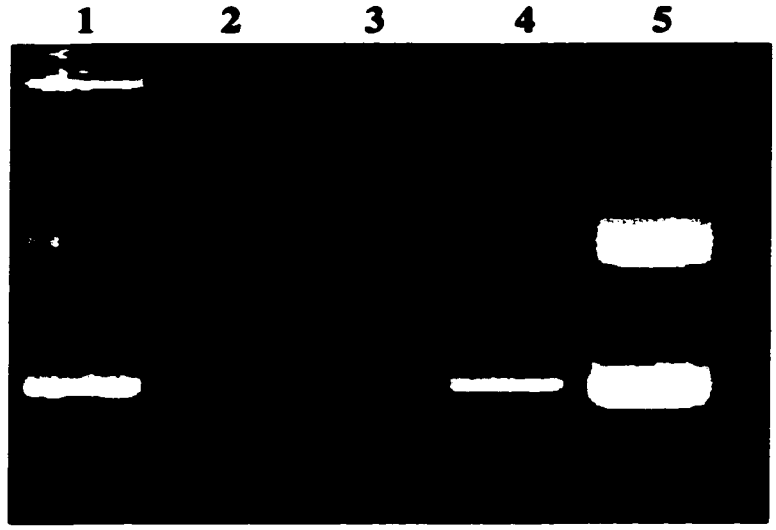
Lane 5: Eluate containing pure plasmid DNA with no other contaminating nucleic acids. The top figure shows purified plasmid pWES4 in lane 5, and the bottom figure shows purified plasmid pBEN in lane 5.

Figure 3.4 Fluorescence in transformed colonies of mycobacteria expressing GFP grown on M-7H11-OADC agar plates containing charcoal.

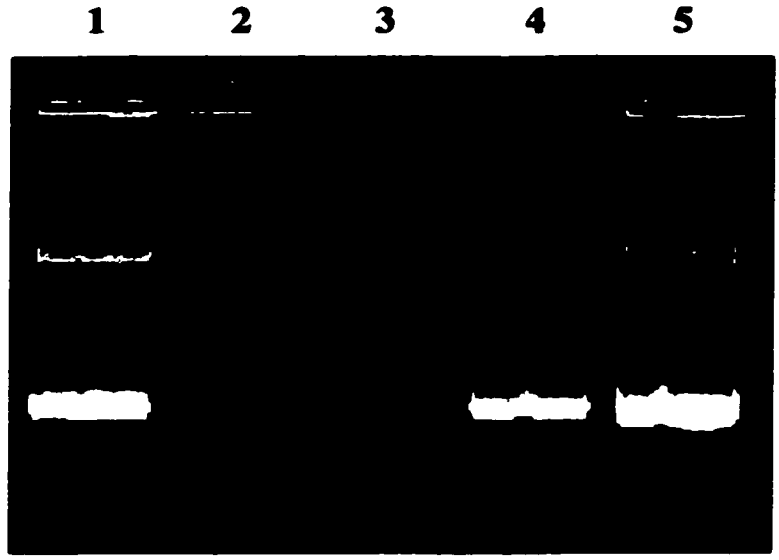
M. smegmatis and *M. terrae* containing the plasmids pWES4 and pBEN encoding, respectively, a wt and a mutant GFP (GFPmut3) were grown in M-7H11-OADC agar containing kanamycin and activated charcoal. The wt GFP is excited at 395 nm and the red-shifted mutant at 501 nm light. The colonies were seen under a 366 nm UV lamp and photographs were taken. Green fluorescence was seen in colonies of mycobacteria containing the plasmid pWES4 expressing a wt GFP. Mycobacteria containing the plasmid pBEN express a red-shifted mutant GFP, which was not excited by the wavelength (366 nm) of the UV lamp and did not fluoresce. Untransformed mycobacteria without the plasmid do not express GFP and were not seen to fluoresce (controls).

(A) Non-fluorescent colonies of *M. smegmatis* (pBEN).
(B) Intense green fluorescent colonies of *M. smegmatis* (pWES4).
(C) Non-fluorescent colonies of untransformed *M. smegmatis* (control).
(D) Green fluorescent colonies of *M. terrae* (pWES4).
(E) Non-fluorescent colonies of *M. terrae* (pBEN).
(F) Non-fluorescent colonies of untransformed *M. terrae* (control)

Figure 3.3



Lane 1-4: Fractions obtained at purification
Lane 5: Pure plasmid pWES4 (5262 bp)



Lane 1-4: Fractions obtained at purification
Lane 5: Pure plasmid pBEN (5254 bp)

Figure 3.4

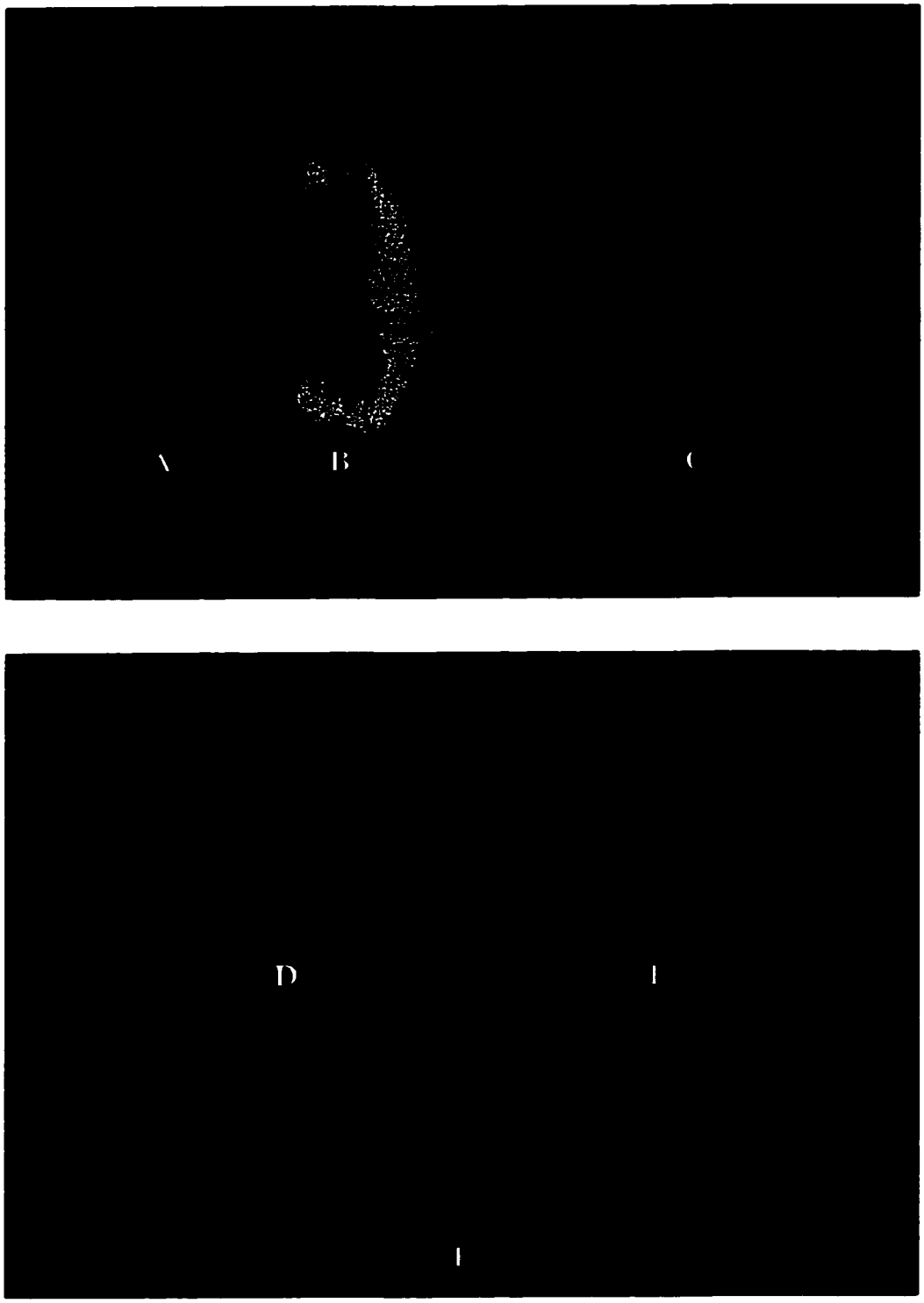
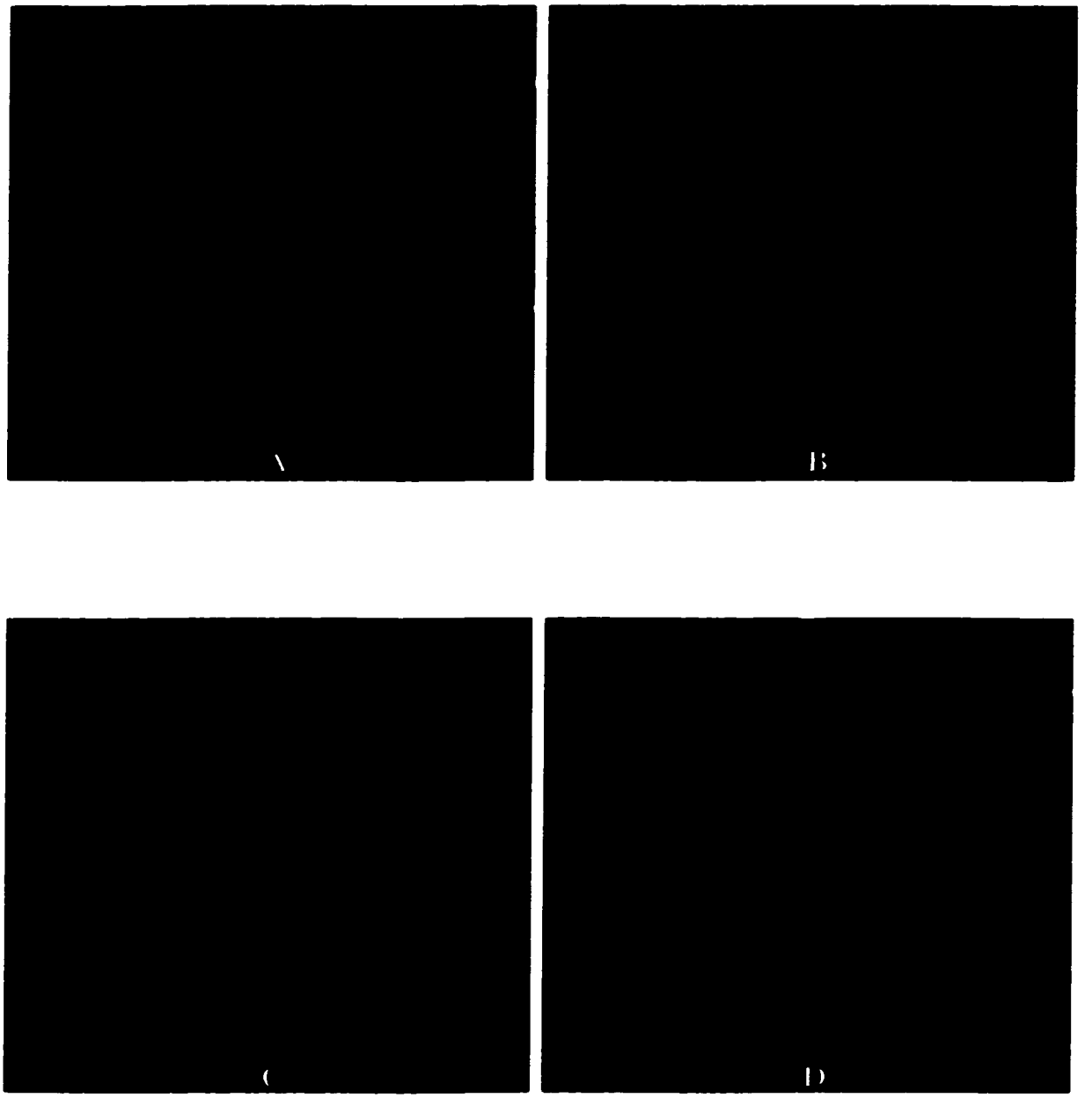


Figure 3.5 Digital imaging micrographs of mycobacteria expressing GFP

Colonies of *M. smegmatis* mc^2155 and *M. terrae* containing the plasmids pWES4 and pBEN encoding respectively, a wt and a mutant GFP were suspended in a drop of saline in a microscope slide and seen under an epifluorescence microscope (Olympus) with an FITC excitation-emission filter system and a ×100 oil-immersion objective. A CCD camera (Sony) was used to scan the digital images that were analyzed and integrated using computer software and printed in color. Clumps and dispersed cells were seen in the micrographs (Magnification, ×2,000).

(A) Green fluorescent *M. smegmatis* (pWES4).
(B) Green fluorescent *M. smegmatis* (pBEN)
(C) Green fluorescent *M. terrae* (pWES4).
(D) Green fiuorescent *M. terrae* (pBEN).

Figure 3.5



3.3.2.2. Effect of Transformation on the Susceptibility of Mycobacteria

Before germicidal assays based on fluorescence could be carried out, the susceptibilities of transformed mycobacteria (pBEN) and untransformed bacteria to the effects of drying and germicides were compared using a quantitative carrier test (Section 2.2.10). With overnight drying *M. smegmatis* (pBEN) and *M. terrae* (pBEN) showed less reduction in CFU than untransformed ones indicating higher a resistance of the transformed mycobacteria to drying (Table 3.3). In either transformed or untransformed bacteria, a higher $\log_{10}$ reduction was observed with *M. smegmatis* than with *M. terrae*.

Table 3.3 Effect of drying at different intervals on the survivability of transformed and untransformed mycobacteria.

Mycobacteria	Zero minute (undried) Log_{10} CFU (A)	Dried-1 hour Log_{10} CFU	Dried-2 hours Log_{10} CFU	Dried-4 hours Log_{10} CFU	Dried overnight Log_{10} CFU (B)	Mean Log_{10} reduction (A-B)
M. smegmatis	7.30	6.45	6.37	5.9	5.02	**2.28**
M. smegmatis (pBEN)	7.38	6.64	6.73	6.34	5.93	**1.45**
M. terrae	7.48	Not done			6.50	**0.98**
M. terrae (pBEN)	7.71	Not done			7.02	**0.69**

Each result is the mean of two determinations. An organic load was not included in this test.

When exposed to sublethal concentrations of hydrogen peroxide and alkaline glutaraldehyde for 10 minutes, the $\log_{10}$ reduction in CFU of *M. smegmatis* (pBEN) was less than that of untransformed *M. smegmatis* by 0.71 and 0.88, respectively. Transformed *M. terrae* (pBEN), on the other hand, showed a $\log_{10}$ reduction that was higher than untransformed *M. terrae* by 0.91 and 0.59, respectively (Table 3.4 and Figure 3.6).

Table 3.4 Comparison of the susceptibility of transformed (+pBEN) with untransformed mycobacteria after exposure to sub-lethal concentrations of germicides.

	Log_{10} reduction		
Mycobacteria (% germicide)	Untransformed (A)	Transformed (+pBEN) (B)	Difference (A-B)
M. smegmatis (0.78% H_2O_2)	3.68 ± 0.25	2.97 ± 0.26	0.71
M. smegmatis (0.08% Alkaline glutaraldehyde)	4.14 ± 0.18	3.26 ± 0.41	0.88
M. terrae (3.75% H_2O_2)	2.48 ± 0.25	3.39 ± 0.35	-0.91
M. terrae (0.8% Alkaline glutaraldehyde)	3.54 ± 0.48	4.13 ± 0.30	-0.59

Results are the mean of 12 determinations of log_{10} reduction in CFU ± SD

When *M. smegmatis* and *M. terrae* with and without the plasmid were tested against two preparations of hydrogen peroxide (7% and 7.5%) and alkaline glutaraldehyde (2.4%) at the use-dilutions and contact period recommended by the manufacturer, all the products achieved a ≥6-log_{10} reduction.

Figure 3.6 Comparison of susceptibility of mycobacteria to germicides with and without the plasmid pBEN.

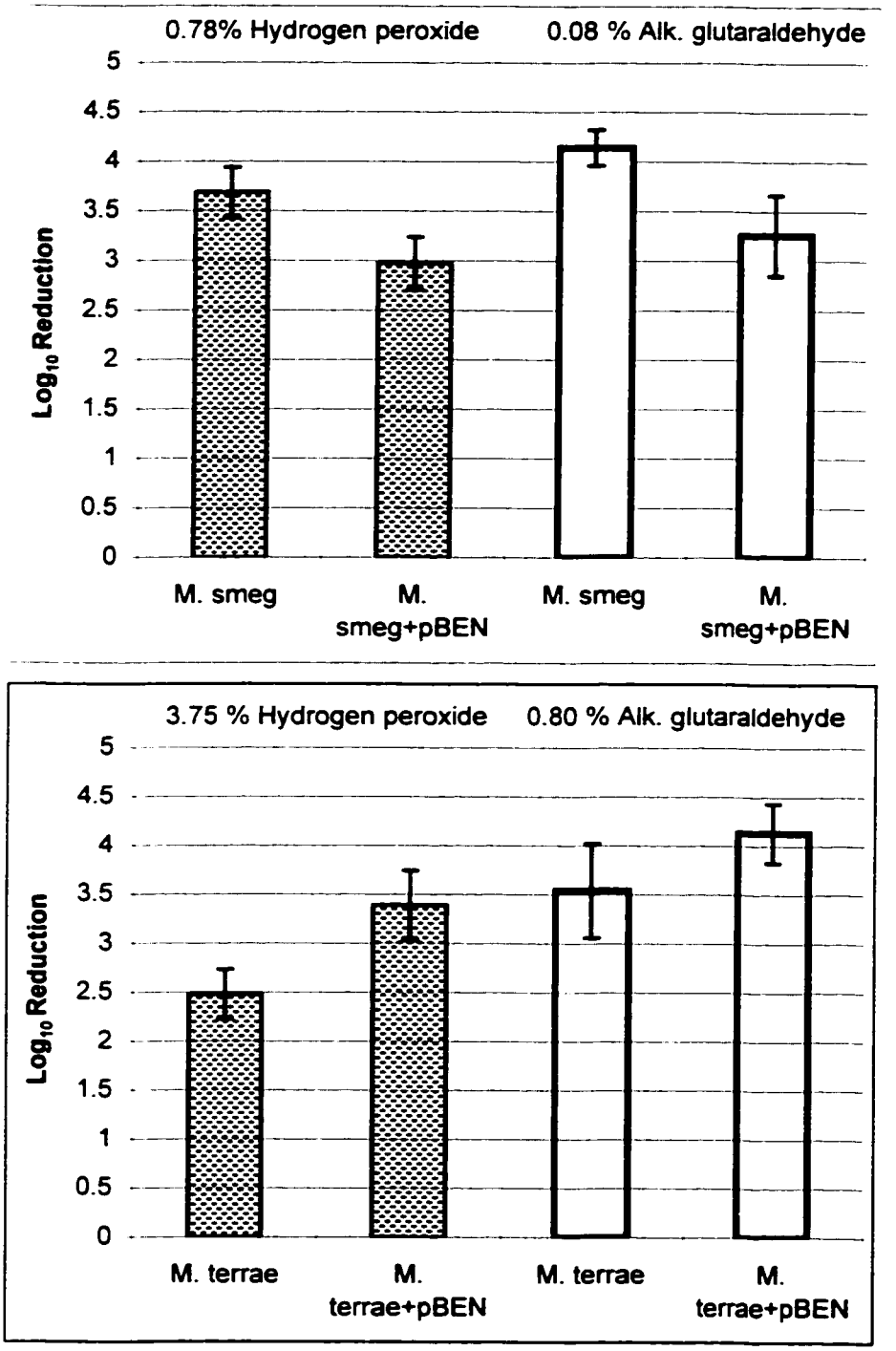
Suspensions were prepared from cultures of *M. smegmatis* and *M. terrae* and the same mycobacteria transformed with the plasmid pBEN. Carriers were inoculated with the suspensions, dried overnight and tested against sub-lethal concentrations of hydrogen peroxide and alkaline glutaraldehyde in a quantitative carrier test.

Calculation of $\log_{10}$ reduction in CFU was done to compare the susceptibility of transformed with untransformed mycobacteria.

(A) When compared with transformed *M. smegmatis* (pBEN), the $\log_{10}$ reduction was higher in untransformed *M. smegmatis* by 0.71 and 0.88 when exposed to hydrogen peroxide (0.78%) and alkaline glutaraldehyde (0.08%), respectively.

(B) When compared with transformed *M. terrae* (pBEN), the $\log_{10}$ reduction was lower in untransformed *M. terrae* by 0.91 and 0.59, when exposed to hydrogen peroxide (3.75%) and alkaline glutaraldehyde (0.8%), respectively.

Figure 3.6


0.78% Hydrogen peroxide
0.08 % Alk. glutaraldehyde
Log10 Reduction
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
M. smeg
M. smeg+pBEN
M. smeg
M. smeg+pBEN
3.75 % Hydrogen peroxide
0.80 % Alk. glutaraldehyde
Log10 Reduction
M. terrae
M. terrae+pBEN
M. terrae
M. terrae+pBEN

3.3.2.3 Measurement of GFP Expression in Mycobacteria

Once GFP expression was observed in transformed mycobacteria, the next phase was to measure the fluorescence (RFU) and to correlate the readings with the growth or the number of viable cells in the same samples. Initially, readings of RFU were made in stored (4°C) samples of transformed mycobacteria to determine the accuracy and sensitivity of the VersaFluor™ fluorometer (BioRad, Hercules, CA). Subsequently, readings were made in incubating cultures of the bacteria to monitor the kinetics of GFP expression with growth.

Fluorescence emission from 2-fold, 5-fold and 10-fold serial dilutions of the same suspensions of *M. smegmatis* (pBEN) and *M. terrae* (pBEN) were measured in disposable cuvettes (Bio-Rad, Hercules, CA) and 4 mL screw-capped clear glass vials (Section 3.2.27). The reduction of RFU in the disposable cuvettes as well as the glass vials correlated well with the serial dilutions until the cell density reached from 10^4 to 10^3 per mL when the readings approached zero in the medium gain setting. When the fluorometer was set at a high gain, the readings were not accurate and those at low gain were too low, even at cell densities of between 10^5 and 10^6 per mL.

Fluorescence readings from cell cultures at a density of approximately 10^6 per mL increased progressively with incubation in M-ADC-TW broth and reached a peak in 3-4 days followed by a slow decrease with time. Broth containing kanamycin at 30 μg/mL allowed the cells to exhibit a higher peak level of fluorescence and there was less reduction of fluorescence with time when compared with corresponding cells grown without kanamycin (Figure 3.7A). As a result, all subsequent tests of fluorescence,

including germicidal assays, were performed with cells incubated with kanamycin. Fluorescence emission from a culture with a high starting titer of cells (~10^7 per mL) increased rapidly to a peak in only 2 days of incubation followed by a slow decrease with time (Figure 3.8).

A culture of untransformed *M. terrae* was examined for fluorescence every 24 hours in triplicates. There was no fluorescence emission above background levels which was initially 0-3 RFU in the three samples followed by a slight increase of up to 14 RFU at day 5 and then a gradual decrease in the next 14 days of incubation.

3.3.2.4 Fluorescence Measurements Following Exposure to Germicides

The GFP-based fluorescence assay was investigated for its ability to monitor the kinetics of GFP expression by *M. terrae* (pBEN) during growth and to observe altered levels of expression due to the inhibitory effects of germicides. RFU of liquid cultures of the tests and controls exposed to germicides and saline, respectively was measured at 24 hour intervals for at least 7 days (Section 3.2.28). The number of CFU in the same cultures (controls and tests) were enumerated in agar plates using membrane filtration at the same time as the fluorescence measurements were made (Section 3.2.29). Several products were tested at different dilutions and contact periods.

A. A suspension (6.85×10^7 in 100 µL) of *M. terrae* (pBEN) was tested against a full-strength (7.5%) hydrogen peroxide-based product for 10 minutes. After the required washing and centrifugation, the cells were resuspended in M-ADC-TW broth and RFU and CFU of control and test cultures were measured daily. RFU from controls increased to reach a peak on the fourth day followed by a slow decrease with time. Log_{10} CFU

Figure 3.7 Kinetics of GFP expression by *M. terrae* (pBEN) under different growth conditions and after exposure to germicides.

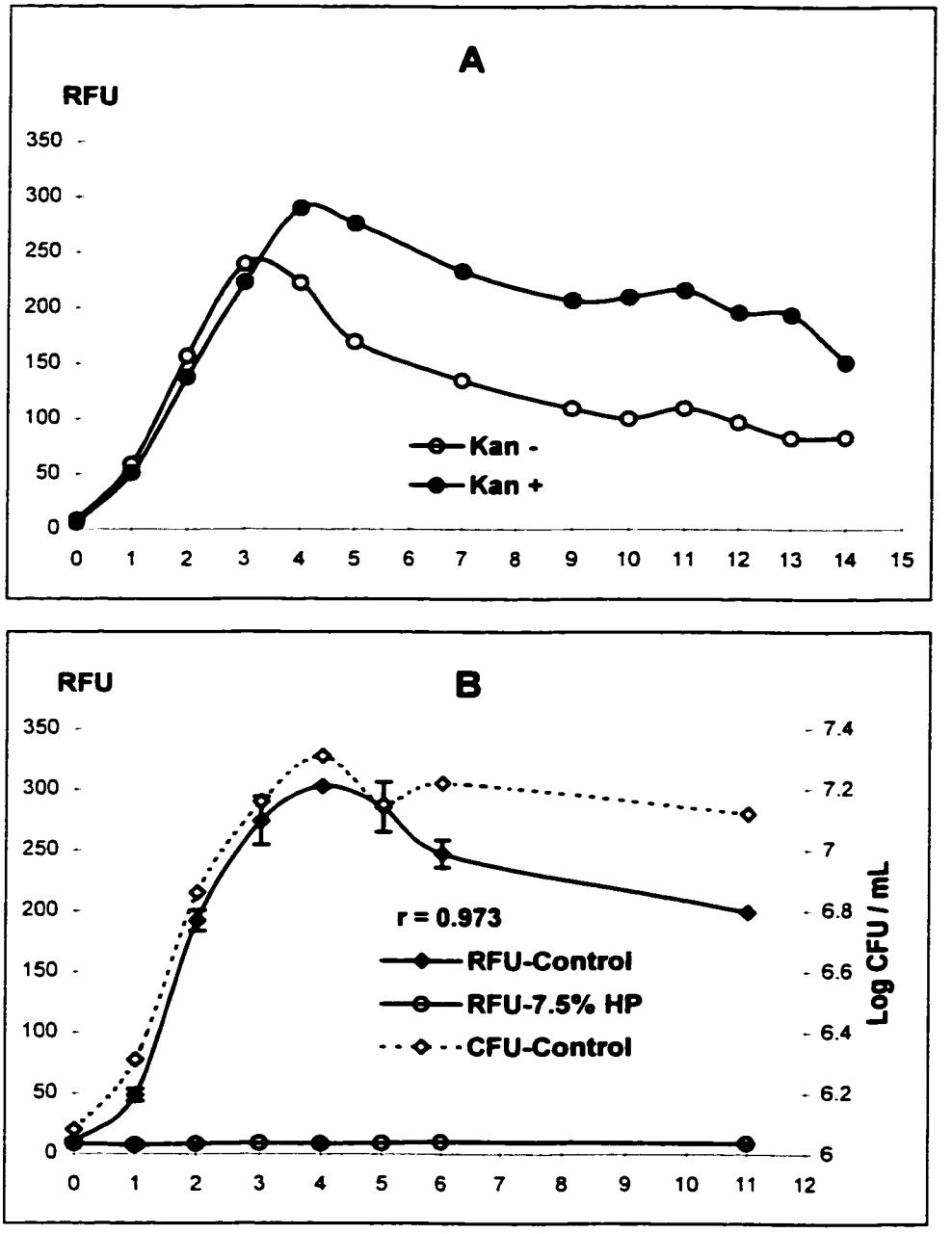
A. A suspension (6.85×10^7 CFU in 100 µL) of *M. terrae* (pBEN) was washed twice with 50 mL 0.85% saline, centrifuged at 2,000 ×*g* at 4°C for 15 minutes and the pellet resuspended in M-ADC-TW broth with (kan+) and without kanamycin (kan-). The cells were incubated in screw-capped 4 mL clear glass vials at 37°C with shaking at 100 cycles per minute. The chart depicts the RFU measured in the vials with (kan+) and without (kan-) kanamycin, every 24 hours with incubation for 14 days.

B. A test suspension of *M. terrae* (pBEN) was exposed to 7.5% hydrogen peroxide (HP) for 10 minutes. At the end of the contact period the test and the control exposed to 0.85% saline were washed, centrifuged, resuspended in M-ADC-TW broth containing kanamycin and incubated. RFU of control and tests were measured as above.

At the same time 100 µL cells from the control and test vials were serially diluted, filtered and the membranes plated on M-7H11-OADC agar containing 30 µg/mL of kanamycin. Following incubation (12-14 days), the plates were counted and $\log_{10}$ CFU per mL was determined.

The chart depicts the RFU of the control and the test, and CFU of the control. There was no growth in the test vial throughout the experiment and therefore these data were not shown. There was a good correlation between growth (CFU) and fluorescence (RFU) in the control ($r = 0.973$). Error bars indicate mean of 2 determinations ± SD.

Figure 3.7


A
RFU
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Kan -
Kan +
B
RFU
350
300
250
200
150
100
50
0
7.4
7.2
7
6.8
6.6
6.4
6.2
6
Log CFU / mL
0 1 2 3 4 5 6 7 8 9 10 11 12
r = 0.973
RFU-Control
RFU-7.5% HP
CFU-Control
-Time in days-

enumerated from the controls increased in parallel to the RFU and a good correlation was observed between the two readings (r=0.973). Fluorescence in the test samples did not increase over time and remained at the baseline (Figure 3.7B). Enumeration of the test samples did not show any growth.

During the procedure of dilution, washing and centrifugation, the CFU in the control culture was reduced by approximately 1.27 $\log_{10}$ from the starting titer (6.85×10^7 in 100 µL) of the suspension. Although reduction of viable or dead cells during the procedure could not be calculated in the tests due to concomitant loss caused by the test germicides, it was assumed to be of the same proportion as observed in the controls.

B. RFU and CFU of control and test cultures exposed to 2-fold dilutions of a 7.5% hydrogen peroxide-based product for a 10 minute contact period were measured daily for 15 days (Figures 3.8 and 3.9). The RFU in the control increased progressively to reach a peak in two days followed by a gradual decrease. There was no increase of RFU in the test exposed to the (7.5%) full-strength product. RFU increased in all the other tests with the sample exposed to the most diluted product (1.5%) appearing approximately 24 hours after the control followed sequentially by the less diluted ones (2.5% and 3.75%). CFU of controls and tests enumerated in agar plates correlated very closely with the RFU values (Figure 3.9).

C. RFU and CFU in controls and tests exposed to 2-fold dilutions of a product based on 2.4% activated alkaline glutaraldehyde for a 20-minute contact period were measured for 11 days (Figure 3.10 and 3.11). Fluorescence in the control increased on the first day followed consecutively by the test exposed to the most diluted product

(0.07%) on the third day, the 0.14% test on the fifth day and 0.27% test on the seventh day. Fluorescence in the 0.48% test increased on the 11th day of observation although a corresponding increase in CFU occurred approximately two days earlier. There was no increase in RFU in the tests exposed to concentrations of 0.8% and 2.4% of the test product. There was a good correlation between the RFU and the corresponding CFU in the control and tests (Figure 3.11 A-F). During visual counting of agar plates, the colonies of tests exposed to glutaraldehyde appeared 2-4 days later than colonies tested against hydrogen peroxide or phenolic germicide.

When the glutaraldehyde based product with the same dilutions were tested with a 10-minute contact time, RFU increased in all the tests and controls in 24 hours (data not shown).

In separate experiments where different products were tested for their intrinsic fluorescence, 2.4% activated alkaline glutaraldehyde (pH 7.5-8.0) had an emission of approximately 200 and 1000 RFU when the product was diluted 100 and 20 times, respectively, in 0.85% saline. When a solution of 1% glycine in saline was used to make the same dilutions of the product, the fluorescence was reduced to 26 and 100 RFU, respectively. This product without activation (pH 4.0) did not record any fluorescence when diluted 12.5 times in saline without glycine indicating that the activating powder was the source of the intense autofluorescence. A 10% acid glutaraldehyde-based product (pH 6.0; no activation required) recorded 6 and 45 RFU when diluted 100 and 12.5 times, respectively, in saline and there was no decrease in the presence of 1% glycine. Fluorescence emissions from hydrogen peroxide and phenolic germicides in use-dilutions, M-ADC-TW broth and saline were within an acceptable range.

In actual experiments, when *M. terrae* (pBEN) was tested with activated alkaline glutaraldehyde for 10 minutes and was diluted 100 times in 0.85% saline on the one hand and with 1% glycine in saline on the other, there was no significant difference in fluorescence emission between the two tests.

D. When *M. terrae* (pBEN) was exposed to different concentrations of an acid glutaraldehyde-based product (10, 3.33, 2.0, 1.11 and 0.59 %) for 20 minutes in five tests, RFU did not increase in any of the cultures except for the one exposed to the most diluted product (1:16 = 0.59%). The increase occurred on the 8^{th} day of incubation and reached control levels just before the 10^{th} day (Figure 3.12A). When the same product was tested with a 10 minute contact time, RFU increased in all the tests within 24 hours.

E. RFU measured in test cultures exposed to four concentrations of a phenolic germicide recorded an increase with only the most diluted product (1:400 = 0.25%) from the 4^{th} day onwards (Figure 3.12B). With 0.5, 1 and 2% concentrations, fluorescence gradually decreased and approached a negative value on the 7^{th} day. A similar observation was made when acid glutaraldehyde (Figure 3.12A) and hydrogen peroxide were used in lethal concentrations and the RFU value went to a negative value during the test period.

F. Fluorescence was increased in all the tests exposed to ethanol at concentrations ranging from 20% to 99% with a 2-minutes contact time. Even within rising values, the RFU was relatively less in the test exposed to 99% ethanol becoming higher with increasing dilutions of the product. After day three, the correlation of fluorescence with the dilutions of the test product was abolished (Figure 3.12C).

Figure 3.8 Kinetics of GFP expression by *M. terrae* (pBEN) after exposure to different concentrations of a hydrogen peroxide-based chemical germicide.

Test suspensions (5.20×10^{8} CFU in 100 µL) of *M. terrae* (pBEN) were exposed to four concentrations (7.5, 3.75, 2.5 and 1.5 %) of a hydrogen peroxide-based (HP) product for 10 minutes. At the end of the contact period the tests and control exposed to 0.85% saline were washed, centrifuged, pelleted and resuspended in M-ADC-TW broth containing kanamycin and incubated in 4 mL glass vials. The chart depicts RFU of control and tests measured in the vials at 24 hour interval using fluorometry. Error bars indicate mean of 2 determinations ± SD.

Figure 3.8

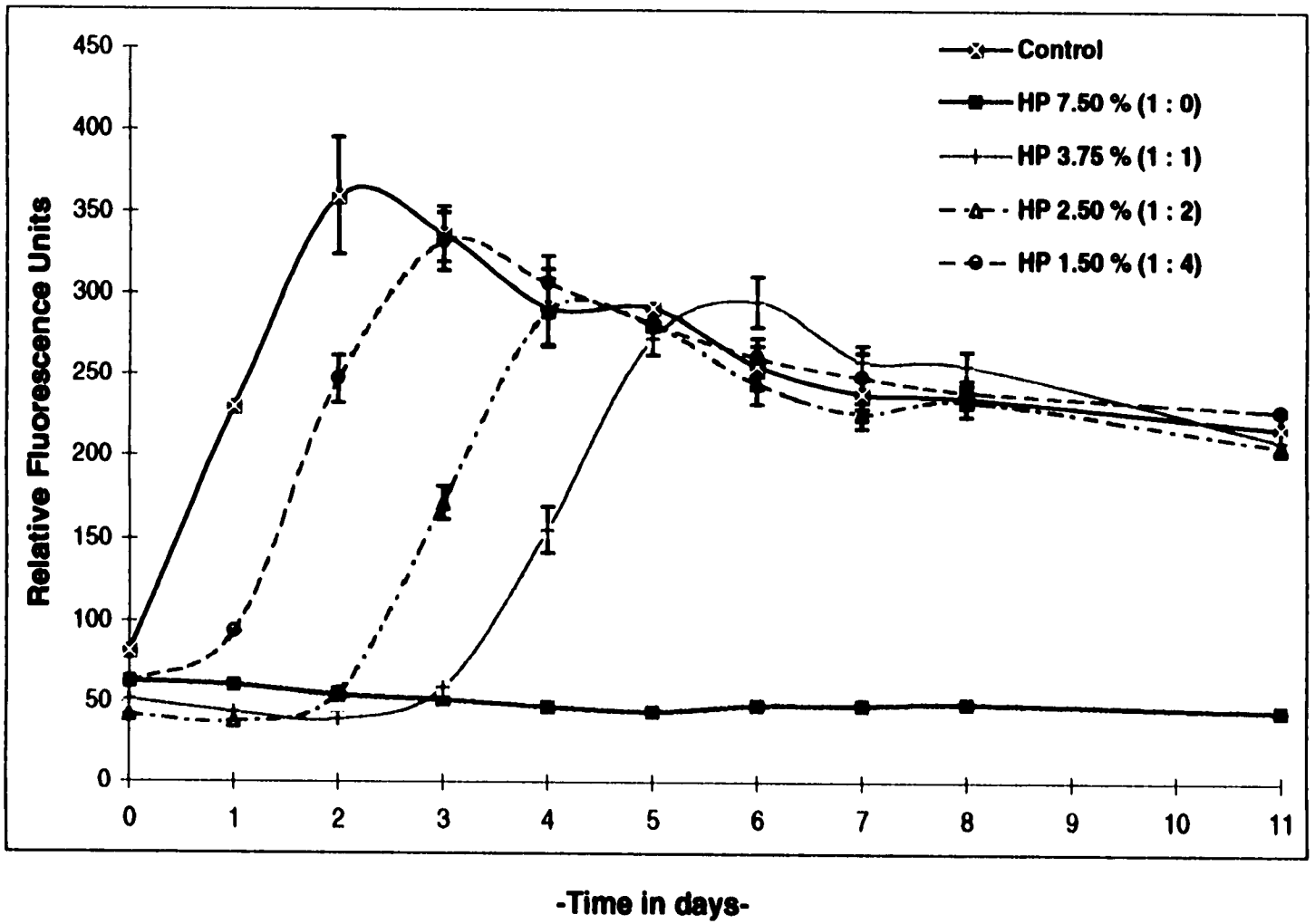

Control
HP 7.50 % (1 : 0)
HP 3.75 % (1 : 1)
HP 2.50 % (1 : 2)
HP 1.50 % (1 : 4)
Relative Fluorescence Units
450
400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
11
-Time in days-

Figure 3.9 Comparison of fluorescence (RFU) and growth (CFU) in *M. terrae* (pBEN) after exposure to hydrogen peroxide-based germicide.

RFU was measured in controls and test samples of *M. terrae* (pBEN) exposed to four concentrations of a hydrogen peroxide (HP) based product with a contact period of 10 minutes (Figure 3.8). At the same time 100 µL cells from the control and test vials were serially diluted, filtered and the membranes plated on M-7H11-OADC agar containing kanamycin. Following incubation (12-14 days), the plates were counted and $\log_{10}$ CFU per mL were determined.

The charts depict the corresponding RFU and CFU measured in the control **(A)** and test vials: **(B)** HP 3.75 %, **(C)** HP 2.50 % and **(D)** HP 1.5 %. The chart does not show the data of the test using full-strength product (7.5 %). The value of CFU and RFU were analyzed using Sigma Stat 3.0 to determine the coefficient of correlation (r value). The r-value indicated a good correlation in the control and all the tests. Error bars in the chart indicate the mean of 2 determinations ± SD. CFU was measured once in each test.

Figure 3.9

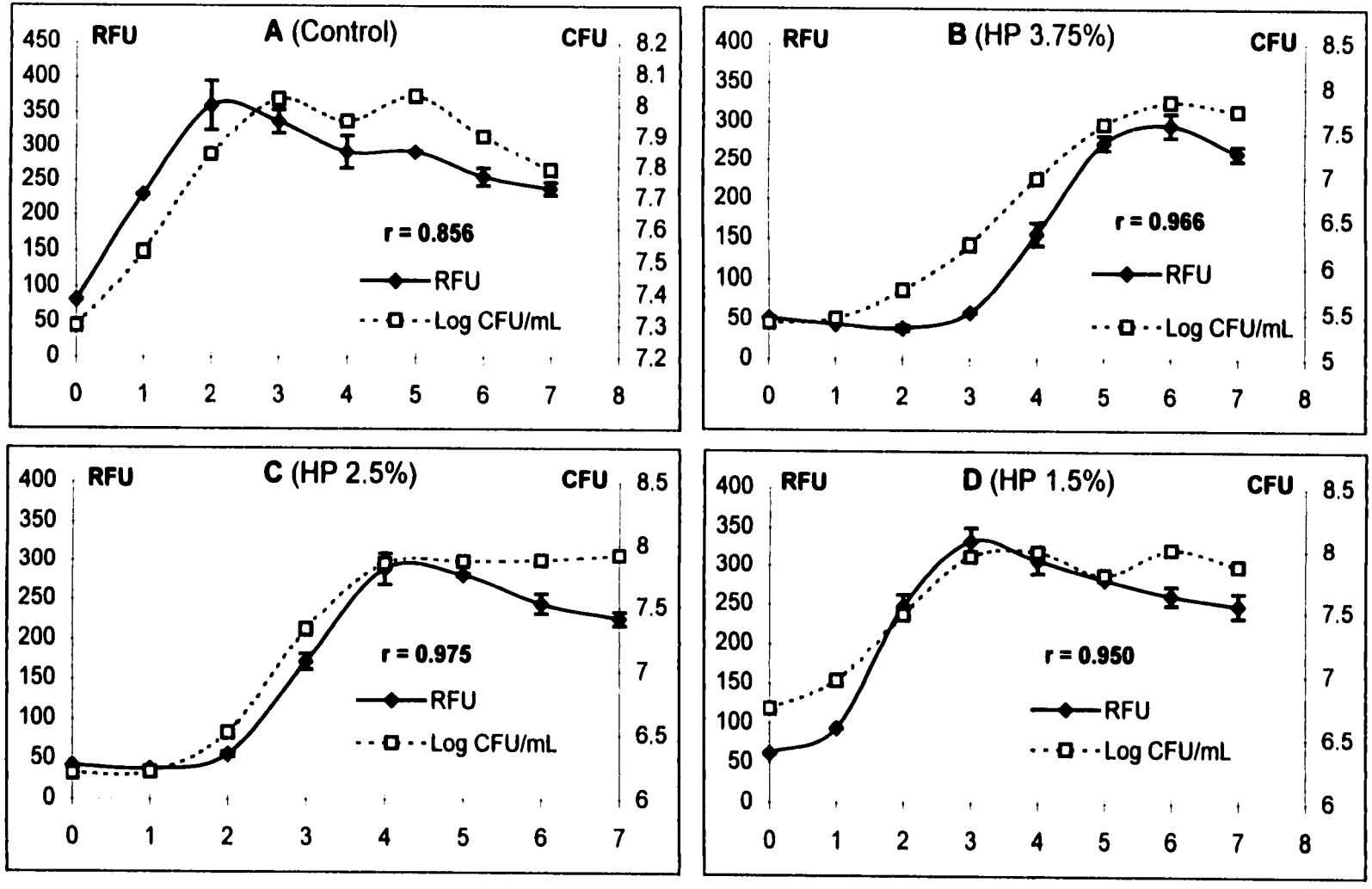

A (Control)
RFU
CFU
r = 0.856
RFU
Log CFU/mL
B (HP 3.75%)
RFU
CFU
r = 0.966
RFU
Log CFU/mL
C (HP 2.5%)
RFU
CFU
r = 0.975
RFU
Log CFU/mL
D (HP 1.5%)
RFU
CFU
r = 0.950
RFU
Log CFU/mL
-Time in Days-

Figure 3.10 Kinetics of GFP expression by *M. terrae* (pBEN) after exposure to different concentrations of an alkaline glutaraldehyde-based chemical germicide.

Test suspensions (~10^8 in 100 µL) of *M. terrae* (pBEN) were exposed to six concentrations of an activated alkaline glutaraldehyde (AlG %: 2.4, 0.8, 0.48, 0.27, 0.14 and 0.07) based product for 20 minutes. At the end of the contact period the tests and controls exposed to 0.85 % saline were diluted and washed in saline, centrifuged, pelleted and resuspended in M-ADC-TW broth containing kanamycin and incubated in 4 mL glass vials. The chart depicts the RFU of controls and tests (AlG) measured directly from the incubating vials at 24 hour intervals for a period of 11 days. Error bars indicate the mean of 2 determinations ± SD.

Figure 3.10

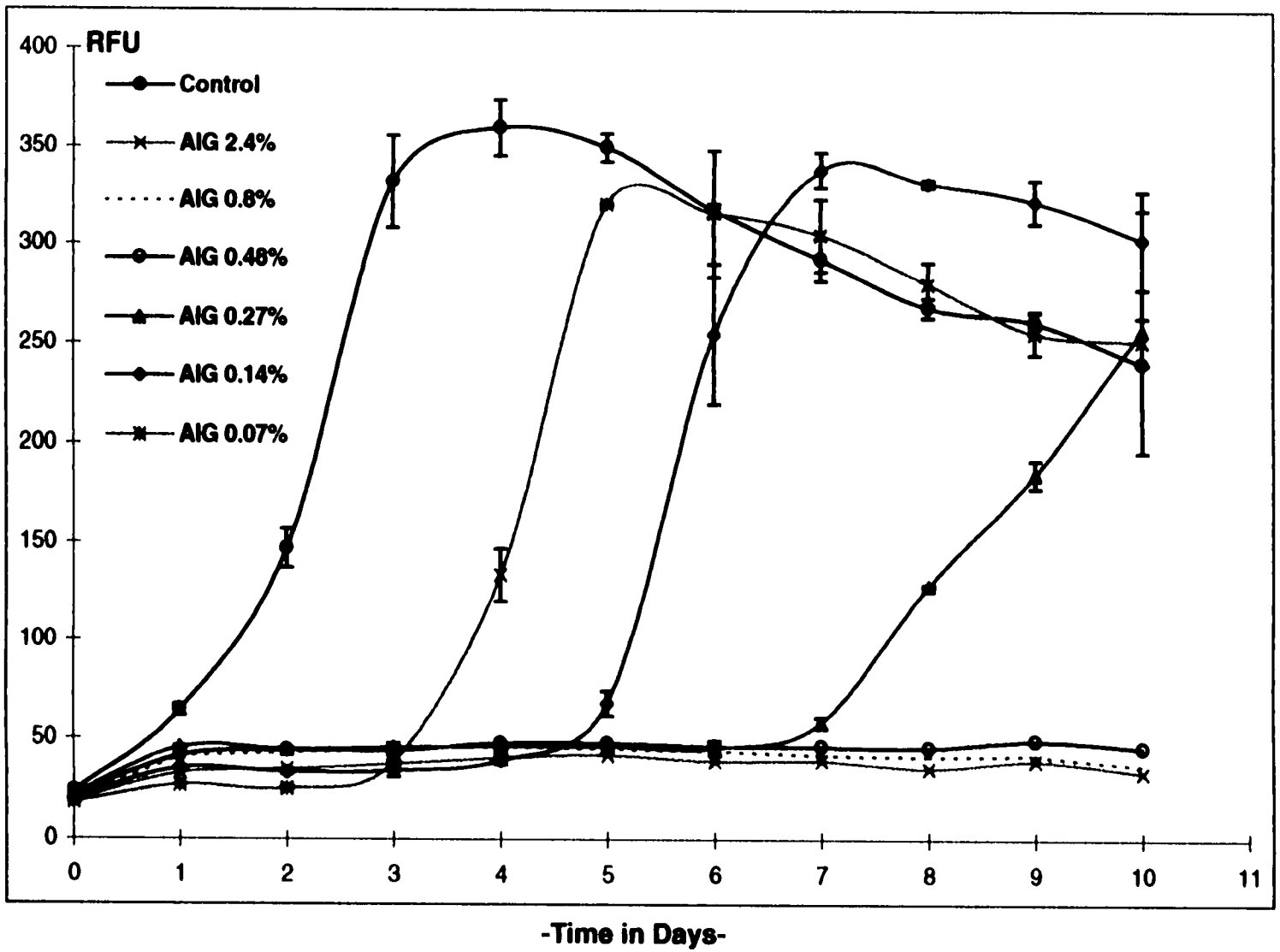

RFU
Control
AIG 2.4%
AIG 0.8%
AIG 0.48%
AIG 0.27%
AIG 0.14%
AIG 0.07%
400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
11
-Time in Days-

Figure 3.11 Comparison of fluorescence (RFU) and growth (CFU) in *M. terrae* (pBEN) after exposure to alkaline glutaraldehyde-based chemical germicide.

Fluorescence (RFU) was measured in controls and test samples of *M. terrae* (pBEN) exposed to six concentrations of an alkaline glutaraldehyde-based (AlG) product with a contact period of 20 minutes (Figure 3.11). At the same time, 100 µL of cells from the control and tests were serially diluted and plated to give countable colonies for determination of CFU.

The charts depict the RFU (marker: hollow circle) and the corresponding $\log_{10}$ CFU per mL (marker: solid black squares) measured directly from the control **(A)** and test vials: **(B)** AlG 0.8%, **(C)** AlG 0.48%, **(D)** AlG 0.27%, **(E)** AlG 0.14% and **(F)** AlG 0.07%. Data of the test using full strength product (AlG 2.4%) was not shown.

The axis in the graphs could not be assigned the same values but were made much smaller in B and C due to the low values of RFU and CFU resulting from exposure to high concentrations of the germicide. The values of CFU and RFU were analyzed using Sigma Stat 3.0 to determine the coefficient of correlation (r-value). The r-value indicated a good correlation in the control and all the tests except B. Error bars in the chart indicate the mean of 2 determinations ± SD. CFU was measured once in each test.

Figure 3.11

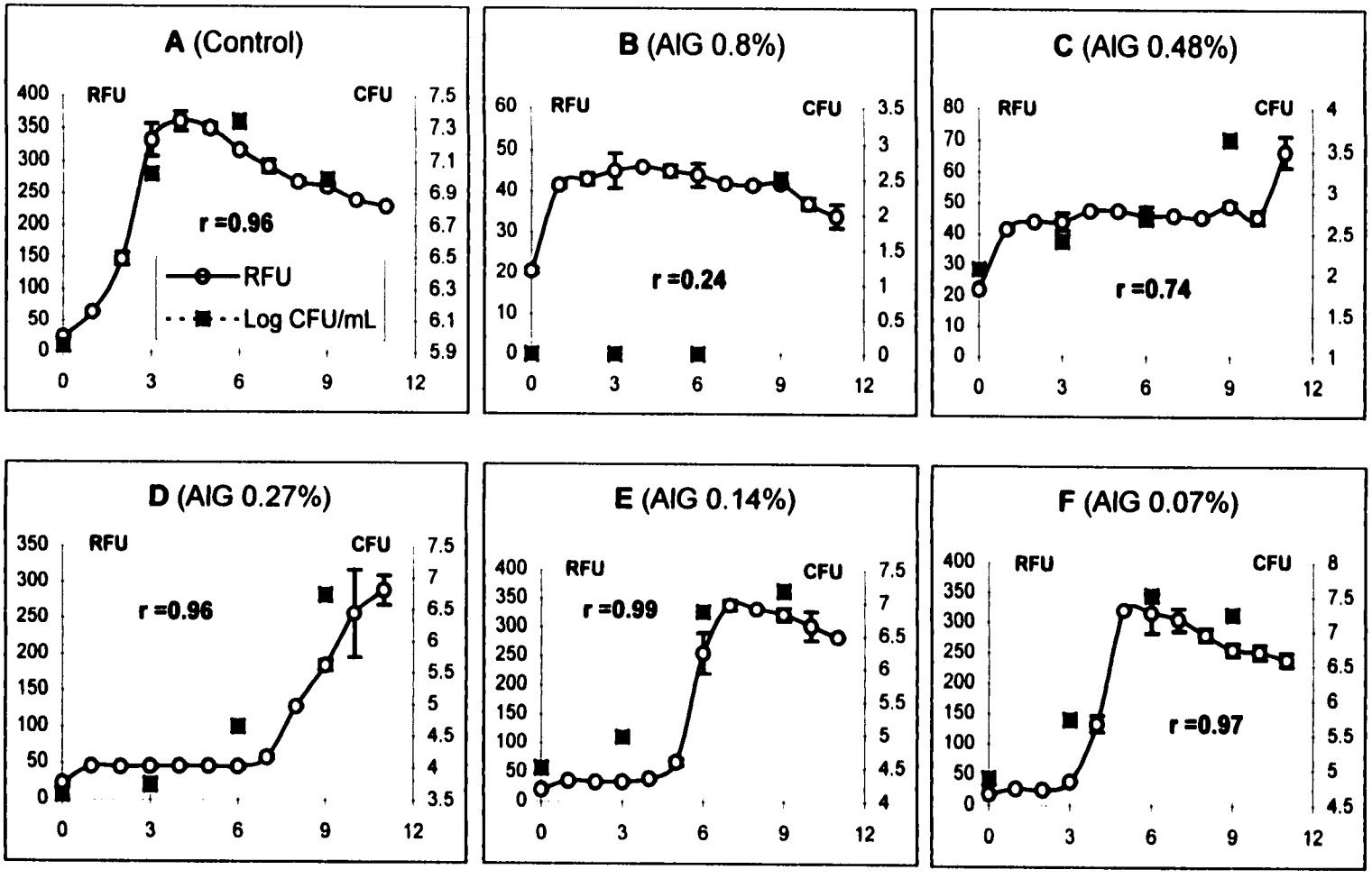
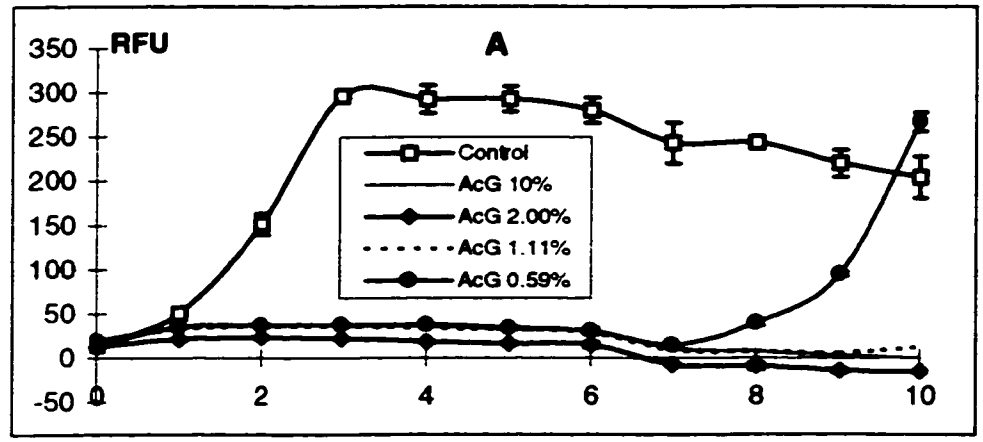

A (Control)
RFU
CFU
r =0.96
RFU
Log CFU/mL
B (AIG 0.8%)
RFU
CFU
r =0.24
C (AIG 0.48%)
RFU
CFU
r =0.74
D (AIG 0.27%)
RFU
CFU
r =0.96
E (AIG 0.14%)
RFU
CFU
r =0.99
F (AIG 0.07%)
RFU
CFU
r =0.97
-Time in Days-

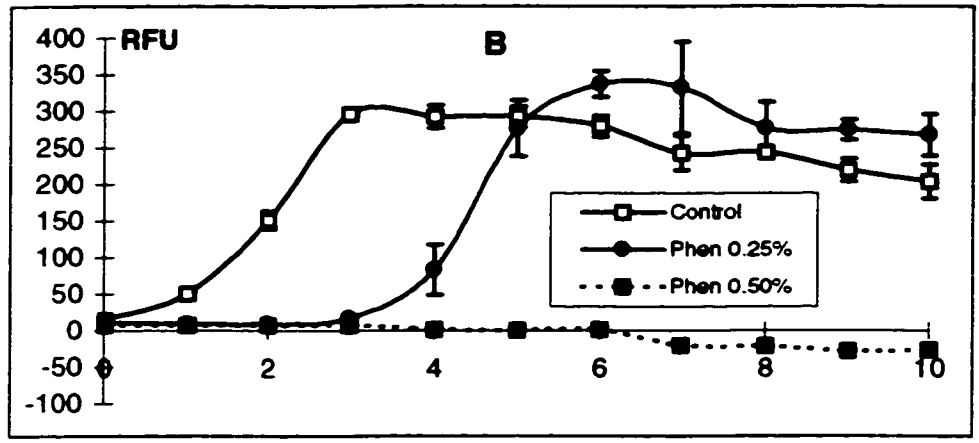
Figure 3.12 Kinetics of GFP expression by *M. terrae* (pBEN) after exposure to germicides based on acid glutaraldehyde, phenolic and ethanol

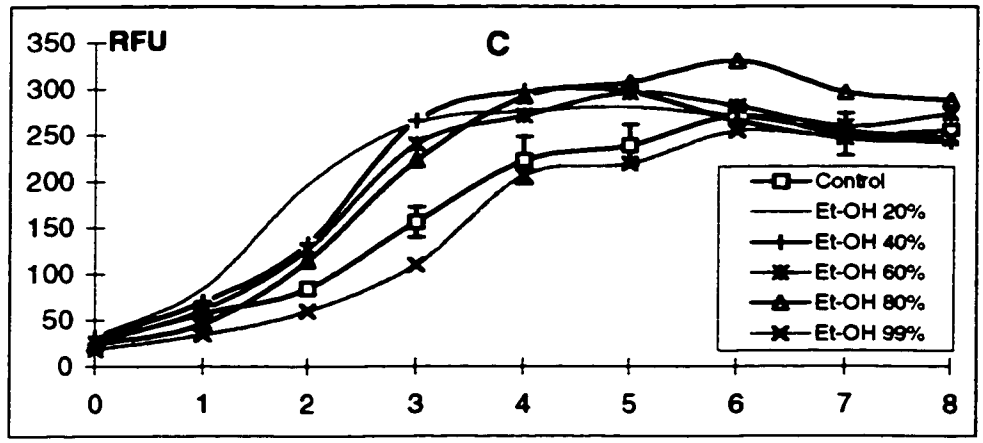
Test suspensions (~10^8 in 100 µL) of *M. terrae* (pBEN) were exposed to different concentrations of germicides based on acid glutaraldehyde, a phenolic and ethanol for a contact period of 20, 10 and 2 minutes, respectively. The controls exposed to 0.85% saline and the tests were diluted and washed in saline, centrifuged, pelleted and resuspended in M-ADC-TW broth containing kanamycin and incubated in 4 mL glass vials. RFU was measured directly from the control and test vials. Error bars in all the charts indicate the mean of 2 determinations ± SD.

(A) Acid glutaraldehyde (AcG %: 10, 2.00, 1.11 and 0.59)
(B) Phenolic (Phen %: 0.50 and 0.25)
(C) Ethanol (Et-OH %: 99, 80, 60, 40 and 20)

Figure 3.12







-Time in Days-

3.4 Discussion

Rapid methods for testing anti-mycobacterial agents are evolving to keep pace with the growing needs of manufacturers, regulators and health-care providers. However, a majority of the rapid methods are not aimed at testing germicides but are geared to the testing of drugs or antibiotics used in the treatment of mycobacterial infections. This study was undertaken in order to develop a rapid method for testing chemical germicides for mycobactericidal activity using reporter genes.

The firefly luciferase gene has been employed for rapid assessment of drug susceptibility of *M. tuberculosis* (Jacobs *et al.*, 1993; Cooksey *et al.*, 1993; Arain *et al.*, 1996), *M. avium* (Cooksey *et al.*, 1995; Arain *et al.*, 1996), *M. bovis* BCG (Arain *et al.*, 1996) and *M. intracellulare* (Arain *et al.*, 1996). Several advantages of the luciferase method were reported in these studies, including the ability to measure luminescence in real time with a high degree of sensitivity and a good correlation of MICs of antimicrobial agents with those obtained by standard assays using very small drug quantities in microtitration plates.

A study where the bacterial luciferase gene from *Vibrio harveyi* was transformed into *M. smegmatis* was one of the few instances where a rapid method was used to screen germicides in addition to testing antibiotics for anti-mycobacterial activity (Andrew and Roberts, 1993). However, it appears that the methods used thus far are not suitable for testing germicides, since elimination of the effects of germicides after the contact period, which is a fundamental requirement, may have been difficult to achieve. Antibiotics, for which these methods were developed initially, were incubated with the recombinant

organisms throughout the test period. Although chemical neutralization was described as a possible means to arrest the action of germicides (Andrew and Roberts, 1993), it does not wash off the germicide creating the possibility of residual inhibitory activity. Chemical neutralizers may in themselves be toxic to some test organisms, interfere with the expression of the reporter gene or the bioluminescent reaction, and their specific activity may not apply to all germicides. Cost and availability are other factors to be taken into consideration in their use.

In the course of developing a bioluminescent system for assessing the viability of mycobacteria, the plasmid pYUB180 (35 kb) containing the luciferase gene, previously used for transforming *M. tuberculosis* (Jacobs *et al.*, 1993), was chosen. In the initial stages of this study, methods used to extract the plasmid pYUB180 from *E. coli* DH5α cells were found to give low yields of plasmid DNA. Although the SDS lysis method for extracting the plasmid from cells grown in Terrific broth appeared to give a better yield compared to the alkali lysis method, the presence of chromosomal DNA and RNA that escaped removal actually resulted in a low quantity of plasmid DNA. Repeated attempts to transform *M. smegmatis* and *M. terrae* with the extracted plasmid pYUB180 using electroporation and calcium chloride were followed by the appearance of only a few colonies that raised doubts regarding the suitability of these cells for germicidal assays. Although a low quantity of plasmid DNA could have resulted in poor transformation, the large size of the plasmid pYUB180 (35 kb) may have also made the transformation of the mycobacteria more difficult. Furthermore, the extremely slow appearance of the colonies of transformed mycobacteria compared to the untransformed ones could indicate an effect

of the plasmid on the growth kinetics that may have significantly altered their susceptibility to germicides. Based on the poor growth and the likelihood of having altered susceptibility to germicides, it was considered that the transformed *M. terrae* would not be suitable as a surrogate organism for assessing mycobactericidal activity of germicides. The overall prospect of developing a luciferase system in *M. terrae* was not encouraging due to further anticipated difficulties including the need for a luciferase substrate, delivery of the substrate successfully through the cell wall of *M. terrae* without any adverse effects to the cells and performing sensitive measurements of light emission. In view of this, an alternative reporter gene system was sought and soon studies with *M. terrae* expressing a red-shifted, high-intensity GFP variant (Cormack *et al.*, 1996) that conferred a green fluorescence to host cells were undertaken. This method was found to meet several crucial requirements for a reporter gene system to rapidly assess the mycobactericidal activity of chemical germicides.

The GFP gene was found to be a more adaptable molecule than luciferase. In this study, measurement of fluorescence in mycobacteria expressing GFP was able to differentiate viable from dead or dying cells in incubating cultures and provided the means for developing an assay for rapid assessment of mycobactericidal activity of chemical germicides.

E. coli DH5α made competent using calcium chloride was easily transformed with the plasmids, pWES4 and pBEN containing the gene encoding GFP. However, GFP was not expressed by transformed *E. coli* as indicated by their failure to fluoresce using epifluorescence microscopy. It appeared that the BCG *hsp*60 promoter does not allow a

heterologous gene such as *gfp* to be expressed in *E. coli*. Similar observations were also made by other investigators (Kremer *et al.*, 1995b). A positive control consisting of the same cells transformed with pEGFP (CLONTECH Laboratories, Inc., Palo Alto, CA, USA) containing a *LacZ* promoter grew in the presence of ampicillin but not in kanamycin and fluoresced bright green. Negative controls without plasmid DNA did not grow in the presence of either antibiotic.

M. smegmatis and *M. terrae* were easily transformed with the plasmids, pWES4 and pBEN using electroporation and a calcium chloride method. Expression of the *gfp* gene under the control of the BCG *hsp*60 promoter could be readily detected by observing the cells using light of appropriate wavelengths.

When irradiated with a hand-held UV lamp, colonies of mycobacteria transformed with pWES4 emitted a bright green fluorescence due to a close match between the excitation wavelength (395 nm) of the wt GFP that was expressed and the wavelength of the UV light (366 nm). Activated Charcoal added to M-7H11-OADC agar facilitated the observation and photography of colonies of fluorescent mycobacteria by absorbing background fluorescence from the plates. Mycobacteria with pBEN expressed a red-shifted GFP, which was not excited by UV light and did not fluoresce. However, mycobacteria with pBEN appeared brighter under the epifluorescence microscope as the excitation (501 nm) and emission (511 nm) spectrum of the red-shifted GFPmut3 was a close match with those provided by the FITC filter system in the microscope. The GFP expressed by *M. terrae* resisted photobleaching to a remarkable extent that allowed microscopic examination and photomicrography to be carried out for longer periods than would be possible with conventional fluorescent dyes. A CCD camera connected to an

imaging software allowed integration and enhancement of the fluorescent images that could be saved on computer disks for future reference and analysis. *M. terrae* (pBEN) was chosen for subsequent studies owing to its higher intensity of fluorescence, and the red-shifted spectral properties that allowed sensitive and high level readings in devices such as a fluorometer or a flow cytometer with standard FITC filter systems.

Comparative fluorometry performed in disposable cuvettes and 4 mL screw-capped clear glass vials demonstrated similar readings for *M. terrae* (pBEN). The glass vials allowed the same cells to be measured repeatedly while being incubated throughout the test period. In this way, the kinetics of GFP expression was monitored consistently without having to discard the samples each time. Measurements of growth (CFU) performed by withdrawing a 100 μL aliquot from the same vials every 24 hours did not show any deleterious affects on the fluorescence readings. A second vial (control) having the same culture from which aliquots of 100 μL were not withdrawn for CFU determination was found to have fluorescence emissions that were very similar throughout the test period (data not shown).

The cellular incorporation of the plasmid pBEN did not appear to alter significantly the susceptibility of *M. terrae* and *M. smegmatis* to the chemical germicides tested. Using a standard quantitative carrier test, *M. terrae* (pBEN) tested with sublethal concentrations of germicides based on hydrogen peroxide (3.75%) and alkaline glutaraldehyde (0.8%) was found to be slightly more sensitive than the untransformed ones by a $\log_{10}$ value of 0.91 and 0.59, respectively. *M. smegmatis* (pBEN) on the other hand appeared to be slightly more resistant than the untransformed ones by a $\log_{10}$ value

of 0.71 and 0.88, respectively (Figure 3.6). When all these species were tested against the same germicides at the recommended use-dilutions and contact periods complete inactivation was achieved.

M. terrae has been considered as a suitable surrogate for assessing the mycobactericidal activity of chemical germicides and, in particular, the tuberculocidal potential of such chemicals. In fact, testing conducted thus far, has found *M. terrae* to be slightly more resistant than *M. tuberculosis* (van Klingeren *et al.*, 1987; Griffiths *et al.*, 1998). Parallel testing of the germicide susceptibility indicated that the transformed *M. terrae* (pBEN) had susceptibility profiles similar enough to that of the parent strain. The transformed strain of *M. terrae* (pBEN) and *M. smegmatis* (pBEN) were found to have a slightly higher resistance to desiccation than the corresponding parent strain which is an advantage when organisms would require to be dried and fixed onto carriers in germicidal tests (Table 3.3).

The kinetics of GFP expression by *M. terrae* (pBEN) were easily monitored by measuring fluorescence directly from the glass vials on a daily basis. In the presence of kanamycin, fluorescence increased to a higher peak level and during the reduction phase it was maintained at a relatively higher level when compared with fluorescence from a similar culture without kanamycin (Figure 3.7A). The plasmid was maintained for prolonged periods of up to several months when the cells were subcultured in broth containing kanamycin due to selection of the plasmid bearing strains by the antibiotic. In the absence of kanamycin it appeared to be maintained for only 5-7 days although further studies are required to confirm a more precise duration for stable expression of GFP.

GFP expression and therefore fluorescence appear to be maintained as long as the host cells are viable. When recombinant organisms were incubated in broth, an increase in fluorescence was observed in the first few days followed by a plateau and a steady fall due to exhaustion of nutrients. Exposure of *M. terrae* (pBEN) to hydrogen peroxide at the recommended use dilution (7.5%) for 10 minutes did not allow fluorescence levels to increase above baseline levels when incubated for 11 days (Figure 3.8 and 3.9). Absence of growth in the same sample indicated that failure to express GFP by non-viable cells prevented any increase in fluorescence. When exposed to the diluted product (1.5, 2.5 and 3.75%), fluorescence increased after a lag period, which varied from several hours to several days depending on the concentrations of the germicide. The diluted product appeared to have inactivated a proportion of the cells. Those that escaped inactivation underwent a phase of recovery during which period GFP was not expressed producing a lag period in fluorescence emission. GFP expression indicated by an increasing level of fluorescence resumed with the onset of replication.

When RFU in controls and the tests were compared with the corresponding CFU, there was good agreement between correlation coefficients (r-values) of the curves (Figure 3.9). Fluorescence assays using different concentrations of acid and alkaline glutaraldehyde, ethanol and a phenolic germicide exhibited a similar pattern of results (Figures: 3.10, 3.11 and 3.12).

A good correlation was observed between RFU and CFU of *M. terrae* (pBEN) in controls and tests exposed to 2-fold dilutions of alkaline glutaraldehyde for a 20-minute contact period (Figure 3.10 and 3.11). Cells that survived even after exposure with alkaline glutaraldehyde required a longer period of recovery than what was observed with

hydrogen peroxide. Even with dilutions as high as 1:32 times (0.07%) of alkaline glutaraldehyde, it required 3-4 days for the RFU to increase, whereas the increase in the control was observed within 24 hours. A similar observation was made in the culture-based quantitative carrier test where cells that survived sublethal concentrations of alkaline glutaraldehyde required longer periods of recovery in agar plates than those exposed to hydrogen peroxide. Cells exposed to full-strength alkaline glutaraldehyde (2.4%) and 0.8% of the product were completely inactivated and RFU remained at or below baseline levels for 11 days. Higher dilutions of the product (0.48, 0.27, 0.14 and 0.07%) allowed RFU to increase after a lag period that varied from 3-10 days.

When full-strength alkaline (2.4%) and acid (10%) glutaraldehyde and their dilutions were used for 10 minutes, fluorescence increased from the next day indicating inadequate mycobactericidal activity of the products for that contact time (data not shown). A comparable observation was made in a quantitative carrier test where numerous colonies appeared in agar plates after exposure of *M. terrae* to similar dilutions of alkaline glutaraldehyde for 10 minutes (data not shown).

Acid glutaraldehyde used at a concentration of 0.59% for 20 minutes was unable to inactivate a proportion of the cells, which was observed as an increase in RFU from the 8^{th} day onwards (Figure 3.12A). When tested at higher concentrations (1.11, 2.0 and 10%), there was no increase in RFU during the test period indicating complete inactivation of the cells. The RFU in these tests gradually decreased over time and approached a negative value that was below those of the baseline fluid. Failure of dead cells to express GFP and gradual decomposition of preexisting GFP appeared to reduce the RFU in these tests. A negative RFU level may also be due to release of products from

dead cells that quenched fluorescence from the suspending medium. The exact mechanism of a negative value requires further investigation.

A phenolic-based germicide used at the recommended use-dilution of 1:200 (0.5%) as well as higher concentrations (1 and 2%) for 10 minutes was able to inactivate the cells as shown by gradual decrease in RFU. The downward shift continued in the negative value as was also observed in tests using acid glutaraldehyde for 20-minutes and hydrogen peroxide for 10 minutes (data not shown). With a higher dilution (1:400 = 0.25%) of the phenolic, RFU increased from the third day onwards indicating poor mycobactericidal activity (Figure 3.12B).

Ethanol used for 2-minutes was unable to achieve complete inactivation of *M. terrae* (pBEN) at all the concentrations tested ranging from 20 to 99% (Figure 3.12C). Ethanol (70%) used for one minute was shown to achieve approximately 3.5 $\log_{10}$ reduction in a suspension test without an organic load (Best *et al.*, 1990). In the present study, a high inoculum (10^7~10^8 cells in 100µL) allowed a large proportion of cells to resist the product with consequent growth of the cells and increasing RFU from the next day onwards. The least RFU was observed with 99% followed successively by 80, 60, 40 and 20% indicating a correlation of fluorescence with the concentration of the test product.

Autofluorescence of different products used in fluorometry was taken into consideration as a possible source of false-positive readings. In our experiments, 100-fold dilutions with 0.85% saline was able to quench very high levels of autofluorescence emitted from activated alkaline glutaraldehyde and was found to be as effective as 1%

glycine. Dilution with saline thus avoids the use of potentially toxic neutralizers some of which may be autofluorescent in addition to being costly or difficult to obtain.

To date, all GFP and most of the luciferase-based reporter gene assays have been developed for evaluating drugs or antibiotics for antimycobacterial activity. The GFP-based assay investigated in our laboratory requires further development that would allow carriers and organic substances to be incorporated in the method. In spite of its current limitations, it provides a rapid and convenient preliminary screen for mycobactericides. Where a product was poorly effective, as shown in the tests using acid and alkaline glutaraldehyde for 10-minutes and ethanol for 2-minutes, a rapid increase in fluorescence allowed assessment of efficacy as early as 24-hours. With more effective products, it required 5-7 days to obtain results of efficacy. The high-intensity, red-shifted GFP provides sensitive measurements as low as 10^4-10^5 cells per mL of sample. Furthermore, the GFP-based assay does not require the addition of substrates or cofactors and the host cells do not require to be lysed or permeabilized enabling direct and repeated measurements of GFP expression in the same samples thus providing a sensitive and real time assessment of the response of mycobacteria to germicides.

4. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The global increase in tuberculosis and NTM infections, many of which are acquired from environmental reservoirs and the partial failure of antibiotics due to the emergence of MDR strains, has intensified the efforts for environmental control of infections. This has provided the impetus to evaluate germicides for their activity against mycobacteria using improved methods and to better define their role in infection control practices. To date, traditional methods that are reliant on culture of mycobacteria remain the standard yardstick for determining mycobactericidal activity of germicides. In the evolution of rapid methods, a GFP-based fluorescent method has been developed in our laboratory. When compared with a culture-based quantitative test a good correlation was observed between the two methods. However, further work is required to adapt the GFP-based approach to a proper carrier-based method of evaluating the mycobactericidal activity of chemical germicides.

In ***section 2*** of this thesis, a quantitative carrier test based on traditional culture on agar plates has been used for the assessment of liquid chemical germicides for mycobactericidal activity. This method provided a quantitative assessment of the activity of germicides used at different dilutions and contact periods. The test also demonstrated the susceptibility of individual species of mycobacteria to the tested germicides. The results obtained were in agreement with the findings of earlier studies. Flat-bottomed glass vials with inserts and septate caps used as carriers in this study allowed convenient inoculation of a known volume of the test bacterium and the quantitative recovery of cells were shown to be accurate and reproducible. The presence of a unique combination of three organic substances conferred the desired degree of protection to mycobacteria

against germicides and simulated in-use conditions of germicide use. Membrane filtration and rinsing with an innocuous diluent provided an effective means of arresting the action of the germicide by separating the test organisms from the germicide and in addition allowed cells to be plated onto semisolid media for accurate enumeration. Tests using the same species and germicides under similar test conditions but at different times produced similar results demonstrating the accuracy and reproducibility of this method. This method however, does not allow for rapid assessment of germicidal activity for the slow growing species of mycobacteria and appears to be unsafe if used for testing pathogenic mycobacteria. The high cost of glassware and media supplements are additional drawbacks.

Reporter genes have in recent years acquired an important role as markers for gene expression and cell biology in varied species including mycobacteria. In ***section 3*** of this thesis, a reporter gene assay was investigated as a rapid alternative to the existing culture method. In view of several drawbacks in the bioluminescence method where the firefly luciferase gene was used, the GFP was investigated as an alternative reporter system to rapidly assess the mycobactericidal activity of chemical germicides.

Investigation with GFP allowed ease of transformation of mycobacterial strains and their expression as fluorescent phenotypes. A red-shifted, high-intensity GFP expressed by *M. terrae* made possible fluorescence measurements in viable cells at a relatively low bacterial density. Direct and repeated measurements of the same sample were made possible using screw-capped glass vials in place of designated cuvettes. This allowed real-time monitoring of the kinetics of GFP expression in viable cells and in

those exposed to germicides. Measurements of fluorescence made in screw capped vials also has enhanced biosafety.

When exposed to germicides based on hydrogen peroxide, alkaline and acid glutaraldehyde, phenolics and ethanol, results of the fluorescence assay were comparable to those obtained by the standard method. A good correlation between fluorescence and the corresponding growth was obtained in the controls and tests exposed to different dilutions of hydrogen peroxide and alkaline glutaraldehyde at 10 and 20 minutes, respectively. Results of the fluorescence assay could be obtained as early as 24 hours although it required 5-7 days to screen a product with sufficient assurance. In contrast, results were obtained in 12-28 days or more with the culture-based quantitative carrier test.

Autofluorescence of germicides and other products used in the fluorescence assay was considered as a potential source of false positive results. Dilution with saline was found to be adequate for quenching high levels of autofluorescence emitted from an alkaline glutaraldehyde-based product without the need for a specific neutralizer. Whether this could be applied for other autofluorescent products requires further investigation.

After the initial cost of the fluorometer, costs related to maintenance and supplies in the fluorescence assay are lower compared to the cost of glassware (filtration units, glass vials, inserts etc.), filter membranes and media supplements used in the culture method as well as to the high cost of reagents used in the luciferase assay. Avoidance of toxic or radio-labelled chemicals as well as aerosols that may be generated in a culture-based method are other advantages of the fluorescence assay.

Although the fluorescence assay is based on a suspension test and does not incorporate carriers or an organic load, it provides a rapid and convenient preliminary screen for germicides. More tests are required to identify potential problems including autofluorescence of germicides and to monitor the performance of this method with different categories of germicides. The suitability of the transformed *M. terrae* (pBEN) as a surrogate organism also needs to be validated further by testing with different categories of germicides. Further development of the fluorescence method is required to increase accuracy and reproducibility of test results, incorporate carriers and organic load, provide maximum recovery of the input inocula and allow a large number of samples to be analyzed in a shorter period of time with minimum cost and labor. Fluorometers equipped for reading microtiter plates would be a suitable alternative to the cuvette type fluorometer used in this study.

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APPENDIX I: LIST OF SOLUTION COMPOSITIONS, CHEMICALS AND SUPPLIERS

PREPARATION	COMPONENTS	QUANTITY	SUPPLIER & CATALOG NO.
Antibiotics:			
Ampicillin (stock solution)	Ampicillin powder	150 mg in 3 mL dd H_2O	ICN Biomedical: 190146
Chloramphenicol (stock solution)	Chloramphenicol powder	34 mg in 1 mL ethanol	Sigma: C-7795
Kanamycin (stock solution)	Prepared kanamycin soln	10 mg kanamycin base	Gibco BRL: 15160-054
Filter sterilize all except chloramphenicol		in 1 mL 0.85% saline	
Calcium Chloride (0.1 M)	$CaCl_2$. $6H_2O$	5.4 g	BDH AnalaR: B 10069 4A
Filter sterilize	dd H_2O	Add up to 200 mL	
Hard water (200 ppm):	Solution I	2 mL	
	Solution II	4 mL	
	dd H_2O	994 mL	
Hard water (400 ppm):	Solution I	4 mL	
	Solution II	4 mL	
	dd H_2O	992 mL	
Hard water solution I	$MgCl_2$. $6H_2O$	6.79 g	BDH AnalaR: B 10149
Autoclave for 20 minutes	$CaCl_2$. $2H_2O$	9.80 g	BDH AnalaR: B 10070 4A
	dd H_2O	100 mL	
Hard water solution II	$NaHCO_3$	5.60 g	Fisher Scientific: BP 328-500
Filter sterilize	dd H_2O	100 mL	
Lysozyme stock solution 100 mg/mL. Filter sterilize	Lysozyme powder	600 mg	Pharmacia: 27-0267-02
	dd H_2O	6 mL	

Media supplement			
Albumin Dextrose Catalase Complex (ADC) Adjust pH to 7.0 with 1N NaOH. Filter sterilize.	Bovine serum albumin fraction V Glucose NaCl Catalase (beef) dd H_2O	5 g 2 g 0.85 g 0.003 g 100 mL	BDH Biochemical: 441555J BDH AnalaR: B 10117 BDH AnalaR: B 30123 Sigma.
Oleic Acid Albumin Dextrose Catalase Complex (OADC) Adjust pH to 7.0 with 1N NaOH. Filter sterilize.	Bovine serum albumin fraction V Glucose NaCl Catalase (beef) Sodium Oleate solution dd H_2O	5 g 2 g 0.85 g 0.004 g 3 mL Add up to 100 mL	BDH Biochemical: 441555J BDH AnalaR: B 10117 BDH AnalaR: B 30123 Sigma
Sodium Oleate solution Incubate at 50° C until solution is clear. Filter sterilize.	Oleic Acid NaOH dd H_2O	0.06 mL 0.06 mL Add up to 3 mL	BDH: B 26264-74 BDH AnalaR:B 10252 or B 30167
Media:			
Luria Bertoni (LB) broth Autoclave for 15 minutes	Tryptose Yeast extract NaCl dd H_2O	10 g 5 g 10 g 1 L	Difco Lab: 0124-17-2 Difco Lab: 0127-17-9 BDH AnalaR: B 30123
LB agar Autoclave for 15 minutes	LB broth agar	1 L 15 g	

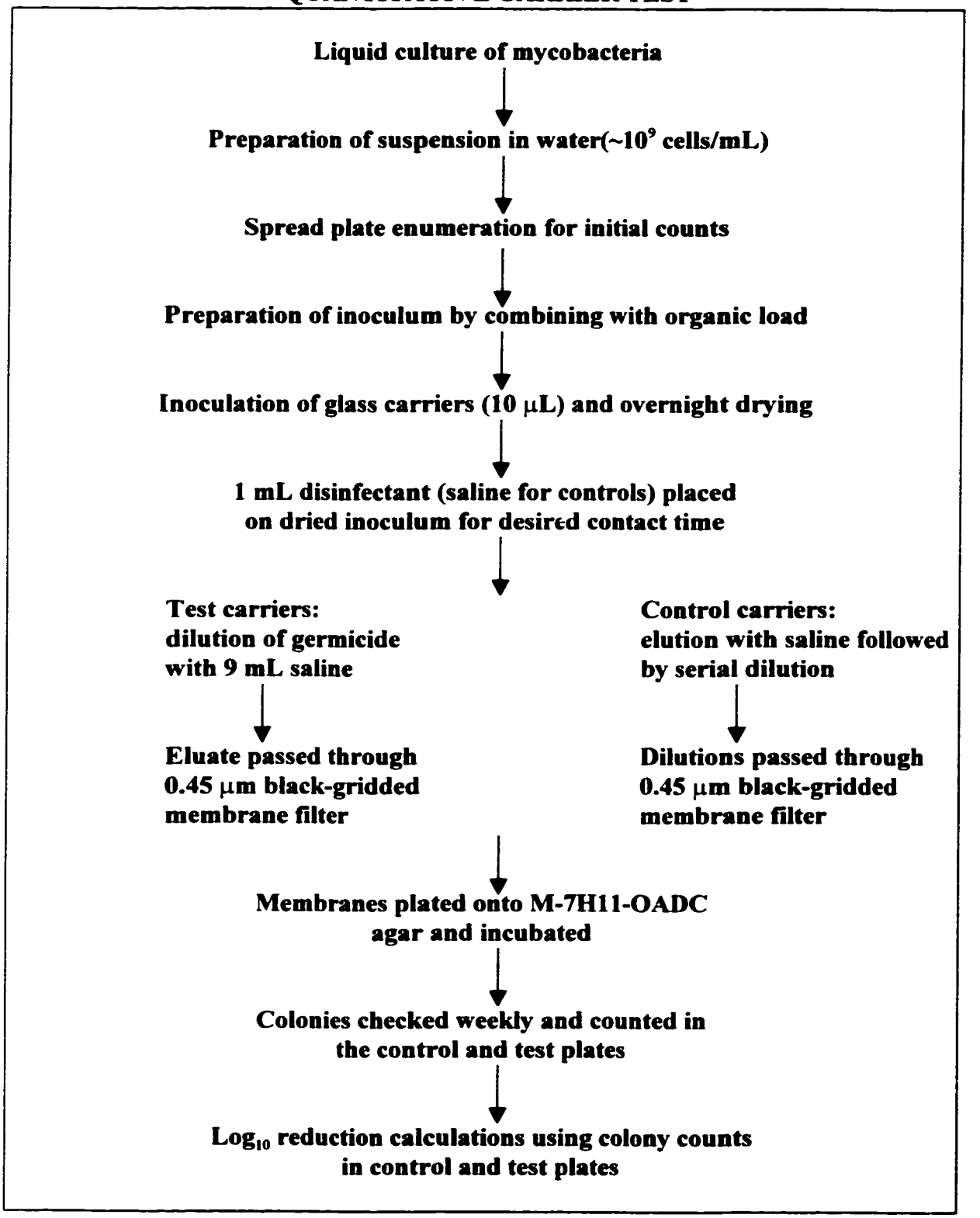
Terrific broth Autoclave I and II separately for 20 minutes. Mix I and II when cooled to 60°C	I. Tryptone Yeast extract Glycerol dd H_2O	6 g 12 g 2 mL 450 mL	Difco Lab: 0123-01-1 Difco Lab: 0127-17-9 BDH AnalaR: B 10118
Salt solution for Terrific broth	II. KH_2PO_4 K_2HPO_4 dd H_2O	2.31 g 12.54 g Add up to 100 mL	BDH AnalaR: B10203
Middlebrook 7H9 broth Autoclave for 10 min. When cooled down to 45°C add ADC.	Bacto® Middlebrook 7H9 dehydrated powder Glycerol or, Tween 80 dd H_2O Bacto® Middlebrook ADC	4.7 g 2 mL 0.5 g 900 mL 100 mL	Difco Lab: 0713-17-9 BDH AnalaR: B 10118 J.T. Baker Chemical Cp.: X 257-7 Difco Lab: 0714-64-0
Mycobacteria 7H11 agar Autoclave for 15 minutes. When cooled down to 50-55°C, add OADC and pour into plates	Bacto® Mycobacteria 7H11 dehydrated powder Glycerol dd H_2O Bacto® Middlebrook OADC (or in-house made OADC)	21 g 5 mL 900 mL 100 mL	Difco Lab: 0838-17 BDH AnalaR: B 10118 Difco Lab: 0722-73-9
Freezing media for mycobacteria	Fetal bovine serum (complement heat inactivated at 50°C Middlebrook 7H9 broth + 10% ADC	20 mL 80 mL	Gibco BRL: 16000-044

SOB Adjust pH to 7.0. Autoclave for 20 minutes.	Tryptone Yeast extract NaCl KCl dd H_2O	20.0 g 5.0 g 0.584 g 0.186 g Add up to 1 L.	Difco Lab: 0123-01-1 Difco Lab: 0127-17-9 BDH AnalaR: B 30123 BDH AnalaR: B 10198-34
SOC Mix pre-sterilized components	SOB 2M Mg^{++} Stock 2M Glucose	98 mL 1 mL 1 mL	
2 M Mg^{++} Stock Autoclave or filter sterilize	$MgCl_2$. $6H_2O$ $MgSO_4$. $7H_2O$ dd H_2O	20.33 g 24.65 g Add up to 100 mL	BDH AnalaR: B 10149-34 BDH AnalaR: B 10151-34
2 M Glucose Filter sterilize	Glucose dd H_2O	36.04 g Add up to 100 mL.	BDH AnalaR: B 10117
Organic load **Tryptone (5%)** **Bovine serum albumin (BSA 5%)** **Mucin (0.4%)** Filter sterilize.	Tryptone BSA Mucin	0.5 g 0.5 g 0.04 g Each component dissolved in 10 mL PBS	Difco Lab: 0123-01-1 Sigma: B-4287 Sigma: M-4503
Plasmid preparation solution I Autoclave for 20 minutes	50 mM glucose 25 mM tris 10 mM EDTA dd H_2O pH	4.50 g 1.51 g 1.86 g Add up to 500 mL 8.0	BDH AnalaR: B 10117 BDH Chemical Ltd: 44151 Sigma: E-5134

Plasmid preparation solution II Heat in a microwave to dissolve. Use fresh each time.	0.2 M NaOH 1% SDS dd H_2O	0.4 g 0.5 g 50 mL	BDH AnalaR: B 10252 or B 30167 BDH: B 30175-34
Plasmid preparation solution III Autoclave for 20 minutes.	Sodium acetate Glacial acetic acid dd H_2O	12.3 g Add until pH is 4.8/5.6 Add up to 50 mL	BDH AnalaR: B 10236 BDH: B 27013-82
SDS (10%) stock solution Heated at 68°C to dissolve. Adjust pH to 7.2	SDS or Sodium lauryl sulphate dd H_2O	5 g Add up to 50 mL	BDH: B 30175-34
STE (also called TEN) Autoclave for 20 minutes. Adjust final pH to 8.0.	0.1 M NaCl 10 mM tris-Cl (pH 8.0) 1 mM EDTA (pH 8.0) dd H_2O	1.752 g 0.473 g 0.112 g Add up to 300 mL	BDH AnalaR: B 30123 or ACS 783 BDH Chemical Ltd: 44151 Sigma: E-5134
Sucrose (10%) in 50 mM tris-Cl Autoclave for 20 minutes. Adjust pH to 8.0	Sucrose 50 mM tris-chloride dd H_2O	10 g 90 mL Add up to 100 mL	BDH: B 10274 BDH Chemical Ltd: 44151
Tris EDTA (TE) Adjust pH to 8.0. Autoclave or filter sterilize.	10 mM Tris-chloride 1 mM EDTA dd H_2O	0.315 g 0.074 g 200 mL	BDH Chemical Ltd.: 44151 Sigma: E-5134
Tris-chloride (50 mM) Adjust pH to 8.0. Autoclave or filter sterilize	Trizma base dd H_2O	0.605 g 100 mL	Sigma: T-1503
Tween 80 (20% stock solution) Heat at 55°C to dissolve.	Tween 80 dd H_2O	20 mL 80 mL	J.T. Baker Chemical Cp.: X 257-7

APPENDIX II

FLOW CHART SHOWING THE MAIN STEPS IN THE QUANTITATIVE CARRIER TEST



APPENDIX III

FLOW CHART SHOWING THE MAIN STEPS IN THE EVOLUTION OF THE GFP-BASED FLUORESCENCE METHOD

Transformation of *E. coli* with plasmid (+*gfp*)

↓

Isolation of plasmid DNA using alkali lysis method

↓

Electroporation of mycobacteria with plasmid DNA (+*gfp*)

↓

Detection of GFP expression (fluorescence) in mycobacteria using epi-fluorescence microscopy and hand held UV lamp (366 nm)

↓

Correlation of fluorescence emission with number of viable cells using fluorometry

↓

Mycobactericidal activity of germicides:

Exposure of *M. terrae* (pBEN) to germicide (tests) and saline (control)
Test and controls diluted 100-fold with saline
Cells were centrifuged and pellet resuspended in 7H9 broth
Cultures incubated in 4 mL screw capped glass vials
Fluorescence (RFU) measured every 24-hours in controls and tests
Altered intensity in tests compared to control fluorescence

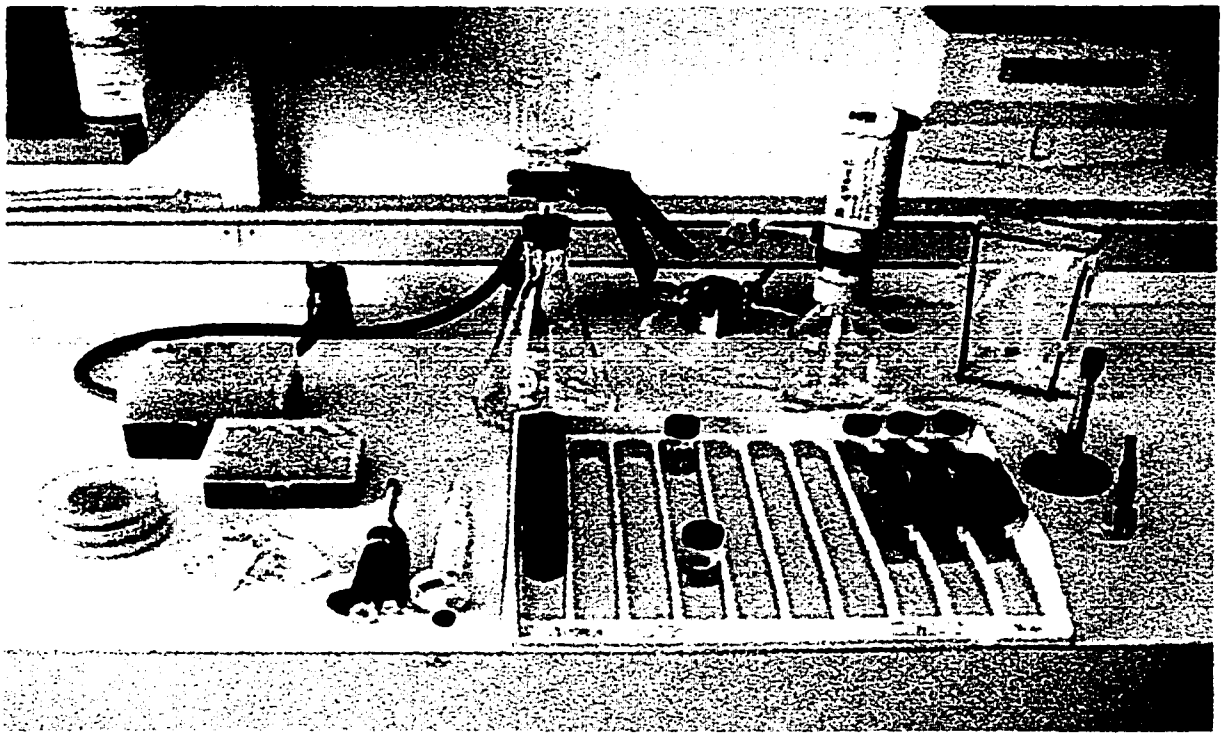
↓

Comparison of fluorescence (RFU) with growth (CFU):

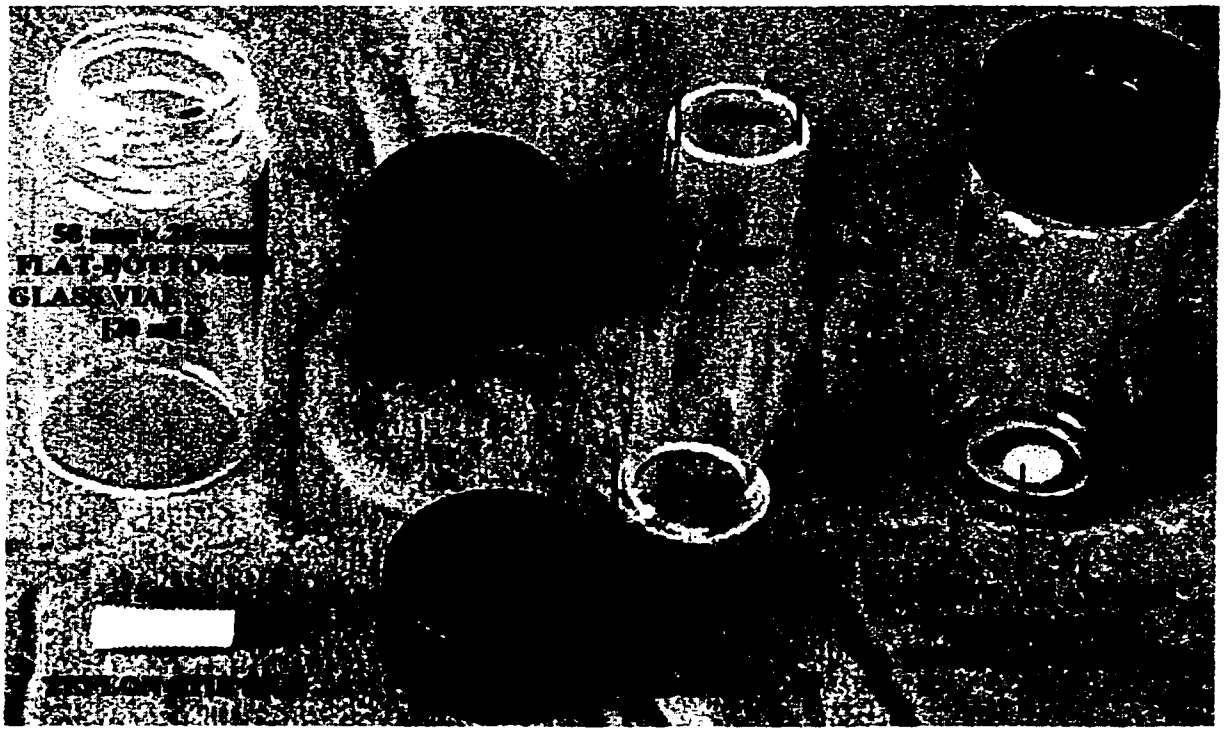
100μL of the test and control samples were serially diluted
The dilutions were filtered on membranes and plated on 7H11 agar
The growth ($\log_{10}$ CFU) in plates were compared with fluorescence (RFU).

APPENDIX IV

A. EQUIPMENT SETUP FOR THE QUANTITATIVE CARRIER TEST.



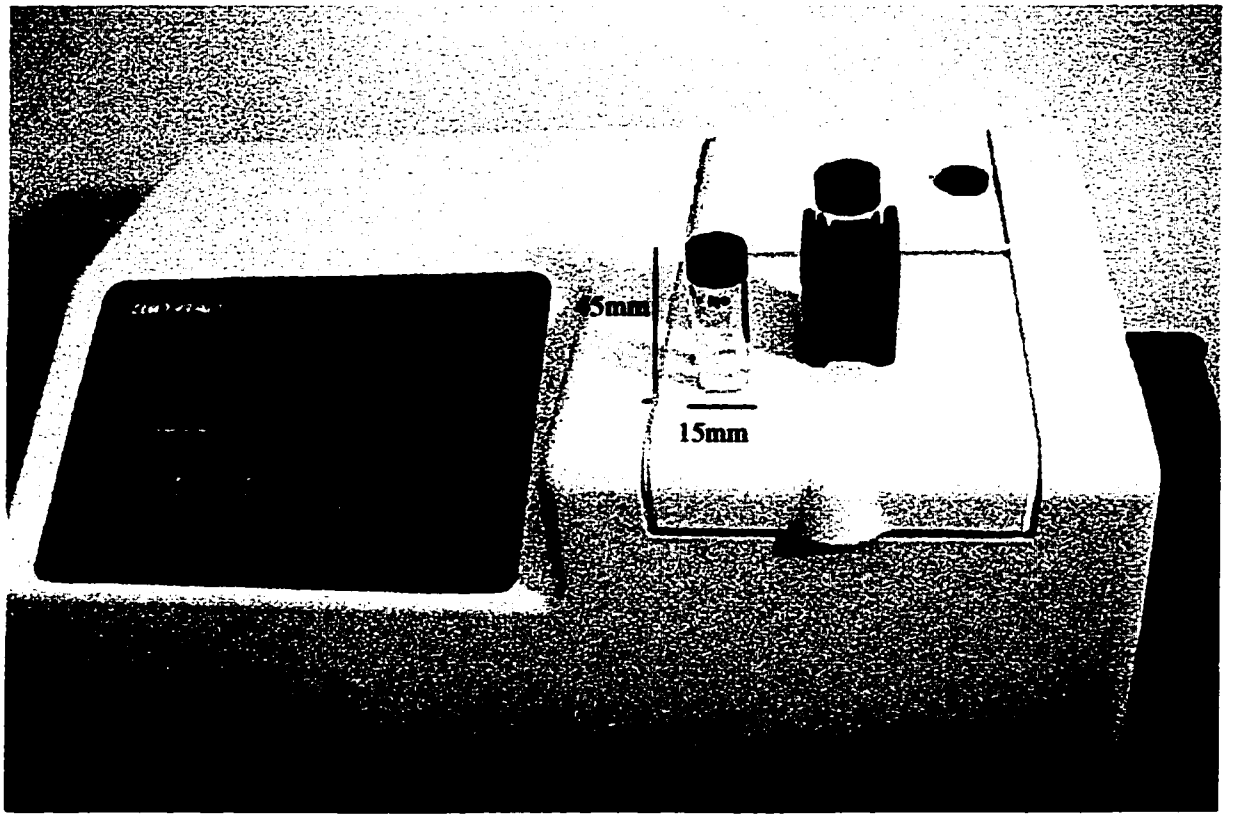
B. PARTS OF A CARRIER FOR INOCULATING MYCOBACTERIA.



APPENDIX V

EQUIPMENT FOR FLUORESCENCE ASSAY

VersaFluor™ Fluorometer System with Cuvette Holder and 4 mL Glass Vials.



CURRICULUM VITAE

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EDUCATION:

1997 - 1999 **UNIVERSITY OF OTTAWA**
Biochemistry, Microbiology, & Immunology Dept.
Ottawa, Ontario, Canada.
- Masters of Science in Microbiology (M.Sc.)

1995 **RESEARCH INSTITUTE OF TUBERCULOSIS**
Tokyo, Japan.
- Diploma in Tuberculosis Control and Epidemiology (DTCE)

1985 - 1986 **UNIVERSITY OF DHAKA**
Institute of Diseases of Chest and Hospital,
Dhaka, Bangladesh.
- Post-graduate Diploma in Tuberculous and Chest Diseases (DTCD)

1978 - 1983 **UNIVERSITY OF DHAKA**
Sir Salimullah Medical College
Dhaka, Bangladesh.
- Bachelors Degree in Medicine and Surgery (M.B; B.S)

(All studies were undertaken by scholarships awarded by the government of Bangladesh and Japan and the University of Ottawa, Canada)

PUBLICATIONS:

- Demonstration of Koch's Bacillus- Theory and Practice. Chest and Heart Bulletin Vol. XV No.1 (1989)
- Tuberculin Reactivity in Open Case of Pulmonary TB. A Random Study at the National TB Control Project. (submitted)

WORK HISTORY:

1997 - 1999 **UNIVERSITY OF OTTAWA**
Department of Biochemistry, Microbiology and Immunology,
Ottawa, Ontario, Canada.
Graduate Student (M. Sc.) and Research Assistant
M.Sc. thesis on:

- Application of reporter gene in the evaluation of mycobactericidal activity of germicides.

1989 - 1996 **NATIONAL TUBERCULOSIS AND LEPROSY CONTROL SERVICES**
Dhaka, Bangladesh
Medical Officer

- Clinical: Diagnosis and management of Tuberculosis and chest diseases.
- Laboratory: Supervision of microbiological work.
- Teaching: Training of medical students and staff.
- Field work: Monitoring and supervision of National TB Control Program in collaboration with the World Health Organization.
- Organizing and participating in workshops, seminars and conferences in collaboration with the World Health Organization, Research Institute of Tuberculosis (Japan) and the International Union Against Tuberculosis and Lung Diseases.

INSTITUTE OF DISEASES OF CHEST & HOSPITAL
Dhaka, Bangladesh

1987 - 1989 ***Medical Registrar***

- A member of the faculty, involved in the teaching and supervision of medical students.
- Management of hospital patients.
- Clinical research.

1985 - 1986 ***Post-Graduate student***

- Received one year post-graduate training in tuberculous and chest diseases.
- Received highest marks in the final examination and awarded the Diploma (DTCD).

RURAL AND INDUSTRIAL HOSPITALS UNDER THE NATIONAL HEALTH SERVICE, BANGLADESH

1984 - 1985 ***Medical Officer***

- Provided medical services, diagnostic and treatment to local patients from rural and industrial areas. Gained experience in tropical and industrial diseases.
- Coordinated control of communicable diseases including cholera and plague.
- Coordinated inspection of food from food and beverage manufacturing plants.
- Participated in the physical examination of national athletes and pilgrims.

MITFORD HOSPITAL
Dhaka, Bangladesh

1983 - 1984 ***Assistant Surgeon***

- Received training in all departments of the hospital with major in surgery.

SUMMARY OF SKILLS:

- **COMPUTER:**

Operating Softwares: MS DOS, Windows 95
Applications: MS Office 97
Internet: Netscape, MS Internet Explorer

- **LABORATORY:**

Environmental Microbiology: Germicidal testing, antibiotic susceptibility assays, testing water samples for mycobacteria.
Molecular Biology: Isolation and purification of DNA, transformation of bacterial cells including mycobacteria, reporter gene studies, PCR and restriction enzyme analysis for identification of mycobacteria. Epifluorescence microscopy and fluorometric studies of organisms.

Cell Culture: Culture of mammalian cell lines.
Virology: Cultivation and quantification of animal viruses. Virucidal testing of germicides.

- **CLINICAL:**

13 years practice (1983-1996) in internal medicine with specialization in pulmonary diseases and tuberculosis.

- **LANGUAGE:**

English and Bengali - Read, write and speak fluently.
Urdu and Hindi - Spoken only.

- **TECHNICAL:**

Repair of electrical and mechanical devices.

MEMBERSHIPS:

- Member of the American Society of Microbiologists
- Member of the Canadian Society of Microbiologists
- Member of the Research Institute of Tuberculosis, Japanese Anti-Tuberculosis Association, Tokyo, Japan
- Member of the Bangladesh Medical Association
- Member of the Bangladesh Medical and Dental Council
- Member of the National Anti-Tuberculosis Association of Bangladesh

- Assistant Editor of the Chest and Heart Association of Bangladesh

MISCELLANEOUS:

- Poster presented at the 49th Annual Meeting of the Canadian Society of Microbiologists. Montreal. June 1999. Title: 'Mycobactericidal Testing of Chemical Germicides: Comparison of Conventional Culture with a Reporter Gene Assay'. (A. A. Zafer, Y. E. Taylor and S. A. Sattar)
- Poster presented at the Ottawa Life Science Conference. World Congress Center in Ottawa, Ontario. 1998.
- Poster presented at the departmental Poster Competition at the Faculty of Medicine, University of Ottawa, Ontario. 1998 and 1999.
- Intensive course in Japanese Language, Tokyo, Japan. 1995.
- WHO Fellowship Training in "Tuberculosis Control Program" at the National Tuberculosis Institute, Bangalore, India. 1994.
- Attended Training of Trainers Course, Dhaka, Bangladesh. 1992.